Wnt signalling in oestrogen-induced lactotroph proliferation

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Adam Giles

Endocrinology & Diabetes Group

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Abstract

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Adam Giles
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The anterior pituitary gland is the major hormonal regulator in the body. The gland contains five secretory cell types whose emergence during development is defined by a tightly regulated array of transcription factors. In adult life, the gland is plastic with the relative proportions of cells varying depending on physiological context. Tumours of the pituitary gland account for 15% of all intracranial tumours in man and are caused by the selective proliferation of one of the secretory cell types. The majority of these (60%) are prolactinomas which consist of very slowly proliferating lactotroph cells, which produce the hormone prolactin. Pituitary tumours are almost never malignant and do not express common genetic markers for cancer, suggesting endogenous proliferative stimuli in the pituitary are the cause of tumour development.

Oestrogen causes lactotroph hyperplasia during pregnancy and increases prolactin secretion. Our group previously showed that Wnt-4 mRNA was upregulated during oestrogen-induced lactotroph hyperplasia in Fischer 344 rats. Wnt molecules are key regulatory proteins controlling differentiation, proliferation and migration in development and adult life. Wnt-4 is involved in the emergence of lactotrophs during development, and has been implicated in pituitary tumour formation. Wnt molecules signal through three pathways. The well studied canonical pathway has been implicated in numerous cancers and centres around gene transcription initiated by translocation of β-Catenin into the nucleus. There are two non-canonical pathways: the Wnt-planar cell polarity (PCP) pathway and the Wnt-calcium pathway which are both poorly understood.

In this thesis, the expression of Wnt-4 was studied in the pituitary, and effects of downstream signalling pathways in response to oestrogen were assessed. Wnt-4 was expressed in all secretory cell types of the pituitary, as well as the marginal zone (MZ), a region of the pituitary that may harbour stem cells. Oestrogen upregulated Wnt-4 protein in the somatolactotroph GH3 cell line, though this could not be replicated in primary tissue. A number of approaches (western blotting, immunofluorescence, reporter gene assays and mutant β-Catenin overexpression) were utilised to show that the canonical pathway was not activated in the pituitary. Wnt-4 had a clear inhibitory effect on calcium oscillations in GH3 cells, showing for the first time a non-canonical effect in the pituitary, though the downstream effects are currently unknown. Attempts made to study the activation of the PCP pathway were inconclusive. Efforts focused on the distribution of key structural and regulatory proteins in the anterior pituitary and the MZ. The MZ was characterised by a single layer of cells at the border of the anterior and intermediate lobes of the pituitary, with high expression of E-Cadherin and Sox 9, though no change in distribution was observed with oestrogen treatment. In the anterior lobe, oestrogen treatment decreased N and E-Cadherin expression, which could be an indicator of PCP pathway activation during oestrogen induced-lactotroph hyperplasia.

Overall, data suggest that Wnt-4 does not directly cause oestrogen-induced lactotroph proliferation, but is likely to play a role in regulating tissue plasticity in the adult gland, as well as in the pathogenesis of pituitary tumours.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other institute of learning.

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Abbreviations

AIP	Aryl hydrocarbon receptor interacting protein
APC	Adenomatous polyposis coli
ACTH	Adrenocorticotrophic hormone
α-MSH	α-melanocyte stimulating hormone
AL	Anterior lobe
Arm repeats	Armadillo repeats
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CaM	Calmodulin
CaMK	Calmodulin dependent kinase
CBP	C-terminal binding protein
CE	Convergent extension
Daam	Dsh associated activator of morphogenesis
DAPI	4',6-Diamidino-2-phenylindole
DCT-FBS	Dextran-charcoal treated FBS
Dkk	Dickkopf
DMEM	Dulbecco's Modified Eagles Medium
DMEM/F12	Nutrient Mixture F12
DMSO	Di-methyl sulphoxide
E2	17β-oestradiol
ERE	Oestrogen response element
FACS	Fluorescence activated cell sorter
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FS cells	Folliculo-stellate cells
FSH	Follicle stimulating hormone
GH	Growth hormone
GFP	Green fluorescent protein
GSK-3β	Glycogen synthase kinase-3 beta

H3	Histone 3
HBSS	Hanks balanced salt solution
HRP	Horseraddish peroxidise
HSC	Hematopoietic stem cell
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL	Intermediate lobe
IMS	Industrial methylated spirit
IP3	inositol 1, 4, 5-triphosphate
LEF	Lymphoid enhancer-binding factor
LH	Lutinising hormone
LRP	Low density lipoprotein related protein
MC	Marginal cells
mβ-Cat	Constitutively active mutant β-Catenin vector
MEN1	Multiple endocrine neoplasia type 1
MZ	Marginal zone
NFAT	Nuclear factor of activated T-cells
NFT	Non-functioning tumour
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PFA	Paraformaldehyde
PKC	Protein Kinase C
PL	Posterior lobe
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PN	Postnatal
PRL	Prolactin
PRLR	Prolactin receptor
Prop1	Prophet of Pit 1
ROCK	Rho Kinase
RP	Rathke's pouch
RT-PCR	Reverse transcriptase polymerase chain reaction
TCF	T-cell specific transcription factor
TNFα	Tumour necrosis factor α
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone
VEGF	Vascular endothelial growth factor

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1.0. Introduction

1.1. The pituitary gland

1.1.1. Basic pituitary function

The pituitary gland is a small organ located at the base of the brain in a small bony cavity called the sella turcica (Davidovici et al., 2008). It secretes hormones which collectively regulate diverse homeostatic mechanisms in vertebrates such as growth, reproduction and metabolism. It consists of two functionally and anatomically distinct regions; the adeonohypophysis which consists of the anterior lobe (AL) and intermediate lobe (IL), and the neurohypophysis, also termed the posterior lobe (PL).

The AL contains 5 different secretory cell types;

- *Somatotrophs* produce growth hormone (GH) which regulates growth and metabolism
- *Lactotrophs* produce prolactin (PRL) which regulates milk production in females
- *Corticotrophs* produce adrenocorticotrophic hormone (ACTH) which regulates glucocorticoid synthesis in the adrenal gland to control metabolic function
- *Thyrotrophs* produce thyroid stimulating hormone (TSH) which regulates the thyroid gland to control skeletal remodelling and metabolism
- *Gonadotrophs* produce luteinizing hormone (LH) and follicle stimulating hormone (FSH) which act on gonads to initiate and maintain reproductive function (Voss and Rosenfeld, 1992).

PRL and GH are monomeric proteins produced by their respective cell types, while ACTH is produced by post-translational modification of pro-opiomelanocortin (POMC) which is produced by corticotroph cells. TSH, FSH and LH are heterodimeric proteins which all contain a common α-subunit bound to a hormone specific β-subunit (TSH-β, FSH-β, LH-β) (Zhu et al., 2007). The anterior pituitary also contains folliculostellate (FS) cells, discussed in more detail in the following section.

The IL consists of melanotrophs which secrete α-melanocyte stimulating hormone (α-MSH) to regulate melanin production from melanocytes. The IL is not found in all mammals, but is present in the adult rodent brain. In humans it is well developed in foetal life, but is involuted in late pregnancy and disappears, with other stimuli

maintaining melanin secretion from melanocytes throughout life (e.g. vitamin D, diacylglycerol, retinoids) (Saland, 2001). The posterior lobe is composed of magnocellular axon terminals. These neurons produce vasopressin (regulates water retention and blood pressure), and oxytocin (controls uterine contraction and lactation), which are transported to the neuron terminals in the PL and released into the circulation (Sladek and Kapoor, 2001).

1.1.2. Pituitary organogenesis

Development of the pituitary gland originates with formation of the Rathke's pouch. In the rat, this arises due to invagination of the oral ectoderm induced by secretion of bone morphogenic protein 4 (BMP-4) at embryonic day 9.5 (e 9.5) from the adjacent ventral diencephalon (Davis and Camper, 2007). A plethora of other signalling molecules including sonic hedgehog (shh), Wnt molecules, fibroblast growth factors and BMP's are expressed in the infundibulum, ventral diencephalon and the Rathke's pouch, and induce extension of the Rathke's pouch into a defined pocket which subsequently closes. The ventral wall of the Rathke's pouch proliferates and becomes the AL, while the IL is formed from a more limited proliferation of the dorsal wall. The infundibulum proliferates down adjacent to the IL and eventually becomes the PL. This basic structure is fully formed by e13.5 (Davis and Camper, 2007; Ericson et al., 1998; Kioussi et al., 2002; Revest et al., 2001; Savage et al., 2003) and is summarised in Figure 1.1 A.

Within the anterior pituitary gland, temporal and spatial expression of transcription factors between e11.5 and e17.5 drives differentiation and proliferation of immature progenitor cells into the mature secretory cell types described previously. For example, Pitx1 and 2 are both expressed from e10.5 and knock out of both genes results in vastly reduced pituitary cell number. Lhx3 and 4 are thought to be regulated by Pitx1 and 2 and are also vital for normal pituitary development (Pellegrini-Bouiller et al., 1999). At e11.5, Tbx19 regulates differentiation of corticotrophs by promoting POMC expression and preventing further differentiation into alternate cell fates (Maira et al., 2003). Prophet of Pit1 (Prop1) is expressed at e12.5 and precedes the expression of Pit1 by a single day (Sornson et al., 1996). Prop1 modulates Pit1 via β-Catenin, which is a vital step in maturation of the lactotroph, somatotroph and thyrotroph lineages (Kioussi et al., 2002). Pit1 is not only important for differentiation of these cell types, but is expressed throughout life in these cells to regulate hormone expression. By an

unknown mechanism, Pit1 activates transcription of the hormone gene specific for that cell type, while simultaneously repressing expression of the other hormone genes (Scully et al., 2000). Gonadotroph expression is driven by SF1 which is first expressed at e13.5. However, mature gonadotrophs are not detected until e17.5, most likely due to temporal control over SF1 mediated Erg1 expression which is thought to be key in gonadotroph proliferation (Zhao et al., 2001). The process is summarized in Figure 1.1 B.

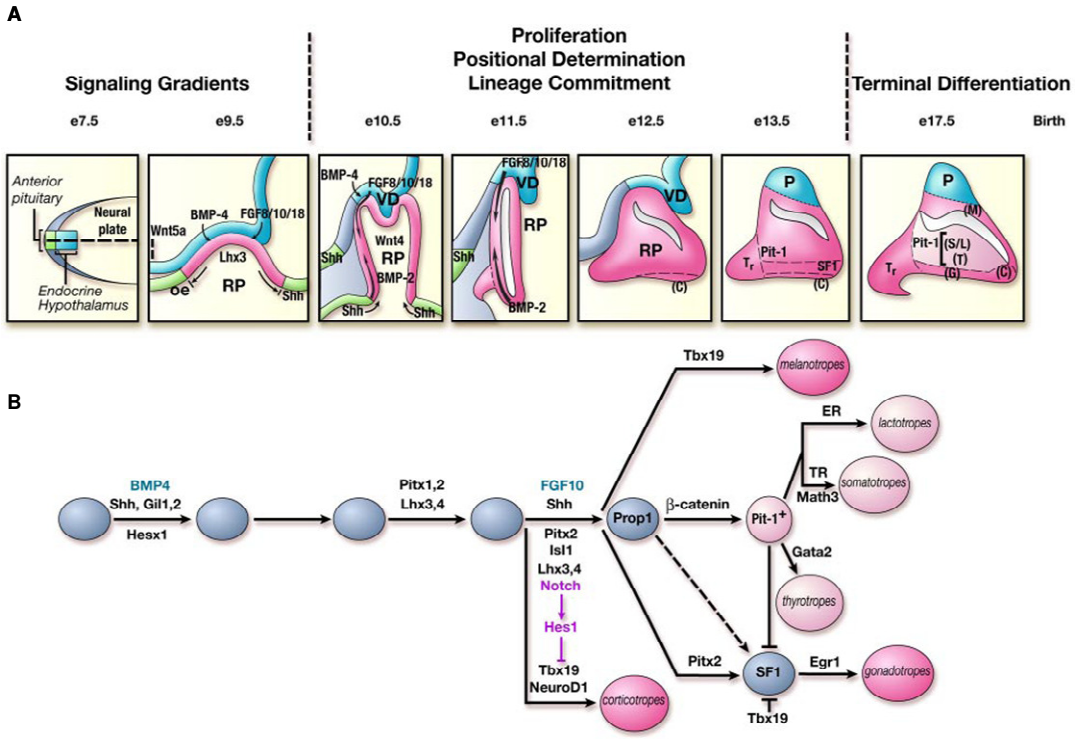


Figure 1.1. Embryonic pituitary development in rat. A - The structural changes that occur during pituitary development between e7.5 and birth. Initial invagination of the oral ectoderm occurs at e9.5 due to BMP-4 expression in the ventral diencephalon (VD). The Rathke's pouch (RP) extends down and closes up between e10.5 and e12.5 and becomes the anterior and intermediate lobes at e13.5. VD descends and becomes the posterior pituitary (P) at e13.5 and pituitary is fully formed by e17.5. **B** - Transcription factors driving expression of mature secretory phenotypes in the anterior pituitary. Pitx1 and 2 regulate Lhx3 and 4 from e10.5. Corticotroph and melanotroph expression is driven by Tbx19, while Prop1 stimulates Pit1 via β-Catenin to induce expression of somatotrophs, thyrotrophs and lactotrophs. SF1 expression mediates gonadotroph differentiation at e17.5, the last of the mature cell types to appear. Figure taken from Zhu *et al.*, Physiol Rev 87, 933-963, 2007.

The anterior pituitary also harbours 2 other cell types, marginal cells (MC's) and folliculostellate (FS) cells. MC's form a planar, single cell layer at the border of the AL and IL, termed the marginal zone (MZ) while FS cells are distributed throughout the anterior pituitary. The MZ has been proposed to harbour stem cells due to the expression of stem cell markers such as nestin and Sox 2, and when cultured these cells can form pituispheres and terminally differentiated hormone secreting cells (Fauquier et al., 2008; Gleiberman et al., 2008). However, it is still unknown if these cells simply play a supportive role in differentiation of mature phenotypes, or are the actual predecessors of mature cells (Vankelecom, 2007).

FS cells are a source of growth factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and follistatin, and have been suggested to regulate cells in the anterior pituitary in a paracrine fashion to modulate hormone secretion (Allaerts and Vankelecom, 2005). The true function of FS cells and cells in the MZ is currently unknown.

1.1.3. Pituitary plasticity and cell networks

Postnatally, the pituitary undergoes a period of rapid proliferation to increase total cell number. The rate of proliferation is highest at postnatal day 2 (PN2), and declines gradually to PN30, after which basal levels of proliferation are maintained throughout life (Carbajo-Perez and Watanabe, 1990). The relative proportions of secretory cell types within the adult pituitary vary depending on the physiological needs of the body. When no unusual demand is being placed on the pituitary, is estimated that 40% of cells in the anterior pituitary are somatotrophs, 35% are lactotrophs, 10% are corticotrophs, 10% are gonatotrophs and 5% are thyrotrophs (Asa et al., 2002).

However, the pituitary is a plastic organ, and the relative proportions of secretory cells can vary throughout adult life according to the physiological demands of the body. For example, during pregnancy, high circulating oestrogen levels result in lactotroph hyperplasia, pituitary enlargement and increased circulating PRL levels (Asa et al., 1982; Elster et al., 1991; Goluboff and Ezrin, 1969; Lloyd et al., 1988), while during puberty, the number of somatotrophs doubles to induce growth (Fauquier et al., 2008). As such, care should be taken to consider the physiological context when considering the relative proportions of cell types in the pituitary. For example, the published proportions of lactotrophs in the rat pituitary have varied from less than 9% to more

than 35% between groups (Levy, 2002) while the percentage of corticotrophs in an unstimulated pituitary has varied between 3% (Taniguchi et al., 1995) and 20% (Castro et al., 1995).

When viewed in a 2D manner, the distribution of cells within the anterior pituitary appears fairly random, with small clusters of similar cell types being the most obvious structural aspect (Asa et al., 2002). However, in the last 10 years evidence has appeared demonstrating clear 3D networks between cells in the pituitary. The first network identified was between FS cells, where it was shown that a calcium signal could be propagated through gap junctions between FS cells across the pituitary (Fauquier et al., 2001). The same group have subsequently demonstrated that GH cells form a continuously linked network throughout the pituitary (Bonnefont et al., 2005) and that each cell type in the pituitary can be categorised by the specific cadherin molecule they express, providing a cell-type specific networking mechanism (Chauvet et al., 2009). Studies on PRL transcription indicate that a co-ordinated pattern of PRL transcription occurs throughout the intact pituitary, which is not seen when cells are dispersed in culture (Harper et al., 2010). This suggests that cells in the pituitary function as part of a network, enabling a pituitary wide response of a particular cell type in response to external stimuli.

Overall, the pituitary is a dynamic organ containing distinct cellular phenotypes in close proximity to one another. During development, the emergence of these cell types is driven by a tightly regulated array of transcriptional queues. In adult life, physiological demands such as pregnancy and puberty exert external influence on the pituitary, altering the proportions of cell types within the pituitary to match hormonal output to physiological requirement. It is likely that these signals are transduced to secretory cells through complex networks that enable a global pituitary response of a specific cell type.

1.2. Pituitary tumours

1.2.1. Pituitary adenomas

Pituitary adenomas account for roughly 10-15% of diagnosed intracranial tumours in man (Dudley et al., 2009) It is estimated that roughly 20% of the population harbours a pituitary adenoma at time of death as judged by meta-analysis and autopsy, though the vast majority of these are too small to exert any clinical significance (Gueorguiev and Grossman, 2009). Adenomas are termed macroadenomas if they are larger than 10mm in diameter, and microadenomas if they are less than 10mm in diameter (Osamura et al., 2008).

Most pituitary adenomas are characterised by excess hormone secretion due to proliferation of one of the secretory cell types in the pituitary. The most common of these are prolactinomas (PRLomas), accounting for roughly 60% of pituitary tumours (discussed further in section 1.3) (Ezzat et al., 2004a). These arise due to proliferation of lactotroph cells which causes excess release of PRL from the pituitary, termed hyperprolactinaemia (Asa and Ezzat, 2002). Other adenomas are termed in a similar manner by the hormone they are producing in excess; GHoma, TSHoma, ACTHoma, FSHoma (Osamura et al., 2008). Roughly 25% of pituitary adenomas do not secrete any hormone and are termed non-functioning tumours (NFT's) (Colao et al., 2008). These tumours are thought to derive from gonadotroph cells as they often secrete the common α-subunit and LH-β and FSH-β subunits, though do not secrete active hormone. However, they can cause secondary endocrine dysfunction by applying pressure on normal pituitary cells and shrinking the pituitary (Greenman and Stern, 2009).

A key facet of pituitary adenomas is that they grow extremely slowly and are almost never malignant. Only 0.2% of pituitary tumours are malignant, and these seem mainly to arise from lactotroph or corticotroph cells (Caron, 2009). Most genetic mutations associated with cancerous tumour growth are absent in pituitary adenomas (e.g. ras, p53, PKC). This is not surprising as mutation of these genes causes uncontrolled, rapid growth and invasion of tumours, where the growth of pituitary tumours is slow and restricted to a single cell type (Asa and Ezzat, 1998). This in turn suggests that

adenoma progression is likely caused by malfunction of the usual endocrine/paracrine control mechanisms that regulate pituitary plasticity (Asa and Ezzat, 1998).

1.2.2. Craniopharyngiomas

Another distinct type of pituitary tumour is the craniopharyngioma. These are more aggressive than other pituitary tumours and mainly affect children aged between 5 and 14. They are often treated surgically, but commonly reoccur, often more aggressively than before surgery (Yang et al., 2010). They arise from a neoplastic transformation of the craniopharyngeal duct which becomes involuted during embryogenesis, and are thought to be caused by mutation of GSK-3β resulting in aberrant Wnt-canonical signalling (discussed in far greater detail in section 1.10.1) (Pettorini et al., 2010).

1.3. Prolactinomas

1.3.1. Incidence and effects

Roughly 60% of all pituitary tumours are prolactinomas (Asa and Ezzat, 2002). Prolactinomas grow extremely slowly and initially exert few debilitating effects so that the patient is often unaware of any problem. The first symptoms are caused by mass effect of the tumour, causing headaches and visual disturbances due to pressure applied to the optic nerve. Excess PRL secretion occurs as the lactotroph population proliferates resulting in hyperprolactinaemia, with symptoms including galactorrhea, oligomenorrhea, loss of libido and sexual dysfunction (Davis et al., 2001; Gurlek et al., 2007a).

1.3.2. Normal prolactin function

PRL is a 23kDa hormone secreted from lactotroph cells. It signals via the PRL receptor (PRLR) which is a single pass trans-membrane protein from cytokine-type 1 receptor family, and transduction of PRL signal is mediated via Jak-Stat, MAPK and PI3K pathways (Ben-Jonathan et al., 2008).

The best known action of PRL is on the mammary gland during pregnancy, where it regulates milk duct side-branching and elongation, as well as stimulating and maintaining milk production (Harris et al., 2004). It also regulates areas of reproduction, including maintenance of the corpus luteum throughout pregnancy, and control over certain mating and maternal behavioural patterns. PRL is also known to exhibit more diverse roles in the regulation of osmotic balance, immune function and angiogenesis (Freeman et al., 2000). Importantly, its secretion is strongly inhibited by dopamine from the hypothalamus, which provides the major regulatory control over PRL secretion (Gonzalez-Iglesias et al., 2007).

