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# **The Role of Histone Deacetylases in Cytokine Regulation in Human Gestational Tissues**

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A thesis submitted in fulfilment of the requirements for the degree  
of Doctor of Philosophy in Biomedical Science, The University of  
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# *Abstract*

Normal pregnancy results in significant immunologic alterations, including the maintenance of a heightened inflammatory state, controlled through the appropriate balance of pro and anti-inflammatory cytokines. Several pathologies of pregnancy such as pre-eclampsia, intrauterine growth restriction and preterm birth have been associated with variations in cytokine expression, both from the placenta and gestational membranes, and in the maternal circulation. Following their investigation as anticancer drugs, histone deacetylase inhibitors were identified as having anti-inflammatory actions, leading to further studies into how histone deacetylases may be involved in cytokine regulation. However, despite the importance of appropriate histone deacetylase activity during development little is known about histone deacetylases (HDACs) in the placenta and membranes.

The overall aim of this research was to investigate the role of histone deacetylases in cytokine regulation in human gestational tissues. HDAC expression was characterised in term placental, amnion and chorion tissues obtained after elective caesarean section. All eighteen human HDACs were expressed, with tissue-specific differences in expression and production identified. A time course was then established to examine the potential links between HDAC activity and cytokine expression and production by placental villous explants *in vitro*. Term explants were maintained at a physiologically relevant oxygen tension with/without an inflammatory stimulus and/or histone deacetylase inhibitor. In the presence of an inflammatory stimulus, HDAC inhibition resulted in mitigation of the normal response, with reduced production of both the proinflammatory cytokine tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and the anti-inflammatory cytokine interleukin-10 (IL-10) and accompanying reductions in TNF $\alpha$  expression. Despite this, levels of other cytokines were not affected, resulting in dysregulated cytokine expression and suggesting that HDAC activity is essential to appropriate cytokine expression. Furthermore both reduced HDAC activity and inflammatory stimulus resulted in selective feedback on HDAC expression and production. These data support a role for HDAC as regulators of cytokine production. Several pathological conditions of pregnancy are already associated with dysregulation of the cytokine network. Modulation of HDAC activity or histone acetylation levels may represent an underlying cause/ consequence of cytokine dysregulation in these conditions, as well as a potential therapeutic strategy

# *Dedication*

To my Husband

Thank you for believing in me

# *Acknowledgements*

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# Table of contents

<b>ABSTRACT .....</b>	<b>II</b>
<b>DEDICATION.....</b>	<b>III</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>IV</b>
<b>TABLE OF CONTENTS.....</b>	<b>V</b>
<b>TABLES.....</b>	<b>VII</b>
<b>FIGURES.....</b>	<b>VIII</b>
<b>ABBREVIATIONS .....</b>	<b>X</b>
<b>CONFERENCES AND SYMPOSIA .....</b>	<b>XIII</b>
<b>PUBLICATIONS .....</b>	<b>XIV</b>
<b>CHAPTER 1 LITERATURE REVIEW .....</b>	<b>1</b>
INTRODUCTION.....	1
CYTOKINES DURING PREGNANCY AND PARTURITION .....	1
<i>Epigenetic regulation?</i> .....	8
EPIGENETICS .....	9
<i>DNA methylation</i> .....	10
<i>Histone Modifications</i> .....	11
<i>Histone Acetylation</i> .....	13
<i>Histone Acetyltransferases (HAT)</i> .....	13
<i>Histone Deacetylases (HDAC)</i> .....	14
<i>Histone Deacetylase Inhibitors (HDACi)</i> .....	38
REGULATION OF CYTOKINES BY HDACS .....	43
<i>NF-<math>\kappa</math>B</i> .....	45
<i>Hypoxia and HIF1<math>\alpha</math></i> .....	46
<i>Interactions</i> .....	50
SUMMARY .....	53
<b>CHAPTER 2 HISTONE DEACETYLASES IN THE PLACENTA AND GESTATIONAL MEMBRANES.....</b>	<b>54</b>
PART A: PRELIMINARY STUDIES .....	54
<i>Global histone acetylation in the placenta, amnion and chorio-decidua</i> .....	54

<i>Histone deacetylase expression and labour</i> .....	60
PART B: CHARACTERISATION OF HISTONE DEACETYLASES IN HUMAN GESTATIONAL TISSUES .....	62
<i>Manuscript submitted to Molecular Human Reproduction</i> .....	62
<b>CHAPTER 3 THE EFFECTS OF HDAC INHIBITION ON CYTOKINE EXPRESSION</b> .....	<b>80</b>
PART A: PRELIMINARY STUDIES .....	80
<i>Histone deacetylase expression in previous studies</i> .....	80
<i>Time course development</i> .....	84
PART B: EFFECTS OF LPS AND TSA ON CYTOKINE EXPRESSION AND PRODUCTION.....	85
<b>CHAPTER 4 HISTONE DEACETYLASE EXPRESSION IN RESPONSE TO LPS AND TSA</b> .....	<b>102</b>
PART A: CLASS I HDACs.....	102
<i>Manuscript submitted to PLoS ONE</i> .....	102
<i>Histone deacetylase inhibition in human placental explants results in concomitant adjustments in HDAC expression</i> .....	102
PART B: CLASS IIA HDAC EXPRESSION.....	116
<b>CHAPTER 5 DISCUSSION AND CONCLUSIONS</b> .....	<b>119</b>
<b>APPENDICES</b> .....	<b>123</b>
<i>Cytokines in physiological and pathological pregnancy</i> .....	123
<i>Methods</i> .....	133
<b>REFERENCES</b> .....	<b>146</b>

# Tables

Table 1: RT-PCR Primer sequences .....	68
Table 2 : RT-PCR Primer sequences .....	90
Table 3: RT-PCR Primer Sequences.....	106
Table 4: IFN- $\gamma$ in pregnancy .....	123
Table 5: IL-1 $\beta$ in pregnancy.....	123
Table 6: IL-1ra in pregnancy.....	125
Table 7: IL-2 in pregnancy.....	125
Table 8: IL-4 in pregnancy.....	126
Table 9: IL-6 in pregnancy.....	126
Table 10: IL-8 in pregnancy.....	128
Table 11: IL10 in pregnancy.....	129
Table 12: IL-12 in pregnancy.....	131
Table 13: TGF- $\beta$ in pregnancy.....	131
Table 14: TNF- $\alpha$ in pregnancy.....	131
Table 15: Complete list of RT-PCR Primers .....	138

# Figures

**All figures are the primary artwork of Sheryl Kirsty Munro.**

Figure 1: Infection-mediated sources of inflammatory signalling during pregnancy .....	4
Figure 2: Epigenetic modifications and the effects on chromatin (Munro et al. 2010) ...	11
Figure 3: The classical family of histone deacetylases (Zn <sup>2+</sup> dependent) .....	15
Figure 4: The non-classical histone deacetylases: the NAD-dependent sirtuins .....	32
Figure 5: Relative density of acetylated histone H3 and H4 in amnion. ....	56
Figure 6: Relative density of acetylated histone H3 and H4 in chorio-decidua.....	57
Figure 7: Relative density of acetylated histone H3 and H4 in placental villous tissue. .	58
Figure 8: Histone deacetylase expression and labour .....	61
Figure 9: Class I HDACs in the placenta and membranes .....	70
Figure 10: Class IIa HDACs in the placenta and membranes .....	71
Figure 11: Class IIb HDACs in the placenta and membranes .....	72
Figure 12: Class IV HDACs in the placenta and membranes.....	72
Figure 13: Class III HDACs (sirtuins) in the placenta and membranes .....	73
Figure 14: HDAC activity in nuclear extracts from placenta and membranes.....	74
Figure 15: HDAC1 and HDAC3 protein in nuclear extracts from placenta and membranes .....	75
Figure 16: Effect of TSA on HDAC expression in amnion, choriodecidua and placental villous explants.....	82
Figure 17: Effect on TSA and LPS on HDAC expression in placental villous explants..	83
Figure 18: Placental villous explant time course .....	84
Figure 19: TNF mRNA expression and protein production by placental villous explants across time.....	92
Figure 20: IL-1 $\beta$ mRNA expression and protein production by placental villous explants across time.....	93
Figure 21: IL-6 (above) and IL-8 (below) mRNA expression and protein production by placental villous explants across time.....	94
Figure 22: IL-10 mRNA expression and protein production by placental villous explants across time.....	95
Figure 23: IL-1ra protein production by placental villous explants across time (above) and by gender (below).....	96
Figure 24: HDAC1 mRNA expression in human placental explants.....	108
Figure 25: HDAC3 mRNA expression in human placental explants.....	109

Figure 26: HDAC1 nuclear protein levels in human placental explants.....	110
Figure 27: HDAC3 nuclear protein levels in human placental explants.....	111
Figure 28: Total histone deacetylase activity in nuclear extracts from human placental explants.....	112
Figure 29: HDAC4 mRNA expression in human placental explants.....	116
Figure 30: HDAC7 mRNA expression in human placental explants.....	117

## Abbreviations

15-PGDH	Hydroxyprostaglandin dehydrogenase 15-(nicotinamide adenine dinucleotide)
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
AZA	5-aza-2' deoxycytidine
BMI	Body mass index
CaMKs	Ca <sup>2+</sup> /calmodulin-dependent protein kinases
cDNA	Complementary DNA
CGNs	Cerebellar granule neurons
CoA	Coenzyme A
CoREST	Corepressor of RE1-silencing transcription factor complex
COX2	Cyclooxygenase-2 / prostaglandin-endoperoxide synthase 2 / PGHS-2
CpG	Cytosine-phosphate-guanine
CPS1	Carbamoyl phosphate synthetase 1
CRM1	Chromosome region maintenance 1/ exportin1 / Xpo1
C-section	Caesarean section
C-TAD	C-terminal transactivation domain
CtBP	E1A C-terminal binding protein
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DTT	Dithiothreitol
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
ER $\alpha$	Estrogen receptor $\alpha$
FGH	Fetal growth restriction
FIH	Factor inhibiting HIF
FOX	Forkhead box protein
GCMa	Glial cells missing homolog 1 protein
GNATs	General control non-derepressible 5 (Gcn5)-related N-acetyltransferases
qPCR	Quantitative real-time PCR
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HDRP	Histone deacetylase 4 and 5 related protein
HI	Heat inactivated
HIF	Heat inducible factor
HP1	Heterochromatin protein 1
HRP	Horseradish peroxidase
Hsp $x$	Heat shock protein $x$
HUB	HDAC6, USP3 and BRCA1 related zinc finger
HUR	Antigen R of patient HU
IAI	Intra-amniotic infection
IFN- $x$	Interferon- $x$
I $\kappa$ B	Inhibitor of $\kappa$ B
IL- $x$	Interleukin- $x$

IUGR	Intrauterine growth restriction
KLF	Kruppel-like factors
L-CoR	Ligand-Dependent Corepressor
LPS	Lipopolysaccharide
LSD1	Lysine specific demethylase 1
MBD	Methyl binding domain
MEF2	Myocyte enhancer factor 2
MITR	MEF2-interacting transcriptional repressor
MMP	Matrix metalloproteinase
mPGES-1	Microsomal prostaglandin E synthase-1
mRNA	Messenger RNA
N-CoR	Nuclear receptor co-repressor 2/ SMRT /Silencing mediator for retinoid and thyroid receptor
NES	Nuclear export signal
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLS	Nuclear localisation signal
NODE	Nanog and Oct4-associated deacetylase complex
NuRD	Nucleosome remodelling and deacetylase complex
NF-Y	Nuclear transcription factor Y
PAGE	Polyacrylamide gel electrophoresis
Pax3	Paired box protein 3
PBMC	Peripheral blood mononuclear cell
PCAF	p300/CBP-associated factor
PE	Pre-eclampsia
PGE2	Prostaglandin E2
PHD	Prolyl hydroxylase domain
PKD	Protein kinase D
PLA2	Phospholipase A2
PLAP	Phospholipase A2 activation protein
PMSF	Phenylmethanesulfonylfluoride
PPROM	Preterm premature rupture of the membranes
PROM	Premature rupture of the membranes/ prelabour rupture of the membranes
PTL	Preterm labour
RFP	RET finger protein
Rpd3	reduced potassium dependency 3
RNA	Ribonucleic acid
RNO	Reactive oxygen species
RNS	Reactive nitrogen species
RPRM	Reprimo
rRNA	Ribosomal RNA
SAHA	Suberoylanilide hydroxamic acid
SDS	Sodium dodecyl sulfate
SE14	SerGlu-containing tetradecpeptide repeats
SGA	Small for gestational age
Sir2	Silent information regulator 2
siRNA	Small interfering RNA
SIRT	Sirtuin / silent information regulator2 (Sir2)-related protein

SMRT	Silencing mediator for retinoid and thyroid receptor / N-CoR Nuclear receptor co-repressor 2
SNP	Single nucleotide polymorphisms
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
TBP-2	Thioredoxin binding protein 2
TCA	Trichloroacetic acid
TD	Term delivery
tDMR	Tissue-specific differentially methylated region
TLR	Toll-like receptor
TNF $\alpha$	Tumour necrosis factor /cachexin /cachectin
TNFR	Tumour necrosis factor receptor
TSA	Trichostatin A
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau
VPA	Valproic acid

## *Conferences and Symposia*

Elements of the research contained in this thesis have previously been presented at the following conferences and symposia:

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**Munro SM**, Mitchell MD, Ponnampalam AP. Histone Deacetylases and Immune Response in the Placenta. Society for Gynecologic Investigation – 59th Annual Scientific Meeting. San Diego, USA, 2012.

**Munro SM**, Mitchell MD, Ponnampalam AP. Histone Deacetylases and Immune Response in the Placenta. National Research Centre for Growth and Development (NRCGD) Science Symposium, Dunedin, New Zealand, 2011.

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Additionally, data has been presented at annual University of Auckland and Liggins Institute student research symposia.

# *Publications*

The following manuscripts have been submitted for publication:

**Histone deacetylases in human gestational membranes exhibit tissue-specific expression**

Sheryl K. Munro, Murray D. Mitchell, Anna P. Ponnampalam

Manuscript submitted to Molecular Human Reproduction

**Histone deacetylase inhibition in human placental explants results in concomitant adjustments in HDAC expression**

S. K. Munro, M. D. Mitchell, A.P. Ponnampalam

Manuscript submitted to PLoS ONE

The following manuscript has been published: *Placenta* 34 (7) , pp. 567-573

**Histone deacetylase inhibition by trichostatin A mitigates LPS induced TNF $\alpha$  and IL-10 production in human placental explants**

S. K. Munro, M. D. Mitchell, A.P. Ponnampalam

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Chapter 3 part B

"Histone deacetylases in human gestational membranes exhibit tissue-specific expression"

Article submitted to Molecular Human Reproduction

Nature of contribution by PhD candidate

Designed and executed the experiments, analysed and interpreted the data, drafted the manuscript

Extent of contribution by PhD candidate (%)

80

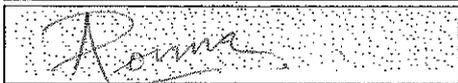
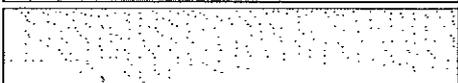
### CO-AUTHORS

Name	Nature of Contribution
Murray Mitchell	Overall supervision and critically revised the manuscript
Anna Ponnampalam	Interpreted the data and drafted the manuscript

### Certification by Co-Authors

The undersigned hereby certify that:

- ❖ the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- ❖ in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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Chapter 4 part B

"Histone deacetylase inhibition by trichostatin A mitigates LPS induced TNF $\alpha$  and IL-10 production in human placental explants"

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Designed and executed the experiments, analysed and interpreted the data, drafted the manuscript

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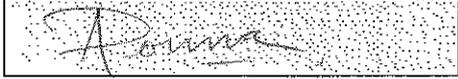
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Chapter 5 part A

"Histone deacetylase inhibition in human placental explants results in concomitant adjustments in HDAC expression"

Article submitted to PLoS ONE

Nature of contribution by PhD candidate

Designed and executed the experiments, analysed and interpreted the data, drafted the manuscript

Extent of contribution by PhD candidate (%)

80

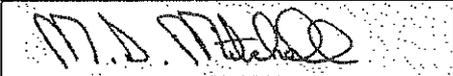
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# *Chapter 1 Literature Review*

## ***Introduction***

It is increasingly being recognised that many health disorders have their origins in events that happened during early development (**Barker et al. 2002**). Altered developmental programming can arise due to early environmental interactions, causing long term effects. For this reason, important reproductive health issues such as intrauterine growth restriction, preeclampsia and preterm delivery are of particular concern (**Tranguch et al. 2005**). Early environmental interactions such as those between the embryo and uterus during implantation and placentation are complex, and impairment can result in placental deficiency -which has been linked to early onset intrauterine growth restriction, recurrent abortion, preeclampsia and preterm delivery (**Wang et al. 2006**). The importance of early interactions are exemplified in mice, where even a transient postponement of blastocyst attachment is sufficient to cause a detrimental ripple effect throughout the pregnancy, with aberrant spacing of embryos, defective placentation, resorption and retarded development of fetuses observed (**Wilcox et al. 1999; Song et al. 2002; Ye et al. 2005**).

## ***Cytokines during pregnancy and parturition***

Cytokines are soluble proteins released by cells, which act non-enzymatically to regulate cellular function (**Nathan et al. 1991**). In addition to other sources and actions they are released by immune cells and affect immune cell differentiation and function (**Bowen et al. 2002**). Cytokines form part of the two way dialogue between the blastocyst and the endometrium during implantation (**Norwitz et al. 2001; Dey et al. 2004; Makker et al. 2006; Pafilis et al. 2007**), and can directly enhance the immune privilege and invasive and proliferative phenotypes of trophoblast (**Bowen et al. 2002**) aiding placentation. Alterations in cytokine expression occur during gestation, with (pre)term labour, infection, preeclampsia and intrauterine growth restriction (IUGR) (**Keelan et al. 2003; Rusterholz et al. 2007; Sharma et al. 2007; Mullins et al. 2012; Weissenbacher et al. 2012**).

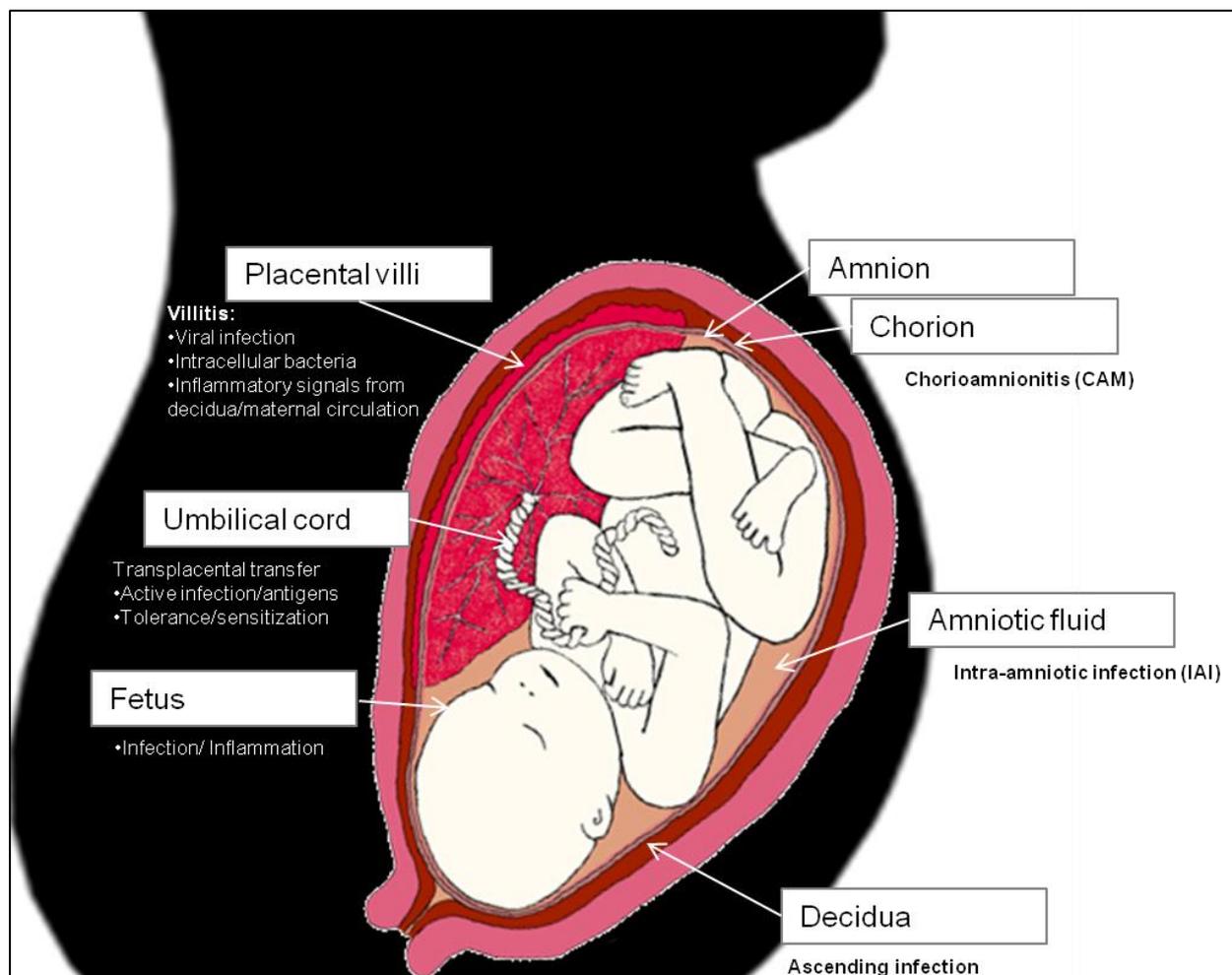
Throughout pregnancy there is controlled mild systemic inflammation, with raised levels of pro-inflammatory cytokines such as tumour necrosis factor (TNF $\alpha$ ), interleukin-6 (IL-6), and IL-1 $\beta$  in healthy pregnant women, as compared to healthy non-pregnant women [Table 4-Table 14] and during which cells of the innate immune system display activated phenotypes (**Sacks et al. 1998; Sacks et al. 1999; Naccasha et al. 2001; Sacks et al. 2003**). It has been proposed that during uncomplicated pregnancy there is a bias toward production of cytokines which promote humoral immunity rather than cell-mediated immunity. An appropriate balance of pro and anti-inflammatory cytokines needs to be maintained throughout pregnancy, and several pathologies have been associated with variations in cytokine expression. Importantly, microbial invasion of the uterine compartment can deregulate the cytokine balance, resulting in preterm labour.

Labour at all gestational ages is an event characterized by activation of the inflammatory cascade (**Romero, Parvizi et al. 1990; Imseis, Greig et al. 1997; Chan et al. 2002; Peltier 2003; Bisits et al. 2005; Lindstrom et al. 2005; Haddad et al. 2006; Romero et al. 2006; Al-Asmakh et al. 2007**), with evidence of inflammatory cells and increased production of proinflammatory cytokines in the cervix, myometrium, chorioamniotic membranes, and amniotic cavity (**Romero et al. 2006; Romero, Espinoza et al. 2007**), as well as by an up-regulation of inflammatory associated genes (**Haddad et al. 2006**). Cytokines play critical roles in parturition, stimulating cervical dilation, rupture of the extra placental membranes and uterine contractility by inducing the production of prostaglandins and matrix metalloproteinases (MMPs) (**Hansen et al. 1999; Brown et al. 2000; Keelan et al. 2003; Olson 2003; Kida et al. 2005**). Cytokines regulate the availability of prostaglandins in the gestational membranes through the induction of phospholipase A2 (PLA2) and prostaglandin-endoperoxide synthase 2 (COX-2) which are necessary for prostaglandin synthesis, and by decreasing expression of the prostaglandin catabolism enzyme hydroxyprostaglandin dehydrogenase 15-(nicotinamide adenine dinucleotide) (15-PGDH) (**Imseis, Zimmerman et al. 1997; Kniss et al. 1997; Hansen et al. 1999; Pomini et al. 1999**). The proinflammatory cytokines also act by promoting the increased expression of matrix metalloproteinases which degrade the extracellular matrix of the cervix and gestational membranes (**Ulug et al. 2001; Arechavaleta-Velasco et al. 2002; Kida et al. 2005; Vadillo-Ortega et al. 2005; Lockwood et al. 2008**).

However these cytokines are also produced by gestational tissues in response to microbial products such as Lipopolysaccharide (LPS) (**Casey et al. 1989; Romero,**

**Brody et al. 1989; Liechty et al. 1991; Romero, Mazor et al. 1991; Svinarich et al. 1996; Huleihel et al. 2004).** Usually infection is ascending, occurring when microorganisms present in the vagina traverse the cervix and colonize the decidua (**Pararas et al. 2006**) but in some cases microorganisms can cross the gestational membranes, resulting in intraamniotic and/or fetal infection (**Romero et al. 2006**) [**Figure 1**]. Indeed gestational tissues collected from preterm births show overt signs of microbial infection in 25-40% of cases (**Romero, Gotsch et al. 2007; Goldenberg et al. 2008**) and elevated amniotic and cervico-vaginal fluid concentrations of the inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  are associated with both reproductive tract infection and subsequent preterm birth [**Table 5-Table 14**] as well as other adverse birth outcomes such as cerebral palsy (**Wu et al. 2000**).

Microbial products are recognised by the Toll-like receptors (TLRs). TLR-2 recognises peptidoglycans, lipoproteins, and zymosan (Gram-positive bacteria, mycoplasmas, and fungi), TLR-3 recognises double-stranded RNA (viruses) and TLR-4 recognises the presence of LPS (Gram-negative bacteria) (**Pasare et al. 2004**). In pregnant women, TLR-2 and TLR-4 are expressed in the amniotic epithelium and are up-regulated in the chorioamniotic membranes with term labour and Intra-amniotic infection (IAI) associated pre-term labour (**Kim et al. 2004**). TLRs are not expressed in the outer trophoblast layers of the placenta suggesting that an inflammatory response will only occur after microbial invasion into the placenta (**Mor et al. 2005**). Recently it was determined that such invasion was common with 27% of placentas having intracellular bacteria present in the basal plate (**Stout et al. 2013**). Interestingly, synergy between different TLRs exists, such that a viral infection (or second type of bacteria, i.e. gram positive vs. negative) can amplify the response to bacteria, or sensitise against existing antigens (**Cardenas et al. 2010; Ilievski et al. 2010; Flores-Herrera et al. 2012**). It has been suggested that exposure to chronic maternal infection may modify fetal innate and regulatory responses independently of in-utero transmission of the pathogens (**Dauby et al. 2012**).



**Figure 1: Infection-mediated sources of inflammatory signalling during pregnancy**

The role of cytokines in inducing labour, has led to extensive investigations into the alterations in cytokine expression occurring during gestation, with (pre)term labour, infection and in different pathologies [Table 4-Table 14]. To date there is lack of consensus between studies which is largely attributed to sample size, source, and confounding factors. The amnion and choriodecidua exhibit distinct patterns of response to LPS and other microbial products with evidence of inflammatory signalling across the layers of the gestational membranes (Zaga et al. 2004; Zaga-Clavellina et al. 2006; Zaga-Clavellina et al. 2007; Thiex et al. 2009), so it is likely that the nature and location of infection may contribute to the pathological outcome. Variables which may influence cytokine levels during pregnancy include maternal BMI, age, and prior preterm delivery status- and as these factors have a greater influence than the stage of gestation (Curry et al. 2008); a large degree of variation exists among the control population. Cytokine production during pregnancy has also been measured in a variety of ways, including determining cytokine production by amniotic fluid derived cells

cultured *ex vivo* (**Sezen et al. 2008**), and directly measuring cytokine concentrations in amniotic fluid (**Romero et al. 2006; Gotsch et al. 2008**), cervico-vaginal fluids (**Imseis, Greig et al. 1997**), maternal plasma (**Holmes et al. 2003; Curry et al. 2008**) and maternal serum (**van der Weiden et al. 2005**) which makes comparisons between studies difficult.

Despite these difficulties, changes in specific cytokine protein levels have been associated with a variety of pregnancy complications. The circulating levels of TNF $\alpha$  and IL-6, which are already more elevated in healthy pregnant women compared to nonpregnant controls, are further raised in patients with preeclampsia (**Greer et al. 1994; Kupferminc et al. 1994; Vince et al. 1995; Conrad et al. 1998**). This increase in proinflammatory cytokines is further exacerbated by the decreased IL-10 production associated with preeclampsia (**Hennessy et al. 1999; Rein et al. 2003; Makris et al. 2006; Borekci et al. 2007; Sharma et al. 2007**). Most studies have however, failed to detect a correlation between earlier circulating TNF $\alpha$  levels and the later onset of preeclampsia (**Meekins et al. 1994; Hamai et al. 1997; Heikkinen et al. 2001; Serin et al. 2002**). This suggests elevated TNF $\alpha$  production could be a consequence rather than cause of the disease, although TNF $\alpha$  levels do correlate with disease progression (**Peracoli et al. 2007**). A recent study identified six cytokine-related genes and five oxidation-related genes up regulated in preeclamptic placentas suggesting a general deregulation of inflammatory processes in this disease (**Lee et al. 2009**). Alterations in TNF $\alpha$  expression have been also associated with a subset of IUGR cases. In IUGR, elevated levels of TNF $\alpha$  have been observed in women with placental dysfunction but not in those with normal placental perfusion (**Bartha et al. 2003**). In a perfusion model system IUGR placentae were shown to secrete higher amounts of TNF $\alpha$  upon angiotensin II stimulation (**Holcberg et al. 2001**) and *in vitro* studies with villous cytotrophoblasts from IUGR pregnancies demonstrated a higher TNF $\alpha$ -induced apoptotic rate when compared with uncomplicated pregnancies (**Kilani et al. 2007**).

IL-10 is a potent anti-inflammatory cytokine and has been shown in animal models to be an inhibitor of cytokine-induced preterm labour (**Terrone et al. 2001; Sadowsky et al. 2003; Rodts-Palenik et al. 2004; Robertson et al. 2006**). IL-10 null mice are also more susceptible to LPS-induced fetal loss (**Murphy, Fast et al. 2005; Robertson et al. 2006**). IL-10 is produced by lymphocytes (**Benjamin et al. 1992; Del Prete et al. 1993**), macrophages (**de Waal Malefyt et al. 1991; Boonstra et al. 2006**), dendritic cells

(**Boonstra et al. 2006**), and stimulated keratinocytes (**Enk et al. 1992; Rivas et al. 1992**). During pregnancy, IL-10 is additionally produced by cytotrophoblast (**Roth et al. 1996; Hanna et al. 2000**), syncytiotrophoblast (**Roth et al. 1996; Hanna et al. 2000**), chorion (**Dudley, Edwin et al. 1997; Marvin et al. 2002**), mononuclear cells/macrophages (**Ekerfelt et al. 2002; Heikkinen et al. 2003; Lidstrom et al. 2003; Gustafsson et al. 2006**), and NK cells (**Vigano et al. 2001; Lidstrom et al. 2003**).

Whether IL-10 changes with pregnancy is subject of debate; some studies have found higher median IL-10 amniotic fluid concentration in women at term than those in the second trimester, while others have seen no changes with advancing gestational age in amniotic fluid, maternal plasma, or in maternal serum [**Table 11**] despite a significantly higher concentration of IL-10 being detected in pregnant than in nonpregnant controls (**Holmes et al. 2003**). Mid-trimester levels of IL-10 have been reported to have no correlation with timing of birth (**Gotsch et al. 2008**), while other studies found IL-10 levels to be higher in patients who subsequently delivered preterm (**Apuzzio et al. 2004**). Some studies have also found labour at term to be associated with significantly higher median amniotic fluid concentrations of IL-10 than women at term not in labour (**Gotsch et al. 2008**) while other groups found no change in amniotic fluid (**Greig et al. 1995; Dudley, Hunter et al. 1997**) or in gestational tissues (**Jones et al. 1997; Hanna et al. 2000; Gustafsson et al. 2006**) or in fact reported a significant reduction in IL-10 expression in the presence of labour when compared to controls (**Simpson et al. 1998; Vives et al. 1999; Hanna et al. 2006**). However IL-10 levels are associated with infection, and higher amniotic fluid concentrations of IL-10 have been detected in the presence of infection /inflammation-associated preterm birth (**Greig et al. 1995; Gotsch et al. 2008**).

IL-10 participates in a negative feedback loop to dampen inflammation (**Moore et al. 2001**) inhibiting the production of proinflammatory cytokines by LPS-activated macrophages (**de Waal Malefyt et al. 1991; Fiorentino et al. 1991**), inhibiting TNF $\alpha$  production (**Bogdan et al. 1991; Gazzinelli et al. 1996; Moore et al. 2001**) and also preventing the release of reactive oxygen intermediates (**Fiorentino et al. 1989; Bogdan et al. 1991**). In the fetal membranes IL-10 has anti-inflammatory activity, suppressing the production of LPS induced TNF $\alpha$  (**Fortunato et al. 1997; Sato et al. 2003**), matrix metalloproteinases (**Roth et al. 1999; Fortunato et al. 2001**), IL-6 and IL-8 (**Fortunato et al. 1996; Fortunato et al. 1998**), but is also reported to have conflicting proinflammatory actions including stimulation of IL-8 production (**Mitchell et**

**al. 2004)** and roles in regulating prostaglandin (PG) biosynthesis within gestational tissues (**Goodwin et al. 1998; Pomini et al. 1999; Brown et al. 2000; Sato et al. 2003; Mitchell et al. 2004; Hanna et al. 2006**).

Following LPS stimulation, there are striking inter-individual differences in IL-10 production, and part of this variability is genetically determined (**Westendorp et al. 1997; Reuss et al. 2002**). The IL-10 gene contains several polymorphisms including microsatellite polymorphisms upstream of the transcription start site, and three single nucleotide polymorphisms (SNPs) –for which only three haplotypes have been found in the Caucasian population (**Eskdale et al. 1995; Eskdale et al. 1996; Turner et al. 1997; Reuss et al. 2002**). Two of these have been the subject of intense investigation (**Anells et al. 2004**) with one haplotype associated with low IL-10 production and the other with higher protein production (**Turner et al. 1997; Reuss et al. 2002**). The latter was proposed to contribute to a reduced inflammatory response, decreasing the risk for adverse neonatal inflammation- associated outcomes (**Dordelmann et al. 2006**). However another study found no major influences on prematurity associated diseases (**Yanamandra et al. 2005**), nor were there statistically significant changes in association with pre-eclampsia (**Makris et al. 2006**). To date no relationships between the maternal IL-10 genotype and spontaneous preterm birth or preterm premature rupture of the membranes (PPROM) has been observed (**Kalish et al. 2004; Kerk et al. 2006; Mattar et al. 2006**), with the exception of one SNP in combination with clinical chorioamnionitis which increased the risk of preterm labour (**Kerk et al. 2006**).

Studies into genetic variation among cytokines continue, and there have been reports of genetic variation in the IL-10 receptor gene (**Simhan et al. 2008**) as well as mutations and SNPs in genes coding for TNF $\alpha$ , IFN- $\gamma$ , and IL-6 (**Chen et al. 1996; Dizon-Townson et al. 1998; Lachmeijer et al. 2001; Heiskanen et al. 2002; Anells et al. 2004; Macones et al. 2004; Mattar et al. 2006; Menon et al. 2006; Canto-Cetina et al. 2007; Molvarec et al. 2008; Moura et al. 2009**). There are ethnic differences in the association of individual SNPs with outcome. Recent data suggests that the combination of TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 maternal gene polymorphisms might contribute to susceptibility to spontaneous preterm birth (**Moura et al. 2009**). Immunological response is most likely the result of interactions between many polymorphic genes (**Hoffjan et al. 2005; Guzman et al. 2008**), and ethnic differences in disease susceptibility, frequencies of certain cytokine polymorphism alleles and clinical heterogeneity of the populations studied will continue to contribute to controversy over

the association of genetic variation and cytokine response (**Hassan et al. 2003; Menon et al. 2006**).

## Epigenetic regulation?

While studies continue into genetic variability of cytokine expression, it is possible that epigenetics may explain some variation seen between tissues and indeed within study populations. It has been previously reported that inhibition of prostaglandin synthesis occurs when the amnion is treated with histone deacetylase (HDAC) inhibitors (HDACi) and a methylation inhibiting agent, a phenomenon not seen in the adjacent chorion (**Mitchell 2006**) suggesting epigenetic regulation of specific genes at a tissue specific level.

Certainly elements of the prostaglandin pathway appear to be under epigenetic regulation. High histone H3 and H4 acetylation, an epigenetic mark associated with transcriptional activation (**Fuks 2005**), have been detected in the proximal region of the COX-2 promoter. Evidence suggests this proximal 1000 bp region is crucial to COX-2 promoter regulation in term amnion (**Mitchell et al. 2008**). Additionally, microsomal prostaglandin E synthase-1 (mPGES-1), which catalyzes the terminal step in the biosynthesis of prostaglandin E2 (PGE2) appears to be transcriptionally induced by HDAC4 (**Chabane et al. 2009**). HDAC inhibition results in the mitigation of IL-1 $\beta$  induced PGE2 synthesis, COX-2 expression, proteoglycan degradation and mPGES-1 expression in chondrocytes (**Chabane et al. 2008; Zayed et al. 2011**).

Other studies have also implicated epigenetic regulation in the control of labour with HDAC inhibitors inhibiting human uterine contractions (**Moynihan et al. 2008**) and delaying the initiation of parturition in mice (**Condon et al. 2003**). Recently it was shown that specific inhibition of HDAC8 in human myometrial tissue *ex vivo*, resulted in inhibition of both spontaneous and oxytocin-augmented contractions through interactions with heat shock protein 20 (Hsp20) (**Karolczak-Bayatti et al. 2011**).

HDACi treatment of chorion explants also results in differential cytokine responses when stimulated, with altered IL-1 $\beta$  but not IL-10, or TNF $\alpha$  (**Sato et al. 2006**), suggesting a potential for epigenetic regulation of cytokine production in gestational tissues. Histone deacetylase inhibition results in unopposed histone acetyltransferase (HAT) activity which would promote the hyper-acetylation of the histones. However

HDACs and HATs, like many other enzymes involved in histone modification have actions which are not specific to the histones and are able to modify other proteins **(Yang and Seto 2007)**. It therefore remains of interest to determine whether altered cytokine profiles seen with HDACi treatment result directly from altered histone acetylation or from the other actions of HDACs.

## ***Epigenetics***

For mainly historical reasons there has been some confusion about what “epigenetics” actually refers to, although a standard definition has been “mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in gene sequence” **(Russo et al. 1996)**. Of these, heritability is perhaps the most restrictive requirement: although neurons undergo stable alterations, since they rarely divide the changes are not heritable in the simplest sense; whereas other alterations seem to be heritable, but are not stability maintained. These issues led to a proposed definition of epigenetics as “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” **(Bird 2007)**, and more recently; an epigenetic trait is a “stably inherited phenotype resulting from changes in a chromosome without alterations in the DNA sequence” **(Berger et al. 2009)**. Changes in chromatin architecture can be mediated by DNA methylation and the post-translational modification of histone tails **(Dolinoy et al. 2007; Jirtle et al. 2007)**. It is thought that non-protein coding RNAs may also regulate chromatin structure, with RNA mediated interplay between the environment, epigenome and transcriptome also occurring, however this is still an emerging area of research **(Mattick et al. 2009)**. Cellular components such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and histone acetyltransferases (HATs) act to maintain or alter these modifications as appropriate. Two of the most well-known epigenetic related phenomena are X-chromosome inactivation and genomic imprinting **(Jirtle et al. 2007)**. A range of environmental factors, including nutrition and exposure to xenobiotic chemicals, can influence the establishment and maintenance of epigenetic patterning and thus long-term gene transcription **(Dolinoy et al. 2007; Jirtle et al. 2007)**. DNA methylation and histone modifications result in alterations to chromatin structure **(Dolinoy et al. 2007; Jirtle et al. 2007)** and are involved in the recruitment of transcription factor complexes **(Berger 2007)**, both of which act to regulate gene expression. Both DNA methylation and histone modifications have been found to be

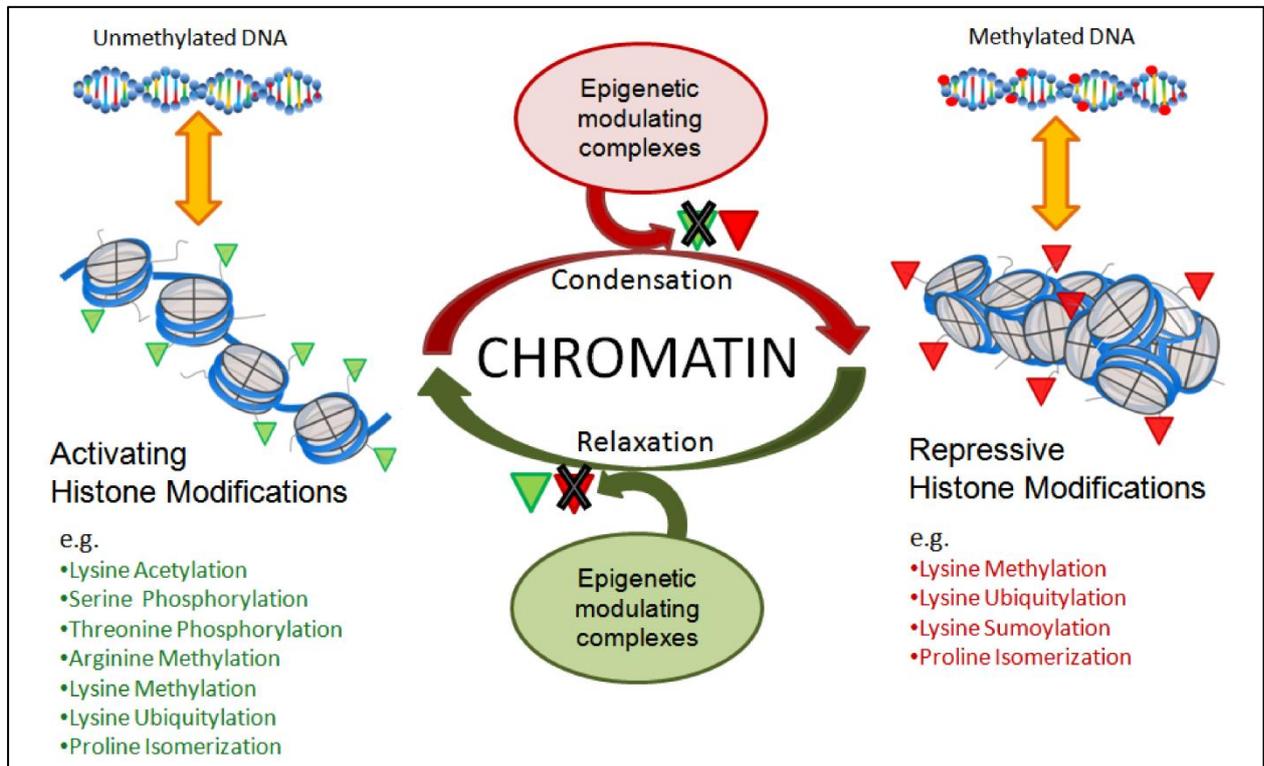
altered in human cancers, within the promoter regions of tumour suppressor genes and oncogenes (**Jirtle et al. 2007**), and it is becoming increasingly clear that such changes are also associated with other disease states (**Guo 2009; Trenkmann et al. 2009; Turunen et al. 2009**).

## DNA methylation

DNA methylation is the covalent modification of post-replicative DNA by the addition of a methyl group to the cytosine ring to form methyl cytosine, catalysed by DNA methyltransferases (DNMTs) (**Ohgane et al. 2008**). Three main DNA methyltransferases (DNMTs) are present in mammals: DNMT1 which maintains methylation by recognising uni-strand methylation after replication, and DNMTs 3a and 3b which predominantly function as *de novo* methyltransferases acting at previously un-methylated sites (**Brero et al. 2006**).

In mammals, DNA methylation is thought to be exclusively associated with cytosine-phosphate-guanine (CpG) dinucleotides, occurring on both strands at the cytosine residue (**Brero et al. 2006**). In vertebrates, large un-methylated GC rich regions can be found at the 5' prime end of many genes; these CpG dense regions are termed CpG islands (**Bird 1987**). DNA methylation at promoter associated CpG islands is strongly linked to repression of transcriptional activity; however the precise role of CpG methylation is an area of on-going research (**Bird 2002**). Approximately 10-20 % of genes display DNA methylation patterns in a tissue-specific manner (**Song, Smith et al. 2005; Eckhardt et al. 2006; Rakyan et al. 2008**) and these so-called tissue-specific differentially methylated regions (tDMRs) have been associated with tissue-specific patterns of gene expression (**Rakyan et al. 2008**). The repressive nature of DNA methylation is thought to result from either the methyl group preventing regulatory molecules binding to a CpG site, or by acting as a recognition signal for methyl-CpG-binding proteins with repressive properties (**Bird 2002**), both of which are mechanisms likely to act in a site-specific manner. However, it should be noted that DNA methylation is not always associated with transcriptional repression. While there is a negative correlation between DNA methylation and gene expression at promoters with a medium or high CpG density, even some low-CpG density promoters show this correlation. In contrast, gene-body methylation positively correlate with gene expression suggesting

novel roles for DNA methylation (**Rakyan et al. 2008**). Further research is required to elucidate the sequence and context-specific nature of this epigenetic modification.



**Figure 2: Epigenetic modifications and the effects on chromatin (Munro et al. 2010)**

## Histone Modifications

Histones are integral to the higher order structure of chromatin. Approximately 146 base pairs of DNA is wrapped around a histone octamer consisting of two sets of the core histones –H2A, H2B, H3, and H4 – to form a nucleosome. These nucleosomes are linked by loops of DNA and the linker histone H1. While the globular domains of histones associate closely with each other, the trailing amino acid “tails” protrude past the surrounding DNA and are subject to post translational modification. New histone proteins are produced during the S (synthesis) phase of the cell cycle when DNA is being replicated (**Lucchini et al. 1995**). The existing histones from the parental strand are randomly segregated between parental and daughter strands during replication and the rest of the histones, synthesised *de novo*, are then deposited (**Sogo et al. 1986**). Two H3-H4 heterodimers are deposited either sequentially, or as a heterotetramer,

followed by the two H2A-H2B dimers, thus forming the octamer (**Worcel et al. 1978; English et al. 2006**).

Many distinct modifications of histones exist, with those identified including lysine acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerisation (**Kouzarides 2007; Lindner 2008**). Modification of histones can be difficult to study as histone modifications are very dynamic. All known histone post translational modifications have now been shown to be reversible, and as some histone modifications have been shown to change within minutes of a stimulus, it is debatable as to which modifications can be considered as truly stable and therefore epigenetic. It is also unknown how the persistence of chromatin state is achieved, and which modifications are therefore truly heritable (**Berger 2007; Kouzarides 2007**).

Histone deacetylation, one of the best characterised histone modifications, is associated with gene silencing, while histone acetylation is associated with transcriptional activation (**Fuks 2005**) [Figure 2]. Histone acetylation patterns are maintained by two opposing groups of enzymes, the histone acetyltransferases (HATs) and histone deacetylases (HDACs) (**Hildmann et al. 2007**). There is also evidence for crosstalk between elements of the DNA methylation machinery and those involved in histone modification, with DNMTs able to recruit complexes containing HDACs (**Fuks 2005**). A group of proteins with methyl binding domains (MBDs) are also able to recognize methylated DNA sequences and recruit HDACs (**Nan et al. 1998; Irvine et al. 2002; Jorgensen et al. 2002**). A reverse regulatory pathway whereby histone acetylation mediates DNA demethylation may also exist (**Szyf et al. 1985; Selker 1998; Cervoni et al. 2001**) as some data show that HDAC inhibition can lead to changes in DNA methylation (**Cosgrove et al. 1990; Chen et al. 1997; Hu et al. 1998; Selker 1998; Hu, Pham et al. 2000; Dobosy et al. 2001; Maass et al. 2002**), although this is not always the case (**Cameron et al. 1999; Singal et al. 2001; El-Osta et al. 2002**). HDACs and HATs, like many other enzymes involved in histone modification, have actions which are not specific to the histones and are able to modify other proteins. Over 80 proteins have been identified which contain acetylated lysine residues, including transcription factors, transcriptional co-regulators, several viral proteins,  $\alpha$ -tubulin, acetyl-CoA synthase, Ku70, and Hsp90 (**Ogryzko et al. 1996; Kouzarides 2000; Sterner et al. 2000; Chan et al. 2001; Yang 2004**). Some HATs also physically associate with non-protein coding

RNA (**Yang and Seto 2007**). Similar actions are seen with other histone modifying enzymes, such as lysine-specific-demethylase-1 (LSD1), which in addition to histone demethylation, has been found to be involved in the demethylation and stabilisation of DNMT1, which is required for the maintenance of global DNA methylation (**Hotz et al. 2009; Wang, Hevi et al. 2009**).

