# Epidrug-mediated reversal of epigenetic changes associated with pituitary adenomas

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## ABSTRACT

The genesis and outgrowth of pituitary adenomas is consequent to the combined contributions of genetic and epigenetic aberrations. Although the relative contribution of each is not known it is likely that particular aberration may be common or particular to an adenoma subtype(s). The impact of subtype specific changes presents significant challenges for clinical management options. In this thesis the potential impact of epigenetic aberrations, that target the Dopamine D2 Receptor (D2R) and the cytokine, Bone Morphogenic Protein 4 (BMP-4) were investigated. In these cases their expression patterns are frequently comprised in a subtype-specific context. This thesis reports that in pituitary cell lines, reduced expression of D2R and that of BMP4 are associated with CpG island methylation and histone modifications indicative of gene silencing. In these cases, incubation of cells with epidrugs, designed to reverse epigenetic silencing, restores their expression. Moreover, epidrug induced re-expression of the D2R is associated with an augmented apoptotic response to dopamine agonist challenges. In challenges, designed to *de-repress* BMP4 silencing, a cell-context-specific response to retinoic acid (RA) is observed. In these cases, epidrug facilitated and RA augmented expression of BMP-4 led to either, increase in cell number, in GH3 cells, or decrease in cell number, in AtT-20 cells, and reflected their cell lineages. However, while epidrugs incubations were only associated with a marginal decrease in methylation, a significant change in histone modifications toward those associated with active genes was apparent. In primary pituitary adenomas change in CpG island methylation associated with either the D2R or BMP4 gene was not apparent, however, for the BMP4 gene, histone modifications associated with either active or silenced genes mirrored their expression patterns as determined by RT-qPCR. These studies show the potential of combined drug challenges as a treatment option, where epidrug renders silenced genes responsive to conventional therapeutic options.

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## ABBREVIATIONS

| | |
|---|---|
| **2OG** | 2-oxoglutarate |
| **5-aza-CdR** | 5-aza-2'-deoxycytidine |
| **9-cis-RA** | 9-cis-Retinoic Acid |
| **ΔΔCT** | Delta delta CT |
| **ACTH** | Adrenocorticotrophic hormone |
| **AIP** | Aryl hydrocarbon receptor interacting protein |
| **All trans-RA** | All trans retinoic acid |
| **Al** | Anterior Lobe |
| **$AMP^r$** | Ampicillin Resistance |
| **ANOVA** | Analysis of variance |
| **ATCC** | American Type Culture Collection |
| **Bc** | Bromocriptine (2-Bromo-α-ergocryptine methanesulfonate salt |
| **BCA** | Bicinchoninic acid |
| **bFGF** | Basic fibroblast growth factor |
| **BMP-2** | Bone Morphogenic Protein 2 |
| **BMP-4** | Bone Morphogenic Protein 4 |
| **bp** | Base pairs |
| **BSA** | Bovine serum albumin |
| **cAMP** | Cyclic adenosine monophosphate |
| **CASP** | Caspase |
| **CDK** | Cyclin dependent kinase |
| **CDH13** | Cadherin 13 |
| **cDNA** | Complementary DNA (deoxyribose nucleic acid) |
| **CFE** | Colony Forming Efficiency |
| **CGI** | CpG Island |
| **CH3** | methyl |
| **ChIP** | Chromatin Immunoprecipitation assay |
| **c-Jun** | Jun Oncogene |
| **C-MYC** | An oncogene similar to myelocytomatosis viral oncogene |
| **$CO_2$** | Carbon dioxide |
| **CpG** | Cytosine-phosphate-Guanine |
| **CREB** | cAMP response element binding protein |
| **CRH** | Corticotropin releasing hormone |
| **CT** | Cycle Threshold |
| **D2R** | Dopamine D2 Receptor |
| **DA** | Dopamine (3-hydroxytyramine) |
| **DAPK** | Death associated protein kinase |
| **DAT** | Dopamine Transporter |
| **DAX-1** | Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 |
| **$ddH_2O$** | Double distilled water |
| **DMEM** | Dulbecco's modified eagle medium |
| **DMR** | Differentially methylated region |
| **DMSO** | Dimethyl sulphoxide |
| **dn** | Dominant Negative |
| **DNA** | Deoxyribonucleic acid |
| **DNMT** | DNA (cytosine-5) Methyltransferase |
| **dNTP** | Deoxyribonucleotide Triphosphate |
| **ds** | Double strand |

| | |
|---|---|
| **EB** | Elution Buffer |
| **ECACC** | European Collection of Cell Culture |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **Egr1** | Early growth response protein 1 |
| **ER** | Estrogen receptor |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **ES cell** | Embryonic stem cell |
| **ETIC** | Eticlopride |
| **FBS** | Fetal Bovine Serum |
| **FDA** | The US Food and Drug Administration |
| **FGF** | Fibroblast Growth factor |
| **FIPA** | Familial isolated pituitary adenoma |
| **FLNA** | Filamin A |
| **FSH** | Follicle Stimulating hormone |
| **GADD45** | Growth Arrest and DNA Damage |
| **GAPDH** | Glyceraldehyde 3 phosphate dehydrogenase |
| **GATA** | Globin transcription factor 1 |
| **GEO** | Gene Expression Omnibus |
| **GH** | Growth hormone |
| **GH3-D2EXP** | D2R expressing GH3 cells |
| **GHR** | Growth Hormone receptor |
| **GHRH** | Growth hormone releasing hormone |
| **GNAS** | G-protein α stimulating polypeptide |
| **GPCRs** | G-protein coupled receptors |
| **H3K9Ac** | Histone-3-Lysine-9-Acetylation |
| **H3K27Me3** | Histone-3-Lysine-27-tri-methylation |
| **HALO** | Haloperidol |
| **HAT** | Histone acetyltransferase |
| **HCP** | High density CGI promoter |
| **HDAC** | Histone deacetylase |
| **HEPA** | High efficiency particulate air |
| **HMGA2** | High Mobility group A2 |
| **HMT** | Histone Methyltransferase |
| **HRP** | Horse radish peroxidase |
| **ICR** | Imprinting control region |
| **IGF-1** | Insulin like growth factor 1 |
| **IHC** | Immunohistochemistry |
| **IK** | Ikaros |
| **IL** | Intermediate Lobe |
| **IPTG** | Isopropyl β-D-1thiogalactopyranoside |
| **LB** | Luria Broth |
| **LH** | Luteinising hormone |
| **Lhx3** | LIM homeobox 3 |
| **LIF** | Leukaemia inhibitor factor |
| **LINE1** | Long interspersed nuclear elements |
| **LOH** | Loss of heterozygosity |
| **LOI** | Loss of imprinting |
| **$m^5C$** | 5-methyl cytosine |
| **5hmC** | 5-hydroxymethylcytosine |
| **MAGE** | Melanoma associated antigen gene |
| **MAPK** | Mitogen activated protein kinases |
| **MB** | Magnetic beads coated in Protein G |

| | |
|---|---|
| **MBDS** | Methyl CpG binding domain protein |
| **MEN** | Multiple Endocrine Neoplasia |
| **M-MLV RT** | Moloney Murine Leukaemia Reverse Transcriptase |
| **miRNA** | Micro RNA |
| **mRNA** | Messenger Ribonucleic acid |
| **MSH** | Melanocyte Stimulating Hormone |
| **NC** | Negative control |
| **NCBI** | National Center for Biotechnology Information |
| **NeuroD1** | Neurogenic differentiation 1 |
| **NFT** | Non-functioning adenoma |
| **NHP** | Normal Human pituitary |
| **NMP** | Normal Mouse Pituitary |
| **NNAT** | Neuronatin |
| **NRP** | Normal Rat Pituitary |
| **NP** | Normal Pituitary |
| **nt** | Nucleotide |
| **OCT** | Octreotide |
| **OD** | Optical Density |
| **Otx1** | Orthodenticle homeobox 1 |
| **P** | Calculated Probability |
| **PAP** | Pituitary adenoma predisposition protein |
| **PBGD** | Porphobilinogen deaminase |
| **PBS** | Phosphate buffer saline |
| **PcG** | Polycomb group |
| **PCR** | Polymerase chain reaction |
| **PIC** | Protease inhibitor cocktail |
| **Pitx2** | Paired-like homeodomain 2 |
| **PL** | Posterior lobe |
| **PMSF** | Phenylmethylsulphonylfluoride |
| **Pol II** | RNA polymerase II |
| **POMC** | Pro-opiomelanocortin |
| **PPARγ** | Peroxisome proliferator activated receptor |
| **PRC** | Polycomb repressive complex |
| **PRL** | Prolactin |
| **Prop-1** | Paired like homeobox 1 |
| **PTAG** | Pituitary Tumour apoptosis gene |
| **PTGs** | Post transcriptional gene silencing |
| **PTTG1** | Pituitary tumour transforming gene |
| **qPCR** | Quantitative PCR |
| **qRTPCR** | Quantitative RT-PCR |
| **RA** | Retinoic acid |
| **RARE** | Retinoic acid response element |
| **RASSFIA** | RAS association domain family |
| **RB** | Retinoblastoma |
| **RIPA** | Radio immunoprecipitation assay |
| **RNA** | Ribonucleic acid |
| **RNAi** | RNA interference |
| **Rpm** | Revs per minute |
| **RT** | Reverse Transcription |
| **RT-PCR** | Reverse transcription PCR |
| **S100A10** | S100 calcium binding protein A10 |
| **SDS** | Sodium dodecyl sulphate |

| | |
|---|---|
| **SEM** | Standard Error of the Mean |
| **SF-1** | Splicing Factor 1 |
| **Shh** | Sonic hedgehog |
| **siNT** | Non-targeting siRNA |
| **siRNA** | Small interfering RNA |
| **Smad** | Mothers against decapentaplegic |
| **sq-PCR** | Semi-quantitative PCR |
| **sqRT-PCR** | Semi-quantitative RT-PCR |
| **SRIF** | Somatostatin release inhibiting factor |
| **ss** | Single Stranded |
| **SSA** | Somatostatin Analogue |
| **SSTR** | Somatostatin Receptor |
| **TE** | Tris (HCl) EDTA buffer |
| **TET-1** | Chromosome ten-eleven translocation 1 |
| **TF** | Transcription factor |
| **TGF-β** | Transforming Growth Factor β |
| **TMB** | 3,3',5,5'-tetramethylbenzidine |
| **T-pit** | T-box transcription factor |
| **TSA** | Trichostatin A |
| **TSG** | Tumour Suppressor gene |
| **TSH** | Thyroid stimulating hormone |
| **Ub** | Ubiquitination |
| **UFC** | Urinary free cortisol |
| **VEGF** | Vascular endothelial growth receptor |
| **WIFI** | Wnt inhibitory factor 1 |
| **Wnt** | Wnt transcription factor/protein |
| **Xgal** | 5-bomo-4-chloro-3-indolyl β-D-galactopyranonide |
| **zeb** | Zebularine also known as 1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one |
| **Zn-15** | Zinc Finger Protein 15 |

## PUBLICATIONS IN PEER REVIEWED JOURNALS

See Appendix VII for further details

1. Haneen Al-Azzawi*, Kiren Yacqub-Usman*, Alan Richardson, Leo Hofland, Richard N Clayton, William E Farrell. Reversal of Endogenous Dopamine Receptor Silencing in Pituitary Cells Augments Receptor-Mediated Apoptosis. Endocrinology (2011) 152: (2) 364-373.
2. Kiren Yacqub-Usman, Alan Richardson, Cuong V. Duong, Richard N Clayton, William E Farrell. The pituitary tumour epigenome: aberrations and prospects for targeted therapy. Nature Reviews Endocrinology (2012) 8:(8) 486-94
3. Kiren Yacqub-Usman, Cuong V. Duong, Richard N Clayton, William E Farrell. Epigenomic Silencing of the BMP-4 Gene in Pituitary Adenomas: A Potential Target for Epidrug-Induced re-expression. Endocrinology (2012) 153:(8) 3603-12
4. Cuong V. Duong, Richard D Emes, Frank Wessely, Kiren Yacqub-Usman, Richard N Clayton, William E Farrell. Quantitative, genome-wide analysis of the DNA methylome in sporadic pituitary adenomas. Endocrine Related Cancer (2012) 19:1-12
5. Kiren Yacqub-Usman, Cuong V. Duong, Richard N Clayton, William E Farrell. Pre-incubation of Pituitary Tumour Cells with the Epidrugs Zebularine and Trichostatin A are Permissive for Retinoic Acid Augmented Expression of the BMP-4 and D2R genes. Endocrinology (2013) 154:(5) 1711-21

*Contributed equally to the publication.

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# *Dedicated to:*

***My Mum: Yasmeen Yacqub***

***My Dad: Yacqub Mirza***

***And***

***My Beautiful Son: Zayan Hashim Usman Farooq***

***An Eternal memory of***
***A Little Angel***

*Today it would be wonderful to see you play or smile*
*But heaven lent you to this world for just a little while*
*And in that short but precious time you brought along much love*
*and all that love is with you now in heaven up above*
*your leaving caused so many tears and such a lot of pain*
*but god needed one more angel so he took you back again.*

# CHAPTER 1: INTRODUCTION

# 1. Pituitary Tumourigenesis

## 1.1 The Pituitary Gland

The pituitary gland is situated at the base of the hypothalamus in the brain, and is often referred to as the “master” gland due to its integral role in the endocrine system. Functionally, by means of interactions with peripheral endocrine organs, and the immune and nervous systems, it regulates basic physiological functions including growth, reproduction and metabolic homeostasis. It is composed of three lobes, the anterior, posterior and the intermediate lobes. The versatile endocrine functions of the gland are carried out by the six cells types in the anterior (AL), intermediate (IL) and the posterior (PL) lobes. These cell types are classified by the hormone they produce and secrete: (1) AL-specific cells such as, corticotrophs producing adrenocorticotropic hormone (ACTH), thyrotrophs secreting thyroid-stimulating hormone (TSH), somatotrophs secreting growth hormone (GH), lactotrophs produce prolactin (PRL), gonadotrophs secreting luteinizing hormone (LH), and follicle stimulating hormone (FSH) as represented in figure 1; (2) IL-specific melanotrophs secreting, melanocyte-stimulating hormones (MSH) and (3) PL-specific hormones such as vasopressin (an anti-diuretic hormone) and oxytocin and these are secreted from the cell bodies of the secretory neurons in the hypothalamus and stored in the axon ending in the posterior lobe. In humans the IL is normally either very small or absent in adulthood and the secretion of MSH by the melanotropes occurs only during fetal life. In mouse, the IL cells maintain a robust proliferative capability even during adulthood and therefore a relatively constant number of IL cells are maintained [1]

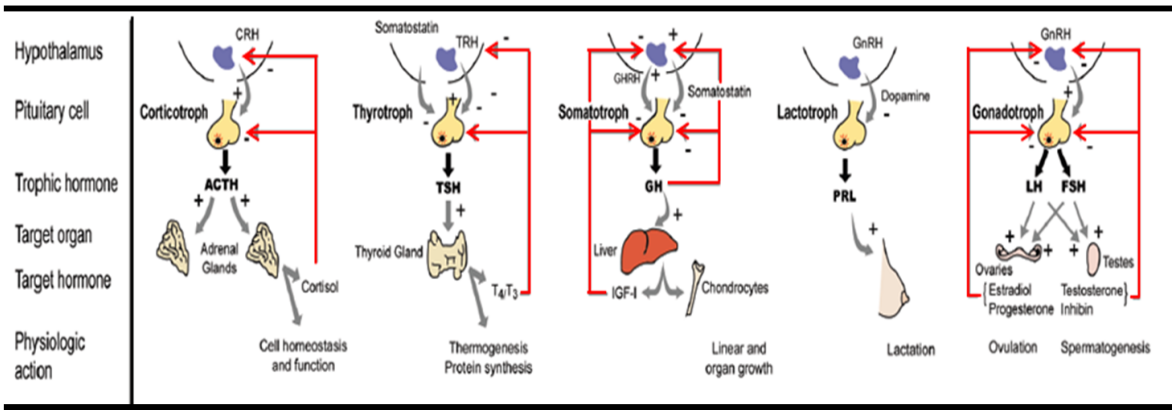



**Figure 1.1: Pituitary gland cell types and the hormones they secrete.** Also shown are their target organs and the higher level control exerted by the hypothalamus. CRH; Corticotropin releasing hormone, ACTH; Adrenocorticotropic hormone, TRH; Thyrotropin releasing hormone, TSH; Thyroid Stimulating Hormone, GHRH; Growth hormone releasing hormone, GH; Growth hormone, GnRH; Gonadotropin releasing hormone, PRL; Prolactin, LH; Luteinising hormone, FSH; Follicle stimulating hormone. Adapted and modified from [2]

During development the adult pituitary gland arises from progenitors of the neuroectodermic primordium (neuronal epithelium and oral ectoderm) known as the rathke's pouch in a temporal and spatial-specific fashion [2, 3]. There are many major pathways implicated in the development of the pituitary gland including the Notch and Wnt regulatory networks and are functional only in the early phases of the pituitary organogenesis and are essential for the emergence of somatotrophs, lactotrophs, thyrotrophs and gonadotrophs [3]. The cell cycle regulator CDK4 (cyclin dependent kinase 4) has also been implicated in post-natal generation of some anterior pituitary cells such as the somatotrophs and lactotrophs [4]. However, the requirement for CDK4 in other primary cells is not clear as the pituitary gland itself, in cdk4-null mice, is smaller [4]. Figure 1.2 shows the major transcription factors and signalling pathways that are implicated in the development of the pituitary gland.

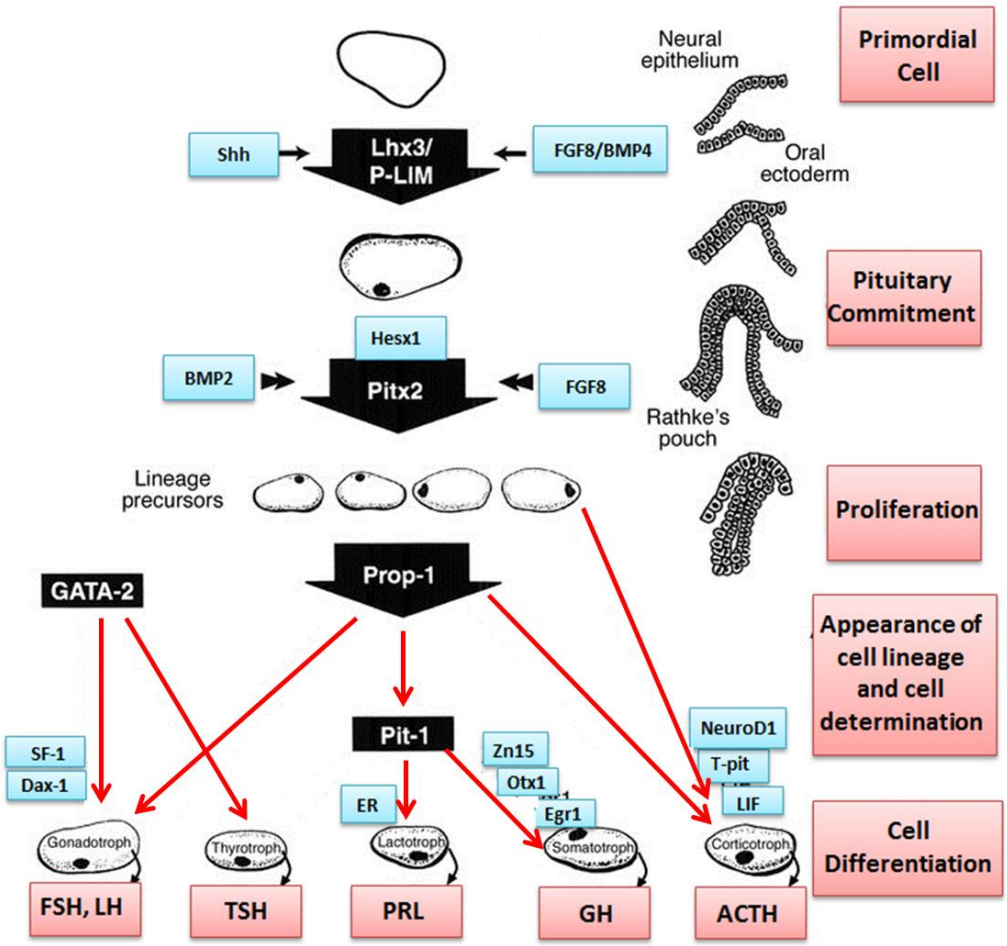



**Figure 1.2: Model for the development of the human anterior pituitary cell lineage** Determined by a temporally controlled cascade of transcription factors. Trophic cells are depicted with transcription factors known to determine cell specific human gene expression. Transcription factors are shown in blue (SF-1, Dax-1, ER, Zn15, Otx1, Egr1, NeuroD1, T-pit, LIF, BMP2, FGF8, Hesx1, Shh, and BMP4) and the black boxes define major temporal factors responsible for each differentiated cell type within the gland (Lhx3/p-LIM, Pitx2, Prop-1, GATA-2 and pit-1). Adapted and modified from [2].

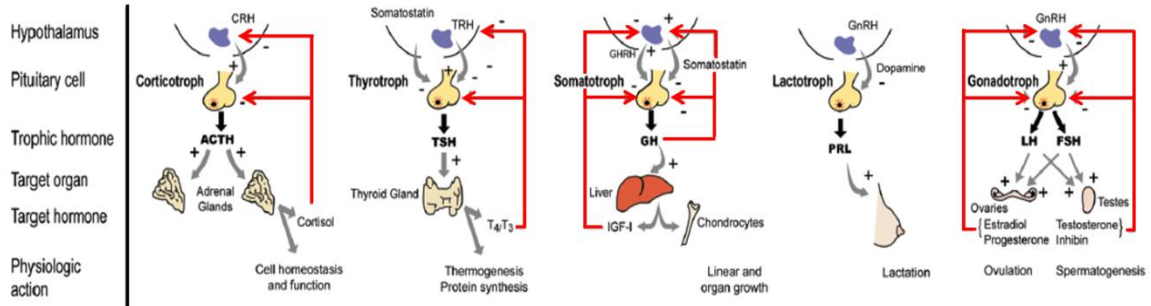
### 1.1.2. Tumours of the pituitary gland

Pituitary Tumours account for approximately 16.7% of all diagnosed intracranial neoplasms (14.4% in autopsy studies and 22.5% in radiological studies) [5]. Without exception each of the distinct cell types within the gland can give rise to a discrete pituitary tumour subtype that is either hormonally active or inactive (non-functional adenomas).

The classification of pituitary neoplasms has undergone a variety of modifications; typically they were separated based on light microscopy. However adenomas are now more commonly classified based upon the hormones that they secrete [6]. Endocrinologically they are considered either active or inactive. Only if the amount of hormone that they secrete exceeds normal levels in the blood and is clinically evident is classed as active. Inactive adenomas contain secretory and cellular components necessary for hormone production; however it is thought that inactive cells either produce undetectable levels of hormone or abnormal hormone that is not recognised by the antibody in radioimmunoassay's, or that these cells have lost the ability to produce any hormone through some acquired genetic defects [5]. Pituitary adenomas can sometimes progress after surgery and can be locally invasive. Ki-67 and p53 expression are referred to as indicators of aggressive behaviour in the World Health Organisation Classification of Endocrine Tumours [7]. A threshold level of greater than 1.3% of Ki-67 labelling index and a positive p53 predicts high aggressiveness and progression of the tumour [8].

Approximately one third of pituitary tumours exhibit local invasive and or recurrent characteristics, although only a small minority progress to pituitary carcinoma which are characterised by metastatic invasion to distinct organs such as liver and lymph nodes[9]. Morbidity is generally caused by inappropriate tissue expansion within the cranium [2]. Despite the fact that these adenomas are slow growing a significant proportion invade downwards into the paranasal sinuses, laterally into the cavernous sinuses, and upwards into the brain. This causes local compressive effects with the associated symptoms, that include headaches, visual

defects and cranial nerve dysfunction. Additional, cell subtype-specific effects are caused by the downstream biochemical complications associated with over-secretion of the various pituitary derived hormones. For example, a consequence of corticotrophinoma development is the excessive secretion of adrenocorticotrophic hormone (ACTH). Corticotrophinomas account for 10-15% of all clinically recognised pituitary adenomas and these ACTH secreting tumours generate a glucocorticoid hypersecretion disorder called Cushing's disease. Cushing's disease leads to adrenal hyperplasia, abnormal fat deposition, thinning of the skin, hypertension, osteoporosis, diabetes and psychological disturbances. In tumours that show aggressive invasion into the brain this can often lead to death. At present, with the exception of prolactinomas, diagnosed pituitary adenomas are treated by surgical intervention, with or without adjuvant radiotherapy [10]. This type of treatment is invasive and can result in undesirable side effects; hence there is need for the development of novel treatment strategies. Figure 1.3 shows the phenotypes associated with each of the major pituitary adenoma subtypes and shown as Table 1.0 below summarising clinically relevant data on the various pituitary adenoma subtypes [2]




| | Cushing's Disease | TSH-cell Adenoma | Acromegaly | Prolactinoma | Non-functioning Adenoma |
|---|---|---|---|---|---|
| Tumor phenotype | Hypercortisolism | Thyroid goiter | Acral enlargement | Galactorrhea | Central effects |
| | Central obesity | Hyperthyroxinemia | Soft tissue swelling | Amenorrhea | Hypogonadism |
| | Striae | | Cardiac hypertrophy | Infertility | Hypergonadism (rare) |
| | Hyperglycemia | | Hypertension | Hypogonadism | Clinically silent |
| | Osteoporosis | | Hyperglycemia | | |
| | Hirsutism | | Sleep apnea | | |

**Figure 1.3: Pituitary gland cell types, the hormones they secrete and the tumours associated with the gland**. Also shown are their target organs and the higher level control exerted by the hypothalamus. The tumour types and clinical phenotypes are discussed in a subsequent section of this thesis. Adapted and modified from [2].

**Table 1.0: Pathological and clinical characteristics of pituitary adenoma subtypes**.

| Cell type | Hormone produced by cell | Hormone function | Tumour type | Incidence | Associated syndromes |
|---|---|---|---|---|---|
| **Corticotroph** | ACTH and other POMC derived peptides | Adrenal cortex; regulation of glucocorticoid synthesis and secretion | Densely granulated (Basophilic); sparsely granulated (chromophobic) | 10-15% | Cushing's disease and Nelson syndrome |
| **Somatotroph** | GH | Liver production of IGF1; bone and muscle growth | Densely granulated (acidophilic); sparsely granulated (chromophobic) | 10-15% | Acromegaly, gigantism |
| **Lactotroph** | PRL | Lactation | Sparsely granulated (densely granulated) | 35% | Amenorrhoea, galactorrhoea. Sexual dysfunction, infertility |
| **Mammo-somatotroph** | GH, PRL | As above | Mamosomatotroph | 5% | Acromegaly, gigantism with hyper prolactinaemia |
| **Thyrotroph** | TSH | Thyroid regulation of thyroid hormone synthesis and secretion | Thyrotroph | 2% | Hypo or hyperthyroidism |
| **Gonadotroph** | FSH, LH | Gonadal regulation of germ-cell development and sex steroid hormones | Gonadotroph, null cell, oncocytoma | 35% | Hypogonadism, mass effects, hypopituitarism* |

*Mass effects resulting in hypopituitarism can occur with any tumour type when it/they are large, but are more characteristic of the gonadotroph tumour type. ACTH-adrenocorticotroph hormone, FSH-follicle stimulating hormone, GH-growth hormone, IGF1- insulin like growth factor, LH-luteinizing hormone, POMC, pro-opiomelanocortin, PRL-prolactin, TSH-thyrotrophin stimulating hormone. Adapted and modified from [2, 11].

### 1.1.3. Factors contributing to pituitary tumourigenesis

On the basis of current pathological investigation criteria, it is not possible to determine whether a tumour will progress to the invasive or malignant phenotype or if it will recur. Unlike other epithelial cancers, pituitary tumours do not follow the classic paradigm of tumour progression, that include (1) initiation; (2) hyperplasia; (3) benign adenoma; (4) aggressive adenoma, and (5) carcinomas. Therefore, there are significant challenges to the deciphering of the aberrations that underlie this tumour type and our current understanding does not permit us to identify the pathogenic changes responsible for the initiation of the disease, or those that promote progression [11].

Although it is apparent that external factor such as hormonal imbalances can trigger pituitary hyperplasia and these changes can in some cases progress to pituitary tumours, it seems unlikely that abnormal regulation of such "extrinsic" signalling pathways is primarily responsible for the pathogenesis of the pituitary tumours[11]. For example, a variety of hormones, e.g. oestrogen and GH-releasing hormone (GHRH), are known to act upon particular pituitary cell subtypes and their subsequent adenoma subtype (i.e. prolactin-secreting lactotrophs and GH-secreting somatotrophs), and promote their proliferative potential [11]. Whilst this oestrogen induced hyperplasia is required to regulate specific normal physiological events e.g. lactation during pregnancy the use of oestrogen containing agents such as the "contraceptive pill" has not been shown to increase the risk of the development of prolactin-secreting tumours (prolactinomas). In addition, in experimental models in which GHRH is ectopically expressed in mice these failed to lead to somatotrophinoma development, at least in the short term [11].

The emerging data suggests that "intrinsic" factors are responsible for the triggering of pituitary tumourigenesis, as inferred by X chromosome inactivation studies in tumours in female patients [12, 13].

These studies suggest that pituitary tumours arise from clonal cell populations, and it is anticipated that proto-oncogene and TSG defects exist within these cells. However, some studies have suggested the presence of multiple independent monoclones in tumour tissue of recurrent tumours [14]. This could suggest that multiple monoclonal expansions are present from the onset of tumour development or alternatively, the individual monoclonal expansions could develop during the life time of the patient. The development of such pituitary monoclonal expansions on a background of diffuse hyperplasia could be reminiscent of the potent mitogenic effects of growth factors, hypothalamic and steroid hormones in the transformation process [2, 15].

Evidence gathered thus far suggests that both intrinsic (oncogene activation, TSG inactivation), and extrinsic factors (growth factors, hypothalamic and steroid hormones) act in concert to promote the development of pituitary tumours [16]. This model has been proposed for pituitary tumourigenesis and is illustrated in figure 1.4. According to this model [16], cells responding to endocrine or paracrine stimuli might expand in a polyclonal manner. As a consequence of this increased proliferation, their susceptibility to acquire activating mutations (in proto-oncogenes) or sustain inactivating mutations (in tumour suppressor genes) is increased and this prompts the emergence of a rapidly expanding monoclonal cell population. At some point in the polyclonal cell expansion, cells susceptible toward acquisition of a transformed phenotype develop, and these represent the cells that comprise the emergence of the monoclonal population. An alternative hypothesis is, a normal cell might acquire a sufficient number of activating mutations or sustain a similar number of inactivating events to prompt a rapidly expanding monoclonal cell population from the onset. Following additional genetic events, this monoclonal expansion may evolve into an invasive pituitary tumour, with further events promoting the progression to the rare metastatic carcinoma phenotype. The “progress” of either of these pathways can be driven by a variety of hormonal stimuli, growth and angiogenic factors, and altered receptor expression.

The development of pituitary tumours is, therefore, a multi-stage process that involves several mutations and / or aberrant epigenetic transition events that are triggered/promoted or facilitated by extrinsic stimuli such as growth factors, hormones, hypothalamic factors, cytokines, angiogenic factors etc.

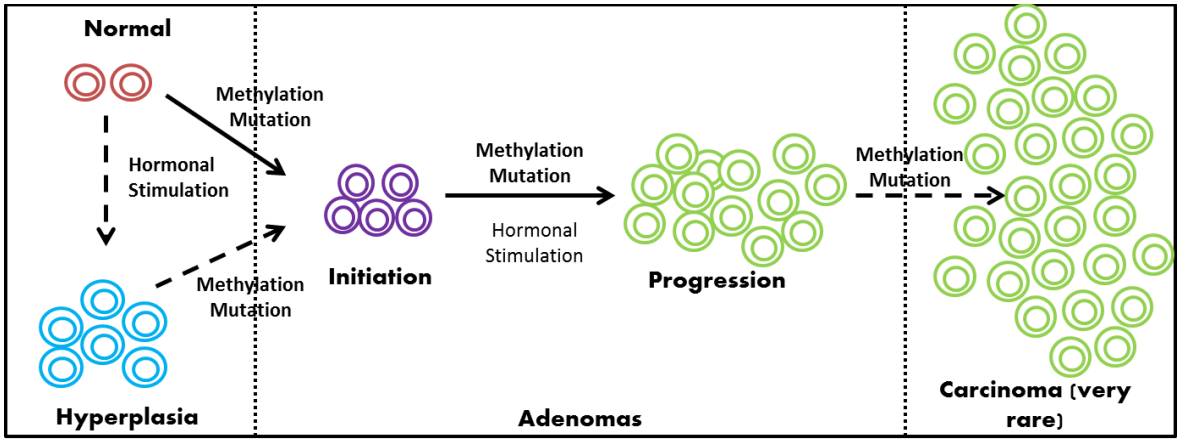



**Figure 1.4. General steps in the development of pituitary tumours.**

Pituitary tumour development is a multi-stage process that involves several mutations and/or aberrant epigenetic transition events. Adapted from [11]

### 1.1.4 Genetic abnormalities

Approximately 50% of pituitary tumours are thought to display aneuploidy [17]. These findings suggest that a significant proportion of pituitary tumours sustain global genetic abnormalities. However, and despite these findings, gene specific abnormalities are infrequent in these adenomas and the majority of these are found in rare familial tumour types [18, 19] and to a moderately high proportion of sporadic GH-secreting somatotrophinomas [20, 21]

#### 1.1.4.1 Oncogenes implicated in pituitary tumours

Genes that are characterised by a gain of function(s) by an activating mutation(s) are typically manifest as either an abnormal protein product or inappropriate expression and that have transforming potential are referred to as oncogenes. The mutation is therefore referred to as an activating mutation and earlier studies focused on these changes.

#### 1.1.4.2 G-protein α stimulating activity polypeptide (GNAS)

The *GNAS1* gene encodes the α subunit of the G protein $G_s$, which couples receptor-activation to intracellular adenylate cyclase.

Activating mutations at the *GNAS1* locus (which encodes the GSα protein), at residue 201 (Arg→Cys or His) or 227 (Gln→Arg or Leu), have been demonstrated in approximately 30% of sporadic GH-secreting pituitary tumours [20, 21]. The so named, *Gsp* mutation, bypasses ligand-dependant cAMP signalling and leads to somatotrophs proliferation via cAMP response element-binding protein (CREB) as a GH-associated transcriptional activator. However, despite the extraordinarily high frequency at which these mutations have been shown to occur in sporadic GH-secreting tumours, other oncogenic mutations commonly associated with non-endocrine

tumours **(e.g. *MYC, FOS* and *MYB*)** do not tend to occur in pituitary tumours [22]. Indeed, on rare occasions when such mutations have occurred, they have been shown to be induced after cellular transformation [22] and are therefore likely to be a consequence of pituitary tumours rather than a causal factor in their development. The role of imprinting at *GNAS1* locus (Gsp) will be discussed in a subsequent section.

### 1.1.4.3 Pituitary tumour transforming gene (PTTG1)

Melmed and colleagues isolated a cDNA encoding a novel pituitary tumour transforming gene termed PTTG1. Over-expression of this gene in the mouse embryonic fibroblast cell line NIH3T3 led to transformation *in vitro* and induced tumours *in vivo* in athymic mice [23]. The same group subsequently demonstrated that majority of human pituitary tumours, irrespective of their subtype, showed increasing levels of the human *PTTG1* mRNA [24]. The level of expression of *PTTG1* also correlated with the aggressive/invasive nature of the tumours [24]. The *PTTG1* protein contains a SH3 docking domain indicating a possible role in intracellular signalling [25], and potently induces the expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) which are both known mediators of cell growth and angiogenesis. Studies indicate that human *PPTG1* is a securin-like protein which inhibits sister chromatid separation during mitosis.

### 1.1.4.4 High-mobility group A2 (HMGA2)

The HMGA protein family consists of a group of small non-histone nuclear chromatin proteins. They are involved in the regulation of chromatin structure [26] and play an important role in the assembly of a multi-protein transcriptional complex that regulates the transcription of the target genes [26]. It has been shown that transgenic mice over expressing the *HMGA2* gene develop

growth hormone and prolactin secreting adenomas [27]. Consistent with the development of prolactin adenomas in *HMGA2* transgenic mice, induction of HMGA2 expression is observed in human prolactinomas in association with gene amplification and or rearrangement in the majority of cases [27], however, and in contradistinction to these findings the HMGA2 gene is not expressed in normal pituitary gland. Moreover, very recent studies from the Fusco group have shown that HMGA protein levels are regulated by epigenetic mechanisms namely through miRNAs interference described in more detail in a subsequent section [28].

#### 1.1.4.5 BMP4

Bone Morphogenetic Protein-4 (BMP-4), a member of the TGF-β family plays a central role during pituitary organogenesis [29, 30] and is overexpressed in different prolactinomas models including dopamine D2 receptor knockout ($D2R^{-/-}$) mice, estradiol treated Fisher rats and in human tumours [31], compared to normal tissue and other pituitary adenoma types. Furthermore, GH3 cells , a rat somatolactotrophinoma cell line, stably transfected with plasmids that express protein to block BMP-4 action have reduced tumorigenicity in nude mice, providing evidence that the BMP-4 stimulatory pathway plays a role in prolactinoma development in vivo [31]. BMPs mediate their effect by binding to a family of membrane tyrosine kinase receptors and subsequently activate a family of receptor substrates, the smad proteins, which act as transcription factors in the nucleus. Proteins such as noggin bind BMP proteins and through sequestration prevent their association with tyrosine kinase receptors, thus controlling the extracellular activating phase of the BMP pathway [31]. Even though BMP4 acts as an oncogene in different prolactinoma models and human tumours, it has an inhibitory role in corticotroph tumourigenesis [32]. In this case enforced expression of BMP-4 in AtT-20 cells inhibited proliferation in *vitro* and blocked tumour cell growth in *vivo* [32]. These findings are thought to reflect the differences in cell lineage (corticotrophs vs somatolactotrophs), where BMP-4 either inhibits or stimulates cell growth respectively. The role

of BMP-4 and mechanisms associated with increased and decreased expression form a specific aspect of this thesis. These findings will be discussed in a subsequent section of this thesis.

### 1.1.4.6 Loss of tumour suppressor genes

Tumour suppressor genes (TSGs) act to restrain cell proliferation by regulating the cell cycle or by maintaining genomic stability. Thus, TSGs are characterised by a loss of function and are recessive at the cellular level in transformed cells. Inactivation of TSGs, in most cases, requires gene deletions or point mutations leading to inactivation of both alleles, i.e requiring two hits in Knudson's hypothesis. These events render the gene either silent or functionally compromised with respect to the encoded protein product. Such inactivation could arise from missense mutations, deletions, inversion or insertion or through epigenetic modifications (discussed in a subsequent section). In the context of pituitary tumours, a number of TSG have been implicated. The mechanisms associated with their loss in pituitary tumours are summarised in the following table (Table 1.1).

**Table 1.1. Genetic mechanisms characterising the loss of tumour suppressor gene expression in pituitary tumours**

| Gene | Genetic Defect | References |
|---|---|---|
| *DAPK* | *Homozygous deletion | [33] |
| *FGFR4* | Truncation | [21, 34] |
| *GH-R* | Inactivating mutation | [35] |
| *GIP2* | Inactivating mutation | [36] |
| *MEN1* | Germline mutation | [18] |

- Also see epigenetic change section.

Numerous studies have addressed the role of known and/or putative tumour suppressor loci in pituitary tumours and these are most commonly associated with promoter hypermethylation [18, 37-41]. Exceptions are mutations in the *AIP*, a gene that encodes the aryl hydrocarbon receptor interacting protein, which have been shown to be a relatively frequent event in individuals with familial low penetrance pituitary adenoma predisposition (PAP) and familial isolated pituitary adenomas (FIPA)[35, 42] but not in sporadic pituitary adenomas. In addition a familial neoplastic syndrome called Multiple Endocrine Neoplasia (MEN1) exists in which family members inherit a mutated MEN-1 allele from one parent [43]. Affected individuals develop mono-clonal tumours of the pancreas; parathyroid and pituitary is to as a somatic mutational event (such as LOH) inactivates the other MEN-1 allele[43]. Indeed, loss of heterozygosity analysis was responsible for identifying LOH on 11q13 which led to the identification of the MEN-1 gene [43-45]. Interestingly, despite this gene being discovered on the basis of its mutation in an inherited disease that leads to the occurrence of pituitary tumours in some affected individuals, there has been relatively little evidence of MEN-1 mutations in sporadic pituitary tumours [46-48]. However, in sporadic pituitary tumours loss of heterozygosity at known or putative tumour suppressors have been reported, with mutations rarely encountered in the retained allele[45, 48].

#### 1.1.4.7 RB1

RB is an important suppressor of E2F-dependent transcription and consequently the G1/S cell cycle transition. Its function is in turn antagonised by sequential phosphorylation by cyclin-D-CDK4 and cyclin-E-CDK2 [11]. RB has been implicated in the pathogenesis of several neoplasms including retinoblastoma and osteosarcoma. Loss of heterozygosity (LOH) at 13q, the locus of the *RB1* gene has been identified in human pituitary adenomas [49, 50], despite the fact that in the intermediate lobe pituitary tumours occur with an almost 100% penetrance in the heterozygous *RB1* knockout mouse[38]. There is also evidence that an independent putative tumour-suppressor

gene at that locus is closely linked with, but distinct from, *RB1* and might be important in pituitary tumourigenesis. Likewise, LOH or homozygous deletion of CDKN2A, which encodes the cyclin dependent kinase inhibitor p16, was shown to be uncommon in pituitary adenomas, although reduced mRNA and protein expression was often detected [41]. These observations suggest that other alterations are driving factors in pituitary tumourigenesis. Indeed, for both of these genes epigenetic change, manifest as methylation of their associated CpG island is a frequent finding (discussed in a subsequent section).

## 1.2 Epigenetics

Epigenetics has been defined as the mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the DNA sequence alone [51].The additional layer of information is much more dynamic than the underlying genetic sequence, and explains how cells that possess the same DNA sequence can become "specialised". For example, epigenetic marks are (with rare exceptions) completely erased following fertilisation, however, the presence of epigenetic differences between cells can be determined as early as the 4-cell stage of embryogenesis [52]. Nevertheless, once established it is important that epigenetic information be maintained (at least in the short-term), and complex systems have evolved to ensure this event occurs. However, it is becoming increasingly apparent that epigenetic regulation can become deregulated, and just as environmental insults have been shown to lead to changes in the genetic "blueprint" that are associated with developmental disorders such as cancer, it is now apparent that epigenetic information is similarly vulnerable to deregulation and change. Therefore, the role that epigenetics play in the development of cancer and other diseases is being actively pursued. The important mechanisms that alter the expression of a gene include DNA methylation, histone modifications, that in turn lead to chromatin remodelling, and the expression of micro RNA (miRNA) [53].

The following sections provides an overview of the major epigenetic modifications apparent during development and their impact on gene expression and also the epigenetic modifications associated with tumourigenesis. Moreover, this chapter will also focus attention on the fact that epigenetic aberrations, unlike genetic mutations, are potentially reversible and their restoration to their pre-silencing status may be achieved through epigenetic therapies, that include the *epidrugs*.

### 1.2.1 DNA Methylation

The chemical modification of cytosine, by the covalent attachment of a methyl group to the 5-carbon position, is a frequent occurrence and leads to the formation of the modified base, 5-methylcytosine ($m^5C$) [54], S-adenosylmethionine acts as a methyl donor in this reaction, and is converted to S-adenosylhomocysteine following loss of its methyl group ($CH_3$) to cytosine (Figure1.5).

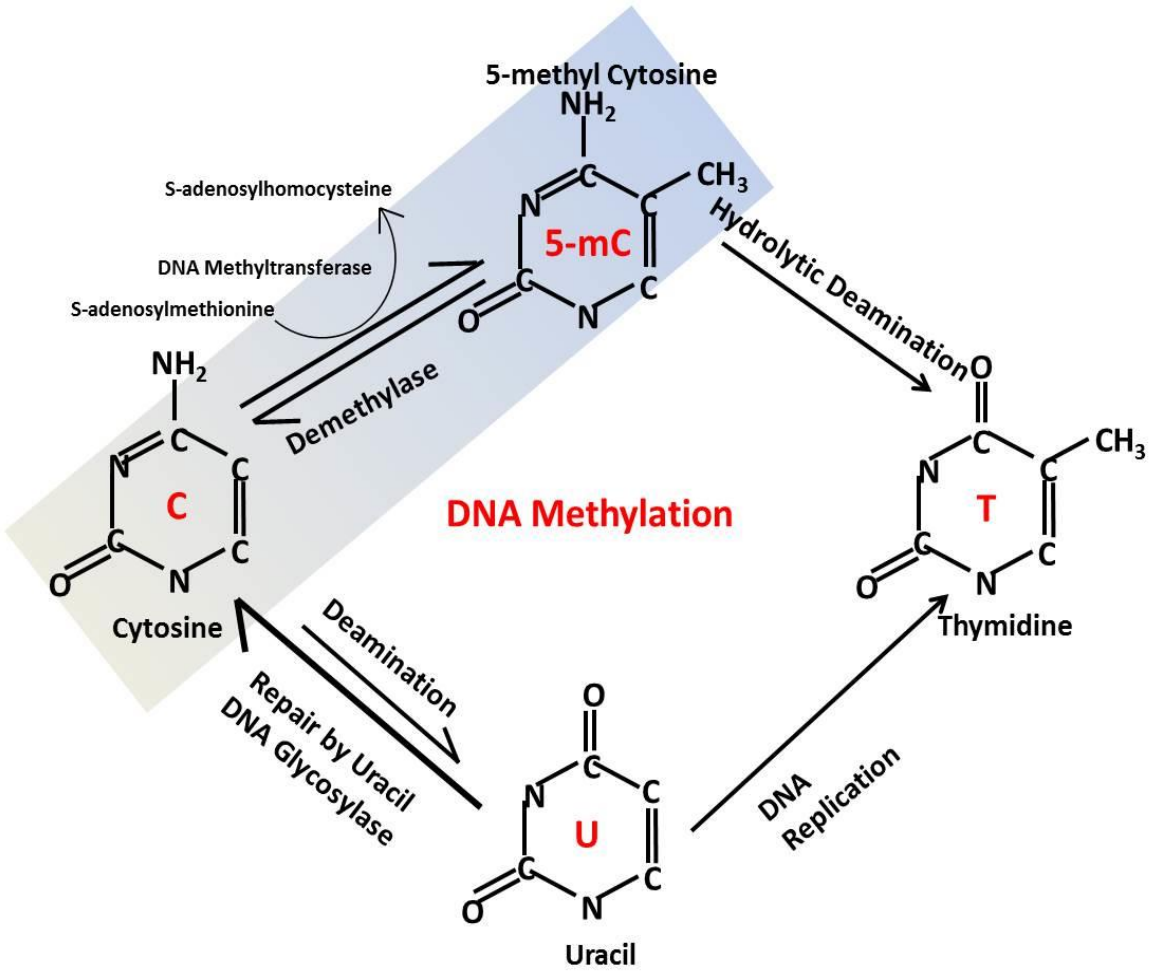



**Figure 1.5: Conversion of cytosine (C) to 5-methylcytosine (m5C)**

Methylation at the 5-position of cytosine involves the transfer of a methyl group from the methyl donor S-adenosylmethionine, generating S-adenosylhomocysteine [55]

DNA methylation is thought to occur almost exclusively in the context of the cytosine-phosphate guanine (CpG) dinucleotide in mammalian cells. In normal cells approximately 70-80% of non CpG islands (CGI) CGI dinucleotides are methylated while CGI, which are usually located within gene promoters, are in general not methylated [56]. In this case, transcription factors access to the underlying gene sequence is facilitated [57]. In contrast, in cancer cells CGI are frequently methylated while non CGI CpG dinucleotides, throughout the genome, showing a decrease in overall methylation. The increase in methylation, at CGI s leads to a decrease or in some cases loss of gene transcription whereas the genome wide loss of methylation is associated with or responsible for genetic instability.

The majority of 5mC thus far identified in the mammalian genome occurs within repetitive DNA sequence [58]. In these cases it is found within interspersed repeat sequences that include, *Alu* elements and also long interspersed nuclear elements; LINE-1 [59, 60]. Transposons constitute more than 45% of the human genome [61], and their inappropriate expression is thought to contribute to genomic instability. DNA methylation also plays an important role in the regulation of gene expression of the X-chromosome, sometimes referred to as X-chromosome inactivation. In this case, one of the two X chromosomes in all somatic cells of placental female mammals is randomly inactivated as a means of dosage compensation [62]. The observation that these chromosomes are heavily methylated led to the suggestion that the acquisition of 5mC is an important event in the initiation of such gene silencing [63]. In a similar manner, the regulatory regions of many genes in the autosomes are associated with DNA methylation. For example, a key regulatory epigenetic mechanism is imposed within the imprinting control regions (ICR) of particular genes. In these cases, methylation may target, in a gene-specific or cell type manner, methylation of either the maternal or the paternal inherited copy of approximately 150 human genes [64]. The resultant imprinting leads to allele specific expression that frequently targets growth promoting and growth inhibiting genes. In addition, the regulatory sequences of approximately 10-20% of mammalian genes also display DNA methylation in a tissue specific and /

or temporal manner [65, 66], suggesting that this modification might play an important role in development processes. Indeed, a wave of demethylation is observed in early stages of embryogenesis [67], and is thought to be critical for ensuring normal development, where a wave of *de novo* re-methylation then follows as shown in figure 1.6. Epigenetic information is also transmitted between generations and plays an important role in trans-generational inheritance.

However, recent studies, have identified a further modification in DNA methylation and characterised as, 5-hydroxmethylcytosine (5hmC) [68]. The conversion of 5mC to 5hmC is catalysed by the chromosome ten-eleven translocation 1 (TET1) protein [69]. TET1 is a 2-oxoglutarate (2OG) and Fe(II)-dependent enzyme that catalyses conversion of 5mC to 5hmC suggesting that this enzyme may have a potential role in epigenetics. Indeed, 5hmC is found at high frequency in several types of mammalian cells, this epigenetic modification may play important roles in cell functions. For example, in cerebellar Purkinje cell DNA, approximately 20% of all cytosine in CpG dinucleotides are present as 5hmC [68]. Data suggested that 5hmC epigenetic marks may be involved in the development of the human brain, and the abnormal 5hmC mark may play a role in the molecular pathogenesis of neurodevelopment disorders [70]. In cancer, several pioneer studies have shown that 5hmC is an epigenetic mechanism. For example, loss of 5hmC has been shown to be an epigenetic hallmark of melanoma [71].