1.3.3. Treatment of prolactinomas

The first option for treating prolactinomas is the use of dopamine agonists such as bromocriptine or cabergoline. These inhibit PRL secretion and lactotroph proliferation, and in most cases this is enough to reduce tumour size and control PRL levels (Gonzalez-Iglesias et al., 2007). Some adenomas are resistant to dopamine agonist treatment, which is more common in macroadenomas than microadenomas as judged by PRL normalisation. This is thought to be due to decreased expression levels of dopamine receptors and decreased affinity for dopamine (Caccavelli et al., 1994). If dopamine agonist treatment is unsuccessful the patient can undergo surgery which is often ineffective in the long term, with many patients redeveloping pituitary dysfunction (Davis et al., 2001).

Prolactinomas are the only type of pituitary tumour where surgery is not the first option, and over 90% of prolactinoma patients are treated successfully with dopamine agonists precluding the need for surgery. While avoiding surgery is of course beneficial to the patient, it does make study of prolactinomas more difficult than other pituitary tumour types due to lack of primary tissue to study.

1.3.4. Oestrogen as a stimulator of lactotroph proliferation

Oestrogen has long been known to exert a proliferative effect on the lactotroph population. This was first observed during pregnancy, where high circulating E2 levels result in lactotroph hyperplasia, pituitary enlargement and increased circulating PRL levels (Asa et al., 1982; Goluboff and Ezrin, 1969; Lloyd et al., 1988). In vitro, E2

induces proliferation of the somatolactotroph GH3 cell line which can be reversed by treatment with anti-oestrogens (Horvath and Kovacs, 1988; Kansra et al., 2005; Lieberman et al., 1982; Song et al., 1989). Many of the effects of oestrogen in vivo have been studied using the oestrogen-sensitive Fischer 344 rat. Lactotroph hyperplasia, subsequently followed by pituitary tumour formation, can be induced by constant treatment with oestrogen for 3-12 weeks (Heaney et al., 1999; Mucha et al., 2007; Phelps and Hymer, 1983; Wiklund et al., 1981). This has provided an extremely useful model to study the effects of oestrogen on the pituitary in vivo. However, despite intense study, the mechanism by which oestrogen exerts this proliferative effect on lactotrophs is unknown.

1.4. Oestrogen signalling

Oestrogen signalling is transmitted via the oestrogen receptor (ER) which leads to the upregulation of E2 target genes (Mosselman et al., 1996). The effects of E2 can vary hugely between tissues and even between cells within the same tissue. Such variance is surprising as signalling is thought to be mediated by just two receptors, ERα and ERβ (Damdimopoulos et al., 2008). The two receptors are members of the nuclear receptor (NR) superfamily of ligand activated transcription factors, vary structurally and are coded for by genes on different chromosomes (Enmark et al., 1997; Gosden et al., 1986; Gruber et al., 2002; Pettersson and Gustafsson, 2001).

ER is normally present in the cytoplasm. E2 diffuses into the cell and binds to the ligand binding (LBD) of ER. ER-ligand complexes form dimers which are then transported to the nucleus where the DNA binding domain (DBD) of ER binds to DNA to initiate transcription of E2 target genes (Nilsson et al., 2001). The DBD consists of 2 zinc fingers which bind to specific sequences on DNA called oestrogen response elements (EREs) to initiate transcription (Whittal et al., 2000). Binding of the ligand to the LBD induces the required conformational change within the DBD to expose the Zn fingers and allow the receptor to bind to the ERE and initiate transcription (Heldring et al., 2007).

Both receptors are expressed in the adult pituitary, with higher expression of ERα than ERβ (Kansra et al., 2005). PRL secretion and lactotroph proliferation are both thought

to be mediated through ERα (Byrnes et al., 2009). ERα is highly expressed in prolactinomas, and there is a correlation between increased ERα expression and decreased dopamine receptor expression, possibly via E2 induced alternate splicing of the dopamine receptor (Wu et al., 2009). This provides a possible explanation for the increased resistance of macroprolactinomas to dopamine agonists.

1.5. Factors implicated in prolactinoma development

1.5.1. Proto-oncogenes

As described previously, very few genes associated with cancerous tumour growth have been identified in prolactinoma development. One exception was a mutation of the RAS gene which was found in one of the very rare, aggressive tumours described previously, which ultimately proved lethal. The group that identified this mutation studied 59 slowly proliferating prolactinomas and found no other examples of the mutation (Cai et al., 1994).

An early promising genetic factor found in pituitary adenomas was a mutation of the α-subunit of the stimulatory G protein, termed gsp. This occurs in 30% of GH-secreting tumours and results in constitutively active adenylate cyclase (Landis et al., 1989; Pertuit et al., 2009; Vallar et al., 1987). However, incidence of gsp within other tumour types is low and no consistent link has been shown between the mutation and tumour development (Davis et al., 2001). No mutations of gsp have been identified in prolactinomas. Some examples of c-myc and c-fos overexpression have been described in prolactinomas, though proliferation rates do not correlate with expression levels indicating other factors must be involved in tumour progression (Boggild et al., 1994).

Another factor implicated in pituitary tumourigenesis was an alternatively spliced form of the FGF-4 receptor, termed pituitary tumour derived (ptd)-FGFR-4. This was expressed in 40% of human pituitary adenomas studied, and could not be detected in normal pituitary tissue. The splice variant contained an N-terminal truncation resulting in a protein of 65kDa as opposed to the 110kDa normal protein. Transfection of ptd-FGFR-4 mRNA into NIH-3T3 cells induced cellular proliferation where injection of

FGFR-1 did not. Transgenic rats were generated using the PRL promoter to drive expression of ptd-FGFR-4 which resulted in pituitary tumours in 90% of rats by 11 months of age (Ezzat et al., 2002).

1.5.2. Tumour suppressor genes

Tumour progression can be caused by down regulation of tumour suppressing genes. A common tumour repressor gene which has been shown to cause pituitary adenomas is retinoblastoma gene (RB). A heterozygotic RB knock out mouse model (Rb+/-) develops aggressive tumours of the anterior and intermediate lobes after a few months of age (Chesnokova et al., 2008; Jacks et al., 1992). However in humans, deletion or mutation of RB are rare, and are generally restricted to aggressive carcinomas and a small proportion of non-functioning tumours and GH-omas (Pei et al., 1995; Simpson et al., 1999). Deletion of cyclin-dependent kinases p18 and p21 also result in tumour progression in mice, though reduced levels of either are rarely found in pituitary adenomas (Yu and Melmed, 2010).

Multiple endocrine neoplasia type 1 (MEN1) syndrome is a genetic disease caused by inactivating mutations of the tumour suppressor gene MEN1 which codes for the protein menin (Pannett and Thakker, 1999). Menin is a nuclear protein which interacts with numerous transcription factors, cytoskeletal proteins and DNA processing and repair proteins to regulate the cell cycle (Piecha et al., 2008). Patients generally develop tumours in 3 organs; the parathyroid gland, the pancreas and the pituitary. At 40 years of age, 40% of MEN-1 sufferers exhibit pituitary tumours, half of which are prolactinomas. It is unknown why mutation of MEN1 causes tumour growth, and especially confounding as to why tumours grow in these organs specifically (Yu and Melmed, 2010).

Another tumour suppressor gene which has been implicated in pituitary adenoma development is aryl hydrocarbon receptor interacting protein (AIP). AIP regulates the trafficking of aryl hydrocarbon receptor (ARH) between the cytoplasm and nucleus where it acts as a transcription factor regulating numerous xenobiotic metabolising enzymes. Toxins such as dioxin are thought to elicit most of their functions through binding to ARH (Karhu and Aaltonen, 2007). Mutations in AIP have been demonstrated in numerous familial circumstances, most notably with mutations being detected in 44% of familial somatotropinomas. However, incidence of mutation in sporadic tumours is far lower, with the mutation being harboured in less than 2% of sporadic

somatotropinomas, and less than 0.3% of other pituitary adenoma sub-types (Yu and Melmed, 2010).

1.5.3. Pituitary tumour transforming gene (pttg)

Much research in the last decade has focused on pituitary tumour transforming gene (pttg). Transcription of pttg produces the active protein PTTG, which was originally isolated from GH4 cells (Pei and Melmed, 1997). Human PTTG is expressed at low levels in many adult tissues, though is absent from the normal pituitary (Zhang et al., 1999b). Numerous studies have shown high expression levels in all pituitary adenoma subtypes (Hunter et al., 2003; McCabe et al., 2002; McCabe et al., 2003; Zhang et al., 1999a), and subsequently in thyroid, colon, breast and liver tumours (Kim et al., 2007).

Much of the pioneering work on PTTG was carried out in E2 induced prolactinomas in the Fischer 344 rat. E2 induces PTTG expression by activating an ERE in the PTTG promoter, and this upregulation precedes lactotroph hyperplasia and adenoma formation (Heaney et al., 1999). PTTG has been shown to upregulate basic FGF (bFGF) and vascular endothelial growth factor (VEGF) in E2 induced prolactinomas which promotes tumour growth and angiogenesis (Ishikawa et al., 2001; McCabe et al., 2002; McCabe et al., 2003).

PTTG has also been identified as the cell cycle regulator securin, which inhibits metaphase to anaphase transition by inhibiting sister chromatid separation (Zou et al., 1999). High levels of PTTG have been associated with genetic instability and aneuploidy in thyroid cancers (Kim et al., 2005). Pituitary tumours exhibit high frequency of aneuploidy, and it is thought that PTTG induced genetic destabilisation contributes to pituitary tumour growth (Heaney et al., 1999).

Further evidence for a role of PTTG in pituitary tumourigenesis comes from knock out and transgenic models in mice. Deletion of PTTG (PTTG-/-) results in pituitary hypoplasia. Furthermore, tumour induction caused by heterozygotic RB knock out (Rb+/-) is prevented when PTTG-/- mice are crossed with Rb+/- mice (Chesnokova et al., 2005; Chesnokova et al., 2007). Over expression of PTTG in the pituitary, driven by the promoter of the α-subunit of glycoprotein hormones (αGSU.PTTG), results in pituitary hyperplasia, which is enhanced when αGSU.PTTG mice are crossed with Rb+/- mice (Donangelo et al., 2006). These data are summarised in Figure 1.2.

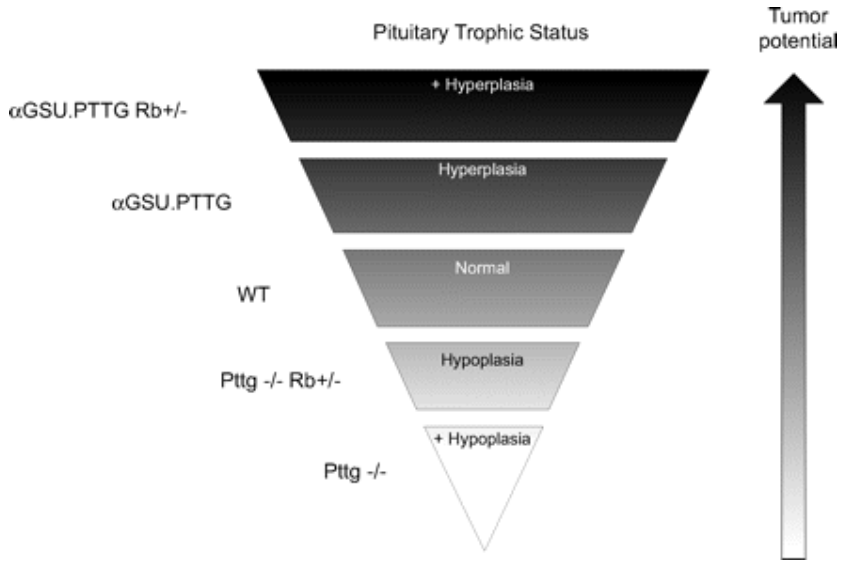


Figure 1.2. PTTG in pituitary tumourigenesis. Mouse models with descending levels of pituitary PTTG are shown on the left, with or without cross-over with tumourigenic RB+/- mouse model. Horizontal bars represent the pituitary trophic status of the pituitary, with arrow (right) indicating the likelihood of tumour development. Figure adapted from Donangelo et al, 2006.

Overall, a number of factors have been implicated in the pathogenesis of pituitary tumours. However, despite intense research, only a small number of mutations have been identified in these factors, they are not found in all sporadic tumours, and are often restricted to familial tumours. Much evidence has focused on the role of PTTG as it is upregulated in pituitary adenomas and has been linked to invasiveness of tumours. However, if this is the case, tumours would be expected to grow faster and invade more than observed in pituitary tumours suggesting other factors are involved in regulating tumour growth. Therefore, it is important to look for new pathways which may be involved in pituitary tumour development.

1.6. Wnt signalling

1.6.1. Wnt signalling in oestrogen-induced lactotroph hyperplasia

In an attempt to identify novel signalling pathways in prolactinoma development, our group conducted microarray analysis on rat pituitaries undergoing lactotroph hyperplasia induced by the synthetic oestrogen diethylstilbestrol (DES). Amongst several genes whose regulation was altered, we noted upregulation of the developmental protein Wnt-4 (discussed in detail in section 1.11). The aim of this thesis is to examine the potential role of Wnt-4 in prolactinoma development.

1.6.2. The Wnt family

Wnt proteins were initially discovered in the 1980's. In mammalian cells, Wnt was identified as the MMTV proto-oncogene Int-1, and in Drosophila it was identified as Wingless. The two were found to be homologous, and the combination of these terms produced the name Wnt (Nusse et al., 1991; Rijsewijk et al., 1987). Wnt molecules signal through Frizzled (Fz) receptors (see section 1.9), and in mammals there are 19 Wnt ligands and 10 Fz receptors (Coudreuse and Korswagen, 2007). Since their discovery, a huge array of actions has been attributed to Wnts. Initially focus was placed on their role in development and cancer, but more recently they have been shown to play numerous roles in adult life as well.

1.7. General roles of Wnt signalling

1.7.1. Roles in development

Wnt proteins exert diverse effects on cells and tissues by driving proliferation, differentiation, apoptosis and cell survival (Willert and Jones, 2006). Wnt signalling has been implicated in virtually every aspect of normal body function, including key developmental process such as sex determination (Tevosian and Manuylov, 2008),

facial morphology (Liu et al., 2010a) and neural crest formation (Kuriyama and Mayor, 2008), as well as development of individual organs e.g. heart (Gessert and Kuhl, 2010), kidney (Schmidt-Ott and Barasch, 2008), lung (Weng and Liu, 2010), pituitary (Treier et al., 1998) and intestine (Clarke, 2006) to name but a few.

1.7.2. Roles in adult life

More recently it has emerged that Wnts play key roles in modulating physiological processes in adult life as well as in development. Examples include regulation of adult T-cells, monocytes and macrophages during the immune response (Staal et al., 2008), control over endothelial cells in the vascular system to induce angiogenesis (Franco et al., 2009) and regulation of the thickness and pigmentation of skin (Yamaguchi et al., 2009).

Over the last 10 years, a role for Wnt signalling in tissue maintenance and repair has been studied through regulation of stem cell niches. The classic examples are the loss of stem cells from the colon after knock out of the Wnt activated transcription factor TCF4 (Korinek et al., 1998), and inhibition of hematopoietic stem cell (HSC) renewal using Wnt inhibitors, with reciprocal differentiation induction of HSCs with Wnt-3A stimulation (Reya et al., 2003; Willert et al., 2003).

1.7.3. Roles in cancer

With such a huge array of growth inducing and developmental effects, it is hardly surprising to find that Wnt signalling has been implicated in numerous cancers. Aberrations in Wnt signalling have been identified in liver (Miyoshi et al., 1998), intestinal (Harada et al., 1999), prostate (Thudi et al., 2010) ovarian (Chen et al., 2010) and renal (Banumathy and Cairns, 2010) cancers. Once again, these are just a few examples found in the literature, aiming to give an indication of the huge variety of effects attributed to Wnt signalling in recent years. More detailed descriptions of Wnt involvement in cancer will be given relative to the specific Wnt signalling pathways in sections 1.10.1/2/3.

1.8. Wnt structure

Wnt molecules are highly conserved between species. Generally they are about 350 amino acids long with a molecular weight of about 40kDa (Yavropoulou and Yovos, 2007). They do not have any functional domains, but contain 22-25 charged cysteine residues which are thought to form intra- and inter-molecular di-sulphide bonds to regulate protein folding (Tanaka et al., 2002).

Despite the numerous charged cysteine residues, Wnt molecules are hydrophobic. This is attributed to addition of a palmitate group (a 16-carbon saturated fatty acid) at a highly conserved cysteine residue corresponding to C77 in mouse Wnt-3A (Willert et al., 2003), while further post-translational modification is observed in the form of multiple glycosylation sites (Coudreuse and Korswagen, 2007). Figure 1.3 shows the evolutionary conservation of these sites between C. elegans, Drosophila and Mouse.

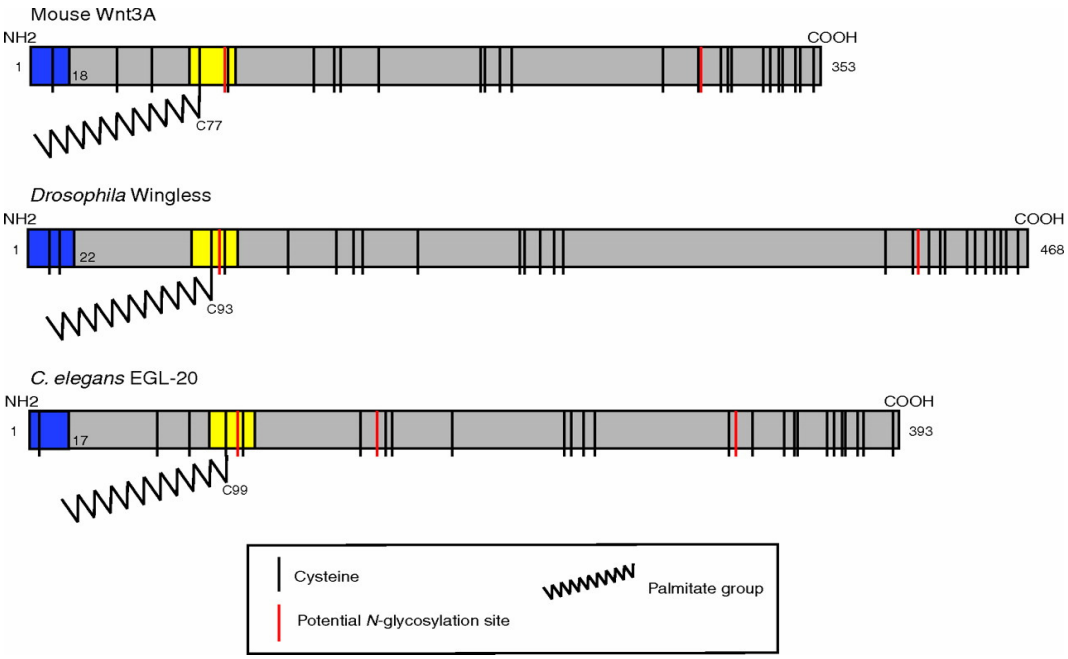


Figure 1.3. A schematic diagram of Wnt-3A from mouse, Wingless from Drosophila and EGL-20 from C.elegans. Wnt molecules express 22-25 cysteine molecules which are conserved between species. A palmitate molecule binds to a highly conserved cysteine (at C77, C93 and C99 in mouse, Drosophila and C.elegens respectively) across the yellow region, while numerous glycosylation sites important for Wnt secretion are present. Adapted from Coudreuse and Korswagen, 2007.

Glycosylation may be involved in intracellular trafficking as levels of glycosylation are higher in Wnts prior to secretion (Kitajewski et al., 1992; Mason et al., 1992), and secretion of Wnt-3A and Wnt-5A is impaired when glycosylation sites are removed. Conversely, palmitoylation seems to be important for Wnt signalling. Removal of the palmitate group from Wnt-3A and Wnt-5A does not affect secretion, but reduces binding affinities to Fz receptors and prevents downstream Wnt signalling events (Komekado et al., 2007; Kurayoshi et al., 2007).

Another possible benefit of palmitoylation may relate to the generation of concentration gradients which mediate Wnt signalling. Unbound Wnt molecules interact with the cell membrane via their palmitate group. However, Wnts have been shown to bind to extracellular lipoproteins which displace them from the cell membrane. This allows them to travel up to 20-30 cell distances away from their point of secretion (Figure 1.4) (Coudreuse and Korswagen, 2007; Neumann and Cohen, 1997), while knock down of lipoproteins severely reduces the range of Wnt signalling (Panakova et al., 2005).