## Histone Acetylation

Acetylation of the histones occurs rapidly after synthesis, with the HATs catalysing the transfer of an acetyl moiety from acetyl coenzyme A (acetyl-CoA) onto the  $\epsilon$ -amino group of lysine residues (**Vidali et al. 1968; DeLange et al. 1969; Bronner et al. 2007**). This initial acetylation is thought to assist with deposition of histones onto DNA, after which the histones are rapidly deacetylated (**Ruiz-Carrillo et al. 1975; Jackson et al. 1976; Annunziato et al. 1983**) and later re-acetylated with new patterns to enable various functions. As well as functions resulting from site-specific acetylation, it is notable that various other modifications occur on lysine residues and thus acetylation can act by precluding other modification; HDACs and HATs therefore act to control the availability of a lysine residue for other covalent modifications (**Yang and Seto 2007**).

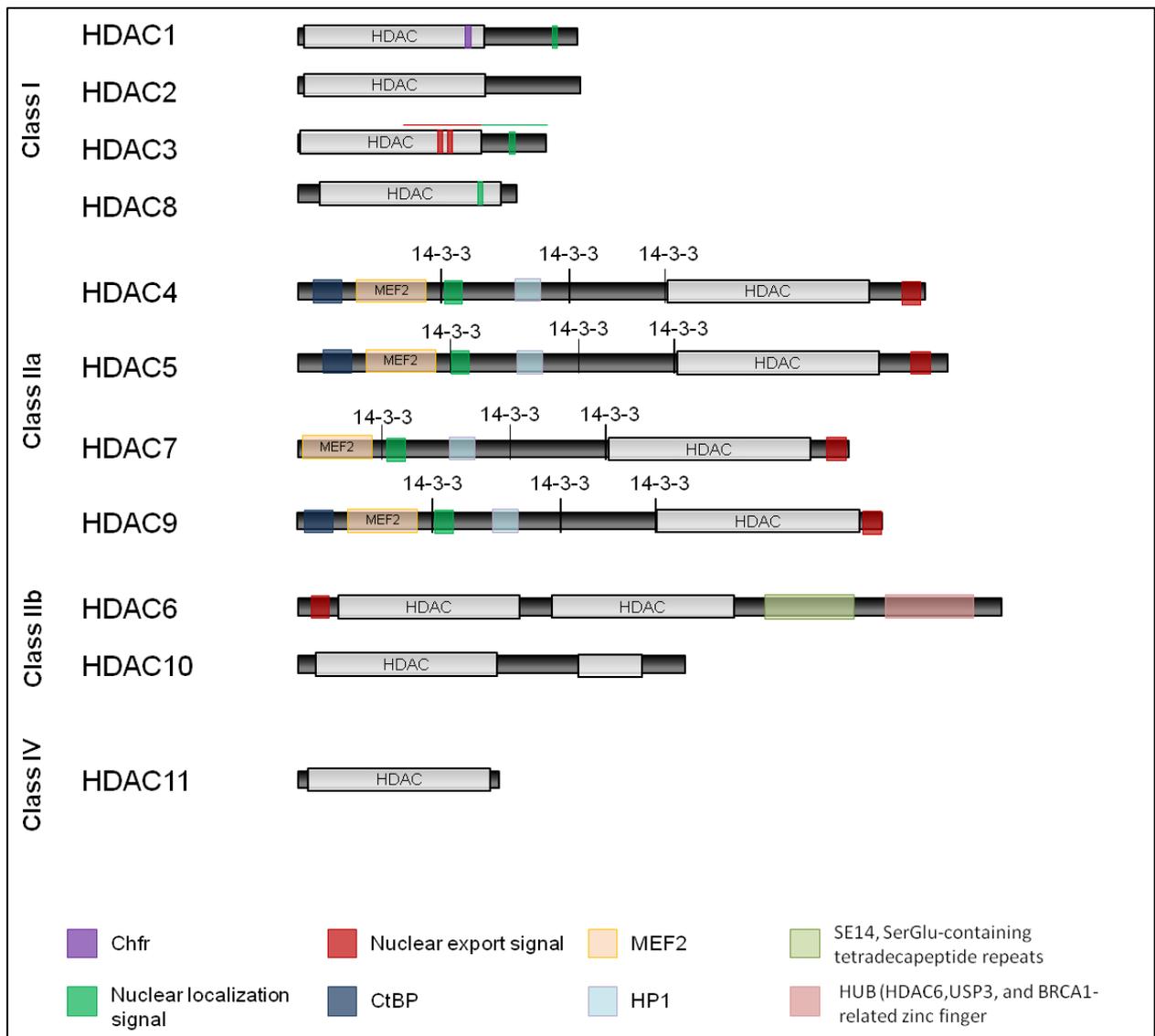
## Histone Acetyltransferases (HAT)

There are several families of HATs. The first family, the general control non-derepressible 5 (Gcn5)-related N-acetyltransferases (GNATs) include GCN5, histone acetyltransferase 1 (HAT1) and p300/CBP-associated factor (PCAF). The p300/CREB binding protein (p300/CBP) family forms the second major family and the MYST proteins founder members (MOZ, Ybf2/Sas3, Sas2 and Tip60) the third. An additional family of transcription factors includes ATF2, TAF1, TFIIC90, and Tip60, all of which have HAT activity, and several HATs remain unclassified. Many other proteins have been reported to possess HAT activity and it is anticipated that additional HATs may remain to be identified (**Bronner et al. 2007; Nagy et al. 2007; Yang and Seto 2007**).

## Histone Deacetylases (HDAC)

The first histone deacetylases (or more correctly lysine deacetylases), were identified in the 1990s. Since histones were identified as the first substrates of these proteins, which catalyse the removal of an acetyl moiety, they were termed histone deacetylases (HDAC) even though some of them do not actually target histones. The first to be cloned was the yeast protein Rpd3 (reduced potassium dependency 3) (**Vidal et al. 1991**). Mammalian HDAC1 was found to be an orthologue of yeast Rpd3 and was cloned independently in 1996 along with the yeast protein Hda1 (**Rundlett et al. 1996; Taunton et al. 1996**). Further proteins including Sir2 (silent information regulator 2) were shown to possess HDAC activity and lead to the classification of eighteen Human HDACs (**Cress et al. 2000; Khochbin et al. 2001; Grozinger et al. 2002; Verdin et al. 2003; Blander et al. 2004; North et al. 2004**).

The HDACs are grouped into four classes within two families, based on sequence similarity and cofactor dependency (**Khochbin et al. 2001; Grozinger et al. 2002; Pandey et al. 2002; Verdin et al. 2003; Gregoretta et al. 2004**). The Classical Family comprises classes I, II and IV, which share sequence similarity and require Zn<sup>2+</sup> for deacetylase activity, while the “Sirtuin” Family (silent information regulator2 (Sir2)-related protein) comprises the class III HDACs, which have no sequence resemblance to members of the classical family and require NAD<sup>+</sup> as the cofactor. Class I consists of HDAC-1, -2, -3, and -8 (**Yang and Seto 2007**) which have ubiquitous expression in many human cell lines and tissues (**Gray et al. 2001**). Class II contains HDAC-4, -5, -6, -7, -9, -10, and can be further subdivided based on sequence homology and domain organisation into Class IIa, consisting of HDAC-4, -5,-7,-9 and Class IIb containing HDAC-6 and -10 (**Yang and Seto 2007**). Class IV consists of HDAC-11, and Class III SIRT1-7 (**Yang and Seto 2007**).



### Figure 3: The classical family of histone deacetylases (Zn<sup>2+</sup> dependent)

The classical HDACs are shown to scale with approximate locations of major domains. The histone deacetylase domain of HDAC1 contains residues essential for interaction with the ubiquitin ligase, Chfr. The class IIa HDACs have domains for interaction with myocyte enhancer factor 2 (MEF2), 14-3-3 proteins, and transcriptional co-repressors such as heterochromatin protein 1 (HP1) and E1A C-terminal binding protein (CtBP). The SE14 domain of HDAC6 is essential to cytoplasmic retention and acetyl-microtubule targeting while the zinc finger motif binds to ubiquitin and possesses ubiquitin ligase activity.

## ***Class I HDACs***

The Class I HDACs, HDAC1, -2, -3, and -8, are yeast Rpd3 homologs, and are composed primarily of a catalytic domain [Figure 3] (Taunton et al. 1996; Yang et al. 1996; Yang et al. 1997; Dangond et al. 1998; Emiliani et al. 1998; Grozinger et al. 1999; Buggy et al. 2000; Hu, Chen et al. 2000; Van den Wyngaert et al. 2000). Class1 HDAC expression is considered to be ubiquitous and primarily nuclear in localization, with Class I HDACs commonly acting in multiprotein complexes (Yang and Seto 2008). Aberrant expression of several class I HDACs in tumour samples has been reported (Halkidou, Gaughan et al. 2004; Zhu et al. 2004; Huang et al. 2005; Song, Noh et al. 2005; Zhang et al. 2005; Wilson et al. 2006; Nakagawa et al. 2007) and class I HDACs are involved in a number of developmental processes.

There is structural homology between the HDACs with a common catalytic mechanism. Despite this functional redundancy between the HDAC isoenzymes is limited in a tissue and stimulus dependent manner, often with different sets of target genes for each HDAC despite common co-repressor complexes (Klampfer et al. 2004; Montgomery et al. 2007; Akhtar et al. 2009; Haberland, Johnson et al. 2009; Haberland, Montgomery et al. 2009; Montgomery et al. 2009). Class I HDACs affect many pathways and HDAC1-3 have been shown to cooperatively affect STAT-dependent signalling (Klampfer et al. 2004; Kramer et al. 2006; Buchwald et al. 2009; Kramer et al. 2009; Spange et al. 2009).

### HDAC1&2:

HDAC1 and HDAC2 appear to have originated from independent gene duplication events in different lineages, also occurring in a common ancestor of all vertebrates (Gregoretti et al. 2004). Human HDAC1 and HDAC2 proteins exhibit 82% sequence similarity suggesting there should be a high degree of functional overlap. Knockout studies in mice however, have revealed a number of distinct and non-redundant biological functions (Brunmeir et al. 2009).

The HDAC1 catalytic domain contains two amino-acid residues essential for the interaction with the ubiquitin ligase, Chfr, which regulates protein degradation (Oh et al. 2009), and HDAC1 also contains a C-terminal nuclear localisation signal (NLS) not found in HDAC2 (Taplick et al. 2001). A C-terminal coiled-coil domain specific to HDAC2 is thought to enable additional protein-protein associations (Gregoretti et al. 2004), while sumoylation of HDAC1 has been suggested to modulate its interaction with

other proteins, though the biological consequences are still subject to debate (**Colombo et al. 2002; David et al. 2002; Gocke et al. 2008**). HDAC1 can be acetylated by p300 resulting in inactivation of its deacetylase activity, and mutations to the critical acetylation target sites abrogate HDAC1 function *in vivo* (**Simone et al. 2004; Qiu et al. 2006**). Interestingly, acetylation of HDAC1 also inhibits the activity of HDAC2 (**Luo et al. 2009**) showing that one deacetylase can trans-regulate the activity of another. HDAC2 is regulated post-translationally by tyrosine nitration and cysteine S-nitrosylation by nitric oxide/reactive nitrogen species (RNS) controlling its release from chromatin, the formation of protein-aldehyde adducts in response to cigarette smoke, or reactive oxygen species (ROS)/aldehydes, serine phosphorylation, the heat shock protein HSP70 and a specific polyubiquitinylation and proteasomal degradation pathway limited to this HDAC (**Kramer et al. 2003; Ito et al. 2004; Marwick et al. 2004; Yang et al. 2006; Colussi et al. 2008; Kee et al. 2008; Nott et al. 2008; Adenuga et al. 2009; Barnes et al. 2009; Brandl et al. 2009; Osoata et al. 2009**). The RNA-binding protein HUR (antigen R of patient HU) which regulates mRNA stability, translation and localization has been shown to bind the 3'-untranslated AU rich region of HDAC2 mRNA suggesting a further means of posttranslational regulation (**Butter et al. 2009**).

Some degree of cross regulation occurs between HDAC1 and HDAC2 with respect to protein levels. In a number of murine tissues and cell lines depletion of either HDAC1 or HDAC2 results in increased protein levels of the other (**Lagger et al. 2002; Zupkovitz et al. 2006; Senese et al. 2007; Yamaguchi et al. 2010**), which appears to be regulated at the (post)translational level as mRNA levels do not appear to be affected (**Zupkovitz et al. 2006; Montgomery et al. 2007; Senese et al. 2007**) and could therefore involve modulation of protein stability.

HDAC1 and 2 exist solely in the nucleus (**Rountree et al. 2000; Taplick et al. 2001**) and are part of some of the same multiprotein complexes, such as Sin3 (**Hassig et al. 1997**) nucleosome remodelling and deacetylase (NuRD) (**Tong et al. 1998**) corepressor of RE1-silencing transcription factor (CoREST) (**Humphrey et al. 2001; You et al. 2001; Hakimi et al. 2002**) and Nanog and Oct4-associated deacetylase (NODE) complex (**Yang and Seto 2008**). As part of these multiprotein complexes, the activity of HDAC1 and HDAC2 has been implicated in the regulation of cell cycle progression (**Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998; Yamaguchi et al. 2010**), differentiation (**Liu, Li et al. 2009; Montgomery et al. 2009**) cellular aging (**Pegoraro et al. 2009**) and cancer (**Ropero et al. 2006**). HDAC1 also forms a complex

with RET finger protein (RFP) and Nuclear transcription factor Y (NF-Y) (**Kato et al. 2009**), regulating the sensitivity of cancer cells to oxidative stress through the repression of thioredoxin binding protein 2 (TBP-2) expression.

While deacetylase activity remains in the absence of either HDAC, deletion of HDAC1, but not of HDAC2 in embryonic stem cells, causes a significant reduction in the HDAC activity of the Sin3A, NuRD, and CoREST co-repressor complexes suggesting HDAC1 is the major catalytic subunit (**Dovey et al. 2010**). Indeed there is a requirement for both HDAC1 and HDAC2 at certain key developmental periods, such as gastrulation. Studies in mice have demonstrated an essential role in embryogenesis for HDAC1 (**Lagger et al. 2002; Montgomery et al. 2007**) and many components of HDAC1/2 complexes, including Sin3A (**Cowley et al. 2005**), SDS3 (**David et al. 2003**), MBD3 (**Hendrich et al. 2001**), and LSD1 (**Wang, Scully et al. 2007; Wang, Hevi et al. 2009**). Germ-line deletion of HDAC1 results in early embryonic lethality while in contrast constitutive HDAC2 knockout mice survive embryogenesis either dying shortly after birth in some models (**Montgomery et al. 2007**) or some surviving to adulthood in other models (**Trivedi et al. 2007; Zimmermann et al. 2007; Guan et al. 2009**). Conditional HDAC1/2 deletions in culture, showed murine embryonic stem cell differentiation but not proliferation is affected by loss of either HDAC1 or HDAC2 (**Dovey et al. 2010**) suggesting it is specific processes that are affected by loss of either homolog.

In contrast, deletion of both HDAC1 and HDAC2 is required in a number of cell types to generate a phenotype (**Montgomery et al. 2007; Haberland, Johnson et al. 2009; Montgomery et al. 2009**) suggesting functional redundancy in many settings, indeed in many cases observed phenotypic effects accompanying loss of either HDAC1 or HDAC2 could originate from a compensatory up-regulation of the other, rather than inactivation of the gene itself. However in oral, prostate, endometrial and gastric cancers high levels of HDAC2 are considered an independent marker of poor prognosis (**Bieliauskas et al. 2008; Chang et al. 2009; Weichert 2009**) suggesting the balance does remain important, and aberrant expression of HDAC2 has also been reported in association with chronically inflammation and dystrophic muscles (**Yoo and Jones 2006; Colussi et al. 2008; Barnes et al. 2009; Buchwald et al. 2009; Hong et al. 2009; Kong et al. 2009**). HDAC2 is also suggested to negatively regulate memory formation and synaptic plasticity (**Guan et al. 2009**).

HDAC1 can regulate a number of pathways and is recruited to the pS2 promoter in the presence of estrogens (**Metivier et al. 2003**). Studies have shown that HDAC1, along

with HDAC4, can interact with ER $\alpha$  and repress its transcriptional activity (**Kawai et al. 2003; Leong et al. 2005; Zhou et al. 2007**). Inhibition of HDAC2 by small interfering RNA (siRNA) is also sufficient to down regulate ER $\alpha$  expression (**Bicaku et al. 2008**).

#### HDAC3:

The HDAC3 subclass exists as a single protein in sequenced organisms from humans to *Drosophila* (**Gregoretto et al. 2004**) and is regarded as distinct from HDAC1 and HDAC2, as it is not found in either Sin3 or NuRD co-repressor complexes (**Knoepfler et al. 1999**). HDAC3 has two reported isoforms—HDAC3A and HDAC3C (**Yang et al. 1997**) differing at the N terminus, and can be found in both the nucleus and cytoplasm. HDAC3A, which has a direct repeat of 101 bp at the 5' end of its cDNA could be generated by posttranscriptional modifications though it could also be an artefact formed during library construction, while HDAC3C either is an alternatively spliced isoform of HDAC3, or it is transcribed from a different transcriptional start site (**Yang et al. 1997**). HDAC3 is part of a multiprotein complex containing SMRT (silencing mediator for retinoid and thyroid receptor) and as part of this complex is essential to the enzymatic activity of class IIa HDACs (**Fischle et al. 2001; Fischle et al. 2002**).

HDAC3 binds to and regulates the human GCMA transcription factor which regulates the expression of syncytin, a placental fusogenic protein mediating trophoblastic fusion (**Yu et al. 2002; Chuang et al. 2006**). The transcriptional coactivator CBP enhances GCMA-mediated transcriptional activation by acting as a transcriptional coactivator and as a HAT, acetylating GCMA to prolong its protein stability (**Chang et al. 2005**). HDAC3 is a key factor in reversing GCMA acetylation thereby regulating trophoblastic fusion in placental morphogenesis (**Chuang et al. 2006**).

#### HDAC8:

HDAC8 appears to have undergone significant functional specialization relative to other class I HDACs having been lost during evolution in some species (**Gregoretto et al. 2004**), and shares greatest homology with HDAC3 (**Buggy et al. 2000**). HDAC8 appears to be restricted to vertebrates (**Gregoretto et al. 2004**) suggesting that it does not play an important role in conserved processes, though it may have developmental or tissue-specific functions consistent with its apparently tissue-specific expression (**de Ruijter et al. 2003**). Initial cloning showed detectable mRNA expression in every tissue tested, with the highest expression in the liver, and Northern blot analysis revealed two transcripts, a more common 1.7kb form and another at 2.4kb particularly prominent in

cancerous cell lines (**Buggy et al. 2000**). However later studies showed that while considered to be a predominantly nuclear and ubiquitous protein, albeit with different expression to HDAC1 and -3 (**Buggy et al. 2000; Hu, Chen et al. 2000; Van den Wyngaert et al. 2000**), in normal human tissues, HDAC8 was in fact exclusively expressed by cells showing smooth muscle differentiation and was mainly detected in their cytosol (**Waltregny et al. 2004**). HDAC8 was identified in visceral and vascular smooth muscle cells, myoepithelial cells, and myofibroblasts with immunocytochemistry strongly suggesting a cytoskeleton-like distribution of the enzyme (**Waltregny et al. 2004**). In human myometrium, specific inhibition of HDAC8 resulted in increased Hsp20 acetylation and inhibition of *ex vivo* contractions, yet there was no change in histone acetylation or global gene expression suggesting little nuclear involvement (**Karolczak-Bayatti et al. 2011**).

### ***Class 2a HDACs***

Class II HDACs are the human homologues of yeast HDA1 and HDA3 (**Fischle et al. 1999; Grozinger et al. 1999; Miska et al. 1999; Verdell et al. 1999; Wang et al. 1999; Kao et al. 2000**). Class IIa consists of HDAC4, -5, -7 and -9, which mainly function as transcriptional co-repressors, possessing other repression domains in addition to their deacetylase domains (**Sparrow et al. 1999; Wang et al. 1999; Kao et al. 2000; Lemercier et al. 2000; Zhou, Richon, Rifkind et al. 2000**). Class IIa HDACS also associate with heterochromatin protein 1 (HP1), which recruits histone methyltransferases thereby coupling histone deacetylation and methylation to repress transcription (**Zhang, McKinsey and Olson 2002**).

Class IIa mammalian HDACs contain a long noncatalytic N terminus and a C-terminal HDAC catalytic domain. The extended long N-terminal domains show sequence similarity and is the hallmark of this subfamily, possessing conserved amino acid motifs specialized for binding an array of proteins, and therefore important for Class IIa HDAC function and regulation (**Fischle et al. 1999; Grozinger et al. 1999; Miska et al. 1999; Verdell et al. 1999; Wang et al. 1999; Kao et al. 2000; Yang et al. 2005; Martin et al. 2007**). This N-terminal adaptor region contains intrinsic nuclear import and export signals for nuclear-cytoplasmic trafficking such as a canonical nuclear localization signal, and controls their sub cellular localization (**McKinsey, Zhang, Lu et al. 2000; McKinsey et al. 2001; Wang et al. 2001**).

Class IIa mammalian HDACs are present in both the nucleus and the cytoplasm (**Miska et al. 1999; Grozinger et al. 2000; McKinsey, Zhang, Lu et al. 2000; Wang et al. 2000; Kao et al. 2001; Dequiedt et al. 2003; Petrie et al. 2003**). They are subject to CRM1-dependent nuclear export and a range of mechanisms promote the sub-cellular distribution of class IIa HDACs and their associated activity (**Fischle et al. 1999; Miska et al. 1999; Grozinger et al. 2000; McKinsey, Zhang, Lu et al. 2000; Wang et al. 2000; Kao et al. 2001**).

Binding partners shown to promote nuclear retention include myocyte enhancer factor 2 (MEF2) and HIF-1 $\alpha$  (**Miska et al. 1999; Verdel et al. 1999; Wang et al. 1999; Lu, McKinsey, Nicol et al. 2000; Borghi et al. 2001; Kao et al. 2001; Wang et al. 2001; Chan et al. 2003; Kato et al. 2004; Martin et al. 2007**). By contrast, 14-3-3 proteins stimulate cytoplasmic retention (**Grozinger et al. 2000; McKinsey, Zhang and Olson 2000; Wang et al. 2000; Kao et al. 2001; McKinsey et al. 2001**), and this interaction is dependent on site specific phosphorylation. One of the major 14-3-3 binding sites overlaps with the Nuclear localization signal thereby impeding importin  $\alpha/\beta$  access (**Grozinger et al. 2000**). 14-3-3 binding is also thought to act in *trans* as a nuclear export signal, and 14-3-3 binding is sufficient to affect the sub-nuclear localization of class IIa HDACs (**McKinsey et al. 2001**) though association with 14-3-3 could in fact be dispensable for CRM1-mediated nuclear export of these HDACs (**Gao et al. 2006**). There have been indications that it may be released from their targeting DNA-binding partners, rather than exit from the nucleus which is the crucial step in the relief of class IIa HDAC transcriptional repression by phosphorylation (**Lu, McKinsey, Nicol et al. 2000; McKinsey, Zhang, Lu et al. 2000**) with 14-3-3-dependent nuclear export of class IIa HDACs only serving as a supporting mechanism ensuring maximal activation of target genes (**McKinsey, Zhang, Lu et al. 2000**).

Phosphorylation of class IIa mammalian HDACs is mediated by Ca<sup>2+</sup>/calmodulin-dependent protein kinases (CaMKs) I, II, and IV (**Grozinger et al. 2000; Lu, McKinsey, Zhang et al. 2000; McKinsey, Zhang, Lu et al. 2000; Kao et al. 2001; Chawla et al. 2003; Davis et al. 2003; Linseman et al. 2003; Bacs et al. 2006; Karamboulas et al. 2006; Bossuyt et al. 2008**) along with other unidentified kinases (**Bertos et al. 2001; Zhao et al. 2001; Zhang, McKinsey, Chang et al. 2002**). HDAC4 can be phosphorylated by extracellular signal-regulated kinase 1/2 in a process stimulated by oncogenic Ras, which then promotes nuclear localisation (**Zhou, Richon, Wang et al. 2000**). Phosphorylation occurs at a number of serine residues distinct from the 14-3-3

binding sites **(Zhou, Richon, Wang et al. 2000; Deng et al. 2005; Paroni et al. 2008)**, with HDAC4, -5 and -9 phosphorylated at a conserved serine residue not present in HDAC7, by Dyrk1B **(Deng et al. 2005)**. In addition to phosphorylation, HDAC4, -5, and -9 are also subject to ubiquitylation **(Hook et al. 2002; Li et al. 2004)** and sumoylation **(Tatham et al. 2001; Kirsh et al. 2002; Petrie et al. 2003)** -though these modifications do not affect the sub cellular localization **(Tatham et al. 2001; Hook et al. 2002; Kirsh et al. 2002; Petrie et al. 2003)**, and proteolytic cleavage **(Bakin et al. 2004; Li et al. 2004; Paroni et al. 2004; Scott et al. 2008)**.

It is likely that other distinct kinases may differentially regulate the sub cellular localization of class IIa members. One recently identified was protein kinase D (PKD), a downstream effector of PKC which was shown to phosphorylate the 14-3-3 binding sites of class IIa HDACs and neutralize their repressive activity **(Vega, Harrison et al. 2004; Dequiedt et al. 2005; Parra et al. 2005; Matthews et al. 2006)**. Interestingly PKD activation is also necessary for inactivation of class IIa HDACs during T-cell apoptosis **(Dequiedt et al. 2005; Parra et al. 2005)**, cardiac hypertrophy **(Vega, Harrison et al. 2004; Bossuyt et al. 2008)**, B-cell receptor signalling **(Matthews et al. 2006)**, skeletal and cardiac muscle remodelling **(Fielitz et al. 2008; Kim, Fielitz et al. 2008)** and angiogenesis **(Ha et al. 2008; Wang et al. 2008)**.

Despite shared regulatory schemes, class IIa members display distinct sub cellular localizations **(Bertos et al. 2001)**. For example, HDAC4 is mainly cytoplasmic in undifferentiated myoblasts, with accumulation in the nucleus upon differentiation into myotubes **(Miska et al. 2001)**. In contrast, HDAC5 and HDAC7 which were located in the nucleus relocate to the cytoplasm during differentiation into myotubes **(McKinsey, Zhang, Lu et al. 2000; Dressel et al. 2001)**. In another example, hypoxia induces the nuclear import of HDAC7, but not HDAC4 or HDAC5 **(Kato et al. 2004)**. In addition, the sub cellular localization of class IIa HDACs is known to be affected by serum starvation **(Dressel et al. 2001)**, membrane depolarization **(Chawla et al. 2003; Linseman et al. 2003)**, and other stimuli **(Kao et al. 2003; Chang et al. 2004; Halkidou, Cook et al. 2004; Harrison et al. 2004)**.

The consensus is that regulation of class IIa members relies on their ability to shuttle between the nucleus and the cytoplasm, in response to specific extracellular signals **(Martin et al. 2007)**. Class IIa HDACs in the nucleus can partner with transcription factors and corepressors to inhibit transcription, while cytoplasmic accumulation of class IIa HDACs makes them unable to impact on transcription, as they are sequestered

away from histones rendering them enzymatically inactive as histone deacetylases (**Fischle et al. 2001; Fischle et al. 2002**). However while Class IIa HDACs are thought to deacetylate nucleosomal histones in the vicinity of target promoters- thereby acting as transcriptional corepressors, it is notorious that compared to class I or class IIb, class IIa HDACs are actually very inefficient histone deacetylases (**Hassig et al. 1998; Fischle et al. 1999; Hu, Chen et al. 2000; Fischle et al. 2002**). Indeed, in place of a catalytic tyrosine residue that is strictly conserved in other group I enzymes, class IIa HDACs have a histidine that compromises their activity towards acetyl-lysines (**Lahm et al. 2007**). This has raised the possibility that class IIa HDACs might in fact not target acetylated proteins *in vivo*, but still-to-be-discovered substrates (**Martin et al. 2009**).

It is known that class IIa HDACs can repress transcription independently of their C-terminal catalytic domain; therefore at least part of their repressive properties need not rely on deacetylation of proximal histones (**Sparrow et al. 1999; Wang et al. 1999; Kao et al. 2000; Lemerrier et al. 2000; Zhou, Richon, Rifkind et al. 2000**). Not binding DNA directly, the repressive activity of class IIa HDACs relies on interactions with sequence-specific DNA binding proteins, which would then dictate the targeting specificity of class IIa HDACs (**Martin et al. 2009**). Indeed, motifs in the N-terminal region of class IIa HDACs can act to recruit co-repressors, such as HP1 or CtBP (**McKinsey et al. 2001; Zhang et al. 2001; Zhang, McKinsey, Chang et al. 2002**). Class IIa HDACs also interact with a range of other proteins (**Yang et al. 2005**) including Calmodulin (**Youn et al. 2000; Berger et al. 2003**), Serum response factor (SRF) (**Davis et al. 2003**), Myocardin (**Cao et al. 2005**), a range of transcription factors [Runx2 and -3 (**Jin et al. 2004; Vega, Matsuda et al. 2004**), (**Ducy et al. 2000; Ito 2004**), TEL (ETV6) (**Petrie et al. 2003**), BCL6 (**Lemerrier et al. 2002**), PLZF (**Lemerrier et al. 2002; Chauchereau et al. 2004**), CREB2 (**Guan et al. 2002**), GATA-1 (**Watanabe et al. 2003**)], and corepressors B-CoR (**Huynh et al. 2000**), N-CoR (**Huang et al. 2000; Kao et al. 2000**), SMRT (**Huang et al. 2000; Kao et al. 2000**), ARR19 (**Jeong et al. 2004**), RIP140 (**Castet et al. 2004**). Class IIa HDACs can also modulate the transcriptional activity of their partners, regulating specific posttranscriptional modifications, such as sumoylation (**Gregoire et al. 2005; Zhao et al. 2005; Gregoire et al. 2006**) or ubiquitylation (**Jin et al. 2004; Jeon et al. 2006**).

## HDAC4

The deacetylase activity of HDAC4 and other class IIa HDACs has been reported to be dependent on associated complexes containing HDAC3 and SMRT (**Fischle et al. 2001; Fischle et al. 2002**), however recent studies suggest class IIa HDACs do possess weak deacetylase activity (**Lahm et al. 2007; Schuetz et al. 2008**). In addition sumoylation of HDAC4 was found to be required for its deacetylase activity (**Kirsh et al. 2002**). Other actions of HDAC4 are likely to be mediated through binding partners, such as the physical association of HDAC4 with Runx2, which inhibits its transcriptional activities (**Kang et al. 2005**). HDAC4 also binds to 53BP1, a p53 binding protein that regulates cell cycling in response to DNA damage and may thereby regulate DNA repair (**Kao et al. 2003; Berns et al. 2004**). HDAC4 along with the class IIb HDAC; HDAC6, has also been linked with HIF-1 $\alpha$  stability and are thought to regulate the acetylation level of the HIF-1 $\alpha$  protein (**Qian et al. 2006**). HDAC4 can also interact with the N-terminus of ER $\alpha$ , stimulating its binding to estrogen-responsive gene promoters and leading to suppression of ER $\alpha$  transcription (**Leong et al. 2005**). HDAC4 also contributes to the transcriptional induction of mPGES-1, which catalyzes the terminal step in the biosynthesis of prostaglandin E2 (**Chabane et al. 2009**).

HDAC4 oligomerizes with HDAC5 and this may account for its unique sensitivity to CaMKII-mediated phosphorylation and nuclear export (**Backs et al. 2006**). Hetero-oligomerization with HDAC4 brings HDAC5 in close proximity of CaMKII, allowing its phosphorylation by the kinase and promoting its nuclear export (**Backs et al. 2008**). In addition to regulation by 14-3-3 proteins, the intracellular localization of HDAC4 is regulated by oncogenic Ras (**Zhou, Richon, Rifkind et al. 2000**) and shows distinct localization compared to other class IIa HDACs during muscle differentiation (**McKinsey, Zhang, Lu et al. 2000; Miska et al. 2001**). Over expression of HDAC4 or inhibition of CaMKII during cardiomyogenesis inhibits the transition of mesoderm to cardiomyoblast (**Karamboulas et al. 2006**), and a high abundance of HDAC4 and -5 suggest additional roles in neurons (**Grozinger et al. 1999**). Expression of HDAC4 can be regulated at the post-transcriptional level by microRNAs (miRNA). During skeletal muscle differentiation, miR-1 promotes myogenesis by targeting HDAC4 (**Chen, Mandel et al. 2006**) and miR140, a cartilage specific miRNA, can bind to HDAC4 mRNA and interfere with its translation (**Tuddenham et al. 2006**). In light of this level of regulation it is not surprising that HDAC4-null mice display skeletal defects (**Vega, Matsuda et al. 2004**). Further regulation is achieved by Caspase 3 cleavage of HDAC4,

which promotes the nuclear localization of the N-terminal fragment (**Liu et al. 2004; Paroni et al. 2004**) which may lead to further actions of HDAC4.

### HDAC5

Like HDAC4, HDAC5 mRNA are highly abundant in the brain (**Grozinger et al. 1999**) with HDAC4 and -5 predominantly cytoplasmic in cerebellar granule neurons (CGNs) cultured under depolarizing conditions. Switching CGNs to non-depolarizing medium induces cytoplasm-to-nucleus translocation of both HDAC4 and -5, and expression of constitutively nuclear mutants correlates with apoptosis -exemplifying the importance of cellular localisation (**Linseman et al. 2003; Bolger et al. 2005**).

HDAC5 activity has been shown to be affected by fluid shear stress in endothelial cells, with fluid shear stress stimulating the phosphorylation of HDAC5, which affects its interactions with the MEF2 transcription factor by promoting its nuclear export (**Wang et al.**). This then leads to subsequent effects on the activity of transcription factor KLF2 and the expression of endothelial nitric oxide synthase (eNOS) (**Wang et al.**). Interestingly, HDAC5 also has effects on MEF2 transcription factor activity in response to cytokines however this involves p65 and NF- $\kappa$ B, rather than phosphorylation (**Kumar et al. 2005**).

HDAC5 is involved in inducing apoptosis (**Huang et al. 2002**) and plays important roles in regulating muscle differentiation. HDAC5 targets myocardin, a transcriptional coactivator important for smooth muscle differentiation (**Cao et al. 2005**), and inactivation of murine HDAC5 and -9 genes leads to cardiac hypertrophy (**Zhang, McKinsey, Chang et al. 2002; Chang et al. 2004; McKinsey et al. 2004**).

### HDAC7

Several alternatively spliced isoforms exist for HDAC7 though the functional significance of these splicing isoforms is as yet unknown and it is likely that different isoforms may have different functions in different tissues (**Zhou et al. 2001**). HDAC7 exists in the nucleus and cytoplasm, but also localizes to mitochondria and may regulate the initiation of apoptosis (**Amerik et al. 2000; Bakin et al. 2004**). Likewise, the protein kinase D1 (PKD1,) which phosphorylates HDAC5 and HDAC7 at their 14-3-3 binding sites (**Vega, Harrison et al. 2004; Parra et al. 2005**), also localises to mitochondria (**Bakin et al. 2004**). In addition to promoting nuclear export, 14-3-3 association was shown to protect HDAC7 from proteosomal degradation (**Li et al. 2004; Martin et al. 2007**), which may contribute to its cytoplasmic accumulation. Experimental design and

culture conditions such as cell confluence, culture media, the presence or absence of growth factors (e.g., VEGF, vascular endothelial cell growth factor) can all influence the localization of HDAC7 (**Margariti et al. 2010**). Like HDAC4, the deacetylase activity of HDAC7 had been reported to be dependent on associated HDAC3 complexes (**Fischle et al. 2001; Fischle et al. 2002**). However characterisation of the catalytic unit of HDAC7 has shown it is capable of independent histone deacetylase activity albeit at a low level, which can be inhibited by HDACis (**Schuetz et al. 2008**). The catalytic domain of HDAC7 has a novel zinc binding motif adjacent to the active site, which is thought to participate in substrate recognition and protein-protein interactions, and which is conserved in class IIa HDACs (**Schuetz et al. 2008**). Variant active site topology in the HDAC7 catalytic domain has also been identified, which results in altered catalytic properties and in an enlarged active site pocket (**Schuetz et al. 2008**). Interestingly the HDACi vorinostat acts to selectively suppress HDAC7, resulting in a down-regulation of HDAC7 protein and an associated down-regulation of HDAC7 mRNA -with no alteration in the half-life of HDAC7 protein (**Dokmanovic, Perez et al. 2007**).

In the human, HDAC7 is highly expressed in thymus, heart and lung. HDAC7 is transiently and predominantly expressed in CD4/CD8 DP thymocytes within the thymus (**Dequiedt et al. 2003**), associating with MEF2D and repressing expression of a series of MEF2-target genes, such as the pro-apoptotic orphan nuclear receptor Nur77 (**Dequiedt et al. 2003; Kasler et al. 2007**). Mice lacking HDAC7 die during mid-gestation from cardiovascular defects (**Vega, Matsuda et al. 2004**) which is thought to largely be due to the importance of HDAC7-MEF2 interactions during development. HDAC7 has also been identified as a negative regulator of osteoblast differentiation through its Runx2 corepressor function (**Jensen et al. 2008**) and has been reported to associate with HIF-1 $\alpha$  and enhance its nuclear localization and transcriptional activity (**Kato et al. 2004**).

HDAC7 is expressed in the vascular endothelium during early embryogenesis where it maintains vascular integrity (**Chang et al. 2006**). Silencing of HDAC7 in endothelial cells alters their morphology and motility and prevents their assembly into tube-like structures (**Mottet et al. 2007**). HDAC7 is critical during angiogenesis, regulating the expression of several genes encoding extracellular matrix and adhesion proteins, including membrane type 1 metalloprotease and the secreted matrix metalloproteinase 10 (MMP-10) (**Ha et al. 2008**). HDAC7 regulation of MMP-10 expression depends on its association with MEF2C, a factor previously implicated in blood vessel development

and vascular integrity (**Lin et al. 1998**). A recent study found that HDAC7 bridges 14-3-3 $\epsilon$ ,  $\zeta$ ,  $\eta$  and  $\beta$ -catenin and stabilizes  $\beta$ -catenin in the cytoplasm of endothelial cells (EC) (**Margariti et al. 2010**). This resulted in inhibition of EC growth and lead to the elongation of G1 phase. It had been previously determined that phosphorylation sites in HDAC7 are critical for VEGF induced EC proliferation (**Ha et al. 2008; Wang et al. 2008**) and that VEGF induces HDAC, and in particular HDAC7 phosphorylation. VEGF-induced degradation of HDAC7 was found to disrupt the HDAC7-  $\beta$ -catenin-14-3-3 complex resulting in nuclear translocation of  $\beta$ -catenin to the nucleus and induction of EC growth (**Margariti et al. 2010**).

HDAC7 also appears to be involved in regulating estrogen-mediated transcription. HDAC7 has been reported to associated with the pS2 promoter in the presence of estrogen, a phenomenon also reported with the class I HDAC, HDAC1, and appears to act redundantly (**Metivier et al. 2003**). Previous studies have shown that HDACs, such as HDAC1 and HDAC4, can interact with estrogen receptor  $\alpha$  (ER $\alpha$ ) and repress its transcriptional activity (**Kawai et al. 2003; Leong et al. 2005**) and a recent study showed that HDAC7 can also bind to ER $\alpha$  and mediate repression- independently of its deacetylase activity (**Malik et al. 2010**). Estrogen dependent recruitment of HDAC7, ER $\alpha$ , and FOXA1 to promoters of Reprimo (RPRM) gene- which is associated with cell cycle inhibition and tumour suppression. Class IIa HDACs are also recruited by various other ER $\alpha$ -associated corepressors, including SMRT, NRIP1, LCoR, MTA1, REA (Prohibitin), and SAFB1/2 (**Huang et al. 2000; Wei et al. 2001; Fischle et al. 2002; Fernandes et al. 2003; Kurtev et al. 2004**).

### HDAC9

The cloning and characterization of HDAC9 was reported in 2001 with at least six different proteins possible due to alternate splicing (**Zhou et al. 2001**). HDAC9 and HDAC9a, which has a shorter C terminal end, act as repressors, possess deacetylase activity and are sensitive to inhibition by TSA and suberoylanilide hydroxamic acid. Another isoform had been previously identified and designated histone deacetylase 4 and 5 related protein (HDRP) - being 50% identical to the N-terminal domain of HDAC4 and -5 (**Zhou, Richon, Rifkind et al. 2000**) or MEF2-interacting transcriptional repressor (MITR) (**Sparrow et al. 1999; McKinsey et al. 2001; Zhang et al. 2001**). HDRP lacks the C-terminal domain and deacetylase activity, but functions as a transcriptional repressor (**Zhou et al. 2001**). Another three isoforms represent each of HDAC9, HDAC9a and HDRP but lacking a nuclear localisation signal, and are thereby

constitutively cytoplasmic (**Zhou et al. 2001; Petrie et al. 2003**). Intracellular localization of HDAC9 is regulated by 14-3-3 and calmodulin-dependent kinase (**McKinsey et al. 2001**). The ratio of HDAC9 and HDAC9a transcripts varies among tissues and combined with different localization due to splicing variation, could result in tissue specific function.

Like other Class IIa HDACs, HDAC9 and HDAC9a interact with MEF2 and repress MEF2-mediated transcription and inactivation of the mouse HDAC5 and HDAC9 genes leads to cardiac hypertrophy (**Zhang, McKinsey, Chang et al. 2002; Chang et al. 2004; McKinsey et al. 2004**). In addition HDAC9 expression is up regulated in senescent fibroblasts (**Mason et al. 2004**).

### ***Class 2b HDACs***

The Class IIb HDACs, HDAC6 and HDAC10, are distinct from Class IIa in several aspects. They possess a second (pseudo) catalytic domain, and are also resistant to the inhibitory effects of trapoxin B and butyrate (**Guardiola et al. 2002; Gurvich et al. 2004**). While both proteins interact with the major cellular phosphatase PP1, and could therefore be involved in the same regulatory networks (**Brush et al. 2004**) there is little functional overlap between these two HDACs (**Guardiola et al. 2002; Matsuyama et al. 2002**).

#### *HDAC6*

HDAC6 was first discovered in the mouse (**Verdel et al. 1999**) and is classified as a member of the class II HDAC (**Yang et al. 2005; Dokmanovic, Clarke et al. 2007**). In contrast to class IIa members, HDAC6 possesses two deacetylase domains and a c-terminal zinc finger motif (**Grozinger et al. 1999; Verdel et al. 1999; Khochbin et al. 2001; Seigneurin-Berny et al. 2001; Boyault et al. 2007; Pandey et al. 2007**). This motif has been given several different names, reflecting its functions. The central part of this motif is similar to regions found in BRAP2 (BRCA1-associated protein 2) and several ubiquitin-specific proteases (USPs). It has been shown that this motif binds to ubiquitin (**Seigneurin-Berny et al. 2001; Hook et al. 2002**) and possesses E3 ligase activity (**Kovacs et al. 2004**), suggesting involvement in ubiquitin-dependent pathways. It has been named the DAUP (deacetylase-ubiquitin-specific protease) domain (**Amerik et al. 2000**), the HUB (HDAC6, USP3, and BRCA1-related) finger (**Bertos et al. 2001**), Zn-UBP (ubiquitin carboxyl-terminal hydrolase like zinc finger) (**Seigneurin-Berny et al.**

**2001**), PAZ (polyubiquitin-associated zinc finger) (**Hook et al. 2002**) and BUZ (bound to ubiquitin zinc finger) (**Kawaguchi et al. 2003**). HDAC6 has been shown to tightly associate with p97, an ATPase known to be involved in endoplasmic reticulum-associated proteosomal degradation (**Seigneurin-Berny et al. 2001**). HDAC6 also directly regulates aggresome formation and cellular management of misfolded proteins, binding polyubiquitinated proteins and regulating their processing and interaction with chaperones (**Hook et al. 2002; Kawaguchi et al. 2003; Boyault et al. 2006**) and requires both its deacetylase and ubiquitin-binding activities to do so. HDAC6 is also present in Lewy bodies associated with neurodegenerative disorders, such as Parkinson's disease (**Kawaguchi et al. 2003**).

HDAC6 was the first HDAC identified as actively maintained in the cytoplasm (**Verdel et al. 2000**) with human HDAC6 harbouring a unique SE14, repeat domain which is pivotal for cytoplasmic retention and highly conserved only in primates (**Bertos et al. 2004**). Under specific circumstances other HDACs can also be found in the cytoplasm, however, only the class III HDAC, SIRT2 (**North et al. 2003**) appears to share any of the HDAC6 activities (**Hubbert et al. 2002; Matsuyama et al. 2002**).

Initially it was believed that HDAC6 localized exclusively to the cytoplasm (**Verdel et al. 2000; Barlow et al. 2001; Bertos et al. 2004**) suggesting histone was not the best physiological substrate (**Zhao et al.**) However a portion of HDAC6 can be nuclear in some cells. In the murine B16 melanoma line, transfer of a portion of HDAC6 from the cytoplasm to the nucleus accompanied butyrate-induced differentiation and cell cycle arrest (**Verdel et al. 2000**) and murine HDAC6 possesses intrinsic nuclear import and export signals for active nucleocytoplasmic trafficking (**Verdel et al. 2000; Bertos et al. 2004**). Experiments performed in breast cancer cells have revealed that HDAC6 is an estrogen target gene (**Yoshida et al. 2004; Zhang et al. 2004; Saji et al. 2005**) and that HDAC6 protein is present in the nuclei of normal breast epithelial cells but is cytoplasmic in adjacent malignant cells (**Yoshida 2004; Yoshida et al. 2004; Zhang et al. 2004**). In breast cancer, HDAC6 expression levels also correlated with better prognosis and response to endocrine therapy (**Yoshida et al. 2004; Zhang et al. 2004; Saji et al. 2005**). As well as being an estrogen target, gene, HDAC6 is also capable of direct interaction with ER $\alpha$  in the cytoplasm, facilitating the non-genomic action of estrogens (**Azuma et al. 2009**), and inhibition of HDAC6 depletes ER $\alpha$  and down regulates estrogen-induced gene transcription (**Saji et al. 2005**).

HDAC6 interacts with several nuclear proteins, including HDAC11 (**Gao, Cueto et al. 2002**), sumoylated p300 (**Girdwood et al. 2003**), transcriptional corepressors such as ETO2 and L-CoR (**Amann et al. 2001; Fernandes et al. 2003; Palijan et al. 2009**), and sequence specific transcription factors such as NF- $\kappa$ B (**Zhang and Kone 2002**) and Runx2 (**Westendorf et al. 2002**), so the sub cellular localization of HDAC6 is likely to be carefully regulated. The deacetylase activity of HDAC6 can be negatively regulated through acetylation by p300 -relieving both HDAC6-mediated down-regulation of p21 transcriptional activity and the suppression of Sp1 transcriptional activity by HDAC6 (**Han et al. 2009**).