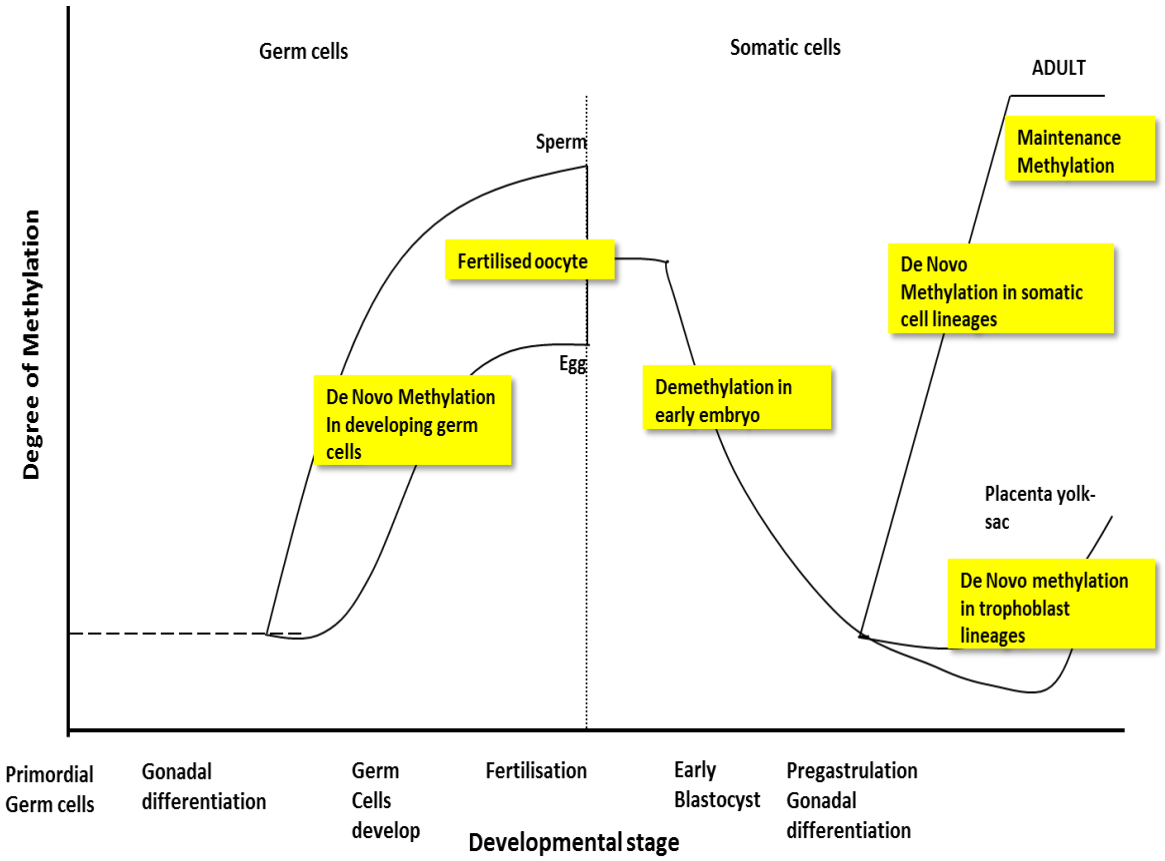



**Figure 1.6: Changes in DNA methylation during mammalian development.** Development stages for gametogenesis show rapid DNA methylation by de novo methylation giving rise to substantially methylated genomes in sperm and egg cells. In the embryo where a wave of genome-wide demethylation occurs at the pre-implantation stage and is succeeded shortly after by large scale de novo methylation beginning at the pregastrulation stage. Adapted from Human Molecular Genetics 2, Strachan and Reid.

### 1.2.1.2 CpG "Islands"

The CpG dinucleotide is under represented through-out the mammalian genome, occurring at approximately one fifth of its expected frequency, however in some regions of the genome the observed frequency of CpG dinucleotides is higher at some, 4-6% [72]. The explanation for these different frequencies is that spontaneous deamination of cytosine coverts this base to uracil. The uracil base mismatch is excised from DNA by the enzyme uracil DNA glycosylase which leads to error free repair. However, the 5-methylcytosine residues are deaminated to thymine which cannot be excised and repaired by this system. As a result 5-methylcystosine residues are hotspots for spontaneous transitions. Indeed, the majority of CpG dinucleotides across the genome are methylated whereas the CpG dinucleotides in CpG enriched regions associated with promoter region are not and accounts for their different frequencies across the genome. The CpG "rich" regions are referred to as CpG islands (CGI). CpG islands are frequently found within stretches of DNA that are approximately 1000 base pairs (bp) long and these are associated with more than half of core gene promoters [73] and also found within intra-genic sequence [74]. It has been suggested that the association between gene silencing is more frequent when the CpG island is found within the promoter region and extending into the first coding exon [75]. In 1987 Gardiner-Garden proposed that a CGI should not be smaller than 200bp with a CG percentage greater than 50% and have a CpG ratio greater than 60% [76]. However in 2002 Takai and Jones proposed an alternative criteria to define a CGI that suggested that at least 500bp, with CG content of 55% and a CpG ratio of 65% constitute the minimum criteria for a CGI ( a representation of normal DNA is shown in figure 1.7) [77].

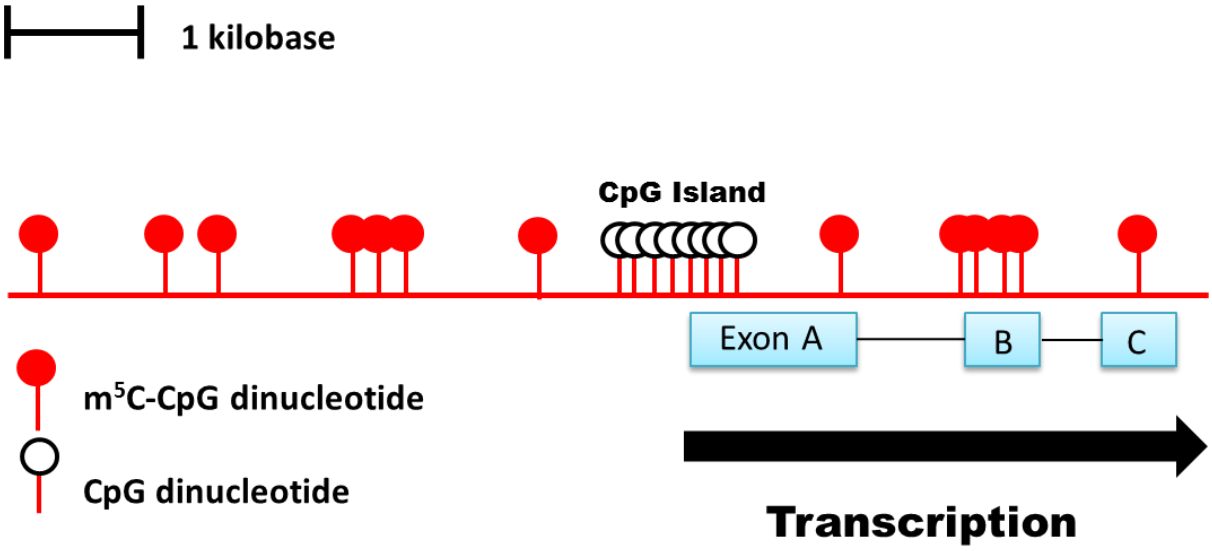



**Figure 1.7: Genomic distribution of CpG dinucleotides and their typical methylation status in mammals**. The red, filled "lollipops" represent methylated CpG sites. The unfilled "lollipops", typically found in clusters, termed CpG islands are in general not methylated. Boxes A, B C represents 3 exons labelled A to C.

#### 1.2.1.3 Establishment and propagation of DNA methylation

Once established, $m^5C$ is a highly stable modification that is maintained in dividing cells and is not easily reversed [78]. It was predicted that two general classes of DNA methylating enzymes exist: *de novo* methylation enzyme that establish DNA methylation patterns at specific sequences early in development, these are DNMT3A/3B, and also the maintenance methylation enzymes (DNMT1) that preserves DNA methylation patterns during cell division[63, 79]. The DNMT1 enzyme recognises hemi-methylated CpG dinucleotides that are the product of semi-conservative DNA replication. Indeed, it has been shown that a conserved group of eukaryotic proteins called DNA (Cytosine-5) methyltransferases (DNMTs) fulfil this role (Figure 1.8).

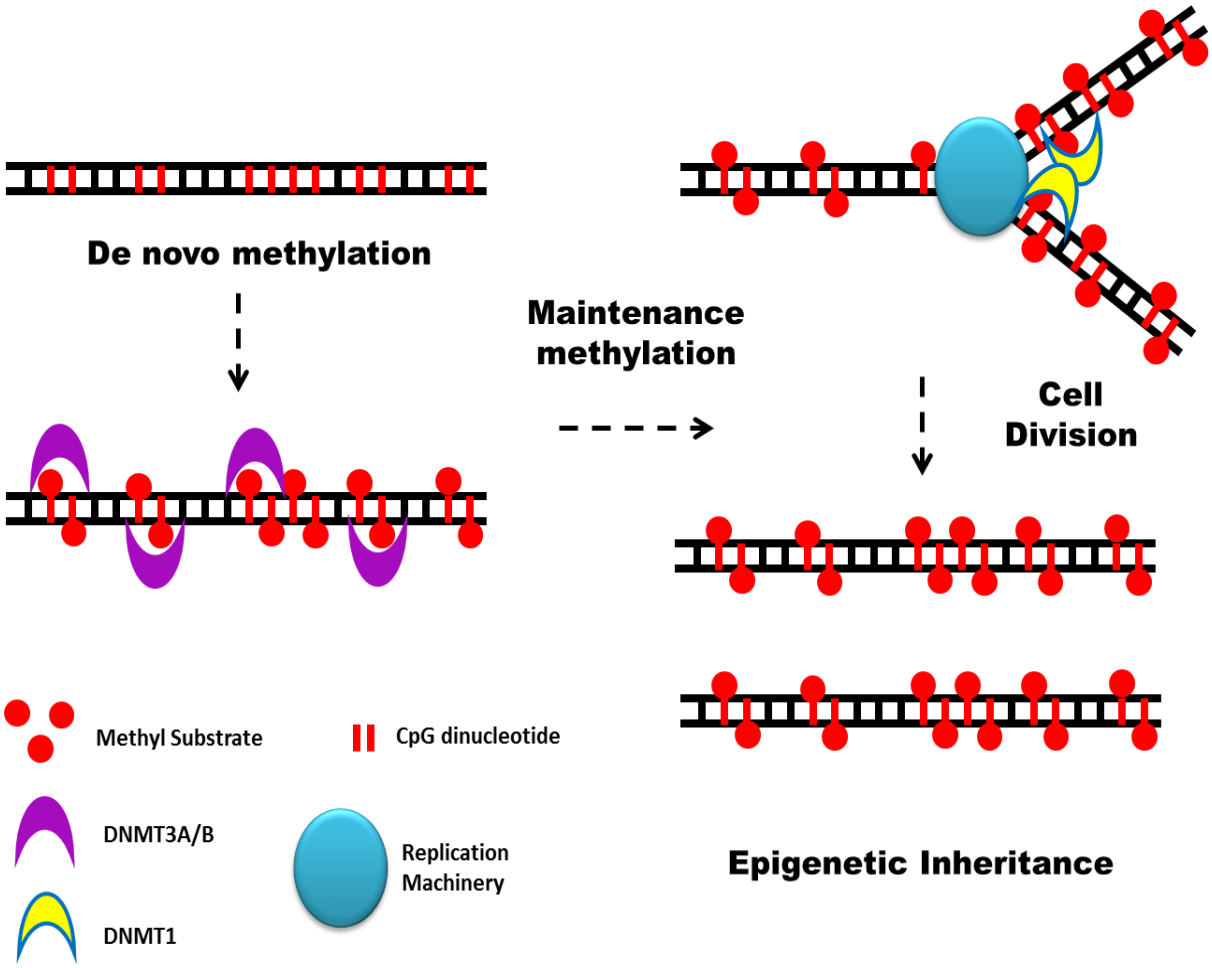



**Figure 1.8: Establishment and maintenance of DNA methylation in mammalian cells.**

DNA methylation in mammals occurs in the nucleotide, CpG. Methyl group can be introduced into unmethylated DNA by the *de novo* methylation enzymes DNMT3A and 3B. However, in the process of DNA replication, the methyl group on the template, parental strand, is recognised and a methyl group introduced on to the opposite (daughter) strand by the enzyme DNMT1. In the presence of DNMT1, hemi-methylated DNA becomes fully methylated and in this way is independent of the DNA sequence itself however, methylation patterns are maintained through successive replications.

DNMT1 was the first DNMT to be reported in 1988 [80]. The 180kDa protein is encoded by the *DNMT1* gene where it was shown that purified DNMT1 methylates hemimethylated DNA at a rate 5-30 fold greater then unmethylated DNA. [81] On this basis it was suggested that this enzyme plays an important role in the maintenance of DNA methylation in mammalian cells. During the G1-G2 phase of the cell cycle DNMT1 is thought to be diffuse throughout the cells. However during the S phase of the cell cycle it is associated with the replication fork consistent with its role in maintaining DNA methylation patterns. [82, 83]. Since then, a number of genetic analyses in various animal models have been carried out to support this role of DNMT1 as a maintenance methylase. For example, extensive studies of ES cells and embryos nullizygous for the murine *Dnmt1* gene have been carried out [84]. These analyses revealed that DNMT1 is essential for development, with lethality seen shortly after gastrulation in *Dnmt1*-null embryos [85, 86]. These mutants show marked loss of genomic $m^5C$ and demethylation of surveyed sequences [84-86]. Data suggests that two additional members of the DNMT family, DNMT3A and DNMT3B, are responsible for the establishment of the 5mC marks. For example, it has been shown that genetic inactivation of *Dnmt3a/3b* in mouse embryonic stems (ES) cells led to the inhibition of DNA methylation patterns being established [87]. However DNA methylation at imprinted regions was maintained in these cells, suggesting that these genes do not play an important role in the maintenance of DNA methylation.

#### 1.2.1.4 DNA Demethylation

Inappropriate changes to the epigenomic landscape are frequently harmful, however these changes are potentially reversible. However, in contrast to the enzymes responsible for methylation those leading to demethylation and the removal of the methyl group from 5mC have not thus far been identified. Indeed, although several DNA methyltransferases have been identified there are still no conclusive reports of *bone fide* DNA demethylating enzymes. Where

DNA demethylation does occur it is through the process that leads to dilution through successive cell generations and during the DNA replication process. In these cases an unmethylated single strand of DNA is synthesised which can result in an unmethylated double strand of DNA molecule in the following generation [88]. For this to occur DNMT1 must be inhibited, since available evidence suggests that DNMT1 recognises hemi-methylated DNA.

#### 1.2.1.5 Abnormal DNA methylation in cancer

The link between DNA methylation and cancer is firmly established [89, 90], and two general phenomena have been shown to occur in relation to DNA methylation in cancer cells. Hypomethylation of CpG sites throughout the genome is a characteristic of tumour cell, however, the target CpGs are not associated with CGI and the resultant hypomethylation leads to chromosomal instability [91]. In addition there is some evidence that hypomethylation of some proto oncogenes leads to their inappropriate expression [92]. Concomitant with the genome-wide hypomethylation is the process of hypermethylation of normally unmethylated CGIs (Figure 1.9). In these cases CGI methylation is associated with or responsible for epigenetic silencing of multiple genes and in particular those defined as tumour suppressor genes (TSGs) [89].

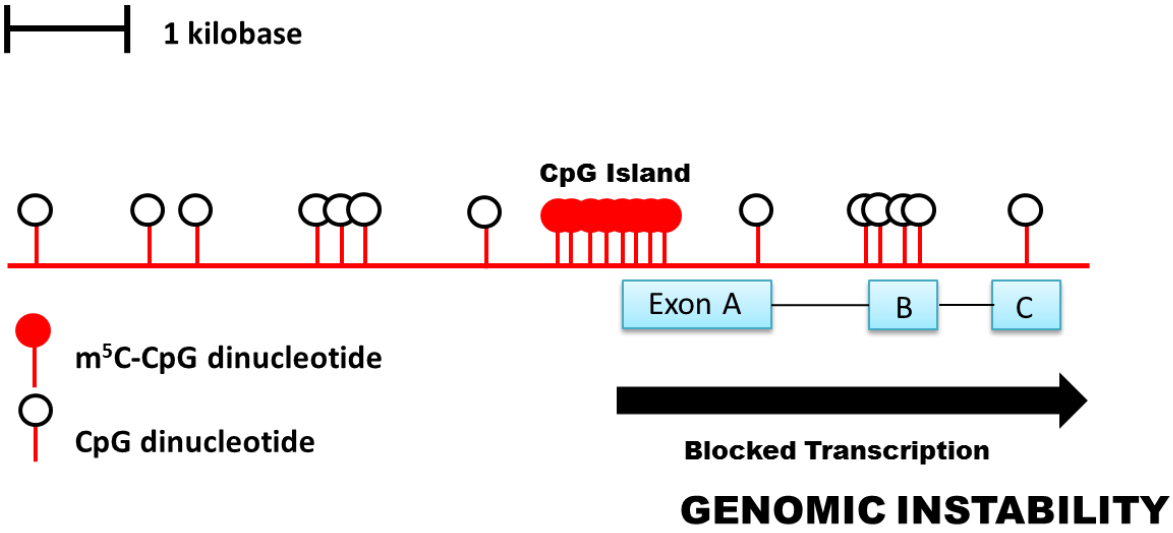



**Figure 1.9. Abnormal DNA methylation at CpG Islands (CGIs) in cancer.**

The pattern of methylation seen in normal cells (Figure 1.7) is reversed in cancer cells. CGIs are hypermethylated and non CGI dinucleotides are hypomethylated. The unfilled circles show the absence of methylation at CpG dinucleotide and the filled circle show these CpG sites as methylated. The Boxes A, B and C show 3 exons within the gene.

### 1.2.1.6 Global Hypomethylation

Genome-wide DNA hypomethylation was the first epigenetic modification to be identified in tumours [93]. Multiple reports have described global, genome-wide hypomethylation of DNA. In these studies majority of the genome-wide hypomethylation was found with DNA repeat elements that comprise of 45% of the genome [92, 94]. As described previously this phenomenon is linked with genomic instability and in some cases the re-activation of oncogenes and of repetitive DNA elements [92, 95]. In cancer cells, data has suggested the link between global hypomethylation, chromatin restructuring, nuclear disorganisation precede and are perhaps causal changes in the activity and expression of the histone modifying enzymes and chromatin regulators [94]. Interestingly, although global hypomethylation is a common feature across multiple tumour types its frequency shows differences between tumour type, tumour stage and the specific sequences harbouring these changes [94]. DNA hypomethylation has been shown to cause the inappropriate activation of several growth promoting genes, including *C-MYC, H-RAS, CCND2* and *SERPINB5* in gastric cancers [96-98] S1000A4 in colon cancer [98] and PAX in endometrial cancer [99]. In addition, many cancer/testis genes that are expressed normally in the healthy testis but not in differentiated cells are often activated in these cells by hypomethylation in cancer. These gene including members of the melanoma-associated antigen (MAGE) gene family [100-102] and microRNA gene *let-7a-3* [103]. These findings suggest that DNA hypomethylation is an early occurrence during the development of cancer.

### 1.2.1.7 CpG island hypermethylation

Promoter associated CGI hypermethylation and gene silencing is a frequent observation, together with global hypomethylation in multiple diseases and in cancer. Although this association has been reported in a large number of publications, it is not clear whether CGI methylation is a cause

or the consequence of gene inactivation [104]. While some studies suggest a causal association between methylation and gene silencing others suggest that silencing precedes methylation and that methylation is necessary to maintain the gene silencing [105]. These aberrations represent a major shift in the distribution of the 5mC mark throughout the genome within disease states. CGI hypermethylation was first reported in retinoblastoma [106, 107], and was associated with epigenetic silencing of the important tumour suppressor gene *RB1* and also that of *CDKN2A*(p16) [108]. Such modifications were then regarded as surrogates for the classic loss [109], of TSGs apparent as inactivating mutation or deletion.

As is the case with genome-wide DNA hypomethylation, it is now becoming clear that CGI hypermethylation frequently, but not invariably, occurs during the earliest stages of cancer development [110]. For example, a series of genes have been identified that exhibit DNA hypermethylation in pre-invasive stages of colon and other cancers. However these genes are infrequently mutated in such cancers (e.g *CDKN2A, SFP1*, *GATA4* and *GATA5*) [111]. One hypothesis is that in stem/precursor cells that the "normal" epigenetic modulation of expression of these genes acts to prevent such cells from proceeding down a differentiation pathway and so remaining immortalized. The activation of these genes, through reversal of epigenetic changes, leads to their differentiation [112]. However, their inappropriate CGI hypermethylation inhibits transcription, thereby preventing differentiation and promoting survival and clonal expansion. In this case one theory is that the resulting pre-invasive stem cell become "addicted" to the survival pathways, and subsequently select for mutations in genetic gatekeeper genes (e.g *APC*) that provide an even stronger progression stimulus [112].

### 1.2.2 Histone Modifications

DNA is rarely in a "naked" configuration and in *vivo* it is associated with proteins called histones that define the chromatin structure. The most important component of the chromatin is the nucleosome which is composed of 147 base pairs of DNA wrapped around an octomer of histone core proteins. This octomer is composed of two copies of each H2A, H2B, H3 and H4. From this fundamental chromatin unit, N- and C- terminals of core histones protrude from this core structure and contact adjacent nucleosomes (Figure 1.10)[113]. Traditionally the structure of the nucleosome was thought primarily to play a role in the packaging of DNA within the nucleus. Although this role is vital, more recent evidence has emerged which confirms that as with DNA methylation, that covalent modification of nucleosome proteins is involved in the epigenetic regulation of transcription [114].

Within the nucleosome, each of the histone proteins, that make up the core octamer, possess a lysine rich amino terminal tail and provides sites for many forms of post-translational modifications including acetylation [115], and methylation [116], as well as ubiquination, phosphorylation, sumoylation, and ADP ribosylation [117] (Figure 1.10). The overall combination of these modifications comprises what is frequently termed the "histone code" and these combinatorial changes determine the expression status of the underlying gene sequences [53]. The "*reading*" or interpretation of this code is thought to determine whether DNA at a particular genomic location is packaged in a highly compacted (heterochromatin) or relaxed (euchromatin) form [113]. Acetylation of histones, associated with an open chromatin structure, is catalysed by the histone acetyltrasnferases (HATs)[115] and shown in figure 1.11. Most descriptions of histone modifications and their influence on gene expression have focused on the changes in acetylation and methylation patterns, especially acetylation of lysine residues in histone N-terminal tails and methylation of lysine and arginine residues in the N terminal tails [118-123] see figure 1.12. The principal modifications associated with either active or silent genes can be summarised as follows:

histone tail acetylation is associated with active genes, as is trimethylation of lysine 4 in the N-terminal of histone 3 [119, 124]; however, trimethylation of lysine 9 and or lysine 27 in the end terminal of histone 3 (H3K9me3 or H3K27me3) or lysine 20 in the N terminal of histone 4 are associated with transcriptional repression [125-127] and shown in figure 1.12. In addition a histone variant H2A.Z has been associated with an active chromatin structure and has been found to be absent from epigenetically inactivated genes [128].

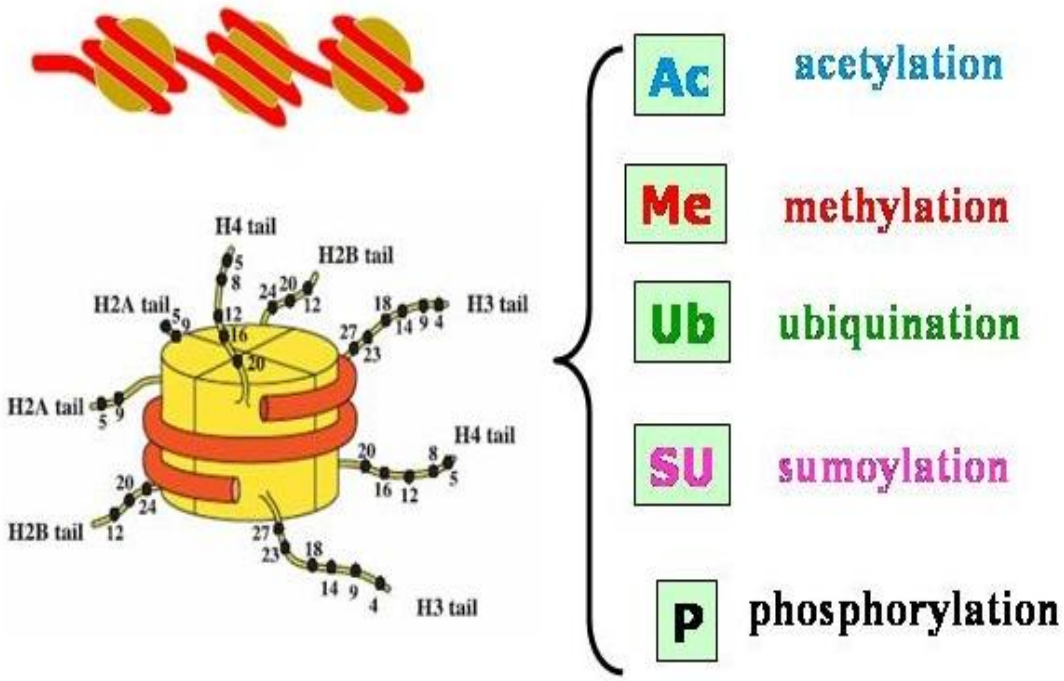



**Figure 1.10: Nucleosome model and the major post translational modifications of histone subunits.**

DNA and histones are assembled into nucleosomes. The nucleosomes consist of 147bp of DNA wrapped around an octomer of four core histone proteins. The four core histone proteins are H2A, H2B, H3 and H4. These four histone proteins contain histone tails that are subject to a number of modifications including acetylation, methylation, ubiquination, sumoylation, phosphorylation. Adapted from http://chemistry.gsu.edu/

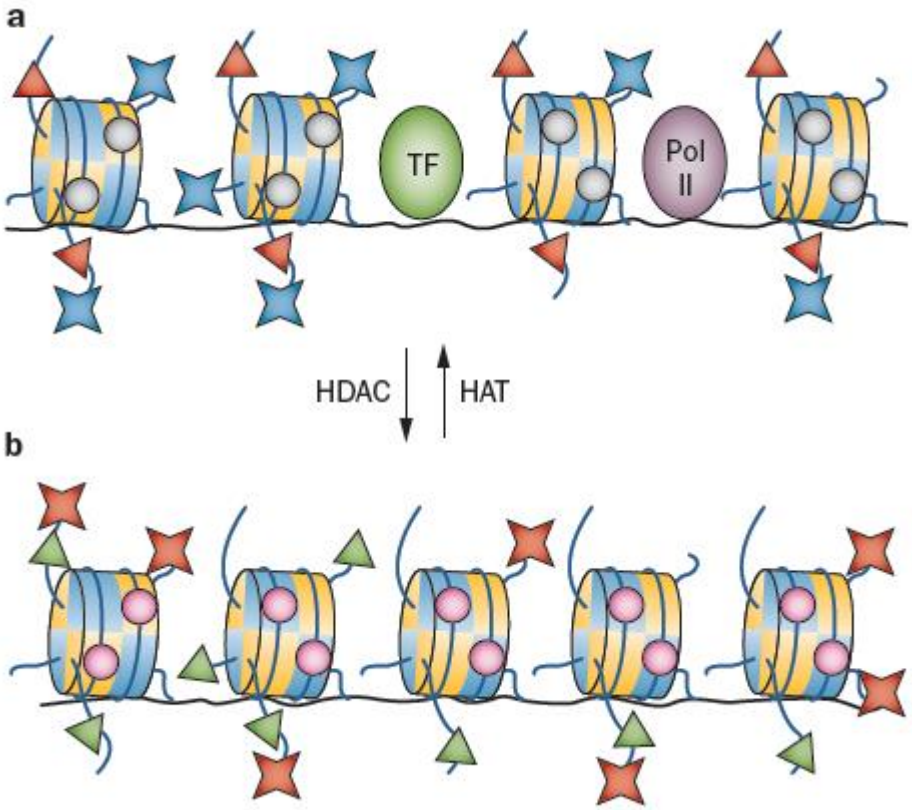



**Figure 1.11: Epigenetic modifications associated with transcriptionally active and transcriptionally silent genes.** A) A transcriptionally active gene with an open relaxed, chromatin structure. DNA with non-methylated CpG islands (grey circles) in the promoter region is coiled around nucleosomes (blue and yellow cylinders). Histone 3 tails are acetylated (red triangles) at lysine 9 (H3K9Ac) or Trimethylated (Blue stars) at lysine 4 (H3K3Me3). This relaxed configuration allows access to transcription factors and associated transcriptional machinery, including RNA polymerase II. B) A silent gene with a closed chromatin structure. Promoter CpG islands are methylated (Pink circles), and the histone 3 tails are Trimethylated (Red stars) at lysine 9 or 27 (H3K9me3/H3K27me3). Accompanying these changes in methylation, the histone tails are hypo acetylated (green triangles) and these patterns are associated with closed chromatin. Abbreviations: Hat, histone acetyl transferase; HDAC, histone deacetylase; Pol II, RNA polymerase II; TF; Transcription factors. Adapted from [53].

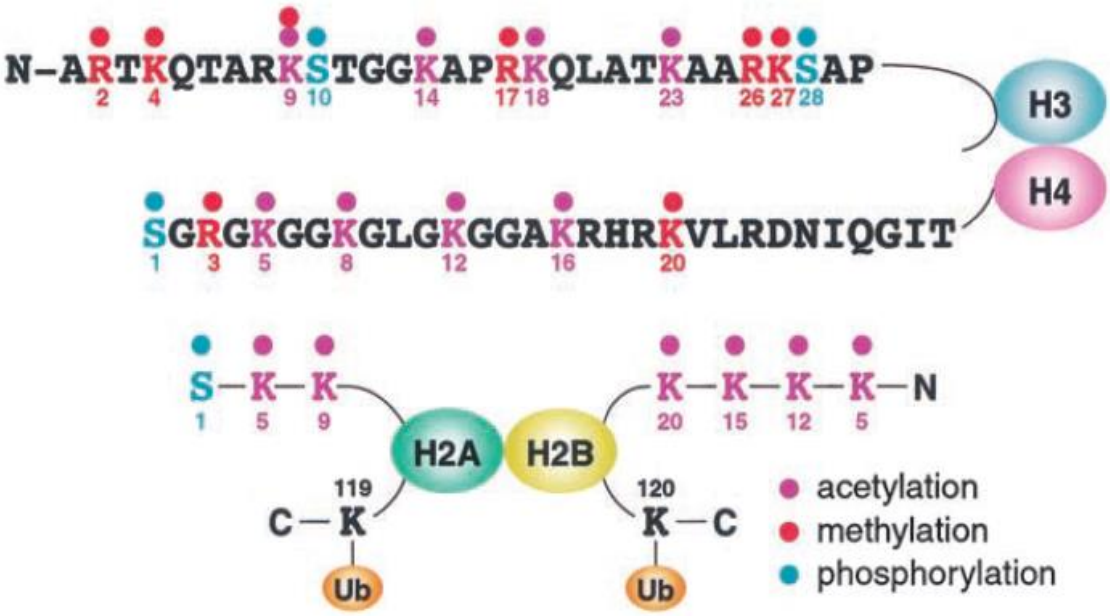



**Figure 1.12**. **Sites of post translational modification on the histone tails**. The modifications shown include acetylation (purple), methylation (red), phosphorylation (green), and ubiquination (orange)[129]. Abbreviations Ub; Ubiquination, A; alanine, G; glycine, K; lysine, L; leucine, P; proline, Q; glutamine, R; arginine, S; serine, T; threonine, V; valine representing amino acids sequence on the histone tail.

### 1.2.2.1 Mechanistic interdependence

The histone changes associated with condensed chromatin and gene silencing are frequently accompanied by, or associated with, DNA methylation. The mechanism by which DNA methylation might directly regulate gene expression is not entirely clear. Indeed, chromatin remodelling is principally mediated through repressive histone modifications as described in figure 1.11. However, the observation that transcriptional silencing is frequently associated with gene promoter methylation might reflect the interdependence of these two processes as shown in figure 1.13. Examples of how these processes are integrated are apparent from multiple studies showing that methylated DNA sequences preferentially bind a family of methyl binding domain proteins that, in turn, recruit HDACs to the site (Figure 1.13) [130]. Similarly, DNMT1, which maintains DNA methylation patterns, also bind HDACs [131-133]. Conversely, at non methylated DNA sequences, the associated histones are acetylated by histone acetyl transferases (HATs) that contribute to an open chromatin structure conducive to gene transcription [89, 134].

Although the interdependence of DNA methylation and histone modification has not been directly investigated in pituitary cells and tumours, the siRNA-mediated knockdown of DNMT3B transcription in pituitary cells provides some insight into this relationship. DNMT3B a de novo DNA methylase, but once knocked down in pituitary cells is associated with increased histone acetylation and decreased methylation [135].

The opposing actions of HATs and HDACs on covalent histone modifications contribute to the reversible nature of acetylation and deaceytlation patterns and to their subsequent effects on gene expression. However other histone tail modifications, such as methylation also show widespread changes in both normal and tumour cells. Methylation of histone tails is mediated by histone methyltransferases (HMTs). Although the principal function of these enzymes is histone modification, they are also able to recruit the DNA-methylating enzyme DNMT1 to these regions

as shown in figure 1.13. In this way, and in contrast to DNA directed histone modifications, histone methylation can drive CpG island methylation.

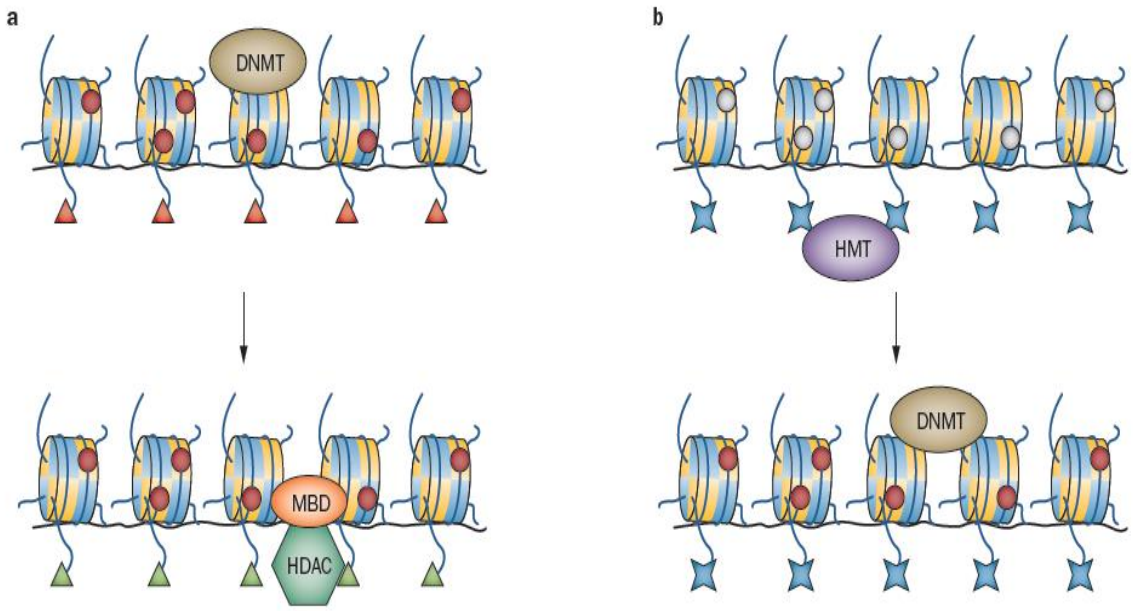



**Figure 1.13**: **Interdependence of DNA methylation and histone modifications.** A) Interactions between DNA methylation and histone deaceytlation. DNA CpG island methylation (red circles) by DNMTs precedes binding of Methyl CpG binding domain protein (MBDs), which recruit HDACs to induce transcriptional repression by mediating deaceytlation (green triangles) of histone tails and chromatin remodelling. B) Interaction between histone 3 lysine methylation and DNA methylation. Non methylated CpG islands in promoters (grey circles) are accompanied by histone 3 trimethylation at lysine 4 and lysine 27 (H3K4 and H3K27me3; blue stars) mediated by the HMTs proceeds de novo DNA methylated (red circles) by DNMTs. Abbreviations; DNMT, DNA Methyltransferase; HDAC, Histone deaceytlase; HMT, histone Methyltransferase; MBD, methyl-CpG binding protein. Adapted from [53].

In addition to histone modifications, a group of proteins call polycomb-group (PcG) proteins have been shown to be associated with gene repression and activation. PcG are conserved chromatin factors that were originally discovered as HOX genes, these genes are a set of transcription factors which are expressed early during embryonic development [136]. PcG maintain the silent state of the HOX gene outside of their expression domain. However a second group of protein termed Trithorax group maintain the active transcription in the appropriate expression domains. PcG and trithorax have long been considered as cellular memory system that stably lock the Hox gene. However genome wide studies have revealed that PcG proteins bind more genes in addition to Hox gene mainly comprising transcription factors involved in diverse cellular functions and developmental pathways [137, 138]. The association of the PcG binding to their target does not necessarily result in gene silencing. PcG have been demonstrated to be dynamically involved in ES cell plasticity and cell fate determination [137, 139]. Genome wide mapping studies have shown that PcG complexes are predominantly bound to genes that encode master development regulator proteins [138]. Many of these regulator proteins are repressed in ES cells and upon differentiation a discrete set of these genes becomes activated which indicates a crucial role of the PcG in a dynamic regulation of stem cell identity and fate determination [137]. H3K27Me3, the histone modification associated with repressed genes is distributed over a large chromosomal region and its distribution correlates with Polycomb repressive complex 2 (PRC2) which recruits the methyltransferase and also distributed up to 20% of gene promoters in ES cells [137]. In these cases, most of these promoters are also marked by the activating histone modification H3K4me3 resulting in bivalent domains [140, 141]. The current hypothesis is that the chromatin states poise genes for activation. However the bivalent status of these genes predisposes them not only for activation but also for repression. Following specific cell fate decisions non-induced bivalent genes tend to lose the active mark and the H3K27Me3 mark is retained (reviewed in [142]). The discovery of the *de novo* bivalent domains has three important implications. First, bivalent domains appear to be the consequence of PRC2 targeting [141]. The second, PcG proteins can

prime genes for both activation and repression during terminal differentiation and third, *de novo* formation of bivalent domains at later developmental stages indicates that the fate of all targets is not predetermined in embryonic stem cells [143].

### 1.2.3 Epigenetic abnormalities in pituitary tumours

In the context of pituitary tumourigenesis, recent studies have shown that epigenetic aberrations play an important role in pituitary tumourigenesis. In these cases the changes have shown particular association with the silencing of TSGs. These findings, therefore, provide an alternative mechanism for the loss of gene expression and to those attributed to genetic abnormalities. In these cases multiple studies now show that DNA methylation within CGI of genes encoding, growth factor regulators, apoptosis inducers, cell adhesion molecules, that either alone or in combination with histone modifications are associated with gene silencing in various types of pituitary tumours.

#### 1.2.3.1 Inappropriate DNA methylation in pituitary tumours

DNA methylation is an important epigenetic mechanism associated with inappropriate gene expression in pituitary tumours and this has been summarised in the following table.

**Table 1.2: DNA methylation associated gene expression defects in pituitary tumours**

| Gene | Epigenetic Defect | Reference |
|---|---|---|
| **CASP8** | DNA hypermethylation | [144] |
| **CDHI** | DNA hypermethylation | [145, 146] |
| **CDH13** | DNA hypermethylation | [145] |
| **CDKN2A (p16)** | DNA hypermethylation | [41] |
| **CDKN1B** | DNA hypermethylation | [147] |
| **DAPK** | DNA hypermethylation | [148] |
| **FGFR2** | DNA hypermethylation | [3] |
| **GADD45** | DNA hypermethylation | [3, 149] |
| **IK1** | DNA hypermethylation | [135] |
| **LGALS3** | DNA hypermethylation | [150] |
| **MAGEA3** | DNA hypomethylation | [151] |
| **MEG3** | DNA hypermethylation | [152, 153] |
| **NNAT** | DNA hypermethylation | [154] |
| **PTAG** | DNA hypermethylation | [155] |
| **RASSF1A** | DNA hypermethylation | [156] |
| **RB1** | DNA hypermethylation | [157] |
| **S100A100** | DNA hypermethylation | [158] |
| **WIFI** | DNA hypermethylation | [159] |

The inappropriate silencing of the CDKN2A (p16) gene is frequently associated with promoter region CGI methylation in pituitary tumours [41]. This appears to be, as in many other types of cancer, an early event in pituitary tumourigenesis [160]. Enforced expression of this gene in pituitary tumour cell line, GH3 inhibit cell proliferation [161], which is consistent with its role as a cell cycle regulator and classification as a TSG. Subsequent studies showed that this gene is infrequently silenced in the somatotrophinomas pituitary tumour subtypes [45], and further studies revealed that in these types of tumours, the promoter of the *RB1* gene was instead methylated [157]. In non-functioning pituitary adenomas, somatotrophinomas and prolactinomas diminished levels of GADD45$_\gamma$ mRNA has been identified [149] and this was correlated later to inappropriate CGI methylation [162]. Likewise, in the majority of pituitary tumours analysed, of gonadotroph origin, transcriptional silencing of MEG3a is also apparent and is associated with CpG methylation [153]. Enforced expression of both GADD45$_\gamma$ and MEG3a in tumour cell lines also led to the inhibition of growth, thus providing support for their role as tumour suppressor genes [151]. Some of the other studies describing methylation associated gene silencing for additional cell cycle regulators, as well as for genes that play a role in apoptosis, invasion and metastasis are shown in table 2. Many of these genes are known or putative TSGs and these observations emphasized the importance of DNA methylation with regards to pituitary tumourigenesis.

### 1.2.3.2 Imprinting defects in pituitary tumours

Imprinting of the maternal and paternal genomes during gametogenesis establishes conditions whereby a specific allele is more abundantly, or in some cases exclusively, expressed in the offspring. It has been suggested that inappropriate DNA methylation at the imprinted loci might play an important role in the development of cancer. For example, loss of imprinting (LOI) and abnormal biallelic expression of the autocrine growth factor IGF2 was first observed in Wilm's tumours [163], and has since been found to be common in lung cancer [164], breast cancer [165], ovarian cancer [166]) and glioma [167].

In the context of the pituitary tumours, approximately 40% of growth hormone secreting tumours harbour somatic mutations in the GNAS1 gene (GSα) [20, 21]. Studies in both human and mice demonstrate that GSα is imprinted in tissue specific manner, being primarily expressed from the maternal allele in some tissues and biallelically expressed in most other tissues [168]. Likewise, in normal human pituitary GSα is near exclusively expressed by the maternal allele as described by Hayward [168]. In this study, 21/22 growth hormone secreting pituitary tumours harboured activating mutations in the maternal allele. Unlike the normal pituitary, even the non-mutated paternal allele contributed to the expression of GSα in the tumours [168]. In the same study, biallelic expression of GSα transcript of gsp(-) tumours was also demonstrated. Also majority of pituitary tumours show transcriptional silencing of MEG3a [153] and it has correlated with the abnormal biallelic methylation of the 5' flanking region of this normally paternally imprinted gene. ZAC, a zinc finger binding protein that induces apoptosis and cell cycle arrest, is another imprinted gene whose expression is significantly reduced in non-functioning pituitary adenoma [169].

Another further example of an imprinted gene is Neuronatin (NNAT). NNAT is one of the most abundant transcripts in the pituitary and is thought to be involved in the development and

maturation of this gland. Its expression is confined to normal pituitary however NNAT has significantly reduced expression in pituitary tumour cell line AtT-20. Re-expression was established using a siRNA approach in a time dependent manner. Re-expression was associated with partial demethylation of NNAT CpG island implying silencing is mediated through DNA hypermethylation[154]. MEG3 methylation is associated with silencing to be confined predominantly to non-functioning pituitary adenomas in the gonadotroph lineage, however this is not the case with methylation associated silencing of NNAT as it does not appear to show subtype specificity[152, 154, 170]. Thus similar to MEG3 NNAT represents a further example of an imprinted gene that is silenced in association with methylation of the normally expressed and unmethylated allele in pituitary tumours which appear to act as a TSG[154].

### 1.2.3.3 Inappropriate covalent histone modification in pituitary tumours

#### Hematopoietic transcription factor Ikaros (IK)

Ikaros (Ik) was described as a transcription factor that recognises regulatory sequences of genes expressed in lymphoid cells [171]. The N terminus encodes zinc finger motifs that recognise cognate DNA-binding sites while the C terminus shared by all Ik isoforms contains the dimerization domain that is required for the formation of homodimers or heterodimers. Isoforms such as Ik6 lack DNA-binding domain can act as dominant negative (dn) regulators of Ik function.

Significant insight into how histone modifications might be inappropriately regulated in pituitary tumours were provided through studies of ikaros (Ik) and its dominant negative isoform Ik6. In normal pituitary, abundant expression of the three functional Ik isoforms (1, 2 and 3) is seen in the anterior pituitary [34] and these play important roles in the regulation of multiple hormones including proopiomelanocortin (POMC), prolactin (PRL), GH and GHRH, as well as cell growth and survival [34, 122]. In contradistinction to these findings, the dn isoform Ik6 was found to be expressed in approximately 50% of pituitary tumours [34]. Ik6 protein was subsequently shown to lead to H3 lysine acetylation-associated transcriptional induction of the anti-apoptotic factor BCL-XL [122]. This suggests that abnormal expression of the dominant negative isoform of Ik (Ik6) might be responsible for the inappropriate epigenetic regulation of cell proliferation and hormone secretion in a proportion of pituitary tumours. These findings were subsequently supported by studies in the rat pituitary cell line GH4 where GH transcriptional suppression and PRL transcriptional activation are, at least in part, mediated by the effects of Ik6 on promoter acetylation [172]. Interestingly, in primary tumours that do not express Ik, as well as in the mouse pituitary cell line AtT-20, transcriptional silencing was shown to be associated with exon 1 CGI methylation and concomitant histone modifications [3], suggesting that Ik itself could be regulated by epigenetic mechanisms.

## Cancer/Testis (C/T) Antigen-MAGEA3

C/T antigen are immunogenic proteins that are expressed in normal gametogenic tissues and in different tumours types. C/T antigen genes such as Melanoma antigen family A, 3 (MAGEA3) are not normally expressed in adult somatic tissues (including the pituitary), and this repression is associated with DNA methylation [151]. However recent studies show that MAGEA3 is inappropriately expressed in pituitary tumours, and this is associated with DNA hypomethylation and histone lysine acetylation [153], suggesting that gene expression induction through epigenetic mechanisms may play an important role in pituitary tumourigenesis. Interestingly, the observation that *MAGEA3* was specifically re-expressed in FGFR2- negative pituitary tumours suggested that such induction was a downstream response of FGFR2 loss. FGFR2 has been known to function as the control of oestrogen-signalling and therefore it is hypothesised that inappropriate oestrogen signalling in pituitary tumours may be responsible for the augmentation [151]. Indeed, in a cell line model it is clear that oestrogen treatment led to the induction of MAGEA3 expression, with associated DNA hypomethylation and histone acetylation in a cell line model. Furthermore, in the same study it was also evident that this response was specifically observed in FGFR2-negative female tumours *in vivo* thus providing additional support for the role of oestrogen signalling played in this response [151].

### 1.2.4 miRNA in Pituitary adenomas

The HMGA protein family, a group of small non-histone nuclear chromatin proteins involved in the regulation of chromatin structure and play an important role in the assembly of transcriptional complexes that in turn regulate transcription of the target genes [26]. HMGA2 has been shown to be overexpressed in transgenic mice developing growth hormone and prolactin secreting adenomas and also in human prolactinomas in association with gene amplification and or rearrangement [27]. However, and in contradistinction the HMGA2 is not expressed in normal pituitary gland. Moreover, very recent studies from the Fusco group have shown that HMGA protein levels are regulated by miRNAs [28]. miRNA are a class of small (19-25 nucleotides) non coding RNAs involved in temporal and tissue specific eukaryotic regulation by binding the 3'-unstranslated region of the target mRNA. These have been shown to induce mRNA degradation or inhibition of its translation. The group analysed the expression levels of HMGA-targeting miRNA (miR-15, -16 and Let 7a) in human primary pituitary adenomas. The expression of these miRNAs showed an inverse correlation with HMGA2 expression [28]. Furthermore, transfection experiments with these specific siRNA, in this case as short-hairpin RNA (shRNA) expression vectors, that target the HMGA transcript led to reduced HMGA levels in GH3 cells and mediated a significant decrease in proliferation. These data suggests that HMGA-targeting miRNAs are able to negatively regulate pituitary cell cycle progression [28, 173].

### 1.2.5 Pharmacological Epigenetic unmasking strategies

The epigenetic aberrations that distinguish tumour cells from their normal counterparts are reversible [174, 175]. Administration of pharmacological agents that reverse epimutations typically causes global re-expression of previously silenced genes, which can be employed to identify the genes subject to this form of regulation. In these studies, gene expression is usually detected by cDNA microarrays and can be used to identify unknown genes, some of which are tumour specific, a previous study of this group shows by knockdown of DNMT1 and microarray analysis the identification of newly expressed genes was possible[158]. Importantly, some genes that are re-expressed following drug treatment were not methylated before intervention, and others that were expressed before treatment showed reduced expression [176, 177]. These findings might reflect reversal of epigenetic silencing of an upstream regulator that could either stimulate or repress its downstream targets, which are identified by microarray. Indeed in pituitary tumours, the expression status of particular IKZF1 (which encodes IK6) and FGFR2 (which encodes FGFR2-IIIb) isoforms and also of DNMT3B affects the expression of their downstream target genes and in some cases, their epimutation status.

A broad range of drugs have been described to target the epigenome and effectively reverse DNA methylation and histone modifications in various tumour types [158, 178-180]. The majority of these drugs directly target the epigenome through inhibition of the enzymes responsible for these aberrations; DNMTs and HDACs. Some researchers have raised concerns about the possible adverse effects of these drugs, particularly at high doses although these seem to be particularly effective in cells that are undergoing cell proliferation [181].

### 1.2.5.1 DNA Methyltransferase Inhibition

Inhibitors of DNA methylation rapidly reactivate the expression of genes that have undergone promoter methylation dependant gene silencing, particularly if this silencing has occurred in a pathological situation. The first described inhibitors of methylation which were FDA (The US Food and Drug Administration) approved is 5-azacytidine (5-Aza) and its deoxy analog (5-Aza-dC) were initially developed as chemotherapeutic cytotoxic agents and was used to treat disorders such as myelodyplastic syndrome [182], It was subsequently discovered that they are also powerful inhibitors of DNA methylation and could induce gene expression and differentiation in cultured cells [54, 183]. Once introduced into a nucleus, both nucleoside analogues are converted to the deoxynucleotide triphosphates and are then incorporated into replicating DNA in place of cytosine. As such, they are primarily active in the S phase of the cell cycle and serve as powerful inhibitors of DNA methylation. DNMTs get trapped on DNA containing modified bases such as azacytosine, 5-fluorocytosine and Zebularine. This results in the formation of heritable demethylated DNA [54, 149].

A disadvantage and an important concern with the use of these nucleoside analogues is that they are chemically unstable in aqueous solution. These agents also suppress the proliferation of blood cells of the myeloid lineage and thereby lead to toxicity problems[184]. However other nucleoside analogue being evaluated in clinical trials is zebularine.

Zebularine is a cytidine analogue originally synthesised as a cytidine deaminase inhibitor. Zebularine effects seem to be more selective for cancer cells then non-malignant cells as cancerous cells have a higher proliferation rate than non-malignant cells [128]. Besides being an effective inhibitor of DNA methylation, Zebularine possesses a number of properties desirable for a therapeutic agent. Zebularine has a very stable half-life [185]. Orally administered Zebularine has been shown to cause demethylation and reactivation of a silenced and hypermethylated p16 (*CDKN2A*) gene in bladder tumour cells grown in nude mice (25). Other studies also show that

zebularine is cytotoxic *in vitro* and *in vivo* (25). Due to its low cytotoxicity Zebularine can be given continuously at low doses to maintain demethylation for a prolonged period [185].

### 1.2.5.2 Histone Deacetylase Inhibition

At present an array of drugs with HDAC inhibitory effects have been described and are currently under clinical trials. However the FDA has only approved one HDAC inhibitor, Vorinostat also known as SAHA for the treatment of cutaneous T-cell lymphomas [186].

The biochemical structures of HDAC inhibitors are extremely heterogeneous. The vast majority of HDAC inhibitors are designed to interfere with the catalytic domain of HDACs and thereby block substrate recognition and induce gene expression. The HDACs described so far greatly vary in structure and origin and can be sub-classed based on their distinct chemical properties as shown in table [128].