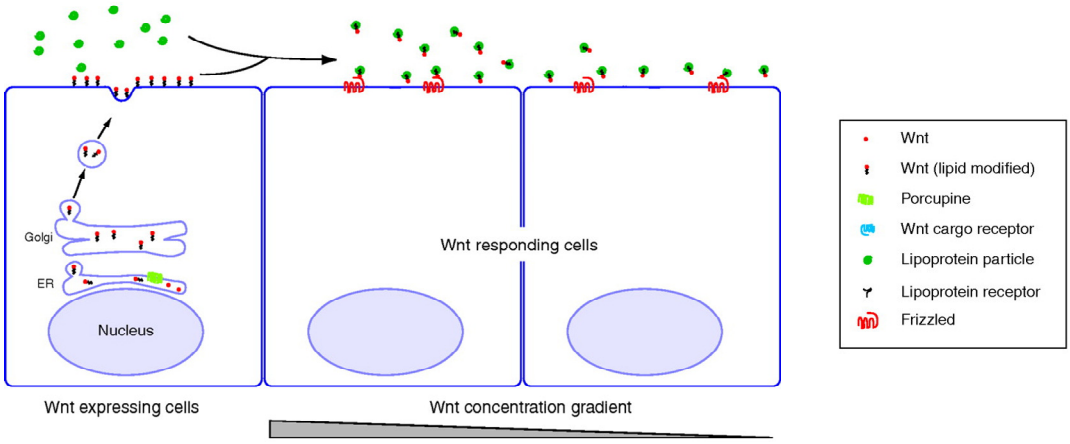


Figure 1.4. Long range Wnt signalling enabled by lipoprotein transport. Wnt molecules are bound in extracellular regions by lipoproteins which counteract Wnt adhesion to the cell membrane via the palmitate group. This allows Wnts to spread out further across their morphogenic field, generating concentration gradients which mediate Wnt signalling. Adapted from Coudreuse and Korswagen, 2007.

1.9. Frizzled receptors

Interestingly, all the diverse effects of Wnt signalling seem to be mediated via Wnt binding to Fz receptors. There are 10 known mammalian Fz receptors containing 7-transmembrane domains and a large, highly conserved extracellular motif containing 10 cysteine residues called cysteine rich domains (CRD) (Lyons et al., 2004). Wnts bind to the CRD of a Fz receptor, and it has been shown that a number of Fz receptors bind multiple Wnts with high affinity (Wu and Nusse, 2002).

The combination of Wnt ligand and Fz receptor interacting determines downstream signalling events. Therefore, the overall effect of Wnt signalling is dependent on the specific expression patterns of Wnt ligands and Fz receptors in a particular tissue. Clearly, with 19 ligands and 10 receptors, there is huge potential for variation in the downstream effects of Wnt signalling, giving an explanation of the hugely variable effects of Wnt molecules described previously (Rao and Kuhl, 2010).

1.10. Wnt signaling pathways

Wnt molecules classically activate 3 signalling pathways; the Wnt-canonical pathway, the Wnt-planar cell polarity (PCP) pathway and the Wnt-calcium pathway. By far the most studied of these is the canonical pathway due to its involvement in numerous cancers. While the canonical pathway has been extensively defined, the PCP and calcium pathways remain poorly understood. As such, Wnt signalling is often discussed in terms of canonical or non-canonical signalling, often with no differentiation made between the 2 non-canonical pathways. Over recent years, more focus has been applied to the non-canonical pathways, and they are slowly becoming accepted as vital pathways to regulate the huge variation in Wnt signalling events.

Traditionally, specific Wnt molecules were assigned to either canonical or non-canonical functions. For instance, Wnt-1, Wnt-3A and Wnt-8 are generally known as canonical Wnts, Wnt-5A and Wnt-11 are considered non-canonical Wnts, while Wnt-2, Wnt-4, Wnt-5B and Wnt-6 have been shown to activate both canonical and non-canonical pathways (Kikuchi and Yamamoto, 2008). This was based on early studies of

Wnt protein function which defined Wnt molecules as canonical or non-canonical depending on their ability to transform C57 MG mammary epithelial cells (Wong et al., 1994). More recently it has become apparent that this system is an oversimplification; Wnt-11 activates the canonical pathway in axis formation in Xenopus (Tao et al., 2005), Wnt-3A activates the calcium pathway in bone formation in mouse (Tu et al., 2007), while Wnt-5A and Wnt-11 have been shown to interact physically to activate canonical signalling in Xenopus embryogenesis (Cha et al., 2008).

In the same manner Fz 4 has been shown to activate canonical signalling in the blood brain barrier (Ye et al., 2009), and also activates non-canonical signalling in retinal angiogenesis (Robitaille et al., 2002). It is becoming clear that analysis of any Wnt ligand or Fz receptor in a biological context requires detailed examination of all Wnt signalling pathways.

1.10.1. The Wnt-Canonical Pathway

1.10.1.1. β-Catenin

Canonical signalling is centred around the stability of cytoplasmic β-Catenin. β-Catenin is a large protein which can interact with numerous proteins through structures known as armadillo repeats (arm repeats) which are made up of two sequential 20aa alpha helices forming a hairpin structure. β-Catenin contains a sequence of 12 arm repeats, which collectively form a positively charged super-helix to which multiple regulatory proteins can bind simultaneously (Huber et al., 1997; Willert and Nusse, 1998).

β-Catenin plays two major roles in cells. At the cell membrane it interacts with the cytoplasmic regions of cadherin molecules to regulate cell-cell adhesion via adherens junctions (see section 1.10.3.2) (Shapiro and Weis, 2009). In the nucleus, β-Catenin interacts with transcription factors T-cell specific transcription factor (TCF) and Lymphoid enhancer-binding factor (LEF) to influence expression of a host of genes which regulate diverse aspects of cell behaviour (Mo et al., 2009).

Free β-Catenin is found in the cytoplasm, and is rapidly destroyed under basal conditions preventing gene transcription. This occurs in 2 steps; firstly adenomatous polyposis coli (APC) and axin bind to β-Catenin via arm repeats to form the “destruction

complex". β-Catenin is then sequentially phosphorylated by casein kinase 1 (CK1) at Ser-33, and glycogen synthase kinase-3β (GSK-3β) at Ser-37 and Thr-41 resulting in its ubiquitination at the proteasome (Mo et al., 2009; Price, 2006; Willert and Jones, 2006). The structure of β-Catenin showing the arm repeats and interactions with key regulating proteins is shown in Figure 1.5.

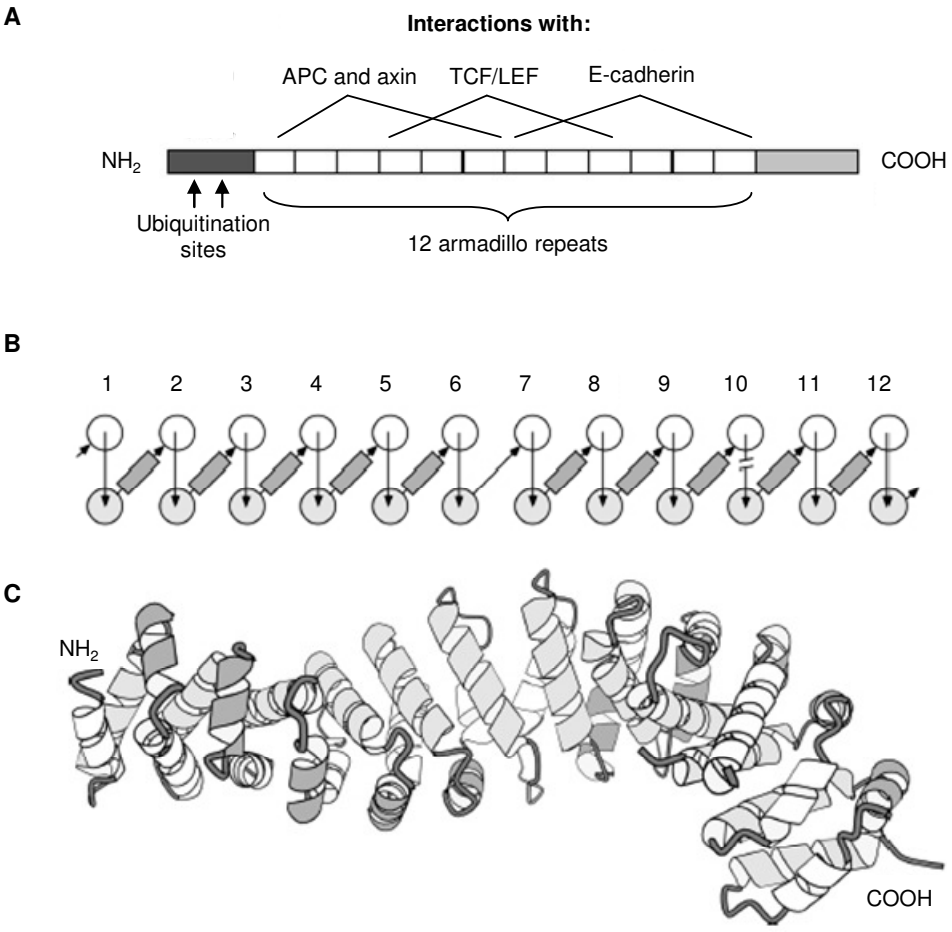


Figure 1.5. β-Catenin structure. A – The primary structure of β-Catenin contains 12 arm repeats which allow interactions with APC, axin, TCF/LEF and E-cadherin. Ubiquitination sites are located at the amino terminus. **B** – Topological representation of the arm repeat region of β-Catenin. α-helicies are represented as either circles or rectangles as viewed from top or side respectively. **C** – Ribbon representation of the arm repeat region of β-Catenin demonstrating the superhelical structure formed from repeating arm repeats. Figure adapted from Willert and Nusse, 1998.

1.10.1.2. Wnt activation of canonical signalling

Binding of Wnt to Fz inhibits ubiquitination of β-Catenin in two ways. Firstly it hyperphosphorylates low density lipoprotein related protein (LRP) which is located adjacent to Fz on the cell membrane and is essential for canonical signalling. Hyperphosphorylated LRP binds axin with high affinity and sequesters it away from the destruction complex to the cell membrane. Axin is expressed at relatively low levels, and so its binding to LRP and removal from the destruction complex rapidly inhibits β-Catenin degradation (Lee et al., 2003).

Secondly, binding of Wnt to Fz recruits the cytoplasmic protein Dishevelled (Dsh) to the membrane. Here Dsh is phosphorylated which allows it to bind and inhibit CK1 and GSK-3β (Lee et al., 2001). These factors allow β-Catenin to accumulate in the cytoplasm, and then translocate into the nucleus where they activate TCF/LEF to elicit transcription of Wnt target genes (Cadigan and Liu, 2006; Chien et al., 2009; Kikuchi and Yamamoto, 2008; van and Nusse, 2009). The mechanism by which β-Catenin translocates to the nucleus is not known, but it does not require a nuclear localisation signal, the most common mechanism by which molecules enter the nucleus (Fagotto et al., 1998)

The TCF/LEF family has four members; LEF-1, TCF-1, TCF-3 and TCF-4. These are normally bound to intranuclear repressors of canonical signalling termed Groucho and C-terminal binding protein (CBP). β-Catenin displaces Groucho and CBP from TCF/LEF by unknown mechanisms, providing β-Catenin with a DNA binding domain to upregulate canonical genes (Ilyas, 2005; Jin, 2008; Jin and Liu, 2008; Staal et al., 2008). The canonical pathway is summarised in Figure 1.6.

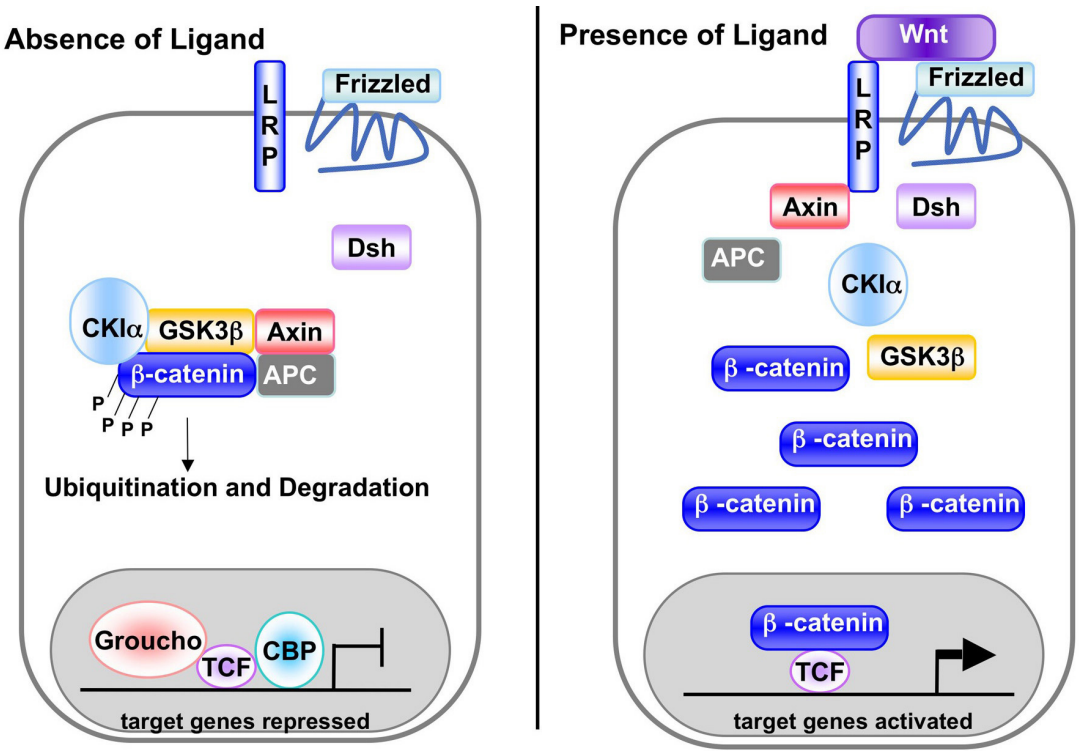


Figure 1.6. The canonical pathway. In the absence of ligand (left) β-Catenin is bound by axin and APC forming the "destruction complex", allowing GSK-3β and CK1 to phosphorylate β-Catenin resulting in its degradation at the proteasome. In the nucleus, TCF transcriptional function is repressed by Groucho and CBP. When Wnt binds to Fz (right), LRP is phosphorylated and sequesters axin to the membrane, and Dsh is bought to the membrane where it inactivates GSK-3β and CK1. β-Catenin accumulates in the cytoplasm, and translocates to the nucleus where it displaces Groucho and CBP from TCF, binds TCF itself, and activates transcription of canonical target genes. Image taken from Wormbook – The online review of C.Elegans biology (Eisenmann D, 5 A.D.)

1.10.1.3. Extracellular inhibition of the canonical pathway

The canonical pathway is inhibited by a number of secreted factors. The best categorised of these is Dickkopf (Dkk), which inhibits Wnt signalling by forming a ternary complex with Wnt, Fz and LRP (Rao and Kuhl, 2010). Dkk was discovered through its ability to inhibit axis duplication in Xenopus embryos overexpressing β-Catenin (Krupnik et al., 1999). It is upregulated by canonical signalling, providing a

negative feedback loop to regulate the canonical pathway (Gonzalez-Sancho et al., 2005).

Other extracellular canonical inhibitors include secreted frizzled related proteins (sFRPs) and Wnt Inhibitory Factors (WIFs). The human sFRP family consists of 5 members which contain a CRD sharing 30-50% homology with the CRD on Fz receptors. Wnt molecules thus bind to sFRPs and are prevented from binding to Fz (Melkonyan et al., 1997). The actions of WIFs are less understood. They do not have a CRD, but instead are defined by five evolutionarily conserved epidermal growth factor (EGF)-like repeats. The function of these EGF repeats is not known but WIFs have been shown to down regulate Wnt signalling in a number of tissues (Ilyas, 2005).

1.10.1.4. Gene regulation by the canonical pathway

The canonical pathway has been shown to regulate over 100 genes. It is thought that β-Catenin directly activates a relatively small number of genes itself, such as c-myc, MMP-7, cyclin D1 and VEGF. These genes themselves regulate pathways that have diverse effects on gene activation themselves, ultimately regulating a much larger number of genes in a secondary or tertiary manner to bring about the effects of the canonical pathway (Vlad et al., 2008).

After β-Catenin has activated target genes, it exits the nucleus by binding to axin and APC which are thought to shuttle between the cytoplasm and nucleus (Cong and Varmus, 2004; Henderson and Fagotto, 2002). However, the mechanisms involved are poorly understood and require much further attention.

1.10.1.5. The canonical pathway in cancer

The canonical pathway was quickly identified as playing a role in cancer and tumour progression. The most widely studied example is in colorectal cancer, where 80% of tumours exhibit mutations of APC resulting in constitutively active β-Catenin, excessive stem cell renewal and tumour formation (Rowan et al., 2000; Saif and Chu, 2010; Takemaru et al., 2008). The Wnt inhibitor Dkk is downregulated in colorectal cancer,

suggesting loss of negative feedback may contribute to cancer progression (Gonzalez-Sancho et al., 2005).

Mutations of β-Catenin have been found in 54% of endometrioid ovarian cancers, as well as in two paediatric cancers, hepatoblastoma (Koch et al., 2004) and Wilms' Kidney tumours (Li et al., 2004). Interestingly, although most hepatoblastoma tumours express nuclear β-Catenin, 80% of them also display upregulated Dkk levels, suggesting its potential role in clinical therapy (Wirths et al., 2003). Dysregulated β-Catenin-LEF coupling has been identified in breast cancer (Gebeshuber et al., 2007), β-Catenin upregulation of the apoptosis inhibitor survivin contributes to the development of small cell lung cancer (Nakashima et al., 2010) and nuclear β-Catenin was found in 23% of metaplastic breast carcinomas (Lacroix-Triki et al., 2010).

1.10.2. The Wnt-calcium pathway

1.10.2.1. Calcium signalling

Calcium is a vital regulator of intracellular signalling in virtually all cells in multicellular organisms. Alterations in intracellular calcium levels activate ubiquitous calcium sensors like protein kinase C (PKC) and calmodulin (CaM) which in turn activate secondary messengers such as calmodulin dependent kinases (CaMK I-IV) and calcineurin which shape subsequent cellular responses (Gwack et al., 2007).

Intracellular calcium levels can be elevated from 2 major calcium sources. Ion channels at the cell membrane can open to allow influx of calcium from the extracellular space, while intracellular stores of calcium can be also be released. There are feedback mechanisms between receptors and channels at both sites. In most non-excitable cells, the majority of intracellular calcium release comes from the inositol 1, 4, 5-triphosphate (IP3)-sensitive calcium channels in the endoplasmic reticulum. Opening of IP3 channels can then induce calcium release from neighboring calcium channels in the ER in a process called "calcium induced calcium release". If release of intracellular calcium is continued and stores become depleted, the store operated calcium entry (SOC) pathway becomes activated at the plasma membrane triggering calcium influx from the extracellular space (Berridge, 2009; Mikoshiba, 2007; Slusarski and Pelegri, 2007).

Changes in intracellular calcium are usually transient as newly released calcium is quickly bound by calcium binding proteins. These can either be calcium buffers whose role is simply to absorb free calcium and transport it back into calcium stores, or can be calcium sensors such as CaM which mediate calcium signalling. As such, intracellular calcium levels can often be observed as dynamic oscillations, and changes in the frequency, amplitude and duration of these oscillations can determine downstream cellular responses (Slusarski and Pelegri, 2007; Uhlen and Fritz, 2010).

1.10.2.2. Wnt induction of calcium transients and calcium enzymes

The Wnt-calcium pathway was first discovered on the finding that injection of Wnt-5A mRNA intro zebrafish embryos induced IP3 mediated intracellular calcium release and doubled the frequency of calcium transients in the subsequently formed blastocyst (Slusarski et al., 1997a; Slusarski et al., 1997b), while mutation of Wnt-5A reduced calcium transients in the same model (Westfall et al., 2003). This calcium release may require the action of Dsh as a gain-of-function Dsh mutant results in increased calcium fluxes in Xenopus embryos, while loss-of-function Dsh mutants cause decreased calcium fluxes (Sheldahl et al., 2003). Dsh is though to activate phospholipase C (PLC) which then initiates calcium release from intracellular stores by activation of IP3 channels (Staal et al., 2008).

It has subsequently been shown that Wnt-induced calcium release was sufficient to activate PKC in mouse embryonic bone formation (Tu et al., 2007) and CaMKII in dorsoventral axis formation in Xenopus embryos (Kuhl et al., 2000). Calcineurin is also activated by the Wnt-calcium pathway, which subsequently activates the transcription factor nuclear factor of activated T-cells (NFAT) which may mediate some effects of the Wnt-calcium pathway (Rao and Kuhl, 2010); Wnt molecules activate NFAT to promote cardiac hypertrophy and cardiac remodeling (Rao and Kuhl, 2010), regulate axis formation in Xenopus (Saneyoshi et al., 2002) and play a key role in bone formation by regulating osteoblast proliferation and differentiation (Fromigue et al., 2010; Stern, 2006). The eventual effects of the Wnt-calcium pathway are thought to include altered cytoskeletal remodeling and cell motility, differentiation, apoptosis and morphogenesis. The Wnt-calcium pathway is summarized in Figure 1.7.