The deacetylase domains of HDAC6 act to deacetylate  $\alpha$ -tubulin (**Hubbert et al. 2002; Matsuyama et al. 2002; Haggarty et al. 2003; North et al. 2003; Palazzo et al. 2003; Zhang et al. 2003**) which stimulates cell motility (**Hubbert et al. 2002; Matsuyama et al. 2002; Haggarty et al. 2003; North et al. 2003; Palazzo et al. 2003; Zhang et al. 2003**). HDAC6 and SIRT2 (the other tubulin deacetylase) can form a tubulin-binding complex (**North et al. 2003; Nahhas et al. 2007; Zhao et al. 2010**), and recent studies have excluded the requirement of adaptor proteins between HDAC6 and tubulin (**Zhao et al. 2010**). SIRT2 however may require HDAC6 for effective interaction with tubulin (**Nahhas et al. 2007**) though as HDAC6 knock-out mice are viable and exhibit hyperacetylated tubulin in most tissues while demonstrating apparently normal development (**Zhang, Kwon et al. 2008**). It is possible that SIRT2 and other HDACs can substitute for some of the cytoplasmic and nuclear functions of HDAC6.

Another substrate of HDAC6 mediated deacetylation is the heat-shock protein Hsp90. HDAC6 interacts with Hsp90, regulating its acetylation and thus the stability of the Hsp90 chaperone complex (**Kovacs et al. 2005; Murphy, Morishima et al. 2005**). HDAC6 inactivation leads to the hyperacetylation of Hsp90, loss of chaperone activity and dissociation from the essential co-chaperone p23. This results in the accumulation of inactive glucocorticoid receptor defective in hormone binding. Inhibition of HDAC6 also enhances Hsp90 mediated maturation of matrix metalloproteinase-2, which in an *in vitro* model was associated with increased breast cancer cell invasion (**Verdel et al. 2003**). Importantly, as a regulator of Hsp90 chaperone functions, HDAC6 may have an indirect impact of the transcriptional activities of various factors (**Kovacs et al. 2005; Murphy, Morishima et al. 2005; Hurst et al. 2006; Kong et al. 2006**).

Further actions of HDAC6 continue to be identified, for example HDAC6 is thought to contribute to the activation of *c-jun* expression (**Hazzalin et al. 2005**), appears to

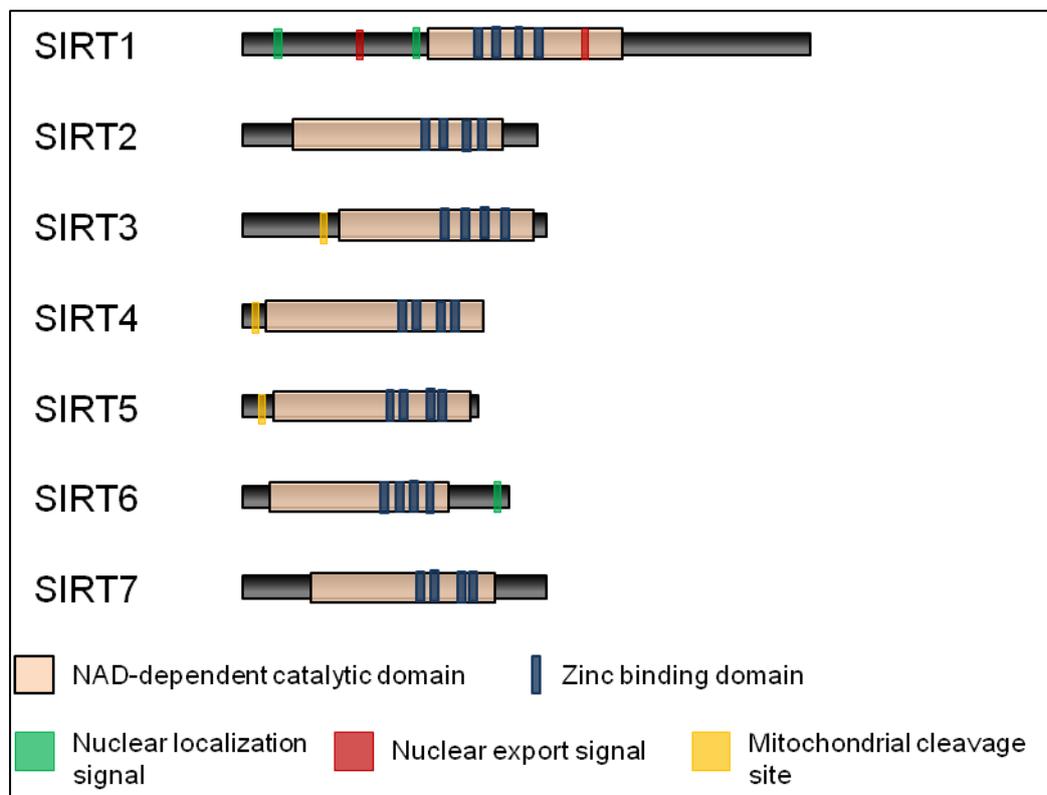
stabilise HIF-1 $\alpha$  (Qian et al. 2006), and deacetylates cortactin (Zhang, Yuan et al. 2007). In addition HDAC6 interacts with p150glued/dynactin, an adaptor protein for dyneins and kinesins (Hubbert et al. 2002; Kawaguchi et al. 2003) and Phospholipase A2 activation protein (PLAP) which controls prostaglandin levels and phospholipase activity (Seigneurin-Berny et al. 2001). Cellular levels of HDAC6 are critical for lymphocyte chemotaxis (not its catalytic activity) (Cabrero et al. 2006) and treatment with a specific HDAC6 inhibitor, has been shown to significantly reduce the exocytosis of IL-1 $\beta$ -containing secretory lysosomes (Carta et al. 2006).

### HDAC10

Discovered in 2002, HDAC10 is the closest relative of HDAC6 (Fischer et al. 2002; Guardiola et al. 2002; Kao et al. 2002; Tong 2002). The HDAC10 catalytic domain has the highest homology with the first HDAC6 catalytic domain while a C-terminal leucine rich region shows limited sequence similarity to the second deacetylase domain of HDAC6 a pseudo-duplication (Fischer et al. 2002; Guardiola et al. 2002; Kao et al. 2002; Tong et al. 2002). HDAC10 is widely expressed and localizes to both the nucleus and the cytoplasm (Fischer et al. 2002; Guardiola et al. 2002; Kao et al. 2002; Tong et al. 2002). HDAC10 function remains elusive however it has been linked to the regulation of the cleavage factor CFIm25 and poly-A polymerase which are involved in pre-mRNA 30-end processing (Shimazu et al. 2007). Like HDAC6, HDAC10 also appears to associate with molecular chaperones, with a recent study identifying HDAC10 in a stable complex with deacetylated heat shock protein 70 (Hsp70) (Lai et al. 2010). HDAC10 also physically associated with, and decreased the repressional activity of two transcriptional regulators, paired box protein 3 (Pax3) and KRAB-associated protein 1 (KAP1), maintaining them in a deacetylated state (Lai et al. 2010).

### **Class 3 HDACs**

The Class III HDACs are members of the Sir2 family and are nicotinamide-adenine dinucleotide (NAD)-dependent deacetylases (Imai et al. 2000; Landry, Sutton et al. 2000; Smith et al. 2000; Grozinger et al. 2002). SIRT1 and SIRT2 can be found both in the nucleus and in the cytoplasm, in a cell and tissue-dependent manner, SIRT3, SIRT4 and SIRT5 are located in the mitochondria, and SIRT6 and SIRT7 are nuclear proteins (Haigis and Guarente 2006).



**Figure 4: The non-classical histone deacetylases: the NAD-dependent sirtuins**

### SIRT1

SIRT1 is predominately distributed in nucleus (**Vaziri et al. 2001; Michan et al. 2007**) and interacts with a number of regulator proteins including p53 (**Luo et al. 2001; Vaziri et al. 2001; Langley et al. 2002**), ku70 (**Cohen et al. 2004**), Tat (**Pagans et al. 2005**), TIP60 (**Wang et al. 2010**), forkhead transcription factor FOXO1 (**Nakae et al. 2006; Potente et al. 2007**), PPAR- $\gamma$  (**Picard et al. 2004**) and p300 (**Bouras et al. 2005**). It is the most well studied sirtuin.

SIRT1 physically associates with, and directly deacetylates p65, suppressing NF- $\kappa$ B transcriptional activity (**Yeung et al. 2004; Chen et al. 2005**), and also regulates ER $\alpha$  expression through basal transcriptional complexes at the ER $\alpha$  promoter (**Yao et al. 2010**). Chemical inhibition of SIRT1 activity has been shown to cause hyperacetylation of NF- $\kappa$ B, leading to enhancement of its activity as well as increased histone acetylation at the NF- $\kappa$ B binding site on the MMP9 promoter (**Nakamaru et al. 2009**).

SIRT1 is involved in many important physiological and pathologic processes (**Imai et al. 2010**) such as apoptosis (**Luo et al. 2001; Cohen et al. 2004**), angiogenesis (**Potente**

et al. 2007), stress resistance (**Brunet et al. 2004**) cell survival, inflammation (**Nayagam et al. 2006**) HIV-1 replication (**Pagans et al. 2005**), cell and tissue differentiation (**Anastasiou et al. 2006**) and energy metabolism (**Picard et al. 2004; Hallows et al. 2006; Gerhart-Hines et al. 2007**).

SIRT1 expression has been reported to be up-regulated by caloric restriction (**Cohen et al. 2004; Crujeiras et al. 2008; Schwer et al. 2008**), (**Nemoto et al. 2004**), and is also responsive to oxidative stress. Oxidative stress has been shown to cause dissociation of SIRT1 mRNA-HuR complexes, resulting in a reduced SIRT1 mRNA half-life and correlating with reduction of both SIRT1 mRNA levels and cell survival (**Abdelmohsen et al. 2007**). Oxidative stress has also been shown to decrease SIRT1 activity without any change in the protein level in vitro (**Nakamaru et al. 2009**) which is thought to result from a reduction in NAD levels *via* PARP-1 activation (**Furukawa et al. 2007**). Oxidative stress induced reductions in placental glucose uptake by placenta can be abolished by SIRT activation suggesting a similar mechanism is occurring (**Lappas et al. 2012**).

SIRT1 modulates stress responses which would otherwise commit cells into programmed cell death or apoptosis, ensuring an appropriate response to the level of stress (**Levine et al. 2006**), Jones and Baylin 2007 (**Anastasiou et al. 2006**), and has been identified as a cancer-specific survival factor. SIRT1 phosphorylation at serine 27 (S27P) results in increased SIRT1 protein stability and is thought to be responsible for elevated SIRT1 protein levels in cancer cells (**Ford et al. 2008**).

A variety of other mechanisms also regulate SIRT1 (**Milner 2009**). A cellular “active regulator of SIRT1” (AROS) was identified in 2007 (**Kim, Kho et al. 2007**) followed by reports of a suppressor later identified as the product of the DBC-1 gene (deleted in breast cancer-1) (**Kim, Chen et al. 2008; Zhao et al. 2008**). Post-translational modification by sumoylation also affects the activity of SIRT1 and is coupled with the cellular response to DNA damage (**Yang, Fu et al. 2007**). SIRT1 mRNA levels and protein stability in the presence of fluorouracil is regulated by p53, which since p53 is itself subject to deacetylation by SIRT1 affords a regulatory feed-back loop between p53 and SIRT1 in response to stress (**Ford et al. 2008**). SIRT1 expression is suggested to be also subject to the microRNA miRNA-34a (**Yamakuchi et al. 2008**).

A recent study identified reductions in SIRT1 mRNA expression and protein production with labour following proinflammatory stimuli, while activation of SIRT1 had anti-inflammatory effects (**Lappas et al. 2011**).

### SIRT2

Although SIRT2 can be also located in the nucleus, it is a predominantly cytoplasmic protein where it co-localizes with microtubules and like HDAC6 acts as a tubulin deacetylase (**North et al. 2003**). SIRT2 is highly expressed in the human brain, with an absence in many brain-tumour cell lines, suggesting it may act as a tumour suppressor and regulate cell cycle progression (**Voelter-Mahlknecht et al. 2005**). SIRT2 also binds to and deacetylates FOXO3a, elevating the expression of FOXO3a target genes and promoting cell death in response to severe stress (**Wang, Nguyen et al. 2007**). Like HDAC1, SIRT2 is subject to acetylation by p300 which results in attenuation of deacetylase activity (**Han et al. 2008**).

### SIRT3

SIRT3 is expressed ubiquitously, particularly in metabolically active tissues, and regulates global mitochondrial lysine acetylation (**Onyango et al. 2002; Lombard et al. 2007**). SIRT3 is a soluble protein located in the mitochondrial matrix and is particularly high in tissues rich in mitochondria: brain, heart, liver, kidney and brown adipose tissue (**Onyango et al. 2002; Schwer et al. 2002; Lombard et al. 2007**). The possibility that SIRT3 might also be present in the nucleus has been raised (**Scher et al. 2007; Hallows et al. 2008; Nakamura et al. 2008; Sundaresan et al. 2008**) with suggestions that SIRT3 might shuttle between compartments in response to stress (**Scher et al. 2007**). Further studies confirmed that both human and mouse SIRT3 localise to the mitochondria (**Lombard et al. 2007; Cooper et al. 2008**) and that both contain a cleavable N-terminal mitochondrial-targeting pre-sequence (**Schwer et al. 2002; Jin et al. 2009**). However a recent study (**Shulga et al. 2010**) has again raised the possibility of a full-length form residing in the nucleus.

Human SIRT3 requires proteolytic processing to gain full deacetylase activity (**Cooper et al. 2008**). The precursor protein contains a mitochondrial-targeting N-terminal peptide which is cleaved off after import, and two conserved arginine residues are essential for this process (**Schwer et al. 2002; Cooper et al. 2008**). SIRT3 expression is activated by caloric expression in adipose tissue (**Hirschey et al. 2010**), and in brown fat is also upregulated by cold exposure, and reduced by an elevated climate (**Shi et al. 2005; Hallows et al. 2006**). SIRT3-deficient mice show a significant reduction in basal ATP levels in multiple tissues as well massive acetylation of mitochondrial proteins and suppression of oxidative phosphorylation (**Ahn et al. 2008**).

A proteomic study in 2006 suggested that as many as 20% of all mitochondrial proteins are regulated by lysine acetylation **(Kim, Sprung et al. 2006)**. Both SIRT3 and 4 have been implicated in nutrient sensing via mitochondrial NAD<sup>+</sup> levels **(Yang, Yang et al. 2007)**. SIRT3 deacetylates and activates both the mitochondrial enzyme acetyl-CoA synthetase 2 (AceCS2) which catalyses the formation of acetyl CoA **(Hallows et al. 2006; Schwer et al. 2006)**, and isocitrate dehydrogenase 2, an enzyme that promotes regeneration of antioxidants **(Schlicker et al. 2008)**. SIRT 3 also deacetylates and activates glutamate dehydrogenase, a central metabolic regulator in the mitochondrial matrix **(Schlicker et al. 2008)**, and deacetylates and inactivates cyclophilin D, promoting the detachment of hexokinase II from the mitochondria **(Shulga et al. 2010)**. SIRT3 also regulates mitochondrial protein synthesis by deacetylation of the ribosomal protein MRPL10 (mitochondrial ribosomal protein L10) **(Yang et al. 2010)**. SIRT3 has been shown to interact with FOX3a, ATP5a and Hsp70 **(Jacobs et al. 2008; Law et al. 2009; Huang et al. 2010)**. Interestingly, increased expression of SIRT3 has been shown to correlate with an extended life span in humans and a variant of the SIRT3 gene is associated with longevity in humans **(Rose et al. 2003; Bellizzi et al. 2005)**.

#### SIRT4

Expression levels of SIRT4 are relatively high in the liver, heart, kidney and brain **(Haigis, Mostoslavsky et al. 2006)** and like SIRT3, SIRT4 is localized in the mitochondrial matrix with its first 28 amino acids cleaved after import **(Haigis, Mostoslavsky et al. 2006; Ahuja et al. 2007)**. However, SIRT4 is a mitochondrial protein for which NAD<sup>+</sup>-dependent deacetylase activity has not been detected *in vitro* **(Verdin et al. 2004; Haigis, Mostoslavsky et al. 2006)**. Rather SIRT4 acts as an ADP-ribosyl transferase. ADP-ribosylation of glutamate dehydrogenase by SIRT4 in pancreatic  $\beta$ -cells represses glutamate dehydrogenase activity resulting in inhibition of insulin secretion **(North et al. 2003; Haigis, Mostoslavsky et al. 2006; Ahuja et al. 2007)**.

#### SIRT5

The third of the mitochondrial sirtuins, SIRT5 is a soluble protein and localizes to the inter-membrane space **(Nakamura et al. 2008)**. In the humans, it is most predominantly expressed in brain, testis, heart muscle cells and in lymphoblasts **(Michishita et al. 2005; Mahlknecht et al. 2006)** and SIRT5 protein exhibits NAD-dependent deacetylase activity on histone H4 peptide *in vitro* **(North et al. 2003)**. However, in contrast to SIRT3, SIRT5 deacetylates none of the mitochondrial matrix proteins tested, but can

deacetylate cytochrome c, a protein of the mitochondrial inter-membrane space (**Schlicker et al. 2008**) thereby regulating oxidative metabolism, as well as apoptosis initiation. SIRT5 may also regulate urea production, as it deacetylates and activates carbamoyl phosphate synthetase 1 (CPS1) which is essential for the urea cycle (**Nakagawa et al. 2009; Ogura et al. 2010**).

### SIRT6

SIRT6 is a nuclear protein (**Voelter-Mahlknecht et al. 2006**) capable of promoting ADP-ribosylation (**Liszt et al. 2005; Mostoslavsky et al. 2006**), and was only recently confirmed as possessing histone deacetylase activity (**Michishita et al. 2008**). SIRT6 had been found to fractionate with chromatin biochemically (**Mostoslavsky et al. 2006**) but initially deacetylase activity could not be detected on several histone substrates. Highly site specific, SIRT6 deacetylates lysine 9 of histone H3 (H3K9Ac), acting at the telomeres to maintain telomere integrity (**Michishita et al. 2008**) and was recently determined to deacetylate lysine 56 of histone H3 (H3K56) (**Michishita et al. 2009; Yang et al. 2009**) maintaining dynamic changes in H3K56 acetylation levels at telomeres over the cell cycle (**Michishita et al. 2009**).

SIRT6 is a critical regulator of transcription, genome stability, telomere integrity, DNA repair, and metabolic homeostasis (**Tennen et al. 2010**). SIRT6 is required for efficient DNA double-strand break repair in the context of chromatin (**McCord et al. 2009**). SIRT6 has a role in base excision DNA repair and promotes the resistance to DNA damage and oxidative stress and suppresses genomic instability in mouse cells. Depletion of SIRT6 protein leads to telomere dysfunction with end-to-end chromosomal fusions and premature cellular senescence (**Mostoslavsky et al. 2006; Michishita et al. 2008**). SIRT6 also negatively regulates aging associated NF- $\kappa$ B-dependent gene expression programs (**Kawahara et al. 2009**). SIRT6-deficient mice are born normally, but develop several acute degenerative processes at around 3 weeks of age, dying before one month of age. These defects include severe metabolic imbalance, reduced serum IGF-1, complete loss of subcutaneous fat, lymphopenia, osteopenia, and an acute and fatal onset of hypoglycemia, leading to death (**Mostoslavsky et al. 2006**).

The C terminus of SIRT6 is essential for proper nuclear localization while The N terminus of SIRT6 is required for deacetylation in cells, contributing to efficient chromatin association and intrinsic catalytic activity- catalytic activity which is required for stable association of SIRT6 with chromatin (**Tennen et al.**). Promoter associated H3K9 deacetylation of NF- $\kappa$ B target genes decreases promoter occupancy by p65

attenuating NF- $\kappa$ B transactivation (**Kawahara et al. 2009**). Like SIRT1, SIRT6 binds p65 but acts through histone deacetylation rather than directly deacetylating p65 (**Yeung et al. 2004; Chen et al. 2005; Wan et al. 2010**). SIRT6 also regulates glucose homeostasis by acting as a corepressor of HIF1 $\alpha$  acting as a safeguard mechanism, down modulating basal transcription of Hif1a target genes under normal nutrient conditions to maintain proper glucose flux toward mitochondrial respiration and to prevent excessive glycolysis (**Zhong et al. 2010**). Recently SIRT6 mRNA expression and protein production were shown to be reduced in preterm fetal membranes both with pre-term spontaneous labour, and following exposure to LPS (**Lim et al. 2013**).

### SIRT7

SIRT7 is a widely expressed nuclear protein and is associated with active rRNA genes (**Ford et al. 2006**), deacetylase activity has not been detected for SIRT7. SIRT7 is positive regulator of Pol I transcription and is required for cell viability in mammals (**Ford et al. 2006**). However the functional characteristics of SIRT7 are essentially unknown and remain to be further elucidated (**Voelter-Mahlknecht et al. 2006**)

## **Class 4 HDACs**

### HDAC11

HDAC 11 was originally considered a class 1 HDAC, but phylogenetic analysis led to its consideration as the defining member of a separate fourth class (**Gregorette et al. 2004**). The Last of the zinc-dependent HDACs to be discovered, HDAC resides in the nucleus and is considered an immunoregulator due to its role in the regulation of interleukin 10 (IL-10) expression (**Georgopoulos 2009; Villagra et al. 2009**). HDAC11 directly interacts at the chromatin level with a distal region of the IL-10 promoter in activated macrophages, and kinetics suggest there may be a secondary means of regulation by HDAC11 (**Villagra et al. 2009**). Modulation of HDAC11 activity and therefore IL-10 protein abundance would shift a response from high immune function to anergy or tolerance (**Georgopoulos 2009**).

## Histone Deacetylase Inhibitors (HDACi)

Several compounds have been identified which can inhibit the function of HDACs, leading to hyperacetylation of the histones as well as other nonspecific effects (**Takai et al. 2007**). Microarray studies indicate that HDACi are selective in their effects on gene expression, altering the expression of only 2 to 10% of genes analysed and down-regulating as many genes as they up-regulate (**Kelly et al. 2005**). Many HDACi function by blocking access to the active site either reversibly, or irreversibly (trapoxin and depudesin) (**de Ruijter et al. 2003**). Inhibitors against the Zinc-dependent HDACs (Classes I, II & IV) can be classified into several main groups, based on chemical structure (**Estiu et al. 2010**). They all share a common pharmacophore pattern consisting of: (i) a zinc binding domain, (ii) a linker domain of appropriate length which mimics the substrate and occupies the active site channel, and (iii) a cap substructure, which interacts with amino acids at the entrance of the active site channel (**Finnin et al. 1999; Somoza et al. 2004; Vannini et al. 2004**). Irreversible HDAC inhibitors act by a different mechanism (**Furumai et al. 2001; Kramer et al. 2001; Marks et al. 2001**).

*In vitro* studies revealed that treatment with pharmacologic class I and II HDAC inhibitors (HDACi) resulted in the induction of antiproliferative, pro-differentiative, or pro-apoptotic genes resulting in cellular growth arrest, terminal differentiation and cell death in transformed cells (**Kijima et al. 1993; Ito et al. 2000; Munster et al. 2001; Suenaga et al. 2002; Bolden et al. 2006; Minucci et al. 2006; Xu et al. 2007**). The effect of HDAC inhibition on gene expression is not thought to be a general one, indeed, DNA micro-arrays using malignant cell lines cultured in the presence of a HDAC inhibitor indicated that only a specific small number of genes (1-7%) showed altered expression (**Van Lint et al. 1996; Mariadason et al. 2000; Della Ragione et al. 2001; Munster et al. 2001; Yan et al. 2001; Yang et al. 2001; Zhu et al. 2001**). There are also roughly equal numbers of genes being activated and repressed following pharmacological inhibition of HDAC activity (**Smith 2008**) providing evidence for a dual role by HDACs and this may depend on the pathway involved- Histone modification or other interactions.

A number of HDAC inhibitors are currently in preclinical to phase II/III trials and biological analysis has revealed differences in isoform selectivity (**Khan et al. 2008**), which may contribute to the responsiveness of different cancers to treatment. Indeed, while originally thought to act equally across the HDACs; it is now recognized that many HDAC inhibitors show significant differences in activity against specific classes of

HDAC, and this is being utilized in the development of isoform specific HDAC inhibitors. The main groups of zinc-dependent HDAC inhibitors are the simple aliphatic carboxylic acids, hydroxamic acids, benzamides, cyclic peptides and depsipeptides (**Estiu et al. 2010**). The hydroxamic acid derivative HDAC inhibitors are pan-HDAC inhibitors and target both class I and class II HDACs, while, aliphatic acids, benzamides and cyclic peptides have inhibitory activity against the class I HDACs but have limited activity against the class II HDACs at higher concentrations (**Furumai et al. 2001; Gottlicher et al. 2001; Glaser et al. 2004; Gurvich et al. 2004**). Indeed class II HDACs were five times less susceptible to inhibition by valproic acid than the class I HDACs and HDAC4 is comparatively less sensitive to inhibition by butyrate (**de Ruijter et al. 2003**). HDAC8 is relatively resistant to TSA, SAHA and MS-275 (**Vannini et al. 2004**). HDAC6 is insensitive to the short-chain fatty acids and cyclic tetrapeptide inhibitors with large cap groups including TPX, FK228, and MS-275 (**Matsuyama et al. 2002**) but can be inhibited by tubacin which doesn't inhibit the other enzymes (**Haggarty et al. 2003**). HDAC6 was identified early on as an exception when it came to HDACi sensitivity, due to its double catalytic domain, likewise HDRP due to its absence of one. Interestingly HDAC6 is resistant to inhibition by the irreversible inhibitor trapoxin (**Furumai et al. 2001**). The cyclic tetrapeptide, FK228, also strongly inhibits HDAC1 and -2 but is relatively weak in inhibiting HDAC4 or -6 (**Furumai et al. 2002**). Specific inhibitors have now been developed for several of the HDACs, utilizing structural properties of HDAC inhibitors that previously demonstrated selectivity (**Kozikowski et al. 2007; Chen et al. 2009; Ontoria et al. 2009; Suzuki 2009; Estiu et al. 2010**).

### ***Hydroxamate inhibitors***

Most of the early HDACi studies utilised hydroxamic acid derivatives, typified by suberoylanilide hydroxamic acid (SAHA) (**Richon et al. 1996; Richon et al. 1998**) and Trichostatin A (TSA) (**Yoshida et al. 1990; Yoshida et al. 1995**). Other Hydroxamic acid derivative HDAC inhibitors include m-carboxycinnamic acid bishydroxamide (CBHA), Scriptaid, Oxamflatin, Pyroxamide, PCI-34051, LAQ824, LBH589, PXD-101, LBH-589 and Cyclic hydroxamic acid containing peptides (CHAPs). Hydroxamic acids are the most potent inhibitors of deacetylase activity (**Schuetz et al. 2008**) however studies are now reassessing the assumption that they are non-selective inhibitors of class I and II enzymes. HDAC7 was recently confirmed to show significant binding to hydroxamate inhibitors (**Schuetz et al. 2008**), however HDAC8 has a much lower affinity to hydroxamic acids than previously thought (**Kapustin et al. 2003; Estiu et al.**

**2010**). Some hydroxamates are currently in clinical use such as SAHA (marketed as “vorinostat”) others like Trichostatin A (TSA), which is frequently used in research, are of limited therapeutic use, as they have poor bioavailability *in vivo* and toxic side effects at high doses (**Takai et al. 2007**).

TSA is a fermentation product of *Streptomyces* and was originally used as an anti-fungal agent (**Tsuji et al. 1976**) before its potent anti-proliferative effect on cancer cells was noted (**Yoshida et al. 1987; Yoshida et al. 1988**). The ability of TSA to inhibit HDAC was first reported in 1990 (**Yoshida et al. 1990**). The zinc-dependent HDACs were found to be sensitive to inhibition by TSA at nanomolar concentrations (**Carmen et al. 1996; Rundlett et al. 1996; Taunton et al. 1996; Yang et al. 1996; Yang et al. 1997; Dangond et al. 1998; Emiliani et al. 1998; Fischle et al. 1999; Grozinger et al. 1999; Miska et al. 1999; Verdel et al. 1999; Wang et al. 1999; Buggy et al. 2000; Hu, Chen et al. 2000; Kao et al. 2000; Van den Wyngaert et al. 2000**), while the sirtuins are relatively insensitive to TSA (**Imai et al. 2000; Landry, Sutton et al. 2000; Smith et al. 2000; Barlow et al. 2001**). SAHA was synthesized as a hybrid polar compound containing a hydroxamic acid, and developed as a strong differentiation inducer. It was like TSA, later found to be a potent inhibitor of partially purified HDACs (**Richon et al. 1998**). In addition to inhibiting HDAC activity, SAHA (vorinostat) has also been reported to selectively suppress expression of HDAC7 with little or no effect on the expression of other class I and class II HDACs examined. The down-regulation of HDAC7 protein by SAHA was associated with down-regulation of HDAC7 mRNA with no alteration in the half-life of HDAC7 protein, and transformed cell lines were the most sensitive to HDACi effects (**Dokmanovic, Perez et al. 2007**).

### ***Short Chain fatty acids***

The short chain fatty acids- or simple aliphatic carboxylic acids are a well-known group of HDAC inhibitors, but are inefficient, operating at a millimolar rather than at micro or nanomolar range. This group comprises butyrate, phenyl butyrate and valproic acid (VPA) (**de Ruijter et al. 2003**). Like the Hydroxamic acids, Sodium butyrate, was found to be a non- specific HDAC inhibitor and induces cell arrest and differentiated phenotype in a number of cancer cells (**Kim et al. 1980; Cioe et al. 1981; Ryan et al. 1987; Graham et al. 1988; Hoessly et al. 1989**). Butyrate is an endogenous metabolite which along with the bioactive lipid mediator sphingosine-1-phosphate can antagonize HDAC activity directly (**Kramer et al. 2003; Heinzl et al. 2007**) (**Atmaca et al. 2007; Hait et al. 2009**). Butyrate can be found in the human colon, resulting from the microbial

fermentation of complex carbohydrates and appears to regulate the physiological differentiation of colonocytes (**Yang and Seto 2007**). Butyrate targets class I and class IIa HDACs and specifically catalyzes proteasomal degradation of HDAC2 (**Gottlicher et al. 2001; Kramer et al. 2003; Heinzl et al. 2007**), while sphingosine-1-phosphate selectively binds to and inhibits HDAC1 and HDAC2 (**Hait et al. 2009**). Like Butyrate, Valproic acid also selectively degrades HDAC2 via the proteasome, acting to increase expression of the E2 ubiquitin-conjugating enzyme Ubc8 (**Kramer et al. 2003**). It has been reported that deacetylases associated with the NCoR corepressor complex are more sensitive to inhibition by valproic acid than enzymes not associated with NCoR (**Gottlicher et al. 2001**), and a recent study revealed that the HDAC7 catalytic domain did not bind to butyrate or valproic acid (**Schuetz et al. 2008**).

### ***Carboxylates***

The carboxylate class (**Chen, Faller et al. 2003**), which is defined as possessing a carboxylate in the metal binding moiety, has generally poor HDAC inhibitory activity in comparison with other inhibitors. Butyrate, a natural product generated in humans by both metabolism of fatty acids and bacterial fermentation of fibre in the colon, was the first identified HDAC inhibitor (**Boffa et al. 1978**). Related compounds such as phenyl butyrate and the anticonvulsant valproic acid (**Phiel et al. 2001**) were later found to be HDAC inhibitors. It has been shown that butyrate possesses some selectivity, which poorly inhibits class IIb HDACs such as HDAC6 and -10 (**Guardiola et al. 2002**).

### ***Benzamide inhibitors***

The benzamides include N-acetyldinaline (CI-994), M344, MCGD-0103 and MS-275, and are effective at  $\mu\text{M}$  ranges. They are relatively selective, for example MS275 is a class I selective inhibitor which blocks the activities of HDAC1, 2, and less efficiently HDAC3 with no inhibition of HDAC6-8 (**Inoue et al. 2006; Schuetz et al. 2008**). Selective inhibitors of HDAC1 and HDAC2 have been developed from rational modifications of the benzamide moiety (**Witter et al. 2007; Methot et al. 2008**).

### ***Cyclic tetrapeptides***

The Cyclic tetrapeptides include Apicidine, Trapoxins, HC-toxin, Chlamydocin, and Depsipeptides (FR901228 or FK228 -"istodax"). Cyclic tetrapeptide antibiotics are characterized by their complicated structure and their high HDAC-inhibitory potential acting in the nM to  $\mu\text{M}$  range. Most are products of bacteria or fungi but some, like

apicidin and depsipeptide have been chemically engineered as combinations of hydroxamic acids and cyclic tetrapeptides **(de Ruijter et al. 2003)**.

### ***Sirtuin inhibitors***

Deacetylation *via the* Class III HDACs, the sirtuins, is dependent on NAD<sup>+</sup> rather than zinc **(Imai et al. 2000; Landry, Slama et al. 2000)**. During deacetylation by SIRTs, NAD<sup>+</sup> is believed to be hydrolyzed, releasing nicotinamide, with the acetyl group of the acetylated lysine substrate being transferred to the cleaved NAD<sup>+</sup>, generating Oacetyl-ADP ribose **(Sauve et al. 2003; Zhao et al. 2004)**. Several classes of sirtuin inhibitors have been reported **(Neugebauer, Sippl et al. 2008)** including the by-product of deacetylation, nicotinamide. Nicotinamide is a potent SIRT inhibitor and is thought to inhibit SIRTs by binding to a conserved pocket adjacent to the NAD<sup>+</sup> however nicotinamide and other nonhydrolyzable NAD analogs such as Carba-NAD are not particularly suitable for *in vivo* studies since they are not cell permeable and they inhibit other NAD-dependent enzymes **(Landry, Slama et al. 2000)**. The sirtuins are less sensitive to both TSA and fatty acids than class I HDACs tested in parallel **(Barlow et al. 2001; Blander et al. 2004)** so high-throughput cell-based screens have been conducted to identify cell-permeable small-molecule inhibitors of the NAD-dependent deacetylase activity of the class II HDACs **(Bedalov et al. 2001; Grozinger et al. 2001)**.

Sirtinol (2-(2-hydroxy-naphthalen-1-ylmethylene)-amino]-N-(1-phenyl-ethyl)-benzamide) and M15 were shown to inhibit both yeast Sir2 and human SIRT2 *in vitro*, while splitomicin inhibits the deacetylase activity of yeast sirtuins and demonstrated some degree of selectivity **(Fulco et al. 2003; Zhao et al. 2003; Yeung et al. 2004)**. As splitomicin is not a very efficient inhibitor for human sirtuins, a series of analogues were developed **(Neugebauer, Uchiechowska et al. 2008)**. Further analogues of sirtinol have also been developed, which are between 2- and 10-fold more potent than sirtinol against human SIRT1 and SIRT2 enzymes **(Mai et al. 2005)**. Current Sirtuin inhibitors include AGK2, Tenovin, Suramin, Cambinol, Salermide, and Thiobarbiturates Sirtuin inhibitors **(Alcain et al. 2009)**.

### ***Endogenous HDAC modulators***

As mentioned, Sodium Butyrate (NaB) can be found in the human colon and is not specific for HDACs, inhibiting the phosphorylation and methylation of proteins as well as DNA methylation **(Takai et al. 2007)**. Other inhibitors of classical HDACs can be found

in food: diallyl disulfide is found in garlic, sulforaphane in broccoli, and resveratrol –a sirtuin activator, as well as related polyphenols can be found in nuts, certain plant leaves, dark cherries, and red grape skins. Curcumin, the principle curcuminoid in indian curry spice, and two other natural products have also been identified which inhibit HAT activity (**Balasubramanyam et al. 2004; Druesne et al. 2004; Myzak et al. 2004; Yang and Seto 2007**). Nitric oxide (NO) was recently identified as an endogenous molecule that specifically inhibits certain class I HDACs in response to brain-derived neurotrophic factor (BDNF) in neurons (**Nott et al. 2008; Nott et al. 2009; Riccio 2010**), regulating the interaction of HDAC2 with chromatin independently of HDAC2 activity. While many posttranslational modifications influence HDAC activity, there is still little evidence directly linking environmental stimuli to changes in nuclear HDAC functions (**Brandl et al. 2009**) though in addition to diet, other influences can produce effects which may modify histone acetylation. Mouse models also suggest the function of class IIa HDAC can be regulated by a range of factors including neuronal stimuli (**Mejat et al. 2005; Belfield et al. 2006**), immune activity (**Dequiedt et al. 2003**), and physical exercise (**Berdeaux et al. 2007**).

## ***Regulation of cytokines by HDACs***

Experiments in a variety of cell types have implicated HDACs in the regulation of several cytokines and pathways, and it is likely that at least some of these operate in the placenta and gestational membranes. In humans, HDAC inhibition selectively alters cytokine mRNA expression and protein production by immune cells (**Leoni et al. 2002; Bode et al. 2007; Roger et al. 2011**). In Th0 cells, HDAC1 and HDAC2 are recruited to the IFN- $\gamma$  locus, repressing transcription (**Chang et al. 2008**). Likewise HDAC1 represses transcription of IL-2 in T-cells (**Wang, Lee et al. 2009**) and IL-12 in macrophages (**Lu et al. 2005**).

Differential inflammatory responses to LPS have also been identified following the use of selective HDAC inhibitors in macrophages (**Halili et al. 2010**) and this may reflect the specific actions of individual HDACs, which can have opposing effects. For example, HDAC1 and HDAC8 can both repress IFN- $\beta$  expression, while HDAC6 acts as coactivator to enhance activity (**Nusinzon et al. 2006**).

In melanoma cells, the transcription factor Sp1 recruits HDAC1 to the IL-1 promoter, regulating IL-1 $\alpha$  expression (**Enya et al. 2008**). HDAC-1 is also enriched at the PDGF-D

promoter in cells exposed to IL-1 $\beta$  and forms a cytokine-inducible gene silencing complex with p65 and IRF-1 (**Liu and Khachigian 2009**). HDAC1-3 are recruited to the IL-4 promoter (**Valapour et al. 2002**), HDAC1 and HDAC5 are recruited to the IL-8 promoter (**Schmeck et al. 2008**) and HDAC11 is recruited to the IL-10 promoter (**Villagra et al. 2009**) repressing transcription. HDAC3 has been specifically implicated in the regulation of TNF $\alpha$  protein production (**Zhu et al. 2010**). Furthermore, selective inhibition of HDAC3 inhibits both TNF $\alpha$  and IL-6 mRNA expression (**Gillespie et al. 2012**). SIRT6 has also been shown to positively regulate TNF $\alpha$  (**Van Gool et al. 2009**).

HDAC7 and HDAC9 interact with FOXP3 and TIP60 to repress IL-2 expression (**Li et al. 2007**), and HDAC3 binds to pro-IL-16 to repress Skp2 expression and subsequently promote cell cycle arrest (**Zhang, Tuzova et al. 2008**). The Glucocorticoid receptor (GR) recruits HDAC2 to the GM-CSF promoter, antagonising the effects of IL-1 $\beta$  (**Ito et al. 2000**), and also binds to IL-5 promoter where it recruits HDAC1, interfering with GATA positive regulation (**Jee et al. 2005**). HDAC2 mediated deacetylation of GR also enables interaction between the receptor and nuclear factor (NF)- $\kappa$ B, promoting its repressor activity upon GMCSF expression (**Ito et al. 2006**). In addition HDACi studies have suggested promoter associated histone acetylation is involved in regulating IL-5 (**Han et al. 2007**) and the IL-12p40 locus (**Bode et al. 2007**).

For most cytokines, receptor binding triggers an intracellular signalling cascade involving one or more signal transducer and activator of transcription (STAT) proteins (**Levy et al. 2002**). HDAC1 modulates IFN $\alpha$ -induced transcription and associates with both STAT1 and STAT2 (**Nusinzon et al. 2003**), inhibition of HDAC activity suppresses the IFN- $\alpha$ -induced innate antiviral response (**Nusinzon et al. 2003**).

HDAC1 can also be recruited to the Id-1 promoter by STAT5, where it deacetylates C/EBP $\beta$  allowing transcriptional activation of the Id-1 inhibitor (**Xu et al. 2003**). HDAC3 enhances STAT5 phosphorylation (**Togi et al. 2009**) and also deacetylates STAT1 allowing its phosphorylation and nuclear translocation (**Kramer et al. 2009**), while HDAC1-3 deacetylate STAT3 to inactivate its transcriptional functions (**Yuan et al. 2005**). Transcription regulation mediated by GATA-1 is diminished in the presence of HDAC3-5 (**Watanoto et al. 2003**), HDAC3 suppresses the transcriptional potential of GATA-2 (**Ozawa et al. 2001**) and phosphorylated GATA-3 recruits HDAC3 and HDAC5 to specific DNA regions (**Chen, Osada et al. 2006**).

## NF- $\kappa$ B

Nuclear factor- $\kappa$ B (NF $\kappa$ B) is a ubiquitous family of dimeric transcription factors that belong to the Rel family. They play a major role in regulating cytokine production, cell proliferation, differentiation, adhesion, survival and apoptosis and can be activated by an array of stimuli such as inflammatory cytokines, oxidant stress, and hemodynamic forces (**Collins et al. 2001; Lappas et al. 2007; Perkins 2007**). Many genes encoding proinflammatory cytokines contain NF- $\kappa$ B recognition elements within their promoters (**Mukaida et al. 1989; Goldfeld et al. 1990; Sanceau et al. 1995**). NF $\kappa$ B is a nonhistone protein which both recruits HATs and HDACs to target gene promoters and is itself a substrate for HATs/HDACs (**Chen et al. 2001; Chen and Greene 2003; Quivy et al. 2004; Yeung et al. 2004; Yang, Wright et al. 2007; Rajendrasozhan et al. 2008**).

There are five distinct subunits: NF- $\kappa$ B1 (p50/105), NF- $\kappa$ B2 (p52/100), RelA (p65), RelB and C-Rel. NF- $\kappa$ B1 and NF- $\kappa$ B2 are synthesized as the precursor proteins, p105 and p100, and proteolytic processing generates their N-terminal DNA binding forms, p50 and p52, respectively. p50 and p52 lack any identifiable transactivation domains and, primarily form homodimers which function as repressors of transcription. RelA, RelB and C-Rel do not require processing, and contain transactivation domains in their carboxyl terminal regions. The classical NF- $\kappa$ B dimer contains RelA and p50, and is responsible for most of the inducible activity of NF $\kappa$ B (**Lernbecher et al. 1993**) although a variety of other Rel-containing dimers are also known to exist (**Baeuerle et al. 1994; Baeuerle et al. 1996**).

In most cell types, NF- $\kappa$ B is found in the cytoplasm in an inactive form, bound to a family of inhibitory proteins termed I $\kappa$ B (inhibitor of  $\kappa$ B) (**Chen et al. 2004; Aguilera et al. 2006; Lappas et al. 2007; Perkins 2007**). In response to a variety of stimuli (including Toll-like receptor (TLR) and tumour necrosis factor receptor (TNFR) superfamily activation) I $\kappa$ Bs are phosphorylated by I $\kappa$ BKinases (IKKs), leading to their ubiquitination and proteasomal degradation, which allows NF $\kappa$ B to translocate to the nucleus and activate target genes (**Doyle et al. 2006; Basak et al. 2008**).

Subcellular localization and proteolytic processing are key elements in many immune signalling networks, including the NF- $\kappa$ B (**Gopal et al. 2006**) and MAPK pathways (**Baek et al. 2002**). The NF- $\kappa$ B subunit p65 (RelA), can be deacetylated by HDAC3 and SIRT1 promoting NF- $\kappa$ B binding to I $\kappa$ B $\alpha$  and its nuclear export (**Chen et al. 2001; Gao**

**et al. 2005; Kasper et al. 2006).** Class IIa HDACs bind I $\kappa$ B $\alpha$  and therefore negatively regulate NF- $\kappa$ B (**Aguilera et al. 2004**).

The class I HDACs 1, -2, and -3, and the class III HDAC SIRT1 have been implicated in the inhibition of NF- $\kappa$ B -mediated transcription (**Chen et al. 2001; Quivy et al. 2004; Yeung et al. 2004**). In unstimulated cells, the p50 subunit of NF- $\kappa$ B binds HDAC1 to repress the transcription of NF- $\kappa$ B target genes. After activation, the p50/p65 heterodimer translocates to the nucleus and displaces p50/HDAC1 (**Zhong et al. 2002**). A similar mechanism has been proposed for HDAC3, in which it associates with TAB, N-CoR and p50 (**Baek et al. 2002**). HDAC2 modulates cytokine signalling by antagonizes cytotoxic effects of NF- $\kappa$ B (**Kaler et al. 2008**). SIRT6 physically interacts with RelA and represses NF- $\kappa$ B-dependent gene expression by deacetylating histone H3K9 (**Kawahara et al. 2009**).

In HeLa cells , treatment with the HDACi apicidin leads to an increase in transcriptional activity of NF- $\kappa$ B and the expression of its target genes, IL-8 and TNF $\alpha$ . (**Kim, Seo et al. 2006**). TNF $\alpha$  expression was induced by apicidin at earlier time points than NF- $\kappa$ B activation or IL-8 expression and results suggested that activation of NF- $\kappa$ B signalling required both the PI3K/PKC signalling pathways and HDAC1 (**Kim, Seo et al. 2006**). However reports on the effects of HDACi on NF- $\kappa$ B activity are conflicting (**Chen et al. 2001; Mayo et al. 2003; Kim, Lee et al. 2006; Takada et al. 2006**).

## Hypoxia and HIF1 $\alpha$

Another pathway which is involved in cytokine signalling, and involves regulation by HDACs is the Hypoxia inducible Factor- $\alpha$  (HIF- $\alpha$ ) pathway. The production of TNF $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$ , but not IL-6, in human placental tissues in explant culture is markedly stimulated by reduced O<sub>2</sub> tension (**Benyo et al. 1997**) and this effect is restricted to tissues obtained after 11 weeks gestation (**Benyo et al. 1997**). Furthermore, villous explants derived from preeclamptic placentas were found to be equally sensitive to the effect of hypoxia on TNF $\alpha$  and IL-1 expression (**Benyo et al. 2001**). Trophoblasts derived from preeclamptic placentas cultured under hypoxic conditions also secrete less IL-10 than normal trophoblast cells (**Bowen et al. 2005**). Normal term placental villous explants studies also showed a clear positive correlation between the amount of IL-10 released and oxygen tension, with 2% oxygen resulting in significantly lower IL-10 levels than both 21% and 8% oxygen (**Royle et al. 2009**). Hypoxia/reoxygenation also

increases TNF $\alpha$  synthesis by placental villous tissue in vitro and is more potent inducer of cytokine secretion than hypoxia alone **(Hung et al. 2004)**.

The Hypoxia inducible Factor (HIF) transcription factor complex is a heterodimer formed by two subunits, one of three distinct HIF- $\alpha$  subunits and the constitutively expressed protein; arylhydrocarbon receptor nuclear translocator (ARNT, HIF $\beta$ ). The three HIF $\alpha$  proteins: HIF-1 $\alpha$ , 2 $\alpha$  and 3 $\alpha$ , appear to have nonredundant roles in mediating adaptive responses to hypoxia **(Carmeliet et al. 1998; Ryan et al. 1998; Tian et al. 1998; Hu et al. 2005; Maynard et al. 2007; Dutta et al. 2008)**. Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is a key mediator of the cellular adaptation to nutrient and oxygen stress, functioning as a direct transcriptional activator of genes involved in erythropoiesis (erythropoietin), angiogenesis (VEGF), and glucose metabolism (GLUT-1 and GLUT-3, lactate dehydrogenase (LDH), phosphoglycerate kinase (PGK-1), glucose-6-phosphate isomerase (GPI), and phosphofructokinase-1 (PFK-1) **(Seagroves et al. 2001; Pugh et al. 2003; Lum et al. 2007; Fong 2008)**. HIF1 $\alpha$  also represses mitochondrial respiration in a coordinated fashion **(Semenza 2003)** by up regulating expression of the pyruvate dehydrogenase kinase (PDK) gene **(Kim, Tchernyshyov et al. 2006; Papandreou et al. 2006)** which phosphorylates and inactivates pyruvate dehydrogenase (PDH) and by inhibition of the cytochrome oxidase subunit Cox4-1 and the coactivator PGC-1b **(Fukuda et al. 2007; Zhang, Gao et al. 2007)**.