**Table 1.3 HDAC inhibitors**

| Name | Chemical nature | Clinical status | Reference |
|---|---|---|---|
| **Sodium phenylbutyrate** | Short-chain fatty acid | Phases I, II | [187] |
| **Sodium butyrate** | Short-chain fatty acid | In Clinical trial | [128] |
| **Valproic acid** | Short-chain fatty acid | Phases I, II | [188] |
| **OSU-HDAC42** | Short-chain fatty acid | Not yet in clinical trial | [189] |
| **Trichostatin A** | Hydroxamic acid | Not yet in clinical trial | [190] |
| **Vorinostat** | Hydroxamic acid | Approved (CTCL)<br>Phases I, II and III | [191] |
| **Panobinostat** | Hydroxamic acid | Phases I, II and III | [128] |
| **Belinostat** | Hydroxamic acid | Phases I, II | [192] |
| **Romidepsin** | Cyclic peptide | Phases I, II | [193] |
| **Entinostat** | Benzamide | Phases I, II | [194] |
| **MGCD-0103** | Benzamide | Phases I, II | [195] |

The short chain fatty acids comprise of one class of HDAC inhibitors of which Valproic acid for example has been shown to be efficient in reducing tumour growth and metastasis formation in breast cancer rat model [187]. Treatment with valproic acid has also resulted in differentiation of

transformed cells [187]. The hydroxamic acids comprise another class of HDAC inhibitors, which inhibit zinc-dependent HDACs, including Trichostatin A (TSA). The zinc binding group is analogous to the acetyl group of the histone N-acetylated lysine. Treating cells with TSA results in high levels of acetylated histones [190]. Differentiating and antiproliferative activities have also been observed when treating murine erythroleukaemia cells with TSA at a nanomolar concentration [190]. Despite the many anticancer effects of TSA the drug is not in clinical trials due to its severe side effects. It could be expected that HDAC inhibitors would have a global effect on gene expression as they are supposed to block several classes of HDAC, This is not the case it seems that HDAC inhibitors only affect a small fraction of the transcriptome [128]. To date interactions between HDACs and a large number of non-histone proteins such as transcription factors, DNA repair enzymes, structural proteins and signal transduction mediators have been shown and the role of HDACs as key players in many different cellular processes is accepted [196-199]. Therefore the various interactions makes it difficult to establish the precise mechanisms of HDACs and in turn develop HDAc inhibitors capable of re-activating tumour suppressor genes without undesirable effects [128] [200].

#### 1.2.5.3 Combination strategies: DNMT inhibitors combined with HDAC inhibitors.

Since DNA methylation and histone deaceytlation are both important in the epigenetic inactivation of tumour suppressor genes it is acceptable to use DNMT inhibitors and HDAC inhibitors in combined treatments strategies. A synergistic effect of combined DNMT inhibitors and HDAC inhibitors has been observed in colon cancer cell lines at hypermethylated tumour suppressor gene loci including CDKI p16 [201], but have yet to be established together in published clinical trials. Initially it has been demonstrated that administration of TSA alone did not re-activate densely methylated tumour suppressor genes p16, however when the cancer cells were treated with DNMT a synergistic effect of the two drugs was observed [202]. Collectively

these findings show that combined therapies that target epimutations offer new therapeutic options.

### 1.2.5.4 Combination strategies: epigenetic therapy and chemotherapy.

Many HDAC inhibitors; TSA, belinostat and vorinostat have been shown to act as synergists with a large number of chemotherapeutic drugs such as paclitaxel, gemcitabine, cisplatin, etoposide and doxorubicin [203] have been investigated in cell line model systems [203-206]. In particular the administration of DNMT1 inhibitors or HDAC inhibitors before chemotherapy seems to be a promising strategy to overcome the development of multidrug resistance as acetylation of core histones provides an open conformation making DNA more accessible to drugs [203]. Pre-treatment of cancer cells with TSA or vorinostat before applying chemotherapeutic drugs; ellipticine, doxorubicin and cisplatin increased the sensitivity of the drugs with more than 10 fold in brain tumour cell line. The effect was specific [203]. Applying the drugs in reverse order initiating with the chemotherapeutic drugs had no effect. The broad capacity of HDAC inhibitors to synergise with various chemotherapeutic drugs indicates that they lower the threshold for cancer cells to undergo apoptosis mediated by the drugs. However further studies on the effects of HDAC inhibitors in combination with chemotherapeutic drugs are needed.

### 1.2.5.5 Combination strategies: epigenetic therapy and radiation therapy.

Radiotherapy has widely been used for the treatment of cancer and the search has been for different compounds to modulate the cellular response such as radiation sensitizer and also to protect against acute and late effects of ionization radiation; radioprotectors [207].

HDAC inhibitors are thought to be able to modulate the effects of ionizing radiation by changing gene expression causing cells cycle arrest, growth inhibition and induction of apoptosis [208]. TSA, valproic acid, vorinostat have been found to enhance the sensitivity towards ionization radiation of different cell lines [209, 210]. The modulation of cell cycle arrest in G-1 phase by inhibition of DNA synthesis in the S phase, induction of apoptosis and down regulation of surviving signals contributes to irradiation sensitivity of the cells, when HDAC inhibitors are administered at high concentrations. However at lower non-toxic concentrations HDAC inhibitors can still modulate insensitivity by affecting the expression of genes involved in response to DNA damage.

## 1.3 Role of Receptor mediated signalling in the pituitary cells

### 1.3.1 Receptors as mediators of endocrine signals

Proliferation and differentiation of cells during development and the maintenance of cellular homeostasis require a continuous flow of information to the cell. This is provided either by the diffusing molecules or by the direct cell-cell or cell-matrix interactions. Cells utilise a wide variety of molecules and signal transduction systems to communicate to one another and exert their effect by interacting with specific receptor proteins that are coupled to one or more intracellular effector systems. The presence of an appropriate receptor therefore defines the population of target cells for a given effector molecule and provides a molecular mechanism by which the effector molecule elicits its biological function [211].

### 1.3.2 Receptors expressed by the pituitary cells

Similar to most other cells in the body, pituitary cells express a wide spectrum of receptors. It would be beyond the scope of this thesis to include a discussion of all of the receptors expressed by the pituitary gland. However, and for completion Table 1.4 summarises the receptors thus far identified as associated with the pituitary gland. In this thesis more detailed consideration will be given to receptors and their ligands that impact on hormone secretion and or growth.

One set of important receptors are the GPCRs, these play important roles in a variety of biological and pathological processes such as development and proliferation, neuromodulation, angiogenesis, metabolic disorders, inflammation, and viral infection. The GPCR is one of the most targeted protein families in pharmaceutical research today representing approximately 35% of the top 20 prescribed FDA-approved drugs [212]. In addition, the GPCRs expressed by the

pituitary gland (Table 1.4) also include Somatostatin (SSTR1-5) and Dopamine (D1-5) receptors, which have been extensively utilised in the clinical treatment of pituitary tumours.

Mutations in any component of the G protein-coupled signal transduction pathway may cause disease including G-protein α-subunits. Genes encoding these molecules are targets for loss and gain of function mutations which therefore result in endocrine, metabolic and developmental disorders (1-6). In particular mutations in GPCR and G-protein genes typically lead to hormonal resistance syndromes. Heterotrimeric guanine nucleotide protein (G protein) are composed of three subunits α, β and γ, the functional specificity of each G protein depending on the α-subunit which differ from one G protein to another [213]. The guanosine diphosphate (GDP)-bound α-subunit binds tightly to βγ and is inactive, whereas the GTP bound form dissociates from βγ and activates the effector proteins. The interaction of an agonist with the specific GPCR causes exchange of GDP to GTP in the α-subunit, while the turn off is timed by GTP hydrolysis. The α-subunit triggered effectors are enzymes of second messenger metabolism and ion channels. They induce short term effects on hormone secretion, neurotransmission and muscle contractions as well as long term effects on gene transcription [214].

The G proteins that have demonstrated to be targets of disease causing mutations in pituitary adenomas is Gsα , and Gsα that mediates adenylyl cyclase activation and cAMP formation. To date, the gene encoding Gsα (GNAS) is the only gene encoding a G protein that has been identified as a target of either gain of function mutation that unequivocally cause endocrine disease. Activating mutations lead to proliferation of endocrine cells in which cAMP is a mitogenic signal leading to somatotrophs, thyroid, adrenal and gonadal adenomas or McCune-Albright syndrome (9).

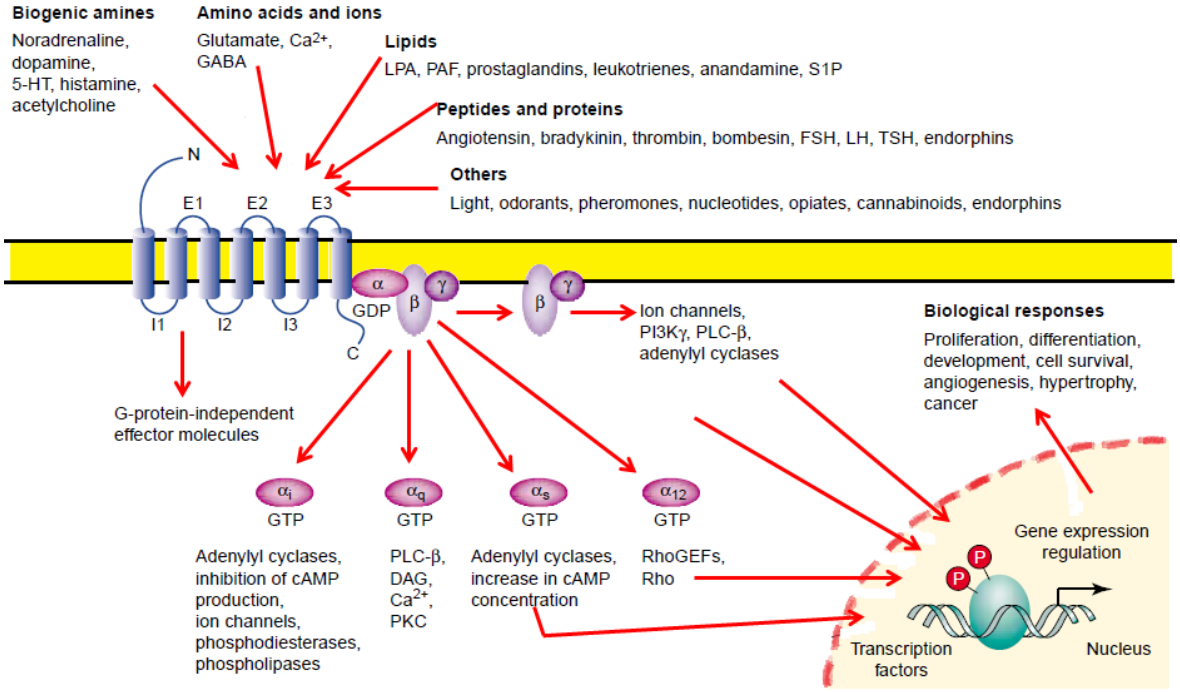



**Figure 1.14 Diversity of G-protein coupled receptors (GPCRs).** A wide variety of ligands including biogenic amines, amino acids, ions, lipids and proteins use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G protein dependent and independent pathways. Such signalling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Adapted and modified from[215].

**Table 1.4: Receptors expressed in the pituitary gland**

| Receptors studied in the pituitary | References |
|---|---|
| **G-Protein receptors (G-PCRs)**<br>Adenosine receptors )A1, A2A, A2B, A3) | [216] |
| Angiotensin (AT1A, AT1B, AT2) | [217] |
| Adiponectin receptors (ADIPOR1, ADIPOR2) | [218] |
| Complement C3a receptor1 (C3aR1) | [219] |
| Complement C5a receptor 1 (C5aR1 or CD88) | [220] |
| Corticotrophin-releasing hormone receptor (CRHR1 & 2) | [221] |
| Dopamine receptor (D1-5) | [222] |
| Endothelin receptor ( ETA, ETB) | [223] |
| Epidermal growth factor receptor (EGFR) | [224] |
| Fibroblast growth factor receptors (FGFR 1,2,3,4,6) | [225] |
| Growth hormone releasing hormone receptor (GHRHR) | [226] |
| Gonadotropin-releasing hormone receptor (GnRHR) | [227] |
| GABA receptor B (GABAB) | [228] |
| Luteinizing hormone receptor (LHR) | [229] |
| Melatonin receptor (MT1 & MT2) | [230] |
| Purinergic receptors (PI-A1, A2A, A2B, A3 & P2Y-P2RY1,2,4,5,6,8,9-14) | [231] |
| Serotonin receptor (5HT1,2,4-7) | [232] |
| Somatostatin receptor (SSTR1-5) | [222] |
| Tachykinin receptor2 (NK2) | [233] |
| Thyrotropin-releasing hormone receptor (TRHR) | [234] |
| Vasopressin receptor (V1B or V3)<br>**Non- G-protein coupled receptors (Non-GPCRs)** | [235] |
| Estrogen receptors (ER-1 & 2) | [236] |
| Insulin Receptor (CD220) | [237] |
| Insulin-like growth factor 1 (IGF-1) | [238] |
| Interleukin-1/6 receptor (CD126-CD130 complex) | [239] |
| GABA receptor A (GABAA) | [240] |
| Purinergic receptors (P2X1-7) | [241] |
| Prolactin receptor (PRL-R) | [242] |
| Ryanodine receptors (RyR3) | [243] |
| Serotonin Receptor (5HT3) | [232] |
| Stromal cell-derived factor-1 (SDF-1 or CXCL12) | [244] |
| Vascular endothelial growth factor receptors (VEGFR1-3) | [245] |

### 1.3.3 Receptor mediated treatment of pituitary tumours

The treatment of pituitary tumours is largely dependent upon the adenoma subtype. Some tumours are removed surgically while others are treated medically and in some cases a combined approach is adopted. Surgery has been the first line of treatment in acromegaly (GH secreting adenomas), non-functioning tumours and corticotroph adenomas that give rise to Cushing's disease [246]. For prolactinomas, the first line of treatment and usually the most successful is medical treatment employing dopamine agonist [247, 248]. Therefore, only in those cases where medical intervention has failed through either drug resistance and/or intolerance would surgical intervention be considered as a therapeutic option [200, 247]. Complications such as hypopituitarism, Cerebrospinal fluid leakage, the dependence upon lifelong hormone replacement therapy and either regrowth or recurrence (especially in Cushing's disease) [246] are the major disadvantages associated with the pituitary surgery.

With availability of dopamine (DA) agonist and Somatostatin analogues (SSA), the medical treatment of pituitary adenomas and in particular for prolactinomas and inhibition of hormone secretion in GH secreting adenomas respectively has shown significant improvement. DA agonists comprise the ergots (Bromocriptine, Pergalide, Metergoline, Lisuride, Terguride and carbogoline) and non-ergot derivatives (the Quinagolide compounds). The efficacy of dopaminergic compounds in the treatment of prolactinomas is well established with almost 90% success rate [249, 250]. Clinically useful formulations of SSA are useful for suppression of GH secretion in acromegaly [251] and in some TSH secreting adenomas [252]. In case of acromegaly, SSAs effectively lower serum GH in more than 90% of patients, normalise IGF-I in 40-60% and some reports show reduction in tumour size in almost half of these patients [253].

### 1.3.4 Expression of D2 and Somatostatin receptors in pituitary tumours

DA agonists and SSA requires the presence of their respective receptors for their activity. Dopamine receptors belong to the G-protein coupled receptor superfamily and at least five subtypes of dopamine receptors have been identified (D1, D2, D3, D4 and D5) [254, 255]. These are further divided into two groups on the basis of their amino acid sequence and their pharmacological properties. The first group are Gα coupled D1 like receptors which stimulate adenylyl cyclase activity and it comprises of D1 and D5 receptors. The second group is Gαi/o-coupled D2 like receptor which inhibit adenylyl cyclase activity and comprises D2, D3 and D4 receptors. D2-like dopamine receptors influence additional signalling mechanisms including the modulation of potassium and calcium channels [256]. In addition, D2 like receptors also exist in two variant forms e.g $D2_{short}$ and $D2_{long}$. Dopamine stimulation via $D2_{short}$ receptor subtype has shown to be greater than that via $D2_{long}$ [257]. It is the D2 receptor that plays a major role in mediating the inhibition of dopamine on PRL release compared to D1, D3, D4 and D5 [258, 259]. The detailed signalling mechanism induced by dopamine and its agonists will be described in a subsequent section.

Somatostatin (SRIF) regulates the secretion of multiple pituitary derived hormones that include GH, PRL and TSH and also has effects on proliferation through binding G protein-coupled SRIF receptors. There are five subtypes of this receptor SST1, SST2, SST3, SST4 and SST5 [260]. Alternative splicing generates two isoforms of SST2 receptor, these being, SST2A and SST2B [260]. Similarly, the expression of SST4 is also rather infrequent in the anterior pituitary. SSA receptor activation mediates cytostatic effects and cell cycle arrest in G1, or apoptosis following SSA treatment of tumour cells both in vitro and in vivo [261]. Activation of SST2 and SST5 are involved in the control of GH secretion [262, 263]. Some reports suggest that the activation of receptors of SST1, 2, 4 and 5 induce cell cycle arrest and apoptosis [264]. Downstream activation of

phosphotyrosine phosphatases (PTPs) by SSTs represent one of the main intracellular mechanisms involved in the antiproliferative effect of SRIF and analogues [265].

Both SST and D2 receptors are expressed in pituitary adenomas. The D2 receptor which is expressed by all types of pituitary tumours, is associated with two or more SST subtypes in different adenoma subtypes [reviewed in [266, 267]. For example SST2 and SST5 are associated with D2 receptors in the majority of GH-secreting adenoma [268-270] while D2 has been shown to associate with SST1, SST2 and SST5 in prolactinomas [270, 271]. Non-functional pituitary adenomas express mainly SST3 to a lesser degree, SST2 and rarely associated with SST1 [270, 272, 273]. D2 and SST5 are almost equally represented in the corticotropes tumours [274, 275]. Finally TSH secreting adenomas, expression of SST1, SST2 and SST5 and D2 receptors have been reported [270, 276]. Figure 1.15 illustrates the spectrum of D2 and SST receptor subtypes in different types of pituitary adenomas.

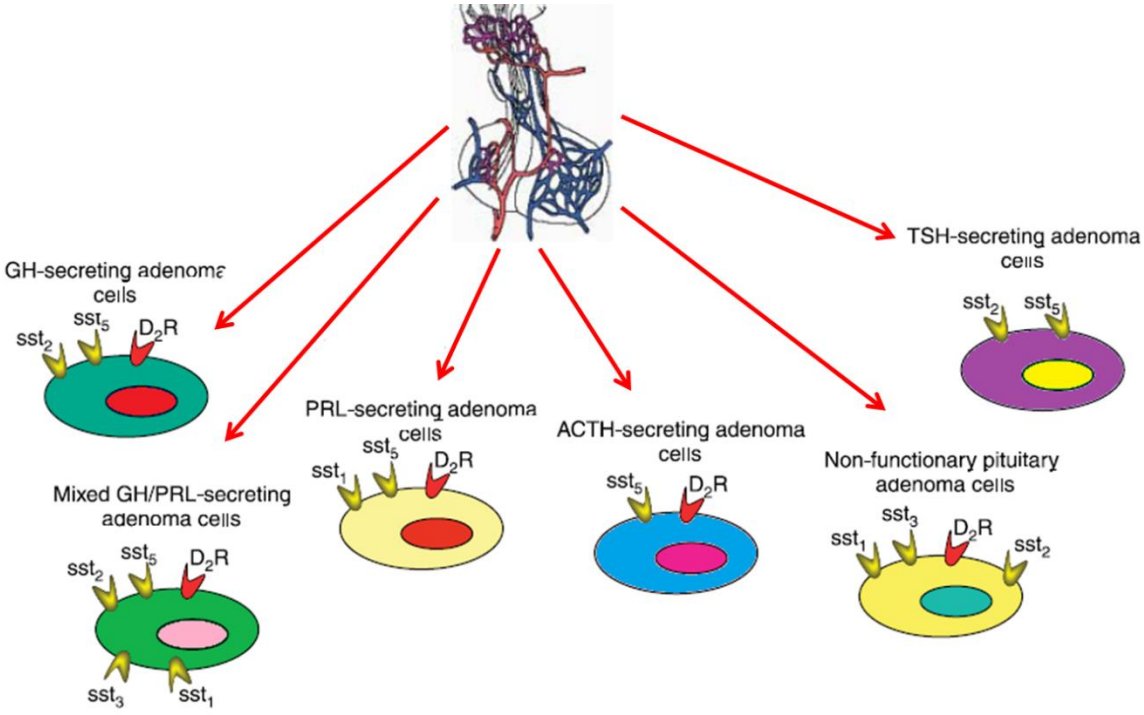



**Figure 1.15: The distribution of Somatostatin and D2 dopamine receptors in the different type of pituitary adenomas**. The D2 is the receptor mostly represented in the pituitary tumours and is preferentially associated with SST2 and 5 in somatotrophinomas, with SST1 and SST5 in prolactinomas, with SST2 and 3 in non-functioning adenomas and SST5 in corticotrophinomas. Adapted from [277].

### 1.3.5 Dopamine D2 receptor mediated signalling in the pituitary cells

Dopamine receptors are important for mediating the effects of dopamine and its agonists on the pituitary cells. Dopamine secreted from the hypophyseal hypothalamic neurons is a principal inhibitory regulator of prolactin (PRL) release by pituitary lactotropes [278]. Similarly, DA agonists inhibit PRL secretion from lactotropes in patients with prolactinomas. In addition DA agonists also cause tumour shrinkage in prolactinomas [248, 279, 280].

In addition to its role in the inhibition of PRL release from the Lactotropes, DA agonist cause shrinkage of pituitary tumours especially of prolactinomas [281]. In normal lactotropes and their corresponding tumours the molecular pathways through which dopamine and more particularly its agonists inhibit proliferation and/or lead to tumour shrinkage are currently under investigation [282, 283]. Studies of dopamine and dopamine agonist mediate effect on pituitary cell proliferation/death have made extensive use of rodent and murine tumour cell lines relative to cultured anterior pituitary cells from these species [284-287]. In these cells, dopamine and its agonist have been shown to induce apoptotic cell death [282-289].

Binding of the DA or its agonist to pituitary D2 receptors mediate the activation or inhibition of a series of effector molecules which determine the downstream signalling necessary for the physiological effects. The intricate details of D2 mediated signalling in normal and tumour cells and their differences especially with respect to antiproliferative and apoptotic functions are poorly understood. However, as discussed earlier the involvement of some of the intracellular messengers have been confirmed in the pituitary cells and these have been illustrated in figure 1.15. Binding of dopamine or its agonists to D2 receptor (in both normal and pituitary tumour cells) inhibits adenylyl cyclase enzyme and phosphatidylinositol metabolism, activates voltage-dependent potassium channels and decreases the activity of voltage-dependent calcium currents, modulates the activity of phospholipase C [277]. In addition to this, the mitogen-activated protein

kinase and extracellular signal-regulated kinase pathway are also involved in the D2 receptor mediated signalling (figure 1.16).

Different types of pituitary tumours show variable D2 expression, localized, in some cases, to both the cytoplasm and nuclei. The significance of nuclear localisation of D2 receptor remains unclear but both isoforms of D2 receptors (D2S and D2L) are thought to be relevant to the signalling pathways involved in the proliferation and cell death of pituitary tumour cells [281]. Decreased expression of D2 isoforms have also been known to lead dopamine agonist resistance in prolactinomas [290].

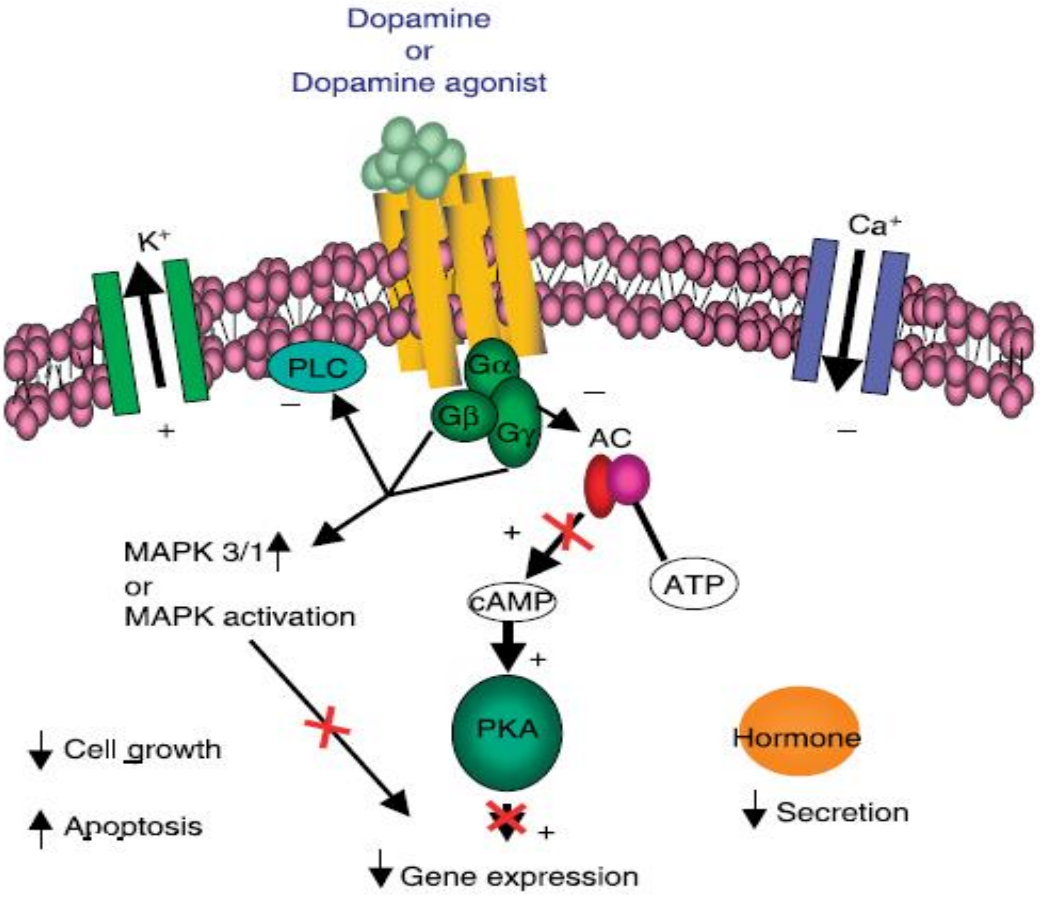



**Figure 1.16: Principal signal transduction associated with the activation of dopamine receptors in pituitary cells.** Dopamine binding to pituitary D2 receptors inhibits adenylyl cyclase, phosphatidylinositol metabolism, activated voltage activated potassium channels and decreases voltage activated L type and T type calcium currents. It modulates the activity of phospholipase C, activates the mitogen-activated protein kinase and extracellular signal regulated kinase pathway. Adapted and modified from [277]

### 1.3.6 Use of dopamine (DA) agonists in the treatment of pituitary tumours

Bromocriptine (see figure 1.17) is a derivative of the ergot alkaloid. The addition of the bromine atom renders this alkaloid a potent dopamine agonist and virtually all of its actions result from stimulation of dopamine receptor.

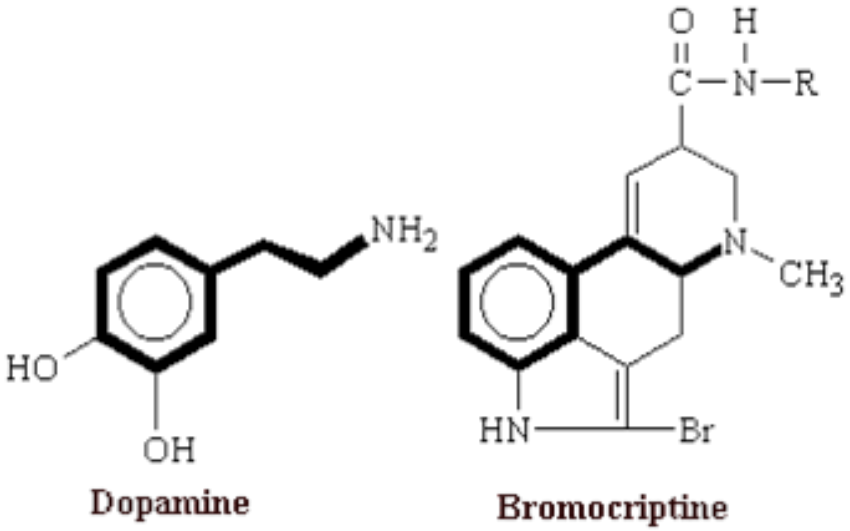



**Figure 1.17: Structure of dopamine and its agonist, Bromocriptine.**

### 1.3.6.1 Use in PRL secreting adenomas (Prolactinomas)

Bromocriptine (BC) was first introduced into clinical practise in 1979, as the first medical treatment for prolactinomas [291]. The success of BC in patients with microprolactinomas is 80-90% normalising serum PRL levels, restoration of gonadal function and also in most cases resulting in tumour shrinkage [292]. However success rate is reduced in patients with macroprolactinomas [260]. In these patients, headache and visual defects improves dramatically even within the first few days of drug treatment.

The second most commonly used drug to treat prolactinomas is the Carbogoline (CAB). This is a D2 receptor selective agonist. The treatment with CAB is associated with normalisation of PRL levels in more than 80% of patients [293]. In addition to its effects on PRL normalisation, CAB treatment has also known to result in tumour shrinkage [248] and improve visual field effects. The only other clinically relevant DA agonist that has been used clinically to treat prolactinomas is Quinagolide. Like BC and Cab, Quinagolide therapy also results in normalisation of PRL levels and tumour shrinkage [280, 294].

### 1.3.6.2 Use in GH secreting adenoma (somatotrophinomas)

The administration of DA agonist inhibits GH secretion in normal subjects [295, 296]. Treatment with DA agonist has been shown to be effective in those GH-tumours which co-secrete PRL [2]. In some studies treatment of patients with BC has resulted in 70% of symptomatic improvement but the effect on tumour shrinkage has only been observed in 10-15% of cases [296]. Very few studies report the effectiveness of Cab in GH secreting adenomas [297-299].

### 1.3.6.3 Use in ACTH-secreting, TSH secreting & non-functional adenoma

The medical treatment of ACTH secreting adenoma (corticotrophinoma) is reserved for patients with failed surgery [300]. The drugs employed in their treatment are adrenal blocking drugs (such as metyrapone, ketoconazole). Even though these are effective in lowering cortisol, they lack effect on tumour size [300]. The use of DA agonists in ACTH secreting adenoma is still experimental and perhaps warrants a more detailed investigation and appraisal in these patients.

Treatment of TSH secreting adenoma does not employ DA agonist as these are ineffective in blocking TSH secretion. They induce tumour shrinkage only in those cases where there was a combined excess of TH and PRL [301]. Similarly, dopamine agonists are not employed in the treatment of non-functional adenoma. The reason for this is thought to be due to the low levels and affinity of D2 receptors expressed on the surface of NFAs [274]. Tumour shrinkage in these types of adenomas have also been associated with expression of short forms of D2 receptor [274]. However, CAB and Quinagolide have been used in treatment of NFAs with varying results [302-304].

### 1.3.7 Signalling pathways induced by Somatostatin or its analogue

As already discussed, the effect of Somatostatin and its analogues (SSA) are mediated through the activation of PTX sensitive Gi/o couple SST (SST1-5) receptors in the pituitary cells [305]. Interaction of Somatostatin with its receptors inhibit secretion of a wide range of hormones such as GH, PRL, TSH and ACTH from the normal pituitaries and also the pituitary tumour cells [253, 262, 306, 307] [308-311]. Activation of SST2 and SST5 play a major role in the control of GH secretion [262, 263], activation of SST1,2,4 and 5 induce cell cycle arrest [264] and activation of 2 and 3 induces apoptosis [264]. In contrast, the expression of SST4 is infrequent in the anterior pituitary.

The exact mechanisms and intracellular pathways of SST subtype mediated signalling in normal and tumour cells and their differences especially with respect to antisecretory, antiproliferative and apoptotic functions are not entirely clear, however the role of some of the intracellular messengers have been confirmed in the pituitary cells. Therefore when SST receptors are bound by ligand or analogues (SRIF or SSA), they initiate a complex set of signalling events in normal and tumour cells which include modulation of several key enzymes (figure 1.18).

The response of cells to SSA treatment is frequently dependent upon the receptor subtype specificity of the ligand. Thus, some bind to multiple different receptor subtype whilst some target specific receptor subtypes. In addition, despite Somatostatin analogues being available with high affinities for specific receptors [252, 268, 304, 312-315] their usefulness is entirely dependent on cells expressing the particular receptor subtypes. The loss or reduced expression of SST receptor is frequently the limiting factor in a proportion of pituitary adenoma [316, 317]. In addition presence of other factors, as example, SSTs internalisation, desensitisation and/or receptor crosstalk, subcellular expression pattern of SST subtypes, presence other intracellular complement proteins (e.g β-arrestin involved in intracellular vesicle trafficking) will impact on cellular response [277, 310, 318].

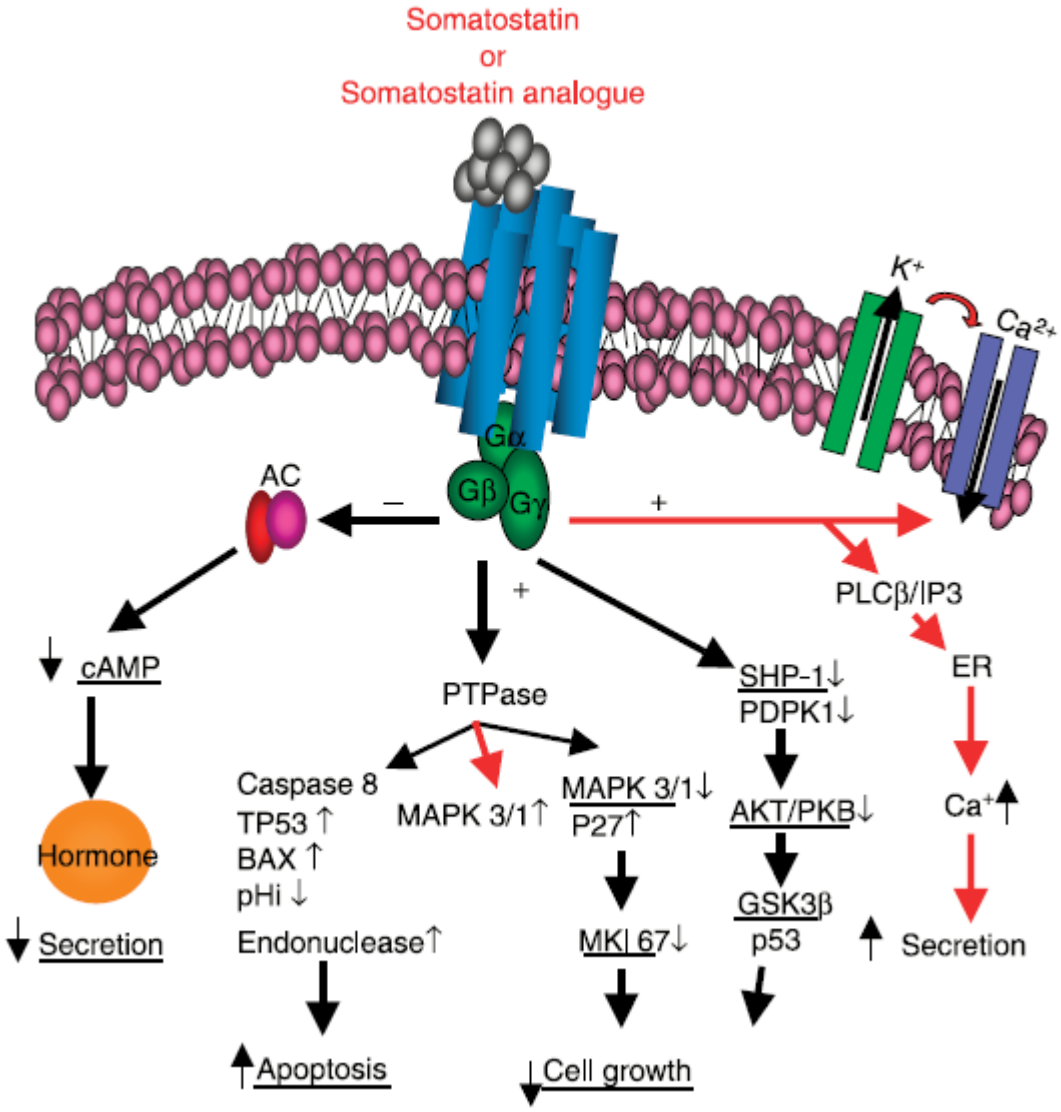



**Figure 1.18: Principal intracellular signalling cascades associated to Somatostatin receptors in pituitary cells.** Somatostatin analogue binding to the Somatostatin receptors inhibits adenylyl cyclase, activates potassium channels and/or inhibits calcium channels. Phosphotyrosine phosphatases and mitogen-activated protein kinase are modulated as well and along the stimulation of phosphotyrosine phosphatases and mitogen activated protein kinase are modulated as well and along the stimulation of phosphotyrosine phosphatase may also produce a cytostatic action. Adapted and modified from [266].

### 1.3.8 Somatostatin analogues (SSA) in the treatment of pituitary tumours

#### 1.3.8.1 Use in GH secreting adenoma

SSA therapy for GH secreting adenomas yielded promising results. SSAs are successful in reducing serum GH and IGF-1 level in 90 % and 40-60% of patients respectively [253]. Tumour shrinkage, as an outcome of SSA therapy was seen in 45% of patients [319]. In patients without prior surgical and/or radiological intervention, SSA therapy was shown to induce tumour shrinkage in 51% of cases [319]. However, in patients where surgical and/or radiological intervention had been unsuccessful tumour shrinkage was apparent in significantly fewer cases at 29% of patients [319]. There are case reports that demonstrate that patients receiving SSA therapy can show tumour shrinkage without any effect on the GH and IGF-I levels [320, 321]. The reason for this was the differential expression of SSTs on tumour membranes in which SST2 being poorly expressed while that of SST3 and SST5 are highly expressed [320, 321]. Another disadvantage with SSA therapy is that tumour shrinkage was found to be reversible once the treatment was discontinued [322]. GH secreting adenoma co express D2 and SST2a frequently [268, 270]. Therefore, a combined treatment strategy employing DA agonists and SSA could be an answer to avoid/prolong the development of resistance in these tumour types [323].

#### 1.3.8.2 Use in ACTH secreting and clinically non-functioning adenoma

The drugs of choice for ACTH secreting adenomas are adrenal blocking drugs. However, combined treatment with CAB and SR (slow release LAN) has been employed in the treatment of ectopic Cushing's syndrome and this combination showed more benefit than CAB alone [300]. Therefore, combined treatment strategy employing both DA agonists and SSA has been proposed but this requires more clinical studies.

Even though, SSTs are expressed by clinically non-functioning pituitary adenoma [272, 273, 324], SSA mediated tumour shrinkage occurs only in 10-13% of cases [325]. However a combined treatment with OCT and CAB was associated with tumour shrinkage in approximately 30% patients [326].

#### 1.3.8.3 Use in TSH secreting adenoma

Somatostatin inhibits the secretion of TSH in physiological conditions and in TSH secreting adenomas. In addition, TSH secreting adenomas express Somatostatin receptors. Therefore, SSAs are considered the only choice for the medical treatment of these adenomas as they are effective in restoring hormone levels and produce tumour shrinkage [306, 327, 328]. OCT has also been employed preoperatively to reduce tumour size for easy removal [329].

### 1.3.9 SSAs with affinities for multiple SSTs in the treatment of pituitary tumours

Following the discovery of the SST, the initial pharmacological strategies were to construct ligands with a high affinity for each receptor subtype but more recently the approaches have been directed towards new compounds that are capable of interacting with more than one SST subtype. These include analogues such as BIM-23244, SOM230 (pasireotide). A bi-specific analogue BIM232144 can activate both SST2 and SST5 receptor and therefore achieves a better control of GH hyper secretion in GH secreting and mixed GH/PRL secreting adenoma [263, 313]. Corticotroph adenomas which express higher levels of SST5 relative to SST2 are more sensitive to universal SST ligand, pasireotide [312]. It has been demonstrated recently that pasireotide can modulate SST trafficking [330] distinct from other SSA analogues and thus providing an alternate

explanation for the differential regulation of SST responsiveness during long term administration of SSA analogues.

### 1.3.10 DA agonists and SSA chimeric molecules Dopastatin in the treatment of pituitary tumours

As illustrated in figure 1.15 the majority of pituitary adenoma co-express SST and D2 receptors on their surface. Depending upon the phenotype of the tumours, the expression of these receptors is variable. Based on this information a new chemical approach has been adapted which consisted of synthesising chimeric molecules containing structural elements of both Somatostatin and DA agonist and direct against both the superfamilies of GPCRs). This was attempted with the view that such molecules will enable co-engagement and activation of both receptors and therefore would increase clinical efficacy [314]. The first molecule of this class were BIM-23A387 (SST2 and D2) and BIM-23A760 (SST2, SST5 and D2 affinity). These molecules were effective in controlling hormone hypersecretion in human GH-secreting adenomas in vitro [314, 331] which were partially responsive to long term treatments with octreotide or lanreotide. In addition, these chimeric compounds were also effective in GH/PRL secreting adenomas in vitro [314]. The use of dopamine Somatostatin chimeric molecules in other types of pituitary tumours is still unknown and requires further studies.

### 1.3.11 Developments in the medical treatment of ACTH secreting adenomas.

The first line treatment for Cushing's syndrome is surgery however additional treatments are necessary if surgery is not successful. This is performed through various medical therapies that have been recently reviewed [332, 333]. Adrenal directed therapy (steroidogenesis inhibitors) may be highly effective but does not treat the underlying tumour or restore normal pituitary secretory dynamics [332]. The most experience with steroidogenesis inhibitors has been acquired with metyrapone and ketoconazole which appear to be more effective and better tolerated than aminoglutethimide [334, 335]. Metyrapone treatment leads to inhibition of aldosterone biosynthesis and accumulation of aldosterone precursors with mineralocorticoid activity. Electrolyte balance and blood pressure levels vary individually with the degree of aldosterone inhibition and 11 deoxycorticosterone stimulation levels however these have many side effects [336].

Pituitary directed therapy targets the underlying cause of the disease and there are several investigational agents under evaluation [16, 335, 337, 338], subsequent studies do not support a routine clinical role for the use of peroxisome proliferator activated receptor –γ (PPAR-γ) agoinsts such as rosiglitazone and pioglitazone [339, 340].

### 1.3.12 Bone Morphogenic Protein (BMP) a transforming Growth Factor-β (TGFβ) in pituitary signalling.

Bone morphogenetic protein (BMP)-4 is a key mediator of anterior pituitary organogenesis. The bone Morphogenic proteins are members of the TGF-β superfamily of multifunctional secretory peptides. To date more than 20 TGF family members have been described and these can be further subdivided into several groups on the basis of their structure and function. Two of the best studied members of this family, BMP-2 and BMP-4, share significant homology and have been shown to play a significant autocrine and paracrine roles in anterior pituitary organogenesis. BMP-4 is thought to have differential role depending on the pituitary cell subtype. It is thought to have growth promoting and inhibiting roles and has been recently reviewed [341].

#### 1.3.12.1 BMP action on Lactosomatotrope cells

The discovery of BMP-4 action on differentiated pituitary lactotroph cells was first reported by Paez-Pereda and group [31]. The BMP-4 molecule was found to be overexpressed in lactotropes adenomas derived from dopamine d2 receptor null mice and also estrogen induced female rat tissue. Increased BMP-4 expression was also detected in prolactinomas compared to its expression levels with other functioning and non-functioning tumour tissue [31]. Biologically BMP-4 only promoted lactotrope cell proliferation but also prolactin production in conjunction with Smad-estrogen receptor interaction[342].

BMP-4 inhibits the transcriptional activity of estrogen receptor at low doses of estradiol, however estrogen stimulates transcriptional activity of BMP-4 specific Smad signalling. The reciprocal regulation via BMP4-Smad/ER interaction promotes specific control of PRL synthesis in lactotrope cells [342]. However suppression of endogenous BMP receptor activity in vivo by the BMP binding protein noggin leads to arrest of the development of Pit-1 lineage pituitary including lactotropes

[343]. It was also shown by the same group that noggin expression is conversely down regulated in prolactinomas in D2R null mice, endogenously expressed BMP-4 promotes growth and PRL productivity by lactotropes through Smad-ER interaction [31].

Furthermore it has recently been found a functional interrelationship between BMP system and Somatostatin receptor expression in relation to PRL secretion [344]. Endogenous BMP actions are involved in the enhancement of PRL production, since BMP-4 and 6 directly increased PRL and also cAMP levels. Secondly, BMPs modulate SSTR sensitivity of GH3 cells in an autocrine/paracrine manner. Importantly, BMP-4 and 6 reduced SSTR2 expression but increased SSTR5 expression [344]. The effect of the SSTR5 preferring agonist Pasireotide which reduced PRL secretion induced by forskolin was facilitated by the presence of BMP-4 and in turn blocked by noggin treatment. These findings indicate that endogenous BMP activity up regulates SSTR5 but down regulates SSTR2 in lactotropes. Thus BMP-4 acts to increase PRL release and furthermore the BMP system plays a regulatory role in SSTR sensitivity of lactotrope tumour cells as demonstrated in figure 1.19 [344].

#### 1.3.12.2 BMP action in Corticotrope cells

Bioactivity of BMP action was also discovered in corticotropes as a negative regulator for the expression of an adrenocorticotropin precursor, proopiomelanocortin (POMC) by Nudi and group [345]. BMP-4 signalling suppresses endogenous POMC expression as well as POMC promoter activity in mouse Corticotrope AtT-20 cells. The transcription factor Pitx1 and Tpit are critical for differentiation of Corticotrope cells. BMP-4 stimulation activates phosphorylation of Smad 1 that is recruited to the POMC promoter, in which Smad1 acts through interactions with the transcription factor Pitx and or Tpit and functionally disrupts transcriptional activity of POMC [32] further demonstrating that BMP-4 inhibits ACTH secretion and cell proliferation in vitro using

Corticotroph cell line AtT-20 [32]. In addition AtT20 cells that were stably transfected with a dominant negative Smad4 and that treated with noggin have increased tumorigenicity in nude mice, showing enhancement of BMP-4 action can inhibit corticotrope tumour growth in vivo [32].

BMP-4 ligands including BMP2, 4, 6 and 7 particularly BMP-4 decreased basal ACTH production in corticotrope cells as demonstrated in figure 1.19 [344]. BMP-4 inhibited Corticotropin releasing hormone (CRH) induced ACTH production and POMC transcription by suppressing CRH induced MAPK activity in these cells [341]. The actions of the CRH peptides are mediated through splicing variant forms including CRH receptor (CRHR) type 1 and type 2. CRHR activation results in GTP/GDP exchange on the G protein subunit leading to the activation of a series of signalling pathways. CRH activates numerous pathways and amongst these cascades BMP-4 suppresses CRH induced phosphorylation of ERK and p38 pathways [344]. This indicates that ERK and p38 activation is likely to occur upstream of cAMP synthesis and that cAMP-PKA and ERK pathways are functionally connected. In addition the cAMP-PKA pathways also contributes to Smad 1/5/8 signalling suggesting that endogenous BMP may act as an auto regulatory machinery to control ACTH overproduction [344].

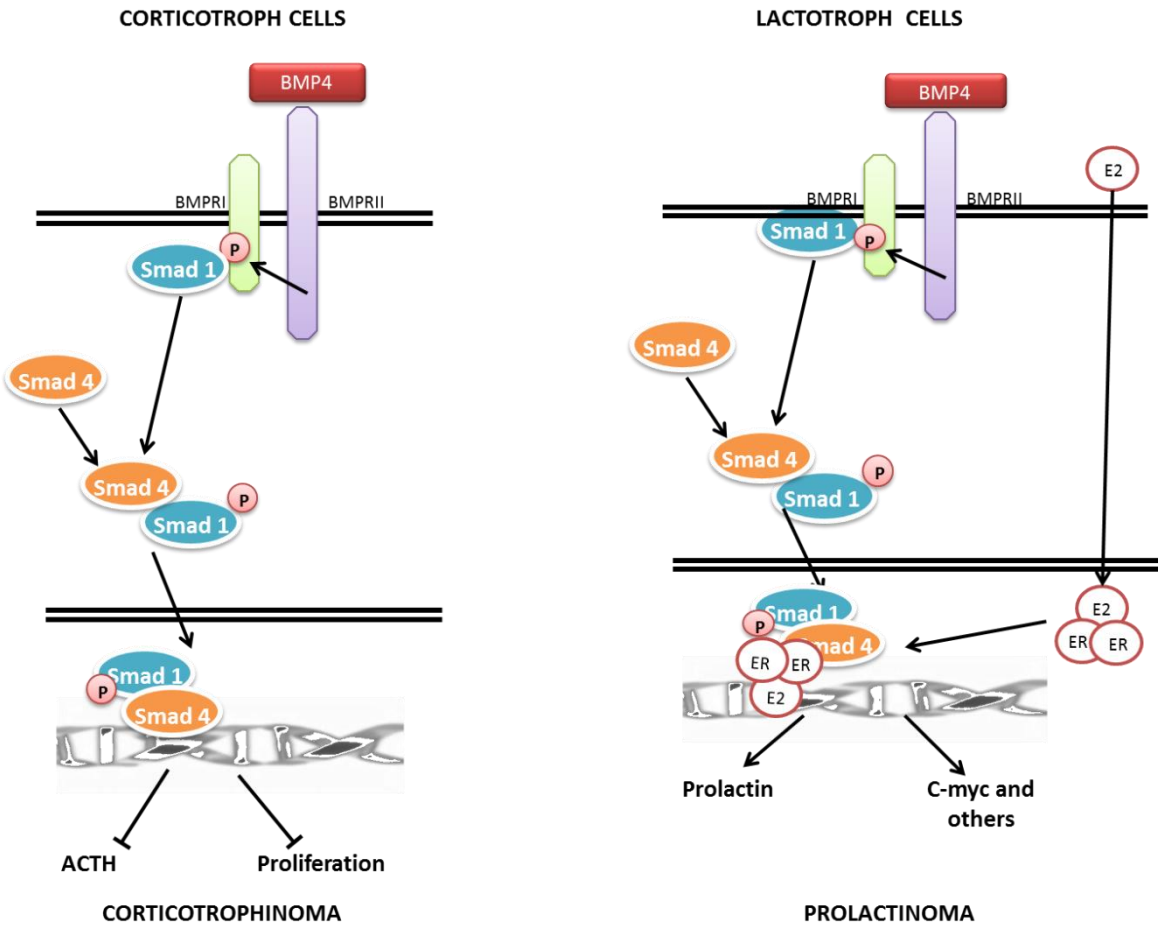



**Figure 1.19: Action of BMP-4 as a bifunctional protein**

Action of BMP4 as a bifunctional protein in prolactinomas and Corticotrophinomas. Differential action of BMP-4 on lactotroph and corticotroph cells. BMP-4 induces prolactin secretion and cell proliferation in lactotroph cells meanwhile inhibits corticotroph cell proliferation and adrenocorticotrophic hormone secretion. Adapted from [346].

### 1.3.13 Retinoic acid in the inhibition of ACTH secretion

The potential of the retinoids and in particular retinoic acid (RA) as a treatment option for pituitary adenomas have been explored by several groups [341, 347-349]. In corticotroph adenoma cell line, At-T20, Ra inhibits ACTH biosynthesis and POMC transcription and mediates the observed decrease in cell proliferation and cell viability [347]. However predating these studies, similar endpoints have been reported in an in vivo nude-mouse model of Cushings syndrome and most likely focused attention on the potential of the retinoids as effectors in pathways leading to inhibition of hormone secretion and tumour growth [31]. Indeed, a subsequent and seminal finding from this group described a significant role for BMP-4 in the genesis of pituitary adenomas [31]. However retinoic acid is in its early days of testing and requires more work before it reaches clinical trials.

# Chapter 2: Aims & Objectives

## 2. AIMS OF THIS STUDY

With limited but important exceptions genetic aberration responsible for the outgrowth or those that characterise human pituitary adenomas are infrequent findings. However, at the outset of my research it was already apparent that epigenetic changes, apparent, at that time, as inappropriate methylation of CpG islands, were a frequent finding. Equally, in a clinical management context, it was also apparent that response to medical intervention strategies was effective in a limited range of adenoma subtypes and even in these cases resistance to therapeutic intervention was a frequent finding.