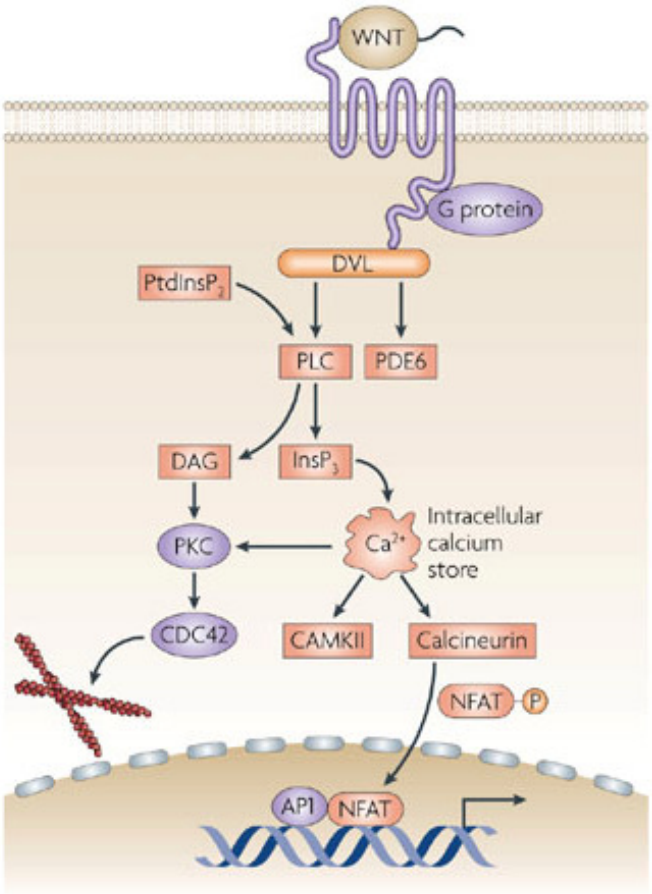


Figure 1.7. The Wnt-calcium pathway. Binding of Wnt to Fz leads to calcium release from intracellular stores through IP3 channels on the endoplasmic reticulum, mediated by Dsh. Increased intracellular calcium levels in turn activate calcium enzymes PKC, calmodulin kinases (eg CamKII) which may regulate cytoskeletal remodelling, and calcineurin. Activation of calcineurin leads to NFAT phosphorylation and translocation to the nucleus where it acts as a transcription factor to upregulate calcium pathway genes. Figure adapted from Staal and Tiessmen, 2008.

The role of the Wnt-calcium pathway in cancer has been poorly studied, however, a few examples in the literature are available. In virtually all cases, studies focus on the actions of Wnt-5A which is considered the main “non-canonical” Wnt; Wnt-5A was increased in a human prostate cancer cell line which was associated with upregulated CamKII signalling and altered cytoskeletal remodeling and cell motility (Wang et al., 2010), Wnt-5A was upregulated in human cutaneous melanoma cells and its over expression was correlated with PKC activation and increased cellular invasion (Weeraratna et al., 2002) and Wnt-5A activated NFAT to promote tumour growth in human breast cancer cells (Leandersson et al., 2006).

1.10.3. The Wnt-planar cell polarity pathway

Planar Cell Polarity (PCP) is the generation of a uniform orientation of a population of cells along a single epithelial plane. Establishment of PCP is found throughout the animal kingdom. It regulates the orientation of wings of a bird, scales on a fish and hairs on mammals. PCP is also evident within the body, such as in the orientation of microvilli extending into the intestine, organisation of mechanosensory hairs in the cochlea, or extension of a single axon branching out from a neurone (Fanto and McNeill, 2004; Rao and Kuhl, 2010; Zallen, 2007).

The most studied example of PCP is the arrangement of bristles on the wing of Drosophila. Orientation of the bristles is dependent of localization of Fz and five other key cytoplasmic proteins which organise themselves along the proximal-distal axis. In particular, Flamingo, Prickle and Dsh bridge the proximal-distal axis on both sides, Fz and Diego localise distally, and Strabismus localises proximally, thus establishing polarity of the cell (Wang, 2009; Widelitz, 2005). The mechanisms by which this occurs are not understood, but have been summarised in Figure 1.8.

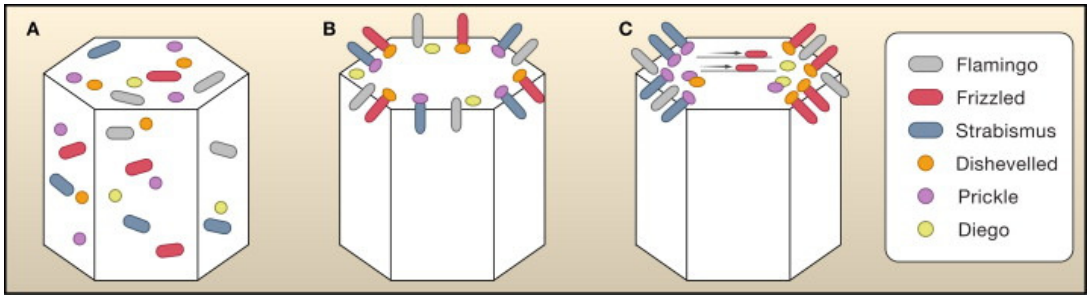


Figure 1.8. Establishment of planar cell polarity in Drosophila. A schematic diagram of a Drosophila wing epithelial cell at early (A/B) and late phase (C) in development. Distal is to the right, proximal is left and apical is up. **A** – The core PCP proteins are distributed homogenously in the cytoplasm. **B** – Flamingo moves to the apical membrane, and recruits Fz and Strabismus, which then recruit and bind Dsh and Prickle respectively. **C** – PCP proteins sort into distal and proximal domains with Fz and Diego located distally, Strabismus located proximally and Flamingo, Prickle and Dsh bridging the axis. The process is modulated by a proximodistally aligned microtubule network (grey lines in C). Figure adapted from Zallen, 2007.

The establishment of PCP in vertebrates is poorly understood, though it involves Dsh recruitment by Fz at the cell membrane, which then activates small GTPases such as Rho A and Rac. Other downstream cellular regulators such as Rho Kinase (ROCK) and JNK are then activated which modulate gene transcription and cytoskeletal reorganization (Kikuchi and Yamamoto, 2008; Rao and Kuhl, 2010). The pathway is summarized in Figure 1.9.

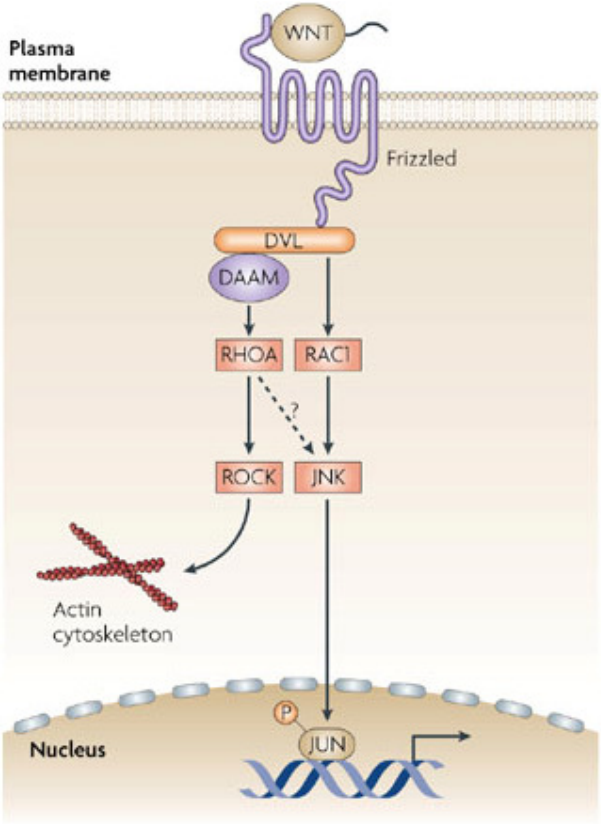


Figure 1.9. The planar cell polarity pathway in vertebrates. Wnt binding to Fz recruits Dsh to the membrane. Dsh activates small GTPases Rho A and Rac, which in turn activate ROCK and JNK downstream effectors to regulate gene transcription and cytoskeletal reorganisation. Figure adapted from Staal and Tiessmen, 2008.

1.10.3.1. The planar cell polarity pathway regulates cell motility in convergent extension

A well studied example of PCP signalling in vertebrates is during convergent extension during gastrulation in Xenopus, where polarised mesenchymal cells derived from the mesoderm interact to lengthen the embryo along the anterior-posterior axis (Yamanaka et al., 2002). In these cells, Prickle is located at the anterior membrane of migrating

cells, while Dsh is located at the posterior membrane, in a manner reminiscent of the proximal and distal distribution of these proteins in Drosophila wing cells described in Figure 1.9 (Wada and Okamoto, 2009; Yin et al., 2008). The PCP pathway is thought to play a key role in convergent extension as mutation of Strabismus results in decreased velocity of dorsally directed cells (Jessen et al., 2002), knock down of Prickle with siRNA results in shortening of the embryo (Carreira-Barbosa et al., 2003) while loss-of-function studies demonstrate that the process is dependent on Wnt-5A and Wnt-11 signalling (Kilian et al., 2003).

1.10.3.2. Planar cell polarity in cancer due to epithelial to mesenchymal transition

A key attribute of mesenchymal cells is that they are not connected to each other via adherens junctions, which allows them to migrate past neighboring cells during convergent extension. This differs from epithelial cells which are connected to each other via adherens junctions (see figure 1.10) and are therefore unable to move past each other (Zallen, 2007).

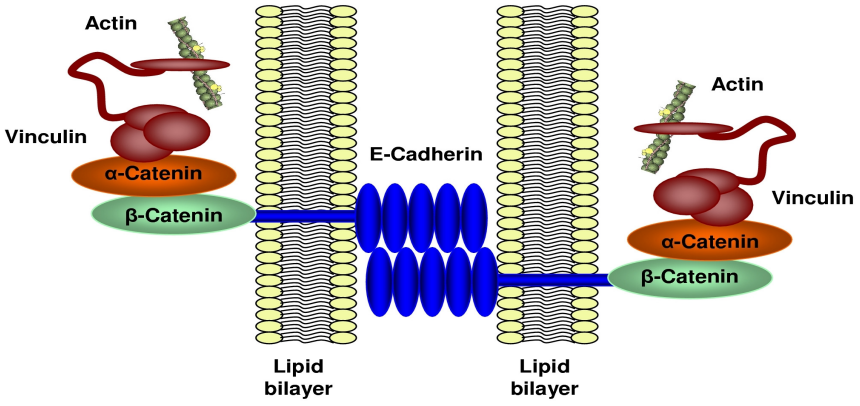


Figure 1.10. Adherens junction structure. Schematic diagram showing 2 adjacent cells bound by an adherens junction. Extracellular regions on E-Cadherin bind to each other in the extracellular space. β-Catenin binds to the intracellular tail of E-Cadherin via arm repeats, and α-Catenin then binds to β-Catenin. The head and tail domains of vinculin separate, activating the protein. The head binds α-Catenin, while the tail binds to actin filaments to modulate actin polymerisation. The junction provides a direct link between cytoskeletal structures of neighboring cells (Pokutta and Weis, 2002).

E-Cadherin is a vital component of adherens junctions. A relatively simple, but very important distinction can be made between static epithelial cells expressing membrane bound E-Cadherin, and mobile mesanchymal cells which express N-Cadherin, but do not express E-Cadherin (Huber et al., 2005). This is important because the loss of adherens junctions and the subsequent ability of cells to migrate away from their original location is a vital step in tumour metastasis. Wnt-PCP signalling has been implicated in this process, which is known as epithelial to mesenchymal transition (Heuberger and Birchmeier, 2010).

Wnt-5A over expression in human gastric cancer cells resulted in increased cell migration and invasion due to activation of Rac and focal adhesion kinase (FAK). Importantly, no effect on proliferation was observed suggesting this pathway does not directly cause tumour growth, but only changes the invasive characteristics of cells (Kurayoshi et al., 2006). In a similar manner, Wnt-5A was overexpressed in human breast cancer cells leading to activation of Jnk and increased cell invasiveness. Canonical signalling was also detected and considered key for tumour cell proliferation, though it had no impact on tumour invasiveness demonstrating 2 Wnt pathways working together to drive cancer (Pukrop et al., 2006). Further examples of PCP induced cell invasiveness in cancer have been documented in renal (Hirata et al., 2010) lung (Zhao et al., 2010), prostate (Yamamoto et al., 2010) and intestinal (Sancho et al., 2009) cancer. In virtually all these cases, Wnt activation of the PCP pathway is observed only in late phases of cancer when patient prognosis is poor, suggesting PCP activation could play a key role in transition from pre-malignant tumour to malignant tumour, and could therefore be a key therapeutic target in cancer research.

1.10.4. Overlap of Wnt signalling pathways

In previous sections, the three Wnt signalling pathways have been described in isolation from one another, however there is a growing body of evidence suggesting complex interplay between the pathways.

For example, the traditional classification of Wnt ligands to a specific pathway can no longer hold true as Wnt-5A (which has been used to study virtually all key aspects of non-canonical signalling) activates Jnk (Pukrop et al., 2006), calcium (Slusarski et al., 1997b) and canonical (Mikels and Nusse, 2006) signalling depending on cellular context. Wnt-5A also activates both the Jnk and calcium pathways in axis formation.

Although they have been studied independently, mutation of Wnt-5A downregulates PCP and calcium pathways to cause identical embryo dysmorphology, suggesting they are part of the same pathway (Kilian et al., 2003; Kuhl et al., 2000). Wnt-3A, known as "the canonical" Wnt, does activate the canonical pathway in most cases, but has also been shown to activate Jnk pathways in Chinese hamster ovary cells to regulate cell migration (Endo et al., 2005), and PKC in embryonic bone development in mice (Tu et al., 2007).

Downstream signalling components from the different pathways have also been shown to interact. CamKII is involved in epithelial-mesenchymal transition during neural crest migration which is usually attributed to the PCP pathway (De et al., 2005) while conversely, Prickle has been shown to regulate intracellular calcium levels during gastrulation (Veeman et al., 2003). Non-canonical pathways have been shown to inhibit canonical signalling numerous times; Wnt-5A promoted β-Catenin degradation in a colon cancer cell line (Topol et al., 2003), overexpression of Wnt-5A reduced invasiveness, proliferation and migration of thyroid tumour cells by promoting membrane localisation of β-Catenin (Kremenevskaja et al., 2005), Wnt-5A down regulation in Xenopus embryos resulted in dorsalisation due to increased nuclear β-Catenin expression (Westfall et al., 2003) and Wnt/Jnk induced upregulation of Dkk caused inhibition of canonical signalling in human non-small cell lung cancer cells (Lee et al., 2004).

β-Catenin is the central player of the canonical pathway, but is also a key component of adherens junctions which are deconstructed during PCP-induced cell migration (Kurayoshi et al., 2006). Adherens junction deconstruction can in turn activate canonical signalling as β-Catenin freed from the cell membrane then accumulates in the cytoplasm, which can then drive cancer cell proliferation (Pukrop et al., 2006). Conversely, β-Catenin can be sequestered away from the nucleus to the cell membrane to inhibit canonical signalling (Bernard et al., 2008).

The cytoplasmic protein Dsh has been implicated in all 3 pathways (Rao and Kuhl, 2010). During canonical signalling, Dsh molecules polymerise to form large structures which bind Fz and LRP at the cell membrane. Axin and GSK-3β then bind to the Dsh/Fz/LRP complex which prevents degradation of β-Catenin (Cliffe et al., 2003; Zeng et al., 2008). In PCP signalling, Dsh binds Fz and Diego at the distal membrane, while Prickle blocks Dsh activation and prevents its localization at the proximal membrane (Jenny et al., 2005). Dsh has also been shown to interact directly with Dsh associated

activator of morphogenesis (Daam) which results in the formation of the Rho-GTP complex which subsequently activates ROCK to regulate cytoskeletal structure (Habas et al., 2001). Dsh also impacts on calcium signalling, as gain-of-function Dsh results in increased calcium flux and translocation of PKC to the membrane, whereas loss-of-function results in decreased calcium flux and reduced PCK membrane translocation (Sheldahl et al., 2003).

The mechanisms by which a specific Wnt pathway is activated in a given context is currently far from understood. It is becoming clear that the Wnt pathways can no longer be considered independent of each other, and highlights a point made earlier that that when studying an aspect of Wnt signalling, attention should be given to the specific Wnts present, the expression of Fz receptors and the downstream effects of all signalling pathways in that context.

1.11. Wnt signalling in the pituitary

Dr Sönke Friedrichsen, a postdoctoral fellow in our lab, conducted microarray analysis on pituitaries undergoing oestrogen-induced lactotroph hyperplasia. Amongst several genes whose regulation was altered, Wnt-4 was found to be upregulated. This early work provided the basis for the current project, and using the microarray data and a substantial amount of data presented here, we recently published a paper in Journal of Cell Science, which has been inserted at the back of this thesis. All the work in this thesis was conducted by myself, except for a very few instances which have been highlighted in the text.

The array data showed a modest increase in Wnt-4 mRNA which was subsequently validated by Q-PCR. This array was conducted on anterior pituitary tissue which therefore contained all the secretory cell types in the gland, albeit in a situation where lactotrophs dominated after oestrogen stimulation (Giles et al., 2011). Therefore the effect cannot be confined to lactotrophs, and indeed, the localisation of Wnt-4 is a key topic examined in this thesis. There is one other similar array study in the literature which examined gene regulation in GH3 cells after oestrogen stimulation (Fujimoto et al., 2004). This study was conducted using an older array which could detect changes of expression in considerably less genes than in our paper (7000 genes against 31000

genes). This study on GH3 cells only detected alterations in expression of 33 genes (26 upregulated and 7 downregulated) where our study detected altered regulation of thousands of genes. None of the genes considered focused on in our study were altered in the GH3 array.

Wnt signalling may be involved in pituitary pathophysiology: Wnt-4 affects specific cell type expansion in the normal developing mouse pituitary. It is expressed from embryonic day 9.5 (e9.5) to e14.5, and Wnt-$4^{-/-}$ mice displayed drastically reduced numbers of TSH and GH positive cells (Treier et al., 1998). PRL positive cells were not analysed in this study, though the near absence of TSH and GH positive cells, and the common delineation of TSH, GH and PRL cells would suggest that lactotrophs were also reduced in number. In a similar study, Potok et al also found reduced cell numbers in the AL of Wnt-$4^{-/-}$ mice which this time did investigate the lactotroph population, though the decrease in cell number was not as great as in the previous report (Potok et al., 2008). This is most likely explained by the fact that tissue used by the Potok group was taken at E18.5 as opposed to E17.5 used by the Treier group, giving an extra day for mature cells to emerge, and for hormone levels to build up to detectable levels.

Wnt-4 is rapidly upregulated by oestrogen during uterine growth in mice, associated with activation of the canonical signalling pathway (Hou et al., 2004). Molecules associated with Wnt signalling, such as the Frizzled receptor, APC, β-Catenin and TCF are expressed in the developing mouse pituitary (Douglas et al., 2001), and β-Catenin has been shown to interact with Prop-1 to control cell fate determination of Pit-1 derived cell lines in the developing pituitary (Olson et al., 2006).

Evidence regarding the downstream effects of Wnt molecules in the adult pituitary is contentious. Semba et al (2001) found frequent nuclear accumulation of β-Catenin in 57% of human pituitary adenomas studied (Semba et al., 2001). However, in a similar study using 54 human pituitary adenomas, Miyakoshi et al (2008) found that although Wnt-4 expression was increased in GH/TSH/PRL-omas, β-Catenin was restricted to the cell membrane and never found in the nucleus, suggesting a non-canonical action of Wnt-4 (Miyakoshi et al., 2008a). The same group also reported that Wnt-4 was specifically expressed in the majority of somatotrophs, and a few thyrotrophs in the untreated rat pituitary, and that oestrogen increased Wnt-4 expression in these cell types (Miyakoshi et al., 2009). The canonical inhibitor Wnt inhibitory factor 1 (WIF-1) was reported to be down-regulated in a series of human pituitary tumours which was associated with increased expression of the canonical target gene cyclin D1

Furthermore, transfection of GH3 cells with WIF-1 decreased cell proliferation suggesting a role for WIF-1 as a potential tumour suppressor (Elston et al., 2008).

A number of Fz receptors have been implicated in the pituitary. Fz 6 is expressed in the pituitary and has been shown to interact with Wnt-4 in kidney cells (Miyakoshi et al., 2008a). Fz 2 and Fz 5 have both been detected in the developing pituitary (Burns et al., 2008; Douglas et al., 2001), while Fz 4 has been detected in the developing mouse brain and the adult mouse pituitary. Taken together, these data suggest Wnt-4 could be involved in oestrogen-induced prolactinoma development.

1.12. Wnt-4

Wnt-4 is a 39kDa molecule containing 25 conserved cysteine residues and 2 N-glycosylation sites (Coudreuse and Korswagen, 2007). Wnt-4 plays important roles in the development of a number of organs. Wnt-4 knock out mice die within 24h of birth through renal failure (Stark et al., 1994) and exhibit poorly developed pituitary glands (as discussed in the previous section) (Treier et al., 1998). Wnt-4 plays a vital role in sex determination by preventing Leydig cell differentiation in the developing ovary (Yu et al., 2006), and Wnt-4 knock out male mice show partial female-male sex reversal (Vainio et al., 1999). Wnt-4 knock out mice also have poorly developed adrenal glands due to incomplete zona glomerulosa formation (Heikkila et al., 2002), and exhibit low numbers of thymocytes (Mulroy et al., 2002).

Wnt-4 is generally considered a non-canonical Wnt due to its inability to transform C57 MG mammary epithelial cells (Wong et al., 1994). Other examples of non-canonical Wnt-4 signalling include activation of PKC in axon guidance in the rat spinal chord (Wolf et al., 2008), activation of P38 and MAPK in mesanchymal stem cells (Chang et al., 2007) and regulation of milk duct side branching through β-Catenin independent pathways (Kim et al., 2009)

However, Wnt-4 also activates the canonical pathway in a number of circumstances. Wnt-4 activation of β-Catenin controls cell growth and survival in MDCK cells through binding to Fz 6 (Lyons et al., 2004), maintains female germ cells in the foetal mouse ovary (Liu et al., 2010b), and regulates renal nephrogenesis in mouse (Park et al.,

2007). As such, when studying the effect of Wnt-4, care must be taken to consider all Wnt signalling pathways.