The activity of HIF-1 $\alpha$  is tightly regulated. Under normoxic conditions, when O<sub>2</sub> availability and demand are balanced, HIF-1 $\alpha$  is hydroxylated by prolyl hydroxylase domain (PHD) oxygenases, at multiple prolyl residues **(Bruick et al. 2001; Epstein et al. 2001)**, including those in the C-terminal transactivation domain (C-TAD), which is the target of factor inhibiting HIF (FIH). The hydroxylated prolines serves as a recognition signal for the von Hippel-Lindau (VHL) ubiquitin ligase, targeting HIF-1 $\alpha$  for polyubiquitination and proteasomal degradation **(Salceda et al. 1997; Huang et al. 1998; Kallio et al. 1999; Cockman et al. 2000; Kamura et al. 2000; Ohh et al. 2000; Tanimoto et al. 2000; Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001; Yu et al. 2001)**. Hydroxylation in the C-Tad region also prevents the recruitment of the coactivator CREB binding protein (CBP)/p300 preventing transcription of inducible target genes **(Mahon et al. 2001; Lando et al. 2002)**. Under hypoxic conditions, the oxygen-dependent PHDs are not active and HIF-1 $\alpha$  protein levels are stabilized **(Aragones et al., 2009)** resulting in HIF-1 $\alpha$  translocation into the nucleus and activation of target gene transcription. However other mechanism of regulation are proposed to

exist, as even under normoxic and normoglycemic conditions, HIF-1 $\alpha$  regulates basal expression of its target genes (**Carmeliet et al.,1998**). The HIF1A promoter was also recently reported to be subject to histone H3 acetylation and RNA polymerase II occupancy which was necessary for transcription (**Kenneth et al. 2009**).

HIF-1 $\alpha$  is not only responsive to oxygen levels, but is rather a mediator of cellular Responses to stress, and is stimulated by reactive oxygen species (ROS) (**Gorlach et al. 2007**) and Nitric oxide( NO) (**Sandau et al. 2001**). Expression of NO synthase has been shown to cause HIF-1 $\alpha$  accumulation, highlighting the role of NO as an intracellular activator of this transcription factor (**Haddad 2002**). HIF-1 $\alpha$  expression and production has subsequently been shown to be up regulated under normoxia in response to growth factors, hormones, coagulation factors, cytokines and vasoactive peptides (such as LPS, TNF $\alpha$ , HGF or IL-18), to different metals and also in response to mechanical stress (**Zelzer et al. 1998; Hellwig-Burgel et al. 1999; Richard et al. 2000; Thornton et al. 2000; Duyndam et al. 2001; Gorlach et al. 2001; Haddad et al. 2001; Fukuda, Hirota et al. 2002; Gao, Jiang et al. 2002; Kim et al. 2002; Page et al. 2002; Stiehl et al. 2002; Treins et al. 2002; Kietzmann et al. 2003; Gao et al. 2004; Tacchini et al. 2004; Frede et al. 2006; Bonello et al. 2007; Diebold et al. 2008; Kim, Shao et al. 2008; Rius et al. 2008; van Uden et al. 2008**).

HIF-1 $\alpha$  has a number of roles in addition to regulating angiogenesis and metabolism, for example, HIF-1 $\alpha$  interacts with Notch1 to maintain undifferentiated cell states (**Gustafsson et al. 2005**) and studies using a HIF Modulator FM19G11, showed alterations in stem cell differentiation status with HIF inhibition (**Moreno-Manzano et al.**). Data also suggests that interplay between HIFs and histone deacetylases (HDACs) is crucial for trophoblast stem cell differentiation. A novel role for Aryl hydrocarbon receptor nuclear translocator (ARNT) in trophoblast differentiation was identified with ARNT influencing HDAC expression, localization and activity in an oxygen-independent fashion. There was misregulated nuclear expression of the class IIa HDACs in particular in ARNT-null cells, along with preferential differentiation into chorionic trophoblasts and syncytiotrophoblast, rather than spongiotrophoblasts and trophoblast giant cells (**Maltepe et al. 2005**). Complete disruption of HIF signalling through loss of the ARNT or HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits results in improper placental development, characterized by a diminished spongiotrophoblast layer and insufficient chorio/allantoic fusion (**Fryer et al. 2006**).

The structurally different HDAC inhibitors TSA, LAQ824, FK228, and sodium butyrate have all been reported to inhibit HIF-1 $\alpha$  in cancer cell lines (**Jeong et al. 2002; Mie Lee et al. 2003; Qian et al. 2004**). TSA induces acetylation of HIF-1 $\alpha$  in the oxygen-dependent degradation (ODD) domain (**Xenaki et al. 2008**), and also increases expression of p53 and VHL leading to degradation of HIF-1 $\alpha$  (**Kim et al. 2001**). HDAC inhibitors also induce degradation of HIF-1 $\alpha$  independently of the VHL pathway by disrupting the HSP70/HSP90 axis function (**Kong et al. 2006**). It was determined that effective HIF-1 $\alpha$  inhibition requires class II HDAC inhibition (**Qian et al. 2006**). HDAC4 and HDAC6 co-immunoprecipitated with HIF-1 $\alpha$ , and specific inhibition of HDAC4 and HDAC6 compromised HIF-1 $\alpha$  stability and transcriptional activity (**Qian et al. 2006**). Inhibition of HDAC4 by siRNA had a positive effect on HIF-1 $\alpha$  acetylation, which correlated with reduced stability (**Qian et al. 2006**).

There are still some controversies as to how HDACs interact with HIF-1 $\alpha$  however this may represent the diverse roles of the individual HDACs in the regulation of HIF-1 $\alpha$  and the differentiation expression of HDACs between cell types. Yeast two-hybrid assays showed binding activity of HDAC7 to HIF-1 $\alpha$  four times as high as that of HDAC4 with no binding for HDAC5 (**Kato et al. 2004**). HDAC7, which translocates from the cytoplasm to the nucleus under hypoxic conditions, forms a complex with HIF-1 $\alpha$  and p300, resulting in increased levels of HIF-1 $\alpha$  target genes (**Kato et al. 2004**). The same study reported there was no association of HDAC4 or HDAC5 with HIF-1 $\alpha$  in mammalian cells, and that this was possibly due to the fact that under normoxic conditions HDAC4 localized to the cytoplasm and HDAC5 localized to the nucleus with no translocation under hypoxia (**Kato et al. 2004**).

However both HDAC4 and HDAC5 are reported to physically associate with HIF-1 $\alpha$  through the inhibitory domain (ID) which is the binding site for factor inhibiting HIF-1 (FIH-1) (**Seo et al. 2009**). HDAC4 and HDAC5 increased the transactivation function of HIF-1 $\alpha$  by promoting dissociation of HIF-1 $\alpha$  from FIH-1 and association with p300 (**Seo et al. 2009**). It was also reported that HDAC4 & 5 enhanced transactivation by HIF-1 $\alpha$  without stabilizing HIF-1 $\alpha$  (**Seo et al. 2009**), though an earlier study had previously determined that acetylation regulated HIF-1 function by targeting the HIF-1 $\alpha$ /p300 complex, not HIF-1 $\alpha$  directly (**Fath et al. 2006**). HIF-1 $\alpha$  is also reported to be stabilized, with an associated up regulation of VEGF, by over expression of HDAC1 (**Kim et al. 2001; Yoo, Kong et al. 2006**), though the mechanism is unclear. Recently it was

reported that SIRT6 acts as a corepressor of HIF-1 $\alpha$ , downregulating basal transcription of HIF1 $\alpha$  target genes under normal nutrient conditions **(Zhong et al. 2010)**. HDAC expression may also be regulated by hypoxia. A study has shown by immunoblotting that there is a global reduction of acetylated H3-K9 in response to hypoxia, as well as increased expression of HDACs 1, 2, 4 and 11 **(Islam et al. 2006)** and hypoacetylated histone H3 is found at the promoters of the hypoxia-repressed genes, novel immunogenic protein 3 (NIP3) and survivin **(Hoffman et al. 2002; Murai et al. 2005)**. Likewise hypoxia can increase HDAC activity **(Safronova et al. 2009)**.

## Interactions

The effect of HDAC activity on the HIF1 $\alpha$  and NF $\kappa$ B pathways and cytokine production is complicated by the fact that HIF1 $\alpha$  and NF $\kappa$ B interact. The HIF-1 $\alpha$  promoter contains numerous consensus sequences for both NF- $\kappa$ B and HIF-1, which allows the maintenance of an autoregulator loop when NF- $\kappa$ B is activated under normoxia **(Tacchini et al. 2004; Gorlach et al. 2008)**. Indeed the NF $\kappa$ B subunits p50 and p65 (RelA) directly interact with a highly conserved classical NF $\kappa$ B consensus site in the HIF1A promoter **(Belaiba et al. 2007; Bonello et al. 2007; Diebold et al. 2008)** and, RelB, c-Rel, and p52 also modulate HIF1A transcription **(Rius et al. 2008; van Uden et al. 2008)**. Upon exposure to short term hypoxia, NF $\kappa$ B subunits translocate to the nucleus, where they interact with the HIF1A promoter **(Belaiba et al. 2007)**.

NF $\kappa$ B activity in cultured cells has been shown to be increased by hypoxic exposure **(Koong et al. 1994)** but this requires substantially lower levels of oxygenation (e.g. 0.02%) than those necessary for HIF activation. Interestingly, the oxygen-sensing HIF prolyl hydroxylases (PHD enzymes) are able to regulate I $\kappa$ B-dependent NF $\kappa$ B signalling via prolyl hydroxylation **(Cummins et al. 2006)** and FIH, which controls HIF transactivation, can hydroxylate p105 (NF $\kappa$ B1) and IKBA in an oxygen-dependent manner **(Cockman et al. 2006)**. Cellular responses to TNF $\alpha$ , colchicine and HGF suggest protein cross-talk between the NF $\kappa$ B and HIF pathway is also occurring **(Jung, Isaacs et al. 2003b; Zhou et al. 2003; Tacchini et al. 2004)**. Additionally, activation of the HIF1A promoter in response to ROS and oxidative stress is dependent on NF $\kappa$ B binding **(Bonello et al. 2007; Rius et al. 2008; van Uden et al. 2008)**, and knockout studies have shown that perturbations of the NF $\kappa$ B pathway can have profound effects on HIF activation **(Rius et al. 2008)**.

Further complication arises from the fact that experiments have shown that LPS the expression of almost all HDACs, albeit to different magnitudes and kinetics (**Aung et al. 2006**), as well as affecting HIF-1 $\alpha$ , NF $\kappa$ B, and the cytokines which feedback on these pathways. Following LPS stimulation of macrophages HDAC1 is gradually upregulated until 8 h, and becomes stabilized by 24 h after stimulation while HDAC6, 10 and 11 are gradually down regulated up to 8 h before beginning to recover after 24 h while HDAC4, 5 and 7 are also down regulated but are upregulated 24 h after LPS stimulation (**Aung et al. 2006**). An upregulation of HDAC1 with LPS exposure is interesting, as proinflammatory signalling such as those activated by LPS, IL-1 $\beta$ , TNF $\alpha$  and IFN $\gamma$  has been shown to induce HDAC1 ubiquitination and proteosomal degradation in a mechanism involving the same intermediaries as NF- $\kappa$ B signalling (**Gopal et al. 2006**). Some of the effects of bacterial components on HDAC expression are very specific, for example, *Legionella pneumophila* infection can increase the expression of HDAC5 but not other HDACs (**Schmeck et al. 2008**). A recent study also showed that LPS increased HDAC activity, with induced HDAC3 activation occurring via mitochondrial ROS and c-Src signalling (**Zhu et al. 2010**).

LPS, is able to up regulate HIF-1 $\alpha$  independently of oxygen tension (**Blouin et al. 2004; Peyssonnaux et al. 2005; Frede et al. 2006; Kim, Kim et al. 2007; Peyssonnaux et al. 2007**) which is thought to occur at least in part through NF $\kappa$ B activation via LPS activation of Toll like receptor TLR-4 (**Peyssonnaux et al. 2007**) (**Peyssonnaux et al. 2007; Vink et al. 2007**). TNF $\alpha$ , IL-1 $\beta$  and other proinflammatory cytokines are known to activate HIF-1 $\alpha$  through a receptor-mediated pathway even under normoxia (**Hellwig-Burgel et al. 1999; Thornton et al. 2000; Albina et al. 2001; Haddad et al. 2001; Sandau et al. 2001; Jung, Isaacs, Lee, Trepel, Liu et al. 2003; Scharte et al. 2003; Gilroy et al. 2004; Westra et al. 2007; van Uden et al. 2008**). Studies revealed a fundamental role of NF- $\kappa$ B in transmitting the TNF $\alpha$  signal (**Jung, Isaacs, Lee, Trepel, Liu et al. 2003; Zhou et al. 2003**). LPS challenge in mice with conditional gene targeting revealed HIF-1 $\alpha$  to be a critical determinant of the sepsis phenotype, promoting high level production of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12 (**Peyssonnaux et al. 2007**). Interestingly bacterial exposure is a stronger stimulus for HIF-1 $\alpha$  stabilization than is hypoxia itself, with bacterial-induced HIF-1 $\alpha$  stabilization readily demonstrated even at normoxia (**Hellwig-Burgel et al. 1999; Thornton et al. 2000; Haddad et al. 2001; Sandau et al. 2001; Jung, Isaacs,**

**Lee, Trepel, Liu et al. 2003; Jung, Isaacs et al. 2003a; Vanden Berghe et al. 2003; Peyssonnaud et al. 2005).**

Data from several studies have suggested a co-operation between COX-2 and HIF-1 $\alpha$  (**Agarwal et al. 2003; Jung, Isaacs et al. 2003a; Zhong et al. 2004; Kaidi et al. 2006**). HIF-1 $\alpha$ , has been observed to be regulated by the COX-2 product PGE2 and it has been shown that this HIF-1 $\alpha$  activation is IL-1 $\beta$ -dependent but independent of hypoxia (**Stasinopoulos et al. 2009**). NF- $\kappa$ B is highly inducible by proinflammatory stimuli in human intrauterine tissues, LPS (**Lappas et al. 2006**), TNF $\alpha$  (**Yan et al. 2002; Lappas et al. 2006**), IL-1 $\beta$  (**Ackerman et al. 2004**) leptin and adiponectin (**Lappas et al. 2005**) have all been shown to increase the activation of NF- $\kappa$ B in human intrauterine cells and tissues. Several of the HIF-regulated cytokines themselves are also able to transcriptionally induce HIF- $\alpha$ , suggesting the presence of a positive feedback loop which may promote the septic response or other, more chronic inflammatory conditions.

## **SUMMARY**

In summary, the appropriate expression of cytokines is important to pregnancy and parturition. A high degree of variation in expression between individuals could reflect changes at an epigenetic level. Certainly the HDACs have been directly implicated in cytokine expression; however these effects may be through histone acetylation changes, and also be a result of direct interaction with transcription factors and other cellular proteins. HDACs interact with the NF- $\kappa$ B and HIF1 $\alpha$  pathways which are both responsive to, and regulate cytokine expression. Further interact occurs through changes in HDAC expression and activity, as well as NF- $\kappa$ B and HIF1 $\alpha$  pathway modulation in responses to inflammatory stimulus such as infection. Many of these interactions are likely to be cell/tissue/stimulus dependent, and it remains to be determined how HDACs interact with cytokine expression in human gestational tissues. Insights into HDAC regulation of cytokine expression may provide therapeutic strategies for conditions associated with dysregulation of the cytokine network, such as preeclampsia and infection mediated preterm labour.

**Our central hypothesis is:** Histone deacetylases are involved in the regulation of cytokine production in human gestational tissues.

### **We hypothesise that:**

There are tissue specific differences in HDAC expression by amnion, chorion and villous placenta

HDAC inhibition results in alterations to cytokine expression and production

Cytokine stimulation by LPS is associated with altered HDAC expression production or activity

# *Chapter 2 Histone Deacetylases in the placenta and gestational membranes*

## ***Part A: Preliminary studies***

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### Global histone acetylation in the placenta, amnion and chorio-decidua

**Aim:** To determine if global histone acetylation in amnion, choriodecidua and villous varies with labour and preterm birth.

**Methods:** (For additional details see appendix ).

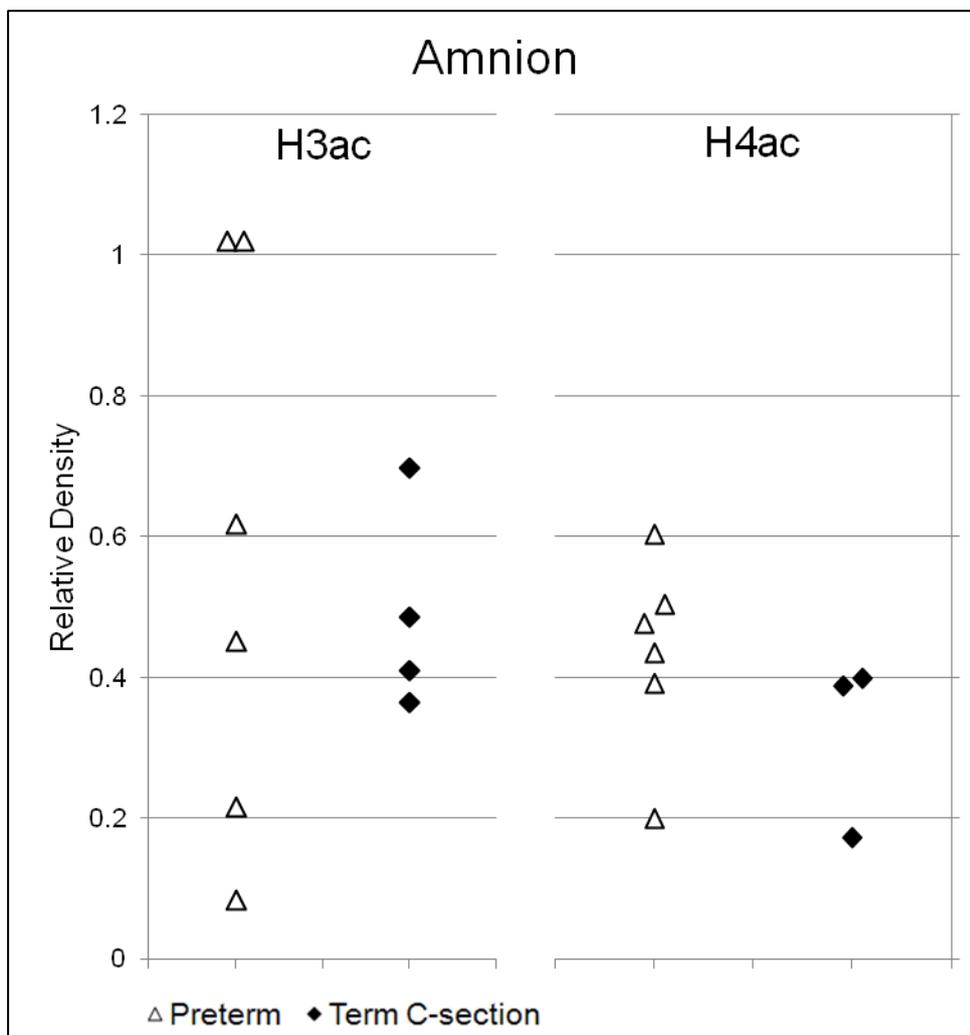
Histone extraction was performed based on a published protocol available for cell lines (**Shechter et al. 2007**), adapted for use with tissue. All steps were performed on ice or at 4°C. Matched amnion, chorio-decidua and placental villous tissue from previous studies and approved for follow up studies were utilised (ethics # 95/225). Frozen tissues were first crushed in the presence of liquid nitrogen. Each tissue sample was then suspended in 1mL of hypotonic lysis buffer (10mM Tris-HCl ph 8.0, 1mM KCL, 1.5mM MgCl<sub>2</sub>, 1mM DTT, 1mM PMSF, protease inhibitor (Roche, New Zealand), “PhosSTOP” Phosphatase Inhibitor Cocktail (Roche) and homogenised before proceeding as directed for cells in the protocol. Briefly, histones were acid extracted in H<sub>2</sub>SO<sub>4</sub>, precipitated with Trichloroacetic acid (TCA) and washed with acetone, before re-suspension in ddH<sub>2</sub>O. A control sample was also prepared in the same manner using placental tissue explants treated for 24 hours with the histone deacetylase inhibitor, sodium butyrate (5mM). Protein concentrations were determined using the Bicinchoninic acid (BCA) assay.

Histones extracts (2µg) were separated using 15% SDS PAGE, even transfer was checked using Coomassie blue to check for protein remaining in the gel, and Ponceau S to reversibly detect protein in the membrane. Following protein electrophoresis (duplicate gels), histones were transferred to nitrocellulose membrane (Amersham Biosciences, Sweden) or, as no housekeeping protein was available as a loading control, gel replicates for each analysis were Coomassie-blue stained. Western blotting was performed using anti-acetyl histone H3 and anti-acetyl histone H4 antibodies (06-599 and 06-866, Millipore, MA, USA). Chemiluminescence detection of HRP-

Conjugated secondary antibodies was performed using Supersignal® West Dura Extended Duration Substrate (Pierce, Thermo Fisher Scientific, USA), and x-ray films. Densitometry was performed using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad) and Quantity One software.

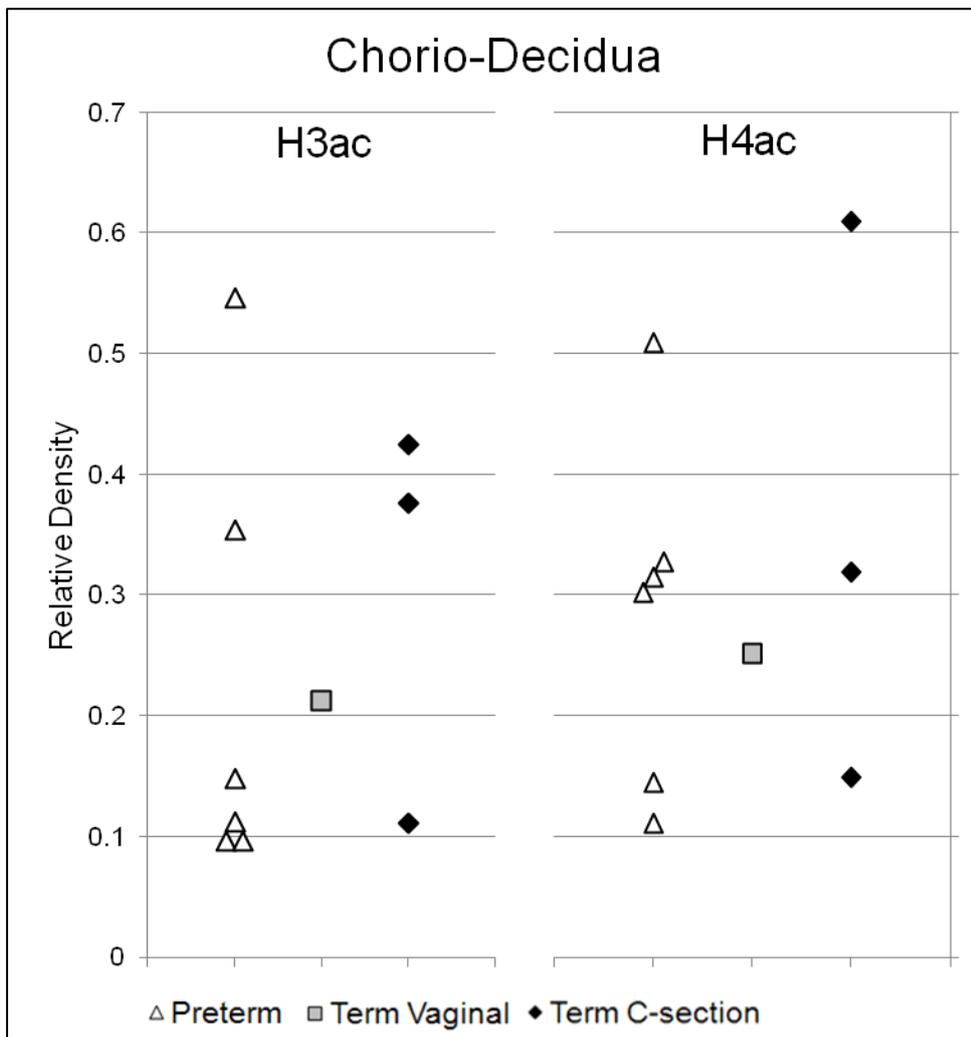
Data were normalised to stained total H3 or H4 protein and calculated as optical density relative to the sodium butyrate treated positive control. Data were analyzed by one-way ANOVA followed by Student t-tests. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). A p value of  $\leq 0.05$  was considered significant.

**Results:** There were no significant differences in global histone H3 or H4 acetylation in amnion [Figure 5] chorio-decidua [Figure 6] or villous tissue [Figure 7] from preterm, term-labouring, or term non-labouring deliveries.



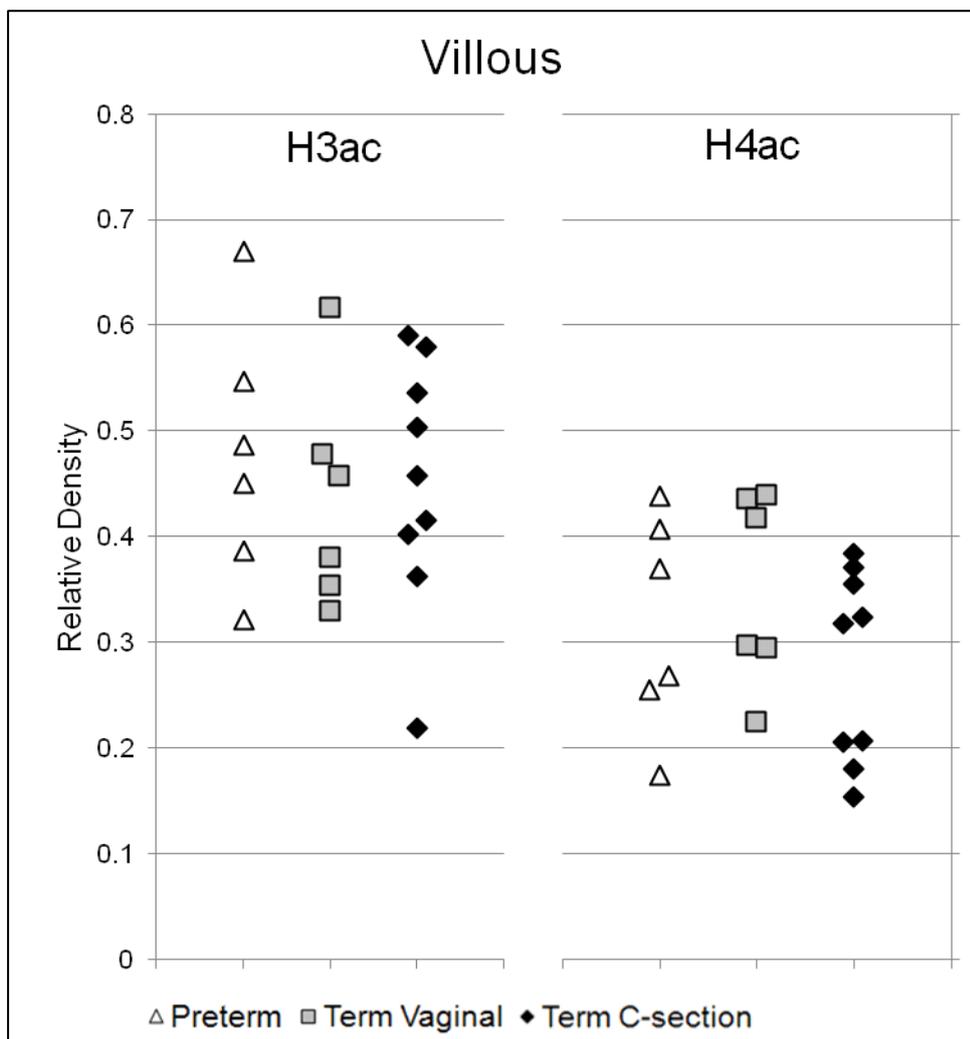
**Figure 5: Relative density of acetylated histone H3 and H4 in amnion.**

*Global histone H3 and H4 acetylation levels in amnion were determined by Western blotting and calculated as optical density relative to the positive control. Data were analyzed by one-way ANOVA followed by Student t-tests. A p value of  $\leq 0.05$  was considered significant.*



**Figure 6: Relative density of acetylated histone H3 and H4 in chorio-decidua.**

*Global histone H3 and H4 acetylation levels in chorio-decidua were determined by Western blotting and calculated as optical density relative to the positive control. Data were analyzed by one-way ANOVA followed by Student t-tests. A p value of  $\leq 0.05$  was considered significant.*



**Figure 7: Relative density of acetylated histone H3 and H4 in placental villous tissue.**

*Global histone H3 and H4 acetylation levels in amnion tissue were determined by Western blotting and calculated as optical density relative to the positive control. Data were analyzed by one-way ANOVA followed by Student t-tests. A p value of  $\leq 0.05$  was considered significant.*

**Discussion:** Term amnion and term chorio-decidua did not always yield sufficient histone for subsequent analysis. In contrast there were no problems with extracting histones from preterm tissues. This is likely due to the relatively low cellularity and greater extracellular matrix components of the amnion in particular, and may also be a function of membrane remodelling with gestation. Sample variation was also higher in the amnion and chorio-decidua which may indicate active modification of acetylation levels is occurring in these tissues. Because only global acetylation levels were examined, localised significant modifications cannot be ruled out. Where statistical comparisons between the three groups were possible, no difference was identified in this study. Global acetylation levels in preterm samples did not appear to correlate with clinical data. Interestingly, clustering of histone H4 acetylation levels in villous tissue seems to suggest two alternate states, but insufficient information is available to speculate as to the variable.

**Conclusions:** There were no significant differences in global histone acetylation detected between term labouring, term without labour, or preterm amnion chorio-decidua and villous tissues. There is limited value to examining global histone acetylation as histone acetylation is labile and globally these changes may be missed. Future studies will concentrate on alterations in histone deacetylases expression and activity.

## Histone deacetylase expression and labour

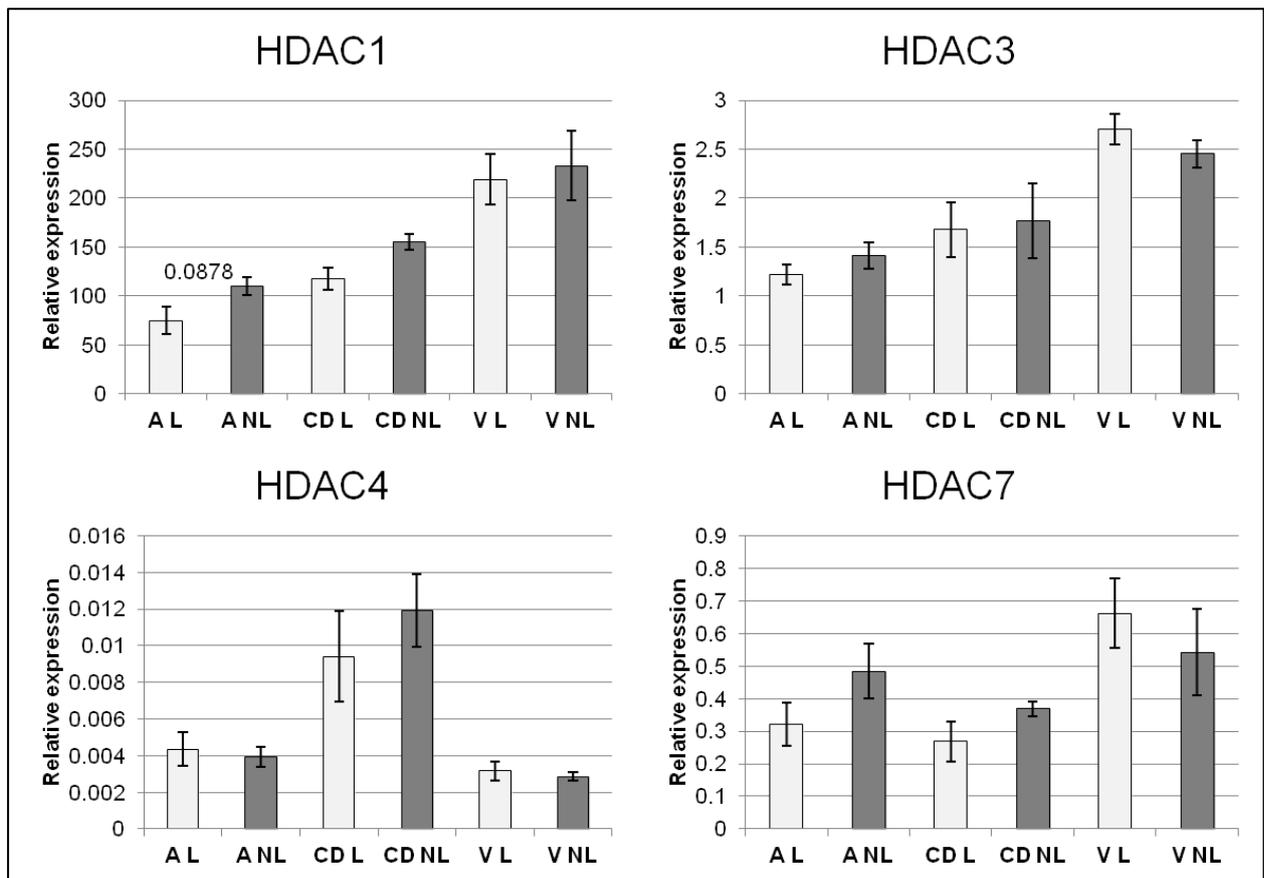
**Aim:** To determine if expression of histone deacetylases in amnion, chorio-decidua and placental villous tissue changes with term labour.

**Methods:** (For additional details see appendix ). Placentas and attached membranes were collected from women delivering at Auckland city hospital with informed written consent and the approval of the NorthernX regional ethics committee. Eight individual placentae from singleton pregnancies of healthy nonsmoking mothers were used in this preliminary study, four after elective caesarean section at term, and four following labour at term. Villous placental tissue, amnion and choriodecidua were sampled within 30 minutes of delivery as previously described (**Keelan et al. 1999**), and frozen for subsequent analysis.

Frozen tissue was crushed in the presence of liquid nitrogen and total RNA was extracted using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). The Total RNA concentration and quality for each sample were determined using a Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Total RNA preparations were then treated with DNase I (Life Technologies) to remove any DNA contaminants and converted to cDNA using SuperScript III and random hexamers (Life Technologies).

Quantitative real-time PCR (qPCR) was performed using Fast SYBR® Green Master Mix (Life Technologies) and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SDS 2.1 software. The qPCR protocol had an initial stage at 95°C for 20s, followed by amplification for 40 cycles; denaturation at 95°C for 1second and annealing/ extension at 60°C for 20 seconds. A dissociation stage was performed for all runs. Class I histone deacetylases HDAC1 and HDAC3 were investigated, along with class IIb histone deacetylases HDAC4 and HDAC7. YWHAZ was used as a housekeeping gene. Primer sequences are given in **Table 1** (section B).

Real-time-PCR results were calculated as absolute values and are given as mean relative expression compared with housekeeping gene; YWHAZ. Data are given as means  $\pm$  SE (n=4). Data were analyzed by one way ANOVA followed by Student's t-tests. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). A p value of  $\leq 0.05$  was considered significant.



**Figure 8: Histone deacetylase expression and labour**

Real-time-PCR results for HDAC1, HDAC3, HDAC4 and HDAC7 in term amnion (A), choriodecidua (CD) and placental villous tissue (V) with and without labour (L / NL). Results were calculated as absolute values and are presented as mean relative expression compared with housekeeping gene; *YWHAZ*,  $\pm$  SE ( $n=4$ ). Significant differences between tissues were calculated by one way ANOVA followed by Students *t*-test.

**Results:** No significant differences between tissues before and after labour were detected [Figure 8]. There was a trend for reduced HDAC1 mRNA expression in amnion after labour ( $p=0.0878$ ).

**Discussion:** While it is likely a larger sample size would yield clearer results, variations such as duration of labour will require extensive sample numbers to control for. Transcriptional changes in cytokine mRNA expression mediated by HDAC promoter activity are likely to happen well in advance of the increased cytokine production seen with labour. After labour of differing durations, alterations in histone deacetylase expression, production and/or activity are of unknown relevance as they are well removed from the event under scrutiny.

**Conclusions:** Given the inherent problems of examining regulatory processes after they have occurred, further investigations will use an *ex vivo* approach.

## ***Part B: Characterisation of histone deacetylases in human gestational tissues***

Manuscript submitted to Molecular Human Reproduction

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Histone deacetylases in human gestational membranes exhibit tissue-specific expression

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## **Abstract**

Despite the importance of appropriate histone deacetylase activity during development, little is known about the role of histone deacetylases (HDAC) in the placenta and membranes. In full-term tissues, modulation of HDAC activity has tissue specific effects on the inflammatory network. As each of the eighteen different HDACs has differing sub cellular locations and sensitivity to HDAC inhibitors, we propose that many of the differences in response to HDAC inhibitors reflect underlying differences in HDACs expression. We have characterised HDAC mRNA expression in normal term human placenta, choriodecidua and amnion. Additionally we examined HDAC activity in nuclear extracts from each tissue type as well as the nuclear protein levels of HDAC1 and HDAC3. We have shown that amnion, choriodecidua and villous placenta, each have distinct HDAC expression profiles. An understanding of the normal expression patterns of the HDACs provides a reference point in understanding disease states linked to inappropriate epigenetic modulation.

Keywords: Histone deacetylase, sirtuin, amnion, choriodecidua, placenta

## Introduction

Gene activity is intrinsically linked to the surrounding chromatin architecture, which can be altered through DNA methylation and the post-translational modification of histone tails (**Dolinoy et al. 2007; Jirtle et al. 2007**). These modifications are also involved in the recruitment of transcription factor complexes (**Berger 2007**). One of these modifications; histone acetylation, is generally associated with transcriptional activation. Histone acetylation levels are maintained through the opposing actions of the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). Inhibition of HDAC activity promotes the hyper-acetylation of the histones via unopposed HAT activity. However, as HDACs associate with, and deacetylate a range of proteins other than histones, HDAC inhibition can have a multitude of other effects (**Yang and Seto 2007**).

Appropriate HDAC activity is essential for embryogenesis. The histone deacetylase inhibitor (HDACi) valproic acid (VPA), an antiepileptic drug, is a known human teratogen (**Ornoy 2009**), readily crossing the human placenta (**Nau et al. 1981; Albani et al. 1984**) resulting in a range of defects to the developing fetus (**Ornoy 2009**). Analogs of VPA which lack histone deacetylase inhibitory activity have been shown to be less teratogenic (**Gurvich et al. 2005**) suggesting that it is the inhibition of histone deacetylase activity itself which causes most of the adverse effects. It has been shown that VPA treatment is sufficient to fully reprogram human first-trimester amniotic fluid stem cells to pluripotency, exemplifying the role HDACs must play in early developmental pathways (**Moschidou et al. 2012**). Genetic variation could play a role in susceptibility to VPA teratogenesis, with the effects of VPA exposure on fetal mice dependent on both maternal and fetal genetic strain (**Downing et al. 2010**). This suggests that the maternal genotype or uterine environment is an important mediator of VPA teratogenicity. Interestingly there are also strain-associated differences in acetylation of histones H3 and H4 in both the embryo and placenta of mice following *in utero* VPA exposure (**Downing et al. 2010**). This differential response to HDAC inhibition suggests genetic variation in histone deacetylase expression could play a large role in response to environmental cues during development.

Despite the importance of appropriate HDAC activity during development, little is known about the role of histone deacetylases in the placenta and membranes. Even small changes in HDAC expression or activity in the placenta or membranes could have far reaching effects if normal function or responses to stimuli are compromised. In full-term

tissues, modulation of HDAC activity has tissue specific effects on the inflammatory network. HDACi treatment of choriodecidual explants by trichostatin A (TSA) results in differential cytokine responses when stimulated, with altered IL-1 $\beta$  but not IL-10, or TNF $\alpha$  (**Sato et al. 2006**) production, but TSA has no effect on cytokine production by the amnion (**Mitchell et al. 2012**). Conversely lipopolysaccharide (LPS) stimulated TNF $\alpha$  and IL-10 production from placental villous explants is altered by TSA treatment but there is no alteration in IL-1 $\beta$  production (**Munro et al. 2013**). Additionally inhibition of prostaglandin synthesis occurs when the amnion is treated with histone deacetylase inhibitors and a methylation inhibition agent, a phenomenon not seen in the adjacent choriodecidua (**Mitchell 2006**).

What then is the normal population of histone deacetylases in the placenta and gestational membranes? There are eighteen human HDACs, grouped into four classes within two families, based on sequence similarity and cofactor dependency (**Khochbin et al. 2001; Grozinger et al. 2002; Pandey et al. 2002; Verdin et al. 2003; Gregoretta et al. 2004**). As each of the different HDACs has differing sub-cellular locations and sensitivity to HDACi, we propose that many of the differences in response to HDAC inhibitors reflect an underlying difference in the population of HDACs expressed. We therefore sought to characterise HDAC expression in normal term human placenta, choriodecidua and amnion. Additionally we examined HDAC activity in nuclear extracts from each tissue type. We also examined the nuclear protein levels of class I HDACs; HDAC1 and HDAC3, which are involved in large multi-protein regulatory complexes(**Hassig et al. 1997; Tong et al. 1998; Fischle et al. 2001; Humphrey et al. 2001; You et al. 2001; Fischle et al. 2002; Hakimi et al. 2002; Yang and Seto 2008**) and are particularly susceptible to inhibition from TSA and VPA (**Khan et al. 2008**).

## Methods

(For additional details see appendix )

Placental tissues and gestational membranes were collected from women undergoing elective caesarean section at term, with the approval of the NorthernX Regional Ethics Committee. Villous placental tissue, amnion and choriondecidua were sampled within 30 minutes of delivery as previously described (**Keelan et al. 1999**), and frozen for subsequent analysis. Unless otherwise stated all reagents for subsequent analysis were used according to the manufacturer's instructions.

### ***RNA extraction and analysis by RT-PCR***

Frozen tissue was crushed in the presence of liquid nitrogen and total RNA was extracted using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). The Total RNA concentration and quality for each sample were determined using a Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Total RNA preparations were then treated with DNase I (Life Technologies) to remove any DNA contaminants and converted to cDNA using SuperScript III and random hexamers (Life Technologies).

Quantitative real-time PCR (qPCR) was performed using Fast SYBR® Green Master Mix (Life Technologies) and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SDS 2.1 software. The qPCR protocol had an initial stage at 95°C for 20s, followed by amplification for 40 cycles; denaturation at 95°C for 1 second and annealing/ extension at 60°C for 20 seconds. A dissociation stage was performed for all runs.

Primers for HDAC 2 were obtained from Primer Bank (**Wang et al. 2003**) (ID#293336690c1). All other HDAC Primers were designed using Primer3 software (**Rozen et al. 2000**) and checked using NetPrimer software (PREMIER Biosoft, Palo Alto ,California USA). Primer sequences are given in

**Table 1.** Purified and sequenced PCR products were used to create standard curves for each primer pair, which were then used to determine absolute levels of PCR product for each target and sample. All samples were analyzed in triplicate.

**Table 1: RT-PCR Primer sequences**

Name	NCBI GENE ID	FORWARD 5'- 3'	REVERSE 3'- 5'	Amplicon Size
<b>HDAC1</b>	3065	GGAAATCTATCGCCCTCACA	TTGCCACAGAACCACCAGTA	206
<b>HDAC2</b>	3066	ATGGCGTACAGTCAAGGAGG	TGCGGATTCTATGAGGCTTCA	112
<b>HDAC3</b>	8841	TGGCTTCTGCTATGTCAACG	TTGATAACCGGCTGGAAAAG	297
<b>HDAC4</b>	9759	CCTCTACACATCGCCATCCT	GGCTGCTCCAGTAAGACCAT	237
<b>HDAC5</b>	10014	GTAGCCATCACCGCAAAACT	GTCCTCCACCAACCTCTTCA	199
<b>HDAC6</b>	10013	TATCTGCCCCAGTACCTTCG	GGACATCCCAATCCACAATC	242
<b>HDAC7</b>	51564	GGCTGCTTTTCAGGATAGTCG	TTCATCAGTTGCTGCGTCAT	162
<b>HDAC8</b>	55869	TTATGACTGCCAGCCACTG	TTCCCAGGACAGCATCATTG	188
<b>HDAC9</b>	9734	GACTCACTCTTCCCCTGCTG	CCAGTTTCTTGCAGTCGTGA	192
<b>HDAC10</b>	83933	CTTCTCCTGGCACCGCTATG	CCGATGGCTGAGTCAAATCC	234
<b>HDAC11</b>	79885	ATCACGCTCGCCATCAAGTT	TCATCCTCTGTGCCCCACTC	224
<b>SIRT1</b>	23411	GCTGATGAACCGCTTGCTAT	ATGGGGTATGGAACCTGGAA	174
<b>SIRT2</b>	22933	GACTTTCGCTCTCCATCCAC	GAGTAGCCCCTTGTCTTCA	201
<b>SIRT3</b>	23410	AGCAACCTCCAGCAGTACGA	CGACACTCTCTCAAGCCATC	216
<b>SIRT4</b>	23409	ACCTGGGAGAAACTCGGAAA	TCAGGACTTGGAACGCTCT	178
<b>SIRT5</b>	23408	ATGCGACCTCTCCAGATTGT	GTCGGAACACCACTTTCTGC	206
<b>SIRT6</b>	51548	GCAGTCTTCCAGTGTGGTGTTC	CGCTCTCAAAGGTGGTGTTCG	140
<b>SIRT7</b>	51547	CCATTTTCTCACTGGCGACT	CCTTTTGTGCGTTTTGTGC	173
<b>YWHAZ</b>	7534	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT	189

### ***Nuclear protein extraction, quantification of HDAC activity and Western blotting***

Frozen tissue was crushed in the presence of liquid nitrogen and nuclear extracts prepared using EpiQuik™ Nuclear Extraction Kit 1 (Epigentek, Farmingdale, NY, USA). Protein concentrations of nuclear extracts were quantified using the RC DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). HDAC activity of extracts was determined using a colorimetric, Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Epigentek). Following protein electrophoresis, nuclear extracts (5µg) were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) and Western blotting was performed using antibodies for HDAC1 and HDAC3 (ab19845 and ab16047 at 1µg/mL, Abcam, Cambridge, UK) and β-actin (A1978 at 1:30000, Sigma-Aldrich, St. Louis, MO, USA). Chemiluminescence detection of HRP-Conjugated secondary antibodies was

performed using Super-Signal West Dura (Thermo Scientific, Illinois) and x-ray films. Densitometry was performed using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad) and Quantity One software.