Therefore, in this thesis the principal questions I wished to address related to mechanisms responsible for resistance to medical intervention. In this case my hypothesis was that sensitivity or resistance would be contingent on the expression status of specific receptors. If this were the case, then I proposed that resistance to conventional therapeutic interventions reflected changes to receptor-associated epigenetic landscapes.

To test this hypothesis it was necessary to use characterised pituitary tumour cells and examine change in these cells and also in primary human pituitary tumours of different subtypes. It was also an aim of this work to reverse epigenetic changes, with the so named epidrugs, and in some experiments determine the consequences for apoptotic responses.

To address these aims receptor pathways associated with dopaminergic regulation (D2 receptor [D2R]) and the cytokine (bone morphogenic protein 4 [BMP-4]) pathway were investigated in pituitary cell lines and primary tumours. The epigenetic aberrations examined included CpG island methylation and chromatin remodelling, in this case manifest as histone tail modifications.

These studies and the data emerging from them prompted me to determine the effects of retinoic acid on the BMP-4 pathway in the context of growth promotion and growth inhibition in a pituitary cell type specific context.

# Chapter 3: Materials & Methods

# Cell Culture Methods

## 3.1 Pituitary Cell Lines:

### AtT-20 Cells

The AtT-20/D16v-F2 (AtT-20) murine pituitary adenoma cell line in the corticotroph lineage was purchased from the European collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK). Cells were passage 13 upon purchase but were designated passage 0 for the purpose of these studies. Cells were not passaged more than 10 times for the studies described in this thesis.

### GH3 Cells

The rodent pituitary adenoma cell line (GH3, ATCC code: CCL-82.1) in the somato-lactotroph cell lineage were purchased from the American Type Culture Collection (Manassas, Virginia). Cells were passage 1 upon arrival and were designated passage 0 for the purpose of these studies. Cells were not passaged more than10 times for the studies described in this thesis.

### MMQ Cells

MMQ cells, a rat pituitary cell line in the lactotroph cell lineage (CRL-10609) were also purchased from the American Type Culture Collection (Manassas, Virginia). Cells were passage 1 upon arrival and were designated passage 0 for the purpose of these studies Cells were not passaged more than 5 times for the studies described in this thesis.

### 3.2 Growth Conditions

The GH3 and AtT-20 cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) with 4500mg/L of glucose, 0.584g/L of L-Glutamine and 0.11g/L of sodium pyruvate (Biosera, Ringmer, UK), and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biosera). The MMQ cell line was grown in Hams-F12 media (Biosera) supplemented with 2.5% FBS and 10% horse serum (Biosera). Cells were incubated in a Thermo Scientific HEPA filtered single chamber water jacketed incubator, at 37$^{o}$C in a humidified 5% $CO_2$ atmosphere.

### 3.3 Antibiotic Supplementation

To minimise the risk of bacterial infection the cell culture medium was supplemented with 4µg/mL of aminoglycoside antibiotic Gentamycin (Sigma-Aldrich, Dorset, UK) and 2µg/mL of antibiotic ampicillin (Sigma).

### 3.4 Detachment of adherent cells

Prior to experimental manipulation or routine passage of cell lines it was first necessary to detach and dissociate adherent cells from each other and from the base of culture flask. This was achieved using a solution of 1.7mM EDTA. Cell dissociation was facilitated by mechanical pipetting to further aid the release of the cells into solution before use. Cells were incubated for 5 minutes at room temperature and then following vigorous pipetting. Cells were neutralised by addition of 1 volume of DMEM containing 10% FBS. Resuspended cells were transferred to a sterile 15mL polypropylene tube (Sarstedt, Leicestershire, UK) and centrifuged at 150 x g for 5 minutes at room temperature in MSE Mistral 2000 centrifuge (UK). The cell pellet was re-suspended in fresh medium by careful pipette mixing.

### 3.5 Culture Vessels

The type of vessels in which the cells were cultured depended on the experimental procedure being undertaken. For general sub-culturing purposes T25 filtered tissue culture flasks (Sarstedt, Leicester, UK) were used, whereas T75 filtered tissue culture flasks were used for the purpose of growing cells to sufficient density for cryogenic storage. A variety of tissue culture plates (96-well, 24-well, 12-well, 6-well; Sarstedt) were used for transfection and drug treatment studies.

### 3.6 Sub-culture

Sub-confluent (70-80%) cells were routinely sub-cultured in sterile conditions every three to four days. Cultured medium was removed and cells were washed with PBS, and then PBS-EDTA was added to cells and further diluted with media post detachment as described above. Diluted cells were then transferred to a 15mL tube and centrifuged. Cells were then resuspended prior to counting with a haemocytometer. Cells were then diluted to 1 $X10^5$ per mL and transferred to the appropriate culture vessel and incubated at 37°C. For routine culture cells were not grown beyond 10 passages and at this point, earlier passage and cryopreserved cells were revived from liquid nitrogen storage.

### 3.7 Cryopreservation of cells

For long term storage cells were cooled to sub-zero temperatures in a solution containing the cryopreservant dimethyl sulphoxide (DMSO). Cells were first grown to approximately 75% confluence in a T75 tissue culture flask and were then released from the adherent surface as described above and transferred to a 15mL polypropylene tube, and centrifuged at 150 x g for 5 minutes. Cell pellets were re-suspended in 900µL of FBS (90%) and 10% DMSO (Sigma), and

transferred to a 2mL cryovial (BD labs). Vials were placed into a “Mr Frosty” freezing container (Nalgene, Neerijse, Belgium) filled to the appropriate level with isopropanol (Sigma) and frozen overnight at -80°C. Frozen vials were then transferred to liquid nitrogen.

### 3.8 Reviving cryopreserved cells

Cells were revived from liquid nitrogen storage by rapid thawing to 37°C. Thawed cell were transferred to a 50mL polypropylene tube and 20mL of DMEM without FBS supplemented was added in a drop wise motion with constant swirling. Cells were then centrifuged 150 x g for 7 minutes and re-suspended in 6mL of DMEM containing 10% FBS. Cells were then transferred to a T25 tissue culture flask and incubated overnight at 37°C. The following day medium was replaced in order to remove dead cells, whilst healthy cells were grown to sufficient density for sub-culturing purposes.

### 3.9 Cell Counting

Disposable haemocytometer chamber (Glasstic® Slide 10 with grids, Hycor Biomedical Inc, California, USA) were used to count cells. Cells were first stained with trypan blue to distinguish dead from live cells and suitable dilutions, such that between 20-50 cells, in each of the squares were enumerated to determine live-cell counts. The average number of cells per millilitre (cell/mL) was determined using the equation below.

Total number of cells per mL = average count per $mm^2$ X $10^4$ X Dilution factor.

## 3.10 Transfections

### Lipofection

Lipofection is a technique used to introduce genetic material into cells via liposome mediated endocytosis. Liposomes are artificially prepared vesicles that are composed of a phospholipid bilayer, a property that allows them to easily fuse with the mammalian cell membrane. This technique is efficient and is capable of transfecting most types of nucleic acids into a wide range of cell types. This technique is simple and reproducible generally showing minimal toxicity. For the studies described in this thesis this technique was used exclusively for the introduction of small interfering RNA (siRNA).

In these cases, Lipofectamine 2000 (Invitrogen (Life Technologies) was used to introduce siRNA into the cell lines. Details of the specific siRNAs used in this study and their targets in rodent cells are provided in the Appendix I.

The day before transfection cells were seeded at a density of 2 $X10^5$ cells per well in a 6 well plate such that the cells would be at approximately 50% confluence at the time of transfection. Lipofectamine 2000 was mixed prior to use and diluted 1:50 in serum free DMEM media (5µL in 250 µL total volume). In a separate tube, 20nM of the siRNA was combined with serum free DMEM (total volume of 250 µl), and both sets of tubes were incubated at room temperature for 20 minutes. After incubation diluted Lipofectamine 2000 and the siRNA mixtures were combined and gently mixed by pipetting. The tubes were then incubated for 20 minutes at room temperature. Meanwhile, culture medium was aspirated from the 6 well plates and wells were washed with serum free DMEM And then replaced with addition of 1.5mL DMEM, and Lipofectamine 2000:nucleic acid complexes. The mixture was added in a drop wise manner to the appropriate well using a 1mL pipette. Plates were gently rocked from side to side and then incubated for 6 hours in a $CO_2$ incubator. After the incubation a further 2mL of DMEM containing

20% FBS was added to each well such that the final concentration of the FBS in the medium was 10% (in a 4mL total volume). For all transfection procedures a “sham” transfection was included where no nucleic acid was added to the Lipofectamine complex. This control was used to determine and account for the effects of Lipofectamine 2000 alone on cell viability. In addition, a non-targeting siRNA control was also included along with the sham and siRNA treated reactions. The non-targeting control siRNA was used to establish the non-specific effects of siRNA *per se* on gene expression.

### 3.11 Incubation of cell lines with pharmacological agents

The most commonly used drugs in this study were 1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one also known as Zebularine and Trichostatin A (Sigma, UK). Zebularine is a cytidine deaminase inhibitor and a DNA demethylating agent. TSA (Trichostatin A) is a histone deaceytlase inhibitor [350-352].

Zebularine was prepared in DMSO and TSA was prepared in ethanol. Both drugs were stored at -20°C.Typically, drugs were stored as stock solutions: Zebularine (1 µM) and TSA (10ng/mL) and diluted prior to use.

24hours prior to drug treatment, 2.5mL of cells, at a concentration of 1 x $10^5$/mL were seeded into 6 well plates. Cells were then treated with the appropriate concentration of drug(s) (see figures and figure legends) every 24 hours for 48 hours. Post Zebularine and/or TSA challenges cells were harvested prior to extracting protein, nucleic acids and chromatin.

In some experiments, bromocriptine (BC) [2-Bromo-α-ergocryptine methanesulfonate salt] and dopamine (DA) [3-Hydroxytyramine] were used. These were obtained from Sigma. Stock solutions were prepared in DMSO and PBS respectively and stored according to manufacturer's instructions. The DA D2R antagonist Eticlopride and Haloperidol were obtained from Calbiochem (Nottingham, UK). The drugs were dissolved in PBS and DMSO respectively. The dose of the antagonists was first optimised relative to the doses of agonists used in specific experiments. In these experiments, antagonists were used at a dose of 25µM since higher doses were found to be cytotoxic. The antagonist used within the study was used to determine the specificity of D2R mediated response in GH3 and MMQ cells.

In some experiments retinoic acid was used to treat cells in culture (Sigma, UK). Two forms of retinoic acid were used in this study 9-cis retinoic acid and all-trans retinoic acid. Both forms of retinoic acid were prepared as stock solutions (1µM) in DMSO and stored at -80°C away from the

light. In the described studies, GH3 cells and MMQ cells were treated with the 9-cis retinoic acid and AtT-20 cells were treated with all-trans retinoic acid.

For the experiments described in this thesis, where cells were challenged with retinoic acid and the pharmacological agents described above (Zebularine and/or TSA) the following protocol was adopted: Cells in culture were first incubated with Zebularine and/or TSA for 48 hours prior to challenge with varying concentration of retinoic acid (see figures and their legends) for a further 24 hours. In cases where cells were treated with retinoic acid alone, cells were first incubated with vehicle (solvent uses to dissolve the drugs) for 48 hours prior to addition of retinoic acid

### 3.12 Colony forming efficiency assay

In some experiments colony formation efficiency assay were used to determine the effectiveness of specific agents on the survival and proliferation of cells. This assay allows enumeration of cells able to form colonies. In this way the assay shows if a specific treatment promotes or inhibits growth, as determined by the number of colonies formed. Colony formation assay are also used to show the tumourigenic properties of cells through their ability to grow in the absence of substrate adherence.

For these experiments cells were first treated with various drug or pharmacological agents relative to vehicle controls. The colony forming efficiency (CFE) assays were carried out in 60mm petri dishes (Sarstedt, Leicester, UK). For each of the petri dishes (post challenge) equal numbers of a single cell suspension ($1.5 \times 10^4$) in complete media was prepared. Molten low melting point soft agar was allowed to cool to $40^{O}$ C and added to the media containing cells that had been pre-warmed to $37^{0}$C. In these cases, the final concentration of soft agar was 0.3%. The dishes had a gridded base (Sarstedt) to facilitate enumeration of colonies. After allowing the soft agar to cool and set agar was overlayed with 5mL of media. In these cases, the concentration of FBS and

horse serum was increased to 20 and 30% respectively and media replaced every three days. Typically cells were cultured for 12 days at 37°C. The number of cells that had formed identifiable colonies after 12 days incubation was counted using a Nikon microscope. A colony was determined as comprising 100 cells or more.

### 3.13 Caspase activation assay

Activated caspases were detected using Caspase-Glo$^{TM}$ assay kits (Promega UK) that specifically detects the terminal caspases (Caspase 3 and 7). Caspase-Glo$^{TM}$ assay is a luminescent assay that utilises the proluminescent caspase substrate and the thermostable luciferase in a single reagent optimised for the caspase activity, luciferase activity and cell lysis. Addition of Caspase-Glo$^{TM}$. Addition of Caspase-Glo reagent to the cells results in cell lysis, followed by caspase cleavage of the substrate to generate amionoluciferin. The liberated aminoluciferin is consumed by the luciferase enzyme generating a “glow-type” luminescent signal. The signal proportional to the specific caspase acticity. This assay system enables detection of caspase activity in a multiwall plate format using cells (treated and untreated) in culture.

Cells were seeded into a 96-well plate at a density of 1X10$^{5}$cells/mL and incubated overnight in growth media. For various duration of time, semi-confluent cultures were challenged with drugs in the absence or presence of specific inhibitors. Duplicate sets of cell treatments were carried out such that one set was utilised for caspase activity while the second was used for cell number correction.

The assay reagents which includes Caspase-Glo substrate, Caspase-Glo buffer and MG-132 (proteasome inhibitor), were combined to make a ready to use assay reagent. 50µl of cuklture media was aspirated from each well to be analysed for caspase activity (originally each well contained 100µl culture media). The ready to use reagent was then added to each well of the 96 well plate containing the treated or the untreated cells in a 1:1 ratio (50µl of culture media:50µl of ready to use Caspase-Glo Reagent). The plates were rocked sideways to mix the content of the wells covered with aluminium foil and incubated at room temperature for 45 minutes. Assay was performed for each type of sample in triplicate wells. At the end of the incubation, 75µl of the content of the wells were transferred to a clean 96 well late and the luminescence was recorded using a plate reader. Data were analysed after correcting for cell numbers.

### 3.14 Primary Tissue material

#### Normal Pituitary Tissue

Pathological normal mouse pituitaries (CD-1) and rat pituitaries (Sprague Dawley) were obtained from a commercial source, Charles River Research laboratories (Kent, UK).

As control normal human pituitaries were also used. These were post-mortem normal pituitaries acquired within 12 hours of death with no evidence of endocrine disease. The normal pituitaries were pulverised under liquid nitrogen using a Biopulveriser stainless steel mortar and pestle device (Biospec, California, USA). The resulting granular admix was then stored as 30-50mg aliquots in sterile tubes at -80°C until required for downstream analysis.

#### Pituitary Tumours

Primary sporadic pituitary tumours were also investigated within this study. They comprised each of the major subtypes and were graded according to a modified Hardy classification [353] The subtypes were as follows: growth hormone (GH)-secreting adenomas, all of which were grade 2 macroadenomas, corticotroph adenomas that were either grade 1 microadenomas or grade 2 macroadenomas, prolactinomas (PRL) that were grades 1, 2 and 3 and non-functioning adenomas (NF) that were grade 2 and grade 3 macroadenomas. Details of each of the tumours used in this study are provided in Appendix II. Tumours were collected from patients during hypophysectomy. Adenoma subtype classification was on the basis of staining for mature hormone (GH, ACTH, FSH, LH and PRL but not for the α-subunit). The non-functional adenomas did not stain for mature hormones. All GH secreting adenomas were classified as pure somatotrophinomas because they did not stain for any mature hormones other than GH. None of

the patients harbouring GH secreting adenomas had received somatostatin analogues or dopamine receptor agonists before surgery and all had elevated GH (3-35mg/mL) and failed to suppress to less than 1ng/mL oral glucose. The prolactinomas were derived from patients where all but one was dopamine agonist intolerant where in this case it was pituitary apoplexy. Prolactin levels were elevated in all cases and ranged from 107-1666 ng/mL. All subtypes had elevated IGF-1 when adjusted for sex and age.

Only those adenomas, in which tumour cells comprised at least 80% of the specimen, as determined at surgery and confirmed by neuropathological assessment, were used in the study. All tumours were freeze fractured using a biopulveriser as described for the normal pituitaries. Tumour tissues were obtained with informed consent, and all studies were performed with institutional ethical approval (Reference number: 10/H1207/46) as shown in Appendix II.

## Molecular Biology Methods

### Cell and Tissue Extractions

### 3.15 Isolation of genomic DNA from cell lines and primary pituitary tissue

The extraction and purification of genomic DNA from cell lines and primary tissue (normal pituitaries and primary pituitary tumours) was performed using a standard lysis and phenol/chloroform procedure. The extractions used the Nucleon DNA extraction kit (GE Healthcare, Buckinghamshire, UK). The protocol for the lysis of samples was dependant on whether samples were derived from cell lines or tissue, but otherwise a common extraction and purification procedure was employed.

**Lysis of cell line samples:** Monolayers of cells (1-3 x $10^5$ cells) were washed once with sterile PBS and harvested into a PBS-EDTA solution. Cells were collected by centrifugation at 150 x g for 5 minutes and the pellets were resuspended in lysis buffer (see Appendix III). Cell samples were incubated overnight at 37°C.

**Lysis of tissue specimens:** 3mL of lysis buffer was added to 10-30mg of tissue and incubated overnight at 56°C.

**Extraction and precipitation of DNA:** After overnight incubations samples were vortexed vigorously and an equal volume of phenol was added to lysates and mixed on a rotary shaker for 10 minutes. The samples were then centrifuged at 914 x g for 10 minutes. The upper aqueous phase was then transferred to a fresh tube, and an equal volume of chloroform added and mixed on a rotary shaker for 10 minutes, and then centrifuged 914 x g for 10 minutes at 4°C. The upper aqueous layer was transferred into a fresh tube and sodium acetate was added to achieve a final

concentration of 0.3M. Equal volumes of 100% ethanol was added and allowed to precipitate overnight at -20°C. This was then centrifuged at 150 x g for 25 minutes at 4°C. The supernatant was discarded and the pellet washed twice with 70% ethanol. After removal of the supernatant the pellet was left at room temperature to dry and resuspended in 50-150µL of double distilled RNase and DNase free water prior to quantification and storage at -20°C.

### 3.16 RNA extraction from cell lines and primary pituitary tissue

Total RNA was extracted using the Guanidinium iso-thiocyanate-phenol-chloroform method previously described (Chomczynski and Sacchi, 1987). Guanidinium isothiocyanate is a chaotropic compound that denatures proteins including ribonucleases, whilst maintaining the integrity of RNA. This can then be used for downstream processes such as cDNA synthesis and reverse transcription PCR (RT-PCR) analysis.

**Isolation from cell lines:** 1-3 x $10^6$ cells were pelleted by centrifugation at 150 x g in a 15mL polypropylene tube then washed in PBS, and re-suspended in 1mL of Guanidinium thiocyanate solution (see appendix III for composition, concentration and storage of stock solution).

**Isolation from tissue:** 1mL of Guanidinium isothiocyanate lysis buffer was added to 10-30mg of tissue and homogenised in a dounce homogeniser to a slurry like consistency. Lysates were then transferred to a 15mL poly propylene tube.

200µl of 2M sodium acetate (pH 4.5) was added and mixed by vortexing. RNA was extracted from the solution by addition of equal volume of phenol and 800µl of 24:1 chloroform isoamyl-alcohol solution. Vigorous shaking ensured that the organic and aqueous phases were adequately mixed, and samples were incubated on ice for 30 minutes. The samples were centrifuged at 914 x g for 20 minutes at 4°C to separate the two phases; the upper aqueous phase was transferred to a fresh sterile tube. A double extraction was carried out by adding a further 800µL of chloroform isoamyl alcohol and shaking vigorously (Appendix III). Samples were incubated on ice for a further 30 minutes. Centrifugation was carried out using the above settings and the upper aqueous phase was transferred to a fresh tube. RNA was precipitated by addition of equal volumes of 99% isopropanol and incubated overnight at -20°C. Precipitated RNA was pelleted by centrifugation at

914 x g for 30 minutes at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol. Following re-centrifugation and removal of the supernatant the pellets were air dried to remove residual ethanol, and dissolved in 10-50µl of sterile RNase and DNase free water (Sigma) depending on the size of the individual pellets. RNA samples were aliquoted out and stored at -80°C.

### 3.17 Purification

During some experiments it was important to further purify the nucleic acid product. This was achieved using The GenElute$^{Tm}$ PCR Clean-up Kit. The kit is designed to rapidly purify single and double stranded PCR amplification products (100bp-10kb) and this achieved purification from excess primers, nucleotides, DNA polymerase, oils and salts.

The protocol comprises relatively few steps. DNA is initially bound to a silica impregnated membrane within the spin column by the addition of a binding buffer. The bound DNA is then washed with a wash buffer and the DNA is released from the membrane in elution buffer by centrifugation. Purified DNA is suitable for several downstream applications including enzymatic digestions, conventional or automated sequencing, ligation, cloning and microarray analysis in this case prior to and following sodium bisulphite conversion (see below).

### 3.18 Quantification of nucleic acid and assessment of purity

The quality of the nucleic acid isolated in any given RNA or DNA sample was estimated by spectrophotometric analysis using the NanoDrop 2000 (ND2000) (Thermo Scientific, Nelson, UK). The NanoDrop is a spectrophotometer that measures micro volumes with a patent sample

retention technology. It allows samples as small as 0.5µL to be measured without the need of cuvettes or capillaries.

The absorbance measurement made on the spectrophotometer will be of all molecules in the sample that absorb at the wavelength of interest. Since RNA, ssDNA and dsDNA all absorb at 260nm, they will contribute to the total absorbance of the sample so some samples will therefore require purification prior to measurement.

The ratio of the absorbance at 260nm and 280nm (260:280) is used to assess the purity of the DNA and RNA. A ratio of ~1.8 is typical for DNA while a ratio of ~ 2.0 is typical for high quality/purity of RNA. Lower ratios suggest the presence of proteins, phenol or other contaminants that absorb strongly at a wavelength of approximately 280nm.

### 3.19 cDNA synthesis

First strand cDNA synthesis was carried out from 1µg of good quality total RNA prepared as described above. For the synthesis, 250ng of random primers (Promega, Southampton, UK ) was added and incubated at 70°C for 5 minutes to melt and denature any secondary structure. Samples were snap frozen before addition of 2.5µL of 5µM dNTP mixture (Appendix III) and 200 units of M-MLV RT (Promega) and a reverse transcriptase buffer added to yield a final reaction volume of 25µL. Samples were then incubated at 37°C for 1 hour and stored at -80°C until required for RT-PCR.

### 3.20 Primer Design

Primer 3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design all PCR primers used throughout this study, other than those used for PCR amplification

of sodium bisulphite converted DNA. Genomic and cDNA sequences were obtained from the *Ensemble* genome browser (www.ensembl.org). Primer sequences with minimum self-complementarity, especially 3' self-complementarity were chosen due to the fact that this is known to aid in the prevention of primer dimer amplification. In addition primers yielding amplicons within a size range 100-300bp were preferred, as these are known to produce more reliable results in quantitative analytical procedures. See Appendix IV for a list of all primer sequence and amplicon sizes used throughout the study. All primers used throughout the study were purchased from Sigma and Invitrogen.

### 3.21 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an in vitro method of DNA synthesis that allows particular regions of DNA (or cDNA in the case of RT-PCR) to be copied and amplified. The DNA template is first denatured by incubation at high temperature: then the temperature is lowered and two oligonucleotide primers that flank the DNA fragment to be amplified are annealed to their complementary sequences on opposite ends of the target sequence. The primers are then extended by *Taq* polymerase and the sequence between the primers is synthesised. Multiple rounds of denaturation, annealing and extension allow for specific amplification of the sequence of interest.

### 3.22 Semi-Quantitative PCR

In reverse transcription (RT)-PCR, cDNA is used as a template in the sqPCR reaction. Primers are designed so that they amplify within the cDNA copy of the mRNA sequence under investigation. That amplification is reflective of gene expression at the transcript level. RT-PCR can be semi-quantitative when equal amounts of cDNA template are used as starting material that is in this

case assessed by the ability of the template to amplify an endogenous control gene with equal efficiencies across all of the samples. In the studies described in this report the endogenous controls were Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or Porphobilinogen deaminase (PBGD) (see appendix IV for primer sequences). Following approximate equalisation of the housekeeping gene cDNA, any observed difference between amplification of the target gene provides an indication of differences in the level of transcript expression.

Semi-Quantitative (sq)RT-PCR amplification were carried out using Promega Go *Taq* Flexi buffer containing a final reaction concentration of 10mM Tris HCl (pH 9.0), 50mM potassium chloride (KCl), and 0.1% Triton-X (Promega, Dorset, UK), 1.5mM magnesium chloride ($MgCl_2$: Promega). 200µM, each of dATP, dCTP, dGTP and dTTP (Fermentas, Belgium), 100µM each of the forward and reverse primers, and 1 Unit of Go*Taq* polymerase (Promega, Southampton, UK). A mastermix was prepared by multiplying the volumes of individual reagents required per reaction by the number of reactions being carried out:

**Table 3.0: PCR Reagents per reaction.**

| Name of reagent | Volumes per Reaction (µl) |
|---|---|
| 5 X Go*Taq* Flexi Buffer | 5 |
| 25mM $MgCl_2$ | 2.5 |
| 100µM Forward Primer | 1 |
| 100µM Reverse Primer | 1 |
| 10mM dNTP's | 1 |
| Taq Polymerase | 0.2 |
| $ddH_20$ | 12.5 |

Once prepared, 24µL aliquots of the master mix were added to each reaction tube. Finally 1 µL of template (DNA or cDNA) or no template control ($ddH_2O$) was added to the appropriate tube giving a final reaction volume of 25µL. Reaction cycles were carried out using GMI G-Storm gradient thermal cycler and also the MJ research PTC-200 thermal (Bio-Rad, Hemel Hempstead, UK). All sqPCR amplifications included a 10 minute denaturation stage at 96°C, followed by 25-35 cycles of denaturation, annealing, and extension steps. A final extension of 10 minutes was included to terminate the reaction as shown in table 3.1 PCR products were electrophoresed on Agarose gels, and visualised by ethidium bromide staining and compared to a known molecular size DNA ladder as described above.

**Table 3.1 Thermal profile of sqPCR.** Thermal profile of sqPCR showing three different segments with temperatures and time per segment.

| | **Segment 1** | **Segment 2** | **Segment 3** |
|---|---|---|---|
| | Denaturation 96°C 10mins | Denaturation 96°C 30sec | Final Elongation 72°C 15mins |
| | | Primer annealing 50-65°C | |
| | | Elongation 72°C 1min | |
| **No of Cycles** | **1** | **35** | **1** |

## 3.23 Electrophoresis

Electrophoresis refers to the separation of charged molecules in an electrical field. Molecules in a mixture are separated from each other on the basis of size, shape or charge. In this study a variety of electrophoresis methods were used to separate and visualise DNA and RNA molecules.

**Agarose gel electrophoresis**

To enable visualisation of the products from sqPCR agarose gel electrophoresis was employed. Low percentage (1-2%) agarose gels were used to separate DNA and RNA fragments. A typical 1% Agarose gel solution was prepared by melting 1g of Agarose (Bioline, London UK ) in 100mL of 1X TAE buffer (diluted from 50X stock; see appendix III) using a microwave oven at high power for 3 minutes. The solution was then allowed to cool before addition of ethidium bromide (Sigma) to achieve a concentration of 0.5µg/mL. The gel was then poured into a pre-assembled Bio-Rad gel casting tray that had been sealed at each end using autoclave tape. A comb with the appropriately sized wells was immersed into the gel, which was then left to cool at room temperature for approximately 30 minutes in order for the gel to polymerise. The comb and autoclave tape were then removed, and the gel was immersed into a Bio-Rad resolving tank containing sufficient 1 X TAE buffer to cover the gel by 2-3mm. 1µL of 6 X loading dye (see appendix III) was added to 5µL of nucleic acid samples, which were transferred to individual wells of the gel. Samples were electrophoresed from the negatively charged cathode to the positively charged anode for 45 minutes at 100V, alongside a GeneRuler 100 base pair DNA ladder (Fermentas, Yorkshire, UK), and nucleic acid fragments were detected and photographed by UV Transillumination using a Syngene gel documentation system (Cambridge, UK).

### 3.24 Quantitative RT PCR (qRT-PCR)

Real time quantitative qPCR allows quantification of PCR products in “real time” during each successive PCR cycle in the course of the reaction. Such reactions are carried out in a thermocycler that permits measurement of the fluorescent detector molecule such as the intercalating dye SYBR green, which fluoresces upon incorporation into double stranded DNA. Increased fluorescence occurs as more dye is incorporated into the DNA as each cycle of the PCR reaction. Detection of fluorescence at each successive cycle allows an amplification plot to be generated. As well as decreasing post-processing steps this methodology minimises experimental error since SYBR Green is not sequence dependant and (unlike other qPCR probes for example TaqMan), it can be used for any reaction.

Quantitative PCR amplification was performed using a Stratagene Mx3005P thermal cycler (Agilent, Cheshire, UK). Reactions were prepared containing 1X Brilliant III SYBR Green QPCR mastermix (Agilent), 400nmol of forward and reverse primer and dd$H_2O$. 11.5uL of the reaction mixture was added to individual wells of 96 well plate or to individual wells of 8 strip tubes (Agilent) and 1uL of either cDNA template or no template control was added to each well. All samples were analysed in triplicate to account for technical variation. The following reaction conditions were carried out, which comprised activation of hot start *Taq* (96°C for 3 minutes) followed by 40 cycles (96°C 30 seconds followed by an annealing and elongation step for 30 seconds) as shown in figure 3.1. A dissociation curve was included at the end of each reaction to ensure that specific amplification has been achieved.

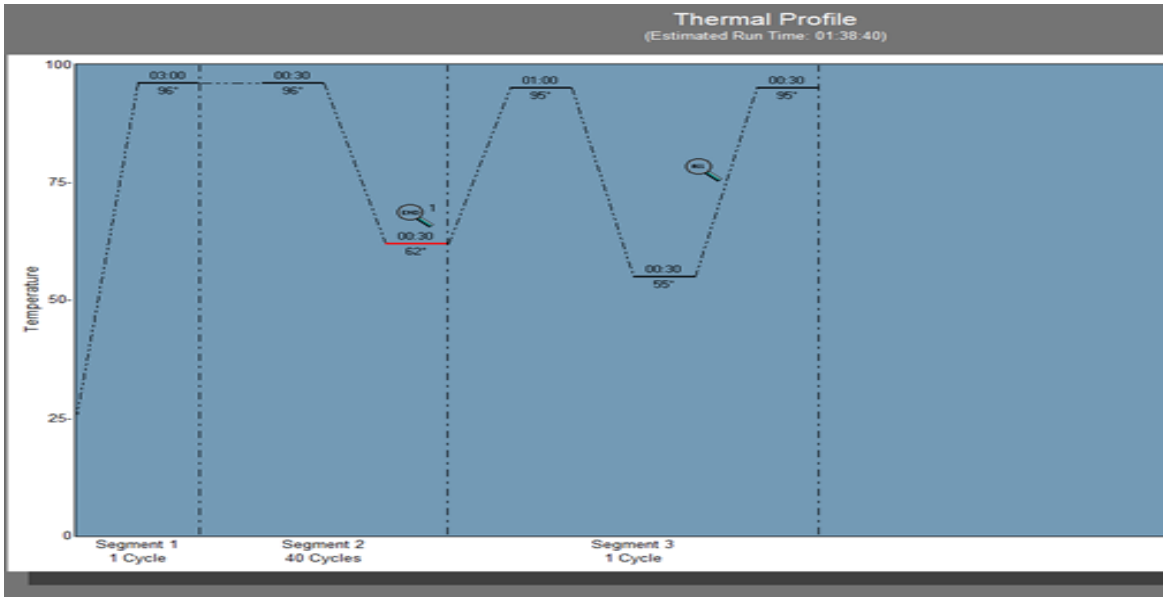



**Figure 3.1 Thermal Profile for QPCR using Stratagene MXpro 3005p**. Thermal profile showing individual stages, temperatures and time in minutes of the stages.

This method is relative, as it requires normalisation to an endogenous control gene. There are two methods by which quantitative (q)RT-PCR can be performed: the relative standard curve and the $2^{-\Delta\Delta CT}$ method. The relative standard curve method uses serial dilutions of cDNA samples, of known concentrations, from which unknown samples are quantified. This is achieved by amplification of the standards alongside the unknowns. It is possible to derive a standard curve for both the target gene and the endogenous control, thereby permitting relative levels of expression to be determined. In these types of experiments it is important to include a standard curve on every plate analysed to counteract potential variability between PCR runs. This method requires minimal validation because the PCR efficiencies of the target and endogenous control do not have to be equivalent as different efficiencies are accounted for by the standard curve.

For samples analysed by the $2^{-\Delta\Delta CT}$ method the comparative differences between the gene of interest and the endogenous control are calculated by a mathematical formula, and as such a standard curve is not required. First, the difference in the cycle threshold (CT) of the gene of interest and endogenous control is calculated (the $\Delta C_T$). Next, subtraction of the control $\Delta C_T$ from

the treated $\Delta C_T$ yields the $\Delta\Delta C_T$. The negative value of subtraction, the $-\Delta\Delta C_T$, is used as the exponent of 2 in the equation and represents the difference in "corrected" number of cycles to threshold. For all genes analysed using this method it is necessary to ensure the template amplification for the target gene occurs at the same efficiency as for the endogenous control. Therefore, during optimisation experiments, the standard curve analyses are carried out as described above, however, the difference in $C_T$ between endogenous control and gene of interest are plotted against log (base 10) input across the dilution range. Regression slopes <0.1 are considered to be acceptable for $2^{-\Delta\Delta CT}$ analyses.

### 3.25 Enzyme Linked Immunosorbent assay (ELISA)

Quantitative expression of intracellular BMP-4 protein was determined by ELISA in the normal pituitary and pituitary adenoma subtypes. An enzyme-linked immunosorbent assays (ELISA) kit was employed for this study (Abcam, Cambridge, UK). It is a plate based assays designed for detecting and quantifying BMP-4 protein. The assay employs an antibody specific for human BMP-4 coated onto a 96 well plate that is able to bind BMP-4. Detection is accomplished by the binding of a second antibody that recognises the bound BMP-4 and where the second antibody is biotinylated. Activity is assessed through incubation with horseradish peroxidase (HRP) conjugated to streptavidin. After extensive washes the addition of substrate produces a measurable coloured end product.

**Preparation of Lysate and Protein extraction:** Proteins were extracted from the primary pituitary adenomas and normal pituitaries into isotonic buffer, this was achieved using the Radio-immunoprecipitation assay (RIPA) buffer. RIPA enables efficient cell lysis and solubilisation while avoiding protein degradation and interference with protein immunoreactivity and biological activity. Tissue samples were lysed in RIPA supplemented with Protease inhibitor cocktail (PIC) including Leupeptin, Pepstatin and phenylmethanesulfonyl fluoride (PMSF). This was achieved by homogenisation in a dounce homogeniser and then centrifuged at 914 x g at 4°C for 10 minutes. Supernatant was collected and stored at -80°C. The amount of protein within the samples was measured using the Bicinchoninic acid (BCA) protein assay reagent. BCA supplemented with 0.8mg/mL copper sulphate was added to a set of known BSA standards and the tumour samples. The BSA standards ranged from 0.1-2.0 mg/ml. These was left to incubate at 37°C for one hour and then measured on a plate reader at 562nm producing a standard curve of absorbance versus concentration a typical example of this is shown in appendix V.

**BMP-4 ELISA:**

All reagents were thawed to room temperature. A constant amount of total protein of 1.5mg/mL was used for each BMP-4 protein determination from the pituitary tumours and normal pituitary samples. BMP-4 standards were supplied by the manufacturer (8.23-6000pg/mL) and the reported sensitivity of the Elisa kit is ~15pg/mL. The ELISA was performed as per kit instructions. Standard and sample solutions were added to the wells in duplicate and wells were covered and incubated at room temperature for 2.5 hours to facilitate binding of BMP-4 protein to the immobilised BMP-4 specific antibody in each of the wells. Solutions were then discarded and wells washed with wash solution. The biotinylated secondary antibody was then added to each well and incubated for 1 hour at room temperature with gentle shaking. Solution was discarded and wash steps were repeated. Horseradish peroxidase (HRP) conjugated with streptavidin was added to each of the wells and incubate for 45 minutes at room temperature with gentle shaking. Solution was discarded and wash steps repeated. A 3,3',5,5'- tetramethylbenzidine (TMB) substrate solution was added to each of the wells and incubated for 30 minutes in the dark and a colour developed in proportion to the amount of BMP4 bound. A stop solution was added which changed the colour from blue to yellow and the intensity of the colour was measured at 450nm a typical example of this is shown in appendix V. A representation of the antibody binding configuration is shown in figure 3.2 below. Interassay and intra-assay variation was measured and was found to be minimal.

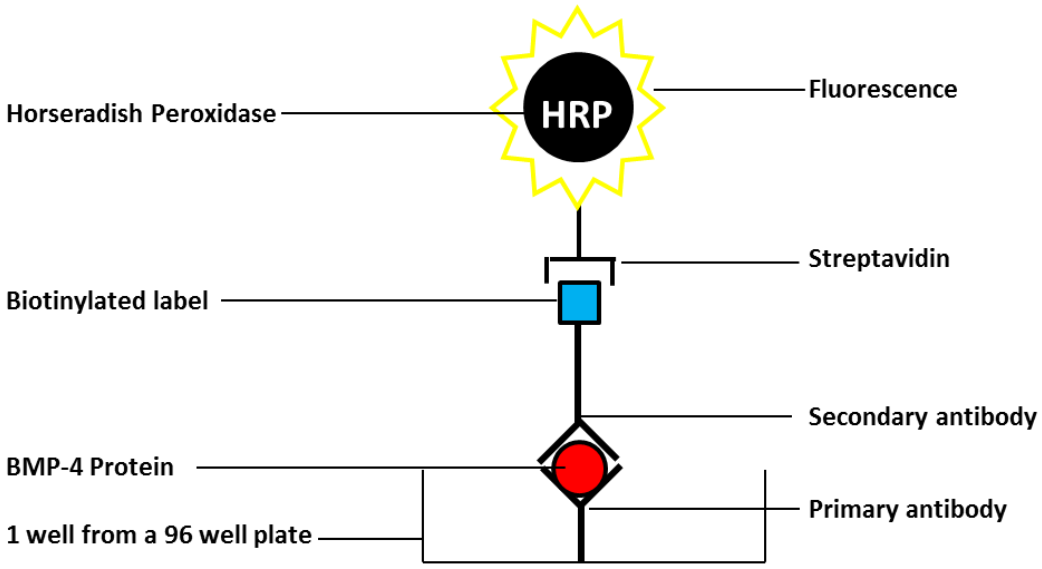



**Figure 3.2: Antibody bound complex of ELISA.** Shows the fluorescence of a single BMP-4 protein bound to the primary antibody in a well of the 96 well plate. The secondary antibody is attached to a biotinylated label. This is added to the bound BMP-4 protein. HRP is added to this with streptavidin conjugated to it. Streptavidin has a high binding affinity to biotin. Once bound produces a measureable colour end product.

**Quantification of BMP-4 protein:**

The mean absorbance for the standards and the samples was calculated. The amount of BMP4 protein measured against the BMP-4 standard samples in pg/ml and was normalised against the total protein concentration present in each sample in nmol/mg (see Appendix V for more detail).

### 3.26 DNA Methylation Analysis

Patterns of DNA methylation were determined by techniques involving sodium bisulphite modification of genomic DNA and as first described by Clark et al [354]. Sodium bisulphite deaminates unmethylated cytosine to uracil, while m5C remains unaffected. PCR amplicons generated from sodium bisulphite converted DNA can therefore be used in downstream procedures to distinguish between methylated and non-methylated CpGs within CGIs (see introduction for more details). Methylation profile of DNA can be determined by DNA sequencing following PCR amplification of sodium bisulphite converted DNA.

### 3.27 Sodium bisulphite modification of genomic DNA

Sodium bisulphite conversation of DNA was performed using a commercial kit. The kit used was EZ DNA Methylation-Gold$^{TM}$ Kit (ZYMO Research, Cambridge, UK). In this protocol 500ng of genomic DNA is first treated with sodium bisulphite, essentially as first described in [354]. For efficient conversion genomic DNA was first denatured by incubating with conversion reagent and heated to 98°C for 10 minutes and then 64°C for a further 3 hours.

The converted DNA and M-binding buffer was added to a column (provided in the kit) that had been inserted into a 2mL eppendorf tube (provided in the kit) and inverted several times. This was then centrifuged at full speed (914 x g) for 30 seconds. The flow-through was discarded and M wash-buffer was then added to the column. This was centrifuged as described above. The flow-through was again discarded. The columns were centrifuged again at full speed to remove any residual wash buffer. The M-desulphonation buffer was then added to the column to desulphonate the DNA and left to incubate at room temperature for 20 minutes. The column was then centrifuged at full speed and all flow-through discarded. The desulphonated column-bound DNA was then washed twice by addition of M-wash buffer and centrifuged at full speed removing

all flow-through. Finally the DNA was eluted into 21µL of elution buffer and then quantified on the Nanodrop ND2000 under the option of single stranded DNA. Samples were stored in -80°C after conversion.

### 3.28 Primer design for sodium bisulphite converted DNA

Primers, that were specific for sodium bisulphite converted DNA were designed for the amplification of bisulphite converted DNA. Typically, for specificity and efficient amplification these were in the size range, 24-32 bases. An example of a sequence for sodium bisulphite converted DNA is shown below:

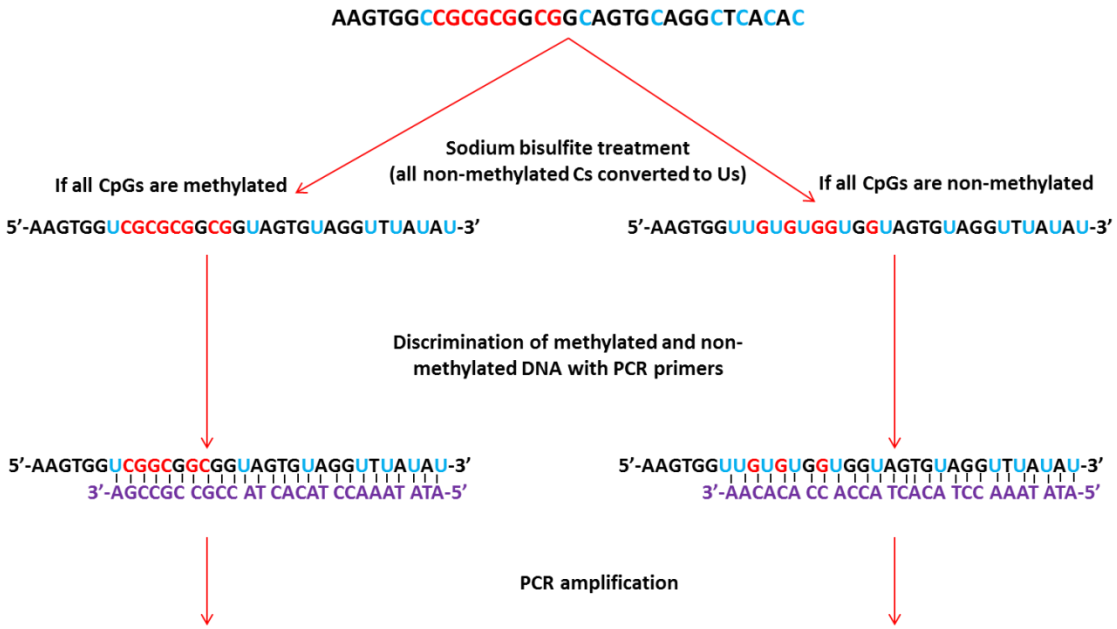



**Figure 3.3 Sodium bisulphite converted sequence.** The stages of sodium bisulphite conversion of a single strand of DNA from unconverted DNA to PCR amplification using sodium bisulphite primers. The red bases show methylated CG's, The blue bases show non-methylated C's that convert to U's. The purple sequence shows the primer sequence to the methylated and non-methylated strand.

The non-methylated cytosine would have been converted to uracil during the bisulphite conversation reaction. The primer is only able to recognise this conversion if a thymidine (T) is incorporated into the design. Methylated Cs, usually in the context of a CpG are not converted and in a sequencing reaction are read as C as shown in figure 3.3 above). Optimal amplicon sizes were between 150-300bp. See Appendix IV for Primer sequence for sodium bisulphite converted DNA.

**3.29 PCR amplification of sodium bisulphite converted DNA**

A touchdown sqPCR was performed using sodium bisulphite converted DNA as template. It was necessary in some cases to perform nested/hemi nested PCR whereby the initial primary PCR product was subjected to a second round of PCR amplification.

**3.30 Purification of DNA from agarose gels**

Prior to T:A cloning of PCR amplicons it was necessary to first resolve them by agarose gel electrophoresis. This step ensured that the fragment corresponded to the right size and that it was separated from primer sequences. Fragments were, therefore, electrophoresed through a 2% agarose gel (as described in a previous section) and isolated and purified using a QIAquick Gel Extraction Kit (QIAGEN, West Sussex). The region of agarose containing the DNA fragment was first visualised with a UV transilluminator and excised from the gel using a sterile scalpel blade. The gel fragment was then transferred to a 1.5mL microcentrifuge tube and the volume of agarose estimated by measuring the mass of the excised fragment. 3 volumes (relative to the volume of the gel fragment) of Buffer QG was added and the agarose was melted by incubation at 50°C on a heat block for 10 minutes. A further 1 gel volume of isopropanol was added and the sample vortexed prior to addition of the DNA containing sample to a column provided within the

kit. The sample was the subject centrifugation and the flow through discarded. This step was repeated. After further wash steps using Buffer PE the column was put into in a 2mL collection tube and elution buffer EB used to release bound DNA as described in the manufacturer's protocol.

**3.31 Ligation**

The ability of *Taq* DNA polymerase to extend PCR amplicons by the template independent addition of a single adenine (A) to the 3'–end, enables the rapid and efficient ligation of PCR products into a T:A cloning vector, the pGEM T-Easy vector (Promega, Southampton, UK) See Appendix VI. The vector is a pre-linearized with a single 3' –terminal thymidine at either ends. The overhang at the insertion site greatly increases efficiency of ligation of PCR products. PCR reactions were first performed in duplicate and then combined in a single tube. 6µL of this reaction mix was resolved on a 1% gel to confirm amplicon size. The remaining reaction was then cleaned on a Gene Elute PCR cleaning column (Sigma-see above). The quantity of the purified PCR product was determined on the Nano-drop (OD 260nm). An appropriate quantity (see calculation below) was then used for ligation into the pGEM vector at a 6:1 insert:vector molar ratio. This was calculated according to the following equation.

$$\frac{\text{ng of vector (50ng)} \times \text{kb size of insert}}{\text{kb size of vector (3kb)}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

50ng of the 3000bp size pGEM vector was typically used in a ligation reaction; therefore at a 6:1 insert:vector molar ratio, approximately 5ng per 100 bases of insert was used. To this reaction 400U of T4 DNA ligase (Promega) and 4.5µL of 10X T4 DNA ligase buffer was added. Ligation reactions were incubated at 4°C for 16 hours.

**Table 3.2 Ligation Reaction**. Ligation reagents for one reaction

| Ligation Reagents | 1 reaction (µL) |
|---|---|
| 10 X T4 Ligase buffer | 4.5 |
| T4 Easy Vector | 0.7 |
| PCR product | 1-4 |
| T4 DNA Ligase | 4 |
| $H_2O$ (10 µL Total) | (~10) |

### 3.32 Transformation

8µL of the total 10µL reaction was added to a 50µL volume of competent cells (See appendix III on growing chemically competent cells) in a sterile 0.5mL microcentrifuge tube, and incubated on ice for 20minutes. The microcentrifuge tube was gently flicked to mix the DNA and cells. The cells were then heat shocked for 2minutes at 42°C in a thermal cycler, followed by a further incubation on ice for 2 minutes. The transformation mixture was then transferred to a sterile 15mL polypropylene tube followed by the addition of 1mL sterile LB medium. (Appendix VI) samples were incubated at 37°C for 2.5 hours in an orbital shaker with vigorous shaking.

### 3.33 Spread Plating

350µL of the bacterial culture was spread onto a LB-agar plate containing 60ug/mL of ampicillin using a sterilised spreader. In addition, 0.1M IPTG (isopropyl β-D-1-thiogalactopyranoside) and 50µL /mL Xgal (5-bromo-4-chloro-3-indolyl β-D-galactopyranonide) were added to agar plates if TA cloning was being carried out, as this allowed for blue-white colony selection. Plates were incubated inverted at 37°C for 16 hours. (See appendix VI)

### 3.34 Screening

All vectors used during this investigation contain the ampicillin resistance gene, $AMP^r$, which meant that inclusion of ampicillin in the LB agar enabled positive selection of transformed colonies. In addition, successfully ligated PCR inserts into the T-Easy vector were screened by a simple colour selection procedure due to the presence of IPTG and Xgal in the LB agar. This substrate is catalysed by the enzyme β-galactosidase (product of the *lac*Z gene), yielding colonies that appear a distinctive blue colour. As the incorporation of a PCR product into the pGEM vector destroys the lacZ gene, β-galactosidase is not produced by successfully transformed cells, which therefore produces white colonies.

To confirm that successfully transformed cells contained an insert, it was necessary to carry out sequencing analysis. Colonies were picked off the agar plate using a sterile pipette tip and lysed in 50µL of $ddH_2O$. This was done by heating at 96°C and then centrifuging at 914 x g for 10 minutes. 5µL of the lysate was subjected to sqPCR amplification using primers that flank the cloning site SP6 reverse and T7 forward (see appendix III). PCR products were purified using the Gene Elute PCR Purification kit (Sigma) and sent to be commercially sequenced (gene service, Cambridge UK) using the T7 forward primer. Typically, 5-10 molecules that were isolated from individual bacterial colonies were sequenced from each of the sample.

### 3.35 Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation assay (ChIP) is a powerful technique allowing analysis of protein modifications within specific genomic regions. ChIP is used to determine changes to epigenetic signatures, chromatin remodelling and transcription regulators that are recruited to specific genomic sites. Although ChIP is a versatile and powerful tool it is technically demanding and for the studies described in this thesis the Active Motifs ChIP-IT Express Enzymatic Kit (Rixensart, Belgium) was employed. There are five main stages to the procedure. In brief intact cells are fixed using formaldehyde, which crosslinks and preserves protein and DNA interactions. The DNA is then sheared using an enzymatic digestion cocktail containing micrococcal nuclease, into small uniform fragments. Specific protein and DNA complexes are immunoprecipitated using antibodies directed against the DNA-binding protein of interest (see below for more detail). Once antibodies have bound the enriched fractions are pulled down with the aid of magnetic beads coated in protein G which has a high binding affinity for the antibody. Following immunoprecipitation cross linking is reversed, the proteins are removed by treatment with proteinase K and DNA is recovered. The DNA is then analysed to determine which DNA fragments were bound to the protein modification of interest. In the case of these studies this was achieved through qPCR. Above steps are shown in figure 3.4 and described in detail below.