1.13. Project Aims

This project aims to study the role of Wnt-4 in oestrogen-induced lactotroph hyperplasia. This will be sub-divided into 2 sections:

1. Wnt-4 expression in the pituitary

    - Is Wnt-4 expressed in the pituitary, and in which cell types?
    - Is Wnt-4 upregulated by oestrogen in the pituitary?
    - Does Wnt-4 affect lactotroph proliferation?

2. Wnt signalling pathway analysis

    - Does E2 or Wnt-4 activate canonical signalling in lactotroph cells?
        - Can lactotroph proliferation be altered by canonical pathway manipulation?
    - Does Wnt-4 activate calcium signalling in lactotroph cells?
        - Are calcium oscillations in lactotroph cells affected by Wnt-4?
        - Does Wnt-4 activate downstream calcium signalling pathways?
    - Does E2 activate PCP signalling in lactotroph cells?
        - Is the expression pattern of PCP proteins altered in pituitaries undergoing E2-induced lactotroph hyperplasia?

1.14. Models used

1.14.1. GH3 cells

GH3 cells are an immortal somatolactotroph cell line which can be used indefinitely in vitro. They produce PRL and proliferate in response to E2 treatment making them a useful model for studying E2-induced lactotroph hyperplasia. Importantly, they lack the dopamine receptor, the major regulatory control over PRL secretion and lactotroph proliferation, demonstrating a key difference between GH3 cells and real lactotrophs. More information regarding the expression of receptors in this cell line can be found in section 2.4.1.

1.14.2. Fischer 344 rats

The Fischer 344 originated through the breeding of #344 rats from a local breeder (Fischer) in 1920, and the rat strain was subsequently inbred at Columbia University. Fischer 344 rats are oestrogen sensitive and will develop prolactinomas in response to constant E2 treatment. The reason for their sensitivity to oestrogen is unknown, and therefore care must be taken when interpreting results. However, they provide an extremely useful model in which to study the proliferative effects of oestrogen in the pituitary.

1.15. Hypothesis

This thesis will assess the hypothesis that oestrogen increases expression of Wnt-4 protein in lactotroph cells, and that Wnt-4 will mediate oestrogen-induced lactotroph hyperplasia by activation of either one, or a combination of, the 3 classical Wnt signalling pathways. This activation could underpin mechanisms causing prolactinoma development in humans.

2.0. Materials and Methods

2.1. Antibodies

2.1.1. Primary antibodies

Wnt-4 (Rabbit, 1:1000 for western blot and 1:50 for immunohistochemistry, SDI, Newark, DE), α-Tubulin (Mouse, 1:25000, Abcam, Cambridge UK), GH (Goat, 1:50, R&D Systems, Abingdon, UK), PRL (Mouse, 1:4000, Pierce, Rockford, IL), TSH (Guinea Pig, 1:100, NIDDK, Bethesda, MD), ACTH (Mouse, 1:200, Novocastra, Milton Keynes, UK), LH (Mouse, 1:1000, kindly donated by Dr.J.F Roser, University of California), R51 PRL (rabbit, 1:500, kindly donated by AS McNeilly, MRC Human Reproductive Sciences Unit, Edinburgh, UK), β-Catenin (mouse, 1:400, BD Transduction Laboratories, Oxford, UK), E-Cadherin (Mouse, 1:200, Transduction Laboratories), N-Cadherin (Mouse, 1:200, Transduction Laboratories) and Sox-9 (Millipore, Billerica, MA).

2.1.2. Secondary antibodies

Donkey anti-rabbit HRP-conjugated (1:2000) and donkey anti-mouse HRP-conjugated (1:25000) (both from Santa Cruz, Heidelberg, Germany), donkey-anti rabbit Alexa Fluor 546 (1:500) and mouse Alexa Fluor 488 (1:1000) (both from Invitrogen, Paisley, UK), donkey anti-goat anti-guinea pig FITC (1:64, Sigma, Dorset, UK), donkey anti-goat FITC (1:500, Santa Cruz) and donkey anti-rabbit Texas Red (1:500, Santa Cruz).

2.2. Plasmids

Super 8x TopFlash is a TCF reporter plasmid containing 8 TCF binding sites linked to a luciferase expression vector (Addgene, Cambridge, MA). Negative control comes in the form of Super 8x FopFlash (Addgene) which contains a point mutation in each of the TCF binding sites, preventing TCF binding, and transcription of luciferase. pNFAT-TA (termed pNFAT) is an NFAT reporter plasmid. Its negative control, pTA, contains the minimal TATA box promoter linked luciferase. pNFAT has the same TATA box promoter linked to luciferase, but with 3 additional NFAT binding sites (both were kind

gifts from Prof Ludwig Neyses, University of Manchester, UK). Mutant β-Catenin (mβ-Cat) was a kind gift of Dr H. Clevers who developed the plasmid (Morin et al., 1997). It contains a point mutation rendering β-Catenin constitutively active. The mutation is a C^{98}→A missense mutation which changes Ser^{33}→Tyrosine. This prevents GSK-3β binding to β-Catenin and targeting it for degradation at the proteosome. This plasmid contains an ampicillin resistance gene and the neomycin phosphotransferase gene which allows for selection of cells containing the plasmid using the antibiotic G-418.

In transient transfection experiments transfection efficiency was measured by co-transfecting the above vectors with pRL-TK Renilla (Promega, Hampshire, UK). In this plasmid, the HSV-thymidine kinase promoter drives constitutive Renilla luciferase expression, providing a quantifiable internal control from which the luciferase value can be normalised.

2.3. Primers

PCR Name	Primers	PCR target size in base pairs
β-Catenin	F - 5' - TGACCTCATGGAGTTGGACA - 3' R - 5' - CGGGCTGTTTCTACGTCATT - 3'	621
Calcineurin	F - 5' - CAGGGTGGTGAAAGCCGTTC - 3' R - 5' - GGATGTCCCCGCAAACTGTG - 3'	230
Fz 2	F - 5' - TCTGGTGGGTGATTCTGTCC - 3' R - 5' - GTAGCAGGCGATGACGATG - 3'	413
Fz 4	F - 5' - TGTGCTGACCTTCCTGATTG - 3' R - 5' - TGCCAAAAACCAAGTGAGTG - 3'	280
Fz 5	F - 5' - CCCCATCATCTTCCTGTCTG - 3' R - 5' - TTTTGGTTGCCCACATAACA - 3'	381
Fz 6	F - 5' - AGAAAATGGAGTTGCGAAGC - 3' R - 5' - ACAGAGGCAGAAGGACGAAG - 3'	190
NFAT 1	F - 5' - ACGATGTGGAGGTGGAAGAC - 3' R - 5' - GGACGCCTCAGAGTTACAGC - 3'	152
NFAT 2	F - 5' - CACCCAATGCTGGCCGAGTC - 3' R - 5' - CTGCTGGCTGGCCGAGGAGG - 3'	300
NFAT 3	F - 5' - AGGTCAGCCTTCTTCCCATT - 3' R - 5' - TGCCCTGTACTTTGTGCTTG - 3'	240
NFAT 4	F - 5' - GACTTTGCTCCCAGAGAACA - 3' R - 5' - GATCCAGTCAACACTAGCTC - 3'	300

2.4. Cell lines

2.4.1. GH3 cells

The GH3 cell line is a well characterised somatolactroph (producing both GH and PRL) cell line, established from a GH producing rat pituitary tumour (Bancroft et al., 1969). They do not produce other pituitary hormones such as TSH, LH, FSH or POMC. They do express a wide variety of receptors including the TRH receptor (Hinkle and Tashjian, Jr., 1973), galanin receptor (Kalkbrenner et al., 1995) and somatostatin receptor (Coleman and Bancroft, 1993) although they lack a functional dopamine receptor (Fischberg and Bancroft, 1995). They undergo proliferation in response to E2 and are thus a useful tool for studying the effects of E2 on lactotrophs.

2.4.2. Human Embryonic Kidney 293 (HEK 293) cells

HEK 293 cells were isolated from a healthy aborted foetus, then transformed by adenovirus to produce an immortal cell line (Graham et al., 1977). They are not considered to be a reliable model of any animal cell type, but they are easy to culture and manipulate making them a very useful tool for molecular biologists.

2.4.3. NIH 3T3 cells expressing Wnt-4

NIH 3T3 cells originate from mouse embryonic fibroblast cells which were continuously cultured until they spontaneously became immortalised (TODARO and GREEN, 1963). A Wnt-4 expression vector was stably transfected into NIH-3T3 cells, using the cytomegalovirus (CMV) promoter to drive constitutive expression of Wnt-4 (Kispert et al., 1998).

2.4.4. LMTK- cells expressing Wnt-3a

LMTK cells are a thymadine kinase-deficient mouse fibroblast cell line initially derived by Kit *et al* (1963). Cells were stably transfected with Wnt-3a expression vector driven by the CMV promoter (ATCC) (KIT et al., 1963).

2.5. Rats

Fischer 344 rats were obtained from Harlan (Indiana, IN) and were subsequently bred under project licence PPL40\2691. Rats were housed according to home office guidelines and killed by a schedule 1 method.

Fischer 344 rats are an inbred, albino strain of rat. A high proportion of rats develop pituitary adenomas in late age (36% in females, 24% in males). They are E2 sensitive, and continuous treatment with E2 for roughly three weeks results in proliferation of the lactotroph population. Continued treatment with E2 results in the development of prolactinomas (Harlan Laboratories F344 datasheet, 2008; Steinmetz et al., 1997).

2.6. Cell Culture

2.6.1. Cell lines

2.6.1.1. General cell culture technique

All procedures were carried out in a Labcaire lamina flow hood to maintain sterility. Any equipment or reagents used in the hood were sterilised by spraying with 70% industrial methylated spirit (IMS) before being taken into the hood. Cells were grown in 10ml culture medium - Phenol Red free Dulbecco's Modified Eagles Medium (DMEM) supplemented with 1g/l glucose (Gibco, UK), 10% Foetal Bovine Serum (FBS) (Gibco) and 1% Glutamax (Gibco). Cells were grown at 37℃ and 5% CO2 in T75 vented flasks

(Corning, Amsterdam, The Netherlands). Regular visual checks were carried out to check viability of cells using an inverted phase contrast microscope.

2.6.1.2. Thawing cells from liquid nitrogen stores

Cells were removed from liquid nitrogen storage and thawed at 37℃ in a water bath. Thawing was carried out as quickly as possible to prevent damage to the cell membrane. Once thawed, 10ml of culture medium was added drop-wise to the cells. Cells were centrifuged at 1200rpm for 5 minutes, before being re-suspended in 10ml culture medium, and then transferred to a T75 flask and incubated as previously described.

2.6.1.3. Splitting cells

At 90% confluence, media was removed from cells and cells were washed with 10ml PBS. For a T75 flask, 1ml 1x Trypsin/EDTA (Lonza, Slough, UK) was added to cells. The flask was incubated at 37℃ and 5% CO_2 for 5 minutes until cells were detached from the flask surface. The reaction was stopped by addition of 5ml culture medium, then cells were centrifuged at 1200rpm for 5 minutes. Cells were resuspended in 10ml culture medium and multiple pipetting of the cell solution was carried out to break up cell clumps. For passaging of cells, resuspended cells were split into new T75 flasks at 1:5 dilutions of culture medium. Where required, resuspended cells were counted using a haemocytometer (Electron Microscopy Sciences, Hatfield PA) and diluted to the appropriate concentration.

2.6.1.4. Cryogenic Freezing of cells

At 90% confluence, cells were trypsinised and spun down as described previously. Cells were resuspended in 3ml freezing medium consisting of 50% FBS, 40% culture medium, 10% Dimethyl sulphoxide (DMSO) (Sigma). 1ml volumes of freezing media/cell solution were put in 2ml cryotubes and placed in a cryo-freezing container (Nalgene, Roskilde, Denmark) at -80℃ for 24 hours. Cells were then stored in liquid nitrogen.

2.6.2. Rat primary cell culture

2.6.2.1. General technique

As with cell lines, all work was carried out in a Labcaire lamina flow hood and any equipment or reagents used in the hood were sterilised by spraying with 70% industrial methylated spirit (IMS) before being taken into the hood. Importantly, a different hood was used to that in which culture of cell lines was carried out to avoid contamination. Primary cells do not adhere to culture vessels as well as cell lines do. Therefore, whenever primary cultures were used, the surface to which they bound was treated with Poly-L-Lysine (Sigma), which is a small polypeptide containing roughly 25 L-Lysine residues. In water, this is positively charged which allows cell surface IgG's to bind more strongly to the culture surface. A suitable volume of Poly-L-Lysine was added to totally cover the culture surface (e.g. 200µl for a 24 well plate) and was incubated for 15 minutes. The surface was then washed 3 times with sterile PBS and left to dry in the hood.

2.6.2.2. Generation of cultures of dispersed pituitary cells

Fischer 344 rats were killed by schedule 1 method, pituitaries were removed and placed in PBS. Pituitaries were placed on a 10cm dish in 40µl per pituitary of 0.1% trypsin and 0.3% bovine serum albumin (BSA) (Sigma) in phenol red-free DMEM supplemented with 4.5g/l glucose: Nutrient Mixture F12 (DMEM/F12) (Gibco). Pituitaries were cut into small pieces with scalpel blades for 3 minutes. Tissue was removed and placed in a 15ml falcon tube, the dish was washed with a further 80µl per pituitary of 0.1% trypsin and 0.3% BSA in DMEM/F12 which was then added to the tissue in the tube. The tube was then placed in a water bath at 37°C for 30 minutes and shaken every 10 minutes to break up cells. 120µl per pituitary of 0.1% trypsin, 0.3% BSA and 0.2% Deoxyribonuclease 1 (Sigma) was then added to the tube, which was then placed back into the water bath for a further 15 minutes. The tube was centrifuged at 1200 rpm for 5 minutes and the supernatant carefully removed. Calcium was removed from the cells to inhibit calcium-dependent adhesion molecules (cadherins) and further disperse cells by re-suspending the pellet in 2ml of 2mM EGTA in Hanks Balanced Salt Solution (HBSS) (Gibco). Tissue was centrifuged at 1200rpm for 5

minutes before tissue was resuspended in 2ml of 1mM EGTA in HBSS. Tissue was centrifuged at 1200rpm for 5 minutes before tissue was re-suspended in 2ml of EGTA-free HBSS. Tissue was centrifuged at 1200rpm for 5 minutes before tissue was re-suspended in 2ml of primary culture medium - DMEM/F12 supplemented with 1% Glutamax (Gibco), 1µg/ml penicillin/streptomycin (Lonza) and 10% FBS. Further dissociation of tissue was carried out by multiple pipetting, then cells were passed through a 40µm cell strainer, counted with a haemocytometer and seeded at appropriate densities in primary culture medium. Cells were allowed to settle for 48 hours before starvation.

2.6.2.3. Generation of live pituitary slices

Fischer 344 rats were killed by schedule 1 method, pituitaries were removed and placed in PBS. For each pituitary, 10ml of 2% Type IX-A, Ultra-low Gelling Temperature Agarose (Sigma) was made up and stored in liquid form at 37°C.

5ml syringes had their nozzle ends cut off, and plunger pulled out so that the syringes were empty. The syringes were inverted and packed in ice. When ready, liquid agarose was poured into the syringes, and as it cooled and set, the pituitaries were placed in the gel. Manipulation of pituitaries was carried out using tweezers to ensure that the pituitaries were not touching the sides of the syringes when the gel fully set. Pituitaries were left for 30 minutes to allow gel to fully set. When ready, the plungers were pushed in so that the gel containing the pituitary was expelled from the syringe. The gel was mounted on a metal base with superglue and sliced in a coronal axis on an Integraslice 7550MM vibratome machine at a frequency of 70Hz, speed of 3mm/s and thickness of 300µm. Slices were placed in primary culture medium and stored in the incubator until required.

2.6.3. Starving cells/pituitary slices

Cells (both cell lines or primary cells) and slices must be starved in serum free medium before an experiment, as steroids or growth factors present in culture medium can affect experimental conditions. Cells/slices were washed in PBS before addition of starving medium. Cell lines were either starved in DMEM supplemented with 1g/l glucose, 1% Glutamax and 5% BSA, or DMEM supplemented with 1g/l glucose, 1%

Glutamax and 10% Dextran-Charcoal treated FBS (DCT-FBS) (Perbio Scientific, Nothumberland, UK) as indicated. DCT-FBS is high quality FBS which has been filtered through carbon-absorbant charcoal filters to remove non-polar material such as hormones, steroids and growth factors. It still retains low levels of growth factors which were necessary to maintain cell viability in experiments over long durations. Importantly however, estradiol is virtually absent after filtration rendering DCT-FBS a suitable starving medium for experiments studying the effects of oestrogen. Primary cell cultures were starved in either DMEM/F12 supplemented with 1% Glutamax, 1µg/ml penicillin/streptomycin and 5% BSA or DMEM/F12 supplemented with 1% Glutamax, 1µg/ml penicillin/streptomycin and 10% DCT-FBS. Pituitary slices were always starved in DMEM/F12 supplemented with 1% Glutamax, 1µg/ml penicillin/streptomycin and 10% DCT-FBS. Cells/slices were left in starving medium for 24h before stimulation.

2.6.4. Stimulating cells/pituitary slices

After 24h starvation, starving medium was removed from cells/slices and stimulation media was added as defined for each experiment. Cells/slices were then placed back in the incubator for the defined time points for that experiment.

2.7. Transfection studies

DNA can be deliberately introduced into cells via the process of transfection. This process can be transient where the foreign DNA does not enter the host cells genome, and is subsequently diluted out by mitosis, or stable where the foreign DNA incorporates permanently into the host cell genome. This incorporation is random and only a few cells will take up the DNA. In stable transfections, the transfected DNA must include a selection gene giving any cell that incorporates the DNA into its genome resistance to a particular selection agent. Only the cells which have incorporated the DNA into their genome will have resistance, and these cells will then proliferate generating a cell population containing the plasmid of interest. In this thesis, all transfections were carried out using the lipid based transfection reagent Fugene 6 (Roche, Burgess Hill, UK). In this protocol, positively charged lipid vesicles are bound by free DNA outside the cell. These vesicles bind to and interact with lipid structures in

the cell membrane allowing incorporation of foreign DNA through the lipid bilayer into the cell.

2.7.1. Transient transfections and luciferase expression assays

HEK 293 and GH3 cells were seeded at appropriate densities (see below) in sterile, white, 96 well plates in 100µl culture medium. GH3 cells were plated at $1x10^4$ cells per well and were transfected with 0.1µg/well of each plasmid with a Fugene:DNA ratio of 3:1. HEK 293 cells were plated at $5x10^3$ cells per well, with 0.05µl/well of test plasmid and 0.01µg/well of Renilla at a Fugene:DNA ratio of 6:1. These conditions result in relatively similar transfection efficiencies between the cell lines as judged by Renilla luciferase expression readout. Transfection media was made up in DMEM supplemented with 1g/L glucose and 1% Glutamax. 20µl transfection medium was then added to each well directly into the usual culture medium containing FBS. Cells were left for 24 hours in the incubator for transfection to occur, cells were then starved and stimulated as required in the normal manner. After appropriate stimulation, the volume in each well was reduced to 50µl, and luminescence was measured using the Dual Glo-Luciferase Assay System (Promega) according to manufacturer's instructions. Briefly, 50µl luciferase reagent was added to each well which first lyses cells and then acts as a substrate for firefly luciferase. Luminescence was detected using a Mithras LB 940 luminometer (Berthold Technologies, Hertfordshire, UK). Next, 50µl Stop and Glo reagent was added to each well which quenches the firefly luciferase signal by up to 10000 times, and subsequently provides a substrate for Renilla luciferase which was read on the luminometer.

2.7.2. Stable transfection of GH3 cells with mutant β-Catenin (mβ-Cat) expression vector

2.7.2.1. Amplification of mβ-Cat plasmid

mβ-Cat is a plasmid expressing a constitutively active form of β-Catenin and was kindly donated by Dr H. Clevers. mβ-Cat was used to transform E.Coli (Sigma). Briefly, 1µg plasmid was added to 30µl competent E.Coli and incubated on ice for 30 mins. E.Coli

were heat shocked for 1 minute at 42℃, before being streaked on an ampicillin resistance agar gel which selects for bacteria containing the plasmid. E.Coli were grown at 37℃ overnight, then a colony was picked and placed in 2 ml LB solution (1% Bacto-tryptone, 0.5% yeast extract and 1% NaCl) containing 100µg/ml ampicillin and shaken for 8 hours at 37℃. 200µl of bacteria solution was removed and placed in 200ml LB solution containing 100µg/ml ampicillin, and was shaken overnight at 37℃. mβ-Cat was then purified from E.Coli population using the Quiagen Maxi Prep kit (Qiagen, Hilden, Germany) according to manufactures instructions.

2.7.2.2. Linearisation of mβ-Cat

The mutant β-Catenin plasmid is a circular DNA fragment. In order for it to integrate into the GH3 genome it must be cut into a linear form which can then enter into the host DNA sequence. If this cut was left to chance it is possible that the cut could occur within mβ-Cat or G-418 resistance regions, rendering the transfection useless. As such, the plasmid was deliberately cut using the restriction enzyme Sma 1 (Roche) at base pair 1127 of the pCL-Neo vector (Figure 2.1).