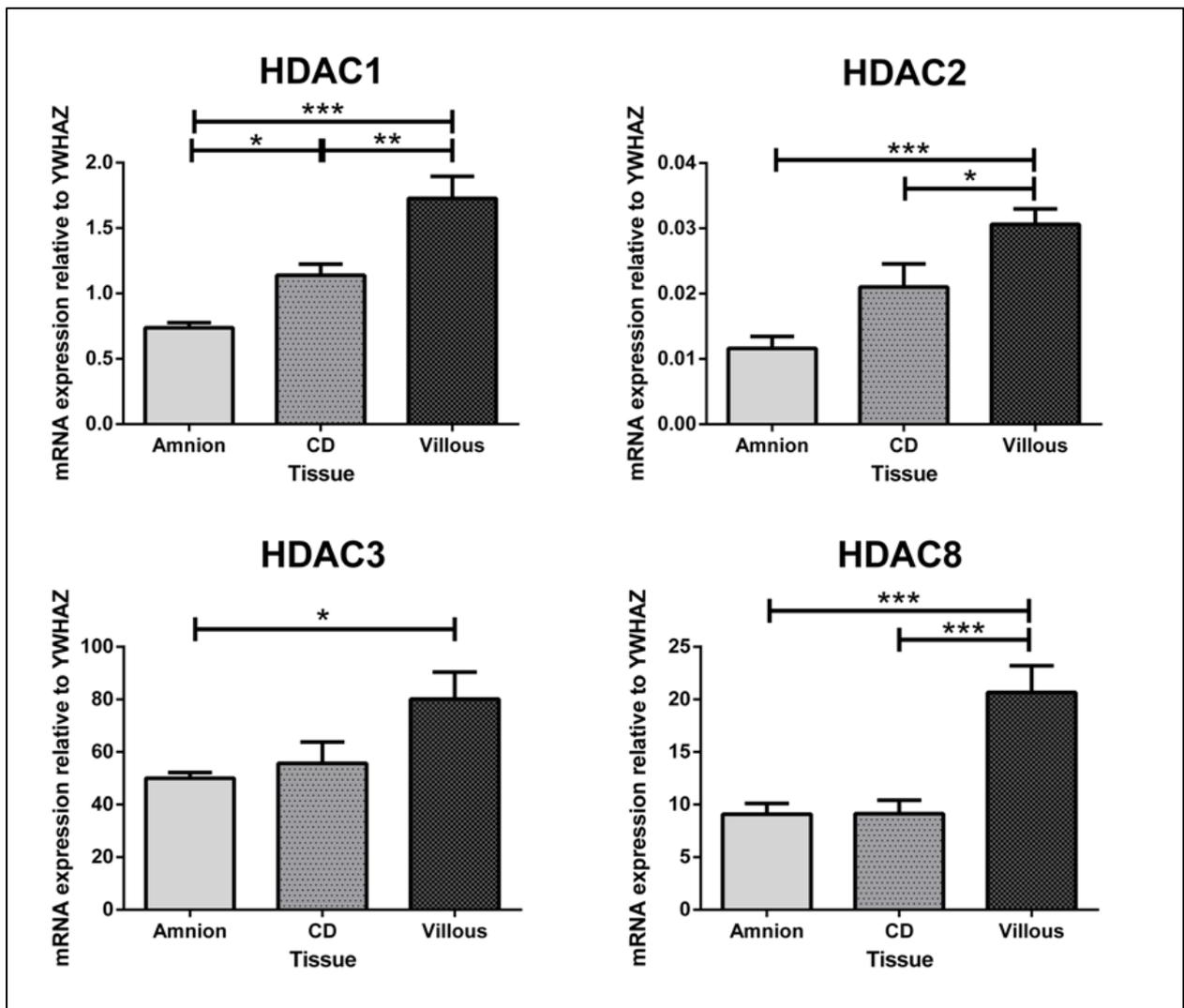
### ***Statistical Analysis***

Real-time-PCR results were calculated as absolute values and are given as mean relative expression compared with housekeeping gene; YWHAZ **[Figure 9-Figure 13]**. HDAC activity was calculated as ng (deacetylated product) per minute, per mg of nuclear extract **[Figure 14]**. Protein production is calculated as density (OD/mm<sup>2</sup>) relative to  $\beta$ -actin loading control, as a proportion of the internal control **[Figure 15]**. All data are given as means  $\pm$  SE (n=8). Data were analyzed by one way ANOVA followed by Tukey's multiple comparisons test. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). A p value of  $\leq 0.05$  was considered significant.

## **Results**

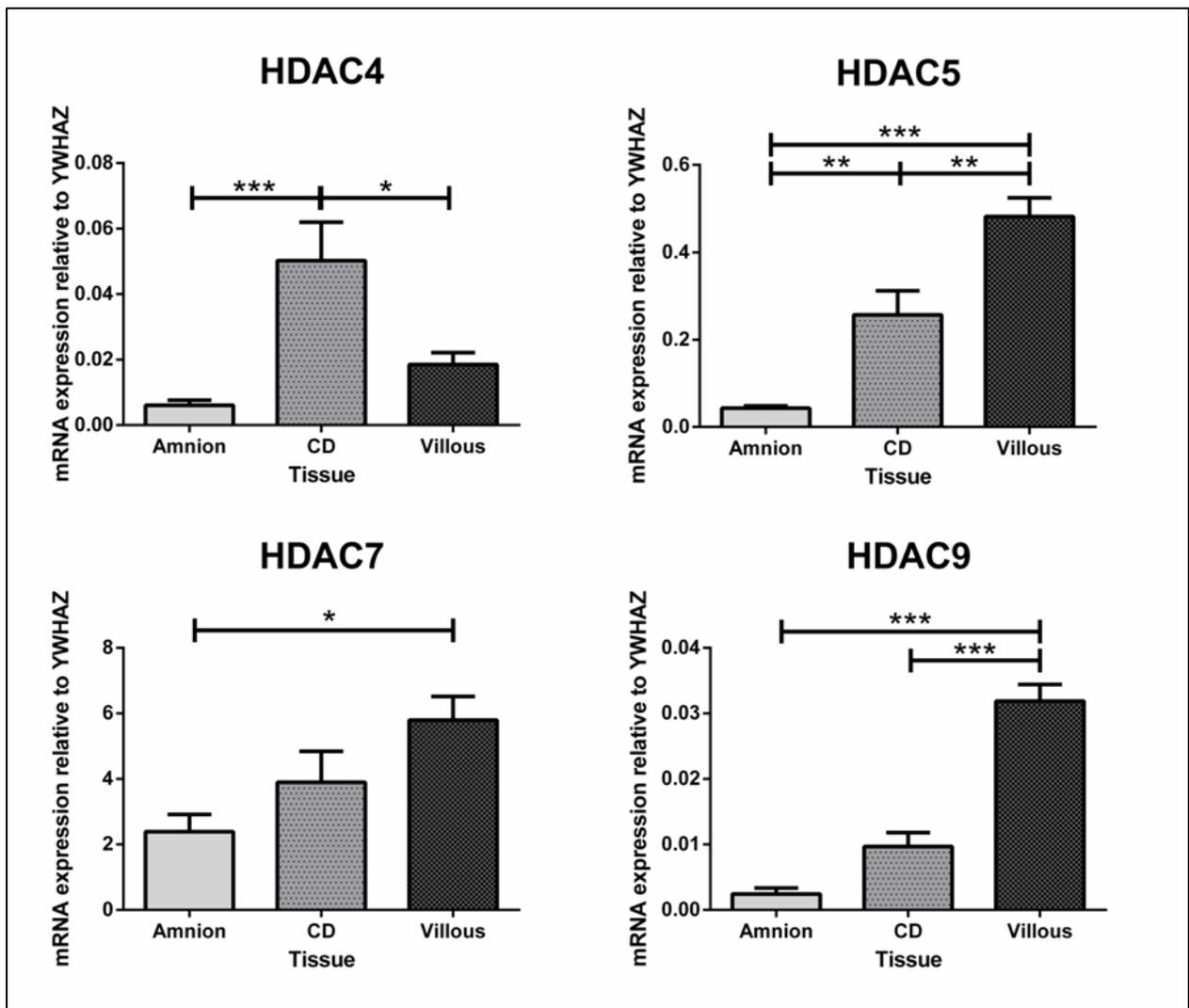
### ***HDAC mRNA expression in the placenta, and membranes***

HDAC mRNA transcript levels in the amnion, choriondecidua, and villous placenta show variation by tissue type, suggesting tissue specific regulation of HDAC expression **[Figure 9-Figure 13]**. There were no statistically significant differences in the mRNA expression of SIRT4 or SIRT7 between the tissues **[Figure 13]**. In general, the amnion has the lowest expression for each of the eighteen human histone deacetylases, with statistical significance reached for fourteen different HDACs when mRNA transcript levels in the amnion are compared with those of the villous placenta. HDAC expression by the chorio-decidua trends toward an intermediate position for most HDACs, with expression higher or equal to that of the amnion, and usually lower than the villous placenta. HDAC4, HDAC10 and HDAC11 are the exceptions to this pattern with mRNA transcript levels in the choriondecidua being equal to or higher than those of the villous placenta **[Figure 10-Figure 12]**.



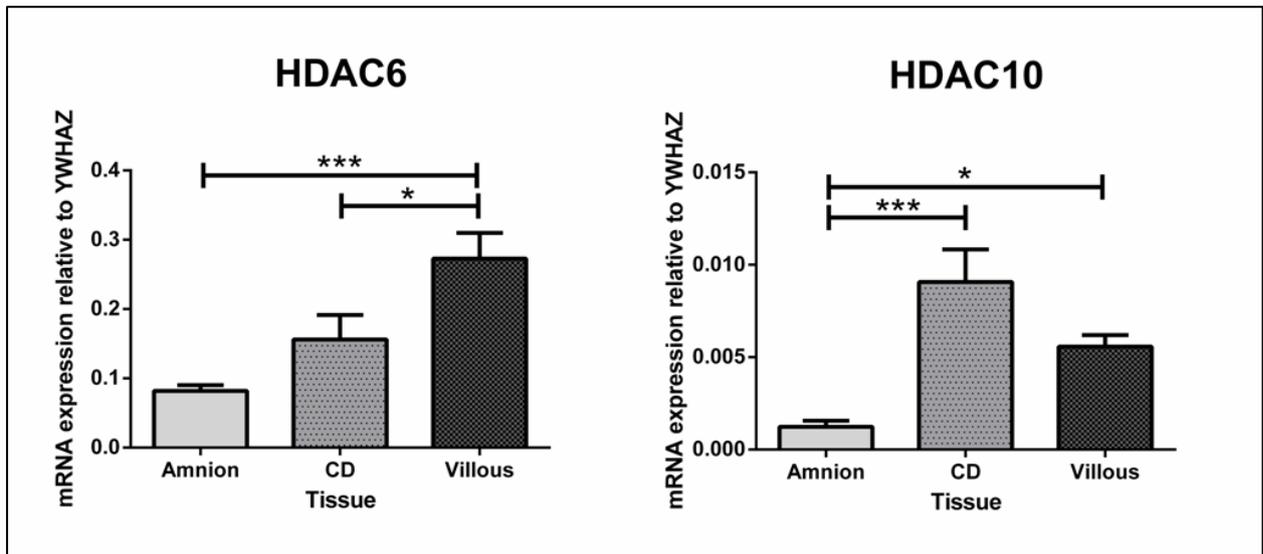
**Figure 9: Class I HDACs in the placenta and membranes**

Real-time-PCR results for HDAC1, HDAC2, HDAC3 and HDAC8 in amnion, choriodecidua (CD) and placental villous tissue. Results were calculated as absolute values and are presented as mean relative expression compared with housekeeping gene; YWHAZ,  $\pm$  SE (n=8). Significant differences between tissues were calculated by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .



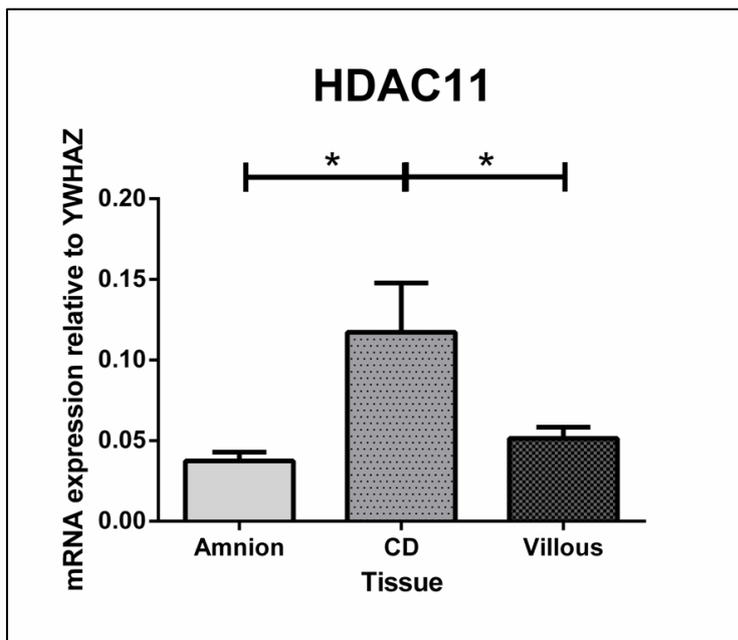
**Figure 10: Class IIa HDACs in the placenta and membranes**

Real-time-PCR results for HDAC4, HDAC5, HDAC7 and HDAC9 in amnion, choriodecidua (CD) and placental villous tissue. Results were calculated as absolute values and are presented as mean relative expression compared with housekeeping gene; YWHAZ,  $\pm$  SE (n=8). Significant differences between tissues were calculated by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .



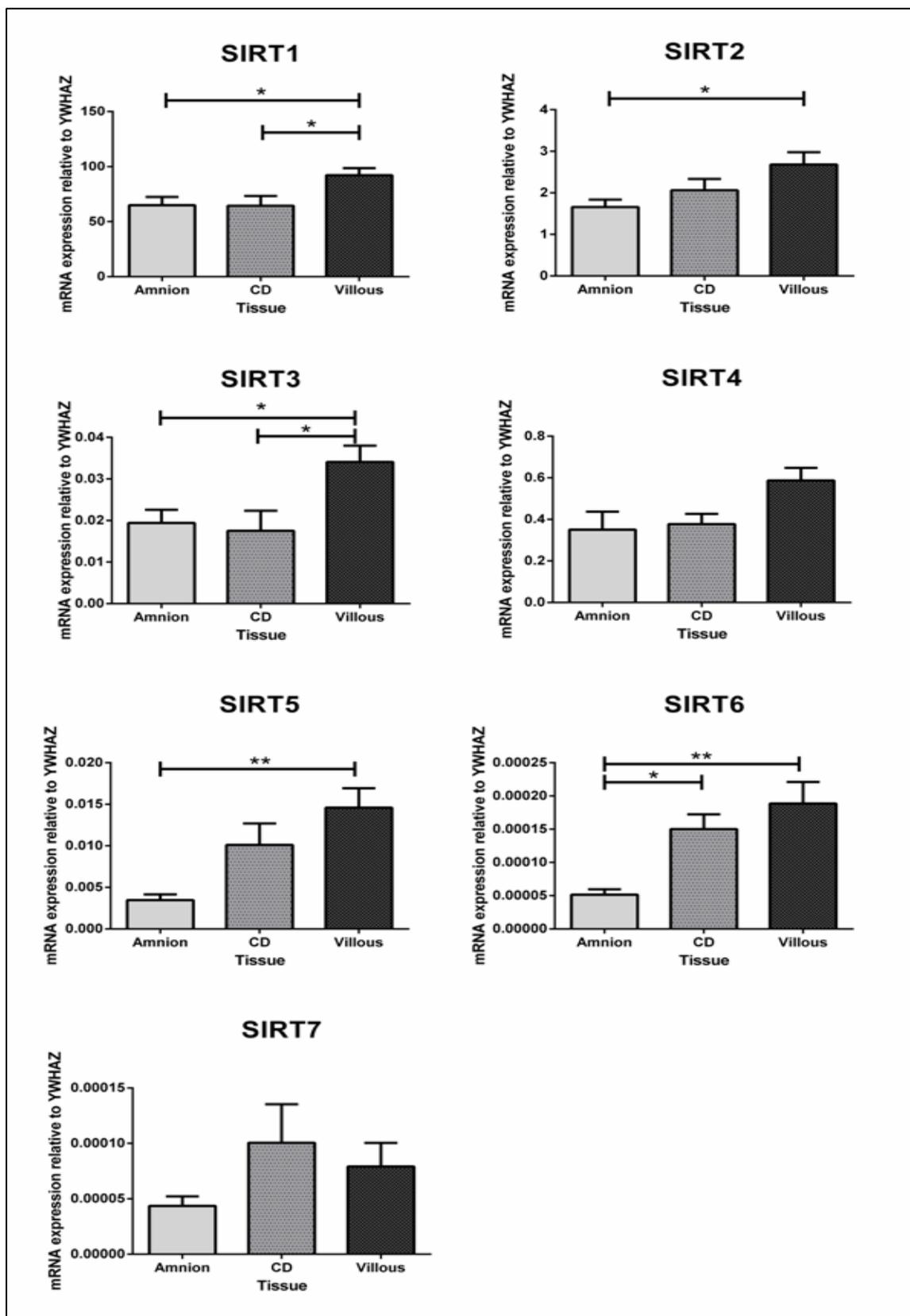
**Figure 11: Class IIb HDACs in the placenta and membranes**

Real-time-PCR results for HDAC6 and HDAC10 in amnion, choriodecidua (CD) and placental villous tissue. Results were calculated as absolute values and are presented as mean relative expression compared with housekeeping gene; YWHAZ,  $\pm$  SE (n=8). Significant differences between tissues were calculated by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .



**Figure 12: Class IV HDACs in the placenta and membranes**

Real-time-PCR results for HDAC11 in amnion, choriodecidua (CD) and placental villous tissue. Results were calculated as absolute values and are presented as mean relative expression compared with housekeeping gene; YWHAZ,  $\pm$  SE (n=8). Significant differences between tissues were calculated by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

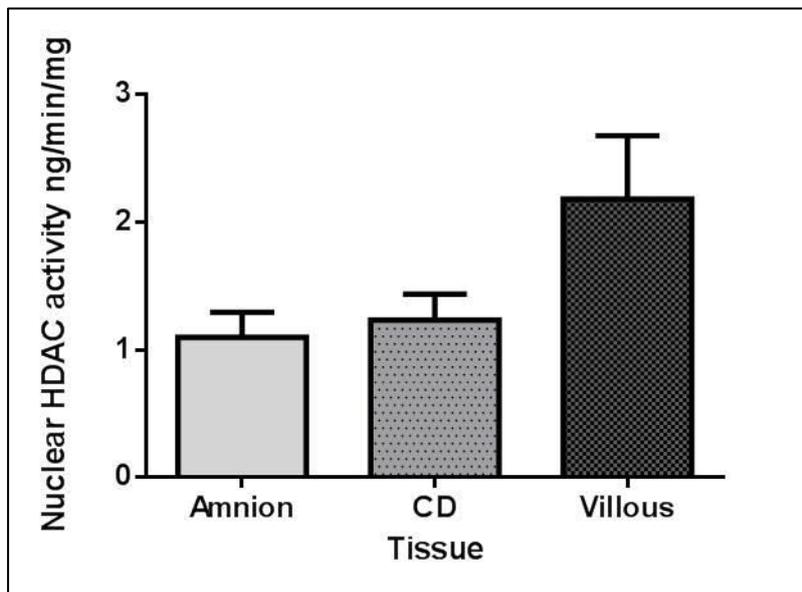


**Figure 13: Class III HDACs (sirtuins) in the placenta and membranes**

Real-time-PCR results for SIRT1-7 in amnion, choriondecidua (CD) and placental villous tissue. Results were calculated as absolute values and are presented as mean relative expression compared with housekeeping gene; YWHAZ,  $\pm$  SE (n=8). Significant differences between tissues were calculated by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

### **HDAC activity of nuclear extracts**

Total HDAC activity measured in nuclear extracts from amnion, choriodecidua and villous placenta reveals limited variation between the tissues [Figure 14]. There is a trend towards increased activity by villous nuclear extracts relative to the membranes ( $p= 0.0748$ ).

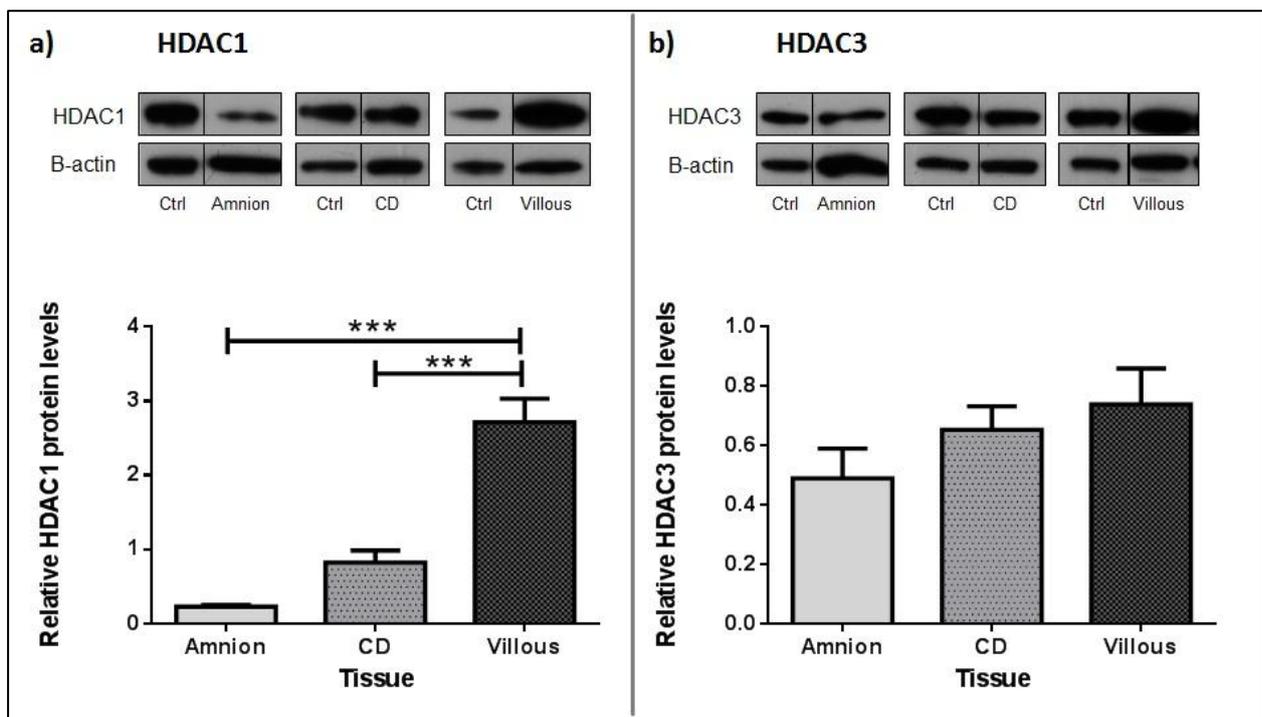


**Figure 14: HDAC activity in nuclear extracts from placenta and membranes**

*HDAC activity was determined by colorimetric detection of deacetylated histone product, following incubation of nuclear extracts with acetylated histone substrate. Activity of nuclear extracts was calculated as ng (deacetylated product) per minute, per mg of nuclear extract. Data were analysed by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .*

### **HDAC1 and HDAC3 nuclear protein**

HDAC1 protein production in nuclear extracts from the villous placenta is significantly elevated when compared to both the amnion and choriodecidua [Figure 15a]. In contrast there are no significant differences in HDAC3 nuclear protein levels [Figure 15b].



**Figure 15: HDAC1 and HDAC3 protein in nuclear extracts from placenta and membranes**

HDAC1 (a) and HDAC3 (b) protein production was calculated as density (OD/mm<sup>2</sup>) relative to  $\beta$ -actin loading control, as a proportion of the internal control (Ctrl). Data are presented as means  $\pm$  SE (n=8). Significant differences between tissues were calculated by one way ANOVA followed by Tukey's multiple comparisons test. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

## Discussion

There are eighteen human HDACs, grouped into four classes within two families, and mRNAs of all eighteen were found to be expressed in human placenta, choriodecidua and amnion. The Classical Family of HDACs is comprised of classes I, II and IV, which share sequence similarity and require Zn<sup>2+</sup> for deacetylase activity. In contrast the Sirtuin Family (silent information regulator2 (Sir2)-related protein) comprises the class III HDACs, which require NAD<sup>+</sup> as the cofactor (Michan et al. 2007).

The Class I HDACs; HDAC1, -2, -3, and -8 are predominantly expressed in the nucleus, though HDAC3 can shuttle to the cytoplasm. However while HDAC8 originally appearing to be ubiquitous and nuclear in location, it has since been reported that it is restricted to cells showing smooth muscle differentiation, where it was mainly detected in the cytosol (Waltregny et al. 2004). Interestingly, HDAC inhibitors have previously been shown to inhibit human uterine contractions (Moynihan et al. 2008) and delay the

initiation of parturition in mice (**Condon et al. 2003**). Recently it was shown that this can be attributed at least in part to the histone-independent actions of HDAC8, which controls the acetylation of heatshock protein 20 (Hsp20). Specific inhibition of HDAC8 in human myometrial tissue *ex vivo* resulted in increased Hsp20 acetylation, no change in histone acetylation or global gene expression, and inhibition of both spontaneous and oxytocin-augmented contractions (**Karolczak-Bayatti et al. 2011**). HDAC3 and HDAC8 are similar in structure (**de Ruijter et al. 2003**) and in our study they had the highest mRNA expression in villous placenta. This was not surprising for HDAC3 as in the placenta HDAC3 is known to bind to and regulates the human GCMA transcription factor which regulates the expression of syncytin, a placental fusogenic protein mediating trophoblastic fusion (**Yu et al. 2002; Chuang et al. 2006**). There were no significant differences in mRNA expression between the amnion and chorion for HDAC3 or HDAC8. Likewise there were no significant differences between the tissue types when examining nuclear HDAC3 protein, however as HDAC3 can localise to the cytoplasm, differences could exist in that compartment.

HDAC1 and HDAC2 are highly similar with 82% sequence identity (**de Ruijter et al. 2003**). They also exhibit a high degree of functional overlap; however knockout studies in mice have revealed a number of distinct and non-redundant biological functions (**Brunmeir et al. 2009; Dovey et al. 2010**). Our results reveal similar patterns of mRNA expression for each at term, with mRNA transcript levels significantly higher in the villous placenta, a trend which is also apparent when examining HDAC1 protein levels. Expression of HDAC1 mRNA in the chorion was intermediate to that of the amnion and villous placenta; however the trend wasn't significant for HDAC2.

Class II contains HDAC4, -5, -6, -7, -9, -10, and can be further subdivided into Class IIa, and Class IIb (**Yang and Seto 2007**). Class IIa consists of HDAC4, -5, -7 and -9, which mainly function as transcriptional co-repressors, possessing other repression domains in addition to their deacetylase domains (**Sparrow et al. 1999; Wang et al. 1999; Kao et al. 2000; Lemercier et al. 2000; Zhou, Richon, Rifkind et al. 2000**). Class IIa members display distinct sub cellular localizations (**Bertos et al. 2001**), with a range of mechanisms and signals promoting either nuclear retention or export (**Fischle et al. 1999; Miska et al. 1999; Grozinger et al. 2000; McKinsey, Zhang, Lu et al. 2000; Wang et al. 2000; Kao et al. 2001**). While HDAC5,-7,and-9 show similar mRNA expression patterns to the Class I HDACs in the placenta and membranes, we found that HDAC4 mRNA expression was significantly elevated in the chorion. It is

possible that the relative abundance of HDAC4 may be due to expression by maternal decidua; however there is little information on normal expression of HDAC4 in the uterus, with the only report on expression in endometrial cancer (**Ahn et al. 2010**). Another possibility is that the observed differences in HDAC4 expression between the tissues are linked to regulation of the prostaglandin pathway. In synovial fibroblasts, HDAC4 contributes to the transcriptional induction of microsomal prostaglandin E synthase-1 (mPGES-1), which catalyzes the terminal step in the biosynthesis of prostaglandin E2 (PGE2) (**Chabane et al. 2009**). Additionally, HDAC inhibition suppresses IL-1 $\beta$  induced PGE2 synthesis, COX-2 expression, proteoglycan degradation and mPGES-1 expression in chondrocytes (**Chabane et al. 2008; Zayed et al. 2011**), and IL-1 $\beta$  induced PGE2 synthesis and COX-2 expression in the amnion (**Mitchell 2006**).

The Class IIb HDACs, HDAC6 and HDAC10, are distinct from Class IIa, with HDAC6 containing a second catalytic domain while HDAC10 contains a pseudo duplication (**Guardiola et al. 2002**). HDAC6 was the first HDAC identified as actively maintained in the cytoplasm (**Verdel et al. 2000**) and its most well characterized role is as an  $\alpha$ -tubulin deacetylase (**Hubbert et al. 2002; Matsuyama et al. 2002; Haggarty et al. 2003; North et al. 2003; Palazzo et al. 2003; Zhang et al. 2003**). HDAC6 also interacts with heat-shock protein Hsp90 regulating its acetylation and thus the stability of the Hsp90 chaperone complex (**Kovacs et al. 2005; Murphy, Morishima et al. 2005**). Inhibition of HDAC6 also enhances Hsp90 mediated maturation of matrix metalloproteinase-2, which was shown to be associated with increased breast cancer cell invasion *in vitro* (**Verdel et al. 2003**). Like HDAC6, HDAC10 also appears to associate with molecular chaperones, with a recent study identifying HDAC10 in a stable complex with deacetylated heat shock protein 70 (Hsp70) (**Liu and Khachigian 2009**). Treatment of cortical neurons with HDAC inhibitors up-regulates Hsp70 (**Marinova et al. 2009**) which is also elevated in a number of pathological conditions of pregnancy (**Chaiworapongsa et al. 2008**). While HDAC6 shows similar expression patterns to the other HDACs, we found that there was a trend for elevated HDAC10 mRNA expression in the choriodecidua as compared to amnion and villous tissue.

The sole member of Class IV is HDAC11, which resides in the nucleus and is considered an immunoregulator due to its role in the regulation of interleukin 10 (IL-10) expression (**Georgopoulos 2009; Villagra et al. 2009**). Like HDAC4, we found

HDAC11 to be significantly elevated in the choriodecidua when compared to the amnion and villous tissue.

The Class III HDACs are the sirtuins, SIRT1-7(Yang and Seto 2007). Their deacetylase activity depends on the cofactor NAD<sup>+</sup>, rather than zinc, and they are inhibited by different compounds than the classical HDACs (Michan et al. 2007; Alcain et al. 2009). SIRT1 and SIRT2 are capable of shuttling between the cytoplasmic and nuclear compartments (North et al. 2007; Tanno et al. 2007). SIRT1 and -2 were the most highly expressed of the sirtuins, and had significantly higher expression in villous tissue. This is supported by a recent study which also identified SIRT1 and SIRT2 mRNA expression as being significantly higher in placenta compared with the membranes, and reported similar gene mRNA expression between amnion and choriodecidua (Lappas et al. 2011). The same study also found reductions in SIRT1 (but not SIRT2) mRNA expression and protein production with labour and proinflammatory stimuli, while activation of SIRT1 had anti-inflammatory effects (Lappas et al. 2011).

SIRT3-5 are mitochondrial HDACs (Brehm et al. 1998; Ropero et al. 2006; Ahuja et al. 2007; Yang, Valvo et al. 2008; Marks et al. 2009) and expression was not dissimilar to the majority of the HDACs investigated in this study. SIRT6 and -7 are predominantly nuclear. SIRT6 is a critical regulator of transcription, genome stability, telomere integrity, DNA repair, and metabolic homeostasis (Tennen et al. 2010) while SIRT7 is associated with active rRNA genes (Ford et al. 2006). SIRT6 mRNA expression and protein production have been shown to be reduced in preterm fetal membranes both with pre-term spontaneous labour, and exposure to LPS (Lim et al. 2013).

There were no significant differences in HDAC activity between tissues, however only total activity was measured, which may have masked tissue specific differences and changes in individual HDAC activity. Class IIa HDACs like HDAC4, are for example, inefficient histone deacetylases compared with Class I and IIb HDACs (Hassig et al. 1998; Fischle et al. 1999; Hu, Chen et al. 2000; Fischle et al. 2002) so differences may not be well recognized with a general HDAC assay. Likewise as activity measurements were limited to nuclear extracts, differences in HDAC subcellular localization would have impacted on the results.

## **Conclusions**

We were able to identify mRNA transcripts for all eighteen histone deacetylases in amnion, choriodecidua and placental villous tissue. In general, the amnion had the lowest expression for each HDAC while choriodecidua expression is equal to or higher than that of the amnion, and villous expression is the highest. Three HDACs had distinctly different patterns of expression with elevated expression in the choriodecidua, equaling or outstripping that of the villous placenta. These HDACs; -4, -10, and -11 are associated with prostaglandin synthesis, heat shock protein 70, and IL-10 regulation respectively, and may therefore underlie different inflammatory response by three tissue types. Nuclear protein distribution for two major HDACs, HDAC1 and -3 appears to be consistent with our findings for mRNA expression. There were no significant differences in HDAC activity between tissues, but as total activity for only the nuclear extracts was measured, differences in HDAC subcellular localization and substrate affinities may have masked results.

We have shown that amnion, choriodecidua and villous placenta, each have distinct HDAC expression profiles. Therefore it is not surprising that the tissues have previously been shown to respond differently to HDAC inhibition. Many diseases, particularly cancers have been associated with altered HDAC expression. An understanding of the normal expression patterns of the HDACs allows us to better understand these disease states, particularly those shown to be responsive to HDACi. With the development of selective HDAC inhibitors, there is a possibility that the effects of inappropriate epigenetic modulation can be corrected in the future.

## **Acknowledgements**

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## **Grants**

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# Chapter 3 The effects of HDAC inhibition on cytokine expression

## **Part A: Preliminary studies**

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### Histone deacetylase expression in previous studies

**Aim:** To determine if changes in cytokine production seen with LPS treatment and histone deacetylase inhibition in previous studies are associated with changes in HDAC expression.

**Methods:** (For additional details see appendix ).

Tissue explants were generated during the course of previous work by our lab, for published data see: (**Sato et al. 2006; Mitchell et al. 2012**). Studies investigated the effect of the DNA methyltransferase inhibitor 5-aza-2' deoxycytidine, and the histone deacetylase inhibitor trichostatin A (TSA) on cytokine production with/without lipopolysaccharide (LPS) stimulation.

#### *1. Effect of TSA on HDAC expression in amnion, choriodecidua and placental villous explants.*

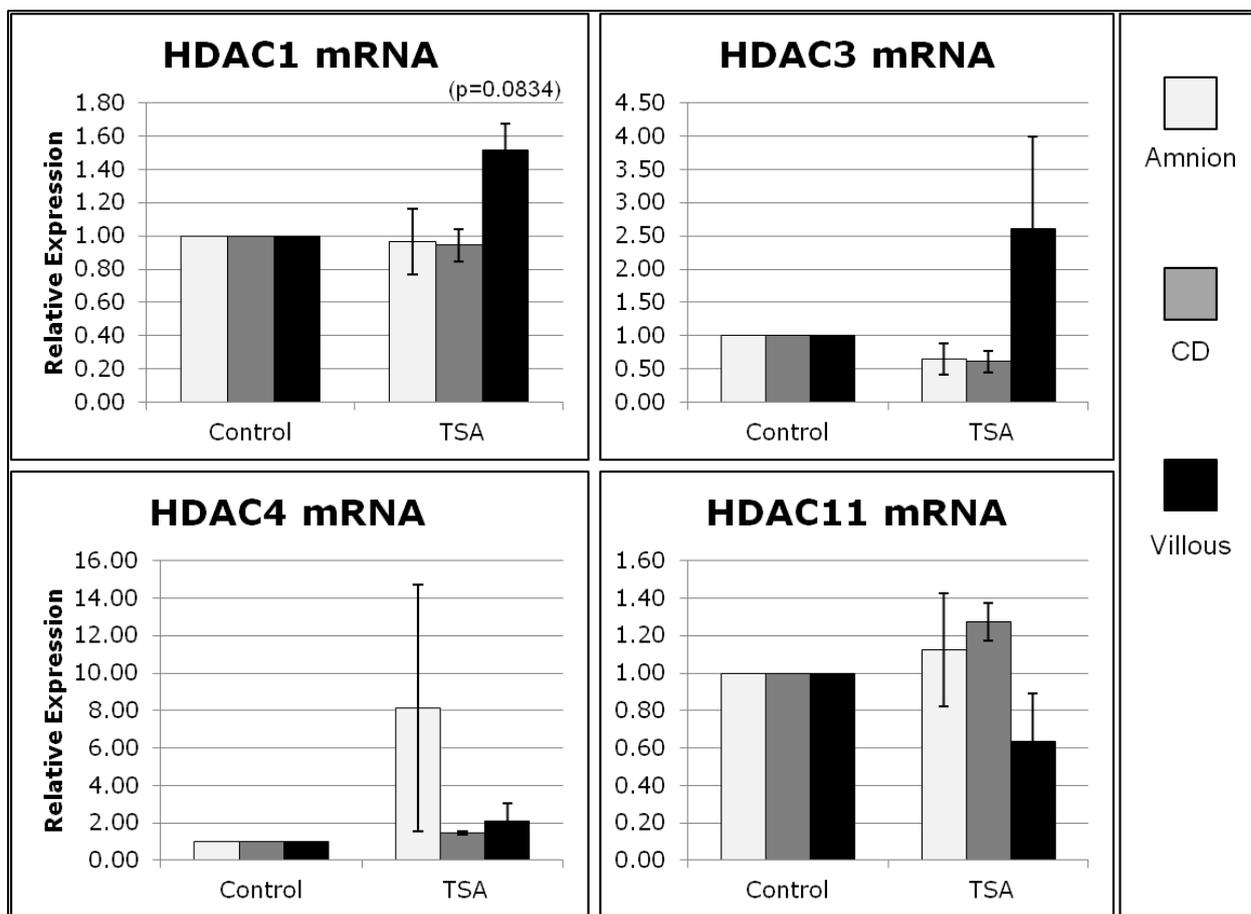
Amnion, choriodecidua and placental explants had been cultured for 24hours with and without 300nM TSA (n=3). Frozen tissue was crushed in the presence of liquid nitrogen and total RNA extraction and quantitative real-time PCR were performed as previously described (Chapter 3).

#### *2. Effect on TSA and LPS on HDAC expression in placental villous explants.*

Placental villous explants (n=4-5) had been cultured for 24hours with and without 300nM TSA and stimulation by LPS (5 µg/mL). Frozen tissue was crushed in the presence of liquid nitrogen and total RNA was extracted as previously described (Chapter 3). Total RNA was quantified on a spectrophotometer (NanoDrop, Thermo Scientific, USA) and DNase treated, before reverse transcription (RT) was performed using Transcriptor First Strand cDNA Synthesis Kits. Quantitative Real-time PCR was performed using a LightCycler 480 Instrument (Roche Applied Science, Penzberg, Germany).

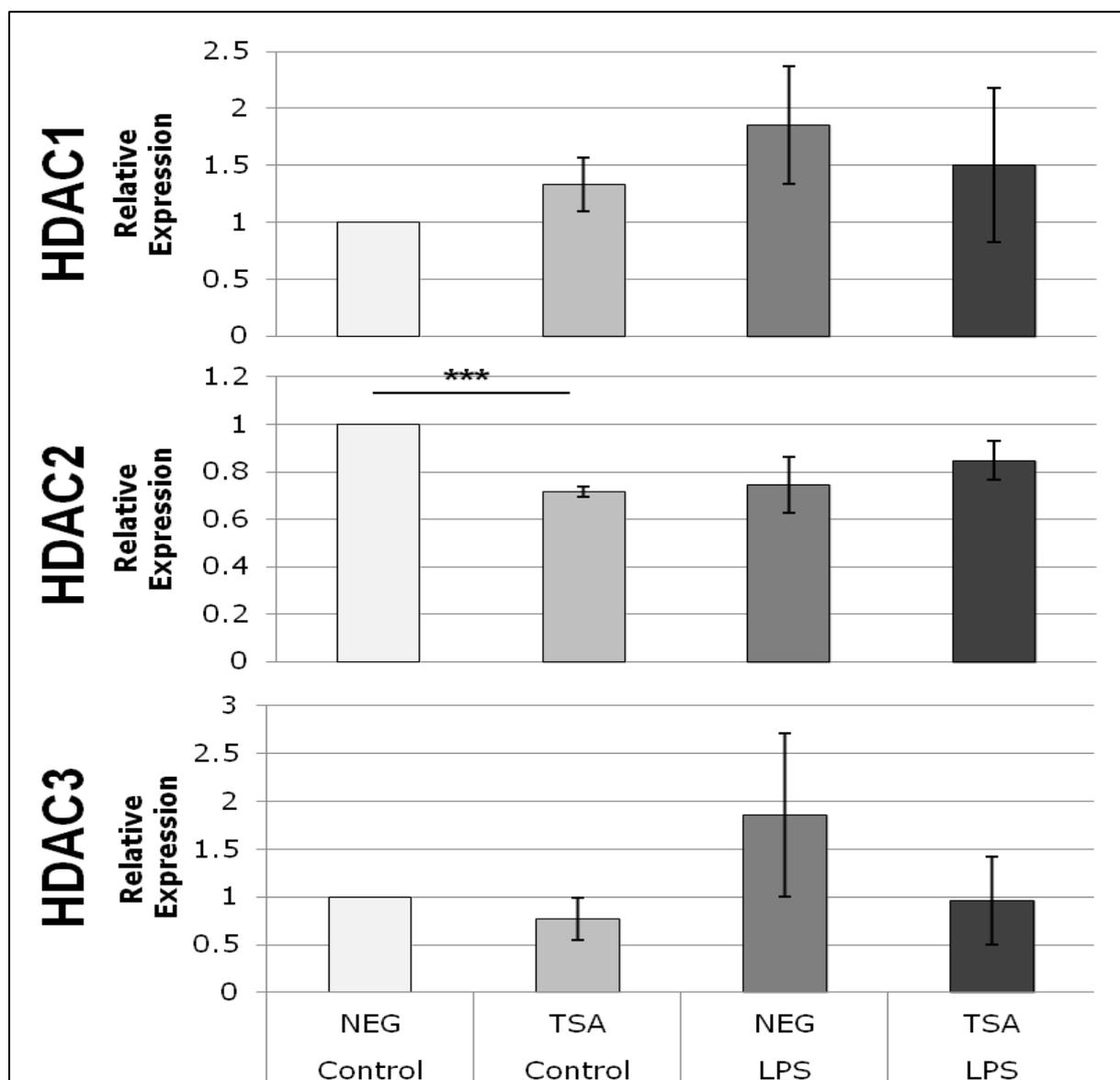
Primer sequences can be found in **Table 1**. Real-time-PCR results were calculated as absolute values and are given as mean relative expression compared with housekeeping gene; YWHAZ. Data are presented as relative change from control (mean  $\pm$  SE). Data were analyzed by one way ANOVA followed by Student's t-tests. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). A p value of  $\leq 0.05$  was considered significant.

**Results:** There was a trend for increased HDAC1 mRNA expression following 24hours TSA treatment in placental villous tissue ( $p=0.0834$ ) but not in matched amnion or chorio-decidua [**Figure 16**]. Villous tissue stimulated with LPS and/or treated with TSA also showed an apparent increase in HDAC1 mRNA with TSA but this was also not statistically significant [**Figure 17**]. However in this second set the related histone deacetylase; HDAC2 was down regulated by TSA after 24 hours treatment ( $p\leq 0.001$ ) [**Figure 17**].



**Figure 16: Effect of TSA on HDAC expression in amnion, choriodecidua and placental villous explants**

Data are presented as relative change from control (mean  $\pm$  SE, n=3). Data were analyzed by one way ANOVA followed by Student's t-tests. Data were analyzed by one way ANOVA followed by Student's t-tests. Statistical significance is given as \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .



**Figure 17: Effect on TSA and LPS on HDAC expression in placental villous explants**

Data are presented as relative change from control (mean  $\pm$  SE.  $n=4-5$ ). Data were analyzed by one way ANOVA followed by Student's *t*-tests. Data were analyzed by one way ANOVA followed by Student's *t*-tests. Statistical significance is given as \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ..

**Discussion:** HDAC2 was significantly decreased in placental explants following TSA treatment while there was a trend towards increased HDAC1. HDAC1 and HDAC2 have each been shown to compensate for the depletion of the other, but only at the protein level, with changes not previously apparent in mRNA (Lagger et al. 2002; Zupkovitz et al. 2006; Montgomery et al. 2007; Senese et al. 2007; Yamaguchi et al. 2010).

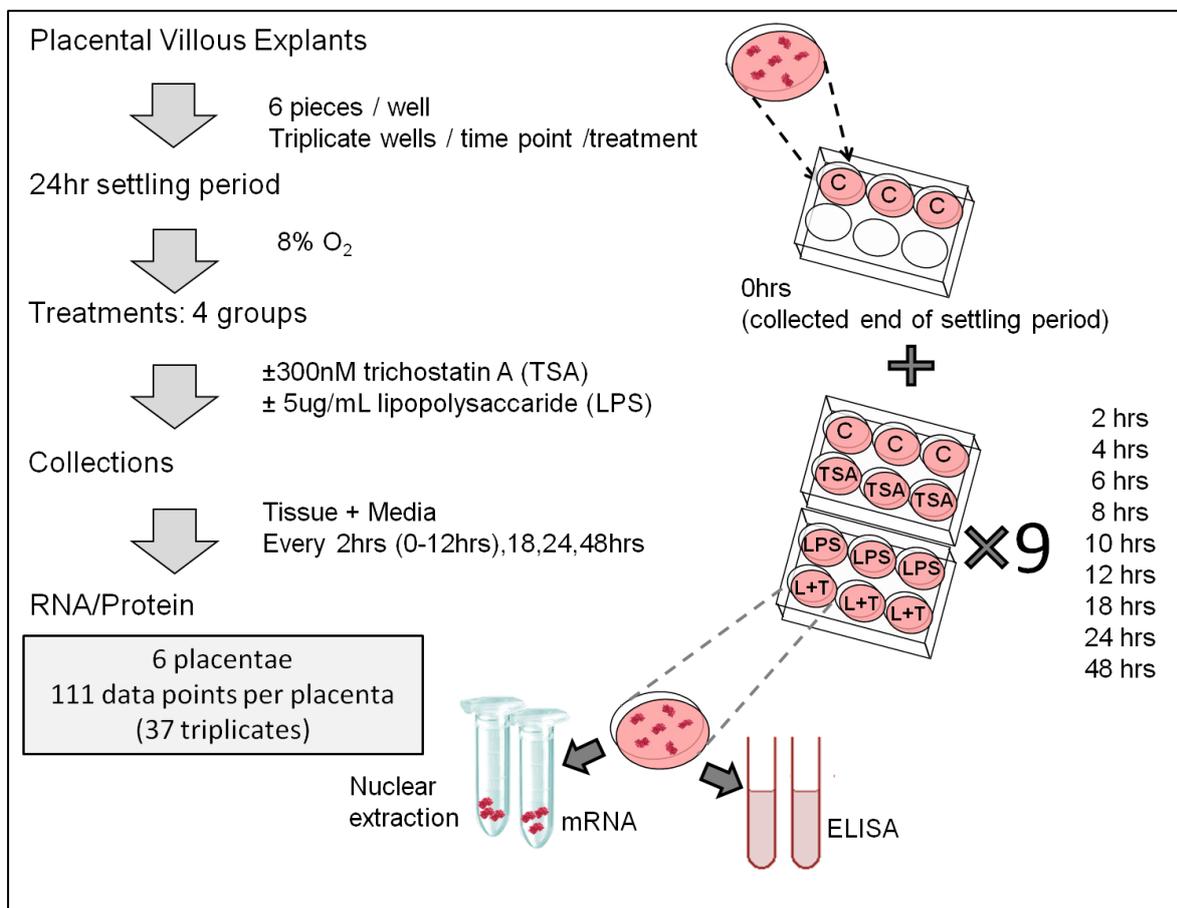
**Conclusion:** Histone deacetylase inhibition of placental explants appears to result in altered HDAC expression. Histone deacetylase expression in placental explants warrants further investigation.