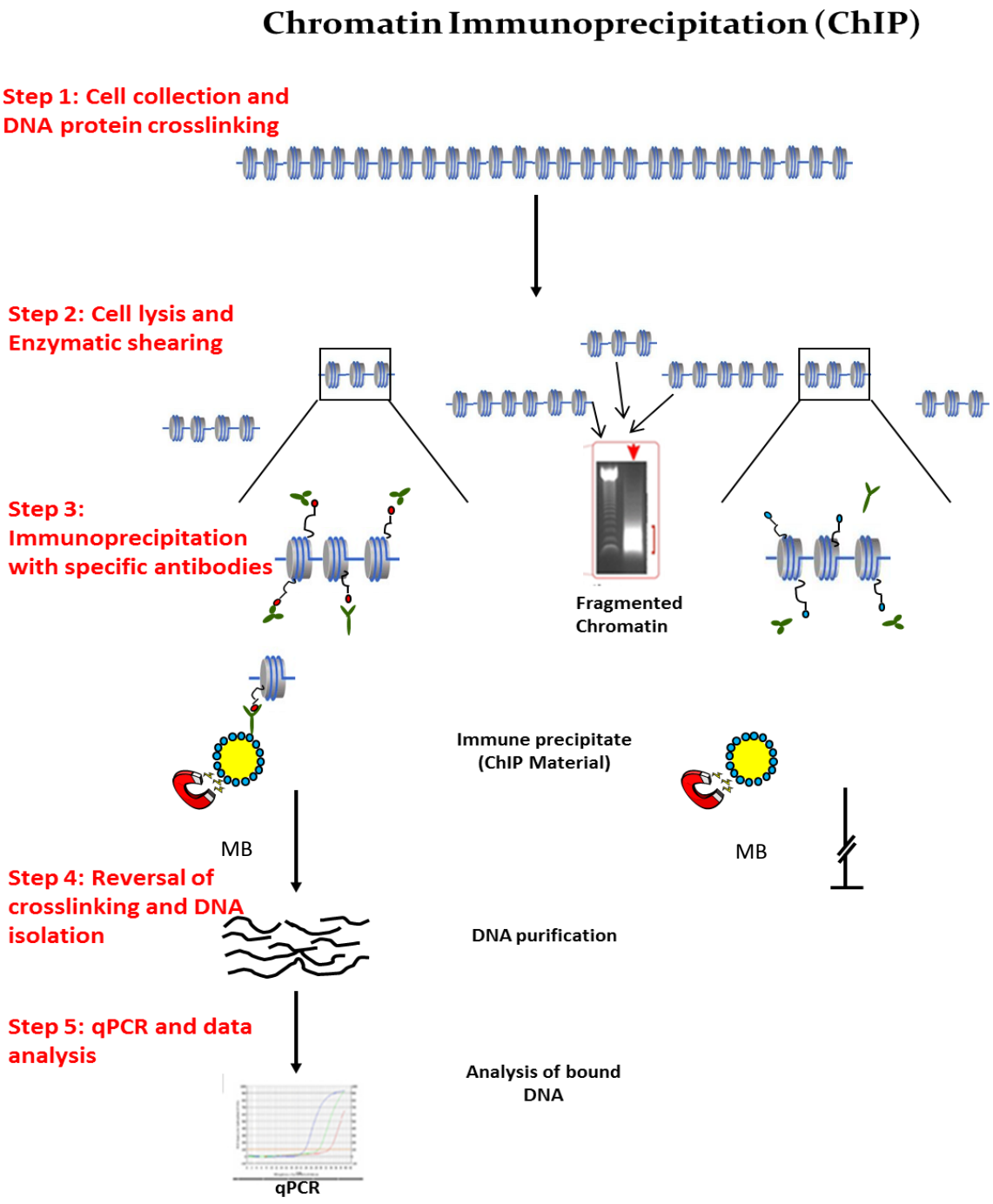



**Figure 3.4: Chromatin Immunoprecipitation Assay representation.** Representation of the ChIP reaction showing the 5 major steps. Demonstration of two different ChIP assays employed from the same preparation. On the left showing and antibody specific to the interaction present and on the right showing antibody that is not specific for the histone tail modification. MB represents magnetic beads coated in protein G. Line with the double break represents no product. Details of each of the step are described below.

**1. Cell collection and DNA-protein crosslinking**

Cells were first grown to 70-80% confluency (approximately 1-1.5 million cells) in a T75 flask. Cells were then detached from the surface of the flask using PBS-EDTA and transferred to a siliconised 50mL tube and centrifuged at 150 x g for 5 minutes. The supernatant was discarded and the pellet washed with ice cold PBS. This was again centrifuged. A solution of DMEM media containing a final concentration of 1% formaldehyde was added to cells and the cell pellets were disassociated by pipetting and incubated on a rotary shaker for 5 minutes. For tissue samples, the 1% formaldehyde-DMEM solution was added to the tissue and homogenised in a dounce homogeniser. 500µL of the glycine solution, supplied with the kit, was added at room temperature and rotated on the rotary shaker for 5 minutes to stop excessive crosslinking and centrifuged as described above.

**2. Cell lysis and enzymatic chromatin shearing**

After the fixation step described above, the cells were first lysed in the lysis buffer supplemented with PIC (Protease Inhibitor Cocktail) and PMSF (Phenylmethanesulfony fluoride) for 30 minutes on ice. During this incubation the enzymatic shearing cocktail was prepared by diluting the supplied enzymatic shearing cocktail (2 x $10^4$ U/ml) to 1:100 with a 50% glycerol solution. For the chromatin shearing step, and to generate fragments in the size range 200-1000bp, it was necessary to perform enzymatic shearing in the presence of micrococcal nuclease. For these fragment sizes it was first necessary to optimise shearing in time course experiments. Therefore, the lysis solution together with the cells were transferred to an ice cold dounce homogeniser and then gently homogenised for 10 strokes on ice to aid nucleic acid release. After transfer to a siliconised microcentrifuge tube and centrifugation (914 x g for 10 minutes at 4°C) the supernatant was discarded and the pellet resuspended in warm digestion buffer supplemented

with PIC and PMSF and incubated at 37°C for 5 minutes. 17µL of the enzymatic shearing cocktail was added to the pre-warmed nuclei and subjected to gentle vortexing and incubated for 12 minutes at 37°C with intermittent vortexing. The enzyme activity was stopped by addition of 7µL of ice cold EDTA and incubated on ice for 10 minutes. All samples were then centrifuged at 914 x g for 10 minutes at 4°C and the sheared chromatin supernatant collected. This could be stored at -20°C for later use.

**3. Immunoprecipitation**

The antibodies used for the described studies were purchased from Abcam (Cambridge, UK). The antibody used to detect active genes recognises the H3K9Ac (histone 3 lysine 9 acetylation) modification [120]. The antibody used to detected silenced genes recognises the H3K27Me3 (histone 3, lysine 27 tri-methylation) modification [355].

After thawing the samples generated in step 2 (see above), 10µL of the chromatin was set aside and represented input DNA. For the samples subject to immunoprecipitation, 20-60µL of sheared chromatin was added to siliconised tubes together with the following reagents; 25 µL protein G beads, 10µL Chip Buffer, 1µL PIC water and modification specific antibody and transferred to a rotary shaker for 4 hours at 4°C. The protein G beads were magnetic beads coated with protein G. The protein G has a binding affinity for the antibody heavy chains and was, therefore, able to pull down the antibody in a complex with the specific histone modification. The DNA-histone complexes were immunoprecipitated and washed. Immunoprecipitation was achieved by placing the tubes on a magnetic stand to pellet the beads and the supernatant was removed without disturbing the beads. The beads containing the complex were washed with ChIP buffers 1 and then 2 several times and pelleted using the magnetic stand.

**4. Reversal of Crosslinking and DNA isolation.**

Following the wash steps, the chromatin was eluted from the antibody that was in turn bound to the protein G beads. This was achieved by addition of 50µL elution buffer and incubating for 15 minutes with intermittent pipette mixing at room temperature. The reverse cross linking buffer was added (50 µL) and tubes immediately transferred to the magnetic stand to allow the beads to pellet. The supernatant (100µL at this point), which contained the chromatin was transferred to a fresh tube.

The immunoprecipitated chromatin and the input chromatin (see step 1) were treated separately. 88µL of ChIP buffer was added to the 10µL of input sample. 2 µL sodium chloride solution was added to both input and immunoprecipitated samples and heated to 95°C for 15 minutes in a thermal cycler. Proteinase K (2µL) was then added to both immunoprecipitated chromatin and Input chromatin and incubated at 37°C for 1 hour. This step effectively digests proteins including the histones that were in complex with the DNA. After the incubation period proteinase K stop solution (2µL) was added to the reaction. The DNA within this solution was then concentrated by binding onto PCR GeneElute columns (as described in a previous section).

**5. Quantitative PCR and data analysis.**

The input DNA was further diluted 1:10 using dd$H_2O$. Primers were designed to amplify a region within 300bp of the known or presumed transcription start site [121, 356]. A qPCR was set up using the DNA isolated by immunoprecipitation of the sheared chromatin and also the Input DNA as a control sample for 50 cycles.

The method used to normalise the ChIP-qPCR data is the percentage input method. It is important to normalise against the input as the input sample represents the total chromatin pre-enrichment. There are several dilution steps subject to immunoprecipitation it is therefore

necessary to use a dilution correction factor for the input. This allows you to calculate equal concentrations of the immunoprecipitated sample and the input. It allows comparison of *"like with like"* samples. The input sample represented 10% of the sample used for immunoprecipitation and therefore necessary to adjust this to 100%. The correction factor of this is to subtract 3.32 CT values (The starting material is 10%, a dilution factor of 10 needed to be taken into consideration, this is 3.32 cycles and was found by $\log_2$ of 10. This was then subtracted from the Ct values of the input as demonstrated in figure 3.5 and 3.6 also explained in detail in the qPCR section above).

The following equation was used to calculate the percentage enrichment relative to the input 100X2^(adjusted input-CT (IP)) see figure 2.5 below for a breakdown of this calculation.

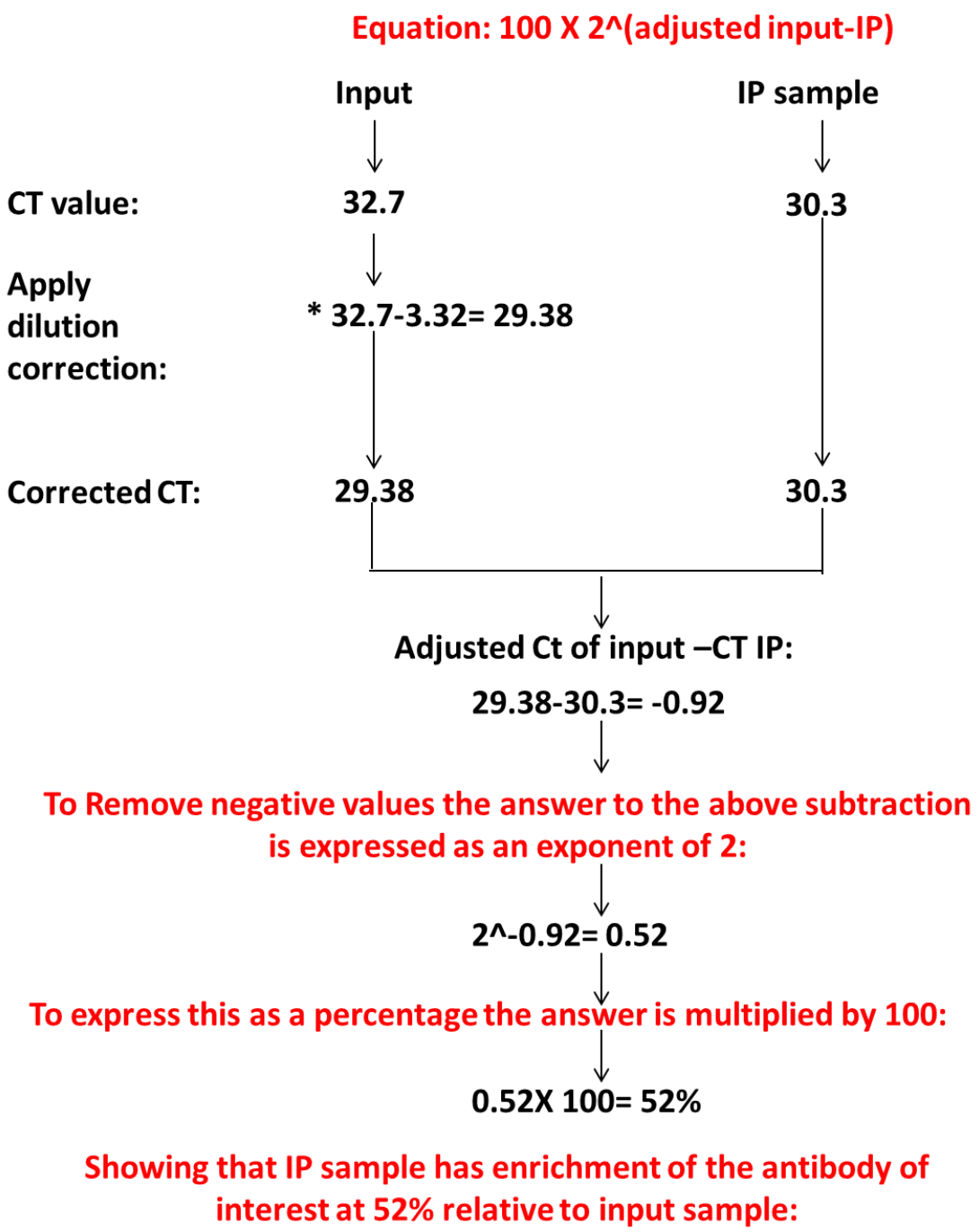



**Figure 3.5: ChIP calculations.** A detailed example of the ChIP calculations used within this study. IP is the immunoprecipitated sample.

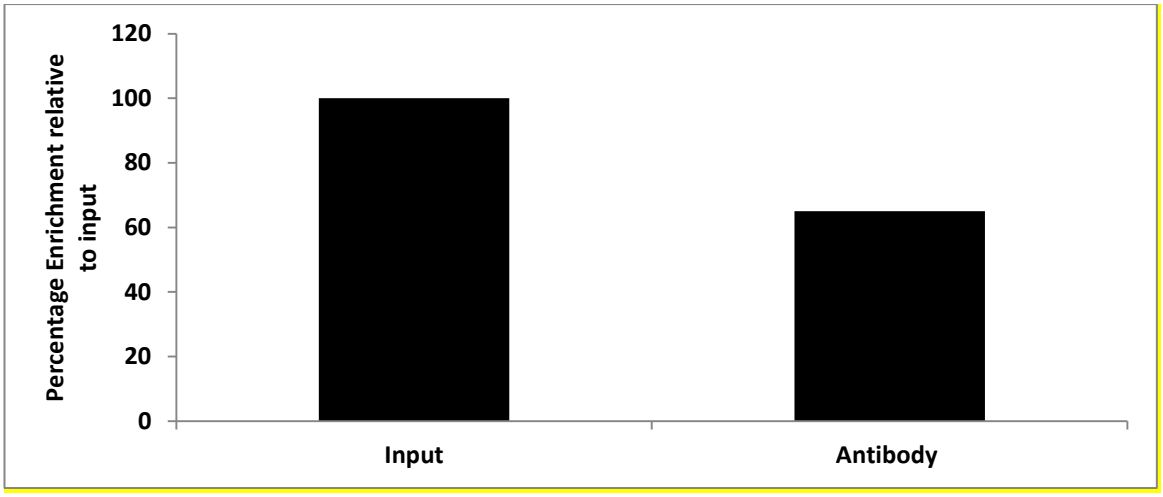



**Figure 3.6 Graph of ChIP calculations.** ChIP calculations of one antibody relative to adjusted input.

# Chapter 4: Reversal of Endogenous Dopamine Receptor silencing in pituitary cells augments receptor mediated apoptosis

# 4. Reversal of Endogenous Dopamine Receptor silencing in pituitary cells augments receptor mediated apoptosis

## 4.1 Aims & Objectives

In this study I investigated potential epigenetic mechanisms responsible for or associated with loss of D2R expression in pituitary cells and the potential for pharmacological strategies to unmask and thereby induce expression of silenced genes. This was investigated using pituitary tumour cell lines that either express or do not express endogenous D2R. In addition the possibility to reinstate and or even augment receptor mediated apoptotic responses was investigated.

## 4.2 Introduction

Dopamine (DA) receptor agonists are considered a first line treatment choice for patients with pituitary prolactinomas where they not only effectively suppress prolactin synthesis and secretion but induce tumour shrinkage [281, 357]. Despite their success a small proportion of patients are intolerant to DA agonist therapy [292, 358], and in cases where resistance is apparent this seems to be related to the number of DA D2 receptors (D2R) expressed by the adenoma [359]. Activation of the D2R by DA and DA agonists lead to a reduced cAMP production through interaction with Gi/Go proteins [259]. The reduction in cAMP levels in normal and tumoural lactotrophs is considered integral to the inhibition of PRL synthesis and release [259, 281, 360-362].

In contrast to the understanding of pathways regulating PRL release within the lactotroph our understanding of receptors and their intracellular pathways responsible for tumour shrinkage and or apoptosis are less clear. Early studies suggested that resistance to DA but not bromocriptine

(BC) mediated apoptosis was a consequence of these cells not expressing a D2R [284]. However more recent studies by Jaubert and colleagues [283] suggest that DA initiated apoptotic responses are mediated through the DA transporter (DAT). Although this transporter represents an attractive alternate mechanism a recent study from our laboratory using the same model system namely the rodent GH3 cell line, reached a different conclusion. In this case, it is showed that both DA and BC elicits an apoptotic response in these cells in the absence of a D2R or a DAT [363]. Moreover characterisation of the intracellular apoptotic pathways engaged by these drugs show them to be distinct. In those studies we showed that a c-Jun N-terminal kinase mediated apoptotic pathway is activated by BC but not by DA. However the DA and BC mediated apoptotic pathways converge to activate the terminal caspase-caspase 3. Indeed, co-incubation experiments (DA and BC) reinforced and extended these findings where we observed a synergistic increase in apoptotic end points [363].

Perhaps surprisingly given its near ubiquitous, but not invariant expression pattern, in the different cell types of the normal pituitary gland and their cognate tumours [357], our understandings of mechanisms responsible for loss or reduced D2R expression are incomplete. However, a caveat to that statement is that a recent investigation, and subsequent to the findings reported in this thesis, suggests that Filamin-A (FLNA) expression may modulate D2R expression [364].

Pituitary adenomas, in common with most other tumour types, display genetic and epigenetics aberrations. Whilst genetic aberration are infrequent in this tumour type aberrations that impact on the epigenomic landscape are a frequent finding and may act in concert with genetic change(s) [91, 365]. Epimutations are apparent as changes in both global and gene specific DNA methylation and histone modifications. In these cases aberrations are frequently associated with or responsible for altered gene expression profiles that characterise the initiation, development, and progression of disease states [91, 181]. In addition, changes to the epigenome may also

impact upon treatment responses, and this is apparent in several different tumour types. For example in ovarian tumours silencing of genes involved in apoptotic pathway are frequent findings [366] as are epigenetic aberrations in specific receptor pathways in primary breast tumours and their cell lines [367] and in these cases can lead to a varying degree of drug resistance.

Multiple studies have used the pituitary tumour cell line, GH3 as a model system to investigate DA and BC mediated apoptosis [283, 285, 289]. These reports show that GH3 cells do not express the dopamine D2 receptor (D2R) whereas, the MMQ cell line does express this receptor. Therefore the initial experiments were devised to determine the presence or absence of the D2R receptor in early passage GH3 cells relative to normal rat pituitary (NRP). Furthermore, my studies were extended to characterise epigenetic changes and apoptotic responses associated with receptor expression patterns.

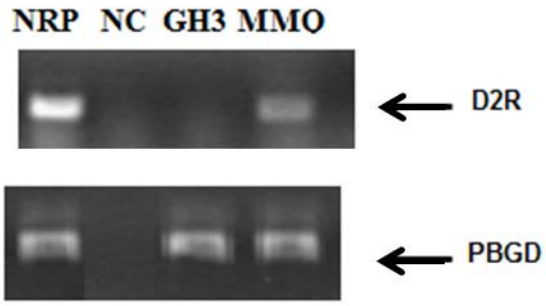
## 4.3 Results

### 4.3.1 Expression analysis of D2R in GH3 and MMQ cells as determined by RT-PCR

Semi quantitative RT-PCR shows the expression of D2R in rodent pituitary cell lines relative to normal rat pituitary. The internal control in this case, was the housekeeping gene PBGD. The expression of D2R at the transcript level in MMQ cells is similar to that seen in NRP. However, the expression of D2R in GH3 is significantly reduced relative to NRP figure 4.1 A.

Quantitative RT-PCR (RT-qPCR) showed that expression of D2R in MMQ is similar to that seen in NRP. However, the expression of D2R in GH3 cells is significantly reduced relative to NRP Figure 4.1B.

**A**




**B**

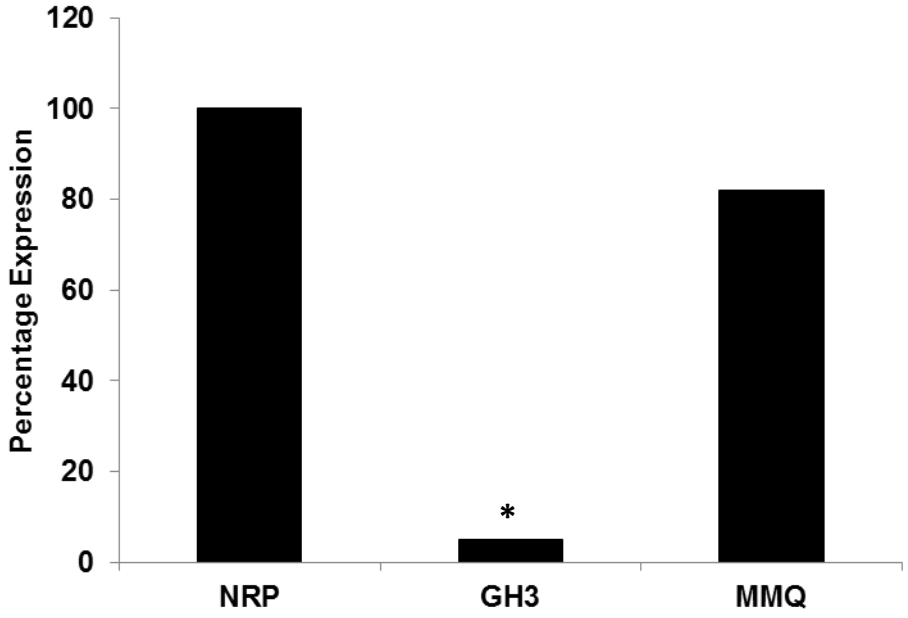



**Figure 4.1: Expression analysis of dopamine D2 receptor (D2R) in normal rat pituitary (NRP), GH3 and MMQ pituitary tumour cell line**. (A) sqRT-PCR (B) qRT-PCR analysis of D2R expression in NRP, GH3 and MMQ cells. PBGD was used as an internal control for PCR. NC, negative control (B) qRT-PCR showing percentage expression relative to normal rat pituitary (NRP) in GH3 and MMQ cells. The results are the mean of three triplicate determinations *, P<0.01 MMP, GH3 Vs NRP. Data were analysed for significance with a paired t test with Welch's correction.

### 4.3.2 Methylation status of the D2R in pituitary cell lines

*In silco* analysis of the rodent D2R using the CpG island searcher http://www.cpgislands.com and also www.ebi.ac.uk, identified a *bona fide* CpG island within this gene. The island extended from -374bp to + 650bp relative to the transcriptional start site and is shown in figure 4.2 A.

A portion of this CpG island was analysed by sodium bisulphite sequencing (as described in the Materials and methods section). The sequencing encompassed 20 CpG dinucleotides. In the NRP across the 20 CpG dinucleotides, 53% of the CpGs across the total clonal population were not methylated (Fig 4.2B). However, and in contrast to these findings, in the GH3 cell line the majority of CpG dinucleotides were methylated and only 5% were not methylated. In MMQ cell line this portion of the CpG island shows a high frequency of methylation at 77% relative to the normal pituitary, however, 23% of the CpGs are not methylated compared to 5% in GH3 cells.

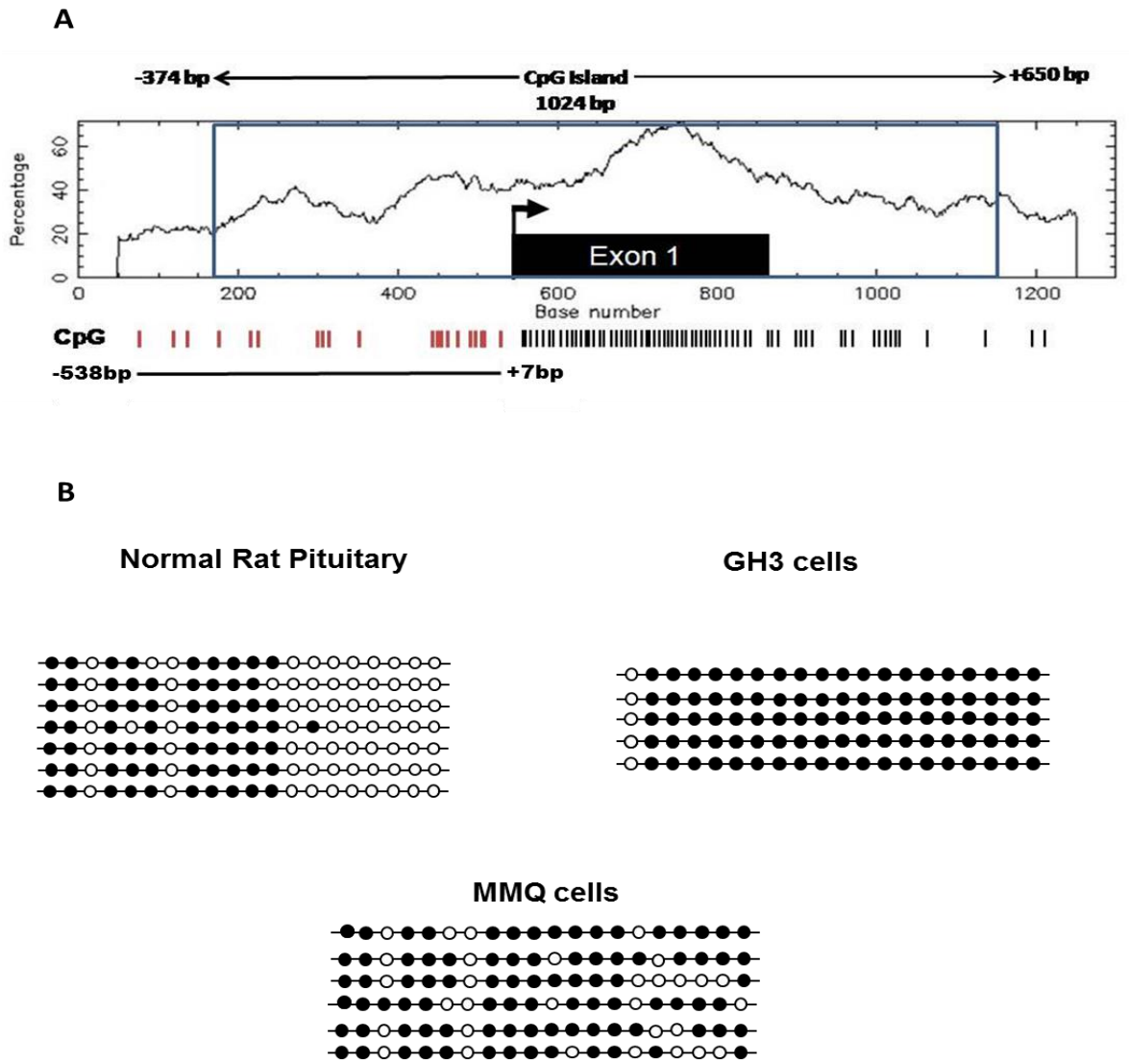



**Figure 4.2 Methylation status of the D2R in pituitary cell lines**. A*). In silico* analysis of rodent D2R CpG island locus region. The CpG island extending from -374bp to +650bp, relative to the transcription start site (Black bent arrow [+1]) was determined using CpG island searcher (www.cpgislands.com) and the figure was drawn using a web based program www.ebi.ac.uk. The observed GC frequency is plotted across the region and is shown relative to transcription start site. Individual CpG dinucleotides are shown as vertical tick marks. The black bar below these tick marks (-538 to +7bp) marks the amplicon that includes the 20 CpG reported for the methylation status. B). beads on a string representation across 20 CpG dinucleotides shown in panel A above. Individual clones from each of the cell lines and NRP are shown. The filled circles show methylated CpG dinucleotide and the unfilled circles show unmethylated CpG dinucleotides.

### 4.3.3 Drug-induced demethylation and re-expression of the D2R in GH3 cells

To determine the contribution and effect of CpG island methylation loss of the D2R expression in GH3 cells, the cells were treated with zebularine, a cytidine analogue either alone or in combination with histone modifying agent TSA. Zebularine as a single agent at the highest dose employed, 3μM, was associated with an increase in the expression of D2R as determined by sqRT-PCR and relative to the housekeeping gene PBGD (figure 4.3A). RT-qPCR results also show that zebularine at this dose was able to augment D2R expression (figure 4.3B). In experiments where higher doses of zebularine were used these were found to be cytotoxic. Combined drug challenges, where the dose of TSA was kept constant (30nM), in the presence of increasing doses of zebularine led to significant increases in D2R transcript expression. However TSA as a single agent was ineffective.

The potential for a mechanistic link between epidrug induced re-expression of the D2R and demethylation of its cognate CpG island was further explored by sodium bisulphite sequencing and following the drug challenges shown in figure 4.3 A and B. GH3 challenges with either zebularine or TSA as single agents was associated with a modest but reproducible decrease in methylation of 10-15% relative to the vehicle treated GH3 cells. For the combined challenges of zebularine and TSA and at the highest doses employed, a 21% decrease in methylation was apparent (figure 4.3B).

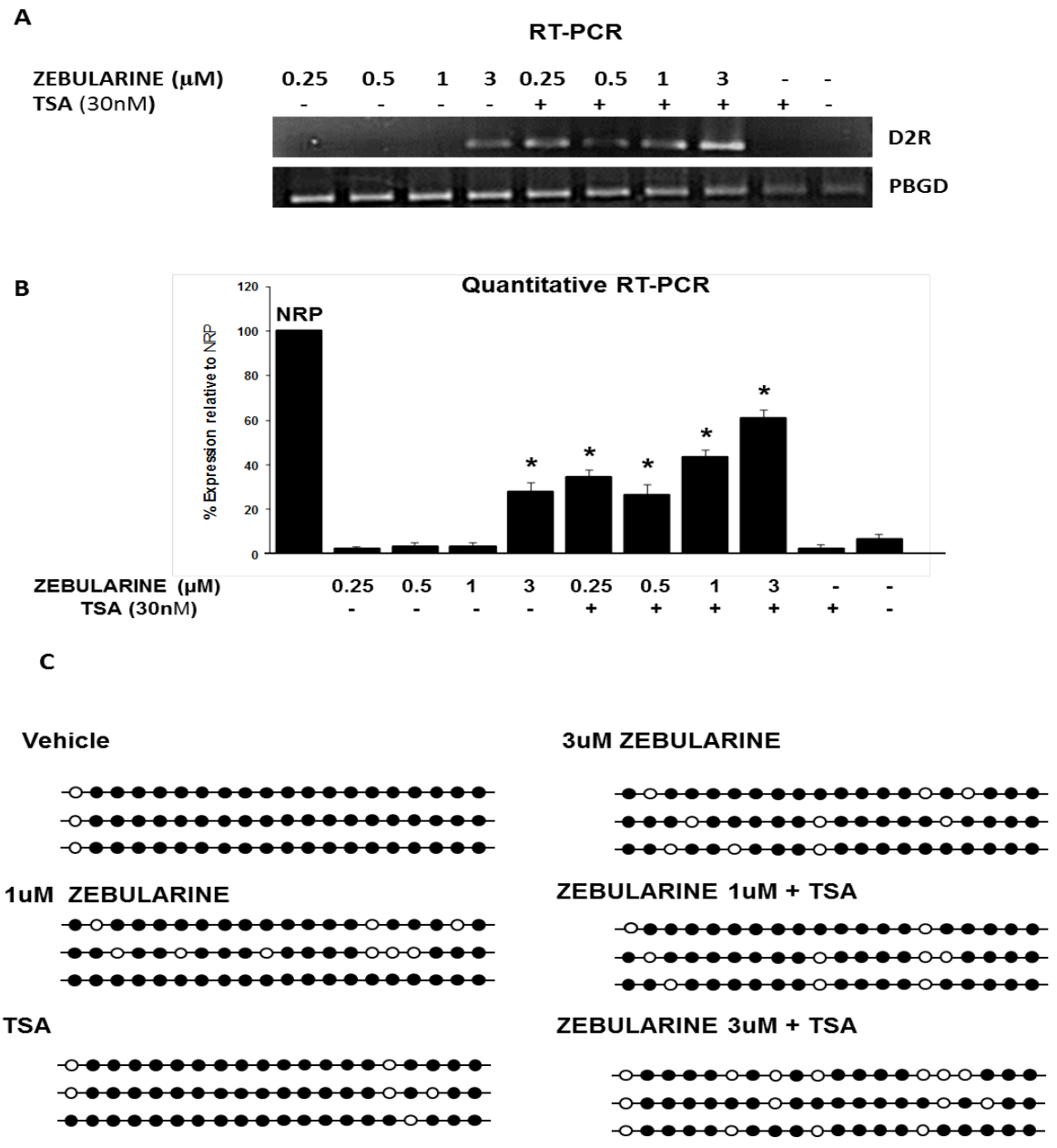



**Figure 4.3 Drug induced demethylation and expression of D2R in GH3 cells**. Expression and methylation status of D2R in GH3 cells to challenges with zebularine (ZEB) or TSA alone or in combined incubations. A) sqRT-PCR and B) qRT-PCR analysis of D2R expression after challenges with drug doses shown in the figure. B) qRT-PCR shows expression as a percentage relative to NRP. C) Sodium bisulphite sequencing across CpG island. Drug challenges are shown in the figure. The beads on a string representation as described previously in figure 4.2B. * P <0.01 vs. vehicle alone. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test.

### 4.3.4 Drug-induced histone modifications in GH3 cells

The epidrug induced (zebularine and TSA) re-expression of the D2R in these cells was robust, however, the observed changes in CpG island methylation were not dramatic. In addition, the necessity to include TSA suggested that aberrations to histone modifications might impact on gene expression. Therefore, ChIP assay were used to investigate the histone tail modifications associated with the drug challenges used with this cell lines. Histone modifications associated with active genes are marked by enrichment of H3K9Ac and silent genes for the H3K27Me3 modification. Across the D2R promoter region GH3 cells show enrichment for  of H3K27Me3 relative to normal pituitary and to that seen in MMQ cells that express the D2R (Figure 4.4A). In contrast, and again relative to normal pituitary and MMQ cells, GH3 cells show a relative depletion in H3K9Ac, the histone modification associated with active genes (Figure 4.4B).

Challenge of GH3 cells with zebularine was associated with a modest decrease in the enrichment of H3K27Me3 relative to GH3 cells challenged with vehicle alone. However no change in the histone modification associated with active genes, H3K9Ac, was apparent.  In combined drug challenges with zebularine and TSA a further decrease in H2K27Me3 was apparent. Furthermore, these challenges also led to a significant increase in H3K9Ac relative to cells treated with vehicle alone. Thus results are consistent with the known role of TSA as an inhibitor of histone tail deaceytlation and promotion of an active chromatin structure.

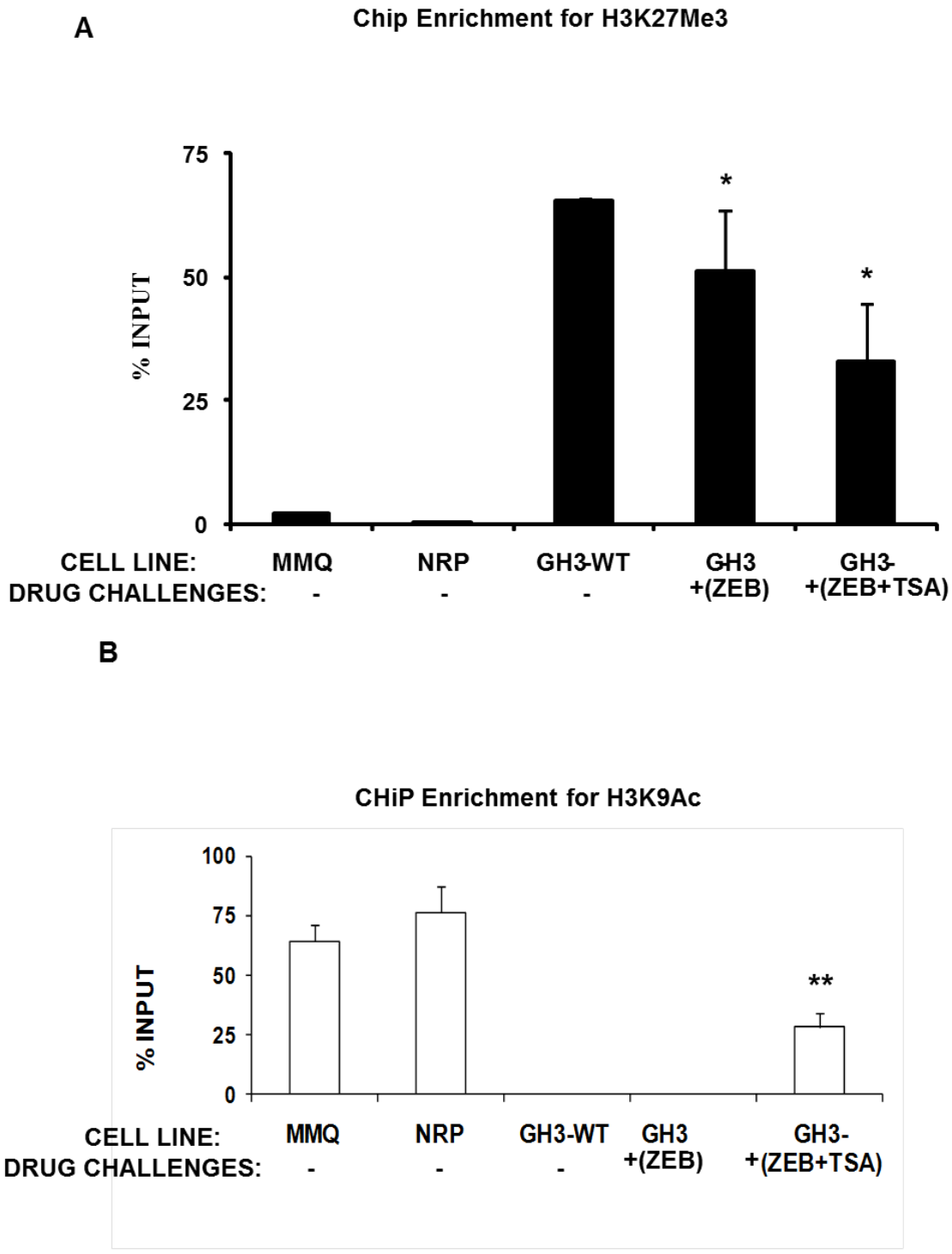



**Figure 4.4 Drug induced histone modifications in GH3 cells.** ChIP analysis of the D2R promoter region. ChIP analyses are shown relative to the input DNA for A) H3K27Me3 or H3K9Ac B). Enrichment in the pituitary cell lines (GH3, MMQ and NRP) are shown after drug challenges with Zeb 3µM and TSA 30nM. * P< 0.05 and ** P< 0.01 vs GH3-WT treated with vehicle alone. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison test.

### 4.3.5 Apoptotic end points in D2 receptor expressing GH3 cells

The co-incubations experiments shown in Figure 4.3 show that 3μM zebularine in combination with 30nM TSA induced robust expression of the D2R in GH3 cells. In these cases, D2R expression was maintained (in the absence of these agents) for a further ~40hrs. (Figure 4.5A). During this "window" and to aid explanation we designated these D2R expressing cell, GH3-D2exp. Culture of these cells, in this case in 96 well plates permitted challenge with the apoptosis inducing agent BC and DA. The findings from these experiments are shown in Fig 4.5C. Relative to wild type GH3 cells the D2R expressing cells (GH3-D2exp) are significantly more sensitive to the apoptosis inducing effects of DA and BC that their non-induced counterparts Figure 4.5A.

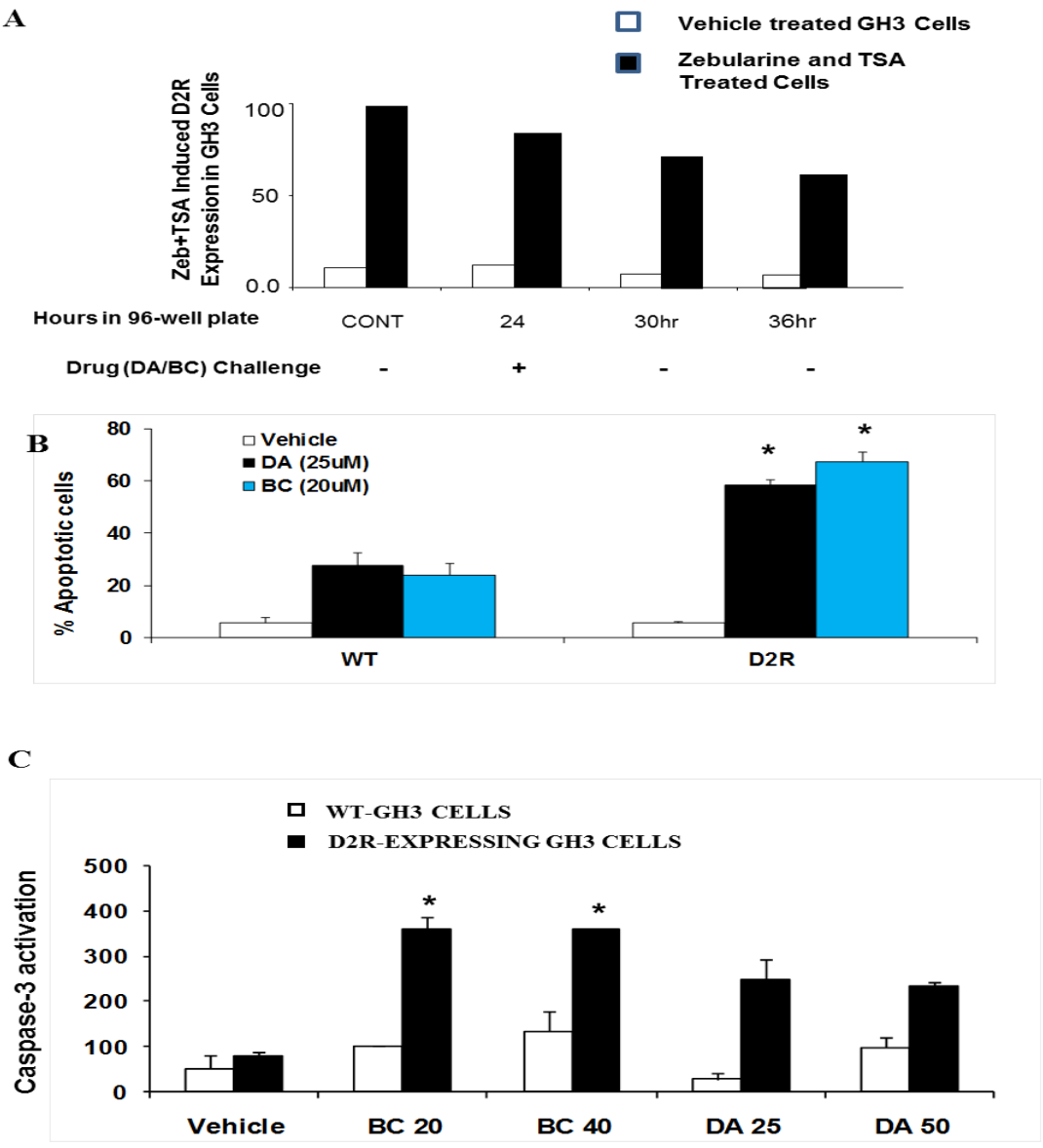



**Figure 4.5 Apoptotic responses of GH3 cells to DA and BC challenges (A-C).** GH3 cells induced to express D2R (GH3exp) and labelled D2R in the figure, relative to cells not expressing D2R (WT), were challenged with DA or BC at the drug concentrations shown in Figure A. B, Apoptotic cell counts (36h post drug challenges) as determined by Hoechst 33342 staining are presented and expressed as a percentage of total cell counts. C) Caspase 3/7 activation 6h after each of the drug challenges. In Panel B white bars represent vehicle treated, Black bars represent DA treated and blue bars represent BC treated. The results are the means of three triplicate determinations in each case *, P< 0.001 GH2-WT treated with BC. Data were analysed for significance with a paired t test with Welch's correction.

### 4.3.6 Specificity of D2R mediated apoptosis in GH3 cells

It was important to assess the specificity of my observations of an augmented apoptotic response as mediated through the re-expression of the endogenous D2R in GH3 cells. This was assessed using a pharmacological and a genetic approach. For the pharmacological approach this was achieved by pre-incubating GH3-D2exp cells with the D2R antagonists, eticlopride or haloperidol before challenging with DA or BC. The antagonist eticlopride and also haloperidol (figure 4.6A) effectively attenuated the augmented apoptotic response apparent in D2R positive cells (GH3-D2exp) cells to either DA or BC challenge and relative to the vehicle treated GH3 cells (GH3-D2veh).

Further support for a D2R specific effect was also apparent in the genetic siRNA knock-down approach. In these cells, D2R expression in D2R expressing cells (GH3-D2exp) was subjected to siRNA mediated knock down relative to cells that were treated with the non-targeting siRNA (siNT). The augmented apoptotic response to both BC or DA in D2R knockdown cells was attenuated in cells incubated with the specific (D2R) siRNA (figure 4.6B).

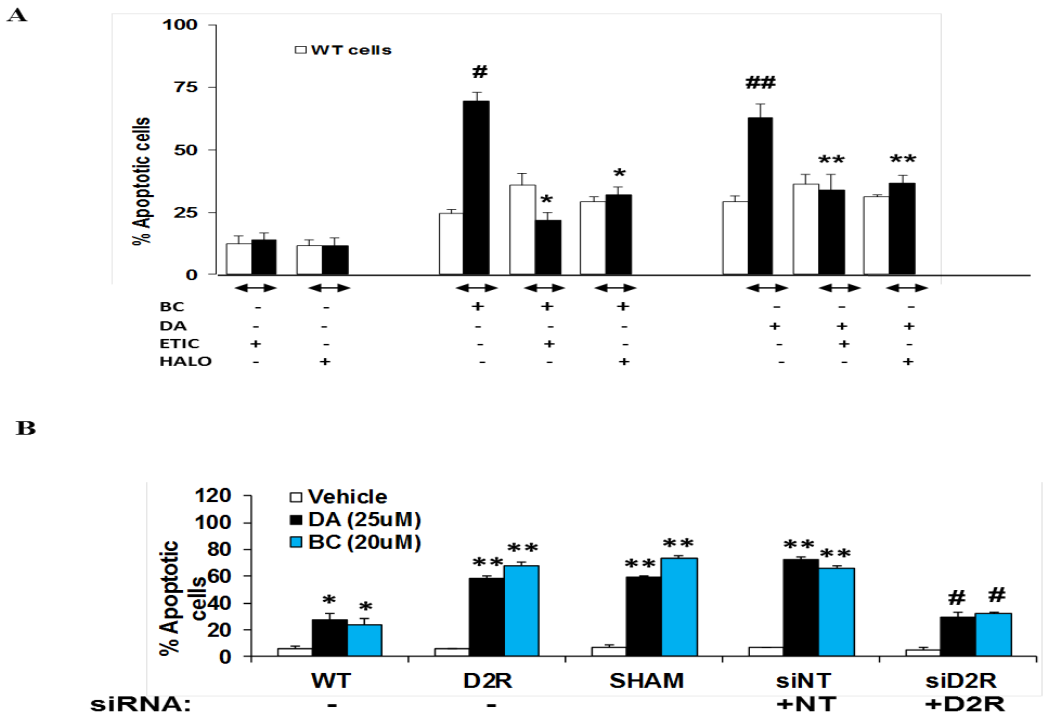



**Figure 4.6 Specificity of D2R mediated apoptosis in GH3 cells to DA and BC challenges**. A) wild-type GH3 (unfilled bars) and GH3 cells expressing D2R (filled bars) were challenged with DA (25µM) or BC (20µM) and pre-incubated with the D2R antagonists eticlopride (ETIC) or haloperidol (HALO). In panel B, white bars represent vehicle treated, Black bars represent DA treated and blue bars represent BC treated cells. #, P < vs. GH3-WT treated with BC. ##, P< vs. GH3-WT cells treated with DA. *, P < vs GH3-D2R cells treated with BC in the absence of either antagonist. **, P < vs GH3-D2R cells treated with DA in the absence of either antagonist.

B) Cells treated with the specific agonist challenges shown in A and transfected with D2R specific siRNA (siD2R) or a non-target siRNA (siNT). As further control, in D2R expressing cells and in the absence of either siRNA, cells were cultured in the absence or presence of the transfection reagent (Lipofectamine) respectively (Sham). *, P < , **, P <0.001 vs GH3-Wt vehicle alone in the absence of a specific siRNA to the D2R. #, P< 0.001 vs GH3-D2R expressing cells transfected with siNT and treated with either BC or DA. In both panels the proportion (as a percentage) of apoptotic cells are shown on the y axis. Data were analysed for significance by 2-way ANOVA with Bonferroni post-test.

### 4.3.7 Specificity of D2R mediated apoptosis in MMQ cells

The MMQ cell line expresses endogenous D2R and provided a means for a more rigorous assessment of potential secondary effects of the epidrugs used thus far in the study. The ability of DA or BC to induce apoptosis in vehicle treated and epidrug (zebularine and TSA) challenged MMQ cells was determined. The results showed that apoptotic responses were not influenced by the epidrug challenges prior to incubation with either DA or BC (figure 4.7A). In these cells and in agreement with observations in the D2R expressing GH3 cells the D2R antagonist attenuate, with approximately equal efficiency, the apoptotic response mediated by either BC or DA.

Similar to the observations in GH3-D2Rexp cells, knock-down of the endogenous D2R transcript by siRNA in MMQ cells attenuated DA and BC mediated apoptosis whereas siNT did not show any effect (figure 4.7B). Collectively these experiments provide convincing evidence that DA and BC mediated apoptosis is achieved principally through the D2R. Furthermore, pre-treatment strategies with the epidrugs zebularine and TSA did not lead to nonspecific sensitisation of these cells to apoptosis inducing agents or augmentation of D2R expression.