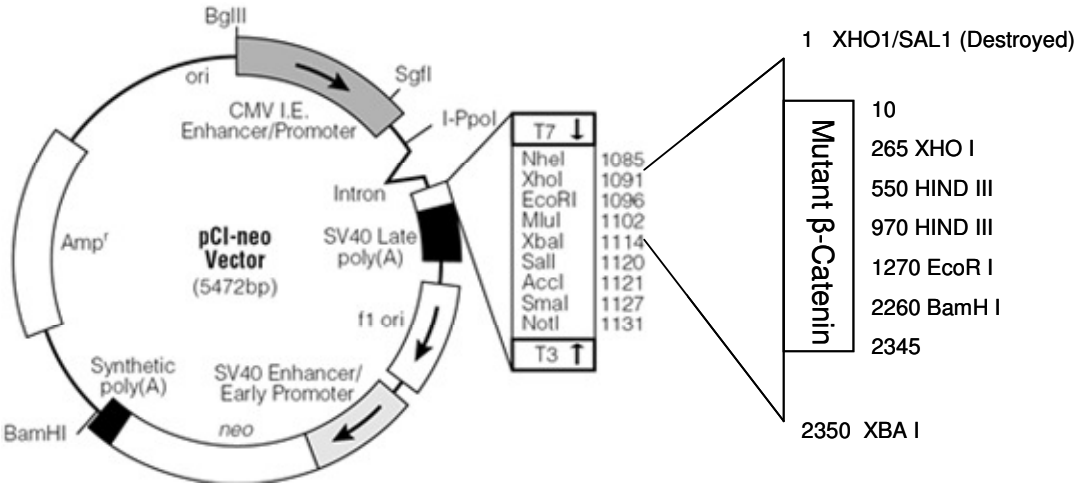


Figure 2.1. Plasmid map of mutant β-Catenin vector. Mutant β-Catenin was inserted into the multiple cloning region of pCL-neo backbone vector at XhoI and XbaI sites. For linearization, plasmid was cut at the SmaI site – bp 1127.

Briefly, 20µg plasmid, 20µl 10x Recommended SuRE/Cut Buffer A (Roche) 8µl SMA I (Roche) and 139.8µl sterile H_2O were incubated overnight at 25℃. The reaction was then terminated by heating the sample to 65℃ for 20 minutes. To purify DNA, 300µl 100% ethanol was added and solution was placed at -20℃ for 1 hour. Solution was centrifuged at 13000g for 10 minutes to form a DNA pellet. Supernatant was carefully removed and pellet was washed in 500µl 70% ethanol, before centrifugation at 13000g for 10 minutes. Supernatant was carefully removed, then sample was allowed to air dry on the bench to ensure total evaporation of ethanol. 40µl ultrapure water was added to the pellet which was left on the bench overnight to dissolve. DNA concentration was measured by nanodrop.

2.7.2.3. Stable transfection and generation of clonal cell lines

GH3 cells were plated at $5x10^5$ cells per well in a 6 well plate in 2ml culture medium. Cells were transfected with Fugene 6 with a Fugene:DNA ratio of 3:1 and 6µl Fugene per well (therefore 2µg DNA per well). Transfection medium was made up to 100µl with DMEM supplemented with 1g/L glucose and 1% Glutamax, and added dropwise to cells to ensure mixing before cells were placed overnight in the incubator. Medium was removed and replaced with standard culture medium supplemented with 600µg/ml G-418 to select for cells containing the plasmid. From then on, these cells were always grown in standard culture medium supplemented with 600µg/ml G-418 (mβ-Cat medium). Medium was replaced every 3-4 days for 2-3 weeks until stably transfected cells had been selected for and were proliferating in distinct colonies. At this point, the cell population was hetrogenous as the insertion site of mβ-Cat plasmid into the host genome is random and would be different for every transfected cell. In order to obtain homogenous cell lines, cells were trypsinised and plated at varying low densities in 10cm dishes and stored in the incubator. Low density plating results in viable cells growing large distances apart allowing for easier selection. Individual cells were allowed to proliferate into colonies which were then removed using a sterile pipette tip, and placed in a 24 well plate in 100µl mβ-Cat medium. After another week, colonies which had been transferred successfully and which were proliferating in distinct colonies were trypsinised and transferred into T-25 flasks. Populations were grown up, transferred into T-75 flasks and then frozen down and stored in liquid nitrogen. 3 clonal cell lines containing the plasmid were generated, termed mβ-Cat 2/3/4.

2.8. Immunofluorescence studies

Immunofluorescence work was carried out on either dispersed cells (immunocytochemistry) or on tissue slices (immunohistochemistry). The staining protocol was similar between the two processes.

2.8.1. Preparation of cells for immunocytochemistry

Primary cells or cell lines were plated and stimulated as previously described. The only addition to the protocol was that cells were seeded on top of a 7mm glass coverslip (Poly-L-Lysine treated in the case of primary cultures) in 24 well plates. Cover slips were sterilised in 70% IMS for a minimum of 4 hours before being placed in the wells and left overnight in the hood to thoroughly dry off. After stimulation, cells were washed twice with cold PBS and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at 4°C. PFA is a cross linking molecule which forms intermolecular bridges through free amino groups. This process preserves subcellular morphology and immobilizes antigens within the cell, while allowing free access of antibodies to all cellular components. Cells were then washed 3 times in PBS before being stored in 70% ethanol until required

2.8.2. Preparation of tissue slices for immunohistochemistry

Rats were killed, pituitaries were dissected out and stored in PBS. Pituitaries were then fixed in 4% PFA for 2 hours at room temperature. Pituitaries were washed twice in PBS before being stored in 70% ethanol. Paraffin is immiscible with water so tissue samples must be thoroughly dehydrated by treatment with increasing concentrations of ethanol. Afterwards, xylene is used as a clearing agent to remove ethanol, and samples are placed in molten paraffin wax which infiltrates the sample and replaces the xylene. This process was carried out in a tissue processor on the following cycle:

70% Ethanol – 2.5 hours
70% Ethanol – 1 hour
90% Ethanol – 1 hour
95% Ethanol – 1 hour
100% Ethanol – 3 cycles, 1 hour each
Xylene – 0.5 hours
Xylene – 2cycles, 1 hour each
Wax – 2 hour
Wax – 3 hour

Tissue was then sliced at 5µm using a microtome, and slices were placed in a 42℃ water bath so that the slices spread out flat on the surface of the water. Slices were then mounted on slides and allowed to dry overnight at 37℃. Wax was then removed by washing with xylene before rehydration of tissue in decreasing concentrations of ethanol as follows:

Xylene – 5 minutes
Xylene – 5 minutes
Dip into 100% ethanol
100% Ethanol – 3 minutes
90% Ethanol – 3 minutes
70% Ethanol – 3 minutes

Antigen retrieval was then carried out by boiling samples in 0.01M sodium citrate buffer (pH6) for 20 minutes. This breaks formalin induced protein cross-links from the fixation process, which can then expose previously hidden antigen binding sites and enhance staining intensity of antibodies. Slices were then washed twice in PBS.

2.8.3. Staining protocol

From this point onwards the protocols were the same for both immunocytochemistry and immunohistochemistry. Samples were blocked for 1 hour in PBS with 20% donkey serum and 5% BSA. Blocking reduces background staining as proteins in the blocking buffer occupy free protein binding sites on the sample not specific to the antibodies used. The first primary antibody was diluted to the appropriate concentration in blocking buffer and added to samples overnight at 4℃. Samples were washed 3x10

minutes in PBS before addition of the 1st secondary antibody diluted in PBS for 2h at room temperature. From this point the protocol was carried out in the dark to prevent photo-bleaching of secondary antibodies. Samples were washed 3x10 minutes and blocking buffer was added again for 1h at room temperature. The second primary antibody was then added overnight at 4°C. Samples were washed 3x10 minutes before the second secondary was added for 2h at room temperature. Samples were washed 3x10 minutes before being treated with 4',6-Diamidino-2-phenylindole (DAPI - 0.1µg/ml, Sigma) for 15 minutes at room temperature. DAPI is a blue fluorescent nucleic acid stain which binds to AT clusters in the minor groove of double stranded DNA and is therefore used to identify nuclei. Samples were washed 3x10 minutes, and then mounted for analysis in Permafluor (Thermo Scientific, Cheshire, UK). Images were collected using a Nikon C1 confocal microscope (Bioimaging Facility, Faculty of Life Sciences, University of Manchester).

2.9. Western Blotting

2.9.1. Generation of lysates

Protein lysates were either generated from cultures of dispersed cells or from live pituitary slices. All steps in lysate generation were carried out at 4°C.

2.9.1.1. Preparation of lysates from dispersed cells

Cells were grown in 6 well plates and stimulated as previously described. Cells were washed twice in PBS before addition of 200µl/well of RIPA buffer (50mµ Tris HCl, 150mM NaCl, 1% NP-40, 0.5% sodium doexycholate and 0.1% SDS in H_2O). Protease inhibitors were also added to RIPA buffer as when cells are disrupted, they release proteases which degrade protein. 1 tablet of Complete, Mini, EDTA free, protease inhibitor cocktail (Roche) was dissolved in every 10ml RIPA buffer just prior to lysis. Cells were scrapped with a sterile cell scraper and the lysate was transferred to an eppendorf and rotated for 30 minutes. Lysate was centrifuged at 12000g for 10 minutes and the supernatant removed and stored at -80°C.

2.9.1.2. Preparation of lysates from live pituitary tissue

After stimulation, slices were washed twice in PBS. Slices were transferred into an eppendorf containing 75µl per pituitary slice of RIPA buffer containing protease inhibitors. Slices were then broken up by being passed 20 times through a 25 gauge syringe needle before being rotated for 30 minutes. Lysate was centrifuged at 12000g for 10 minutes and the supernatant removed and stored at -80°C.

2.9.2. Protein assay

The protein level of the lysates was measured using the D_c protein assay (Biorad, Hemel Hempstead, UK) according to manufacturer's instructions. In the assay, an acidic dye is added to the protein solution which undergoes a differential colour change based upon the protein concentration. Protein standards at 0.4, 0.8, 1.2 and 1.6mg/ml BSA were made up and run alongside samples. A standard curve was drawn from the premade standards which could then be extrapolated to determine the protein concentration in the samples.

2.9.3. Western blotting

Equal amounts of protein (generally 10-20µg/lane) were mixed with 5x laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and heated to 95°C for 5 minutes. The SDS and heating denatures proteins allowing them to separate based more on size than shape. SDS also binds to regular positive charges on the proteins giving the protein an overall negative charge ensuring proteins migrate at a similar rate. Glycerol makes the sample denser so that it sinks to the bottom of the lane, while bromophenol blue acts as a marker of migration, travelling just ahead of the protein. Samples were then separated through an SDS-10% polyacrylamide gel (30% Bis/acrylamide, 1.5M Tris-HCl pH 8.8, 10% SDS, 10% APS, 0.1% Temed) before being transferred onto nitrocellulose membrane using methanol transfer buffer (25mM Tris, 200mM glycine, 10% methanol). Membranes were blocked for 1 hour at room temperature in blocking buffer; 0.05% Tris-Buffered Saline Tween-20 (TBST) (150mM NaCl, 2mM KCl, 25mM Tris Base, 0.05% Tween-20) with 5% non-fat dry milk (NTDM). The membrane was exposed to primary antibody diluted in

blocking buffer, and rocked overnight at 4°C. The membrane was washed 3 x 10 minutes in TBST, then a horseradish peroxidise (HRP) conjugated secondary antibody was added. The membrane was rocked for 1.5 hours at room temperature before 3 x 10 minutes washes in TBST. The membrane was treated with EZ-ECL reagent (Pierce) which contains p-iodophenol. This enhances the reaction by which HRP oxidises luminol to 3-aminophthalate, and light is emitted as a bi-product of the reaction. This light was detected using BioMax XR Film (Kodak, Hemel Hempstead, UK) using a Compact x 2 Automatic Film Processor (X-ograph Ltd, Gloucestershire, UK).

2.10. Proliferation assays

Cells were seeded in clear, 96 well plates and stimulated as previously described. To measure cell number, 15µl of CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was added to 100µl medium in each well. The plate was then returned incubated at 37°C and 5% CO_2 for 2 hours. The MTS solution contains a tetrazolium compound which is bioreduced by metabolically active cells to produce a coloured product. The depth of colour is directly proportional to the number of cells in each well, and measuring the absorbance at 490nm therefore gives information on the relative proportions of cells between wells.

2.11. Reverse Transcriptase - Polymerase chain reaction (RT-PCR)

2.11.1. PCR templates

In this thesis, PCR was carried out on either complementary DNA (cDNA) generated from mRNA isolated from cells, genomic DNA or plasmid preparation.

2.11.1.1. Generation of cDNA

Cells were cultured and stimulated according to experimental design. Cells were lysed and mRNA was isolated using RNeasy Mini Kit (Quiagen) according to manufacturer's instructions. Briefly cells were lysed and homogenised, and lysate was then added in a high-salt buffer system to the RNeasy silica membrane. mRNA binds to the membrane, contaminants were washed away and mRNA was eluted in water. When handling RNA, all equipment and surrounding bench space was cleaned with RNase Away (Sigma) to prevent degradation of RNA by RNases. mRNA was then converted to cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to manufacturer's instructions. This kit contains recombinant reverse transcriptase to transcribe mRNA into cDNA, as well as proofreading enzymes to ensure accurate cDNA synthesis.

2.11.1.2. Isolation of genomic DNA

$2x10^6$ cells were isolated and lysed overnight at 55°C in 300µl DNA extraction buffer (300mM NaCl, 10mM Tris HCl pH8, 0.5% SDS, 5mM EDTA). Proteinase K was added overnight at 150µg/ml to digest protein and isolate DNA. Protein was then washed away by adding NaCl to a final concentration greater than 2M. Sample was shaken for 5 minutes, centrifuged at 13000rpm for 10 minutes, and supernatant was decanted into a fresh eppendorph. 250µl isopropanol was added and mixed thoroughly, and then the sample was incubated at -20°C for 2 hours to precipitate DNA. Sample was centrifuged at 13000rpm for 10 minutes and supernatant carefully removed. The pellet was washed in 500µl 70% ethanol and centrifuged again at 13000rpm for 10 minutes. Supernatant was removed and the pellet was allowed to air dry thoroughly to ensure removal of all the ethanol. The pellet was then re-suspended in sterile H_2O and left overnight on the bench to dissolve. The amount of DNA present was then quantified using a nanodrop.

2.11.1.3. Plasmid template preparation

mβ-Cat plasmid was used as a PCR template - see section 2.7.2.1.

2.11.2. PCR reaction

PCR was carried out using Biotaq DNA Polymerase Kit (Bioline, London, UK) according to manufacturer's instructions in a TC-512 PCR machine (Techne, Staffordshire, UK). 50ng DNA template was used for each reaction. The following basic program was used with annealing time and number of repeats of the cycle being determined for each pair of primers:

Initial denaturation – 94°C for 5 mins

Denaturation – 94°C for 30 secs
Annealing – x°C for 30 secs
Extension – 72°C for 1 min
} Repeat y number of times

Final extension – 72°C for 5 mins

2.11.3. Sequencing of PCR product

After PCR, sample was run on a gel to make sure the DNA product was clean. DNA was purified from the PCR reaction using the QIAquick PCR Purification Kit (Quiagen) according to manufacturers instructions and DNA was quantified. Briefly sample was subjected to high salt concentrations and through a silica membrane to which DNA binds and impurities pass through. DNA was then eluted in water and DNA quantification was carried out. For each sequencing reaction, 4pM of primer and 30ng DNA were mixed in a final volume of 10µl sterile H_2O. Sequencing was carried out with the assistance of the University of Manchester DNA Sequencing Facility using a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) and the sequence produced was analysed using Chromas Lite software.

2.12. Live cell calcium imaging

GH3 cells were seeded in glass bottom dishes (Iwaki, Japan) and left to settle for 24 hours. Cells were loaded with Fluo-4 calcium dye (Invitrogen) for 30 minutes, and then

the dish was transferred onto the stage of a Zeiss Axiovert 200 microscope with an attached XL incubator (humid conditions, 37°C, 5% CO_2). Fluo-4 fluoresces upon binding to calcium and therefore the intensity of fluorescence coming from a single cell at any one time gives an indication of the intracellular concentration of calcium within that cell. Cells were either stimulated with vehicle (DCT-FBS), Wnt-4 recombinant protein (Novus Biologicals, Littleton, CO) or Bay-K-8644 (Sigma) which is an L-type calcium channel agonist, causing calcium influx into cells. Images were taken every 3 seconds, areas of interest were drawn around fluorescent cells and mean intensity throughout the experiment was calculated using Kinetic Imaging AQM6 software (Andor, UK).

Calcium oscillations are extremely sensitive and can be influenced by numerous external factors such as temperature and movement. It is vital when performing live cell calcium imaging to ensure that the physical transition between stimuli does not affect calcium oscillations. In an attempt to reduce any effect of stimulus addition, cells were mounted on a microscope stage that was enclosed within an incubator maintained in humid conditions at 37°C and 5% CO_2. Stimuli added were kept at 37°C and were added in a concentrated form so that a relatively small volume could be added to the well to reduce flow movement caused by addition of new fluid. In all cases, 2ml medium was initially placed in the well, and 100µl of concentrated stimulus was carefully pipetted into the well at the appropriate time.

2.13. Flow cytometry

2.13.1. Cell cycle analysis

GH3 cells were seeded and cultured as previously described. Cells were trypsinised, and $1x10^6$ cells were resuspended in 200µl ice cold PBS. 2ml ice cold ethanol was then added to cells whilst vortexing, then cells were left for 2h at 4°C. Cells were centrifuged at 400g for 10 minutes, ethanol was removed and cells were resuspended in 400µl PBS. RNAse was added to a final concentration of 100µg/ml and propidium iodide was added to a final concentration of 40µg/ml. Cells were incubated for 30 minutes at 37°C, then analysed using a 613/20nm band pass filter on a Beckman Coulter Cyan ADP 220 using Summit V. 4.3 software.

2.13.2. Production of FACS enriched lactotroph population

Rat pituitaries were harvested and dissected as described previously. Cells were incubated in culture medium at $2x10^6$ cells/ml at 37℃ for 2 hours. Cells were washed twice by centrifuging for 1500rpm for 5 minutes, then resuspending the pellet in 1ml wash buffer (EBSS with 25mM Hepes, pH= 7.4, with 4mg/ml BSA). Cells were sorted using forward scatter and side scatter on a BD Biosciences FACS Aria, and two populations were sorted and isolated. Cells were then plated at required density for further experimental use.

3.0. Results - Wnt-4 expression and regulation in the pituitary

3.1. Introduction

Prolactinomas represent approximately 60% of all pituitary tumours in man (Gurlek et al., 2007b). They result from abnormal lactotroph cell proliferation, but they usually display only very slow growth, and the pathogenesis of prolactinoma formation and progression has remained elusive. Classical oncogenic mechanisms seem unlikely to be involved in most cases, and none of the common genetic mutations causing cancer have been found to operate in prolactinomas so far (Levy, 2008). A number of genes have been implicated in pituitary adenoma development (e.g. PTTG, VEGF, gsp) however the origins of many tumours are still unknown.

Oestrogen has long been known to exert a proliferative effect on the lactotroph population. High circulating oestrogen levels during pregnancy result in lactotroph hyperplasia, pituitary enlargement and increased circulating PRL levels (Melmed, 2003). The effects of E2 on lactotroph proliferation can be studied in vivo using the oestrogen-sensitive Fischer 344 rat. In this model, lactotroph hyperplasia, and eventual prolactinoma formation, can be induced by treatment with oestrogen or the synthetic oestrogen diethylstilbestrol (DES) (Heaney et al., 1999; Mucha et al., 2007).

In previous work from our group, microarray analysis was carried out on pituitaries undergoing oestrogen-induced lactotroph hyperplasia in an attempt to identify novel genes that may be involved in pituitary adenoma development. Amongst several genes whose regulation was found to be altered, we detected upregulation of the developmental protein Wnt-4.

Wnts exert diverse effects on cells and tissues by driving proliferation, differentiation, apoptosis and cell survival (Willert and Jones, 2006). They play key roles in the development of a number of systems and organs, and have been repeatedly implicated in tumour growth and cancer progression (Kikuchi and Yamamoto, 2008).

Wnt-4 has been implicated in pituitary organogenesis, as knock out of Wnt-4 results in decreased numbers of Pit-1 derived cell lineages (somatotrophs, lactotrophs and thyrotrophs) in mice (Potok et al., 2008; Treier et al., 1998). Wnt-4 is also expressed in the adult pituitary, and was upregulated by oestrogen treatment (Miyakoshi et al., 2009). Furthermore, Wnt-4 was upregulated in prolactinomas, as well as other types of

functioning pituitary adenoma (Miyakoshi et al., 2008a). Taken together, these data suggest that Wnt-4 is a good candidate for the mediation of oestrogen-induced lactotroph hyperplasia and prolactinoma development.

3.2. Aims

The aims of this chapter are as follows:

- To induce lactotroph hyperplasia in Fischer 344 rats by oestrogen treatment.
    - This will provide a useful, in vivo model in which to study the effects of oestrogen on the pituitary, and will be used throughout this thesis
- To assess which cells in the pituitary express Wnt-4:
    - Is Wnt-4 expressed in GH3 cells?
    - Is Wnt-4 expressed in primary lactotrophs?
    - Do other cell types in the pituitary express Wnt-4?
    - Is the distribution of Wnt-4 in the pituitary altered by oestrogen treatment?
- To assess whether Wnt-4 is upregulated in the pituitary in response to oestrogen
- To assess which Frizzled receptors are present on lactotroph cells
- To assess whether Wnt-4 has a proliferative effect on GH3 cells

3.3. Oestrogen treatment of Fischer 344 rats induces pituitary hyperplasia and lactotroph proliferation

To study the effects of oestrogen on lactotroph hyperplasia, Fischer 344 rats were treated with E2 for 3 weeks. Rats were implanted with mini-osmotic pumps calibrated to deliver E2 at a rate of 125µg/kg/day. In similar studies, a delivery rate of 10-20µg/kg/day is generally used to replicate physiological levels of E2 in ovariectomised rats. As such, the dose administered in our study represents a supraphysiological dose which aims to induce lactotroph hyperplasia.