## Time course development

### Background:

- HDAC inhibition of LPS stimulated placental explants results in decreased TNF $\alpha$  protein after 24hours culture in room air/5% CO $_2$  (**Mitchell et al. 2012**)
- The effect of TSA on cytokine mRNA expression is not known
- LPS and TSA have been reported to alter HDAC expression in other cell types (**Gray et al. 1998; Gray et al. 2000; Aung et al. 2006**).
- We have found changes in HDAC expression with TSA (see previous study)
- Cytokine production has been shown to alter with oxygen tension (**Reti et al. 2007; Royle et al. 2009; Peltier et al. 2011**).
- Term placenta is recommended to be cultured at 8% oxygen (**Miller et al. 2005; Sullivan et al. 2006**).

**Aim:** To develop a time course of the effects of TSA treatment and LPS stimulation on placental villous explants at 8% O $_2$ .



**Figure 18: Placental villous explant time course**

**Results:** Chapter 4 part B and Chapter 5

## ***Part B: Effects of LPS and TSA on cytokine expression and production***

Placenta, 34 (7) , pp. 567-573

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Histone deacetylase inhibition by trichostatin A mitigates LPS induced TNF $\alpha$  and IL-10 production in human placental explants

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## Abstract

**Introduction:** Cytokine expression by the placenta is known to change across pregnancy, and is altered in a number of pathologies; however the precise mechanisms of cytokine regulation in gestational tissues are not well understood. It has been previously reported that cytokine protein production in gestational tissues is regulated in a tissue-specific manner and appears to be epigenetically regulated.

**Methods:** In this study we investigated changes in cytokine mRNA expression and protein production by term placental explants maintained at 8% O<sub>2</sub> in the presence or absence of lipopolysaccharide (LPS) (5 µg/mL) and the histone deacetylase inhibitor trichostatin A (TSA) (300nM).

**Results:** As expected, exposure to LPS stimulated gene expression and protein production of the proinflammatory cytokines IL-1β, IL-6, IL-8 and TNFα, as well as the anti-inflammatory cytokine IL-10. While TSA alone had little effect, TSA co-treatment mitigated the effects of LPS on TNFα and IL-10 protein production with an accompanying reduction in TNFα mRNA transcript levels detected. However, TSA had no significant effect on LPS induced IL-1 β, IL-1ra, IL-6 or IL-8 mRNA expression or protein production.

**Discussion:** The data from this study show that TSA selectively mitigates the stimulatory effect of LPS on TNFα mRNA expression, TNFα protein production and IL-10 protein production. As there is no compensatory effect on IL-1β, IL-1ra, IL-6, or IL-8 mRNA expression or protein production, this results in a dysregulation of the cytokine balance.

**Conclusions:** Insights into HDAC regulation of cytokine expression may provide novel therapeutic strategies for conditions associated with dysregulation of the cytokine network, such as preeclampsia and infection mediated preterm labour.

**Keywords:** cytokine, epigenetic, HDAC, placenta, trichostatin A, TNFα, LPS

## Introduction

Early environmental interactions, such as those between the embryo and uterus during implantation and placentation, can determine our physiological responses to future stresses and stimuli. Impaired interactions can result in placental deficiency -which has been linked to early onset intrauterine growth restriction (IUGR), recurrent abortion, preeclampsia and preterm delivery with deleterious consequences (**Tranguch et al. 2005; Wang et al. 2006**). As cell signaling molecules and immunomodulators, the roles of cytokines in pregnancy, parturition and disease have come under scrutiny. Alterations in cytokine expression occur during gestation, with (pre)term labour, infection, preeclampsia and IUGR (**Keelan et al. 2003; Rusterholz et al. 2007; Sharma et al. 2007; Mullins et al. 2012; Weissenbacher et al. 2012**). There have been correlations made between genetic polymorphisms and altered cytokine expression, particularly with respect to preterm birth (**Moura et al. 2009; Harper et al. 2011**), however many polymorphisms are only implicated in the context of a secondary challenge such as bacterial vaginosis or maternal smoking (**Macones et al. 2004; Tsai et al. 2008**). This interplay between pathologic processes, environmental risk factors, and genetic risk, implicates epigenetics as a mechanism of cytokine regulation during pregnancy.

One of best studied epigenetic modifications; histone acetylation (**Dolinoy et al. 2007; Jirtle et al. 2007**), is generally associated with transcriptional activation, and levels are maintained through the opposing actions of the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). Chemical inhibition of HDAC activity, promotes the hyper-acetylation of the histones via unopposed HAT activity. However as HDACs also deacetylate and associate with a range of other proteins, HDAC inhibition can have a multitude of other effects (**Yang and Seto 2007**).

In humans, HDAC inhibition selectively alters cytokine mRNA expression and protein production by immune cells (**Leoni et al. 2002; Bode et al. 2007; Roger et al. 2011**). Cytokine protein production by gestational tissues, in response to stimulation, can also be modulated with HDAC inhibitor treatment, in a tissue specific manner (**Sato et al. 2006; Mitchell et al. 2012**). Interestingly, TNF $\alpha$  production by placental villous tissue in response to LPS was found to be reduced following HDAC inhibition (**Mitchell et al. 2012**), while other cytokines were unaffected. Similar effects on TNF $\alpha$  protein were not seen in amnion (**Mitchell et al. 2012**) or chorio-decidual explants (**Sato et al. 2006**). Both previous studies were limited to changes in protein levels only at a single time

point of 24hrs, and explants were cultured in room air (21% oxygen). Studies have shown that cytokine levels and interactions can be affected by oxygen levels in culture (**Reti et al. 2007; Royle et al. 2009; Peltier et al. 2011**). We therefore sought to determine if the observed changes in TNF $\alpha$  (and lack of change in other cytokines) could be replicated at a more physiologically relevant oxygen level for term placenta; 8% (**Miller et al. 2005; Sullivan et al. 2006**). We also sought to determine if alterations in cytokine protein production correlated to earlier mRNA levels as would be expected if HDAC inhibition resulted in altered cytokine gene activity. In addition, by measuring mRNA and protein levels across multiple time points we could examine the kinetics of cytokine mRNA expression and protein production. This would also allow us to rule out a time shift in peak production being responsible for observations of an increase/decrease, as might arise by sampling one time point only

**Methods** (For additional details see appendix )

### ***Reagents.***

Lipopolysaccharide (LPS), trichostatin A (TSA), bovine gamma globulins and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gibco® DMEM / F-12 with GlutaMAX™, Gibco® Antibiotic-Antimycotic, TRIzol® reagent and DNase I were from Life Technologies (Carlsbad, CA, USA). Transcriptor First Strand cDNA Synthesis Kit and LightCycler® 480 SYBR Green I Master mix were from Roche Applied Science, Penzberg, Germany). Human IL-1 $\beta$ , TNF $\alpha$ , IL-6, IL-8 and IL-10, ELISA kits were purchased from BD Bio Sciences (Franklin Lakes, NJ, USA). IL-1ra kits were purchased from R&D systems (Minneapolis, MN USA).

### ***Explant culture and treatment***

Placental tissues were collected from women undergoing elective caesarean section at term, with the approval of the NorthernX Regional Ethics Committee. Six individual placentae from singleton pregnancies of healthy nonsmoking mothers were used in the study (4 males, 2 females). Median maternal age was 36 years (SD=3 years), and mean gestational length was 39 weeks. Tissue processing commenced within 15 min of delivery and involved two rinses in PBS and Gibco® DMEM / F-12 with GlutaMAX™ supplemented with 0.1% bovine gamma globulin, and Gibco® Antibiotic-Antimycotic (Amphotericin B, Streptomycin, Penicillin). Placental villous tissue explants of approximately 20 mg (wet weight) were obtained by blunt dissection and removal of

visible connective tissue and vessels. In triplicate, villous tissues explants were then cultured at 37°C in 6-well plates (six pieces per well), media as above, in a humidified incubation box within an O<sub>2</sub> control glove box (Coy Laboratory Products; Detroit, MI, USA). The glove box utilizes glove ports and an airlock to allow access to the chamber interior without disruption of the experimental environment. Explants were cultured for 24 hours under conditions of 5% CO<sub>2</sub> and 8% O<sub>2</sub> (displacement by N<sub>2</sub>) before the treatment period commenced.

The treatment period consisted of the addition of 300nM TSA, which inhibits histone deacetylase activity, or DMSO carrier in fresh media, in the presence or absence of LPS (5 µg/mL). Doses were chosen to be consistent with published literature (**Sato et al. 2006; Mitchell et al. 2012**), and treatment times included a range between 0-12 hours (2 hour intervals) as well as 18, 24 and 48 hours. All treatments and collections were performed within the glove box at 8% O<sub>2</sub>. Following treatment the wet weight of the tissue in each well was determined and both tissue and media frozen for subsequent analysis.

#### ***Analysis of cytokine mRNA by Real-time PCR***

High-throughput disruption of tissue while frozen was performed using the TissueLyser II (QIAGEN, Hilden, Germany) followed by total RNA extraction using TRIzol® reagent according to the manufacturer's instructions. Total RNA was quantified on a spectrophotometer (NanoDrop, Thermo Scientific, USA) and DNase treated, before reverse transcription (RT) was performed using Transcriptor First Strand cDNA Synthesis Kits. Quantitative Real-time PCR was performed using a LightCycler 480 Instrument (Roche Applied Science, Penzberg, Germany). Each reaction was performed in triplicate and normalized against the geometric mean of three housekeeping genes: YWHAZ, RPL13A and ACTB. Primer sequences are listed in **Table 2**.

#### ***Analysis of cytokine production by ELISA***

Measurements of IL-1β, IL-1ra, IL-6, IL-8, IL-10 and TNFα protein in culture medium were determined using human enzyme-linked immunosorbent assay kits according to manufacturers' specifications. Production rates of cytokines were normalized to wet tissue weight.

## Statistical Analysis

Real-time-PCR results are given as mean relative expression  $\pm$  SE. Production rates of cytokines were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE. Statistical significance was determined by two-way repeated-measures ANOVA, matched across treatment and time, followed by Tukey's multiple comparisons test and was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). A p value of  $\leq 0.05$  was considered significant.

**Table 2 : RT-PCR Primer sequences**

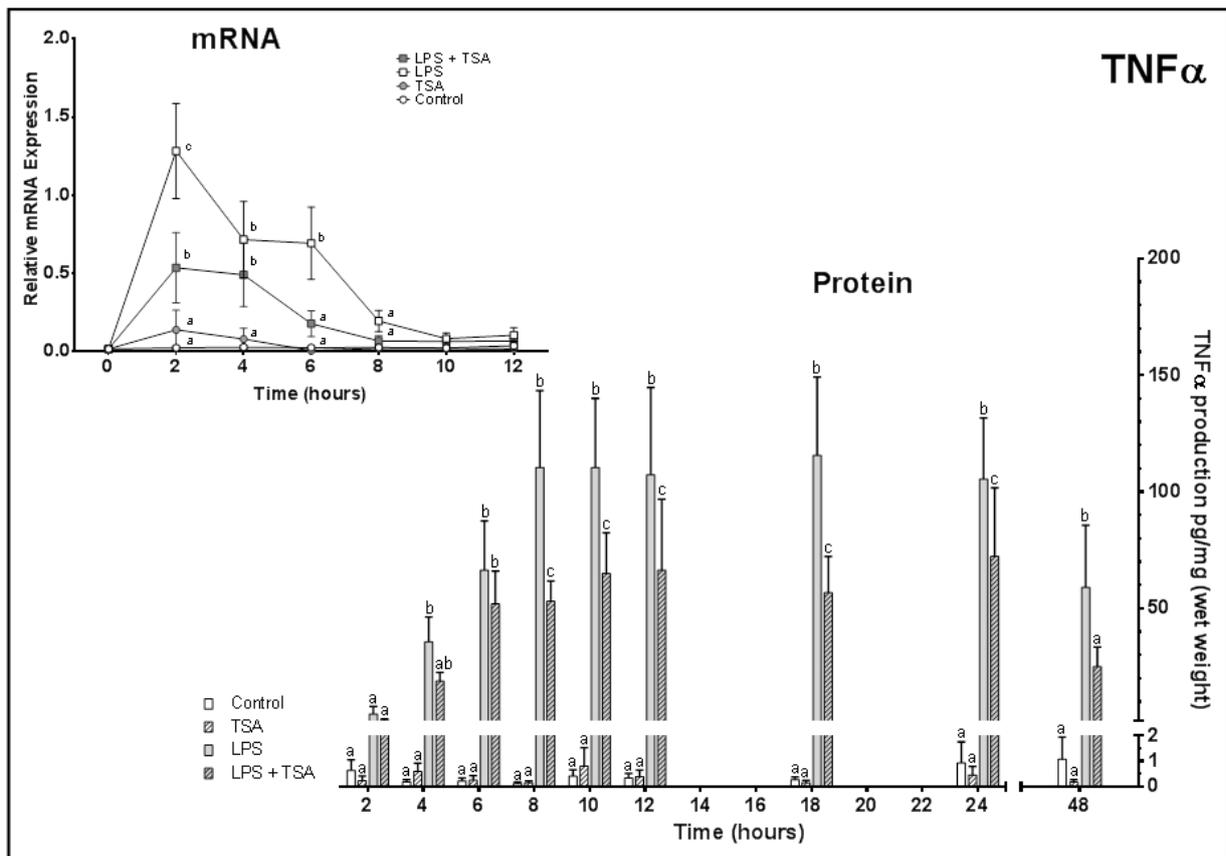
	<b>Fwd. 5'- 3'</b>	<b>Rev 3'- 5'</b>	<b>Amplicon Size (bp)</b>
<b>IL-1<math>\beta</math></b>	GAAGCTGATGGCCCTAAACA	GCATCTTCCTCAGCTTGTCC	160
<b>IL-6</b>	AAATTCGGTACATCCTCGACGG	GGAAGGTTTCAGGTTGTTTTCTGC	112
<b>IL-8</b>	ACCACCGGAAGGAACCATCTCAC	ACTGCACCTTCACACAGAGCTGC	105
<b>IL-10</b>	CAGGGCACCCAGTCTGAGAA	CACATGCGCCTTGATGTCTG	264
<b>TNF</b>	TGCTTGTTTCCTCAGCCTCTTCT	TATCTCTCAGCTCCACGCCATT	277
<b>IL-1ra</b>	AGCCTTCAGAATCTGGGATG	TGTTCTCGCTCAGGTCAGTG	242
<b>YWHAZ</b>	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT	189
<b>RPL13A</b>	GCCCTACGACAAGAAAAAGCG	TACTTCCAGCCAACCTCGTGA	117
<b>ACTB</b>	GCGGACTATGACTTAGTTGCG	CATCTTGTTTTCTGCGCAACTT	67

## Results

### *Effect of LPS on basal cytokine expression/production by human villous placental explants*

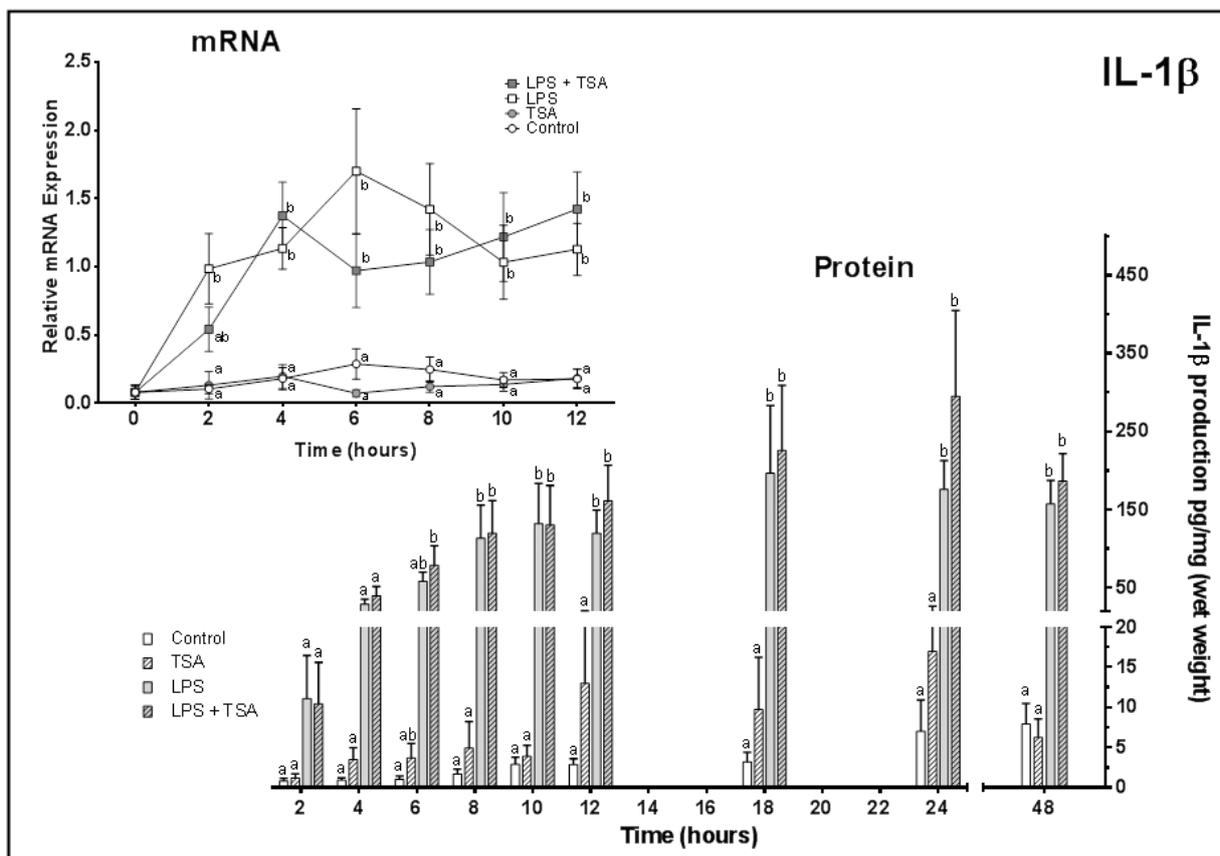
As expected, LPS treatment significantly increased the production of the proinflammatory cytokines. The earliest response was from TNF $\alpha$  which showed peak mRNA expression at 2 hours post-treatment but was a transient increase, with mRNA levels then declining back towards basal levels **[Figure 19]**. No statistically significant difference in TNF $\alpha$  mRNA remained between the LPS treated and control groups by 8 hours post-treatment. In contrast, LPS significantly increased TNF $\alpha$  protein production by 4 hours post-treatment, and TNF $\alpha$  protein levels remained elevated throughout the 48 hour time course. IL-1 $\beta$  mRNA was also significantly increased 2 hours post-treatment **[Figure 20]**, though elevated levels were maintained, and this accompanied by a gradual increase in protein, which was significantly higher than basal control by 8 hours post-treatment. IL-8 mRNA was the next to be significantly elevated in the LPS treated group, at 4 hours post-treatment, followed by IL-6 at 6 hours post-treatment **[Figure 21]**. Like IL-1 $\beta$ , mRNA expression of IL-6 and IL-8 were maintained at the elevated level across the time period investigated. LPS induced increases in IL-6 protein levels were statistically significant from 10 hours post-treatment, and IL8 from 12 hours onward.

mRNA transcripts of the anti-inflammatory cytokine IL-10 were not detectable prior to 12 hours post-treatment. There were however significant increases in IL-10 mRNA detected between 12 and 48 hours, and a significant difference in IL-10 protein production was apparent from 10 hours post treatment **[Figure 22]**. In general there was no significant effect of LPS stimulation on IL-1ra mRNA expression (data not shown) or protein production **[Figure 23]**. However, this was the only cytokine examined for which the trends of the individual placentae were not in agreement. While no treatment effects were detectable in IL-1ra mRNA expression, we observed a clear trend towards increased IL-1ra protein production following LPS treatment in the two placentae from female neonates while the placentae from the four males showed no effect of LPS **[Figure 23]**. While reports have been made of gender differences in IL-1ra expression and gene polymorphisms (**Sadeghi et al. 2005; Elsmen et al. 2006; Bessler et al. 2007**), statistical examination of gender effects is regrettably outside the scope of this study due to limitations of sample size.



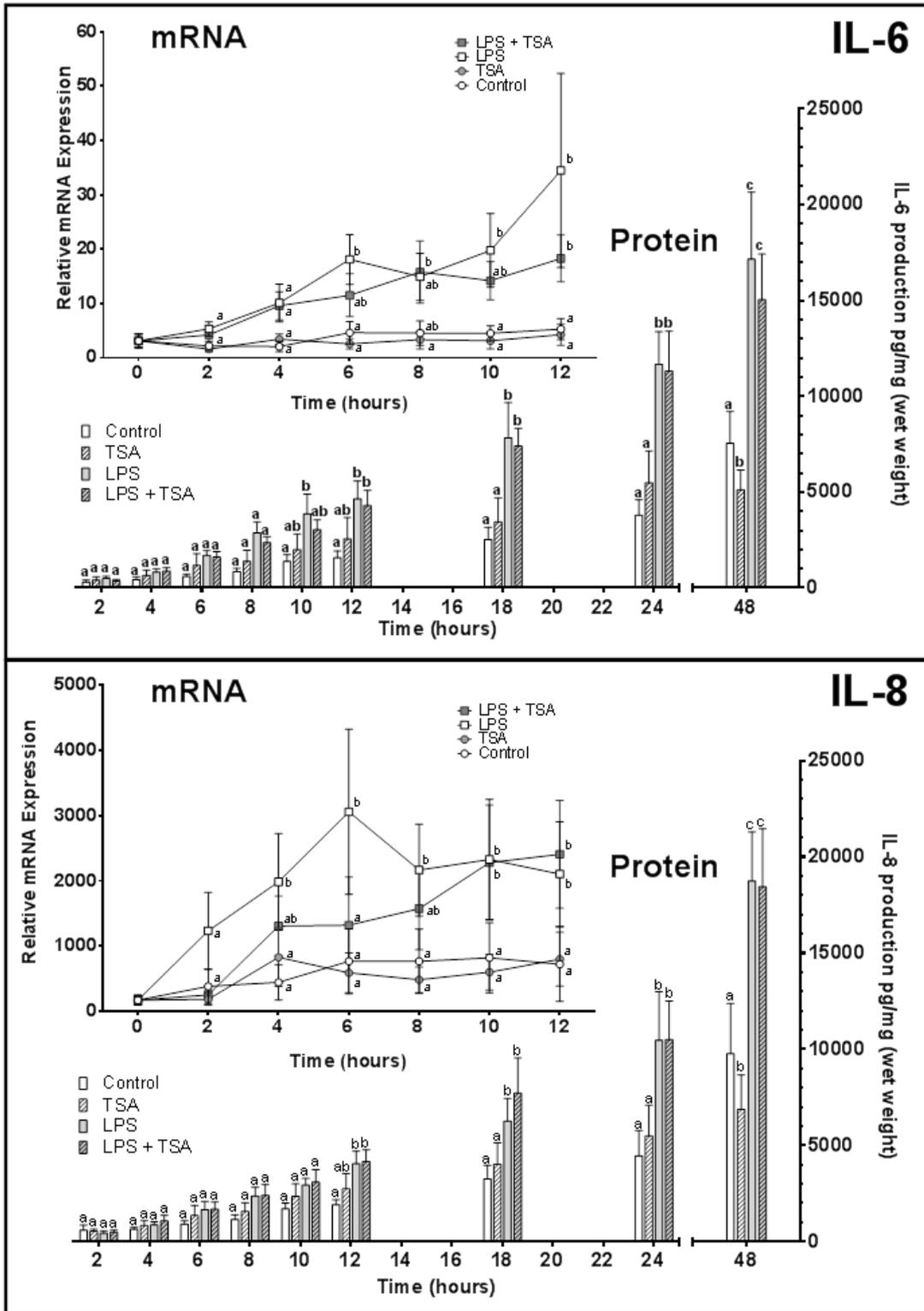
**Figure 19: TNF mRNA expression and protein production by placental villous explants across time.**

Real-time-PCR results are given as mean relative expression  $\pm$  SE. TNF $\alpha$  protein levels in media were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE.  $n=6$  placentae. Statistical comparisons within time points and between treatments are shown. Results of pairwise analysis are shown. Shared lower case letters indicate no statistical difference between groups. A  $p$  value of  $\leq 0.05$  was considered significant



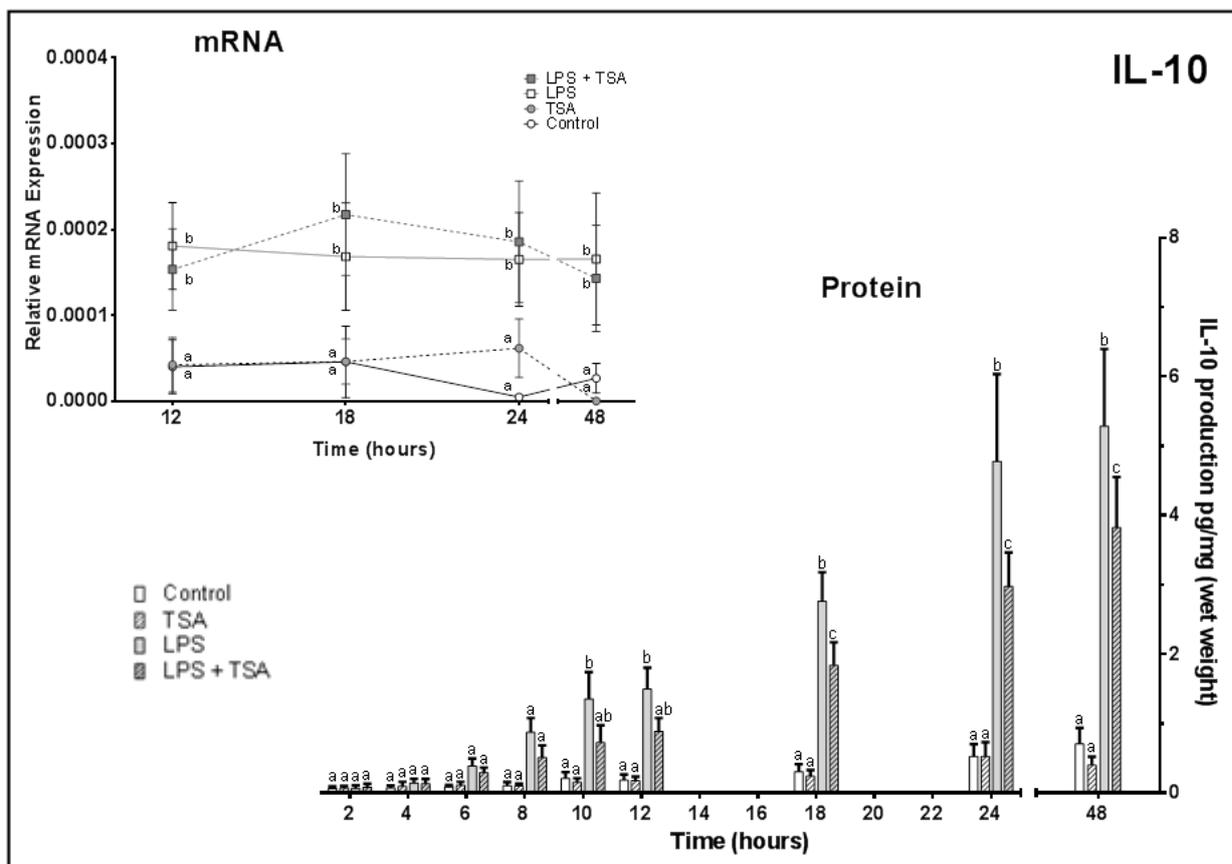
**Figure 20: IL-1 $\beta$  mRNA expression and protein production by placental villous explants across time.**

Real-time-PCR results are given as mean relative expression  $\pm$  SE. IL-1 $\beta$  protein levels in media were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE.  $n=6$  placentae. Statistical comparisons within time points and between treatments are shown. Results of pairwise analysis are shown. Shared lower case letters indicate no statistical difference between groups. A  $p$  value of  $\leq 0.05$  was considered significant



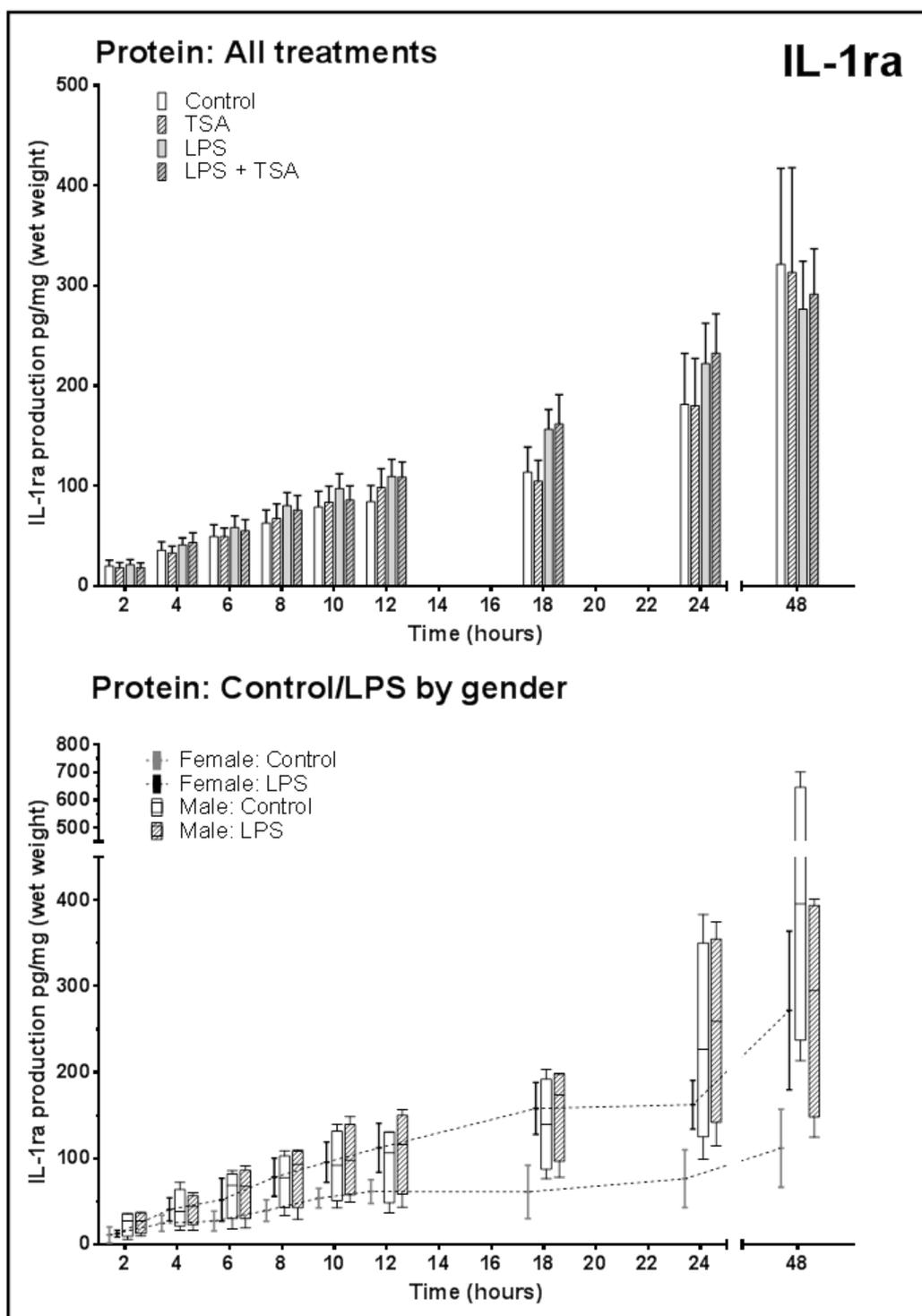
**Figure 21: IL-6 (above) and IL-8 (below) mRNA expression and protein production by placental villous explants across time.**

Real-time-PCR results are given as mean relative expression  $\pm$  SE. Protein levels in media were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE.  $n=6$  placentae. Statistical comparisons within time points and between treatments are shown. Results of pairwise analysis are shown. Shared lower case letters indicate no statistical difference between groups. A  $p$  value of  $\leq 0.05$  was considered significant.



**Figure 22: IL-10 mRNA expression and protein production by placental villous explants across time.**

Real-time-PCR results are given as mean relative expression  $\pm$  SE. IL-10 protein levels in media were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE.  $n=6$  placentae. Statistical comparisons within time points and between treatments are shown. Results of pairwise analysis are shown. Shared lower case letters indicate no statistical difference between groups. A  $p$  value of  $\leq 0.05$  was considered significant



**Figure 23: IL-1ra protein production by placental villous explants across time (above) and by gender (below).**

Protein production rates of cytokines were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE. Shared lower case letters indicate no statistical difference between groups. A  $p$  value of  $\leq 0.05$  was considered significant **Above**; All four treatment groups across time (males and females combined)  $n=6$  placentae. **Below**; Control and LPS groups split by gender, males ( $n=4$ ), females ( $n=2$ ). Boxes represent 25-75% quartile and median. Whiskers represent min/max. Trend lines through mean values are shown for females. Statistical comparisons were not performed

### ***Effect of TSA treatment on basal cytokine expression/production by human villous placental explants***

For all cytokines measured, TSA had no significant effects on basal mRNA expression. There was a small but statistically significant reduction in IL-6 and IL-8 protein production relative to basal levels following 48 hours of TSA treatment **[Figure 21]**.

### ***Effect of combined TSA treatment and LPS stimulation on cytokine expression/production by human villous placental explants***

While TSA co-treatment had no significant effect on IL-1 $\beta$ , IL-6 or IL-8 mRNA expression or protein production **[Figure 20 and Figure 21]**, it mitigated the LPS induced increase in both expression and production of TNF $\alpha$  **[Figure 19]**. Co-treatment with TSA resulted in reduced TNF $\alpha$  mRNA levels compared to LPS alone; this effect was statistically significant at 2 and 6 hours and a clear trend at 4 hours. Furthermore TNF $\alpha$  mRNA levels at 2 and 4 hours in the co-treatment group were still significantly elevated relative to the control group, but by 6 hours the effect of LPS had been reduced, with no difference between the control and combined treatment groups. The LPS induced increase in TNF $\alpha$  production was mitigated by TSA 8 hours after treatment; however levels were still significantly higher than control until 48 hours post-treatment.

Despite no detectable changes in IL-10 mRNA with TSA co-treatment relative to the LPS stimulated group, LPS stimulated increases in IL-10 protein production were reduced **[Figure 22]**. Significant reductions in IL-10 protein production relative to LPS stimulated levels were apparent at 18, 24 and 48 hours post treatment. Prior to this the effects of co-treatment with TSA were still distinguishable, as while the co-treatment group was not significantly different to the LPS group, neither was it significantly different to the control group. In contrast, no co-treatment effect was discernible for IL1- $\alpha$  mRNA expression or protein production.

## Discussion

It has been previously reported that cytokine protein production by gestational tissues is regulated in a tissue-specific manner and appears to be epigenetically regulated. TSA co-treatment increased IL-1 $\beta$  protein production in chorio-decidua, decreased TNF $\alpha$  protein production in villous tissue, yet had no effect on amnion (**Sato et al. 2006; Mitchell et al. 2012**). However neither study examined mRNA expression so a change in gene transcription could not be confirmed. In the current study, we examined the kinetics of cytokine mRNA expression and protein production by villous explants following LPS stimulation and TSA treatment. We also confirmed that the previously reported reduction in TNF $\alpha$  protein production by villus explants (**Mitchell et al. 2012**) also occurs at physiological oxygen levels, and showed this reduction was maintained across the time period studied.

The results showed that the production of TNF $\alpha$  with or without TSA treatment correlated with TNF $\alpha$  mRNA levels. This implies that transcriptional rather than translational regulation was affected by TSA, though does not confirm histone acetylation is directly involved. While histone acetylation appears to correspond with both the competence of cells to express TNF $\alpha$  and with active transcription (**Sullivan et al. 2007**), in our study there was no effect with TSA treatment alone, so increased acetylation is not sufficient to induce TNF $\alpha$  expression in the placenta. Indeed in contrast with the accepted role of HDACs as repressors of gene expression via histone deacetylation, HDAC inhibitors have anti-inflammatory effects in a range of disease models reducing the production of proinflammatory cytokines that drive disease (**Halili et al. 2009**). Similarly, the results from this study imply that in the placenta, HDAC activity is required for an efficient response to LPS, as can be seen with the mitigation of TNF $\alpha$  expression and production following HDAC inhibition. Intensive study of the transcriptional regulation of TNF $\alpha$  in immune cells has identified defined cell type and stimulus-specific enhancer complexes involving the proximal TNF $\alpha$  promoter (**Taylor et al. 2008**). It is therefore likely that the observed effects of HDAC inhibition result through changes in transcription factor recruitment and activity.

Interestingly, mitigation of LPS-induced IL-10 protein production with TSA co-treatment was also apparent; however we were unable to determine if this was reflected in mRNA prior to 12 hours as transcripts were undetectable. It has previously been reported that basal levels of IL-10 protein in human placenta are greatly reduced at term when

compared with the first and second trimesters; this was accompanied by a loss of detectable IL-10 mRNA (**Hanna et al. 2000**). Interestingly the authors were able to detect IL-10 mRNA in term placentae collected following labour suggesting re-expression of IL-10 mRNA occurs, but did not find statistically significant differences in IL-10 protein levels between pre- and post-labour (**Hanna et al. 2000**). Basal levels of IL-10 protein (and TNF $\alpha$  protein) detected in our study were comparable to those reported for another recent placental explant study which used the same tissue weights and oxygen level (**Royle et al. 2009**).

IL-10 and TNF $\alpha$  protein production are closely linked. Neutralization of endogenous TNF $\alpha$  in chorio-decidual explants results in decreased IL10 production (34). In contrast modulation of IL-10 levels has the reverse effect, so that reduction of endogenous IL-10 results in increased TNF $\alpha$  and exogenous IL-10 addition results in decreased TNF $\alpha$  (34). As expected, IL-10 protein levels have increased with LPS treatment, and while IL-10 protein levels appear to still be rising after 48 hours, TNF $\alpha$  protein levels have reduced by this time. The decrease of LPS induced IL-10 protein with TSA treatment corresponds with the reduction of TNF $\alpha$  protein in this treatment group. We cannot therefore rule out the reduction in TNF $\alpha$  rather than the HDAC inhibitor treatment being the direct cause of the IL-10 reduction.

Our results show that IL-1 $\beta$  mRNA expression and protein production were not affected by TSA treatment, which is supported by other studies. While human peripheral blood mononuclear cells (PBMCs) stimulated with LPS in the presence of the related HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) released less TNF $\alpha$  and IL-1 $\beta$  protein, the reduction was only apparent in TNF $\alpha$  and not IL-1 $\beta$  mRNA (**Leoni et al. 2002**). In another study, TSA and SAHA were found to inhibit induction of interleukin-12 protein p40 mRNA and protein upon Toll-like receptor (TLR) stimulation, in murine dendritic cells, as well as human and murine macrophages. While TLR agonist induced TNF $\alpha$  mRNA and protein was affected after longer HDAC inhibition times, IL-1 $\beta$  was once again unaffected by TSA or SAHA (**Bode et al. 2007**). While a previous study of LPS stimulated human chorio-decidual explants showed massive increases in IL-1 $\beta$  protein production following TSA treatment, these explants were also co-treated with 5-aza-2' deoxycytidine (AZA) (**Sato et al. 2006**). AZA, a DNA methyltransferase inhibitor, was also shown to have a significant effect on IL-1 $\beta$  when used alone. This suggests that TSA enhancement of IL-1 $\beta$  protein production in this context was dependent on prior changes in DNA methylation.

While in this study we did not find any differences in LPS stimulated IL-6 or IL-8 mRNA following TSA treatment, other studies have found that circulating IL-6 levels in mice can be reduced by oral administration of SAHA (21) and that TSA treatment of human PBMCs results in reduced IL-6 mRNA expression (39). Furthermore, selective inhibition of HDAC3; a specific member of the HDAC family able to regulate TNF $\alpha$  protein production (**Zhu et al. 2010**), inhibits both TNF $\alpha$  and IL-6 mRNA expression (**Gillespie et al. 2012**). Interestingly, in contrast to TSA, selective HDAC3 inhibitor treatment reduced IL-6 mRNA expression only in the PBMCs isolated from patients with Rheumatoid arthritis, with no effect on healthy PMSCs (**Gillespie et al. 2012**). Differential inflammatory responses to LPS have also been identified following the use of selective HDAC inhibitors in macrophages (**Halili et al. 2010**) and this may reflect specific actions of individual HDACs. Hydroxamic acids such as TSA and SAHA are potent nonspecific inhibitors of deacetylase activity and show little selectivity towards individual HDACs (**Schuetz et al. 2008; Bertrand 2010**). A number of approaches are being used to develop selective inhibitors towards specific HDACs (**Bertrand 2010**) and utilization of these may provide greater insight into cytokine regulation.

A combination of different chromatin conformations/histone modifications, transcription factor recruitment and the actions of specific HDAC family members are likely to act together to regulate cytokines epigenetically. Such levels of control mean that activation of a single receptor may activate only a subset of its targets under differing circumstances (**Foster et al. 2009**). While this means an inflammatory response to a given stimulus can be modified in a cytokine/tissue/time specific manner depending on the environmental cues, such changes are not desirable if they result in unbalanced expression and disease.

## Conclusions

The data from this study show that TSA selectively mitigates the stimulatory effect of LPS on TNF $\alpha$  mRNA expression, TNF $\alpha$  protein production and IL-10 protein production. As there is no compensatory effect on IL-1 $\beta$ , IL-1ra, IL-6, or IL-8 mRNA expression or protein production, this results in a dysregulation of the cytokine balance – but only when there is existing stimulation of the cytokines such as with LPS exposure. Cytokines exhibit multiple mechanisms for control of expression in different contexts and separating primary and secondary effects is difficult, but further work with selective

inhibitors may help identify parts of the cytokine network that can become “uncoupled” and under what circumstances. Several conditions are already associated with dysregulation of the cytokine network, such as preeclampsia, IUGR and infection mediated preterm labour. Modulation of HDAC activity or histone acetylation levels may represent an underlying cause/ consequence of cytokine dysregulation in these conditions, as well as a potential therapeutic strategy. One of the future challenges will be separating the differential responses of neighboring (and interacting) tissues and following up observations with studies earlier in gestation when problems first arise.

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# *Chapter 4 Histone deacetylase expression in response to LPS and TSA*

## **Part A: Class I HDACs**

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Histone deacetylase inhibition in human placental explants results in concomitant adjustments in HDAC expression

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## Abstract

**Introduction:** Cytokine protein production by the placenta and gestational membranes can be modulated with histone deacetylase inhibitor treatment in response to infection. Histone deacetylases directly associate with many components of the inflammatory cascade, and are recruited to the promoters of several cytokines under different conditions. In this study we aimed to examine the dynamics of HDAC1 and HDAC3 in human placental explants across time, with and without stimulation and histone deacetylase inhibition.

**Methods:** We investigated changes in HDAC1 and HDAC3 mRNA expression and nuclear protein, as well as total HDAC activity were determined in human term placental explants maintained at 8% O<sub>2</sub> in the presence or absence of lipopolysaccharide (LPS) (5 µg/mL) and the histone deacetylase inhibitor trichostatin A (TSA) (300nM).

**Results:** Histone deacetylase inhibition resulted in feedback on the mRNA expression of HDAC1. Nuclear protein levels of both HDAC1 and HDAC3 were also significantly elevated by TSA, both alone and in combination with LPS. In contrast LPS decreased HDAC1 mRNA expression, had no effect on HDAC1 or HDAC3 nuclear protein levels, and there was a trend towards increased total HDAC activity.

**Discussion:** The data from this study show that HDAC inhibition can feed back on the expression and production of select histone deacetylases in human placenta. If inflammatory stimuli such as LPS exposure are increasing HDAC activity, this may explain the ability of HDAC inhibitors to act as anti-inflammatory agents.

**Conclusions:** There is mounting evidence for a role in placental cytokine regulation by HDAC1 and HDAC3; however as TSA feedback loops appear to be present, further studies would benefit from targeted knockdown of specific histone deacetylases.

**Keywords:**

Cytokine/HDAC1/ HDAC3/ histone deacetylase/ lipopolysaccharide/  
placenta/trichostatin A

## Introduction

DNA methylation and histone modifications result in alterations to chromatin structure **(Dolinoy et al. 2007; Jirtle et al. 2007)** and are involved in the recruitment of transcription factor complexes **(Berger 2007)**, both of which act to regulate gene expression. Histone acetylation levels are maintained through the opposing actions of the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). Inhibition of HDAC activity promotes the hyper-acetylation of the histones via unopposed HAT activity. However, as HDACs associate with, and deacetylate a range of proteins other than histones, HDAC inhibition can have a multitude of other effects **(Yang and Seto 2007)**.

Alterations in cytokine expression occur during gestation, with (pre)term labour, infection, preeclampsia and IUGR **(Keelan et al. 2003; Rusterholz et al. 2007; Sharma et al. 2007; Mullins et al. 2012; Weissenbacher et al. 2012)**. HDAC inhibition has been shown to selectively alter cytokine mRNA expression and protein production by immune cells **(Leoni et al. 2002; Bode et al. 2007; Roger et al. 2011)**. Cytokine protein production by the human placenta and gestational membranes, in response to stimulation, can also be modulated with HDAC inhibitor treatment **(Sato et al. 2006; Mitchell et al. 2012; Munro et al. 2013)**.

Histone deacetylases directly associate with many components of the inflammatory cascade, and are recruited to the promoters of several cytokines under different conditions. In particular, HDAC1 and HDAC3 regulate the activities of the NF- $\kappa$ B transcription factor interacting with the p50 and p65 subunits to negatively regulate gene expression **(Ashburner et al. 2001; Chen et al. 2001; Baek et al. 2002; Zhong et al. 2002; Gao et al. 2005; Kasper et al. 2006; Shaw et al. 2006; Ziesche et al. 2013)**. HDAC1 and HDAC3 also both associate with I $\kappa$ B $\beta$ , independently of its interactions with NF- $\kappa$ B **(Viatour et al. 2003)**. HDAC1 has been shown to be recruited to the IL-1 promoter **(Enya et al. 2008)** IL-2 promoter **(Wang, Lee et al. 2009)** IL-4 promoter **(Valapour et al. 2002)** IL-5 promoter **(Jee et al. 2005)** IL-8 promoter **(Schmeck et al. 2008)** and the IL-12 promoter **(Lu et al. 2005)** repressing transcription. Recently, HDAC3 has been identified as a specific member of the HDAC family able to regulate TNF- $\alpha$  production **(Zhu et al. 2010)**. Both HDAC1 and HDAC3 also associate with signal transducer and activator of transcription (STAT) proteins **(Nusinzon et al. 2003; Xu et al. 2003; Yuan et al. 2005; Togi et al. 2009)**. HDAC1 is

suggested to be the major catalytic subunit for the deacetylase activity of the Sin3A, NuRD, and CoREST co-repressor complexes (**Dovey et al. 2010**). HDAC3 is part of a multiprotein complex containing SMRT/N-CoR (silencing mediator for retinoid and thyroid receptor) and as part of this complex is essential to the enzymatic activity of class IIa HDACs (**Fischle et al. 2001; Fischle et al. 2002**).

We have previously developed a time course for cytokine expression and production by human placental villous explants in the presence and absence of lipopolysaccharide (LPS) stimulation and trichostatin A (TSA); a general inhibitor for class I and II HDACs (**Munro et al. 2013**). We found that TSA co-treatment mitigated the effects of LPS on TNF $\alpha$  and IL-10 protein production. In this study we aimed to examine HDAC1 and HDAC3 mRNA, nuclear protein, and HDAC activity across time to investigate how they correlated with altered cytokine production in this model.