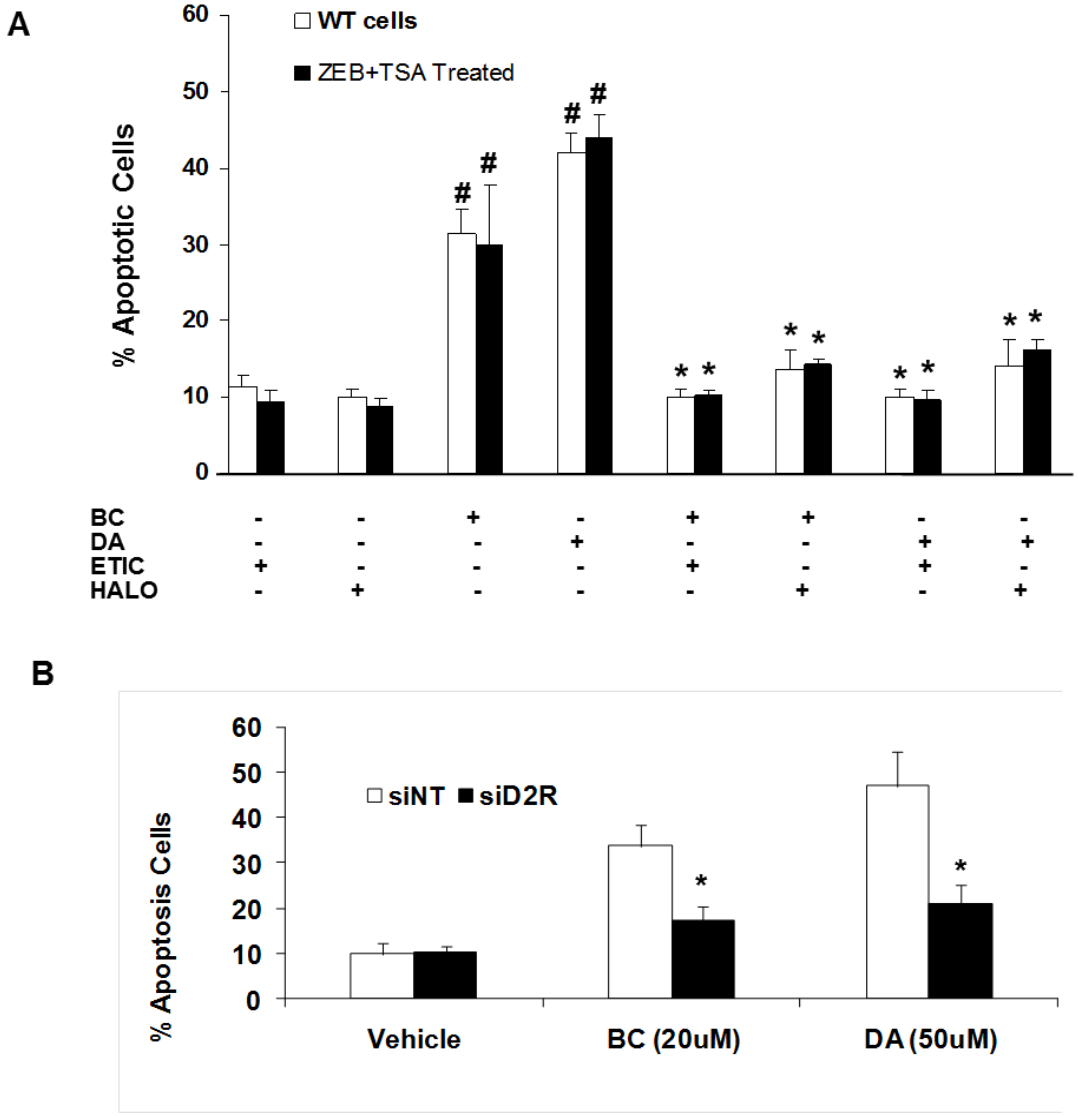


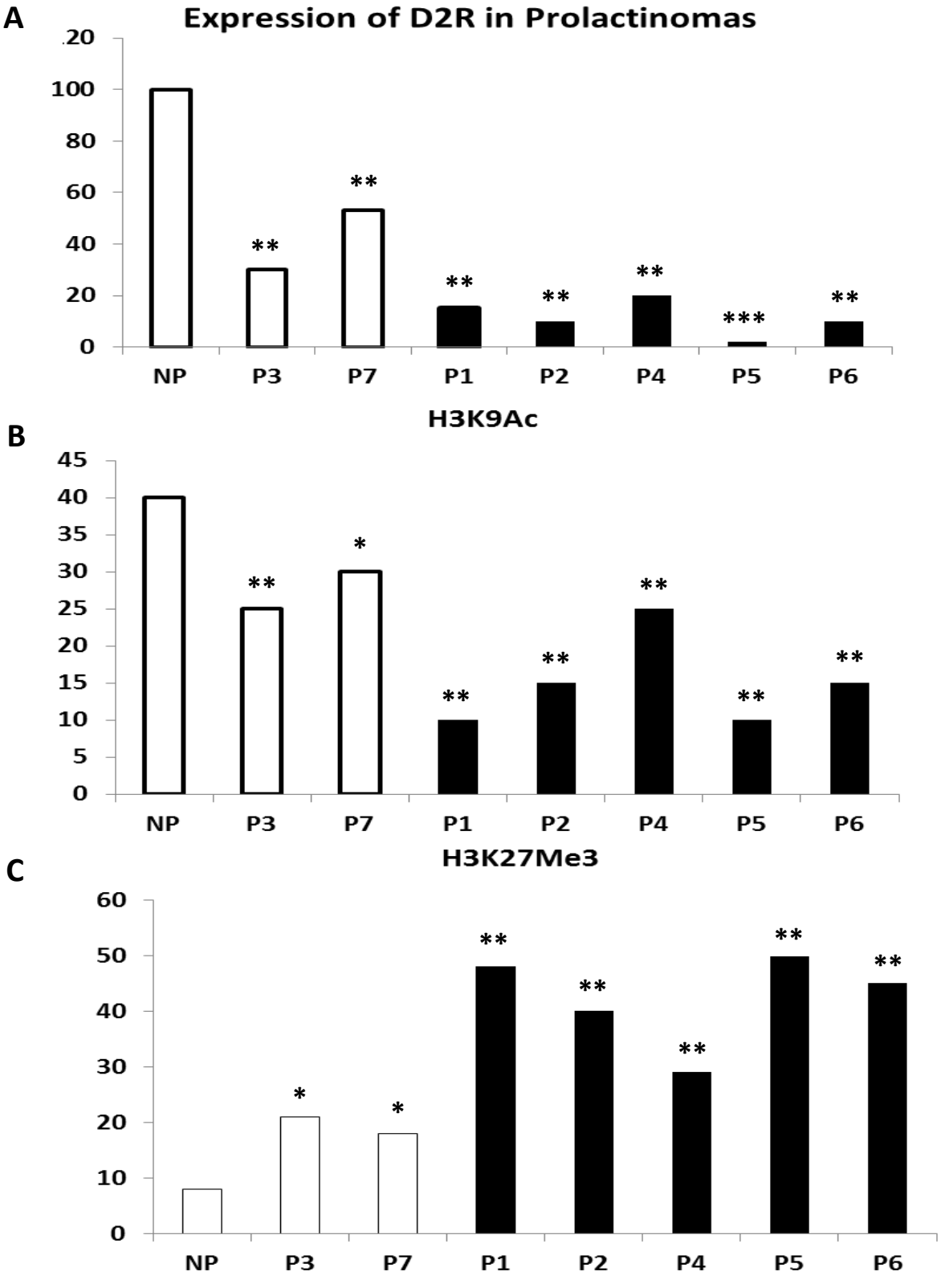

**Figure 4.7 Specificity of apoptotic response of MMQ cells to DA and BC challenges**. The experiments on MMQ cells (that express endogenous D2R) were performed essentially as described in figure 4.6. #, P <  vs MMQ cells treated with either BC (20µM) or Da (25µM) in the presence or absence of Zeb and TSA. *, P < 0.001 relative to MMQ cells challenged cells with BC or DA in the absence of either antagonist. B *, P < 0.001 vs. MMQ cells treated with BC (20µM) or DA (50µM) and siNT. In both panels the proportion (as a percentage) of apoptosis cells are shown on the y axis. Data were analysed for significance by two-way ANOVA with Bonferroni post-test.

### 4.3.8 The expression of D2R in primary pituitary adenomas:

In the absence of characterised human prolactinoma cells line(s) GH3 cells are regarded as a model system. The potential of this cell line reflecting changes apparent in primary adenomas was investigated. The expression of D2R at the transcript level was assessed in a small subset of prolactinomas, that were resistant to medical intervention, and relative to post-mortem normal pituitaries. Five of seven adenomas showed significantly reduced D2R expression at the transcript level and relative to normal pituitaries (Figure 4.8 A).

ChIP analysis of these adenomas was performed essentially as described for the pituitary cells lines. The prolactinomas showing reduced expression of D2R showed lower levels of H3K9Ac relative to post-mortem pituitaries and to adenomas expressing D2R. Similarly, prolactinomas that showed reduced expression of D2R also showed higher levels of H3K27Me3 relative to normal pituitary and prolactinomas that expressed D2R. (Figures 4.8 B and C)

A Correlation analysis of these adenomas was performed. There is a positive correlation between expression of D2R relative to normal pituitary and enrichment of H3K9AC relative to percentage input. There is a negative correlation between expression of D2R relative to normal pituitary and enrichment of H3K27Me3 relative to percentage input (Figures 4.8D and E).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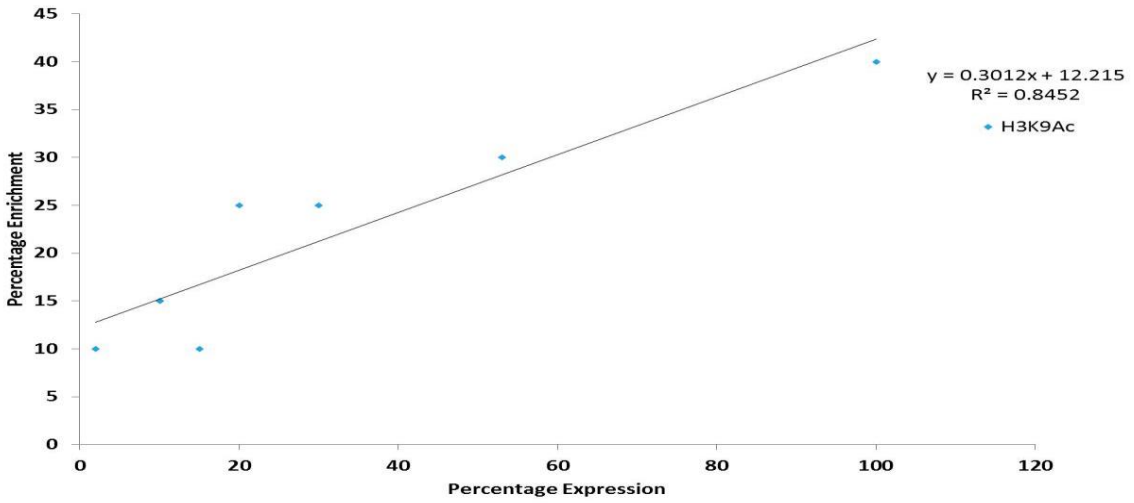


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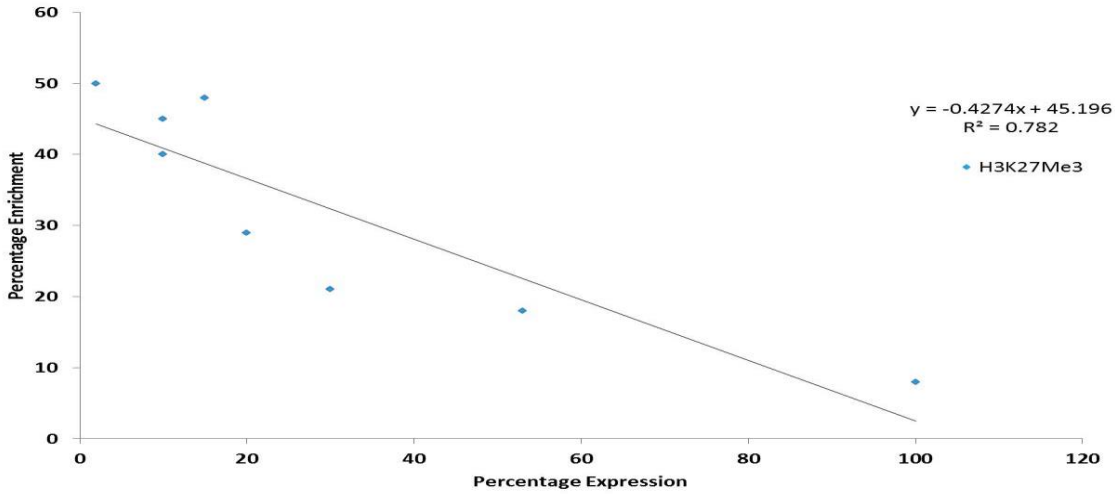



**Figure 4.8 Expression analysis and histone modification analysis in primary pituitary adenomas.** Expression analysis is shown relative to normal pituitary and ChIP analysis is shown relative to the input DNA. A) Expression analysis of seven prolactinomas relative to normal pituitary. The unfilled bars show expressers of D2R and the filled bars show reduced expression of D2R. Percentage expression of 30% and above is classed as expressers. B) H3K9Ac and C) H3K27Me3 enrichment of prolactinomas are shown relative to the input DNA. Correlation between Expression of D2R and percentage enrichment of D) H3K9Ac and E) H3K27Me3 in seven prolactinomas. All tumours used are not responsive to Dopamine treatment.

## 4.4 Discussion & Conclusions

Recent reports, using the pituitary tumour cell line GH3, have explored the apoptotic pathways activated after challenge with either DA [283], or its agonists, BC [363]. Despite the absence of the D2R in these cells, and in contradistinction to earlier report that DA-mediated apoptosis is dependent upon expression of a functional receptor [284]. These studies describe engagement and activation of *bona fide* apoptotic programs. Although the studies described by Jaubert and colleagues concluded that DA mediates its apoptotic effects through a DAT [283] our own studies were not able to confirm this finding. Although our studies showed apoptosis to D2R agonists we were aware that in the absence of a detectable D2R or DAT the mechanisms by which DA induced apoptosis had not been definitively identified [363]. Therefore, to extend these previous studies and also to address the contribution of the D2R to agonist induced apoptosis potential mechanisms responsible for the loss of the D2R in these cells were explored. In addition strategies to induce re- expression of the endogenous receptor and the effects of receptor occupancy on apoptotic end points were also explored.

I show here, for the first time, hypermethylation of a portion of the D2R CpG island in GH3 cells relative to that seen in NRP and in MMQ cells that express D2R and suggests a relationship between loss of D2R expression and methylation. To investigate this potential relationship between epigenetic change and gene silencing, GH3 cells were subjected to pharmacological epidrug challenges designed to inhibit DNA methylation and histone deaceytlation. Although incubation of GH3 cells with single agents alone was responsible for a marginal but reproducible decrease in methylation across the CpG island, re-expression of the D2R transcript was only apparent to zebularine, as a single agent challenge at dose of the order of ~3μM. However co-incubations with both agents (zebularine and TSA) was responsible for a synergistic increase in D2R expression and more of a significant decrease in CpG island methylation. Studies in multiple other tumour types and their cell lines have shown that zebularine principally through DNA

demethylation, reactivates gene expression and that the cognate CpG islands are partially de-methylated following drug challenges [368-370].

Histone modifications either alone or in concert with changes in DNA methylation patterns can lead to either gene activation or repression dependent on which residue(s) are modified and also the specific modification(s) [91]. As example, acetylation of histone 3 lysine 9 (H3K9Ac) is frequently associated with active genes [120]. However, trimethylation of histone 3 lysine 27 (H3K27Me3) is associated with promoters that are transcriptionally repressed [355]. In GH3 cells that fail to express D2R, and relative to NRP and MMQ cells, that express this receptor, the histone modifications, as determined by ChIP analysis, are consistent with repressed genes. In contradistinction to these findings in rodent pituitaries and MMQ cells enrichment for H3K9Ac and depletion of H3K27me3 is apparent. However, combined drug challenges of GH3 cells with zebularine and TSA resulted in an enrichment of H3K9Ac and a decrease in the enriched fraction of H3K27Me3. These findings are consistent with these agents leading to histone modifications and decrease in CpG methylation. Furthermore these changes to the epigenomic landscape lead to re-expression of D2R in these cells. A single more recent study has also demonstrated induced re-expression of the endogenous D2R in GH3 cells. These studies, from the Spada group used retinoic acid to induce re-expression of this receptor in primary human pituitary tumours, however this agent was without effect in GH3 cells. A recent report of retinoic acid in promyelocytic leukaemia cells has also shown that retinoic acid is responsible for induction of change in H3 acetylation and gene expression [371]. The effects of retinoic acid as modifier of the epigenomic landscape are explored and discussed in a subsequent section of this thesis.

The re-expression of endogenous D2R in GH3 cells using the epidrugs zebularine and TSA provided an opportunity to examine the apoptotic end points in these cells and relative to those seen in cells that do not express the endogenous receptor. These studies, in cells that differed only in the D2R status, showed an augment and presumed D2R mediated response to drug

challenges with either DA or BC. In this case the agents have been shown previously to induce apoptosis in these cell lines [283, 363].

A caveat associated with the findings thus far described is that even in the same cell line nucleotide analogues do not necessarily have the same effect on the transcriptome in a particular tumour or tumour cell line and are reviewed in [128]. For example Flotho and colleagues [176] showed that after individual treatment of the same cell line with the nucleoside analogs, decitabine, azacitidine and zebularine the identified unmasked genes showed little overlap with each other and a considerable number of genes were also down regulated. Similar conclusions were reached when comparing the differential effects of decitabine, a DNMT knockout model system, and the HDAC inhibitor TSA [177]. In these cases, the effects on gene expression did not seem to be dependent on dosage and duration which would be expected if the drugs exert their effects solely by incorporation into the DNA during the replicative S phase of the cell cycle. These reports, as example only, prompted me to further investigate the specificity of these observations in greater detail.

The specificity of a D2R mediated and augmented apoptosis response to DA and or DA agonist BC was addressed through pharmacological (receptor antagonists) and genetic (RNA interference) approaches. In these studies, inhibition or interference of dopaminergic signalling pathways was initially investigated in GH3 cells, where endogenous D2R expression had been unmasked and also in MMQ cell line that expressed endogenous functional D2R [372]. In D2R expressing GH3 cells the augmented apoptotic response to either DA or BC challenges was attenuated through pre-incubation with the D2R antagonists, haloperidol or eticlopride. This was reinforced using siRNA mediated knockdown of the D2R in these cells, whereas a non-target siRNA (siNT) was without effect. In MMQ cells, receptor antagonist experiments also attenuated apoptotic responses to DA and BC. In these cells, pre-challenge with zebularine and TSA did not augment apoptosis, however this end point was effectively attenuated by receptor antagonists. Finally and similar to the

findings apparent in D2R expressing GH3, siRNA mediated knockdown of endogenous D2R in MMQ cells also attenuated DA and BC induced apoptosis. Although these studies support the conclusion that re-expression of the D2R in GH3 cells is indeed specific for the observed augmented apoptotic response there may be other aberrations in this apoptotic pathway that we have not identified.

The findings from GH3 cell line as a model system for primary pituitary adenomas was also investigated. In these cases, the first line treatment is dopamine agonists. However, a proportion of prolactinomas are resistance to this intervention and is frequently attributed to reduced D2R number. In these cases or in cases of dopaminergic intolerance these tumours are removed surgically. These prolactinomas, albeit small in numbers were used. Five of seven adenomas showed reduced expression of the D2R. These adenomas that showed reduced expression of D2R also showed depletion for the H3K9Ac modification and enrichment for the histone modification associated with silenced genes, H3K27Me3. However, in the two prolactinomas that expressed the D2R and in normal pituitary, higher levels of H3K9Ac and lower levels of H3K27Me3 were apparent. These results and the concordance between non-expressing prolactinomas and GH3 cell lines provide the impetus toward a more detailed investigation of larger numbers of primary tumours.

Following publication of the findings reported here in the Spada group have shown that FLNA transcript expression is reduced in tumours that also show a decrease in D2R expression. Furthermore these findings are confined to DA insensitive prolactinomas. This group proposes loss of FLNA expression may be a possible mechanism involved in the reduction of D2R, indicating that FLNA is perhaps required to maintain D2R expression. This could possibly lead to an avenue of future studies and further work to investigate the levels of FLNA associated with the prolactinomas or GH3 cells used within the study. Although alterations in the FLNA promoter

methylation were not investigated by this group it is possible that FLNA expression is also subject to epigenetic modifications. [364].

This study, although principally in a model system shows important, albeit potential, consequence for clinical management of primary pituitary adenomas that irrespective of subtype express to varying degrees DA receptor subtypes [222, 292, 357-360, 373, 374]. In this context several reports in different tumour types now describe strategies where epigenetic therapy is combined with chemotherapy or radiotherapy with improved treatment outcomes. These possibilities have been subject to recent review [128]. In addition, this approach, that is where DNMT and HDAC inhibitors are used in combination with, as example anti-estrogens are effective in restoration of estrogen receptor alpha responsiveness to previous ER negative and antiestrogen resistant tumour cells [375-377].

In conclusion the finding from this part of the study uncovers the epigenetic aberrations that are responsible for the D2R receptor silencing in the pituitary cell line GH3 and possibly the primary pituitary adenomas. Restoration of functional receptor through an epigenetic therapy strategy re-establishes a functional dopaminergic pathway that is sensitive to DA and DA agonist challenges. These results prove useful and encouraging combined treatment approaches for the medical management of prolactinomas and other pituitary tumour subtypes through the activation of the Dopamine D2 receptor.

# Chapter 5: Epigenomic Silencing of the BMP-4 Gene in Pituitary Adenomas: A Potential Target for Epidrug-Induced Re-expression

# 5. Epigenomic Silencing of the BMP-4 Gene in Pituitary Adenomas: A Potential Target for Epidrug-Induced Re-expression

## 5.1 Aims & Objectives

The molecular aberrations responsible for inappropriate expression of BMP-4 in sporadic pituitary tumours are not known. Equally the way in which modifications of the epigenetic landscape might impact upon BMP-4 expression has not been described. These types of changes, apparent as CpG island methylation and/or histone modifications were examined in each of the major pituitary adenoma subtypes. Furthermore, to gain mechanistic insight, the causal links between identified aberrations and expression were also investigated. For these studies a pharmacological unmasking strategy was employed, using the so named “epidrugs” that inhibit DNA methylation and histone deaceytlation were employed.

## 5.2 Introduction

The bone morphogenetic protein (BMP) are members of the TGF-β superfamily of multifunctional secretory peptides [378, 379]. To date, more than 20 TGF-β family members have been described, and these can be further subdivided into several groups on the basis of their structure and function [380]. Two of the best studied members of this family are BMP2 and BMP4. These share significant homology and have been shown to play significant autocrine and paracrine roles in the anterior pituitary organogenesis [343, 381]. BMP-4 is also a mediator in the pathogenesis of spontaneous prolactinomas [31] where overexpressing of this cytokine was first described in a dopamine receptor type 2 deficient mouse (D2R2-/-). The pituitary adenomas were confined to female offspring where an extracellular binding protein and negative regulator of BMP-4, noggin, is reciprocally down regulated. Furthermore, in the same report and reinforcing these findings, overexpression of BMP-4 was also apparent in estradiol-induced rat prolactinomas and in human prolactinomas relative to normal tissue and to other pituitary adenoma subtypes [31].

In normal pituitaries, BMP-4 expression, as determined by immunohistochemistry (IHC), is principally confined to the somatotrophs, corticotroph and thyrotroph cell populations and is barely detectable in lactotroph cells [32, 382]. Subsequent to identification of BMP-4 overexpression in prolactinomas, reduced IHC expression of this protein was reported in a significant proportion of corticotroph adenomas from patients with Cushings disease[32]. Furthermore, a single report has also described reduced expression of BMP-4 as determined by RT-PCR, in a gonadotrophinomas, that secretes FSH, and in non-functioning pituitary adenomas [383].

The adenoma subtype-specific differential expression of BMP-4 is somewhat unusual and suggests that this cytokine is bifunctional. If this is the case, then the consequence of BMP-4 expression are perhaps dependent on cellular context and/or the repertoire of transcription factors within a particular cell type. The bifunctional, cell-type-specific roles are also apparent in pituitary tumour cell lines Thus, in GH3 cells BMP-4 stimulates and noggin inhibits in *vivo* tumour growth and in *vitro* proliferation, and these cells are in lactosomatotroph cell lineage [31]. Contrary to this, in AtT-20 cells, in the corticotroph cell lineage, BMP-4 inhibits ACTH secretion and in vitro cell proliferation [32]. In addition, in these cells, transfected with a dominant negative form of Smad-4 or with BMP-4 extracellular inhibitor noggin, increased tumorigenicity in a nude mice model is apparent [32]. These findings, therefore, provide convincing evidence in a cell-type-specific context that BMP-4 is indeed bifunctional, a characteristic exhibited by several other proteins in other tumour types [194, 384, 385].

Since genetic mutations are an infrequent occurrence in this tumour type it was important to determine if epigenetic changes were responsible for the inappropriate expression of BMP-4 and to investigate if these changes, if apparent could be reversed. For these studies it was necessary to investigate change in primary pituitary adenomas and also in pituitary tumour cell line as a model systems.

## 5.3 Results

### 5.3.1 Expression of BMP-4 in primary pituitary adenomas

The expression of BMP-4 was determined in the major pituitary adenoma subtypes at the transcript and protein level (qRT-PCR and ELISA) and as described in the materials and methods section. The expression of BMP-4 at the transcript level was variable within each of the subtypes (Fig 5.1A). A significant proportion, 14 out of 16 non-functioning adenomas, four of four GH secreting adenomas and five of seven corticotrophinomas showed reduced transcript expression relative to the normal pituitaries. However four of nine prolactinomas showed increased expression of BMP-4 relative to normal pituitaries and the other tumour subtypes (Fig 5.1A). In these cases GAPDH was used as the internal control housekeeping gene.

Quantitative expression of BMP4 at the protein level was determined by ELISA. The result, as determined by BMP-4 ELISA mirrored the findings apparent at the transcript level (Fig 5.1B). However, in the corticotrophinomas although BMP-4 protein was reduced relative to the prolactinomas a proportion showed increase relative to normal pituitaries.

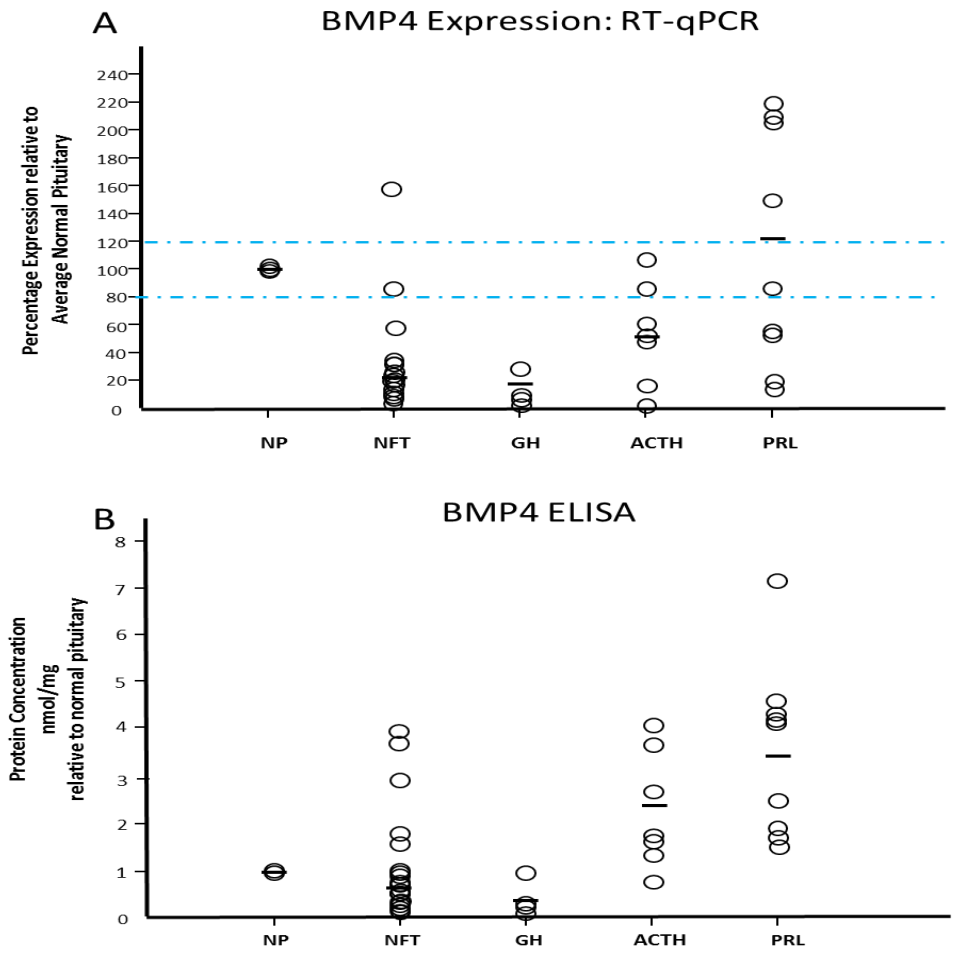



**Figure 5.1: Expression analysis of BMP-4 in primary pituitary tumours:** From left to right normal pituitaries (NP), non-functioning adenomas (NFT), growth hormone secreting tumours (GH), corticotrophinomas (ACTH) and prolactinomas (PRL). **A**) RT-qPCR analysis of BMP-4 transcript expression in primary pituitary tumours and normal pituitary tissue. The percentage expression is shown relative to the mean of 4 normal pituitaries **B**) ELISA analysis of BMP-4 protein expression. The percentage expression is relative to the mean of two normal pituitaries. All experiments were performed thrice with triple determinations. The bar in each column represents the mean of the individual determinations for the normal pituitaries and each of the adenoma subtypes. For RT-qPCR, the dotted lines represent three times the upper and lower SD of the mean expression in the normal pituitaries. Individual adenomas, showing increase or substantial reduction in expression, where categorised on the basis of expression above or below these limits respectively (see materials and methods).

### 5.3.2 Methylation status of the BMP-4 CpG islands in primary pituitary adenomas

*In silico* analysis of the human BMP-4 gene identified two CpG islands, one encompassing exon 1 and the upstream region of the gene and the other encompassing exon 3. Sodium bisulphite sequencing which included at least 10 CpG dinucleotides in each of these islands in normal pituitaries and five or more adenomas of each subtype, failed to show changes in methylation status. In all cases and irrespective of expression status methylation varied between 4-7%. These findings suggest that change in methylation status are not responsible for differential expression of BMP-4 in primary pituitary adenomas as shown in figure 5.2A and B.

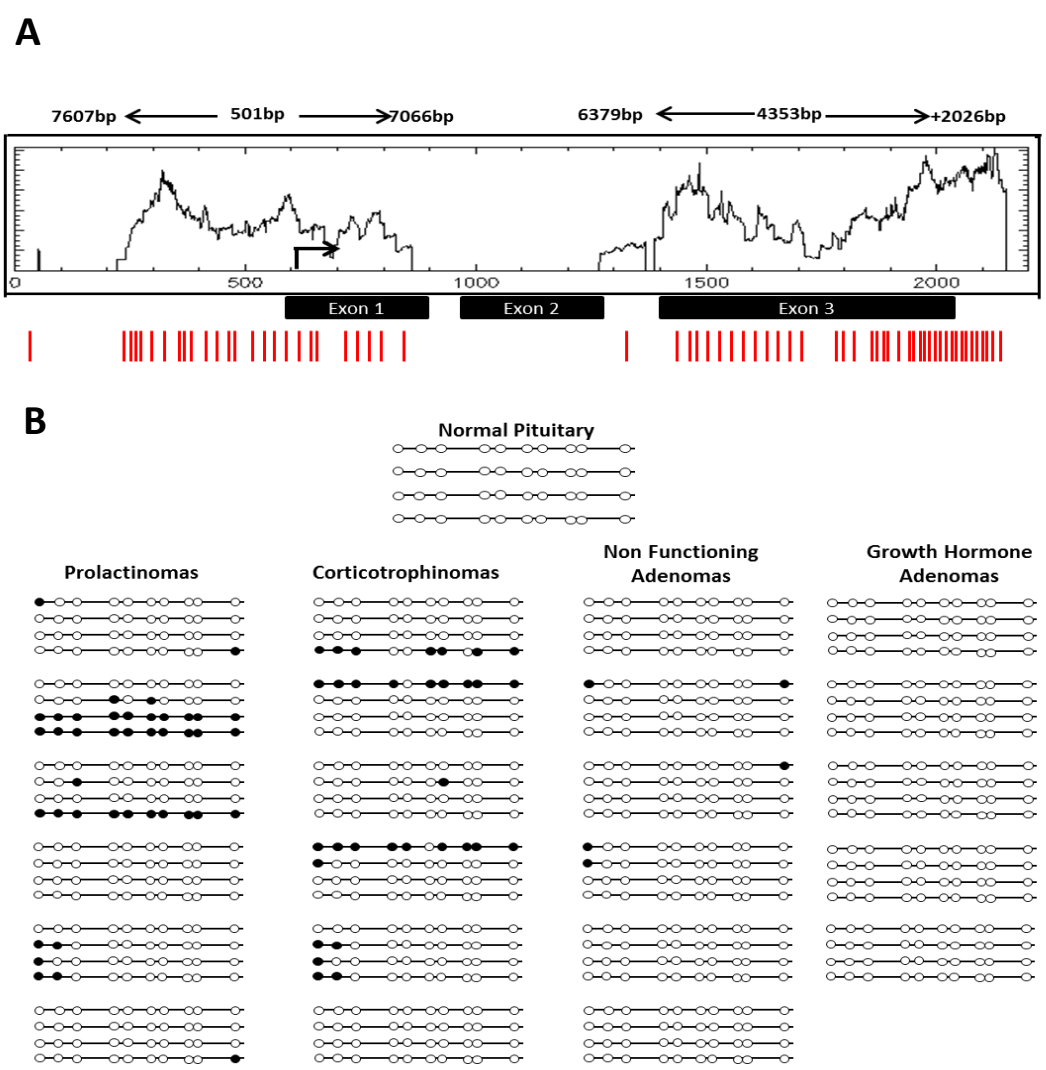
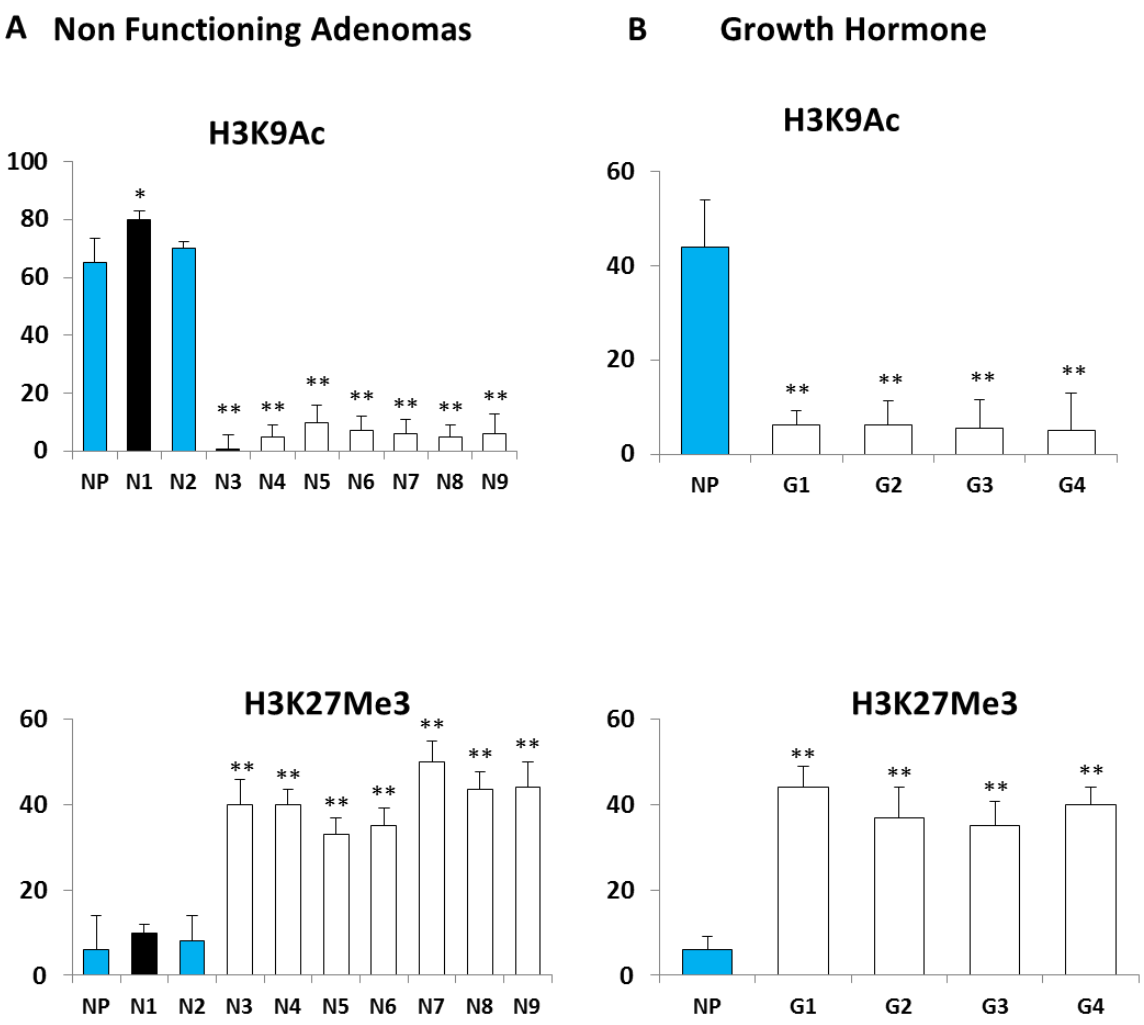



**Figure 5.2: Methylation of BMP4 CpG island in primary pituitary adenomas.** A) *In silico* analysis of the human BMP4 CpG island locus region. Two CpG islands are identified and shown relative to the transcription start site (black bent arrow) and were determined using CpG island searcher and drawn using a web based program (www.ebi.ac.uk). The CpG island plots shows CpG islands associated with Exon 1 and 3. Exon 1 showed barely detectable methylation of the CpGs within its associated island (data not shown). Broadly similar findings were evident within the exon 3 associated CpG in some tumours (see figure above). The observed GC percentage is plotted across the region and is shown relative to the transcription start sites. B) beads on a string representation across 10 CpG dinucleotides shown in exon 3. Individual clones from each six primary pituitary tumour subtypes (and five GH secreting adenomas) and relative to normal pituitary are shown. The filled and unfilled circles (beads) represent methylated and unmethylated CpGs, respectively.

### 5.3.3 Chromatin status of BMP-4 promoter region in primary pituitary adenomas

To determine the potential impact of chromatin modifications on expression of BMP-4 ChIP analysis was employed on samples where I had previously determined expression (figure 5.1) and CpG island methylation status (Figure 5.2). The modifications examined are those associated with active genes, H3K9Ac, or silent gene, H3K27Me3 as described in the previous chapter (chapter 4). For each of the individual adenomas showing loss or significantly reduced expression of BMP-4 as determined by qRT-PCR (white bars) ChIP analysis showed enrichment for H3K27Me3 and depletion for H3K9Ac relative to normal pituitaries (Fig 5.3 A-D). In tumours showing similar (Blue bars) or increased expression (Black bars) of BMP-4, histone modifications were similar to those apparent in post-mortem normal pituitaries. Thus in the majority of adenomas available for analysis, at the histone modifications sites examined, increase in H3K27me3 and decrease in H3K9Ac appear specific for silencing or significantly reduced expression. However two adenomas that express BMP4 (Blue bars) at levels similar to normal pituitaries also show chromatin modifications that characterise loss or reduced expression.


A Non Functioning Adenomas
B Growth Hormone
H3K9Ac
100
80
60
40
20
0
NP N1 N2 N3 N4 N5 N6 N7 N8 N9
H3K9Ac
60
40
20
0
NP G1 G2 G3 G4
H3K27Me3
60
40
20
0
NP N1 N2 N3 N4 N5 N6 N7 N8 N9
H3K27Me3
60
40
20
0
NP G1 G2 G3 G4

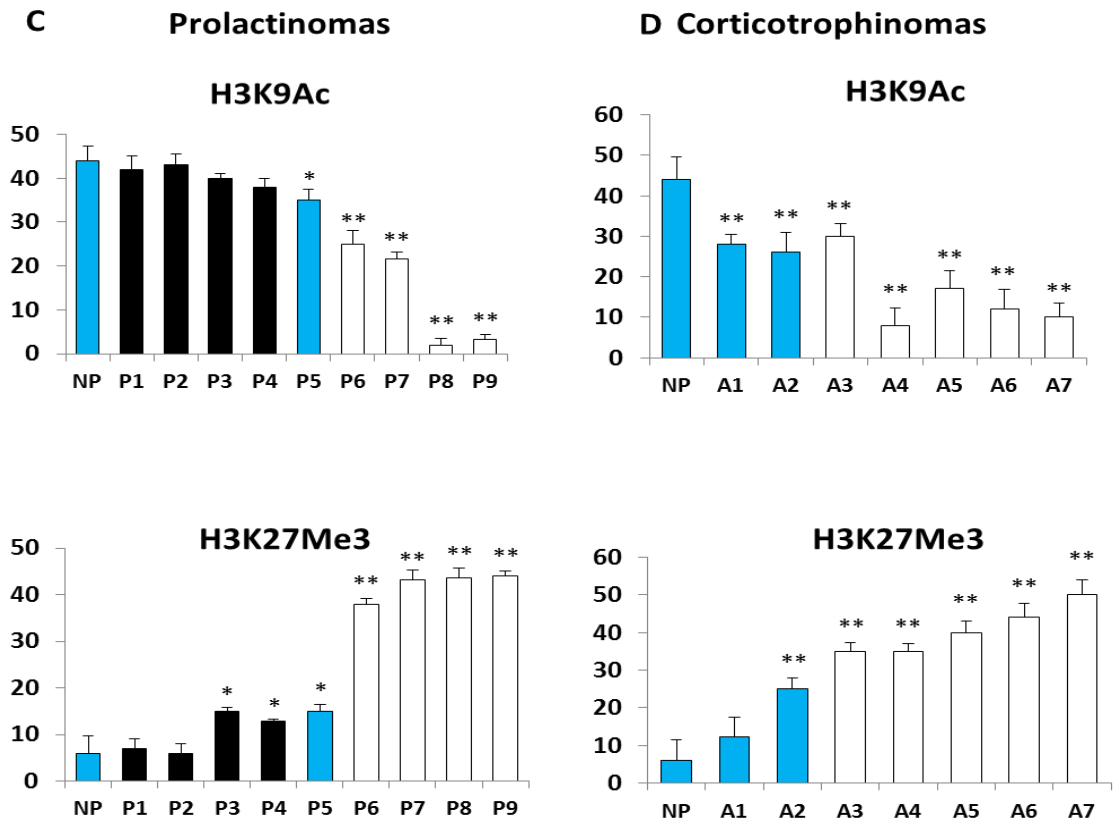



**Figure 5.3: ChIP Analysis of the BMP-4 gene in primary pituitary adenomas:** In each panel and from left to right the bars represent normal pituitaries (NP) and in panel A) human non-functioning, B) growth hormone secreting, C) prolactinomas, and D) corticotrophinomas. Enrichment is expressed relative to the modification seen in input DNA and determined for, H3K9Ac and H3K27Me3. All experiments, in available adenomas, were preformed thrice with triple determination. Error bars show SEM. In each panel the bars represent enrichment in the normal pituitaries and tumour cohort shown in Fig.5.1. The blue bars represent normal pituitaries and tumours showing similar expression levels as determined by qRT-PCR, the black and unfilled bars represent adenomas where expression is either above or below the limits described in figure 5.1 respectively and relative to normal pituitaries. Not all of the tumours shown in Fig 5.1 were examined due to limitations in tumour specimen. ChIP data was assessed relative to normal pituitaries. *, $P < 0.05$, ** $< 0.01$ vs normal pituitaries. Data were analysed for significance by Mann Whitney U test. All experiments were done thrice with triplicate determinations. Error bars show SEM.

### 5.3.4 Expression of BMP4 in pituitary tumour cell lines

The expression of BMP4 was analysed at the transcript level in pituitary tumour cell lines relative to normal rat/mouse pituitary. The internal control used was PGBD which showed similar levels of cDNA in NRP/NMP as the pituitary tumour cell lines. The expression of BMP-4 at the transcript level in GH3, MMQ and AtT-20 is significantly reduced relative to normal pituitaries (Figure 5.4)

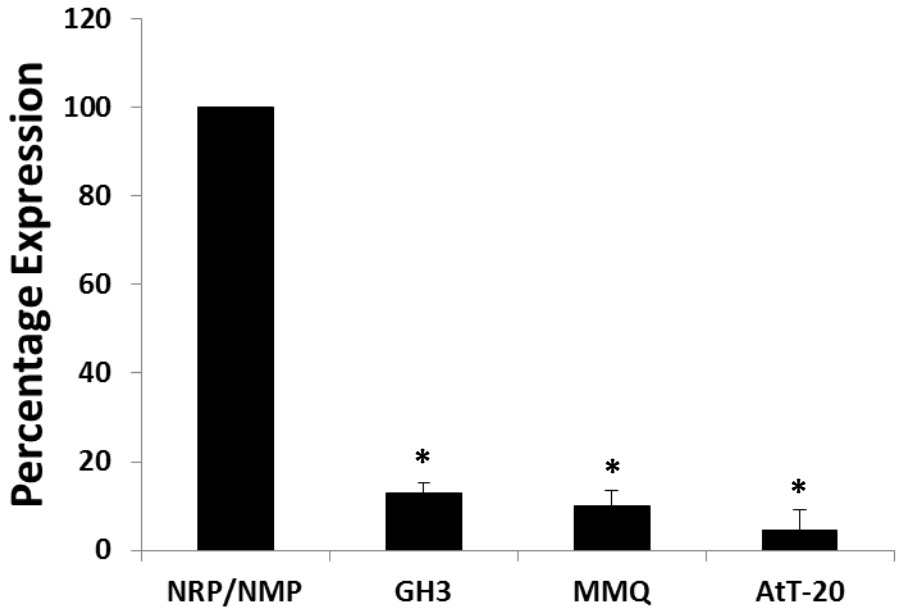



**Figure 5.4: Expression analysis of BMP4 in pituitary tumour cell lines.** The expression of BMP-4 in GH3, MMQ and AtT-20 cells, as determined by RT-qPCR, is shown as a percentage and relative to their respective normal pituitaries. All experiments were performed thrice with triplicate determination. Error bars show SEM. *, $P<0.001$ vs normal pituitary. Data were analysed for significance with a paired t test with Welch's correction

### 5.3.5 Methylation status of the BMP-4 CpG Island in pituitary cell lines

*In silico* analysis of the murine and rodent BMP-4 genes identifies bona fide CpG islands in rat (Figure 5.5A) and the mouse homolog (Figure 5.5 B) of the BMP-4 gene. In rodent cell the BMP-4 gene has one single CpG island extending upstream from exon 1 into the presumed promoter region. A similar analysis of the murine gene identified two CpG island, one that is predominantly within an intronic region between exon 1 and 2, and the second encompassing exon 3.

Sodium bisulphite sequencing of GH3 and MMQ cells in the somatolactotroph and lactotroph (Fig 5.5 A) and of AtT-20 cells, in the corticotroph cell lineage (Fig 5.5B) were compared to the sequence of their respective normal pituitary counterparts. Sodium bisulphite sequencing of normal pituitaries did not reveal methylation with the CpG islands in either species. However in contrast to these findings, in GH3, MMQ and AtT-20 cells, these regions were heavily methylated. However, in AtT-20 cells, CpG methylation was confined to the exon 3 CpG island.

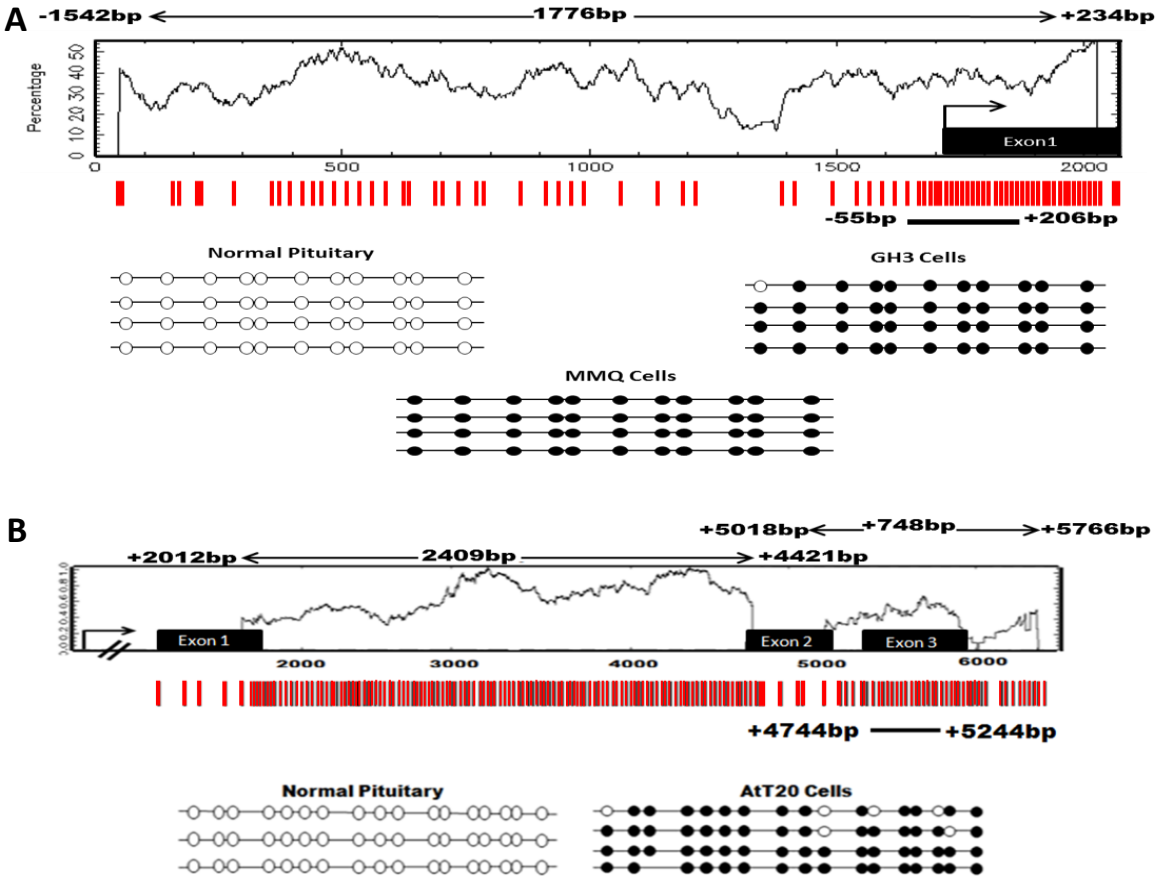
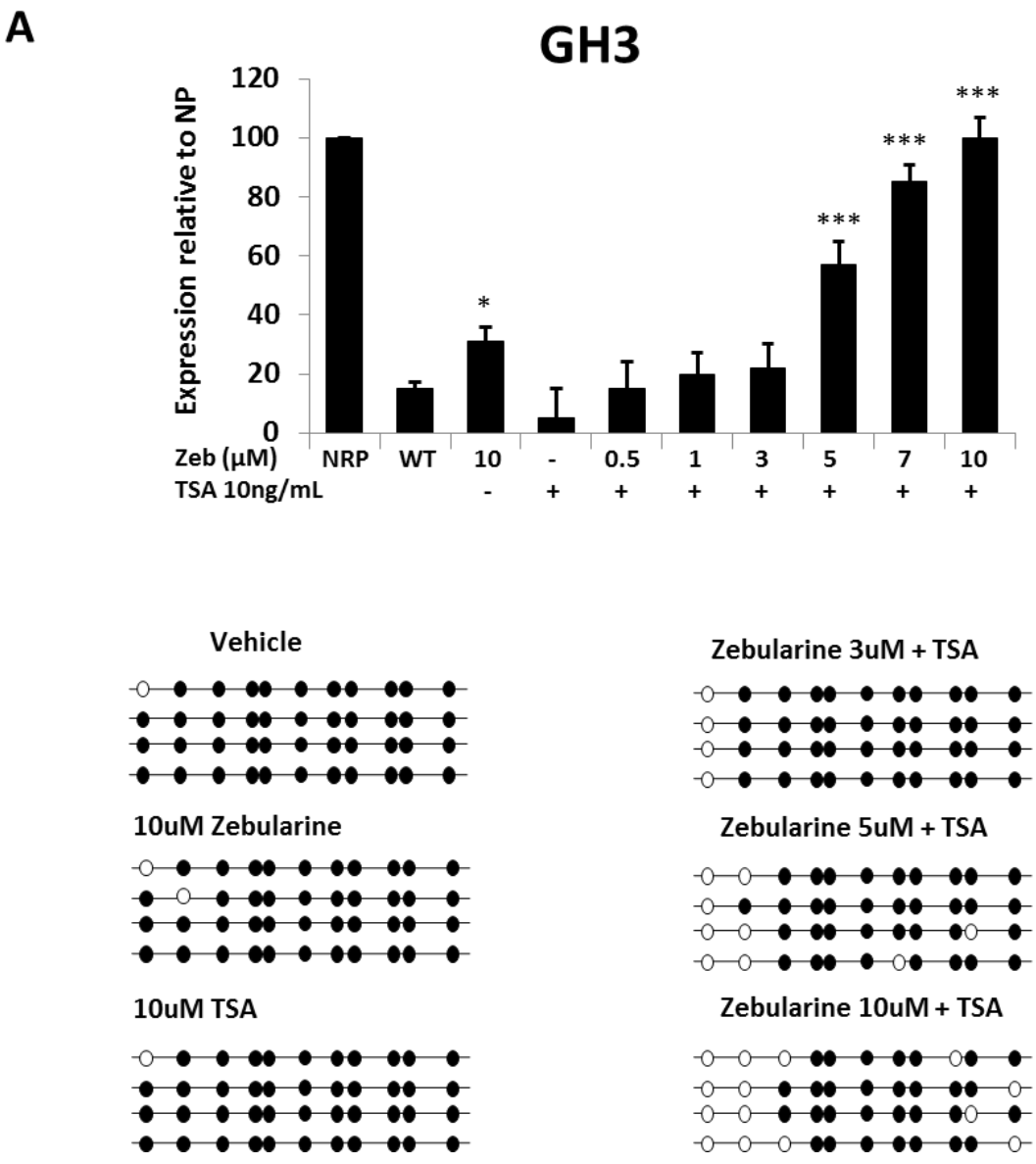



**Figure 5.5*: In silico* analysis of the BMP-4 CpG island locus regions:** Panel **A** shows that the rat CpG Island extends from -1542bp to +234bp, relative to the transcription start site (black bent arrow) and was determined using CpG island searcher and drawn using a web based program (www.ebi.ac.uk). The observed GC percentage is plotted across the region and the vertical tick marks signify the individual CpGs. The horizontal black bar underlines the 11 CpG dinucleotides interrogated for methylation status and these are shown as a beads-on a-string representation. The filled and unfilled circles (beads) represent methylated and unmethylated CpGs, respectively for individual clones in normal rat pituitary and GH3 cells. Panel **B** shows a similar analysis of the mouse CpG island locus region to that described in panel **A.** The CpG islands extending from +2012bp to +4421bp and from +5018 to +5766bp, relative to the transcription start site (black bent arrow). Sodium bisulphite sequencing of individual islands showed methylation to be confined to the downstream CpG island. In this case the beads- on- string representation is across 17 CpGs in this island in normal mouse pituitary and At-T20 cells.