After treatment, rats were killed and pituitaries and uteri harvested and weighed. As expected, pituitary weight increased just over 2-fold from 11mg to 23mg, while uterus weight (the standard recognised bioassay for oestrogen effect) doubled from 0.6g to 1.2g (Figure 3.1).

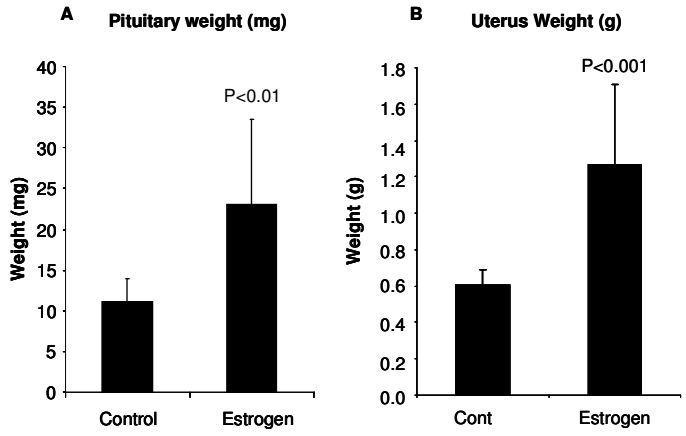


Figure 3.1. Oestrogen induces pituitary and uterus hyperplasia in Fischer 344 rats. Fischer 344 rats were implanted with Azlet mini pumps delivering either vehicle (polyethylene glycol 400 - PEG) or E2 (125µg/kg/day) for 3 weeks. Rats were killed by schedule 1 method and pituitaries (A) and uteri (B) were weighed. Statistical analysis was carried out using Student t-Test (n=8).

Table 3.1 shows the individual measurements for each animal in the study. Most of the E2 treated animals underwent the expected enlargement of pituitary gland and uterus (highlighted in tan), however, 2 animals (3B and 2C – highlighted in green) did not. The reason for this is unknown though it is thought to relate to improper preparation of the

mini-pumps and therefore restricted delivery of E2. Pituitaries from this experiment were to be used for either western blot or immunohistochemical (IHC) analysis. As relatively less material can be generated for western blot protocols, the pituitaries with the biggest pituitary weight increase were used for western blotting, while the rest were used for immunofluorescence.

Animal Number	Cage Letter	Treatment	Pituitary Weight (mg)	Uterus weight (g)	Future experimental design
0	A	E2	16.60	1.24	IHC
1	A	Control	11.60	0.71	Western Blot
2	A	E2	33.50	1.86	Western Blot
3	A	Control	13.90	0.53	IHC
0	B	Control	9.60	0.63	Western Blot
1	B	E2	43.50	1.75	Western Blot
2	B	Control	13.50	0.53	IHC
3	B	E2	16.20	0.67	IHC
0	C	Control	5.60	0.72	IHC
1	C	E2	22.90	1.15	IHC
2	C	E2	14.60	0.76	IHC
3	C	Control	12.40	0.51	IHC
0	D	Control	9.00	0.62	Western Blot
1	D	E2	14.10	1.13	IHC
2	D	Control	13.00	0.6	IHC
3	D	E2	23.30	1.6	Western Blot

Table 3.1. Three weeks E2 treatment of Fischer 344 rats induces uterus and pituitary hyperplasia. Fischer 344 rats were implanted with Azlet mini pumps delivering either vehicle (PEG) or E2 (125µg/kg/day) for 3 weeks. Rats were killed and pituitaries and uteri were dissected out and weighed. Control animals are shown in white, E2 treated animals which exhibited the expected increases in pituitary and uterus weight are highlighted in tan, E2 treated animals which did not exhibit increased pituitary and uterus weights are highlighted in green.

Pituitary PRL content was assessed by western blotting (Figure 3.2 A). Quantification of the PRL band compared against the α-Tubulin loading control revealed a 2-fold increase in PRL in E2 treated rats (Figure 3.2 B). Furthermore, IHC analysis of the anterior pituitary gland showed that in control animals, 46% of cells expressed PRL (cells counted = 856) in keeping with published data, which increased to 65% of cells (cells counted = 1028) in E2 treated pituitaries (discussed in greater detail in section 3.5.1). Together, these data demonstrate that E2 treatment induced lactotroph hyperplasia and PRL secretion. Attempts were made to quantify the levels of serum E2 in our rats to demonstrate successful administration of stimulus. This work is usually carried out by our collaborator Prof Alan McNeilly in Edinburgh, however, technical problems with the assay prevented this work being carried out in time for publication of this work.

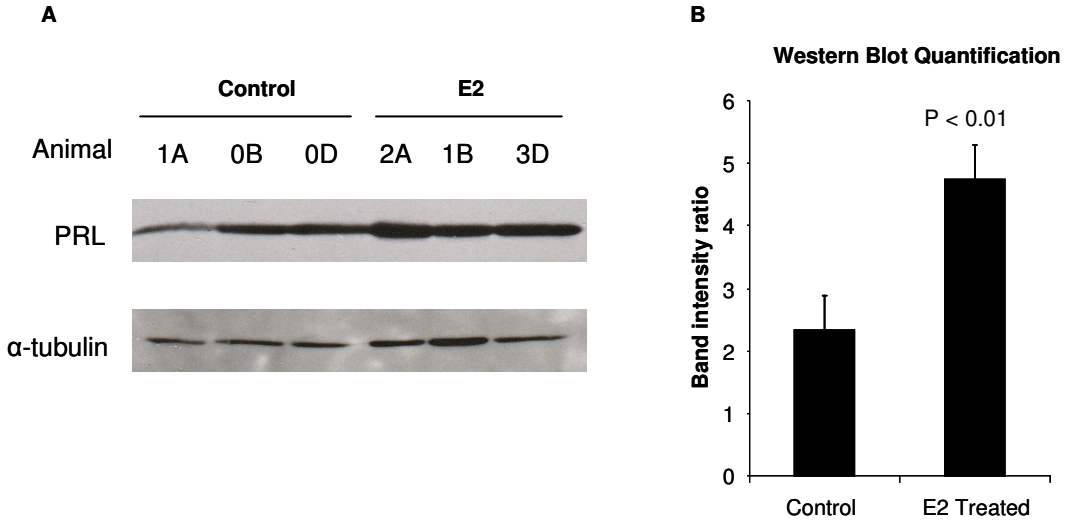


Figure 3.2. Three weeks E2 treatment of Fischer 344 rats increases pituitary PRL levels. Western blot for PRL and α-Tubulin loading control on anterior pituitary lysates from Fischer 344 rats treated with either vehicle (polyethylene glycol 400) or E2 (125µg/kg/day) for 3 weeks. Each blot represents a lysate generated from a single animal selected for western blotting in Table 3.1. (A). Quantification of PRL band intensity relative to α-Tubulin band intensity (B). Data represent means ±SEM. Statistical analysis carried out using Student T-Test (n=3).

3.4. Validation of Wnt-4 antibody

Wnt proteins have been traditionally difficult to purify, and until recently few adequate tools were available to study the effects of Wnt molecules. As such, it is important to ensure that any tool we use is validated to a reasonable degree. To judge the specificity of our antibody, we stained rat kidney, a well characterised positive control for Wnt-4, with our antibody and compared it to published findings. We demonstrated specific expression of Wnt-4 in cortical renal tubules while the renal cortices were negative for Wnt-4 (Figure 3.3 A and B). This staining pattern was in keeping with previously observed expression patterns in this tissue (Figure 3.3 D) (Terada et al., 2003). Secondary only staining demonstrated the specificity of our secondary antibody (Figure 3.3 C). Figure 3.3 E shows a western blot for Wnt-4 demonstrating a clean band at 37kDa, the predicted molecular weight of Wnt-4, further highlighting the specificity of the antibody.

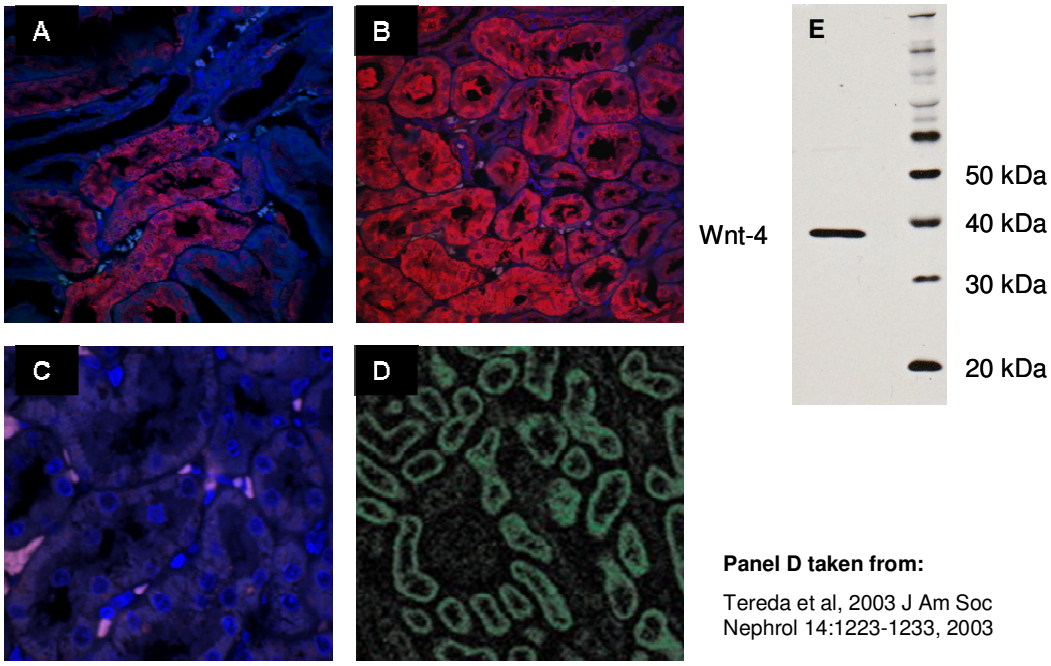


Figure 3.3. Validation of Wnt-4 antibody specificity. Specific Wnt-4 staining in cortical renal tubules (A and B), consistent with previously published material (D) and secondary only control (C). Western blot on a lysate generated from NIH-3T3 cell line constitutively expressing Wnt-4 showing highly specific antibody binding to a protein of the correct predicted size (E).

3.5. Wnt-4 expression in the pituitary

3.5.1. Wnt-4 expression in the anterior pituitary

Dual staining immunocytochemistry (ICC) for PRL and Wnt-4 was carried out on GH3 cells and dispersed cultures of primary pituitary cells. As expected, only some GH3 cells expressed PRL as the GH3 cell line is a somatolactotroph cell line containing both GH expressing and PRL expressing, as well as GH and PRL co-expressing cells. In contrast, Wnt-4 was expressed in all GH3 cells studied (Figure 3.4 top row). In cultures of dispersed pituitary cells, roughly half the cells present expressed PRL in agreement with published data. Wnt-4 expression was not ubiquitous, as would be expected from a primary culture expressing different cell phenotypes. Some cells co-expressed Wnt-4 and PRL, while some cells expressed Wnt-4 alone and some expressed PRL alone.

This indicates that Wnt-4 is not specifically confined to the lactotroph population, and that not all lactotrophs express Wnt-4 (Figure 3.4 bottom row).

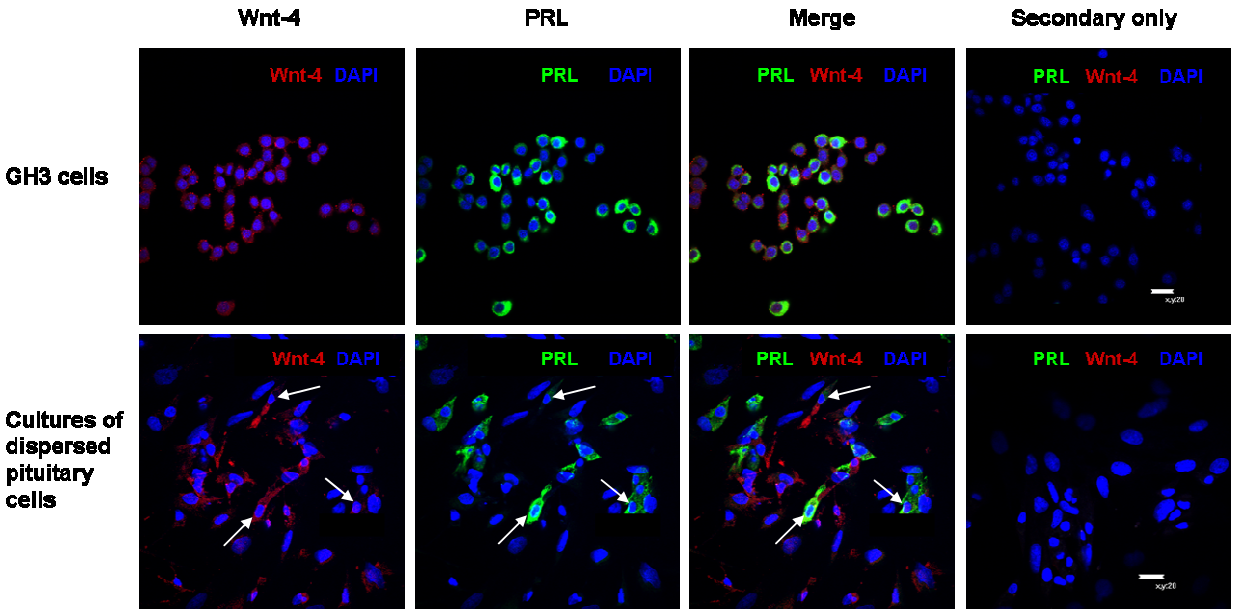


Figure 3.4. Wnt-4 expression in GH3 cells and primary pituitary lactotrophs. ICC of GH3 cells (top row) and cultures of dispersed primary pituitary cells (bottom row). Images show Wnt-4 in red (1st column), PRL in green (2nd column) and DAPI in blue with merged images for PRL and Wnt-4 in the 3rd column. Examples of primary cells co-expressing Wnt-4 and PRL are highlighted by white arrows. Secondary antibody only staining is shown in the 4th column. White bar represents 20µM.

In order to assess which endocrine cell types in the pituitary express Wnt-4, adult female rat pituitary tissue was analysed by dual immunofluorescence staining for Wnt-4 with GH, PRL, ACTH, LH-β and TSH-β. Examples were found where all the secretory cell types co-localised with Wnt-4 (as seen by yellow staining in the merged images) indicating all the secretory cell types in the pituitary expressed Wnt-4 (Figure 3.5.).

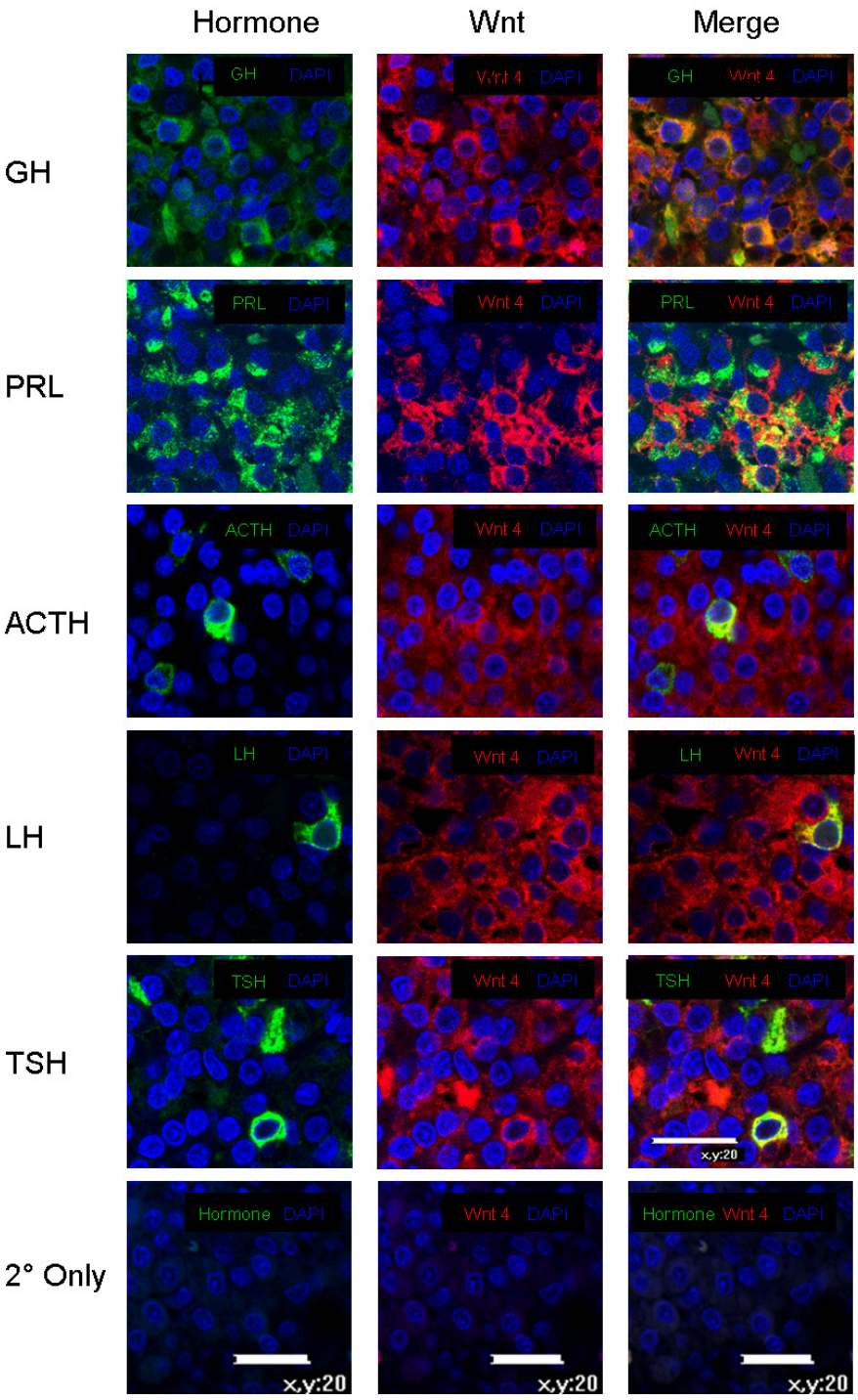


Figure 3.5. Wnt-4 expression in the anterior pituitary. Adult female pituitary tissue was co-stained for Wnt-4 (red) with GH, PRL, ACTH, LH-β and TSH-β (all green). Hormone staining is shown in the left column, Wnt-4 staining is shown in the central column and merged images in the right column. Secondary only staining is shown in the bottom row,

The relative proportions of Wnt-4 expressing cells and secretory cell types in control and E2 treated animal pituitaries were quantified. Overall, Wnt-4 expression was widespread with roughly 70% (±8%) of cells expressing Wnt-4 in controls, which decreased to 56% (±5% - $P < 0.05$) of cells in E2 treated animals. The relative proportions of secretory cell types were roughly consistent with previously published material. In E2 treated animals, the proportion of lactotrophs increased to 65% (±9% - $P < 0.05$) in keeping with lactotroph hyperplasia. As the proportion of lactotrophs was increased, the proportions of the other secretory cell types should decrease. Although this is the case in TSH, ACTH and LH secreting cells, the proportion of GH secreting cells increased slightly to 49% (±6%) of cells. This could be because images used for cell counting were chosen at random meaning regions with high incidence of GH producing cells could skew the counting. It is also possible that a number of cells in E2-treated pituitaries are somatolactotroph cells, expressing both GH and PRL, which would therefore be counted twice using the staining protocol in this experiment.

The proportion of each hormone secreting cell type co-expressing Wnt-4 was also quantified. Somatotrophs exhibit the highest rate of co-localisation, with 93% of GH cells expressing Wnt-4 in controls, while 79% of gonadotrophs expressed Wnt-4. Less than half of lactotrophs, thyrotrophs and corticotrophs (26%, 48% and 24% respectively) expressed Wnt-4, and no significant change in the proportion of cells co-expressing Wnt-4 was detected in E2 treated rats (Table 3.2).

	% of total cells expressing		% of hormone cells co-expressing Wnt-4	
	Control	**E2**	**Control**	**E2**
GH	45.7 ± 7.2	48.7 ± 6.1	93.0 ± 3.1	98.1 ± 2.4
PRL	45.8 ± 3.8	65.0 ± 9.2 $P < 0.05$	26.0 ± 8.4	33.6 ± 9.1
TSH	5.73 ± 2.9	5.17 ± 1.8	48.1 ± 4.3	53.0 ± 6.7
ACTH	5.38 ± 0.9	3.96 ± 2.1	23.5 ± 9.1	21.8 ± 4.3
LH	3.67 ± 1.6	3.23 ± 1.1	79.3 ± 11.1	73.1 ± 6.4
Wnt-4	70.3 ± 8.1	56.4 ± 4.6 $P < 0.05$	N/A	N/A

Table 3.2. The proportion of Wnt-4 and hormone expressing cells in the pituitary. The relative proportions of each secretory cell type in control and E2 treated pituitaries were quantified, and the proportion of hormone cells co-expressing Wnt-4 was also quantified. In each case, a minimum of 800 individual cells were counted from randomly chosen images, and for each hormone cell type, a large enough number of cells was counted to ensure at least 50 secretory cells had been counted. Percentage errors representing SEM are given in each case. Statistics carried out using Student T-Test. Where P values are not given, no significance was detected.