**Methods** (For additional details see appendix )

### ***Explant culture and treatment***

Placental tissues were collected from women undergoing elective caesarean section at term, with the approval of the NorthernX Regional Ethics Committee. Six individual placentae from singleton pregnancies of healthy non-smoking mothers were used in the study. Median maternal age was 36 years (SD=3 years), and mean gestational length was 39 weeks. As previously described (**Munro et al. In Press**), placental villous tissue explants of approximately 20 mg (wet weight) were cultured in triplicate at 37°C for 24 hours under conditions of 5% CO<sub>2</sub> and 8% O<sub>2</sub> (displacement by N<sub>2</sub>) in Gibco® DMEM/F-12 with GlutaMAX™ (Life Technologies; Carlsbad, CA, USA) supplemented with 0.1% bovine gamma globulin (Sigma-Aldrich; St. Louis, MO, USA ) and Gibco® Antibiotic-Antimycotic (Life Technologies ). After this 24 hour settling period, explants were exposed to 300nM trichostatin A (TSA) (Sigma-Aldrich), which inhibits histone deacetylase activity, or DMSO carrier in fresh media, in the presence or absence of 5  $\mu$ g/mL lipopolysaccharide (LPS) (Sigma-Aldrich) before being collected and frozen at 2 hour intervals across 12 hours for subsequent analysis.

### **RNA extraction and analysis by RT-PCR**

High-throughput disruption of tissue while frozen was performed using the TissueLyser II (QIAGEN, Hilden, Germany) followed by total RNA extraction using TRIzol® reagent according to the manufacturer's instructions. Total RNA was quantified on a spectrophotometer Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA) and DNase treated, before reverse transcription (RT) was performed using Transcriptor First Strand cDNA Synthesis Kits (Roche Applied Science, Penzberg, Germany). Quantitative Real-time PCR was performed using LightCycler® 480 SYBR Green I Master mix and LightCycler® 480 Instrument (Roche Applied Science). Purified and sequenced PCR products were used to create standard curves for each primer pair, which were then used to determine absolute levels of PCR product for each target and sample. Each reaction was performed in triplicate and normalized against the geometric mean of three housekeeping genes: YWHAZ, RPL13A and ACTB, before being presented as change from control. Primer sequences are listed in **Table 3**.

**Table 3: RT-PCR Primer Sequences**

Name	FORWARD 5'- 3'	REVERSE 3'- 5'	Amplicon Size
HDAC1	GGAAATCTATCGCCCTCACA	TTGCCACAGAACCACCAGTA	206
HDAC3	TGGCTTCTGCTATGTCAACG	TTGATAACCGGCTGGAAAAG	297
ACTB	GCGGACTATGACTTAGTTGCG	CATCTTGTTTTCTGCGCAACTT	67
RPL13A	GCCCTACGACAAGAAAAAGCG	TACTTCCAGCCAACCTCGTGA	117
YWHAZ	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT	189

### **Nuclear protein extraction, quantification of HDAC activity and Western blotting**

High-throughput disruption of tissue while frozen was performed using the TissueLyser II (QIAGEN, Hilden, Germany) and nuclear extracts prepared using EpiQuik™ Nuclear Extraction Kit 1 (Epigentek, Farmingdale, NY, USA). Protein concentrations of nuclear extracts were quantified using the RC DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). HDAC activity of extracts was determined using a colorimetric, Epigenase™

HDAC Activity/Inhibition Direct Assay Kit (Epigentek). Following protein electrophoresis, nuclear extracts (5µg) were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) and Western blotting was performed using antibodies for HDAC1 and HDAC3 (ab19845 and ab16047 at 1µg/mL, Abcam, Cambridge, UK) and β-actin (A1978 at 1:30000, Sigma-Aldrich, St. Louis, MO, USA). Chemiluminescence detection of HRP-Conjugated secondary antibodies was performed using Super-Signal West Dura (Thermo Scientific, Illinois) and x-ray films. Densitometry was performed using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad) and Quantity One software.

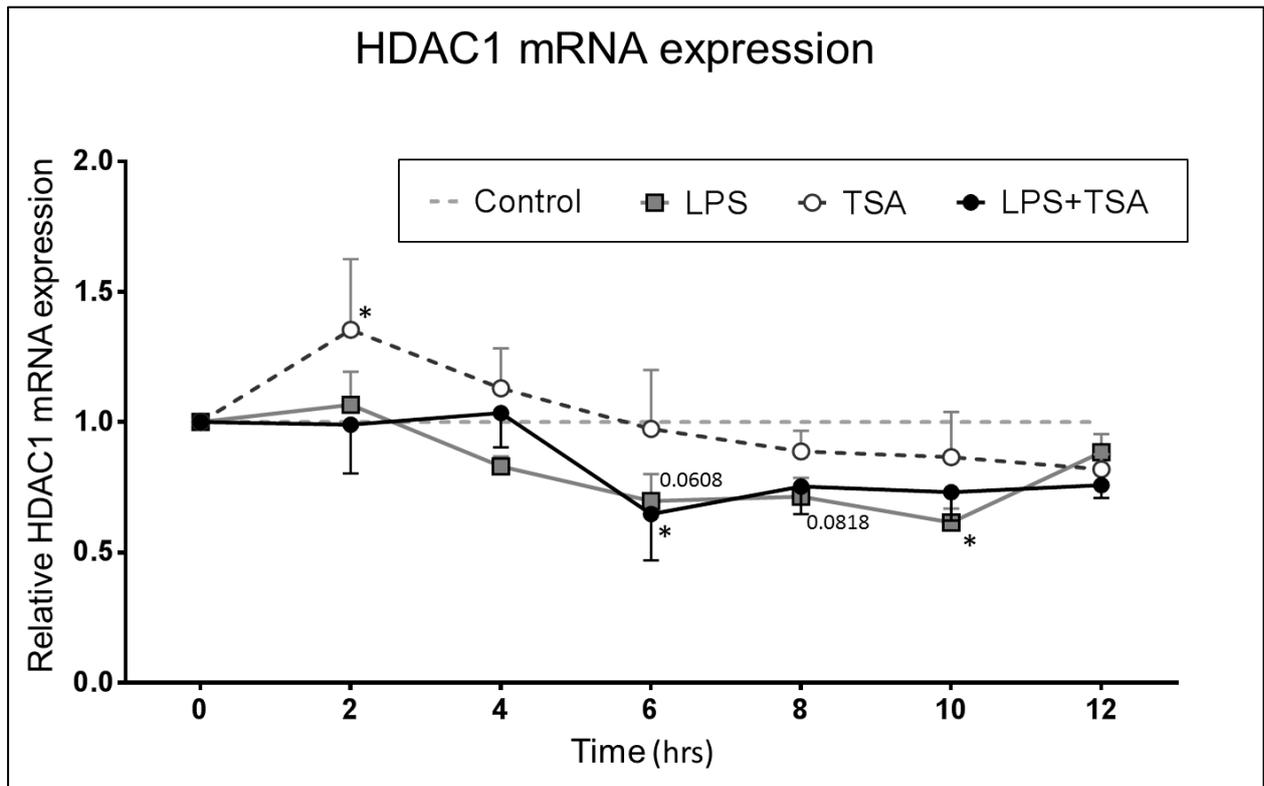
### ***Statistical Analysis***

Real-time-PCR results were first calculated as absolute values before normalization against the geometric mean of three housekeeping genes: YWHAZ, RPL13A and ACTB. HDAC activity was calculated as ng (deacetylated product) per minute, per mg of nuclear protein. Protein production was calculated as density (OD/mm<sup>2</sup>) relative to β-actin loading control, as a proportion of the internal control. All data are presented as relative change from control at each time point, and are given as means ± SE (n=6). Statistical significance was determined by two-way repeated-measures ANOVA, matched across treatment and time, followed by Dunnett's multiple comparisons test. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). A p value of ≤0.05 was considered significant.

## **Results**

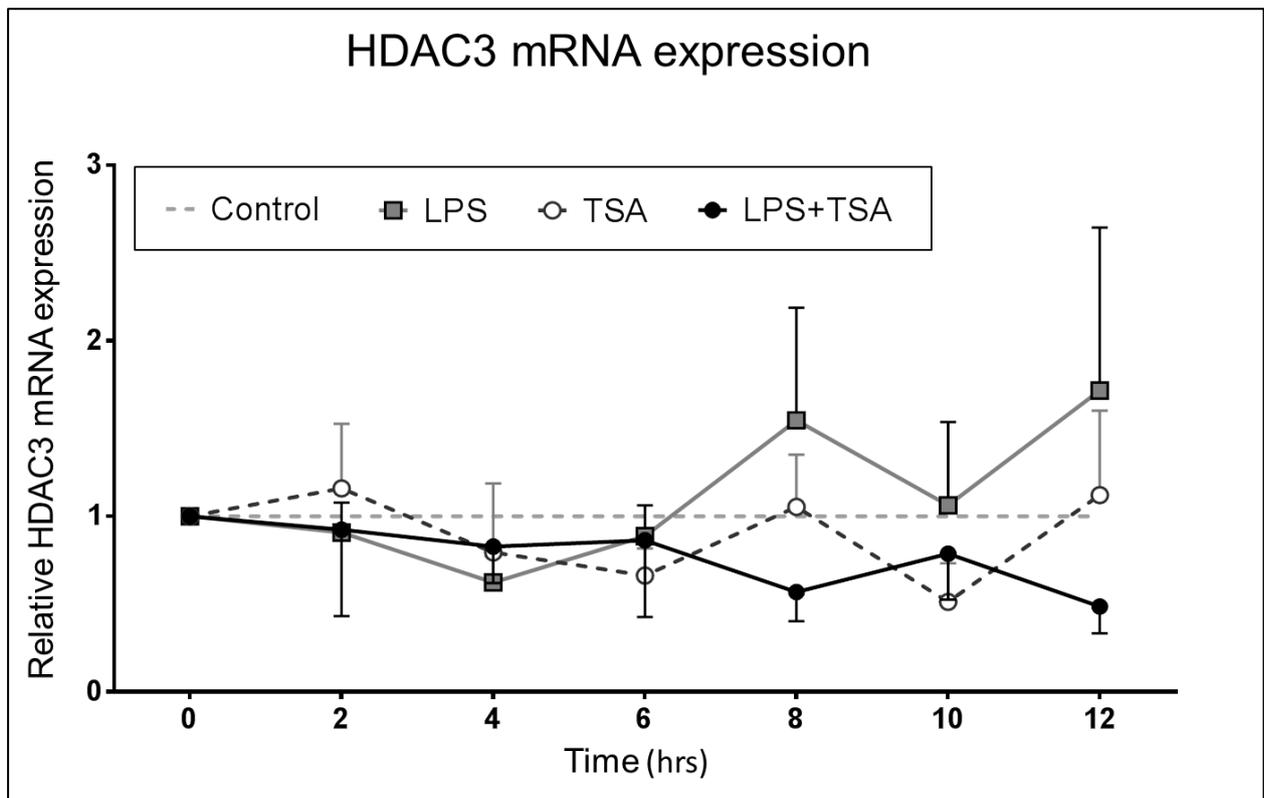
### ***Effect of LPS and TSA treatments on HDAC1 and HDAC3 transcription***

Both time (p=0.0387\*) and treatment (p=0.0049\*\*) had significant effects on HDAC1 mRNA expression in human placental explants **[Figure 24]**. LPS treatment generally resulted in decreased HDAC1 mRNA expression relative to control (6hrs p=0.0608, 8hrs p= 0.0818, 10hrs p=0.0118 \*), while HDAC inhibition by TSA treatment resulted in a transient increase in HDAC1 mRNA expression (2hrs p=0.0225\*). Co-treatment did not affect HDAC1mRNA levels till 6 hours post treatment, when they were significantly reduced relative to control (6hrs p=0.0234\*). In contrast, neither TSA nor LPS treatment alone or in combination resulted in significant alterations to HDAC3 mRNA expression **[Figure 25]**.



**Figure 24: HDAC1 mRNA expression in human placental explants**

*Real-time-PCR results were first calculated as absolute values before normalization against the geometric mean of three housekeeping genes: YWHAZ, RPL13A and ACTB. Data are presented as relative change from control at each time point, and are given as means  $\pm$  SE (n=6). A p value of  $\leq 0.05$  was considered significant.*

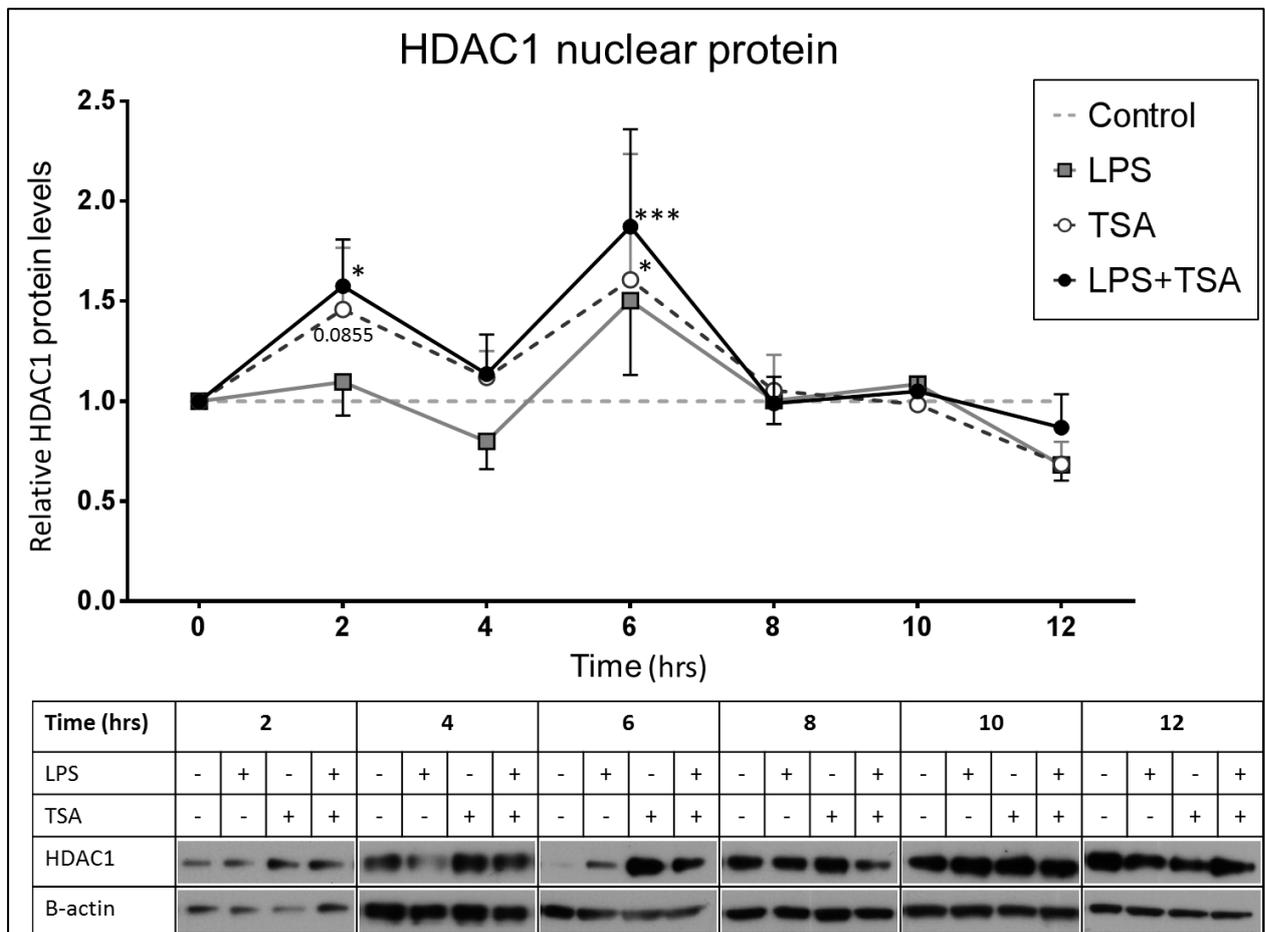


**Figure 25: HDAC3 mRNA expression in human placental explants**

Real-time-PCR results were first calculated as absolute values before normalization against the geometric mean of three housekeeping genes: *YWHAZ*, *RPL13A* and *ACTB*. Data are presented as relative change from control at each time point, and are given as means  $\pm$  SE ( $n=6$ ). A  $p$  value of  $\leq 0.05$  was considered significant.

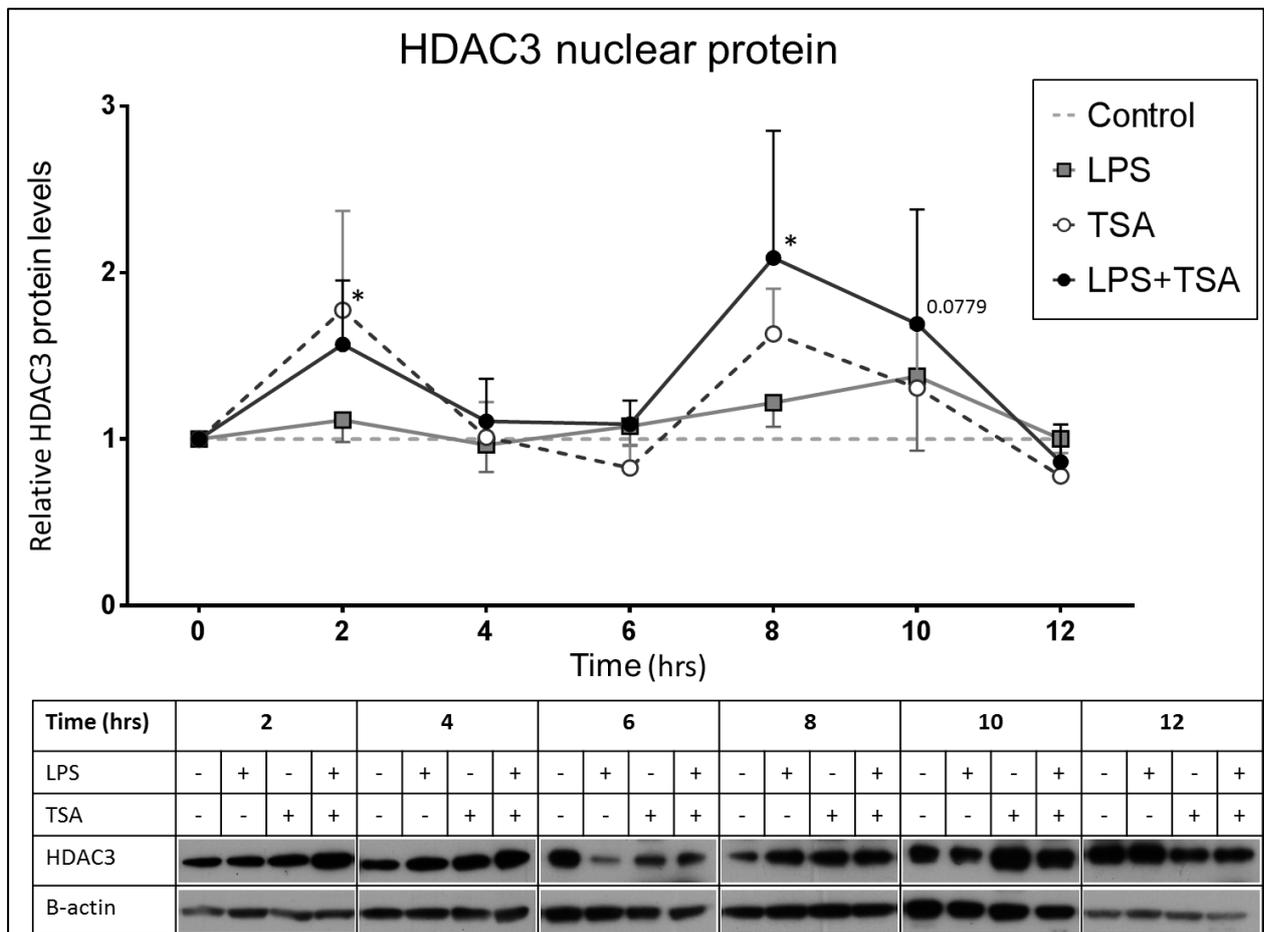
#### **Effect of LPS and TSA treatments on HDAC1 and HDAC3 protein levels in the nucleus**

There were significant effects of treatment on HDAC1 and HDAC3 nuclear protein levels (HDAC1  $p=0.0022^{**}$ , HDAC3  $p=0.0031^{**}$ ). TSA increased HDAC1 protein levels [Figure 26], both alone and in combination with LPS, peaking at 2 and 6 hours post treatment (Control-TSA: 2hrs  $p=0.0855$ , 6hrs  $p=0.0147^*$ , Control-LPS+TSA: 2hrs  $p=0.0222^*$ , 6hrs  $p=0.0003^{***}$ ). Similarly HDAC3 nuclear protein levels were increased by TSA, peaking at 2 hours and again at approximately 8 hours post treatment (Control-TSA: 2hrs  $p=0.0418^*$ , Control-LPS+TSA: 8 hrs.  $p=0.0024^{**}$ , 10hrs  $p=0.0779$ ) [Figure 27]. LPS treatment alone did not significantly alter HDAC1 or HDAC3 nuclear protein levels.



**Figure 26: HDAC1 nuclear protein levels in human placental explants**

Protein production was calculated as density ( $OD/mm^2$ ) relative to  $\beta$ -actin loading control, as a proportion of the internal control. Data are presented as relative change from control at each time point, and are given as means  $\pm$  SE ( $n=6$ ). Statistical significance is given as \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

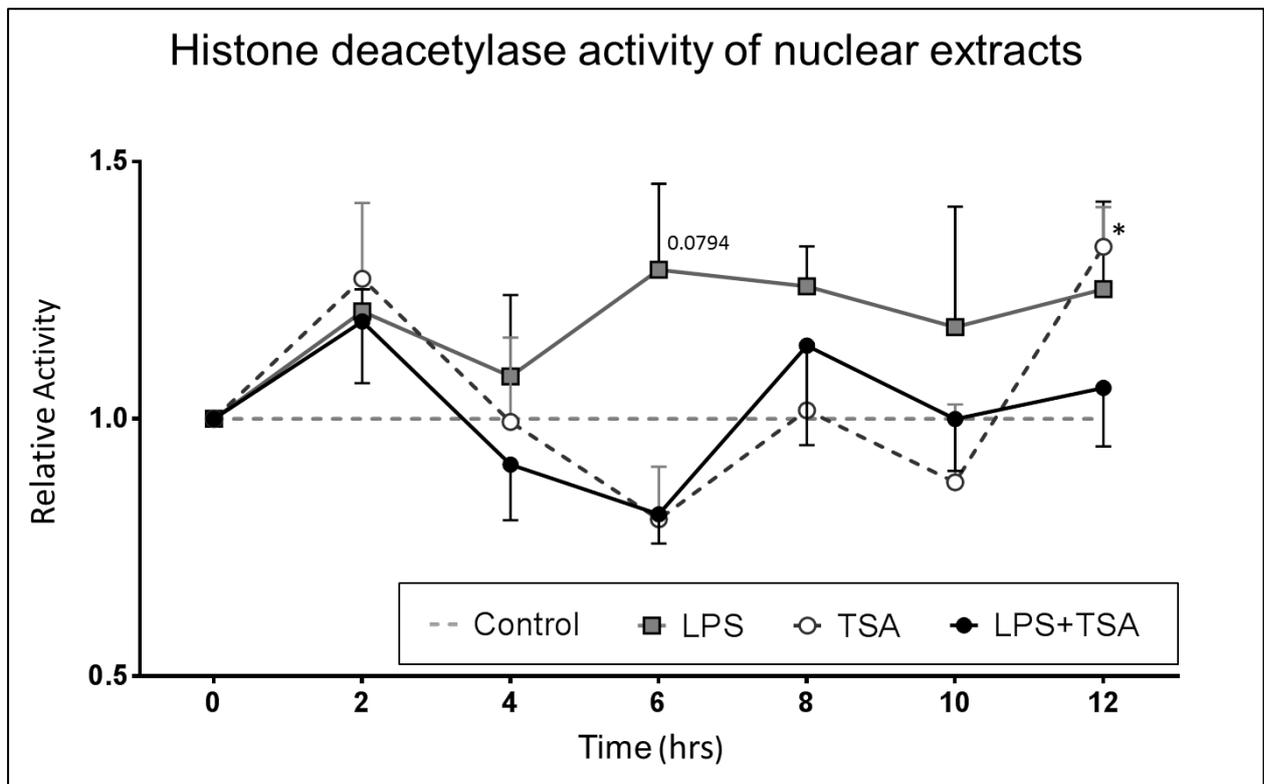


**Figure 27: HDAC3 nuclear protein levels in human placental explants**

Protein production was calculated as density ( $OD/mm^2$ ) relative to  $\beta$ -actin loading control, as a proportion of the internal control. Data are presented as relative change from control at each time point, and are given as means  $\pm$  SE ( $n=6$ ). Statistical significance is given as \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

**Effect of LPS and TSA treatments on total nuclear histone deacetylase activity**

Treatment had significant effects on the total histone deacetylase activity of the placental nuclear extracts ( $p=0.0075^{**}$ ) [Figure 28]. There was a trend towards increased deacetylase activity compared to controls following LPS treatment (6hours  $p=0.0794$ ). TSA treatment resulted in elevated activity relative to controls at 12hours post treatment ( $0.0347^*$ ).



**Figure 28: Total histone deacetylase activity in nuclear extracts from human placental explants.**

HDAC activity was calculated as ng (deacetylated product) per minute, per mg of nuclear protein. Data are presented as relative change from control at each time point, and are given as means  $\pm$  SE (n=6). Statistical significance is given as \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

## Discussion

In this study we examined HDAC1 and HDAC3 mRNA, nuclear protein, and total HDAC activity in placental villous explants in the presence and absence of LPS stimulation and TSA treatment. The HDAC inhibitor TSA is a general inhibitor for class I and II HDACs. It has also been shown to reactivate methylation-silenced genes even in the absence of DNA methyltransferase inhibitors (**Maass et al. 2002**). Studies have also reported reduced global DNA methylation, reduced DNMT1 protein levels (**Arzenani et al.; Januchowski et al. 2007**), altered DNMT1 nuclear dynamics and interactions with chromatin (**Arzenani et al.**) and reduced DNMT3b mRNA levels (**Xiong et al. 2005**).

Data from this study showed that HDAC inhibition by TSA treatment also feeds back on HDAC1 mRNA expression, resulting in a transient increase in HDAC1 mRNA expression at 2 hours, before returning to baseline. Other studies have also reported a TSA feedback loop, which appears to be dependent on growth phase. TSA inhibition

has been shown to feedback on HDAC1 mRNA levels. TSA inhibition of the hepatocellular carcinoma cell line Hep 3B resulted in increased HDAC1mRNA, but only when the cells were at low density or in exponential growth. HDAC1 mRNA expression returned to basal levels at higher densities and became unresponsive to TSA (**Gray et al. 1998; Gray et al. 2000**).

Our results also showed that LPS treatment generally resulted in decreased HDAC1 mRNA expression relative to control. This is in contrast to a study in macrophages which found that LPS stimulation up regulated HDAC1 mRNA till 8hours post treatment with a return to baseline by 24hours (**Aung et al. 2006**). This may be due to the mixed cell population present in tissue as opposed to purified cells in culture, but also culture conditions differed, with our study being undertaken at 8% oxygen (physiologically relevant for term placentae (**Miller et al. 2005; Sullivan et al. 2006**) and under serum free conditions. Additionally our results are presented relative to controls, whereas the study in macrophages only appears to have measured HDAC1 mRNA across time under LPS stimulation (**Aung et al. 2006**). It is possible that under their culture conditions HDAC1 was also increasing over time basally, as a result of cell cycle activity and media change.

In the present study co-treatment of TSA and LPS resulted in no alteration in HDAC1 mRNA from control levels till 6 hours post treatment, when they were significantly reduced relative to control, with the effect of LPS stimulation appearing to dominate over the TSA effects. Neither TSA nor LPS treatment resulted in significant alterations to HDAC3 mRNA expression, and this may reflect the dual nuclear/cytoplasmic localization of HDAC3 resulting in extensive regulation at the protein level rather than mRNA.

In addition to transiently increasing HDAC1 mRNA, we found that TSA increased nuclear protein levels of both HDAC1 and HDAC3 with an initial peak at 2 hours, before a secondary increase 4-6 hours later. Interestingly TSA treatment of Hep3B cells for 24 hours has been shown to result in the cytoplasmic export of HDAC1 but not HDAC3 (**Arzenani et al.**), which may account for the return to baseline of HDAC1 nuclear protein by 8 hours post treatment, when HDAC3 is still elevated. LPS treatment did not significantly alter HDAC1 or HDAC3 nuclear protein levels; however this may reflect the short duration of the study. LPS has also been shown to deplete cellular levels of HDAC1 and not HDAC3 (**Vashisht Gopal et al. 2006**) however doses of LPS were higher than we used in our study. Pro-inflammatory signalling activated by IL-1 $\beta$ , TNF $\alpha$

or IFN $\gamma$  also induced HDAC1 ubiquitination and proteosomal degradation (**Gopal et al. 2006; Vashisht Gopal et al. 2006**); however effects were transient in some cell types. We previously showed that significant changes in TNF $\alpha$  and IL-1 $\beta$  with LPS stimulation in our model did not occur till 4 and 6 hours post treatment respectively (**Munro et al. 2013**), so would be unlikely to significantly affect degradation in the time period investigated.

The most striking observation in this study was the lack of a pronounced decrease in total histone deacetylase activity with TSA treatment. One potential explanation may be the nuclear extraction process. Repression of histone deacetylase activity is traditionally measured by proxy, by looking for increases in global histone acetylation in the cells or tissue of interest. Activity assays allow for intrinsic histone deacetylase activity to be measured in vitro, and addition of HDAC inhibitors at variable concentrations during the assay allows for effective concentrations to be determined. Assays for our control and LPS treated tissues give an idea of the current HDAC activity levels at that point in time. However with TSA treated tissues, we cannot rule out the possibility that the nuclear extraction process has relieved the repression of TSA on the histone deacetylases, such that the assay is measuring the potential activity, rather being representative of the actual conditions in the tissue. Certainly fluctuations in mean activity do correlate well with HDAC1 and HDAC3 nuclear protein levels at that time. Additionally total activity is measured, and the assay used a histone substrate, therefore the combined effects of individual HDAC subpopulations, sensitivity to TSA, and substrate preference could be masking results.

There was a trend for increased total HDAC activity with LPS stimulation. This is interesting as it would explain how HDAC inhibition can mitigate some of the effects of LPS stimulation. LPS increases HDAC activity in cardiomyocytes and the effects have been specifically linked to HDAC3 with siRNA studies (**Zhu et al. 2010**). We did not observe any changes in either HDAC3 mRNA or nuclear protein levels with LPS stimulation; however it is possible that a change in activity is independent of protein levels. Certainly HDAC1 activity can be modulated through post translational modification (**Simone et al. 2004; Qiu et al. 2006**), and it is possible that HDAC3 is similarly affected. In contrast to increased HDAC activity with LPS treatment, another study, also in cardiac myocytes, reported that TNF $\alpha$  inhibited HDAC activity, with the presence of HDAC1 repressing TNF $\alpha$  induced NF- $\kappa$ B gene transcription resulting in cell

death independently of HDAC1 localisation (**Gang et al.**). Mutations which inhibited the catalytic activity of HDAC1 restored NF- $\kappa$ B gene transcription.

## Conclusions

Histone deacetylase inhibitors offer attractive solutions for the treatment of inflammatory conditions. Dysregulated or untimely cytokine expression is linked to preterm labour, preeclampsia and IUGR (**Keelan et al. 2003; Rusterholz et al. 2007; Sharma et al. 2007; Mullins et al. 2012**) and we have previously reported that cytokine protein production by the placenta and gestational membranes, in response to stimulation, can be modulated with HDAC inhibitor treatment (**Sato et al. 2006; Mitchell et al. 2012; Munro et al. 2013**). If inflammatory stimuli such as LPS exposure are increasing HDAC activity, this may explain the ability of HDAC inhibitors to act as anti-inflammatory agents (**Leoni et al. 2002; Bode et al. 2007; Roger et al. 2011**). However many of the HDAC inhibitors used in such studies to date have broad actions affecting entire classes of HDACs. Concerning is the fact that in several instances HDAC expression or production has been increased to compensate with the loss of activity. It is known for example, that class IIa HDACs can repress transcription independently of their C-terminal catalytic domain (**Zhou, Richon, Rifkind et al. 2000**), so what effect would increased HDAC protein levels have, even if not “active”? We conclude that there is mounting evidence for a role in placental cytokine regulation by HDAC1 and HDAC3; however as TSA feedback loops appear to be present, further studies would benefit from targeted knockdown of specific histone deacetylases.

## Acknowledgements

We thank P. Raghuraman for her valuable assistance with protein work. We also thank P. Logan, D De Silva, and Z. Vincent for their help and support. We acknowledge and thank ADHB staff for assisting with patient consents and collection of placentae.

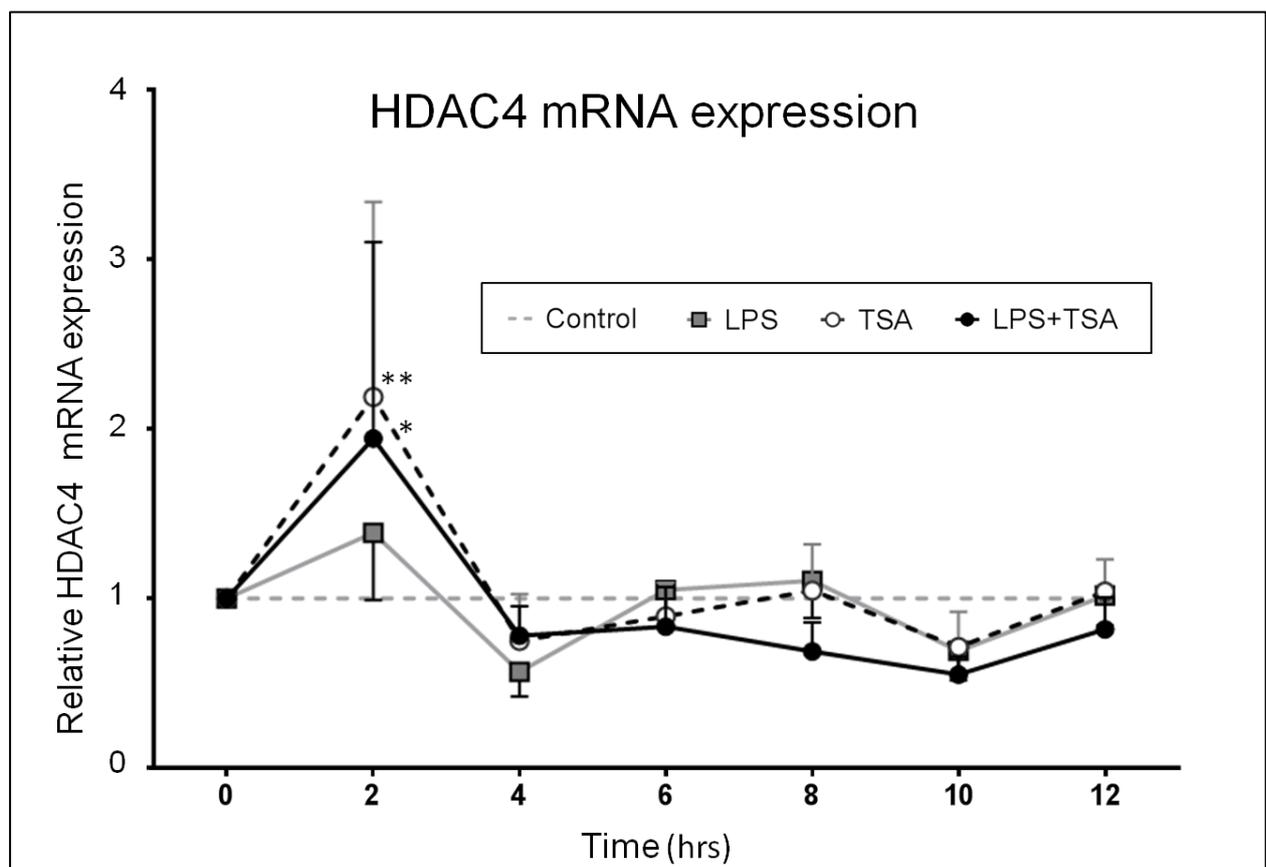
## Part B: Class IIa HDAC expression

**Background:** We have identified changes in Class I HDAC expression with TSA treatment suggesting a feedback mechanism.

**Aim:** To determine if other HDACs associated with cytokine pathways were similarly affected by TSA treatment.

**Methods:** qPCR, as previously described in part A.

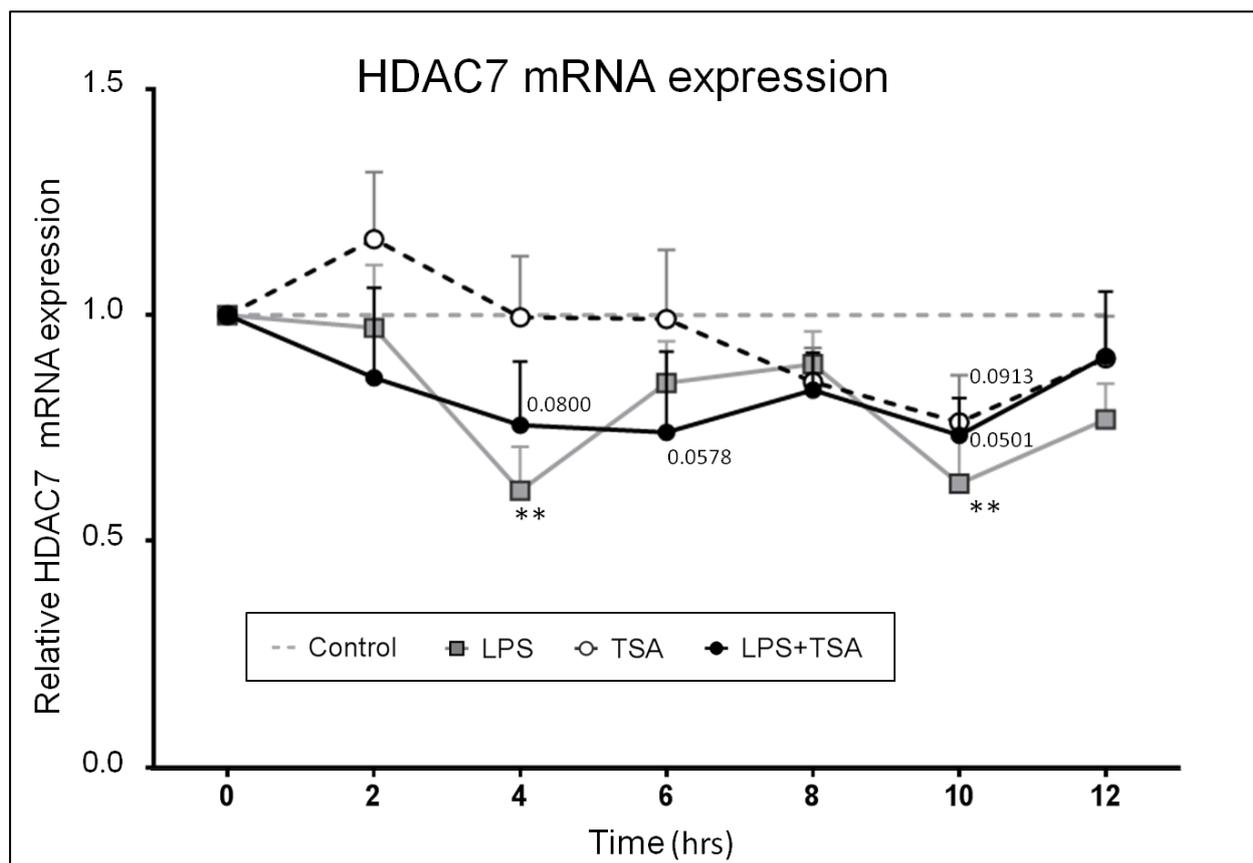
**Results:** There was a significant transient increase in HDAC4 mRNA expression at 2 hours post treatment in association with TSA treatment both with ( $p \leq 0.05$ ) and without ( $p \leq 0.01$ ) LPS stimulation [Figure 29]. HDAC7 mRNA expression was not significantly altered by TSA treatment, but was significantly reduced by LPS; 4hrs  $p \leq 0.01$ , 10hours  $p \leq 0.01$  [Figure 30]. There was a trend for reduced HDAC7 mRNA expression with LPS and TSA co-treatment (6hrs  $p = 0.0578$ , 10hrs  $p = 0.0501$ ).



**Figure 29: HDAC4 mRNA expression in human placental explants**

Real-time-PCR results were first calculated as absolute values before normalization against the geometric mean of three housekeeping genes: *YWHAZ*, *RPL13A* and *ACTB*. Data are presented as

relative change from control at each time point, and are given as means  $\pm$  SE (n=6). A p value of  $\leq 0.05$  was considered significant.



**Figure 30: HDAC7 mRNA expression in human placental explants**

Real-time-PCR results were first calculated as absolute values before normalization against the geometric mean of three housekeeping genes: *YWHAZ*, *RPL13A* and *ACTB*. Data are presented as relative change from control at each time point, and are given as means  $\pm$  SE (n=6). A p value of  $\leq 0.05$  was considered significant.

**Discussion:** the histone deacetylase activities of both HDAC 4 and HDAC7 have been linked to multiprotein complexes with HDAC3 (Fischle et al. 2001; Fischle et al. 2002). Both HDAC4 and HDAC7 are also linked to HIF-1 $\alpha$ . HDAC4 stabilises HIF-1 $\alpha$  (Qian et al. 2006), and TSA treatment or siRNA knockdown have been shown to increase HIF-1 $\alpha$  acetylation and increases expression of p53 and VHL leading to HIF-1 $\alpha$  degradation (Kim et al. 2001; Xenaki et al. 2008). HDAC7, which translocates from the cytoplasm to the nucleus under hypoxic conditions, forms a complex with HIF-1 $\alpha$  and p300, resulting in increased levels of HIF-1 $\alpha$  target genes (Kato et al. 2004).

TNF $\alpha$ , IL-1 and IL-10 expression by placental cells and tissues derived from preeclamptic placentas are sensitive to the effects of hypoxia (**Benyo et al. 2001; Bowen et al. 2005**) as are normal term tissues (**Benyo et al. 1997; Hung et al. 2004; Royle et al. 2009**). It is possible that HDAC4 and HDAC7 are influencing cytokine expression in our placental explant model as a result of interactions with HIF1 $\alpha$ .

**Conclusions:** As with HDAC1, HDAC4 mRNA expression appears to be sensitive to TSA, and HDAC7 exhibits similar reductions in expression with LPS as those seen with HDAC1. These two HDACs are closely linked to the stability and actions of HIF-1 $\alpha$  and further investigation into their expression particularly in pre-eclamptic placentae could yield new insights into the mechanisms of the disease.

## Chapter 5 Discussion and Conclusions

Normal pregnancy results in significant immunologic alterations, including the maintenance of a heightened inflammatory state, controlled through the appropriate balance of pro- and anti-inflammatory cytokines. Several pathologies of pregnancy such as pre-eclampsia, intrauterine growth restriction and preterm birth have been associated with variations in cytokine expression, both from the placenta and gestational membranes, and in the maternal circulation. Following their investigation as anticancer drugs, histone deacetylase inhibitors were identified as having anti-inflammatory actions, leading to further studies into how histone deacetylases may be involved in cytokine regulation. However, despite the importance of appropriate histone deacetylase activity during development little is known about histone deacetylases (HDACs) in the placenta and membranes.

When we began this study there were no reports available on histone deacetylase expression in the placenta and membranes. We have since confirmed that all eighteen human HDACS are expressed in term placenta, amnion and choriodecidua. In general, the amnion has the lowest expression for each HDAC while choriodecidual expression is equal to or higher than that of the amnion, and villous expression is the highest. However three HDACs had distinctly different patterns of expression with elevated expression in the choriodecidua, equaling or outstripping that of the villous placenta. These HDACs; -4, -10, and -11 are associated with prostaglandin synthesis, heat shock protein 70, and IL-10 regulation respectively. We have shown that amnion, choriodecidua and villous placenta, each have distinct HDAC expression profiles. Therefore it is not surprising that the tissues have previously been shown to respond differently to HDAC inhibition.

While it has been shown that HDAC inhibition can reduce LPS stimulated production of TNF $\alpha$ , nothing was known about the kinetics of this effect or whether it was transcriptionally or translationally affected by TSA. We established a time course to examine the potential links between HDAC activity and cytokine expression and production by placental villous explants *in vitro*. Term explants were maintained at a physiologically relevant oxygen tension with/without an inflammatory stimulus and/or histone deacetylase inhibitor. In the presence of an inflammatory stimulus, HDAC inhibition resulted in mitigation of the normal response, with reduced production of both the proinflammatory cytokine tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and the anti-inflammatory

cytokine interleukin-10 (IL-10) and accompanying reductions in TNF $\alpha$  expression. As there is no compensatory effect on IL-1 $\beta$ , IL-1ra, IL-6, or IL-8 mRNA expression or protein production, the results is dysregulation of the cytokine balance. Interestingly TSA only affected LPS stimulated cytokine production, and had no effect basally. This suggests a similar mechanism to the cancer two-hit hypothesis where functionally redundancy or cellular check processes prevent aberrant expression up to a point, until something else goes wrong resulting in uncontrolled processes. In the case of cytokine expression, an alteration in HDAC expression/production/activity may have little effect in an unstressed environment. In the context of maternal chronic inflammation or antigen exposure, aberrant histone deacetylase expression could drastically change the cytokine profile in response to these stimuli and manifest as a disease state.

Indeed it appears inflammatory stimuli directly affect HDAC expression. We showed that LPS exposure results in gradual decreases in HDAC1 and HDAC7 mRNA expression. There is also a trend for increased HDAC activity following LPS treatment. If bacterial exposure can increase HDAC activity, this may explain the anti-inflammatory actions of HDAC inhibition, which would be counteracting this activity increase. However we also identified the ability of TSA to transiently increase HDAC1 and HDAC4 expression, and HDAC1 and HDAC3 nuclear protein. It remains to be seen what the mechanism of this feedback loop is. In the context of HDAC inhibition, it is possible that some of the effects are not due to decreased HDAC activity, but rather increased HDAC protein produced to compensate. Even if chemical inhibition holds, many of the functions of the class IIa HDACs in particular are through interactions with co-repressors which are independent of their functional domain. As many of the HDAC inhibitors used in such studies to date have broad actions affecting entire classes of HDACs we hope to further investigate the effects of HDAC inhibition using more selective inhibitors

Cytokines exhibit multiple mechanisms for control of expression in different contexts and separating primary and secondary effects is difficult, but further work with selective inhibitors may help identify parts of the cytokine network that can become “uncoupled” and under what circumstances. It is not known to what extent cytokine dysregulation in conditions such as preeclampsia is causative or correlative, but as histone deacetylase inhibition can uncouple cytokine expression in normal tissues, so might increased histone deacetylase expression and activity underlie the differences in cytokine expression seen with pre-eclampsia.

Just as modulation of HDAC activity or histone acetylation levels may represent an underlying cause/ consequence of cytokine dysregulation in many conditions, it also becomes a potential therapeutic strategy, with increasing numbers of specific HDAC inhibitors being developed. One of the future challenges will be separating the differential responses of neighboring (and interacting) tissues and following up observations with studies earlier in gestation when problems first arise.

Indeed one of the biggest limitations in these studies has been the use of tissue, which will have variable cell populations between samples. This was also the biggest problem statistically, as it meant there was an inherent level of variation amongst control tissues. Of particular interest would be cell type specific investigation of histone deacetylase expression in choriodecidua. How much for example, of the expression of HDAC4, HDAC10, and HDAC11 which is much greater in these tissues, can be attributed to the maternal decidua? Additionally what are the gestational affects? For example, one of the only known functions of HDAC11 is as an inhibitor of IL-10. Does the high level of HDAC11 expression contribute to inflammatory conditions in the chorion by limiting IL-10 expression? Or is the increased expression equally contributed to by both tissues at term in order to promote a proinflammatory bias and labour?