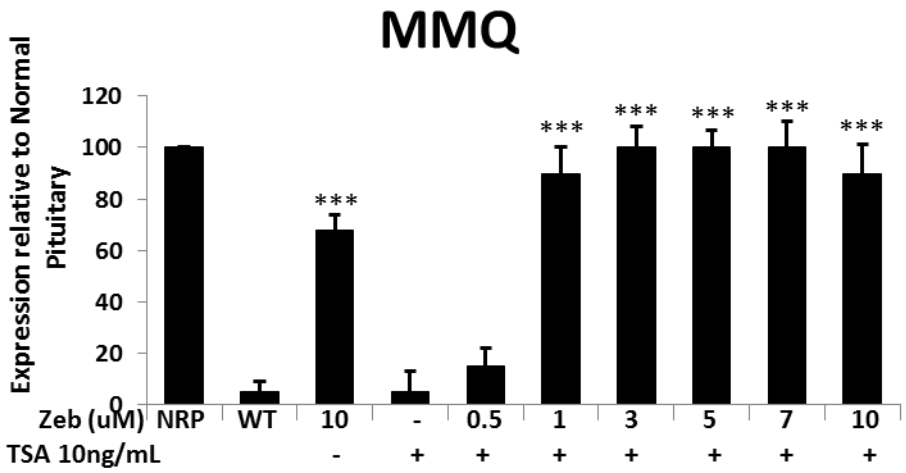
### 5.3.6 Drug-Induced demethylation studies in pituitary cell lines

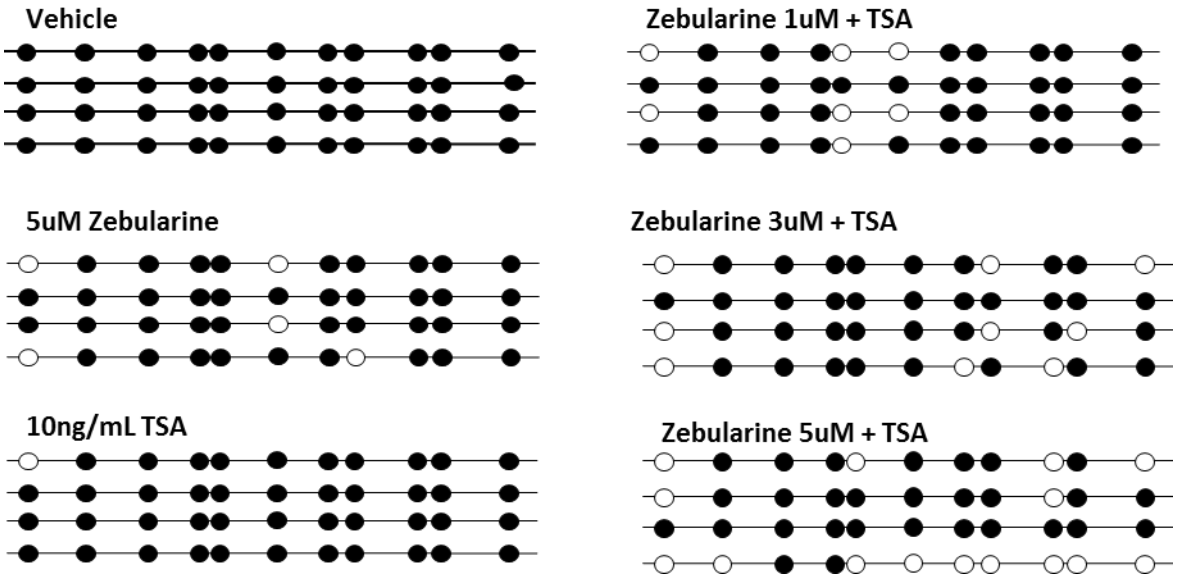
In GH3, MMQ and AtT-20 cell lines, increased CpG island methylation was associated with significantly reduced expression of BMP4 as determined by qRT-PCR and relative to their respective normal pituitaries (Fig 5.6 A-C). In cell lines incubated with zebularine, a modest but reproducible statistically significant increase in BMP4 expression was apparent in GH3 cells. At the highest concentration of zebularine used (10μM) alone there is an increase in the BMP4 expression in MMQ cells, however, this single agent was not effective in AtT-20 cells. However in all three cell lines GH3 (figure 5.6 A), MMQ cells (figure 5.6B) and AtT-20 cells (figure 5.6C) combined challenges with zebularine and TSA induced robust re-expression of BMP4 whereas TSA alone showed limited effects on BMP4 expression.

Sodium bisulphite sequencing of GH3, MMQ and AtT-20 cells showed that single agent drug challenges was associated with barely detectable change (decrease) in CpG island methylation (figure 5.6 A-C). However, combined drug challenges with zebularine and TSA were associated with a decrease in the CpG dinucleotide methylation. In these cases at the highest dose employed, a 30-40% decrease in methylation was apparent in the cell lines.


A
GH3
Expression relative to NP
120
100
80
60
40
20
0
*
***
***
***
Zeb (μM)
NRP
WT
10
-
0.5
1
3
5
7
10
TSA 10ng/mL
-
+
+
+
+
+
+
+
Vehicle
10uM Zebularine
10uM TSA
Zebularine 3uM + TSA
Zebularine 5uM + TSA
Zebularine 10uM + TSA

**B**


MMQ
Expression relative to Normal Pituitary
120
100
80
60
40
20
0
***
Zeb (uM) NRP WT 10 - 0.5 1 3 5 7 10
TSA 10ng/mL - + + + + + + +


Vehicle
Zebularine 1uM + TSA
5uM Zebularine
Zebularine 3uM + TSA
10ng/mL TSA
Zebularine 5uM + TSA

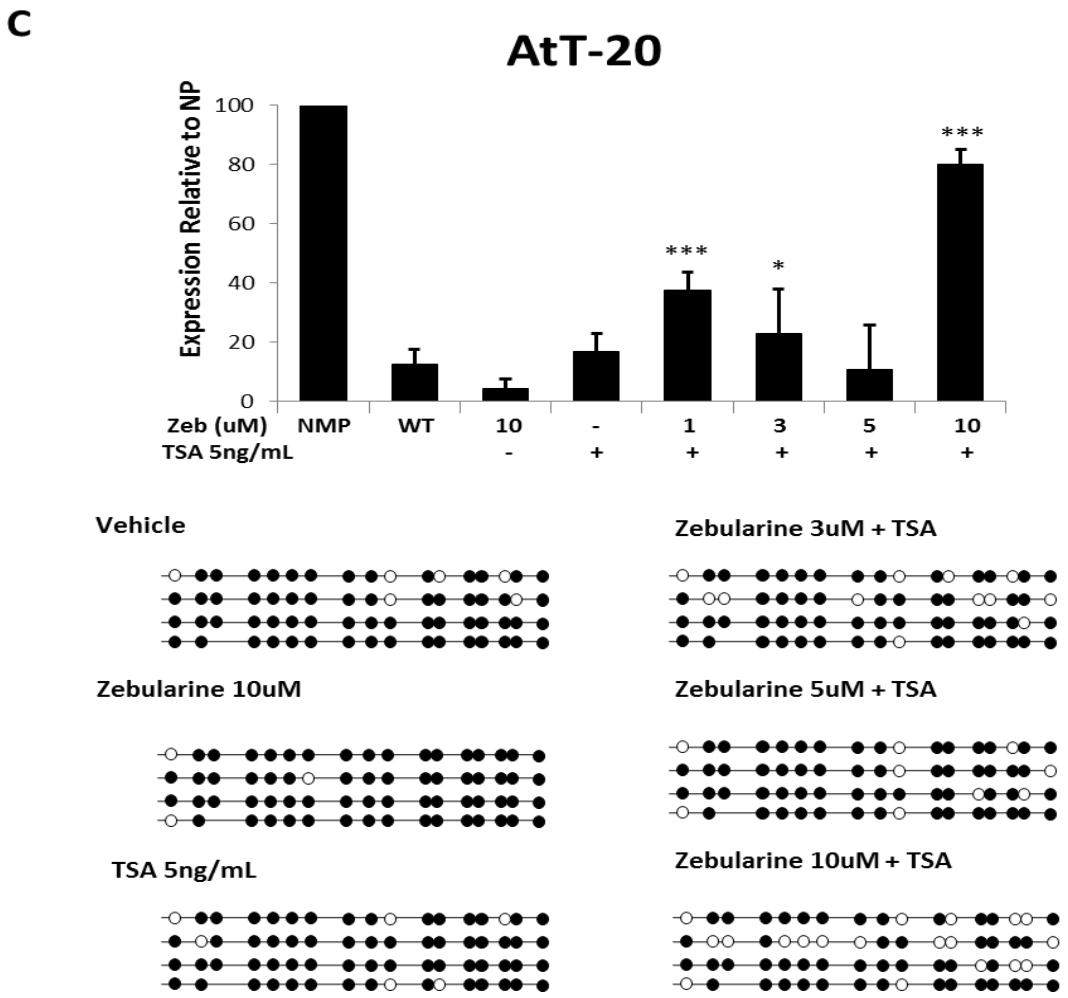



**Figure 5.6: Expression and methylation status of BMP-4 in pituitary cell lines**: Panel **A**, GH3 cells, panel **B**, MMQ and panel **C** AtT-20 cells following challenge with zebularine (Zeb) or TSA alone or in combined incubations. In each case, the upper sections of panels shows RT-qPCR analysis of BMP-4 expression in response to the challenges and drug doses shown in the figures. BMP-4 expression is shown as a percentage relative to expression seen in their respective normal pituitaries. The lower part of each panel shows sodium bisulphite sequencing across the CpG islands. The beads on a string, in each case, represent analysis of four individual clones following each treatment. The filled beads represent CpG dinucleotide methylation and unfilled beads represent unmethylated dinucleotides. Drug-induced re-expression was assessed relative to vehicle treated cells. *, $P < 0.05$, ** <0.01, *** <0.001 *vs* vehicle alone. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. All experiments were done thrice with triplicate determinations. Error bars show SEM.

### 5.3.7 BMP-4 histone modifications post drug challenges

To determine the potential impact of chromatin modifications on BMP-4 expression, ChIP analysis was employed. In GH3, MMQ and AtT20 cell lines and in these cases BMP-4 associated histone modification indicative of gene silencing was apparent. Figure 5.7 shows reduced H3K9Ac and increased H3K27Me3 of the BMP4 gene in the cell lines relative to input and compared to their normal pituitary counterparts. In all three cell lines the histone mark associated with active gene H3K9Ac was enriched after incubations with zebularine or TSA as single agents, however, in combined drug challenges significant synergy is apparent. In GH3 cells, single agent challenges led to a modest reduction in the histone modification associated with silenced gene H3K27Me3. However in MMQ and AtT20 cells single agent drug incubations were ineffective.

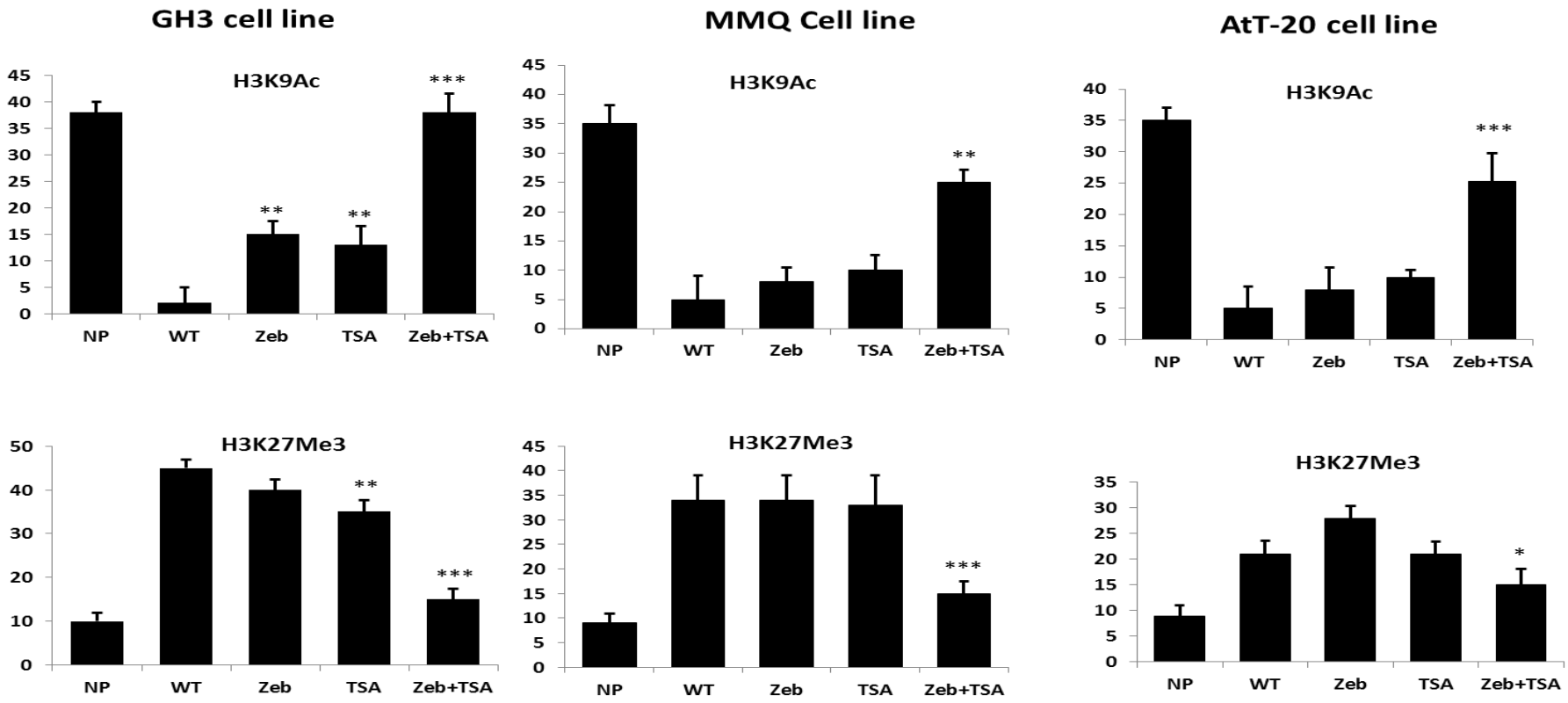



**Figure 5.7: ChIP Analysis of BMP-4 promoter region in pituitary cell lines**. Analyses are shown relative to the modification seen in input DNA for the H3K9Ac and H3K27Me3. Enrichment in the pituitary cell line GH3, MMQ and At-T20, left and right side of the figure respectively, are shown after the drug challenges shown in figure 5.6 and relative to enrichment observed in normal pituitary. *P,* < ** 0.01, *** <0.001 *vs* normal pituitary. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. All experiments were preformed thrice with triplicate determination. Error bars show SEM

## 5.4 Discussion & Conclusion

Under normal physiological conditions, the pituitary gland retains the capacity to respond to signals that lead to reversible changes in growth patterns. The ability to respond in this was is shared by cell populations before and after differentiation, as is their capacity for transdifferentiation, and has led some authors to propose that the gland is to be considered as the “plastic pituitary” [2, 386]. In this context, the growth-promoting and or differentiation inducing signals are counterbalanced by those that mediate inhibition of these pathways. Thus, and as example, dopamine acting through the D2R is a tonic inhibitor of prolactin secretion synthesis and lactotroph proliferation [2, 387, 388]. It is perhaps intuitive, therefore, that knockout of the D2R in a mouse model is the critical change responsible for the genesis and outgrowth of pituitary adenomas that are predominantly in the lactotroph lineage [388].

Mechanistic insight of tumourigenesis in this model has been the identification of BMP-4 as a positive regulator of lactotroph cell proliferation and its increased expression in human prolactinoma [31]. However, in contradistinction to these findings, subsequent studies showed reduced expression of BMP-4 in corticotroph adenomas derived from patients with Cushing’s disease [32]. Furthermore, enforced expression of BMP-4 in AtT-20 cells, that are in the corticotroph cell lineage, suppresses their growth. The apparent bifunctional role of BMP-4 in a cell-type specific context is not without precedent and has been described for several other genes in other tumour types [194, 384, 385]. In these cases, the same protein may exert its action as an oncogene or as a tumour suppressor gene and perhaps explains increased expression of BMP-4 in some pituitary adenoma subtypes and a decrease in others [31, 32]. An example of heterogeneity of expression levels within different cell types in the pituitary is the somatostatin receptor. Somatostatin and its analogue mediate through the activation of PTX sensitive Gi/o coupled somatostatin receptors in pituitary cells [305]. Interaction of somatostatin with its receptor inhibits secretion of a wide range of hormones. Somatostatin usefulness is entirely dependent on

cells expressing the particular receptor subtypes, loss or reduced expression is frequently the limiting factor [316, 317]. SSTR has differential expression in subtype specific adenomas. SST2 and SST5 have a bifunctional role in pituitary adenomas. SST2 is expressed in Growth hormone secreting tumours and non-function adenomas. Whereas its expression is low in Prolactinomas and Cushing adenomas. SST5 is expressed in Prolactinomas and Cushing adenomas and its expression is low in the other subtypes [320, 321]. This was a very important key factor in drug treatments using SSA. Pharmacological strategies were based on the construction of ligands with high affinity for each of the two receptor subtype directed towards new compounds that were capable of interacting with more than one SST subtype. This led to the discovery of BIM-23244, SOM230 (Pasireotide). Pasireotide was a bi-specific compound that could activate both SST2 and SST5 and therefore achieve better control of Growth hormone, adrenocorticotroph hormone releasing, Prolactin hormone and clinically inactive non-functioning adenomas [330].

In this investigation of BMP-4 at the transcript and protein level differential expression of BMP4 across and within different pituitary adenomas is apparent. In agreement with previous reports, increased expression of BMP-4 is apparent in prolactinomas relative to normal pituitaries and to the other adenoma subtypes [31]. However, and in contrast to a previous report [31], only a proportion of prolactinomas overexpressed BMP-4 relative to the normal pituitaries used in this study, suggesting that aberrations other than overexpression of this cytokine are most likely responsible or sufficient for tumour outgrowth. In corticotroph adenomas, variable expression of BMP-4 transcript was apparent, where the majority of adenomas show reduced expression, as determined by qRT-PCR relative to the normal pituitaries used within this study. Similar findings, but in this case as determined by IHC, have been reported previously [32]. However although the present study of BMP-4 protein expression in these adenomas shows significantly reduced expression relative to the prolactinomas, the expression levels are similar to that seen in normal pituitaries and reflect previous reports in which expression was determined by western blot analysis [31]. In the other adenoma subtypes, that is, non-functioning adenomas and

somatotrophinomas, the majority of adenomas show reduced expression of BMP-4 relative to normal pituitaries, and this was apparent at both transcript and protein level. A single previous report has also described reduced BMP-4 expression in non-functioning adenomas and FSH-secreting gonadotrophinomas, and in this case expression was determined at the transcript level [383]. Moreover, It is important to consider the normality of the normal pituitaries used. Previous work within the group through Immunohistochemistry and Pyrosequencing, shows data suggestive that the normal pituitaries used have a normal homogenous population of pituitary cells [154, 389].

This study of BMP4 expression was designed to identify potential discordance between transcript and protein expression in pituitary adenomas, a phenomenon not previously investigated for this cytokine. However the broadly similar transcript and protein expression patterns from the individual pituitary adenomas mitigate against post-translation modifications as responsible for either increased or decreased expression being consequent to effects on protein seen half-life. Equally, the observed decrease in BMP4 protein seen in some adenomas is not a consequence of loss through a protein degradation pathway. However for other proteins and in pituitary tumour context, reduced expression of the cell cycle regulator p27 is apparent and reported to be mediated through protein-degradation pathway instead of low mRNA transcript expression [322].

Genetic defects in this tumour type, and as previously discussed, are infrequent whereas epigenetic change is a more common finding [390], Changes to the epigenome are frequently apparent as DNA methylation and or histone modifications. In the same tumour cohort, where expression of BMP4 was determined, no change in methylation profiles across *bona fide* CpG island were detected. However, analysis of histone tail modifications associated with silent [355] and expressed genes [120], showed clearly discernible patterns associated with reduced BMP-4 transcript. In these cases reduced expression is associated with enrichment for the histone modification, H3K27Me3 and depletion of H3K9Ac relative to normal pituitaries and tumours

expressing BMP-4. These types of modifications have also been described in previous studies in relation to reduced expression of D2R in the pituitary tumour cell line GH3 [121]. In the adenomas where increased expression of BMP-4 is apparent, histone modifications are similar to those seen in normal pituitaries and are consistent with the gene being transcriptionally competent. In these cases the observed transcriptional competence suggests that other aberrations in signal transduction pathways are responsible for the observed increase of BMP-4.

To gain an appreciation of the causal impact of epigenetic aberrations on BMP-4 expression a cell model system was used, and comprised the pituitary tumour cell lines described herein and a strategy we and others have used previously [121, 151, 391]. The cell line GH3, MMQ and AtT-20 expressed low but reproducibly detectable BMP-4. However in contrast to the majority of human tumours that show reduced BMP-4 expression, the cell lines associated CpG islands were heavily methylated. In addition these regions showed enrichment for the histone marks associated with silent genes, H3K27Me3 and concomitant depletion of H3K9Ac, a modification frequently associated with active genes. Moreover, incubations of the cells with the demethylating agent zebularine or the histone deaceytlase inhibitor TSA, as single agent, resulted in a modest change in expression. However, combined drug challenges resulted in robust BMP-4 expression in these cells. In these cases, the combined drug challenges were responsible for a partial decrease in CpG island methylation and marked reversal of histone modification towards the pattern apparent in their normal pituitary counterparts that express BMP-4. Collectively, these findings suggest that significantly reduced expression of BMP-4 in these cell lines was consequent to changes within their respective CpG islands and histone modifications, a phenomenon I had noted previously for the expression of the dopamine D2 receptor in GH3 cells [121]. These findings would support, albeit indirectly, that similar causal mechanisms of silencing are most likely operative in the human pituitary adenomas showing reduced expression of BMP-4. A caveat associated with these conclusions is that silencing and or reduced expression of BMP-4 in human tumours is not associated with CpG island methylation however they do show histone modifications similar to

those seen in the tumour cell lines. Although all of these experiments were performed on early passage cells, it is possible that methylation is an in vitro phenomenon or represents a species specific epimutation. Thus, although models are useful, there is a need to be mindful of potential pitfalls through extrapolation across species boundaries. Equally, epidrug challenges will impact on perhaps multiple genes harbouring epigenetic change. Thus, although I observed epidrug-mediated chromatin modifications to BMP-4 that are coincident with re-expression and suggests that the changes are specific, it did not preclude changes at other epigenetically modified loci that may also impact on BMP-4 expression.

The clinical impact and consequences of variable BMP-4 expression in vivo are not yet known. Indeed, the physiological impact of aberrant BMP-4 expression will also be dependent on cell type, cognate receptor number, and also local level inhibitors such as noggin. However, several studies, primarily in these cell line model systems, show that BMP-4 most likely modulates responsiveness to drugs commonly used in clinical management and that target the Somatostatin receptors. These studies have been subjected to recent review [392, 393] and provide further evidence for the bifunctional characteristics of BMP-4 in a cellular context. Thus BMP-4 enhances prolactin production and proliferation of GH3 cells and modulates SSTR sensitivity through up regulation of SSTR5 and concomitant reduction in SSTR2 expression [393]. However, in AtT-20 cells, in the corticotroph cell lineage, BMP-4 signalling suppresses ACTH secretion and cell proliferation [32, 344]. In the context of the data presented in this section of the study, epidrug-augmented expression of BMP-4, in combination with conventional therapies, might offer new avenues for treatments for these tumours.

# Chapter 6: Pre-incubation of Pituitary Tumour Cells with the Epidrugs Zebularine and Trichostatin A are permissive for Retinoic Acid Augmented Expression of the BMP-4 and D2R genes

# 6. Pre-incubation of Pituitary Tumour Cells with the Epidrugs Zebularine and Trichostatin A are permissive for Retinoic Acid Augmented Expression of the BMP-4 and D2R genes

## 6.1 Aims & Objectives

The purpose of the study was to gain a further insight into the relationship and the interplay between RA mediated expression of BMP-4 and D2R and their epigenetic silencing in the pituitary cell lines AtT-20 and GH3.

## 6.2 Introduction

As discussed and reviewed in the preliminary introduction to this thesis our understanding of the pathogenic mechanisms responsible for the evolution and outgrowth of human pituitary adenomas are far from complete. Moreover, each of the differentiated cell types within this gland can give rise to a tumour that in some cases shows distinct, subtype-specific, genetic and or epigenetic aberrations [386, 394, 395]. Indeed, the impact of these changes within and between subtypes presents significant challenges for their management and the efficacy of the various treatment options.

Studies from several groups have explored the potential of the retinoids and in particular retinoic acid (RA) as a treatment option for the pituitary adenomas [347-349, 396]. As example, and in the corticotroph adenoma cell line, AtT-20, RA inhibits ACTH biosynthesis and POMC transcription and is also responsible for the observed decrease in cell proliferation and cell viability [348]. Pre-dating these studies in AtT-20 cells, similar endpoints, in this case in an *in vivo* nude-mouse model of Cushings disease, were reported and most likely focused attention on the potential of the retinoids as effectors in the pathways leading to inhibition of hormone secretion and tumour

growth [347]. Indeed, subsequent and seminal findings from this group describing a significant role of BMP-4 in the genesis of pituitary adenomas [31].

The identification of BMP-4 and the first report describing its differential expression in the genesis of sporadic pituitary adenomas emanated from investigation of the dopamine D2 receptor deficient mice. In the same report, and across a cohort of primary human pituitary adenoma subtypes, significant increase in BMP-4 was apparent and confined to prolactinomas [31]. Moreover, a subsequent investigation from the same group described reduced expression of the BMP-4 in primary human corticotroph adenomas [348]. These studies were also first to establish the causal link between RA challenge and the subsequent BMP-4 mediated effects that are described above [348]. Differential, subtype specific expression of BMP4 has been reported by other groups and confirm earlier findings of increase in transcript and protein primary prolactinoma [356] whereas most other subtypes showed reduced expression of this cytokine [356, 383].

In addition to the RA induced and BMP-4 mediated effects apparent in corticotroph tumour cells, novel data describing the effects of this retinoid on expression of the dopamine D2 receptor (D2R) have also been reported [373]. The likely impetus of these studies reflects the observations that a proportion of adenomas in the lactotroph lineage are resistant to dopaminergic drugs and is perhaps consequent to deficiency of the D2R [397, 398]. In these cases, a likely scenario is that an activated RA receptor, through its retinoic acid response element (RARE) in the D2R promoter will perhaps restore the receptor expression [399]. If this were the case then RA would re-establish or augment the anti-secretory and anti-proliferative actions of dopamine, through increase in receptor number, in this and perhaps other adenoma subtypes. While these studies show, in a proportion of primary adenomas, that RA challenges increase D2R expression it is without effect in GH3 cells in the somatolactotroph cell lineage [373]. Paradoxically, dopamine challenge of primary adenomas, where RA induced re-expression of D2R was effective, did not lead to

augmentation of the anti-proliferative effects. However in GH3 cells it does lead to decrease in proliferation and increase in apoptosis [373].

My own studies, described in this thesis and that are also now published, have shown that reduced expression of BMP-4 in pituitary tumour cell lines and primary adenomas consequent to epigenetic silencing [356]. Similarly, loss or significantly reduced expression of the D2R in pituitary tumour cell lines is also associated with epigenetic silencing [121]. In these cell lines (AtT-20, GH3 or MMQ) epidrug mediated reversal of these aberrations restore BMP-4 and D2R expression [121, 356]. However, the epigenetic silencing of the BMP-4 and the D2R gene raises interesting question in the context of the observation that RA mediates and or augments expression of the BMP-4 gene in AtT-20 cells [348]. Equally, although the RA does not lead to increased expression of the D2R in GH3 cells it is associated with increased expression of this receptor in primary adenomas [373]. I considered that a possible explanation for these findings is that, RA challenge *per se* might lead to reversal of epigenetic silencing or that this retinoid may, through activation of their cognate receptor(s), either directly or indirectly "override" epigenetic silencing and thereby increase expression of BMP-4 and or that of the D2R gene in these cell lines.

To address the possibilities described above experiments were designed to determine the effects of RA in the presence and absence of epidrug mediated reversal of epigenetic changes. Since these types of studies are reliant on cell that undergo active cell division they were performed on the pituitary tumour cell lines described throughout this thesis.

## 6.3 Results

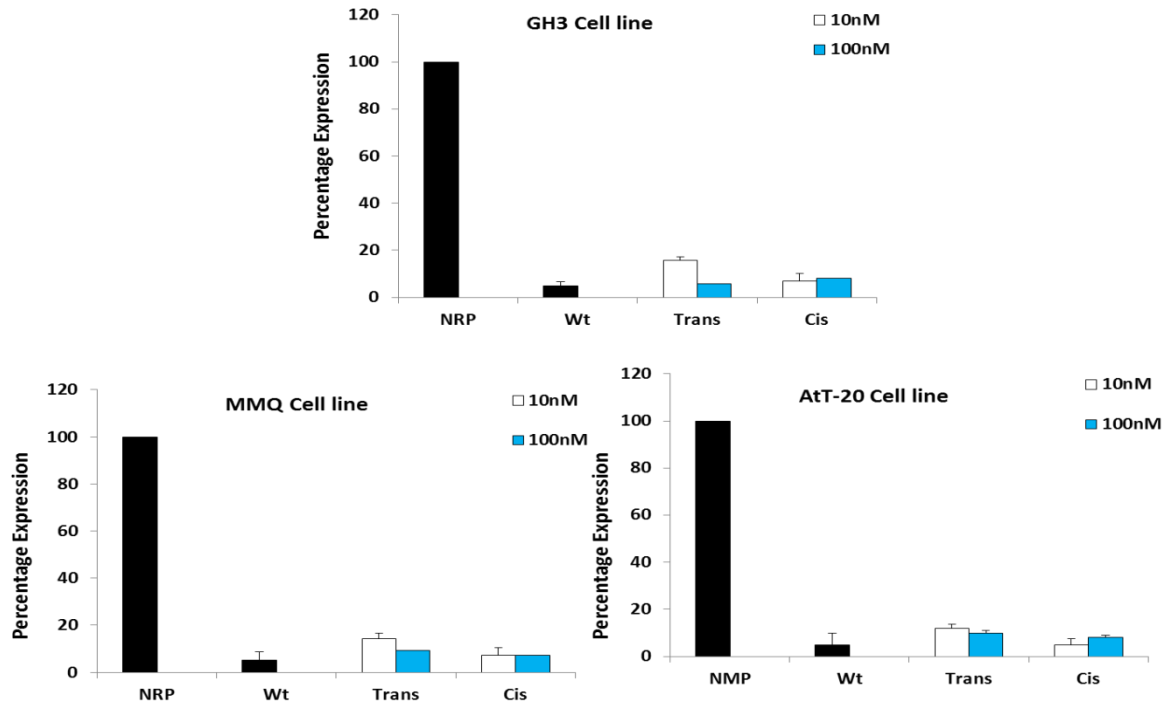
### 6.3.1 Retinoic acid induced expression of BMP-4 and D2R in pituitary cell lines

The expression of BMP4 and D2R was first determined following retinoic acid challenges by qRT-PCR. The internal control used for all the cell lines was PBGD.

The expression of BMP-4 was determined following challenge with retinoic acid in At-T20, MMQ and GH3 cell lines. In a dose response experiment at the highest and lowest dose used in previous studies [348], that is (10nM to 100nM) nether isoform, all-trans RA or 9-cis RA, induced significant increase in BMP-4 expression as determined by qRT-PCR (Figure 6.1A).

However, in these studies a slight but reproducible increase in the D2R transcript expression was detected in GH3 cells following these incubations at the highest dose employed, but this did not achieve statistical significance (Figure 6.1B). In contrast to these findings RA challenges induced robust expression of D2R in MMQ cells in the lactotroph cell lineage to levels similar to those apparent in normal pituitaries (Figure 6.1B).

**A BMP-4**




**B D2R**

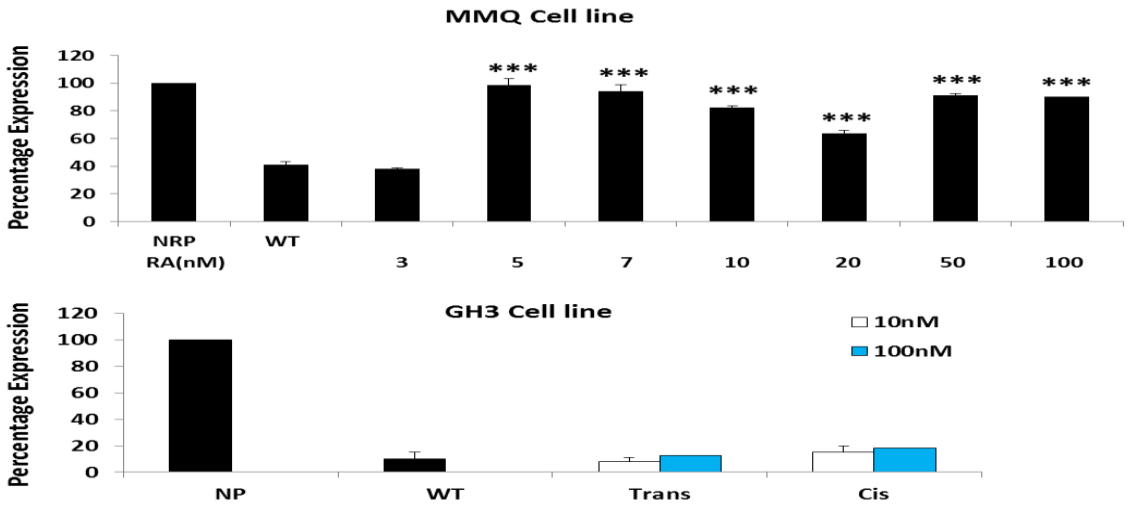



**Figure 6.1: Expression Analysis post Retinoic acid challenge:** A) Expression analysis of BMP4 in GH3, MMQ and AtT-20 cell line relative to normal rat or mouse pituitary respectively. Expression is shown as a percentage relative to the normal pituitaries. B) Expression analysis of Dopamine D2 receptor in MMQ and GH3 cells. Both panels show treatment with the two isoforms of retinoic acid, Trans, (all trans retinoic acid), Cis,: (9-Cis retinoic acid) at the doses shown in the figure. All experiments were performed thrice with triplicate determination. Error bars show SEM. P ***<0.001 relative to untreated cells.

### 6.3.2 Retinoic Acid induced expression of BMP-4 and D2R in epidrug challenged cells

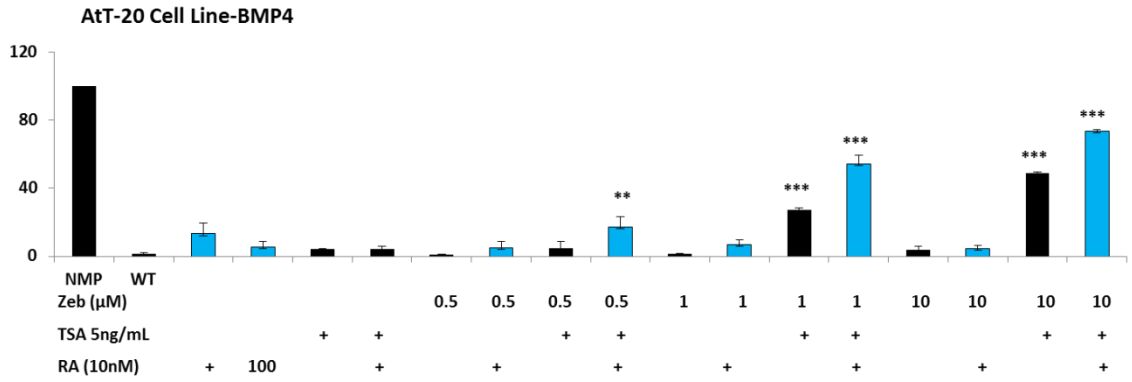
In previous studies described in this thesis and published I had shown that the BMP-4 gene is epigenetically silenced in AtT-20 and GH3 cells as is the D2R in GH3 cells [121, 356]. On the basis of these findings and these studies that show that RA does not induce expression of these genes in pituitary cell lines it was important to determine if epigenetic modifications were responsible for rendering these genes refractory to RA challenge. To address this possibility, AtT-20 and GH3 cells were incubated with increasing doses of zebularine and with a constant TSA concentration shown to be effective in my previous studies. Response to RA was determined in the presence and absence of epidrug challenges.

In AtT20 cells, epidrug co-incubation challenges led to significant increase of BMP-4 expression at zebularine concentrations of 1µM or more, whereas the lower doses were ineffective (figure 6.2A). Moreover, the dose-dependent epidrug-mediated, increase in BMP-4 expression was significantly augmented by RA. In AtT-20 cells, the most significant, RA mediated increase in BMP-4 expression was apparent in epidrug challenged cells, where zebularine (1µM) was used at submaximal concentration. On the basis of these findings, this dose of zebularine in combination with TSA was used in other experiments.

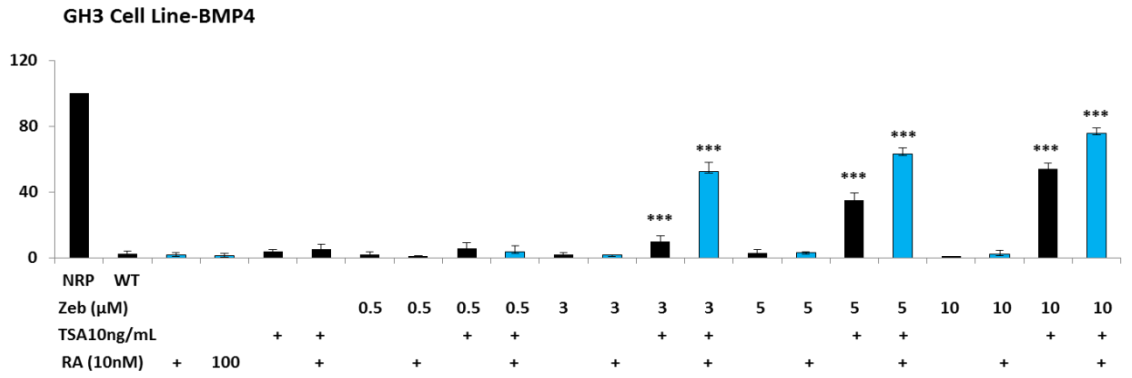
Similar findings of epidrug-mediated and RA-augmented expression of BMP-4 were also apparent in GH3 cells (figure 6.2B). The most significant increase in BMP-4 expression was apparent to RA challenge and epidrugs where the zebularine concentration was 3µM. This concentration, in combination with TSA was used in further experiments in the GH3 cell line.

Similar findings and conclusions were reached for epidrug-mediated and RA-augmented expression of D2R in GH3 cells (figure 6.2C).

A




B




C

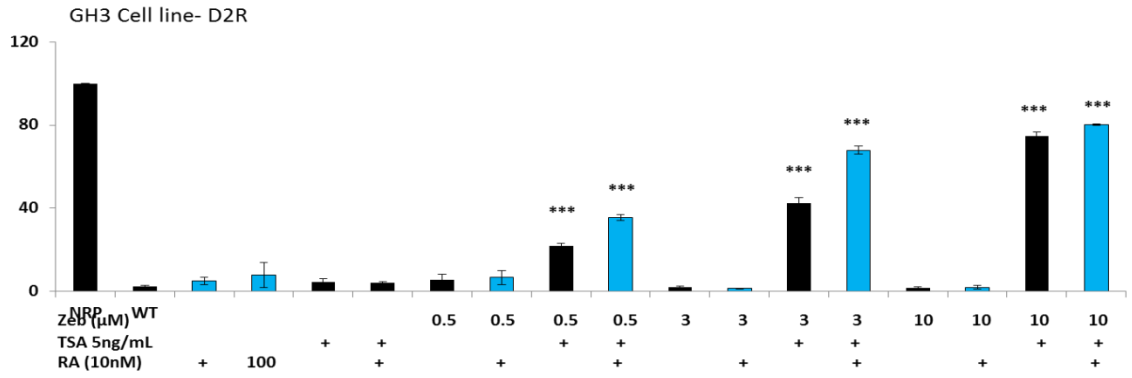



**Figure 6.2: Expression status of BMP4 and D2R transcript in cell lines post epidrug and RA challenges.** Percentage expression of BMP4 in AtT-20 cells (Figure 6.2A), GH3 (Figure 6.2B) and the percentage expression of D2R in GH3 cells (Figure 6.2C). Transcript expression is shown relative to respective normal pituitaries and in these cases set at 100%. Drug treatments are shown in the figure. Blue bars represent retinoic acid treated cells. All experiments were done thrice with triplicate determinations in each experiment and the error bars show the SEM. P, <0.001 vs. vehicle treated cells.

### 6.3.3 Epidrug-mediated demethylation of the BMP-4 and D2R associated CpG islands in pituitary cell lines

The BMP-4 associated CpG islands, in AtT-20 (Fig 6.3A), GH3 cells (Fig 6.3B) and that associated with the D2R CpG island (Fig 6.3C) are differentially methylated relative to their respective normal counterparts. Single agent challenges with, zebularine, TSA or RA are associated with barely detectable changes in the methylation of these gene (figure 6.3A-C). However and as described in a previous section of this thesis co-incubation with the epidrugs (zebularine and TSA) is associated with a modest decrease in methylation. In these cases at the highest concentration of epidrug employed a ~30% decrease in methylation is apparent relative to vehicle treated controls. However RA challenge of epidrug treated cells did not lead to further decrease in methylation (figure 6.3A-C).

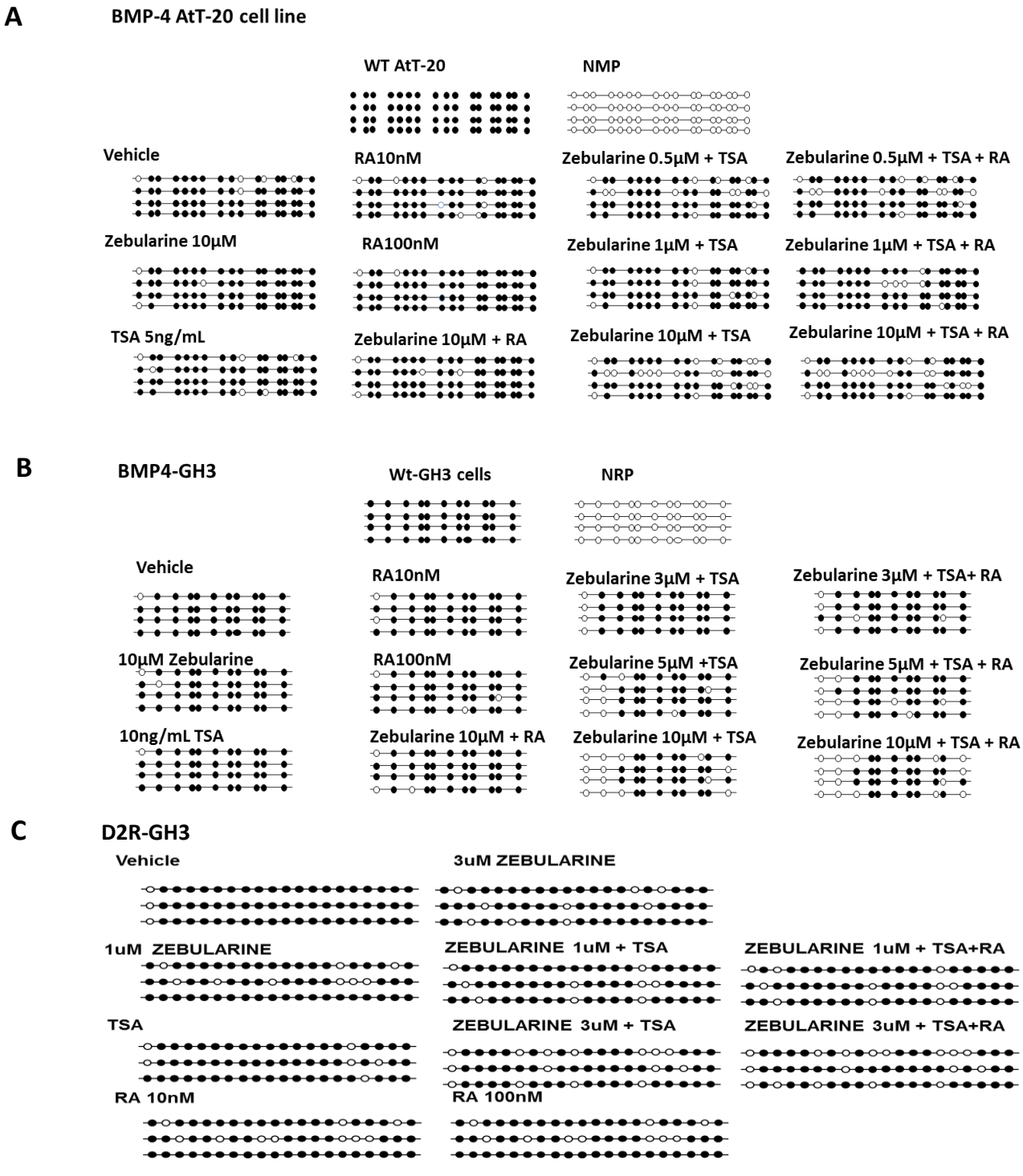



**Figure 6.3: DNA methylation of the BMP-4 and D2R associated CpG islands in pituitary cell lines.**

The BMP-4 associated CpG Island in AtT-20 cells (panel A), GH3 cells (panel B) and of the D2R associated CpG island in GH3 cells (panel C). Drug treatments are shown in each of the figure panels. The filled beads represent methylated CpG dinucleotides and the unfilled beads represent non methylated CpG dinucleotides. All experiments were performed thrice.

### 6.3.4 Epidrug-mediated histone modifications of the BMP-4 and D2R genes in pituitary cell lines

In contrast to their normal pituitary counterparts, AtT-20 (fig 6.4A) and GH3 cells (fig6.4B), where BMP4 expression is barely detectable show histone modifications associated with repressed gene. Similar histone modifications are also apparent for the D2R gene in GH3 cell line (fig 6.4C) relative to their normal pituitary counterparts. In these cases, the promoter regions show enrichment for the histone modification, H3K27Me3 and a depletion of the histone modification, H3K9Ac relative to their normal counterparts. Co-incubations experiments with the epidrugs, zebularine and TSA led to significant change in histone modifications in these cell lines (figure 6.4A-C). In these cases epidrugs induce enrichment of the H3K9Ac modification and depletion in the H3K27Me3 modification. However, the RA-augmented increase in BMP-4 and D2R transcript expression, apparent in epidrug treated cells, is not associated with further change in either enrichment of H3K9Ac or depletion of H3K27Me3. These findings suggest that epidrug mediated modifications to the epigenome are ***permissive*** and sufficient for RA augmented response.

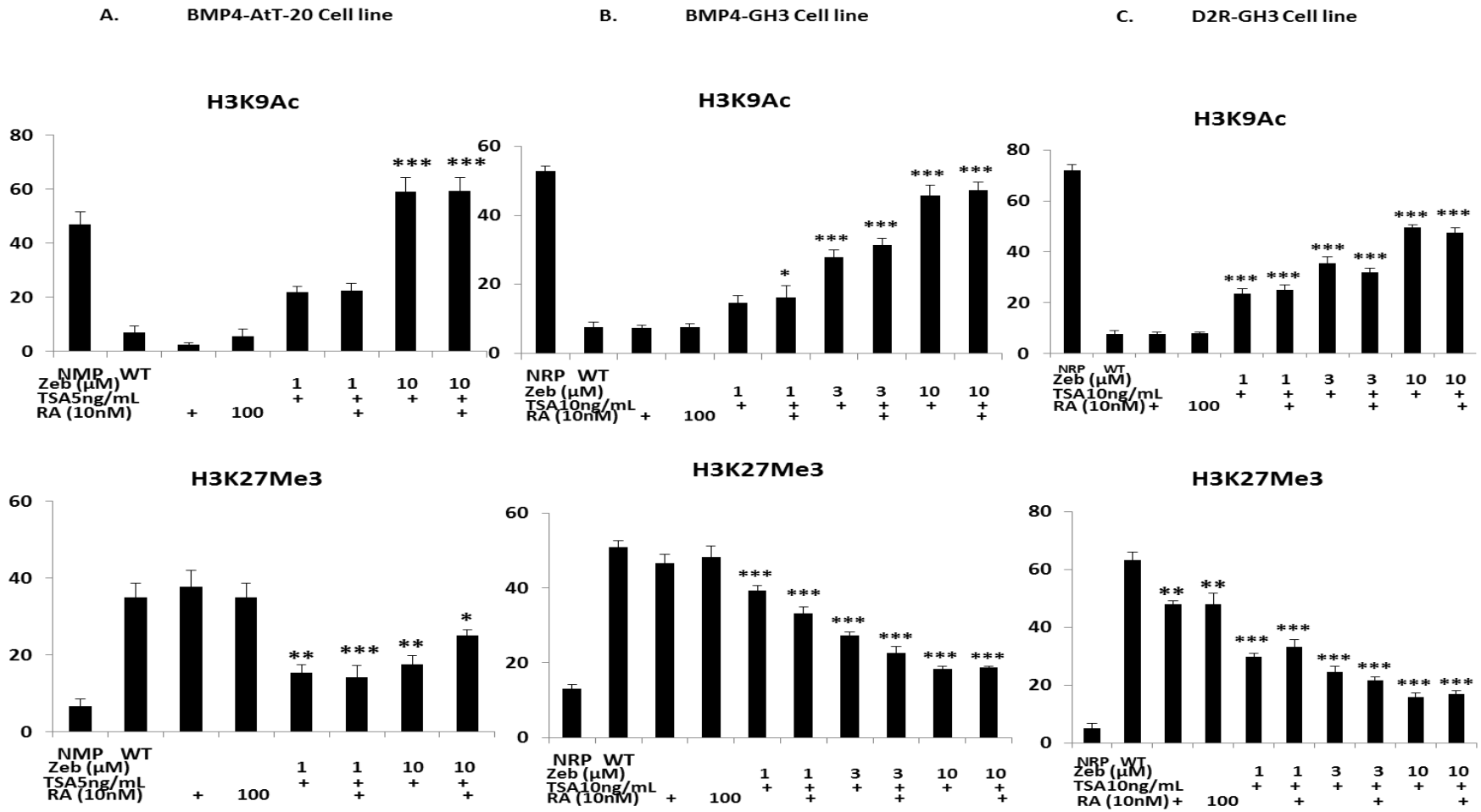



**Figure 6.4: ChIP analysis of the BMP-4 and D2R promoter region in pituitary cell lines.** ChIP analyses are shown relative to input for H3K9Ac and H3K27Me3. Enrichment, in the pituitary cell line AtT-20 and GH3 are shown for BMP-4 promoter region in panel A and B and for D2R in GH3 in panel C following drug challenges. Cells lines were challenged with RA in the presence of sub-maximal concentrations of zebularine and TSA and are shown in each panel. Error bars show SEM. P * <0.05, ** <0.01, *** <0.001 relative to vehicle treated cells.

### 6.3.5 Growth promoting and growth inhibiting activity of BMP-4 in pituitary cell lines

The role of BMP-4 as a mediator in proliferative responses was determined in proliferation assay and by soft agar colony forming efficiency (CFE) experiments. To this end, for the proliferation assays, GH3 and AtT-20 cells were incubated with sub-maximal combinations of epidrugs in the presence or absence of RA. Viable cell counts were made at 24 hour intervals over 4 days. Figure 6.5A shows that in GH3 cells, relative to vehicle treated cells, a significant increase in cell number is apparent in cell treated with epidrugs. This increase in cell number was further augmented when epidrug challenged co-incubated with RA, however, RA alone was ineffective. In AtT20 cells (figure 6.5B) the opposite response was apparent. In this case, epidrug mediated and RA augmented a decrease in growth rate. RA as a single agent was without effect.

Soft agar CFE provided further support for these observations where submaximal challenges with epidrugs did not lead to a significant change in colony numbers relative vehicle treated cells. However, in the epidrug treated cells challenged with RA a significant increase (GH3 cells) or decrease (AtT-20 cells) in the number of colonies formed is apparent (figure 6.5C).

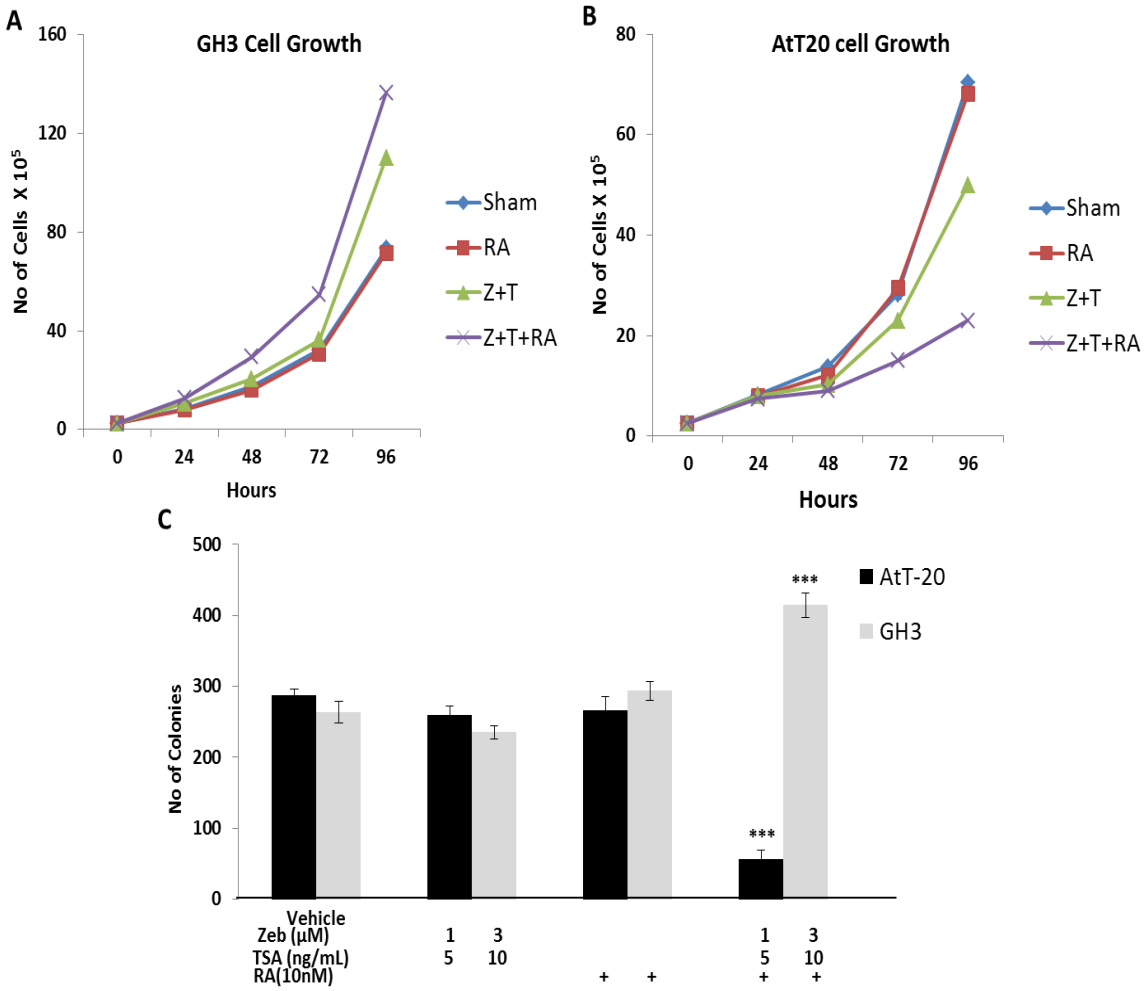



**Figure 6.5 Proliferation and soft agar colony forming efficiency (CFE) assay of drug challenged pituitary cell lines.** The proliferation of GH3 (Panel A) and AtT-20 cells (Panel B), as determined by viable cell counts are shown in cells subjected to drug challenges that induced significant change in CFE shown in Panel C. In epidrug treated GH3 cells a significant increase in the cell number, relative to vehicle treated cells was not apparent until the 96hr time point (P,0.001). In combined challenges with RA significant increase was first apparent at 48hr time point (P<0.05) and increased at 73hr (P<0.01) and 96hrs (P<0.001). No difference in cell number was apparent following RA challenge alone (Panel A). In AtT-20 cell line (Panel B), epidrug challenges resulted in a significant decrease in proliferation at similar time points as those apparent in Panel A. RA alone was without any effect on cell number, but co-incubations with epidrugs led to further decrease in cell number that was apparent at an earlier time point of 48hr. CFE was determined following the challenges shown in Panel C and the differences determined relative to vehicle treated cells.

### 6.3.6 Specificity of BMP-4 as a mediator in the proliferative response

The specificity of BMP-4 as a mediator in the observed proliferative responses was assessed by knock down experiments of the BMP-4 antagonist noggin. It was important to confirm significant and specific siRNA mediated knock down of noggin (siRNA) in GH3 cells relative to that seen in cells transfected with a non-target control (siNT). In these experiments, incubation of cells in the presence or absence of epidrugs had no further effect on noggin transcript levels as determined by qRT-PCR (figure 6.6A). The effects of these manipulations on BMP-4 expression were also determined (figure 6.6B). These experiments showed that knock-down of noggin, in the absence of epidrug challenges was sufficient to a marginal increase BMP-4 however, the increase was significantly augmented in epidrug challenged cells.

Finally the effect of these manipulations on the proliferation response of GH3 cells was determined. Sham transfected and non-target siRNA (siNT) transfected cells showed similar growth profiles over four days (figure 6.6C). Relative to these cells, an increase, of similar magnitude, in cell number was observed in cell challenged with either the siRNA or with the epidrugs. This increase, however, was further augmented when noggin knock down cells were incubated in the presence of the epidrugs.

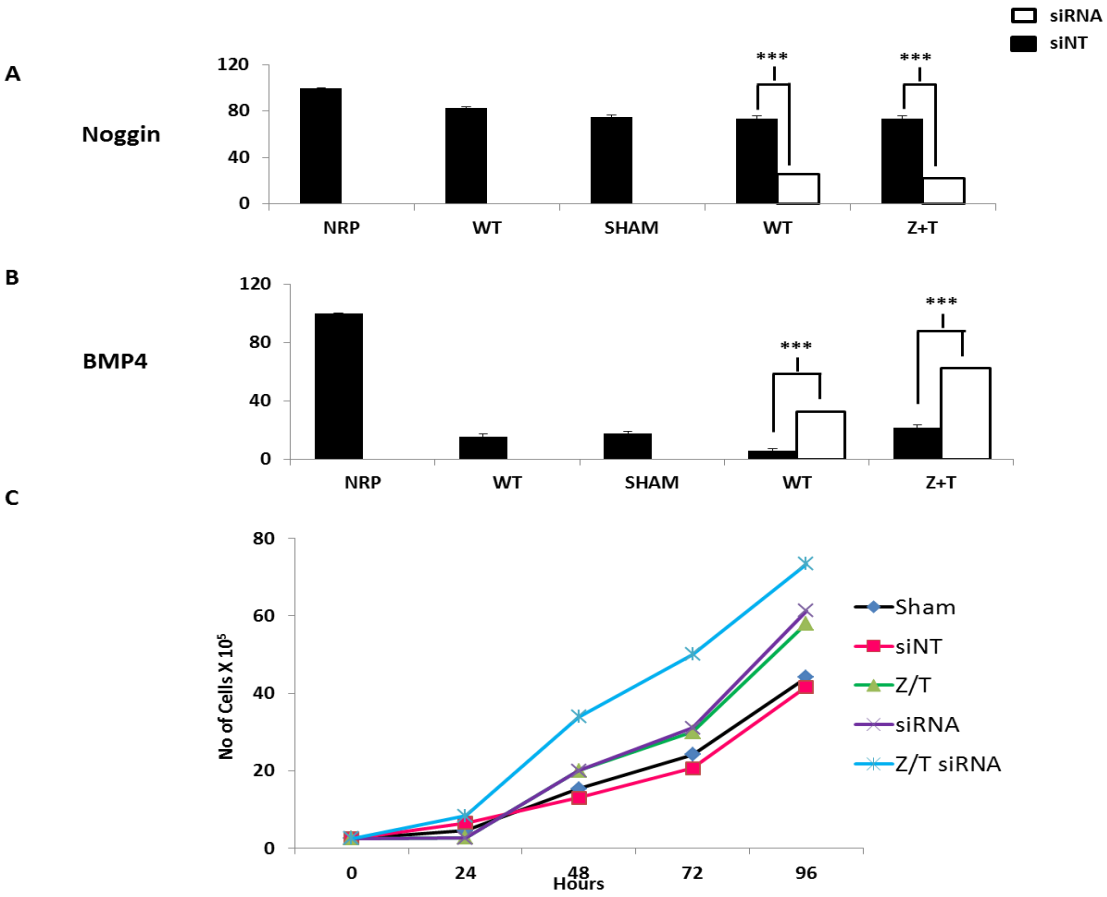



**Figure 6.6: BMP-4 expression and proliferation response following siRNA mediated knock-down of the BMP-4 antagonist noggin**. siRNA mediated knock-down of noggin (siRNA) relative to non-target control siRNA (siNT) leads to significant (*** P<0.001) reduction in noggin expression as determined by qRT-PCR in GH3 cells (Panel A). No further effects on noggin transcript levels were apparent in cells challenged with epidrugs in the absence or presence of RA, and at the doses shown in figure 6.4. qRT-PCR of BMP-4 in noggin knock-down GH3 cells (Panel B) is associated with increase in BMP-4 transcript relative to sham transfected cells. Further increase in BMP-4 is apparent in cells subjected to co-challenge with epidrug in the absence or presence of RA. In four day proliferation assay and relative to sham and siNT transfected cells, specific knock-down of noggin (siRNA) alone and also epidrug challenges led to similar increase in cell number that were significant at 72hr (P<0.01) and 96hr (P<0.001), (Panel C). In cells co-incubated with siRNA, epidrug and RA a significant increase in cell numbers was apparent at earlier time points (48hr, P < 0.001) and maintained through the 72hr and 96hr time points (P <0.001).

## 6.4 Discussion & Conclusion

The identification of molecular aberrations responsible for the pituitary outgrowth and those conferring resistance to medical intervention presents significant challenge. Similar challenges are apparent in the identification of effective drug therapies that suppress inappropriate hormone secretion and inhibit tumour growth. None the less, these dual pharmacologic outcomes are achieved through use of dopamine analogues for most but not all prolactinomas [400]. However, other drug based approaches, that target the somatostatin receptor (SSTR) or the peroxisome proliferator activated receptor γ (PPARγ), principally in GH and ACTH secreting adenomas respectively, achieve benefit in a significantly smaller proportion of cases [324, 332, 333, 401]. More recently, attention has focused on RA as a potential therapeutic option where it was initially shown to prevent experimental Cushings syndrome [347]. Subsequent studies showed that the inhibitory effects on ACTH secretion and cell proliferation were mediated, at least in part, through induction of BMP-4 [348]. Moreover, the potential of RA as a treatment option in Cushing's disease has also been addressed, in a small number of patients, in a proof of concept study. Long term treatment with RA was well tolerated in the majority of cases and urinary free cortisol (UFC) was normalised in four of the seven patients investigated, suggesting that RA represents a promising medical approach in Cushings disease [402].

My studies show that RA challenges *per se* does not induce expression of the endogenous BMP-4 gene in either AtT-20 or GH3 cell lines or induce change or modification to the BMP-4 associated epigenome, which in these cases are indicative of gene silencing. A possible explanation for the discordance between our findings and those reported by Giacomini and colleagues is their use of transfected BMP-4 receptor construct that will not recapitulate the epigenetic modifications of the endogenous gene [348]. An alternative explanation is that differences might reflect clonal variants of these cell lines, however, the findings of BMP-4 gene silencing in primary pituitary

adenomas that is also associated with epigenetic modifications would argue against this being a clonal or cell culture artefact [356].

Early studies showed RA induces D2R expression through a consensus RARE in its promoter region [399]. In the current investigations we too found, in the MMQ cell line, that the D2R gene is responsive to RA. However, GH3 cells were resistant to this challenge and similar conclusions in this cell line have been reported by other investigators [373]. Our interpretation is that resistance, as with the BMP-4 gene, can be explained by epigenetic modification associated with the D2R gene in this cell line. Supporting this interpretation are our findings of histone modification associated with silent gene in GH3 cells and modifications associated with expressed gene in MMQ cells [121]. However, and as further explanation, a recent study has proposed that expression of the D2R is dependent on co-expression of Filamin A, and is reported as essential for receptor expression in primary lactotroph adenomas [364].

Recent studies including those described within this thesis have shown epidrug mediated re-expression of silenced genes and has been subject to recent review [394]. Given that my studies show that this approach is effective for re-expression of D2R and BMP-4 in pituitary tumour cell lines [121, 356] it was reasonable to determine the consequence of epidrug-mediated chromatin remodelling as *permissive* for RA induced expression of the BMP-4 and of the D2R gene. In GH3 and AtT-20 cells, co-incubations with increasing doses of the demethylating agent, zebularine together with the histone deaceytlase inhibitor TSA, but not with either agent alone, led to a dose dependent increase in BMP-4 expression and these findings are consistent with earlier findings in this thesis and published reports from our group [121, 356]. In cells incubated with epidrugs together with RA a significant increase in BMP-4 expression is observed. Similar findings, of an epidrug facilitated, and RA augmented increase of the D2R transcript in GH3 cell line is also apparent. Epidrug challenges, but not RA (either alone or in co-incubations), were associated with limited change to gene associated CpG island methylation status. However, these epidrug

challenges were responsible for significant increase and decrease in histone modification associated with active and repressed genes respectively and reflect my previous findings and reports from our group [121, 356]. The observation that resistance to RA in the absence of epidrugs challenges suggests the latter is permissive and the former promotes or augments expression of these genes in pituitary cells.

By way of explanation for the observed increase of BMP-4 expression in prolactinomas and decrease in most other pituitary adenoma subtypes we recently proposed a bifunctional role for this cytokine (this thesis) and my published study [356]. In these cases, BMP-4 expression would exert either a growth-promoting or growth-inhibiting role in prolactinoma or in other adenoma subtypes respectively. In GH3 cells in the somatolactotroph lineage and AtT-20 cells in the corticotroph lineage our studies now provide further support for the bi-functionality of this cytokine. The observation of an epidrug facilitated and RA augmented increase in cell number and CFE was confined to GH3 cells. In contrast to these findings, in AtT-20 cells, drug challenges inhibited proliferation and CFE, however the decrease in cell number was not associated with a decrease in cell viability. These findings provide further evidence for an RA-induced and BMP-4 mediated growth modifying responses, where growth promotion and growth inhibition are observed in GH3 and AtT-20 cells respectively.

A direct in vitro approach, with respect to the growth-promoting and growth inhibiting actions of BMP-4 *per se* has been adopted by other investigators. In these cases, incubation of pituitary tumour cell lines with exogenous BMP-4 promotes, in GH3 cells [31], or inhibits in AtT-20 proliferation [348]. Our findings add to this body of evidence, where we show that RA induces expression of the endogenous BMP-4 gene in these cells exerts similar effects. A caveat associated with this conclusion is that RA challenge in the presence of epidrugs may also induce expression of other endogenous and perhaps epigenetically silenced growth regulatory genes. However and arguing against this possibility are the findings from the siRNA mediated knock-

down of the BMP-4 antagonist noggin. These studies in GH3 cells show that siRNA mediated knock-down of noggin, in cells incubated with epidrugs, is associated with an increase in cell number as determined by proliferation assays.

Noggin is reported to exert its antagonistic effects through extracellular sequestration of BMP-4 thereby inhibiting binding to its cognate receptor [31, 379]. In this context an unexpected finding from our studies is that knock-down of noggin *per se* induced expression of the endogenous BMP-4 gene in the absence of epidrug induced modifications. However, further increase in transcript expression is apparent when knock-down is combined with epidrug challenges. Furthermore, the 4 day proliferation assays reflected the growth promoting properties of re-expressed and epidrug augmented expression of BMP-4 in this cell line. While these findings do not preclude the characterised role of noggin as a BMP-4 antagonist they perhaps identify additional or perhaps cell type specific mechanisms of BMP-4 regulation. It is perhaps worthy of note that in the D2R knock-out mouse of pituitary tumourigenesis, loss of noggin expression as determined by differential display was accompanied by increase in BMP-4 expression [31]. These findings suggest that their expression is perhaps interdependent. Ideally similar studies to be performed in AtT-20 cells would be ideal however these cells do not express the noggin transcript.

Although the efficacy of current treatment modalities, in the context of BMP-4 expression, are incomplete emerging data shows that BMP-4 may modulates responsiveness to drugs commonly used in this tumour type. In these cases, the expression of the BMP-4 in the signalling cascades engaged through activation or restoration of the somatostatin, D2R and PPARγ receptors has been described [121, 344, 393, 403, 404] and has been subjected to recent review [53, 341]. Thus, the reversal of the epigenetic silencing in combination with classic therapeutic option may improve clinical management strategies. Indeed, in preclinical trails, and as an example only, the histone deaceytlase inhibitor valproic acid in combination with retinoic acid has been shown to be effective both *in vitro* and *in vivo* in patients with acute myeloid leukaemia [405].

# Chapter 7: Conclusion & future work

# 7. Conclusion

Under normal physiological conditions the pituitary gland retains the capacity to respond to signals that lead to reversible changes in growth patterns. The ability to respond in this way is shared by cell populations before and after differentiation and has led some authors to suggest the gland be regarded or termed, “the plastic pituitary” [2, 386]. On the basis of our current understanding, genetic mutations, leading to activation of oncogenes and loss of TSG, are an infrequent finding in this tumour type [16, 386, 390]. However, and in common with many other tumour types epigenetic aberrations are common findings [53, 390, 391]. Indeed multiple reports now describe methylation mediated gene silencing that targets CpG islands [53, 406, 407]. In the majority of these reports genes have been identified using candidate gene approaches and have focused on those in tumour suppressor and or apoptotic pathways [389, 408]. However, more recent reports have used techniques or technologies that either directly or indirectly identified novel genes [31, 158, 389, 408]. In these cases investigators have used techniques that include, differential display, siRNA mediated knockdown strategies and BeadArray analyses [389].

While early studies of pituitary tumours and indeed that performed in many other tumour types focused on CpG island methylation as a mechanism leading to or responsible for gene silencing other epigenetic mechanisms, associated with or responsible for gene silencing, have received less attention. However, where studied, chromatin remodelling, associated with histone modifications and expression of miRNA impact on gene expression or transcript translation in many tumour types including those of pituitary origin [121, 390, 394, 406]. What is more, emerging data would suggest that the demarcation between genetic aberration and epigenetic changes may not be as distinct as we first thought. In this case emerging data from the Fusco laboratory suggest that inappropriate expression of The High Mobility Group A proteins (HMGA),

either through amplification of this gene locus or in addition to decreased expression of microRNA (miRNA) that target HMGA1 and HMGA2 are now described [28, 37, 409].

The treatment options, for the management of patients with pituitary adenoma, are largely dependent on the adenoma subtype. Indeed, the majority are treated surgically whereas those of lactotroph origin, the prolactinoma, are treated with dopamine agonists and in some cases somatotrophinoma, that secrete GH are treated with somatostatin receptor analogues [357, 358]. However, recent studies targeting other receptor types, including somatostatin, PPARγ and BMP-4 receptor have been described [341, 357, 402, 410-412]. In these cases, as indeed is the case for the D2R, some patients and or their adenomas are resistant to these medical intervention strategies [332, 333, 358, 401, 413]. Furthermore, these interventions are frequently designed to not only inhibit inappropriate hormone secretion but also induce tumour shrinkage. It is, therefore, perhaps not surprising that these dual functional end-points are rarely achieved [357, 358]. Although resistance to medical therapy is clearly multi-factorial, and includes in some cases patients who are intolerant to the intervention, a body of literature would suggest that the absence or reduced expression of cognate receptors as responsible for resistance [283, 359, 363, 410]. To our knowledge, and particularly at the inception of this research project, few studies had determined mechanisms or aberrations responsible for loss or reduced expression of clinically relevant receptors in this adenoma subtype. We also considered that if aberrations were identified, and given the knowledge that changes to the epigenomic landscape are reversible, this new knowledge would provide new, perhaps novel, therapeutic options, and perhaps across multiple pituitary adenoma subtypes.

For the receptor directed studies of pituitary tumours my research initially focused on the D2R and subsequently the BMP-4 receptor and mechanisms/aberrations associated with their expression patterns in pituitary adenoma. Research was initially directed toward two key epigenetic modifications, namely CpG island methylation, and histone modifications associated

with gene silencing. In these cases it was recognised that prolactinoma are rarely treated surgically making the availability, or rather, the lack of primary adenoma a limiting factor. Furthermore, primary pituitary show limited proliferative potential *in vitro*. These limitations were partially overcome through use of characterised cell lines in the somato-lactotroph cell lineage, GH3 and in the corticotroph lineage, namely AtT20 cells. Our confidence in these cell lines as models of GH (somatotroph) and ACTH (corticotroph) "like" adenoma, respectively, was based on studies by multiple other investigators and their published reports [31, 283, 348, 363]. However, a caveat associated with these conclusions is that we were, in some cases, extrapolating across species boundaries.

In the context of CpG island methylation in pituitary tumour cell line, my studies showed that loss or reduced expression of the D2 and BMP4 receptors is associated with increase in methylation relative to their normal pituitary counterparts. Challenge of these cells line with drugs that inhibit methylation and histone deacetylation restored expression of D2R and BMP4, however, despite robust transcript expression, as determined by RT-qPCR, the decrease in CpG island methylation was marginal. These findings, at least in part, prompted studies of histone modifications as determined by ChIP analysis. In this case, and again for both genes, histone modifications were those associated with gene silencing and epidrug challenges led to significant change to these modifications. Thus, relative to the marginal, epidrug-induced changes to DNA methylation, the change in histone modifications were far less equivocal. Indeed the findings strongly supported epigenetic change(s) as responsible for gene silencing and that epidrug incubation restored receptor expression.

The silencing and subsequent epidrug-induced re-expression of the D2R in GH3 cells provided opportunity, in follow on studies, to determine dopamine-agonist mediated apoptotic responses. These studies did show a significantly augmented apoptotic response and suggest that reduced receptor number in GH3 cells and perhaps in primary tumours might, at least in part, be

responsible for a failure to respond to dopamine agonists or perhaps explain a blunted response in these adenomas. Indeed in primary tumours, although the number available to us for analysis was limited, histone modifications do appear to reflect receptor expression status, at least as determined by RT-qPCR. While this study was in progress the Spada group showed other mechanisms associated with reduced D2R receptor number and these findings [364] warrant more detailed investigation in a larger adenoma cohort and in the context of epigenetic changes.

My investigations of BMP-4 expression in pituitary cell lines (GH3 and AtT-20) also showed loss or significantly reduced expression of this cytokine. In these cases, as with the D2R in GH3 cells, loss was associated with CpG island methylation and histone modifications. Again, epidrugs restored BMP-4 expression, and as with the epidrug-induced modifications to the D2R epigenome, these were apparent as significant change in histone modification and marginal change in CpG island methylation. In agreement with previous reports, BMP-4 was increased in prolactinoma and decreased in most other adenoma subtypes and these observations suggested that BMP-4 was perhaps bifunctional. In these cases, and dependent on cellular context, this cytokine might either promote or inhibit proliferation. In contrast to pituitary cell lines, primary tumours showing reduced or barely detectable BMP-4 expression were not methylated across their CpG island, however, they did show histone modification associated with gene silencing. In tumours that showed elevated BMP-4 expression, relative to normal pituitary, histone modification reflected those apparent in “transcriptionally competent” genes.

Although my studies had shown that the D2R and BMP-4 were rendered “transcriptionally incompetent” through changes to their epigenome other groups had suggested that the BMP-4 gene is responsive to RA challenge [32, 347]. These findings suggested that RA might, perhaps, either override epigenetic silencing or perhaps lead to its reversal. It, therefore, seemed appropriate to investigate this possibility in some detail. My studies clearly demonstrated that RA challenge *per se* was ineffective as an agent that could override epigenetic silencing. However,

epidrugs were permissive, that is obligate for RA augment BMP-4 expression. I was also able to show in these cell lines that BMP-4 could promote or inhibit growth as determined by drug induced expression of this cytokine in GH3 and AtT-20 cells respectively. These findings provide exciting new opportunities in adenoma subtypes, other than prolactinoma, to use epidrugs in combination with RA to inhibit adenoma growth and hormone secretion in this case in corticotrophinoma.

## 7.1 Future Work

The data presented within this thesis so far presents future scope of work to address some of the following:

1. Investigate the effects of epidrug mediated D2R alongside commonly used drugs in clinical management of pituitary adenomas irrespective of the subtypes they express.
2. The expression of D2R is thought to be dependent on the levels of Filamin A. Filamin A is reported as an essential receptor expression in primary adenomas. Investigate the levels of D2R in relation of Filamin A in GH3 cells and also in primary pituitary adenomas. It is also important to investigate the epigenomic landscape associated with Filamin A and the effects of this on the D2R expression.
3. To investigate the effects of epidrugs and retinoic acid on hormone secretion/ production in conditioned media of cell lines.
4. To investigate the impact and consequence of variable expression of BMP-4 in vivo.
5. Investigate the epidrug mediated BMP-4 expression in responsiveness to drugs commonly used in clinical management and target this towards the somatostatin receptor and receptor sensitivity.
6. To investigate the link between DNA methylation and histone modification in vitro. This could be done by investigating transcription factors associated with DNA methylation alongside the histone conformation in genes that have DNA methylations against those that do not.
7. To investigate the link between genetics and epigenetics. This could be done by investigating microRNA's associated with HMGA1 and HMGA2, further investigating epigenetic aberrations associated with silencing of microRNA's.

# Chapter 8: References

## 8. Reference list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# Appendix

## Appendix I: siRNA Transfection.

**siRNA calculations for transfection:**

Smartpool of siRNA were obtained as lyophilized powder from Dharmacon. These were reconstituted in the recommended volume of siRNA dilution buffer (provided by Dharmacon) to make 20µM working stock solution (see below). These were then stored as s 20µL aliquots at -20°C. The stock solutions were used in the concentrations shown in the Table below to determine optimal, gene specific knock down. In these cases the siRNA solutions were combined with FBS-free DMEM prior to mixing with the Lipofectamine 2000 transfection reagent:

| | siRNA (20µM stock) | FBS free- DMEM |
|---|---|---|
| **20nM** | 2µl | 248 µl |
| **40nM** | 4 µl | 246 µl |
| **100nM** | 10 µl | 240 µl |

**The Smartpool siRNA used in this thesis are shown in the following Table:**

| Name | Target Sequence | Mol. Wt.(g/mol) |
|---|---|---|
| **Noggin (1)** | ACACUGACGUACAUCAUUG | 13,414.9 |
| **Noggin (2)** | GGCAUUGACAUAUGAGACA | 13,414 |
| **Noggin (3)** | GAAAGAGGCUCGUCCACGC | 13,474 |
| **Noggin (4)** | GAACAUCCAGACCCUAUCU | 13,429.9 |

**Appendix II: Primary pituitary adenomas used within the study and a copy of theEthical approval granted for the work.**

| Study Number | Tumour Number | Classification | Grade |
|---|---|---|---|
| GH1 | **16** | **Somatotrophinoma** | **2** |
| GH2 | **23** | **Somatotrophinoma** | **2** |
| GH3 | **27** | **Somatotrophinoma** | **2** |
| GH4 | **529** | **Somatotrophinoma** | **2** |
| GH5 | **596** | **Somatotrophinoma** | **2** |
| ACTH1 | **4** | **Corticotrophinoma** | **1** |
| ACTH2 | **10** | **Corticotrophinoma** | **2** |
| ACTH3 | **22** | **Corticotrophinoma** | **1** |
| ACTH4 | **32** | **Corticotrophinoma** | **1** |
| ACTH5 | **59** | **Corticotrophinoma** | **2** |
| ACTH6 | **195** | **Corticotrophinoma** | **2** |
| ACTH7 | **207** | **Corticotrophinoma** | **1** |
| PRL1 | **25** | **Prolactinoma** | **2** |
| PRL2 | **29** | **Prolactinoma** | **2** |
| PRL3 | **46** | **Prolactinoma** | **1** |
| PRL4 | **56** | **Prolactinoma** | **1** |
| PRL5 | **57** | **Prolactinoma** | **1** |
| PRL6 | **396** | **Prolactinoma** | **1** |
| PRL7 | **535** | **Prolactinoma** | **1** |
| PRL8 | **536** | **Prolactinoma** | **1** |
| PRL9 | **698** | **Prolactinoma** | **3** |
| NF1 | **7** | **Non-functioning** | **3** |
| NF2 | **8** | **Non-functioning** | **2** |
| NF3 | **9** | **Non-functioning** | **2** |
| NF4 | **12** | **Non-functioning** | **2** |
| NF5 | **30** | **Non-functioning** | **3** |
| NF6 | **31** | **Non-functioning** | **3** |
| NF7 | **40** | **Non-functioning** | **2** |
| NF8 | **42** | **Non-functioning** | **3** |
| NF9 | **47** | **Non-functioning** | **3** |
| NF10 | **58** | **Non-functioning** | **2** |
| NF11 | **63** | **Non-functioning** | **2** |
| NF12 | **64** | **Non-functioning** | **2** |
| NF13 | **75** | **Non-functioning** | **3** |
| NF14 | **81** | **Non-functioning** | **3** |

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**Co-ordinator:** **Mrs Rosa Downing**

Date: 08 July 2010

Professor Richard Nigel Clayton
Professor of Medicine, UHNS
University Hospital of North Staffordshire
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Springfield Unit City General
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ST4 6QG

Dear Professor Clayton

**Study Title:** **Molecular Basis of Human Pituitary Tumourigenesis**
**REC reference number:** **10/H1207/46**

Thank you for your letter of 05 July 210, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Ethical review of research sites**

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

**Conditions of the favourable opinion**

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

*For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

*Sponsors are not required to notify the Committee of approvals from host organisations.*

**It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).**

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| Document | Version | Date |
|---|---|---|
| Investigator CV | | |
| Protocol | 1 | 18 November 2009 |
| Letter from Professor Ilana Crome | | 12 January 2010 |
| REC application | | 18 November 2009 |
| Covering Letter | | 05 July 2010 |
| Participant Information Sheet: LREC Project number 1268 | | |
| Participant Information Sheet: Part 1 | 4 | 01 July 2010 |
| Response to Request for Further Information | | |
| Participant Information Sheet: Part 2 | 1 | 14 May 2010 |
| Participant Information Sheet: 10/H1204/6 | 2 | 16 January 2010 |
| Participant Consent Form: Part 1 | 3 | 21 April 2010 |
| Participant Consent Form | 4 | 01 July 2010 |
| Participant Consent Form: Part 2 | 1 | 23 April 2010 |
| Participant Consent Form: 10/H1204/6 | 2 | 16 December 2010 |
| Letter from Professor Crome enclosing Peer Review | | 12 January 2010 |
| Letter of unfavourable opinion from North Staffs | | 05 March 2010 |
| Committee Member Comments | | |

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**After ethical review**

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

# Appendix III: Reagents

All reagents were purchased from Sigma-Aldrich unless stated otherwise.

**RNA extraction reagents**

The reagents stated below are used in the extraction of RNA in addition to those stated in the materials and methods:

**Lysis buffer: 5M Guanidinium thiocyanate:**

5M Guanidinium isothiocyanate

0.5% (w/v) Sarkosyl (N-lauroylsarcosine)

25mM sodium citrate

8% (v/v) β-mercaptoethanol

Mix well and autoclave

**Chloroform Isoamyl-alcohol (24:1)**

Add 239.6mL of chloroform to 10.4mL of isoamyl-alcohol (250ml total volume), mix well and store at 4°C

**2M Sodium acetate pH4.0:**

Add 82.03g of sodium acetate to 200mL of $H_2O$. Adjust the pH to 4.0 using glacial acetic acid, adjust the volume to 500mL by adding $H_2O$ and autoclave.

**70% Ethanol**

Take 70mL of 100% ethanol and add 30mL of $H_2O$.

**cDNA synthesis reagents:**

All reagents arrived ready prepared (Promega, Southampton, UK). However the dNTP's require diluting.

**Dilution of dNTP (Bioline, London, UK)**

The dNTP's are purchased as a 100mM stock and, therefore, comprise 25mM of each of the four bases. For the majority of experiments the working concentration for each base was 5mM. Therefore 200µL of the supplied dNTP was added to 800µL of $ddH_2O$. Aliquots were stored at -20°C and thawed on no more than three occasions.

**DNA extraction Reagents:**

The regents used for DNA extraction in addition to those in the materials and methods are as follows:

**Lysis buffer: TE buffer (pH8.0), 0.5% SDS pH 7.2 and 200µg/mL of Proteinase K**

**TE buffer (pH 8.0)**

10mM Tris HCl (pH 8.0)

1mM EDTA (pH 8.0)

Mix well and autoclave. The amount of TE buffer added to the reaction will vary between samples and between experiments. Cells require 1.5mL of TE buffer whereas tissue requires 3mL of TE buffer.

**20% SDS**

10g of SDS added to 50mL of $H_2O$ and rotate on the rotary shaker at room temperature for 2 hours.

**Proteinase K**

Obtained from Sigma in a powdered form. Add $H_20$ to give a concentration of 10mg/mL. Add desired amount to give 200µg/mL final concentration in lysis buffer.

**Gel Electrophoresis**

Products from RNA extraction, DNA extraction and PCR were in some cases resolved by gel electrophoresis. In these cases the following reagents were used:

**TAE buffer (50X)**

To 800ml ddH2O add 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA. Make up to 1X final volume using $dH_2O$.

**10X Tris-Borate-EDTA (TBE) electrophoresis buffer (10X):**

108g Tris base

55g Boric acid

40mL 0.5M EDTA (pH8.0)

Mix well and store at room temperature.

**Loading buffer:**

1mg/ml Bromophenol blue in 50% glycerol. Store aliquots at -20°C

Loading dye:

0.25% Bromophenol blue

0.25% Xylene cyanol FF

40% Sucrose.

Make up to required volume in $dH_2O$. Loading buffer is X6 therefore when loading 5µL of PCR product add 1µL of loading buffer. Mix well and load onto gel. If adding more or even less sample scale up or down the loading buffer quantities.

**Appendix IV: Primer Sequencing and amplicon size**

| Gene/Primer Name | Species | Forward (5'→3') | Reverse (5'→3') | Amplicon size (bp) |
|---|---|---|---|---|
| **RT-PCR** | | | | |
| PBGD | Rat | CAGCATCGCTACCACAGTGTC | ATGTCCGGTAACGGCGGC | 126 |
| BMP4 | Rat | TTCCGAGCGACGCACTGCC | TGAGCGGCGTCCTCCCGC | 240 |
| D2R | Rat | GCCATTGTTCTCGGTGTGTT | CGGAACTCGATGTTGAAGGT | 176 |
| Noggin | Rat | CTACGCCCTGGTGGTGGT | TCCTTAGGGTCAAAGATAGGGT | 141 |
| PBGD | Mouse | CGGGAAAACCCTTGTGATGC | TTCTTCTGGGTGCAAAATCTGG | 285 |
| BMP4 | Mouse | GTTTTCTGTCAAGACACCAT | CAGACTGGAGCCGGTAAAG | 292 |
| Noggin | Mouse | GACCTGGCTTTCTGGTTCCT | TTCCTTTTGCTTGCCTTTTT | 159 |
| GAPDH | Human | CGACCACTTTGTCAAGCTCA | GGGTCTTACTCCTTGGAGGC | 102 |
| BMP4 | Human | AGGGAGGGAGGGAAGGAG | AGCAGGACTTGGCATAATAAAA | 227 |
| D2R | Human | ATCTCCTGCCCACTCCTCTT | GTTTGGTGTTGACTCGCTTG | 184 |
| Noggin | Human | CCAGAGGCATGGAGCGCTGCC | AGCAGCGTCTCGTTCAGATCC | 205 |
| **Sodium bisulphite** | | | | |
| BMP4 | Rat | TATAATGAGATTTTGGAGTAG | ACTAAAACTCCAACTAC | 405 |
| Nested BMP4 | Rat | TGTAATGTATTTGGTTAGGT | ACCTAACCAAATACATTACA | 183 |
| D2R | Rat | GTATAAGAGGGGATTAGTTT | ACCTTCTTTATCATTCCCATCTTAAA | 545 |
| Noggin | Rat | TAGAGGGTGGTGGAATT | AACTATAATCACAAACCTTCTAC | 352 |
| Nested Noggin | Rat | GGTGGGGATTTATTAAGT | AAATAAACAACTACTTCAACAA | 226 |
| BMP4 | Mouse | TATTTTTGTTAGGTTG | TTAGGTATTAAATTAGTATGGTT | 167 |
| Hemi Nested BMP4 | Mouse | GTAGTTTAAATATTTGTAGAAGT | | 138 |

| | | | | |
|---|---|---|---|---|
| Noggin | Mouse | TGAGGTTTATTAGGGG | ACTCATACTACCTACCCTAC | 271 |
| Hemi Nested Noggin | Mouse | GATGTGTAGATAGTGTTGG | | 245 |
| BMP4 | Human | GTTATTTGAAGTTAGAGGATTTGG | ACTATCATTAAAAACAATATTTAACC | 266 |
| Hemi-nested BMP4 | Human | GAGAGGTATTTAAAAAGGAAAG | | 143 |
| Noggin | Human | GAAATTTAAAGGTTTGGAT | AAAAAAACACACAAATTAAC | 369 |
| Hemi nested Noggin | Human | AAGTGTTTTTAGAATTAGTTTAG | | 243 |
| **ChIP** | | | | |
| BMP4 | Rat | GGGAGCCAATCTTGAACAAA | ACTCCTAGGGGCTGGAAGAA | 157 |
| D2R | Rat | ACAGTGCAGAGATAGTTCTG | GGACAGCTCCGCGGAATCA | 133 |
| Noggin | Rat | CAGCAGCGTCTCGTTCAGAT | CTACACATCCGCCCAGCAC | 108 |
| BMP4 | Mouse | GACTGGGGAGGAAGGGAAG | CGTCTTAGGCTGGGGTCTCT | 156 |
| Noggin | Mouse | | | |
| BMP4 | Human | AGGAAGGAAGATGCGAGAAGG | CCTGGGGACCTCTGAACG | 243 |
| D2R | Human | ACTCAGTGTCACGGGGAGAGGAGGA | TAGCCTCCTCGCCACTTAGA | 211 |
| Noggin | Human | GCTTGGACCCTGCGAGAC | ACTTCCCTCCGCCTGCTC | 119 |
| | | | | |

## Appendix V: BMP-4 ELISA, Protein extraction and analysis

### ELISA

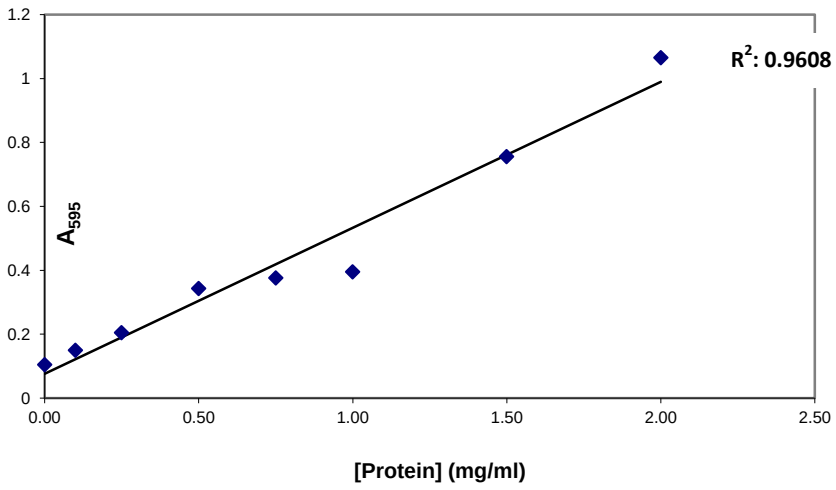
Elisa was performed on primary pituitary adenomas and normal pituitary tissue. The following stages state the preparation and analysis of the proteins used for this kit.

**An example of protein BSA standards, A) readings at A562nm, B) Linear graph showing protein concentration against absorbance:**

**A)**

| [Protein] (BSA) (mg/ml) | A562 |
|---|---|
| **0.00** | 0.1045 |
| **0.100** | 0.15 |
| **0.25** | 0.205 |
| **0.50** | 0.3435 |
| **0.75** | 0.3765 |
| **1.00** | 0.3955 |
| **1.50** | 0.7555 |
| **2.00** | 1.065 |

**B)**




**Measurement of the protein concentration of sample 1 and 2 against the protein BSA standards taking any dilution factor into considerations:**

| **Protein concentration** | | | | |
|---|---|---|---|---|
| | | | | |
| | | **Sample dilution factor =** | **2** | |
| | | | | |
| | **Sample1** ($A_{595}$) | **Sample2** ($A_{595}$) | **Mean** ($A_{595}$) | **[Protein]** (mg/ml) |
| **Sample 1** | 0.819 | 0.822 | 0.8205 | **3.260514** |
| **Sample 2** | 0.918 | 0.903 | 0.9105 | **3.654887** |

**BMP-4 ELISA**

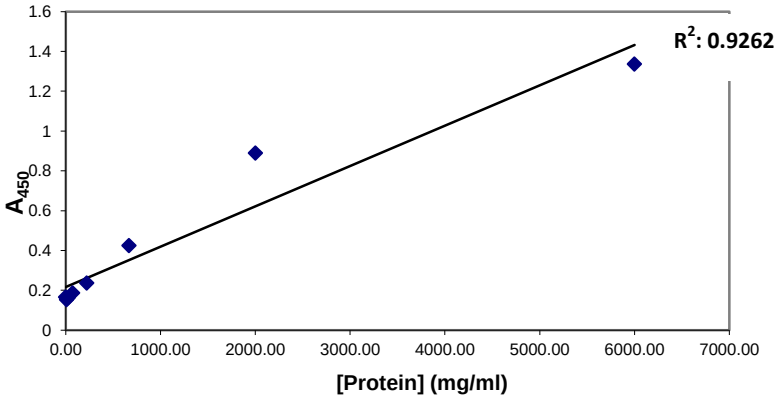
The ELISA was performed as per kit instructions and also as described in the materials and methods section. The amount of BMP-4 protein was analysed similar to the BSA stated above using the BMP-4 standards ranging from 8.23-6000pg/mL as shown below:

**BMP-4 standards A) readings at 450nm, B): Linear graph showing protein concentration against absorbance:**

**A)**

| [Protein] pg/ml) | A450 |
|---|---|
| **0.00** | 0.166 |
| **8.230** | 0.153 |
| **24.69** | 0.162 |
| **74.07** | 0.186 |
| **222.20** | 0.237 |
| **666.70** | 0.425 |
| **2000.00** | 0.89 |
| **6000.00** | 1.337 |

**B)**




**Measurement of the BMP-4 protein concentration of sample 1 and 2 against the protein standards taking any dilution factor into considerations:**

| **Protein concentration** | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| **Sample dilution** | | | | | |
| **factor =** | | | | **5** | |
| | | | | | |
| | | | | | |
| | **Sample1** | **Sample2** | **Sample3** | **Mean** | **[Protein]** |
| | ($A_{595}$) | ($A_{595}$) | ($A_{595}$) | ($A_{595}$) | (pg/ml) |
| **Sample 1** | 1.021 | 1.012 | | 1.0165 | **19733.14** |
| **Sample 2** | 1.01 | 1.235 | | 1.1225 | **22348.07** |
| | | | | | |

**Normalised BMP-4 protein in nmol/mg against total protein:**

| | BMP4 | BCA Protein | Normalized BMP-4 | |
|---|---|---|---|---|
| | **pg/ml** | **mg/ml** | **pmol/mg** | **nmol/mg** |
| **Sample 1** | 19733.14 | 3.26 | 6052.15 | **6.052157** |
| **Sample 2** | 22348.06 | 3.65 | 6114.57 | **6.114571** |

## Appendix VI: Bacterial Transformation

**DNA sequencing analysis:**

DNA sequencing was performed commercially. In this case sequencing of native DNA or of DNA subject to sodium bisulphite conversion was performed. Converted DNA and amplified PCR product was first eluted from Gene Elute PCR column prior sub-cloning into the T:A vector prior to bacterial cloning and transformation (and as described in the M&M).

**LB Medium:**

Luria Broth is used for maintenance and propagation of E.coli. LB broth comprises Bacto-tryptone, Yeast and NaCl.

To 500mL of dd$H_2O$ add 12.5g of LB broth mix well and autoclave immediately

**LB agar plates containing ampicillin, IPTG and X-Gal:**

Dissolve 12.5g of LB and 7.5g agar in 500mL of dd$H_2O$. Mix well and autoclave immediately. Cool to 50 °C before adding 60µl/mL ampicillin, 0.5mM IPTG, 0.08mg/ml X-gal. Mix solution avoiding frothing and pour into 90mm petri dishes at a depth of 3-5mm. Allow to set on the bench and further incubating at 37°C for complete setting. Once plates have set store at 4°C until ready to use.

**Bacterial Transformation**

**Preparation of competent cells**

Chemically Competent JM109 cells (Promega) were routinely prepared for transformation purposes. A colony from an LB plate was inoculated into 5mL of LB media and incubated for 16 hours at 37°C with shaking in the orbital shaker (150-200rpm). The entire 5mL culture was used to inoculate 250mL of LB medium containing 20mM $MgSO_4$ in a sterile glass flask and incubated at 37°C again with shaking. The culture was allowed to grow to an absorbance ($OD_{600}$) of 0.4-0.6 (typically 3-5 hours). The cells were collected by centrifugation at 914 x g for 10 minutes at 4°C using IEC Centra-8R Centrifuge (International equipment company, USA) a temperature controlled centrifuge. The supernatant was discarded and the cells were re-suspended in 10mL of ice cold chemical buffer 1: TFB1 (see below) and incubated on ice for 30 minutes. Cells were then centrifuged at 914 x g for 10 minutes at 4°C. The supernatant was removed again and cells were re-suspended in chemical buffer 2: TFB2 (see below) and incubated for a further 30 minutes on ice. This was then aliquoted into 0.5mL cold microcentrifuge tubes. These aliquoted chemically competent cells were then flash frozen by liquid nitrogen and were placed at -80°C until ready to use, and were used once without refreezing.

**TFB1:**

30mM Potassium acetate

10mM $CaCl_2$

50mM $MnCl_2$

100mM RbCl

15% glycerol

Adjust pH to 5.8 with 1M acetic acid, Filter sterilise and store at room temperature.

**TFB2:**

10mM MOPS (4-Morpholinepropanesulfonic acid) pH6.5

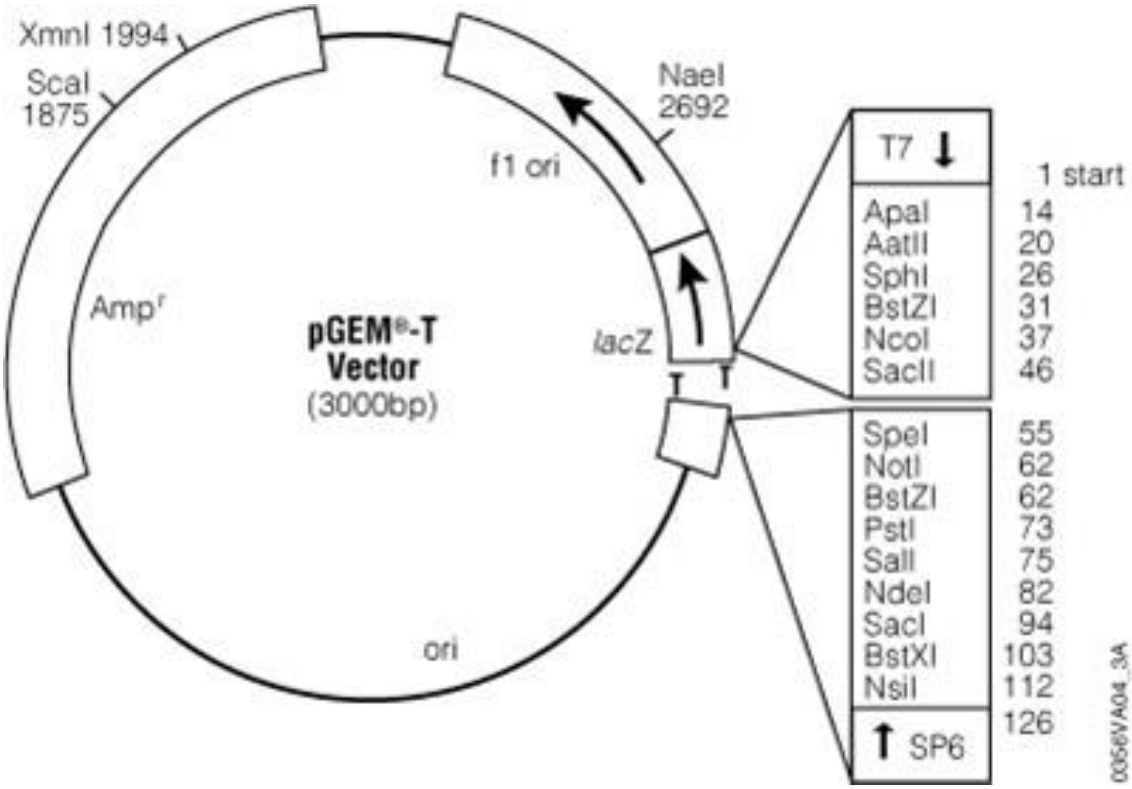
75mM $CaCl_2$

10mM RbCl

15% glycerol

Adjust to pH 6.5 with 1M KOH, filter sterilise and store at room temperature.

**Vector Map: pGEM-T Vector:**



# Appendix VII: Publications in peer reviewed journals