Our results for HDAC inhibition of TNF $\alpha$  expression are also limited by cell type effects. While at term we can be confident that LPS stimulated TNF $\alpha$  expression and production are mitigated, TNF $\alpha$  expression in the placenta is temporal, with alterations in the cells types contributing to expression with gestation. One further advantage of moving to singular cell types for future studies would be the ability to carry out immunoprecipitation experiments. This would allow HDAC promoter and protein binding to be examined providing more mechanistic insight into HDAC–cytokine pathway interactions.

Our studies into LPS and TSA affects were confined to villous tissue, rather than the chorion and amnion which are more associated with inflammation in infection mediated preterm labour. This was largely a logistical decision in order to get sufficient tissue to cover the range of the time course and still ensure sufficient RNA yields in these ground-breaking studies. However the contribution of villitis to pathologies of pregnancy cannot be ignored, particularly as villitis is overrepresented in cases of stillbirth (**Hulthen Varli et al. 2012**) and villitis of unknown etiology is an important cause of intrauterine growth restriction (**Redline 2007**). Ascending infections during pregnancy spread through the maternal decidua, and intracellular infections of the placental basal plate are common occurrences. We therefore feel that inflammatory processes of the

placenta are highly relevant to pathological conditions of pregnancy, with a large potential for antigen exposure and therefore potentially inappropriate activation of the inflammatory cascade.

We conclude that there is mounting evidence for a role in placental cytokine regulation by histone deacetylases. We have contributed data on normal HDAC expression in the placenta and membranes to the field, and shown that histone deacetylase inhibition results in dysregulated cytokine expression in placenta with transcription effects on TNF $\alpha$  and on the histone deacetylases themselves. We welcome further investigations into histone deacetylase expression in pathologies of pregnancy as it is likely that they are linked to aberrant cytokine expression in gestational tissues.

# APPENDICES

## Cytokines in physiological and pathological pregnancy

Below is an overview of investigations into cytokine levels detected in various tissues and fluids during pregnancy [Table 4-Table 14]. NC denotes no statistically significant change. Variable indicates gestational effects are apparent within the study groups such that direction of effect is highly dependent on time of comparison.

Source	Condition	Effect	References
Maternal serum	Gestation	NC	(Ziganshina et al. 2013)
	Miscarriage	Decreased	(Calleja-Agius et al. 2011)
	SGA	Increased	(Georgiou et al. 2011)
Maternal plasma	Gestation	Increased	(Curry et al. 2008)
Circulating white blood cells	Gestation	Variable	(Hanna et al. 2000; Wilke et al. 2006)
	Preterm	Variable	(Wilke et al. 2006)
	Preterm birth (vs. PTL, TD)	NC	(Wilke et al. 2006)
	FGR	Increased	(Raghupathy et al. 2012a)
Placenta	FGR	NC	(Amu et al. 2006)

Source	Condition	Effect	References
Maternal serum	Pregnancy	Increased	(Austgulen et al. 1994)
	Impending Labour	Increased	(Unal et al. 2011)
	Preeclampsia	NC	(Kimya et al. 1997)
	Preeclampsia	NC	(Casart et al. 2007; Szarka et al. 2010)
		Increased	(Kalinderis et al. 2011)
SGA	NC	(Georgiou et al. 2011)	
Maternal plasma	Pregnancy	Decreased	(Conrad et al. 1998)
	Labour	NC	(Laham et al. 1996b)
	Subsequent PE	NC	(Djurovic et al. 2002)
	PE	NC	(Conrad et al. 1998)
Circulating white blood cells	Gestation	NC	(Ziganshina et al. 2013)
	PE	Increased	(Luppi et al. 2006)
Maternal placental perfusate	PE	Increased	(Amash et al. 2012)

<b>IL-1<math>\beta</math> in pregnancy continued</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Cervico-vaginal fluid	IAI	Increased	(Rizzo et al. 1996)
	Preterm Birth	Increased	(Gonzalez Bosquet et al. 2005)
	Gestation	Increased	(Tanaka et al. 1998)
	Labour	Increased	(Cox et al. 1993; Tanaka et al. 1998)
Amniotic fluid	Labour	NC	(Laham et al. 1996b)
	Preterm birth (IAI)	Increased	(Romero, Mazor et al. 1992)
	Preterm Birth	Increased	(Hillier et al. 1993; Carroll et al. 1995)
	Preterm Birth (Caucasian)	NC	(Velez et al. 2008)
	Preterm Birth (African American)	Increased	(Velez et al. 2008)
	PE	NC	(Nakabayashi et al. 1998)
Amnion	Labour	Increased	(Dudley et al. 1996; Laham et al. 1996b; Keelan et al. 1999)
	Preterm Birth	Increased	(Keelan et al. 1999)
	+LPS	NC	(Laham et al. 1996b)
Chorio-decidua	Labour	NC	(Keelan et al. 1999)
		Increased	(Dudley et al. 1996; Laham et al. 1996b)
	Preterm Birth	Increased	(Keelan et al. 1999)
	+LPS	NC	(Laham et al. 1996b)
Placenta	Labour	NC	(Keelan et al. 1999)
		Increased	(Laham et al. 1996b; Hanna et al. 2000)
	Gestation	NC	(Naruse et al. 2010)
	Preterm Birth	NC	(Keelan et al. 1999)
	PE	NC	(Rein et al. 2003)
		Increased	(Rinehart et al. 1999)
	FGR	Decreased	(Amu et al. 2006)
	+LPS	Increased	(Laham et al. 1996b)
+HI bacteria	Increased	(Griesinger et al. 2001)	

<b>Table 6: IL-1ra in pregnancy</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Maternal serum	Pregnancy	Increased	(Austgulen et al. 1994)
		Decreased	(Szarka et al. 2010)
Maternal plasma	Labour	Increased	(Austgulen et al. 1994; Kimya et al. 1997)
	Subsequent PE	Increased	(Kimya et al. 1997)
	PE	Increased	(Szarka et al. 2010)
Cervico-vaginal fluid	Impending labour	Decreased	(Heng et al. 2008)
	Impending PROM	Decreased	(Heng et al. 2008)
	IAI	Increased	(Rizzo et al. 1996)
Amniotic fluid	Gestation	Increased	(Bry et al. 1995)
	Labour	NC	(Romero, Sepulveda et al. 1992)
	Female fetus	Increased	(Bry et al. 1994; Romero et al. 1994; Bry et al. 1995)
	Subsequent preterm	Increased	(Sezen et al. 2008)
	+LPS	NC	(Sezen et al. 2008)
Fetal plasma	Gestation	Increased	(Romero et al. 1994)

<b>Table 7: IL-2 in pregnancy</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Maternal serum	Subsequent PE	Increased	(Eneroth et al. 1998)
	PE	NC	(Borekci et al. 2007)
	PE	Increased	(Szarka et al. 2010)
	SGA	Decreased	(Georgiou et al. 2011)
Maternal plasma	Gestation	Decreased	(Curry et al. 2008)
Circulating white blood cells	Pregnancy	NC	(Hanna et al. 2000; Wilke et al. 2006)
	Gestation	Variable	(Wilke et al. 2006)
	Preterm	Decreased	(Wilke et al. 2006)
	Preterm birth (vs. PTL, TD)	NC	(Wilke et al. 2006)
	SGA	Increased	(Marzi et al. 1996)
Amniotic fluid	Gestation	NC	(Weissenbacher et al. 2012)
Placenta	PE	NC	(Rein et al. 2003)

Source	Condition	Effect	References
Maternal serum	Gestation	Increased	(Ziganshina et al. 2013)
	PE	Increased	(Szarka et al. 2010)
	SGA	Decreased	(Georgiou et al. 2011)
Circulating white blood cells	Gestation	Variable	(Wilke et al. 2006) (Hanna et al. 2000)
Amniotic fluid	Gestation	Decreased	(Weissenbacher et al. 2012)
Placenta	FGR	NC	(Amu et al. 2006)

Source	Condition	Effect	References	
Maternal serum	Pregnancy	Increased	(Austgulen et al. 1994)	
	Impending labour	Increased	(Unal et al. 2011)	
	Labour		NC	(Laham, Rice, Bishop, Hansen et al. 1993)
			Increased	(Opsjln et al. 1993; Hebisch et al. 2001)
	Gestation		NC	(Ziganshina et al. 2013)
			Increased	(Opsjln et al. 1993)
	Preterm labour	Increased	(Laham, Rice, Bishop, Hansen et al. 1993)	
	Miscarriage	Decreased	(Calleja-Agius et al. 2011)	
	PE		NC	(Borekci et al. 2007; Vitoratos et al. 2010)
			Increased	(Johnson et al. 2002; Madazli et al. 2003; Jonsson et al. 2006; Casart et al. 2007; Sharma et al. 2007; Szarka et al. 2010; Kalinderis et al. 2011)
	PE severity	Increased	(Sharma et al. 2007)	
	FGR	NC	(Bartha et al. 2003)	
SGA	NC	(Georgiou et al. 2011)		
PE (post-partum)	Increased	(Vitoratos et al. 2010)		
Maternal plasma	pregnancy	NC	(Conrad et al. 1998)	
	Gestation	Increased	(Curry et al. 2008)	
	Subsequent PE	NC	(Djurovic et al. 2002)	
	PE	Increased	(Kupfermenc et al. 1996; Conrad et al. 1998; Freeman et al. 2004; Bernardi et al. 2008; Kronborg et al. 2011)	
Circulating white blood cells	PE	Increased	(Luppi et al. 2006)	
Cervico-vaginal fluid	Preterm Birth	Increased	(Inglis et al. 1994; Gonzalez Bosquet et al. 2005)	
	IAI	Increased	(Rizzo et al. 1996; Rizzo et al. 1998; Holst et al. 2005)	

<b>IL-6 in pregnancy continued</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Amniotic fluid	Gestation	NC	(Bamberg et al. 2011; Weissenbacher et al. 2012)
		Increased	(Opsjln et al. 1993)
	Labour	Increased	(Opsjln et al. 1993; Saito et al. 1993; Laham et al. 1996a; Olah et al. 1996; Hebisch et al. 2001; Fukuda, Masuzaki et al. 2002)
	Culture	Increased	(Sezen et al. 2008)
	PPROM	Increased	(Fukuda, Masuzaki et al. 2002)
	Subsequent PTL	Increased	(Wenstrom et al. 1998)
	Preterm labour	NC	(Hillier et al. 1993; Laham et al. 1996a)
Amniotic fluid continued	Preterm birth	NC	(Velez et al. 2008)
	Preterm birth (IAI)	Increased	(Romero, Avila et al. 1990; Santhanam et al. 1991; Saito et al. 1993)
	Subsequent preterm, cultured	Decreased	(Sezen et al. 2008)
	Subsequent PE	NC	(Heikkinen et al. 2001)
	PE	Decreased	(Kupfermenc et al. 1996)
		Increased	(Nakabayashi et al. 1998)
+LPS	NC	(Sezen et al. 2008)	
Amnion	Labour	Increased	(Keelan et al. 1999) (Laham et al. 1996a)
	Time in culture	NC	(Laham et al. 1996a)
	Preterm Birth	Increased	(Keelan et al. 1999)
	+LPS	NC	(Laham et al. 1996a)
Amnion + Chorion	+/- IL-1 $\beta$	Increased	(Al-Asmakh et al. 2007)
	Decreased O <sub>2</sub>	Decreased	(Al-Asmakh et al. 2007)
Chorio-decidua	Labour	Increased	(Laham et al. 1996a; Simpson et al. 1998; Keelan et al. 1999; Haddad et al. 2006)
	Time in culture	Increased	(Laham et al. 1996a)
	Preterm Birth	Increased	(Keelan et al. 1999)
	+LPS	Increased	(Laham et al. 1996a)
	+LPS/IL-1 $\beta$ /TNF- $\alpha$	Increased	(Simpson et al. 1998)
Placenta	Labour	NC	(Keelan et al. 1999) (Laham et al. 1996a)
		Increased	(Laham et al. 1996a)
	Preterm Birth	NC	(Keelan et al. 1999)
	PE	NC	(Marusic et al. 2013)
		Increased	(Rein et al. 2003; Bowen et al. 2005)
	Decreased O <sub>2</sub>	Increased	(Bowen et al. 2005)
	FGR	NC	(Amu et al. 2006)
		Increased	(Street et al. 2006)
	+LPS	Increased	(Laham et al. 1996a)
+HI bacteria	Increased	(Griesinger et al. 2001)	

<b>Table 10: IL-8 in pregnancy</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Maternal serum	Pregnancy	Increased	(Sharma et al. 2007)
	Gestation	NC	(Ziganshina et al. 2013)
	Labour	Increased	(Hebisch et al. 2001)
	PE	Increased	(Johnson et al. 2002; Jonsson et al. 2006; Sharma et al. 2007; Szarka et al. 2010)
	SGA	Decreased	(Georgiou et al. 2011)
		Increased	(Laskowska et al. 2007)
Maternal plasma	Gestation	NC	(Laham, Rice, Bishop, Ransome et al. 1993)
	Labour	NC	(Laham, Rice, Bishop, Ransome et al. 1993)
Circulating white blood cells	PE	Increased	(Luppi et al. 2006)
	FGR	Increased	(Raghupathy et al. 2012b)
Cervico-vaginal fluid	Gestation	Increased	(Tanaka et al. 1998)
	Labour	Increased	(Tanaka et al. 1998)
	Preterm Birth	Increased	(Gonzalez Bosquet et al. 2005)
	IAI	Increased	(Hitti et al. 2001; Holst et al. 2005)
Amniotic fluid	Gestation	NC	(Weissenbacher et al. 2012) (Bamberg et al. 2011)
		Increased	(Laham, Rice, Bishop, Ransome et al. 1993)
	Labour	Increased	(Romero, Ceska et al. 1991; Laham, Rice, Bishop, Ransome et al. 1993; Saito et al. 1993; Hebisch et al. 2001)
	Preterm birth (IAI)	Increased	(Romero, Ceska et al. 1991; Saito et al. 1993)
	Preterm birth	Increased	(Velez et al. 2008)
	Subsequent PE	NC	(Heikkinen et al. 2001)
	PE	Increased	(Nakabayashi et al. 1998)
Amnion	Labour	NC	(Laham et al. 1999)
		Increased	(Keelan et al. 1999)
	Preterm birth	NC	(Laham et al. 1999)
		Increased	(Keelan et al. 1999)
	+LPS/ cytokines	NC	(Laham et al. 1997)
Amnion + Chorion	Labour	Increased	(Haddad et al. 2006)
Chorio-decidua	Labour	NC	(Laham et al. 1999)
		Increased	(Keelan et al. 1999)
	Preterm birth	Increased	(Keelan et al. 1999; Laham et al. 1999)
	+LPS	Increased	(Laham et al. 1997)
	+ cytokines	NC	(Laham et al. 1997)

<b>IL-8 in pregnancy continued</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Placenta	Gestation	Increased	(Naruse et al. 2010)
	Labour	NC	(Keelan et al. 1999)
			(Laham et al. 1999)
	Preterm birth	NC	(Keelan et al. 1999; Laham et al. 1999)
	PE	Increased	(Bowen et al. 2005)
	Decreased O <sub>2</sub>	Increased	(Bowen et al. 2005)
	FGR	NC	(Amu et al. 2006)
		Increased	(Hahn-Zoric et al. 2002)
	+LPS	Increased	(Laham et al. 1997; Shimoya et al. 1999; Ma et al. 2006)
+TNF $\alpha$	Increased	(Laham et al. 1997)	
+HI bacteria	Increased	(Griesinger et al. 2001)	

<b>Table 11: IL10 in pregnancy</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Maternal Serum	Pregnancy	Decreased	(Szarka et al. 2010)
	Gestation	NC	(van der Weiden et al. 2005)
		Decreased	(Ziganshina et al. 2013)
	Miscarriage	Decreased	(Calleja-Agius et al. 2011)
	Threatened miscarriage	Variable	(Ziganshina et al. 2013)
	PE	NC	(Djurovic et al. 2002; Makris et al. 2006)
		Decreased	(Hennessy et al. 1999; Borekci et al. 2007; Sharma et al. 2007)
		Increased	(Madazli et al. 2003; Bakheit et al. 2009; Szarka et al. 2010)
Eclampsia	Increased	(Borekci et al. 2007)	
SGA	Decreased	(Georgiou et al. 2011)	
Maternal Plasma	Gestation	NC	(Holmes et al. 2003)
	Pregnancy	NC	(Conrad et al. 1998)
	PE	NC	(Conrad et al. 1998)
Circulating white blood cells	Pregnancy	Variable	(Hanna et al. 2000)
	Placental insufficiency	Decreased	(Raghupathy et al. 2012b)
Amniotic fluid	Gestation	NC	(Heyborne et al. 1994; Dudley, Hunter et al. 1997; Gotsch et al. 2008; Weissenbacher et al. 2012)
	Labour	NC	(Greig et al. 1995; Dudley, Hunter et al. 1997)
		Increased	(Gotsch et al. 2008)
Culture	Increased	(Sezen et al. 2008)	

<b>IL-10 in pregnancy continued</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Amniotic fluid continued	Subsequent PE	NC	(Heikkinen et al. 2001)
	Subsequent Preterm (cultured)	Increased	(Sezen et al. 2008)
	Subsequent preterm Birth	NC	(Gotsch et al. 2008)
		Increased	(Apuzzio et al. 2004)
	Preterm birth	NC	(Velez et al. 2008)
	Preterm birth (vs. PTL, TD)	Increased	(Gotsch et al. 2008)
	SGA	NC	(Spong et al. 1996)
		Increased	(Heyborne et al. 1994)
	Term IAI	Increased	(Gotsch et al. 2008)
	Preterm IAI	Increased	(Greig et al. 1995; Gotsch et al. 2008)
+LPS	NC	(Sezen et al. 2008)	
Amnion	Labour	NC	(Trautman et al. 1997)
	+IL-1 $\beta$	NC	(Trautman et al. 1997)
Chorio-decidua	Labour	NC (mRNA)	(Trautman et al. 1997)
		Decreased	(Simpson et al. 1998)
	+LPS/IL-1 $\beta$ /TNF $\alpha$	Increased	(Simpson et al. 1998)
Chorion	+IL-1 $\beta$	NC	(Trautman et al. 1997)
Decidua	Labour	NC	(Jones et al. 1997; Vives et al. 1999)
	+IL-1 $\beta$	Increased	(Trautman et al. 1997)
Placenta	Gestation	Decreased	(Hanna et al. 2000)
	Labour	NC (mRNA)	(Vives et al. 1999)
		Increased (mRNA)	(Hanna et al. 2000)
		NC (protein)	(Hanna et al. 2000)
		NC	(Marusic et al. 2013)
	PE	Decreased	(Hennessy et al. 1999; Rein et al. 2003; Makris et al. 2006)
		Increased	(Rinehart et al. 1999; Bowen et al. 2005)
		Increased O <sub>2</sub>	Decreased
	IUGR	Decreased	(Amu et al. 2006)
	+HI bacteria	Increased	(Griesinger et al. 2001)

Source	Condition	Effect	References
Maternal serum	SGA	Decreased	(Georgiou et al. 2011)
Maternal plasma	Gestation	Increased	(Curry et al. 2008)
Circulating white blood cells	Placental insufficiency	Increased	(Raghupathy et al. 2012b)
Amniotic fluid	Gestation	Increased	(Weissenbacher et al. 2012)
Placenta	Gestation	NC	(Naruse et al. 2010)
	FGR	Decreased	(Amu et al. 2006)

Source	Condition	Effect	References
Maternal serum	PE	Increased	(Madazli et al. 2003; Szarka et al. 2010)
Circulating white blood cells	Pregnancy	NC	(Wilke et al. 2006)
Amniotic fluid	Subsequent PE	NC	(Heikkinen et al. 2001)
Placenta	FGR	NC	(Hahn-Zoric et al. 2002)
		Increased	(Amu et al. 2006)

Source	Condition	Effect	References	
Maternal serum	Pregnancy	Decreased	(Szarka et al. 2010)	
	Gestation	NC	(Ziganshina et al. 2013)	
	Impending labour	Increased	(Unal et al. 2011)	
	Miscarriage	Decreased	(Calleja-Agius et al. 2011)	
	PE	Increased	(Kupferminc et al. 1994; Vince et al. 1995; Conrad et al. 1998; Johnson et al. 2002; Hayashi et al. 2005; Laskowska et al. 2006; Sharma et al. 2007; Cackovic et al. 2008; Vitoratos et al. 2010)	
	PE severity	Increased	(Sharma et al. 2007)	
	FGR	Increased	(Bartha et al. 2003)	
	SGA		NC	(Georgiou et al. 2011)
			Increased	(Schipper et al. 2005)
PE (post-partum)	Increased	(Vitoratos et al. 2010)		
Maternal plasma	Pregnancy	NC	(Conrad et al. 1998)	
	Gestation	NC	(Curry et al. 2008)	
	Subsequent PE	NC	(Djurovic et al. 2002)	
	PE	Increased	(Conrad et al. 1998; Bernardi et al. 2008; Kronborg et al. 2011)	

<b>TNF-<math>\alpha</math> in pregnancy continued</b>			
<b>Source</b>	<b>Condition</b>	<b>Effect</b>	<b>References</b>
Circulating white blood cells	IUGR with insufficiency	Increased	<b>(Raghupathy et al. 2012b)</b>
	PE	Increased	<b>(Chen et al. 1996)</b>
Cervico-vaginal fluid	Preterm birth (vs. PTL, TD)	Increased	<b>(Inglis et al. 1994);</b>
	IAI	Increased	<b>(Rizzo et al. 1996)</b>
Amniotic fluid	Gestation	Variable	<b>(Weissenbacher et al. 2012) (Opsjln et al. 1993; Bamberg et al. 2011)</b>
	Labour	Increased	<b>(Opsjln et al. 1993)</b>
	Preterm birth (Caucasian)	NC	<b>(Velez et al. 2008)</b>
	Preterm birth (African American)	Increased	<b>(Velez et al. 2008)</b>
	Preterm birth	Increased	<b>(Romero, Manogue et al. 1989)</b>
	Subsequent PE	NC	<b>(Nakabayashi et al. 1998; Heikkinen et al. 2001)</b>
	IUGR	Increased	<b>(Heyborne et al. 1994)</b>
Placenta	Gestation	Variable	<b>(Chen et al. 1991; Pijnenborg et al. 1998)</b>
	Labour	Increased	<b>(Hanna et al. 2000)</b>
	PE	NC	<b>(Rein et al. 2003; Hayashi et al. 2005; Marusic et al. 2013)</b>
		Increased	<b>(Wang et al. 1996; Rinehart et al. 1999)</b>
	FGR	NC	<b>(Amu et al. 2006)</b>
		Increased	<b>(Almasry et al. 2012)</b>
	SGA	Increased	<b>(Holcberg et al. 2001)</b>
+HI bacteria	NC	<b>(Griesinger et al. 2001)</b>	

## Methods

Due to page restrictions associated with manuscript submission and publication, the foregoing experimental methods provided in Chapters 2-4 were kept brief.

Experimental methods have been collated in the following appendix, along with additional detail and validations for the benefit of readers unfamiliar with the techniques used, or requiring additional detail to replicate experiments in their entirety.

This appendix is intended to be supplementary to, not replace the foregoing methods sections. As there are some variations in the techniques used between different experiments, details in Chapters 2-4 should be noted before the appendix is consulted.

## ***Tissue Collection and Culture***

Preliminary investigations utilized placental tissue and gestational membranes previously collected and stored at -80°C, either directly after delivery or following culture. Ms Munro was involved in the ongoing collection and processing of tissue for this tissue bank.

Subsequent experiments utilized placental tissue collected for the PEARL (Placental Epigenetics and Regulation of Labour) study, launched during the course of this PhD, and for which more extensive demographic data is available. Ms Munro was involved in the establishment of a tissue bank from these tissues, and was solely responsible for the setting up and subsequent processing of the placental villous explants for time course development. Assistance was provided for the collection of discrete time points only (18, 24 hrs).

All placentas and attached membranes were collected from women delivering at Auckland city hospital with informed written consent and the approval of the NorthernX regional ethics committee within 30 min of delivery.

### *PEARL study exclusion criteria:*

- Smoking
- Drug/Alcohol consumption (excluding dietary supplements)
- Pregnancy complications (high blood pressure/gestational diabetes/IUGR/preeclampsia)
- Multiple pregnancy
- Meconium or Infection diagnosed at birth
- Induced or premature labour

Following collection, the gestational membranes were manually separated into amnion and chorion (with attached maternal decidua) and cut away from the placenta. Following two washes in sterile and pre-warmed (37°C) PBS, strips of each membrane (orientated from break point to placental margin) were snap-frozen and stored at -80°C. Placental Villous tissue was collected using surgical scissors, working from the maternal side. The basal tissue was first cut away from a target cotyledon and discarded, then tissue sampled from the interior. For tissue banking five points were taken in a “W” shape across the placenta to give a representative sample with varying distance from the umbilical insertion site. For explants, all cotyledons were sampled to gain sufficient

tissue. Tissue was teased apart by blunt dissection to remove visible connective tissue and vessels, and washed in PBS twice before snap freezing.

For explant culture, all tissues were transferred to warmed (37°C) Gibco® DMEM/F-12 with GlutaMAX™ media (Life Technologies, Carlsbad, CA, USA), supplemented with 0.1% bovine gamma globulin, and Gibco® Antibiotic-Antimycotic (Amphotericin B, Streptomycin, Penicillin) directly after washing. Tissue was then further dissected to uniform size and randomly distributed across culture wells. Median size for villous explants was 20 mg (wet weight), while membrane discs of standard size were created using a sterile cork borer. Following settling and treatment incubations, the wet weight of the tissue in each well was determined and both tissue and media frozen for subsequent analysis.

Amnion, choriodecidual and placental explants utilised for preliminary studies were generated during the course of previous work by our lab, for published data see: **(Sato et al. 2006; Mitchell et al. 2012)**. Studies investigated the effect of the DNA methyltransferase inhibitor 5-aza-2' deoxycytidine, and the histone deacetylase inhibitor trichostatin A (TSA) on cytokine production with/without lipopolysaccharide (LPS) stimulation. After 24hrs equilibrium, tissue utilised had been cultured for a further 24hrs with and without 300nM TSA and/or 5 µg/mL LPS in a standard 37°C incubator in room air supplemented with 5% CO<sub>2</sub>.

For time course studies, placental explants were cultured at 37°C in 6-well plates (six pieces per well) in triplicate, media as above, in a humidified incubation box within an O<sub>2</sub> control glove box (Coy Laboratory Products; Detroit, MI, USA). The glove box utilizes glove ports and an airlock to allow access to the chamber interior without disruption of the experimental environment. Explants were cultured for 24 hrs under conditions of 5% CO<sub>2</sub> and 8% O<sub>2</sub> (displacement by N<sub>2</sub>) before the treatment period commenced. The treatment period consisted of the addition of 300nM TSA, which inhibits histone deacetylase activity, or DMSO carrier in fresh media, in the presence or absence of LPS (5 µg/mL) with treatment durations of 0-12 hrs (2 hour intervals) as well as 18, 24 and 48 hrs. All treatments and collections were performed within the glove box at 8% O<sub>2</sub>. Following treatment the wet weight of the tissue in each well was determined and both tissue and media frozen for subsequent analysis.

## ***Tissue Processing***

All tissues were processed from frozen and maintained in that state. Tissue-banked samples and banked explants were crushed to a powder in the presence of liquid nitrogen with pestle and mortar before being transferred to fresh 1.5mL tubes and resuspended in either TRIzol® reagent (RNA extraction) or lysis buffer (protein analysis). For time-course samples, in order to maintain uniformity across the 111 samples per placenta, high-throughput disruption of tissue while frozen was performed using the TissueLyser II (QIAGEN, Hilden, Germany). Adapter plates and stainless steel balls were pre-frozen resulting in a crushing action in each sample tube similar to pestle and mortar. Villous samples were crushed at 30Hz for 4 cycles of 30 seconds duration, and components were re-cooled on dry ice between cycles.

## ***RNA extraction***

Crushed tissues were resuspended in 800µL TRIzol® reagent and 10µL 20x diluted glycogen (Life Technologies, Carlsbad, CA, USA) and allowed to dissociate at room temperature for 5 min. 160µL chloroform was then added to each tube and capped tubes shaken vigorously by hand for 15s and incubated for a further 2-3min at room temperature. Tubes were then centrifuged at 12000g for 15min at 4°C and the aqueous phase transferred to a fresh tube. 400uL isopropyl alcohol was added per tube, mixed and then tubes incubated for at least 20min at 4°C before a second centrifugation at 12000g for 15min at 4°C. The supernatant was then discarded, and the pellet washed with 75% EtOH in DEPC treated water and vortexed. Following a spin for 5min, 4°C at 10000g the supernatant was once again discarded, and then the wash and spin repeated. After removal of the 2nd wash, a further spin assisted in the removal of remaining traces of liquid by pipette. Pellets were air-dried at room temperature for 15min and resuspended in DEPC treated water before transfer to a clean tube, analysis and storage at -80°C. The total RNA concentration and quality for each sample were determined using a Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA) with a 280/260 ratio between 1.8 and 2.0 considered acceptable for further analysis .

## **Reverse Transcription**

**Protocol#1** (for use with ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA))

Thin walled PCR tubes were set up containing 1µg RNA, 1µL 10x DNase Buffer and 1µL DNase I (Life Technologies, Carlsbad, CA, USA) with sufficient DEPC treated water to bring the volume of each tube to 10µL. Tubes were then incubated at room temperature for 15min followed by the addition of 1µL EDTA (as supplied with DNase1) and 10min at 65°C before being held at 4°C. 1µL each of random hexamers and dNTP mix were then added to each tube followed by a 5min incubation at 65°C before returning tubes to 4°C. 7µL Master mix (containing 4µL 5x RT buffer (15mM MgCl<sub>2</sub>), and 1µL each of 0.1mM DTT, RNaseOUT™ and SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) per tube) was then added to each tube (final volume 20uL) Reverse transcription was carried out in a PCR machine with the following protocol: 5min at 25°C, 60min at 50°C, 15min at 70°C, Hold at 4°C, before resultant cDNA was stored at -20°C

**Protocol#2** (for use with LightCycler® 480 Instrument (Roche Applied Science, Penzberg , Germany)).

As before, 1µg RNA was first DNase I treated then incubated at room temperature for 15min before heating to 65°C for 10min, however volumes were made to 11uL and there was no subsequent EDTA addition required. 9uL master-mix was then added to give a final volume of 20µL (see below, Transcriptor First Strand cDNA Synthesis Kit, Roche Applied Science, Penzberg , Germany). Reverse transcription was carried out in a PCR machine with the following protocol: 10min at 25°C, 30min at 55°C, 5min at 85°C, Hold at 4°C, before resultant cDNA was stored at -20°C

<b>Roche Master Mix</b>	<b>µL per tube</b>
5x Transcriptor RT buffer	4
Protector RNase Inhibitor	0.5
Transcriptor Reverse Transcriptase	0.5
Random hexamers	2
dNTP Mix	2

## **Real Time PCR**

Primers for HDAC 2 were obtained from Primer Bank (Wang et al. 2003) (ID#293336690c1). All other HDAC/SIRT Primers were designed using Primer3 software (Rozen et al. 2000) and checked using NetPrimer software (PREMIER Biosoft, Palo Alto ,California USA). Primers for cytokines and housekeeping genes were already available within the LAB. All Primers were tested against cDNA from placenta and melt curve analysis done to check for dimers. After agarose gel electrophoresis to access product size, PCR products were extracted from duplicate wells and purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sent for sequencing to confirm correct target amplification. Purified and sequenced PCR products were used to create standard curves for each primer pair, which could then used to determine absolute levels of PCR product for each target and sample. All samples were analyzed in triplicate.

Table 15: Complete list of RT-PCR Primers

<b>Primers</b>	<b>FORWARD 5'- 3'</b>	<b>REVERSE 3'- 5'</b>	<b>Amplicon Size</b>
HDAC1	GGAAATCTATCGCCCTCACA	TTGCCACAGAACCACCAGTA	206
HDAC2	ATGGCGTACAGTCAAGGAGG	TGCGGATTCTATGAGGCTTCA	112
HDAC3	TGGCTTCTGCTATGTCAACG	TTGATAACCGGCTGGAAAAG	297
HDAC4	CCTCTACACATCGCCATCCT	GGCTGCTCCAGTAAGACCAT	237
HDAC5	GTAGCCATCACCGCAAAACT	GTCCTCCACCAACCTCTTCA	199
HDAC6	TATCTGCCCCAGTACCTTCG	GGACATCCCAATCCACAATC	242
HDAC7	GGCTGCTTTCAGGATAGTCG	TTCATCAGTTGCTGCGTCAT	162
HDAC8	TTATGACTGCCAGCCACTG	TTCCCAGGACAGCATCATTG	188
HDAC9	GACTCACTCTTCCCCTGCTG	CCAGTTTCTTGCAGTCGTGA	192
HDAC10	CTTCTCCTGGCACCCTATG	CCGATGGCTGAGTCAAATCC	234
HDAC11	ATCACGCTCGCCATCAAGTT	TCATCCTCTGTGCCCCACTC	224
IL-1 $\beta$	GAAGCTGATGGCCCTAAACA	GCATCTTCTCAGCTTGTCC	160
IL-6	AAATTCGGTACATCCTCGACGG	GGAAGGTTCAAGTTGTTTTCTGC	112
IL-8	ACCACCGGAAGGAACCATCTCAC	ACTGCACCTTCACACAGAGCTGC	105
IL-10	CAGGGCACCCAGTCTGAGAA	CACATGCGCCTTGATGTCTG	264
IL-1ra	AGCCTTCAGAATCTGGGATG	TGTTCTCGCTCAGGTCAGTG	242
SIRT1	GCTGATGAACCGCTTGCTAT	ATGGGGTATGGAACCTGGAA	174
SIRT2	GACTTTCGCTCTCCATCCAC	GAGTAGCCCCTTGTCCTTCA	201
SIRT3	AGCAACCTCCAGCAGTACGA	CGACACTCTCTCAAGCCCATC	216
SIRT4	ACCTGGGAGAAACTCGGAAA	TCAGGACTTGGAACGCTCT	178

SIRT5	ATGCGACCTCTCCAGATTGT	GTCGGAACACCACTTTCTGC	206
SIRT6	GCAGTCTTCCAGTGTGGTGTTC	CGCTCTCAAAGGTGGTGTTCG	140
SIRT7	CCATTTTCTCACTGGCGACT	CCTTTTGTGCGTTTTGTGC	173
TNF $\alpha$	TGCTTGTTCTCAGCCTCTTCT	TATCTCTCAGCTCCACGCCATT	277
ACTB	GCGGACTATGACTTAGTTGCG	CATCTTGTTTTCTGCGCAACTT	67
RPL13A	GCCCTACGACAAGAAAAAGCG	TACTTCCAGCCAACCTCGTGA	117
YWHAZ	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT	189

HDAC characterization and preliminary experiments where specified were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SDS 2.1 software. The Quantitative real-time PCR (qPCR) protocol had an initial stage at 95°C for 20s, followed by amplification for 40 cycles; denaturation at 95°C for 1second and annealing/extension at 60°C for 20 seconds. A dissociation stage was performed for all runs. Each wells contained 2 $\mu$ L of neat cDNA and 8 $\mu$ L master mix consisting of 0.5 $\mu$ L of forward and reverse primer (working stock of 5 $\mu$ M), 2 $\mu$ L DEPC treated water and 5 $\mu$ L Fast SYBR® Green Master Mix (Life Technologies).

Subsequent experiments utilised the LightCycler® 480 Instrument and LightCycler® 480 SYBR Green I Master mix (Roche Applied Science, Penzberg , Germany). Use of the Transcriptor First Strand cDNA Synthesis Kit to generate cDNA allowed for further dilution of the cDNA (10x with PCR grade water) reducing contaminants from the conversion process. As before well volumes were kept at 10 $\mu$ L. The Quantitative real-time PCR (qPCR) protocol had an initial stage at 95°C for 5min, followed by amplification for 50 cycles; denaturation at 95°C for 10 seconds , annealing/extension at 60°C for 20 seconds and acquisition at 72°C. A melting curve 65°C-98°C was performed for all runs.

### ***Histone Extraction***

Histone extraction was performed based on a published protocol available for cell lines (Shechter et al. 2007), adapted for use with tissue. All steps were performed on ice or at 4°C. Matched amnion, chorio-decidua and placental villous tissue from previous studies and approved for follow up studies were utilised (ethics # 95/225). Frozen tissues were first crushed in the presence of liquid nitrogen. Each tissue sample was then suspended in 1mL of hypotonic lysis buffer (10mM Tris-HCl ph 8.0, 1mM KCL, 1.5mM MgCl<sub>2</sub>, 1mM DTT, 1mM PMSF, protease inhibitor (Roche Applied Science, Penzberg , Germany), “PhosSTOP” Phosphatase Inhibitor Cocktail (Roche) and homogenised before

proceeding as directed for cells in the protocol. Briefly, histones were acid extracted in  $H_2SO_4$ , precipitated with Trichloroacetic acid (TCA) and washed with acetone, before re-suspension in ddH<sub>2</sub>O. A control sample was also prepared in the same manner using placental tissue explants treated for 24 hrs with the histone deacetylase inhibitor, sodium butyrate (5mM). Protein concentrations were determined using the Bicinchoninic acid (BCA) assay.

Histones extracts (2 $\mu$ g) were separated using 15% SDS PAGE, even transfer was checked using Coomassie blue to check for protein remaining in the gel, and Ponceau S to reversibly detect protein in the membrane. Following protein electrophoresis (duplicate gels), histones were transferred to nitrocellulose membrane (Amersham Biosciences, Sweden) or, as no housekeeping protein was available as a loading control, gel replicates for each analysis were Coomassie-blue stained. Western blotting was performed using anti-acetyl histone H3 and anti-acetyl histone H4 antibodies (06-599 and 06-866, Millipore, MA, USA). Chemiluminescence detection of HRP-Conjugated secondary antibodies was performed using Supersignal® West Dura Extended Duration Substrate (Pierce, Thermo Fisher Scientific, USA), and x-ray films. Densitometry was performed using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad) and Quantity One software.

Data were normalised to stained total H3 or H4 protein and calculated as optical density relative to the sodium butyrate treated positive control. Data were analyzed by one-way ANOVA followed by Student t-tests. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). A p value of  $\leq 0.05$  was considered significant.

### ***Nuclear Protein Extraction***

Nuclear extracts were prepared using the EpiQuik™ Nuclear Extraction Kit (Catalog # OP-0002 Epigentek, Farmingdale, NY, USA) which is optimised for use with the Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Epigentek) and provided standardised conditions across the samples analysed. The kit utilises pre-lysis and lysis buffers. Tissue was first crushed, and then resuspended in the pre-lysis buffer at a ratio of 5 $\mu$ L buffer per milligram of tissue, with the addition of DTT (1:1000).

Following 15 min incubation on ice, samples were centrifuged at 12,000 rpm at 4°C for 10 min and the supernatant removed. The nuclear pellet was then resuspended in the lysis buffer containing DTT and protease inhibitor cocktail (5uL buffer per mg) and incubated on ice for a further 15 min with a 5 second vortex every 3 min. The suspension was then centrifuged for 10 min at 14,000 rpm at 4°C and the supernatant transferred into a new micro-centrifuge vial. Protein concentrations of nuclear extracts were quantified using the RC DC™ Protein Assay (Bio-Rad, Hercules, CA, USA).

## ***Protein Quantification***

### *BCA assay (Histone extracts)*

Protein standards were prepared ranging from 31.25 µg/mL to 2000 µg/mL protein using bovine serum albumin (BSA) and the sample buffer (MilliQ-H<sub>2</sub>O for histones). A BSA Stock Standard of 4mg/mL bovine serum albumin in MilliQ-H<sub>2</sub>O was used for the dilutions. 25µL of each standard and sample were added to a 96 well flat bottomed plate, followed by 200µL BCA reagent to each well (1part copper sulphate reagent (4g/100mL MilliQ-H<sub>2</sub>O), 50parts bicinchinonic acid solution (Pierce™, Thermofisher Scientific Waltham, MA, USA). After 30min incubation at room temperature absorbance was measured at 562nm.

### *RCDC Microfuge Tube Assay Protocol (Nuclear extracts\*)*

\*EpiQuik Nuclear extraction buffer reacts significantly with BCA and DC assays

5µL of DC Reagent S was added to each 250 µL of DC Reagent A needed for the run (Reagent A'). Protein standards were prepared ranging from 0.2 mg/mL to 1.5 mg/mL protein using bovine serum albumin and the sample buffer. 25 µl of each standard and samples were pipetted into clean, dry microfuge tubes and 125 µL RC Reagent I then added into each tube and mixed by vortex. Tubes were incubated for 1 minute at room temperature. 125 µL RC Reagent II was then added into each tube and mixed by vortex, followed by Centrifugation at 15,000xg for 3-5 min. The supernatant was removed by inversion and 127 µL Reagent A' added to each to each microfuge tube and mixed by vortex. Tubes were incubated at room temperature for 5 min, or until precipitate was completely dissolved. Tubes were then vortexed, 1 mL of DC Reagent B added to each tube followed immediately by an additional vortex. After the 15 min incubation at room temperature, absorbances were read at 750 nm.

## ***HDAC Activity Assay***

HDAC activity of extracts was determined using a colorimetric, Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (# P-4034 Epigentek, Farmingdale, NY, USA). In this assay, an acetylated histone HDAC substrate is stably coated onto the microplate wells. Active HDACs bind to the substrate and removes acetyl groups from the substrate. The HDAC-deacetylated products can be recognized with a specific antibody. The ratio or amount of deacetylated products, which is proportional to the enzyme activity, can then be colorimetrically measured by reading the absorbance in a microplate spectrophotometer at 450 nm (reference wavelength 655nm). The activity of the HDAC enzyme is proportional to the OD intensity measured.

Input materials can be nuclear extracts or purified HDAC enzymes; for these experiments, nuclear extracts were obtained using the EpiQuik™ Nuclear Extraction Kit and maximum volumes were loaded per well (4µL). The HDAC assay standard is deacetylated histones and also acts as an internal control for antibody activity. Briefly, wells were set up with nuclear extract or left blank and then incubated with substrate and assay buffer for 90 min at 37°C. Standards were incubated with assay buffer but not substrate. After incubation wells were washed and capture antibody added. Capture body was incubated for 60 min at room temperature followed by washing and 30 min incubation with detection antibody. Wells were then washed and developer solution incubated in wells for up to 10 min before stop solution was added and plates were read.

### *HDAC Activity Calculation*

First the average duplicate readings for the sample wells and blank wells were determined. A standard curve of optical density (OD) versus standard concentration was then created and the slope determined, and used to determine the amount of deacetylated product in each well:

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The specific activity could then be calculated using the substrate incubation time, and the amount of nuclear protein (determined by RCDC for each nuclear extract) added per well:

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### ***Western Blotting of Nuclear extracts***

5µg of each nuclear extract with added NuPAGE® LDS Sample Buffer (4X) (Life Technologies, Carlsbad, CA, USA) was first separated by protein electrophoresis using NuPAGE® Bis-Tris Polyacrylamide gels (4-12% gradient), with the NuPAGE® MOPS SDS Running Buffer for 50min at 200V. SeeBlue® Plus2 Pre-stained Protein Standard was run alongside samples (Life Technologies). Following protein electrophoresis, nuclear extracts (5µg) were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using NuPAGE® Transfer Buffer with 10% MeOH for 2 hrs at 50V on ice. Membranes were then blocked for 1hour at room temperature using 3% BSA in 0.1% PBST. Western blotting was performed using antibodies for HDAC1 and HDAC3 (ab19845 and ab16047at 1µg/mL, Abcam, Cambridge, UK) overnight at 4°C. Following 4 x 5min washes in 0.1% PBST, membranes were incubated with goat anti-rabbit secondary at 1:10000 for 2 hrs at room temperature (A0545, Sigma-Aldrich, St. Louis, MO, USA). Membranes were then washed and chemiluminescence detection of HRP-Conjugated secondary antibodies was performed using Super-Signal West Dura (Thermo Scientific, Illinois) and x-ray films. β-actin was used as a loading control, and was detected using anti- β-actin (A1978) at 1:30000 for 1 hr at room temperature followed by goat anti-mouse (A4416) at 1:10000 for 2 hrs. (Sigma-Aldrich, St. Louis, MO, USA).

Densitometry was performed using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad) and Quantity One software. Protein production was calculated as density (OD/mm<sup>2</sup>) relative to β-actin loading control, as a proportion of the internal control.

## **ELISA**

Human IL-1 $\beta$ , TNF $\alpha$ , IL-6, IL-8 and IL-10, ELISA kits were purchased from BD Bio Sciences (Franklin Lakes, NJ, USA). IL-1ra kits were purchased from R&D systems (Minneapolis, MN USA).

ELISA plates were coated with capture antibody the day prior to running the assay. For BD kits the capture antibody was diluted in a coating buffer consisting of 0.1M Sodium Carbonate pH 9.5 (7.13g NaHCO<sub>3</sub>, 1.59g Na<sub>2</sub>CO<sub>3</sub>; made to 1L and adjusted to pH9.5 with 10N NaOH). BD kit plates were stored overnight at 4°C. For IL-1ra (R&D) the capture antibody was diluted in PBS and kept at room temperature overnight. All kits had a standard coating volume of 100 $\mu$ L per well. Plates were washed the next day 3x with 300 $\mu$ L wash buffer (0.05% PBST) and blocked for 1hour at RT (BD: 10% FBS in PBS, R&D: 1% BSA in PBS) before a further 3 washes were applied. Samples and standards were then added. All samples and standards were diluted as required in reserved experimental media (untreated) and treated media blanks were also run in parallel to sample media. Incubation time was 2 hrs at room temperature. Plates were then washed, 100 $\mu$ L (each/combined) detection antibody and Streptavidin-HRP applied, followed by further washes (wash quantities differ between kits, and IL1 $\beta$  and IL-1ra require two step detection antibody and Streptavidin-HRP addition). Substrate solution consisting of Tetramethylbenzidine (TMB) and Hydrogen peroxide was then added (100 $\mu$ L) and plates incubated for 20-30min at room temperature in the dark. The reaction was stopped with 2N H<sub>2</sub>SO<sub>4</sub> and the optical density of each well determined using a microplate reader set to 450nm with wavelength correction is of 570nm. Absorbances of the standards were used to generate a standard curve using a 4 Parameter Logistic (4PL) nonlinear regression model and the amount of cytokine in each well determined. Production rates of cytokines were then normalized to wet tissue weight

## **Statistics**

Real-time-PCR results for characterization and preliminary data were calculated as absolute values and are given as mean relative expression compared with housekeeping gene; YWHAZ (encodes 14-3-3 protein zeta/delta). Data were analyzed by one way ANOVA followed by Student's t-tests.

Real-time-PCR results for cytokine analysis across the Time courses were first calculated as absolute values before normalization against the geometric mean of three housekeeping genes: YWHAZ, RPL13A (encodes 60S ribosomal protein L13a) and ACTB ( $\beta$ -actin). Production rates of cytokines were calculated as picograms per milligram wet tissue weight and are presented as means  $\pm$  SE. Statistical significance for both were determined by two-way repeated-measures ANOVA, matched across treatment and time, followed by Tukey's multiple comparisons test.

Real-time-PCR results for cytokine analysis across the Time courses were also calculated as absolute values and normalization against the geometric mean of YWHAZ, RPL13A and ACTB, and are presented as relative change from control at each time point. HDAC activity was calculated as ng (deacetylated product) per minute, per mg of nuclear extract. Protein production was calculated as density (OD/mm<sup>2</sup>) relative to  $\beta$ -actin loading control, as a proportion of the internal control. HDAC protein production and activity are also presented as relative change from control at each time point. Statistical significance was determined by two-way repeated-measures ANOVA, matched across treatment and time, followed by Dunnett's multiple comparisons test.

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). A p value of  $\leq 0.05$  was considered significant.

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