3.5.2. Wnt-4 expression in the marginal zone

The marginal zone (MZ) is a single layer of cells which lies between the intermediate lobe and the anterior lobe of the pituitary, and is thought to harbour stem cells. These cells may drive cell proliferation of a defined secretory cell type in response to physiological demands.

The MZ can be identified using DAPI staining due to the differences in nuclei dispersion between the anterior and intermediate lobes. Nuclei are most tightly packed in the anterior lobe and are more spread apart in the intermediate lobe (Figure 3.6 A and C). Cells in the marginal zone are reported to specifically express E-Cadherin (Garcia-Lavandeira et al., 2009), and staining sections with E-Cadherin makes the border between the 2 regions even more apparent (Figure 3.6 B). As previously shown, Wnt-4 was widely expressed in the anterior lobe, and was also expressed at high levels in the intermediate lobe. However, it was also highly expressed in a single cell layer along the marginal zone (Figure 3.6 D). Wnt-4 expression patterns in control rats and E2 treated rats were compared but no difference in staining pattern was observed.

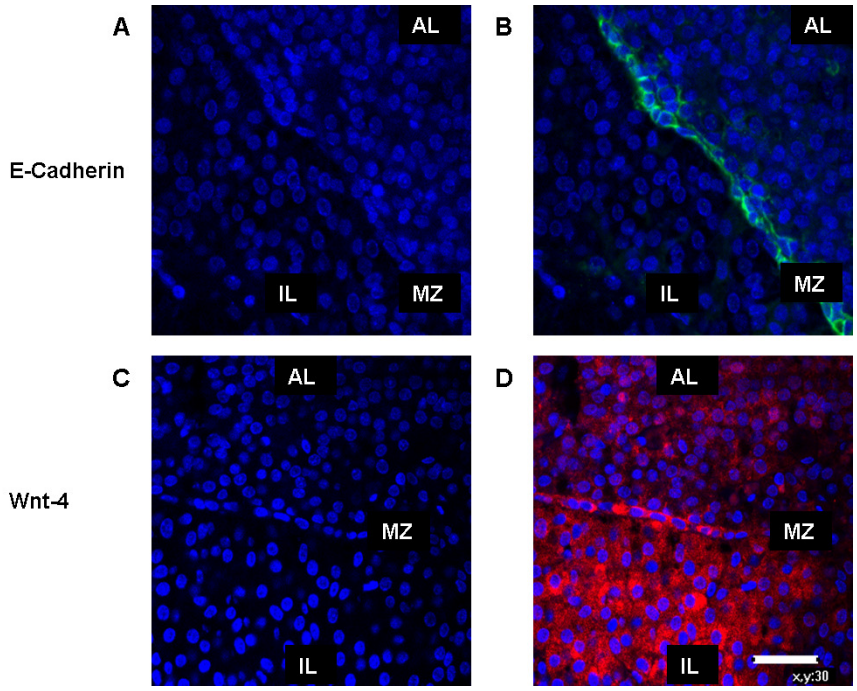


Figure 3.6. Wnt-4 and E-Cadherin are expressed in the marginal zone. IHC staining for E-Cadherin (A and B) and Wnt-4 (C and D) in normal adult female pituitary tissue showing regions encompassing the anterior lobe (AL), the marginal zone (MZ) and the intermediate lobe (IL). **A** and **C** show DAPI only staining demonstrating the different dispersion of cell nuclei between regions, and **B** and **D** show staining for protein of interest (E-Cadherin in green and Wnt-4 in red). White bar represents 30µm.

3.6. Is Wnt-4 upregulated in the pituitary by oestrogen?

3.6.1. Regulation of Wnt-4 protein by oestrogen in GH3 cells

Having identified that Wnt-4 was expressed in the lactotroph population in vivo, and that the proportion of lactotrophs was increased after E2 treatment, we sought to assess whether Wnt-4 regulation could be altered by E2 in vitro. Figure 3.7 shows that Wnt-4 protein was upregulated in GH3 cells treated for 72, 120 and 168h with E2.

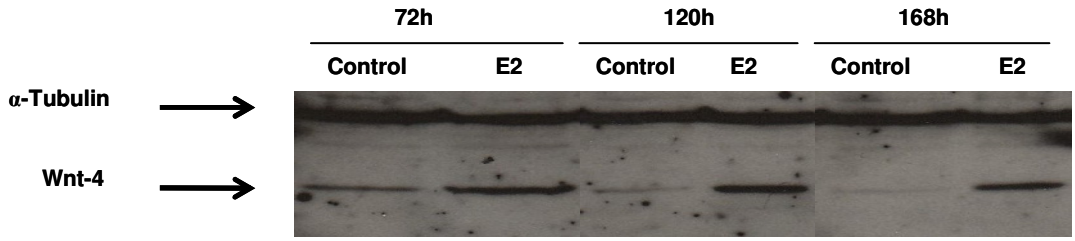


Figure 3.7. Wnt-4 upregulation by oestrogen in GH3 cells. GH3 cells were treated for 72, 120 and 168h with either control (BSA) or E2 (10nM). Blots were probed for Wnt-4 and α-Tubulin loading control. Data are representative of 3 repeated experiments.

3.6.2. Wnt-4 protein levels are unaffected by oestrogen in primary pituitary cell cultures

To see if this upregulation could be replicated in primary cells, cultures of dispersed pituitary cells were treated for 72 and 120h with E2. These cultures contain all the different secretory cell types of the pituitary, consisting mainly of lactotrophs and somatotrophs. As expected from the IHC data, Wnt-4 was expressed in unstimulated cells, however no upregulation was observed when treated with E2 (Figure 3.8).

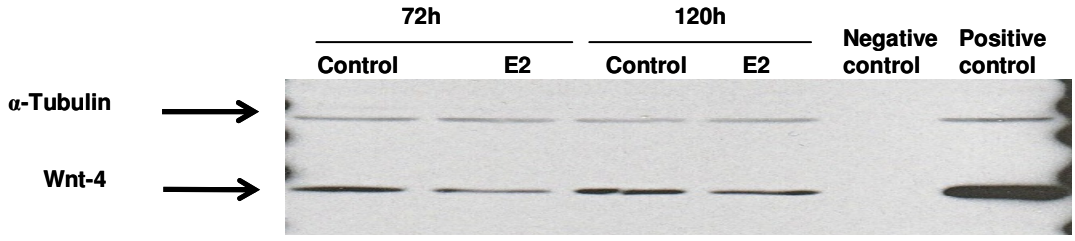


Figure 3.8. Wnt-4 is not upregulated by oestrogen in primary pituitary cell cultures. Primary cultures of dispersed pituitary cells were generated from adult female Fisher 344 rats and treated for 72 and 120h with either control (DCT-FBS) or 10nM E2. Blots were probed for Wnt-4 and α-Tubulin loading control. Wnt-4 positive control was generated from NIH-3T3 cell line constitutively expressing Wnt-4, negative control contained water only. Data are representative of 3 repeated experiments.

3.6.3. Wnt-4 expression is unaltered by oestrogen in intact pituitary tissue

Continuous networks have been demonstrated to exist in the pituitary linking cells of a particular cell type to each other. Though a functional network between lactotroph cells has not been demonstrated, pulses of PRL promoter activity between lactotroph cells are synchronised in intact pituitary tissue, and coupling is lost when pituitary cells are dispersed (Harper et al., 2010). This suggests that a lactotroph cell network does exist, and therefore dispersion of primary cultures as in Figure 3.8 may disrupt such a network.

Instead of dispersing pituitaries into single cell cultures, pituitaries were sliced to a thickness of 300µm and cultured for 24, 72 and 120h. This process is distinct from slicing tissue for histological purposes as the tissue is kept alive in culture after slicing. The protocol has been validated in our group, and tissue viability was maintained through the course of the experiment (Harper et al., 2010). Wnt-4 protein was expressed in control pituitary slices, but was not upregulated by E2 at any of the time points studied (Figure 3.9).

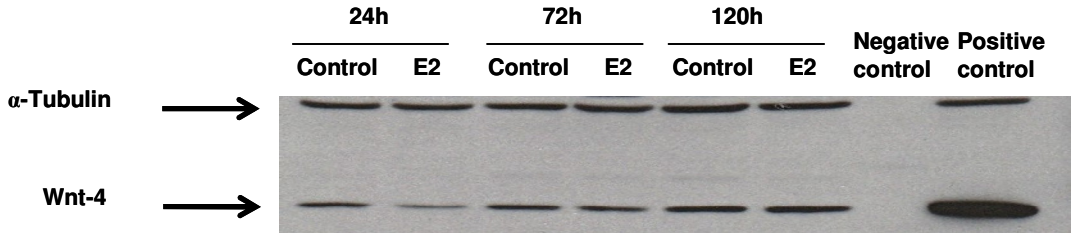


Figure 3.9. Wnt-4 is not upregulated in pituitary slices treated with E2. Adult female Fischer 344 rats were killed and pituitaries were dissected and sliced at a thickness of 300µm. Slices were then cultured for 24, 72 and 120h in either control medium (DCT-FBS) or 10nM E2. Blots were probed for Wnt-4 and α-Tubulin loading control. Wnt-4 positive control was generated from NIH-3T3 cell line constitutively expressing Wnt-4, negative control contained water only. Data are representative of 3 repeated experiments.

3.6.4. Frizzled receptor expression in lactotroph cells

Having demonstrated that Wnt-4 is present in the lactotroph population of the adult pituitary gland, we sought to assess the expression pattern of Fz receptors in GH3 cells and primary lactotroph cells. As described in section 1.11 Fz receptors 2, 4, 5 and 6 have been demonstrated in the rodent brain and pituitary during development and in adult life, and Fz 6 has been shown to transduce Wnt-4 signalling in the kidney. In order to assess the expression of these receptors specifically in the lactotroph population, rather than in a heterogeneous cell population obtained by dispersing the whole pituitary, we carried out FACS sorting on dispersed pituitary cells.

By using a simple forward scatter and side scatter analysis, cells were divided into 2 populations, P1 and P2. Immunocytochemical analysis revealed that the smaller P1 population was 70% positive for GH and PRL co-expressing cells, while the P2 population was 80% positive for PRL with less than 1% of cells positive for GH (Figure 3.10A). FACS sorting and analysis was carried out by Dr Frederic Madec. cDNA was generated from GH3 cells and the FACS P2 lactotroph enriched population and used to run PCR for the above receptors. Genomic DNA (gDNA) was used to optimise the primers and ensure DNA products were of the correct size. Fz-2, 4, 5 and 6 were all expressed in GH3 cells, while Fz-2 and Fz-4 were expressed in the FACS sorted lactotroph population. No band was present for Fz-5 or Fz-6 indicating these receptors were not present in primary lactotroph cells (Figure 3.10 B).

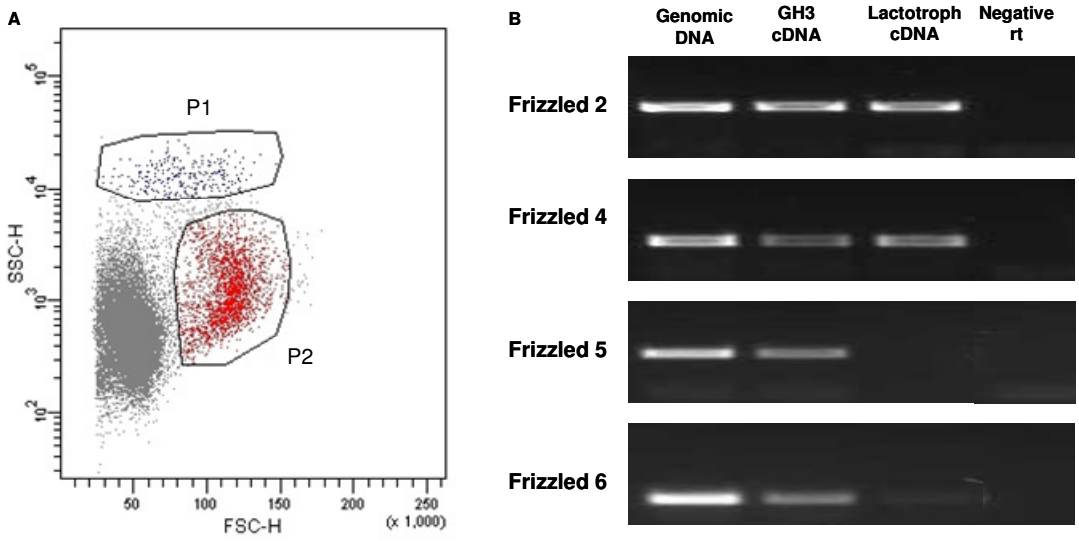


Figure 3.10. Fz receptor expression in GH3 cells and FACS sorted primary lactotroph cells. A - FACS sorting of primary pituitary cells using forward scatter (x-axis) and side scatter (y-axis) isolated 2 cell populations - P1 population was 70% positive for GH and PRL co-expressing cells, P2 population was 79% positive for PRL expressing cells. **B -** PCR for Fz 2, 4, 5 and 6 was run on rat genomic DNA and cDNA generated from GH3 cells and FACS P2 lactotroph enriched cells. Negative control is presented as PCR run on cDNA samples generated without reverse transcriptase.

3.7. Does Wnt-4 affect GH3 cell proliferation?

Wnt-4 was expressed in GH3 cells and was upregulated by E2 in vitro. We sought to assess whether Wnt-4 had any direct effect on GH3 cell proliferation. GH3 cells were treated for 120h with Wnt-4 recombinant protein and cell number was assessed using MTS assay. Results obtained were unexpectedly variable over 7 separate experiments run using an identical protocol (Figure 3.11). In the 7 experiments run, 3 experiments showed that Wnt-4 increased the rate of proliferation (experiments 1, 3 and 7), 3 experiments showed that Wnt-4 decreased the rate of proliferation (experiments 2, 4 and 5) while one experiment (experiment 6) showed no significant change.

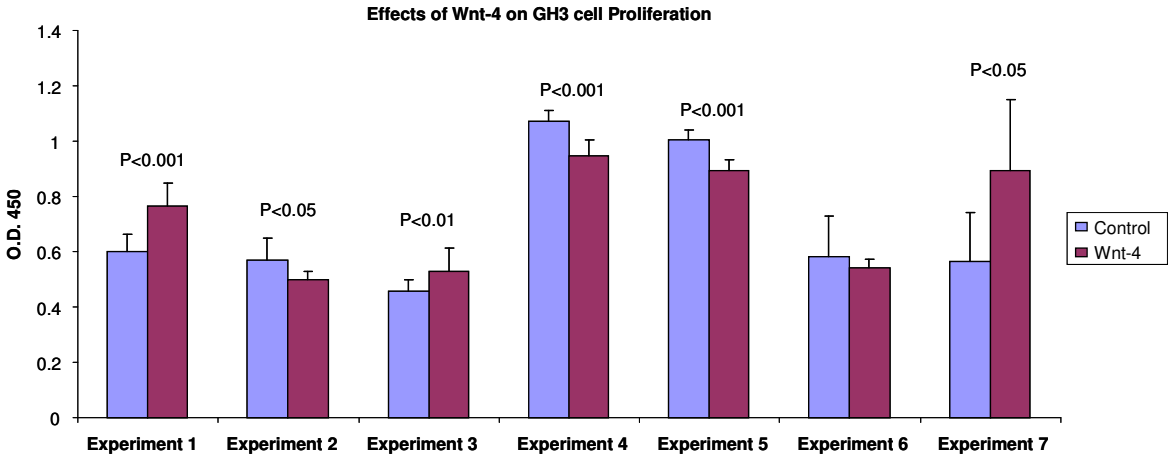


Figure 3.11. Wnt-4 has a variable effect on GH3 cell proliferation. GH3 cells were plated at $1x10^4$ cells per well in a 96 well plate and stimulated for 120h with either control (DCT-FBS) or recombinant Wnt-4 protein (200ng/ml). Cell number was quantified using MTS assay. Data represent means ±SEM. Statistical analysis was carried out using standard t-Test (n=10 for each condition).

In a proliferation assay, the initial seeding density of cells is of vital importance. Due to the relatively small number of cells seeded per well ($1x10^4$), any variation between the initial cell number plated can have a large effect 5 days later when the final cell number is quantified. The seeding densities in experiments 6 and 7 from Figure 3.11 were measured at day 0 to ensure the variability in results was not related to uneven seeding. They were found to be the same, suggesting that the differences in final cell number occurred in the latter part of the experiment (Figure 3.12).

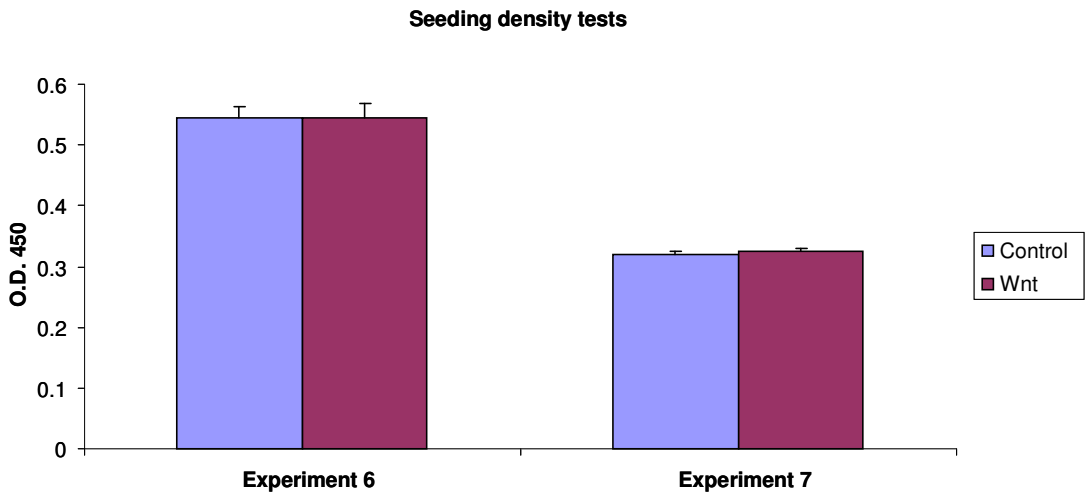


Figure 3.12. Seeding protocol is not the cause of variable proliferation assay result. GH3 cells were plated at $1x10^4$ cells per well in a 96 well plate and allowed to settle for 24h after which cell number was quantified by MTS assay. Data represent means ±SEM. Statistical analysis was carried out using standard t-Test (n=10 for each condition).

The above proliferation assays were run over the course of a few months and utilised GH3 cells from a number of different passages. We therefore tested whether passage number of the GH3 cells could affect the proliferation rate. GH3 cells from 2 different passage numbers (P27 and P37) were stimulated for 120h with both DCT-FBS and FBS. A seeding density test was carried out at day 0 showing that cells were seeded at the same density. After 120h in both conditions, P37 GH3 cells had proliferated more than P27 GH3 cells (Figure 3.13). This highlights the heterogeneous nature of GH3 cells and in future care should be taken to ensure that cells of a consistent passage number are utilised between experiments of this nature.

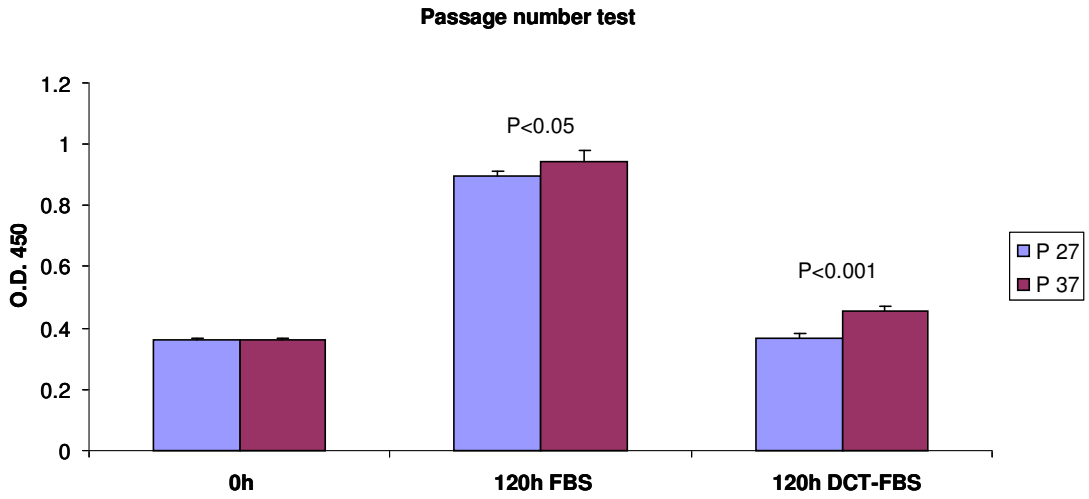


Figure 3.13. GH3 cell passage number affects basal rate of proliferation. GH3 cells from passage number 27 and 37 were plated at $1x10^4$ cells per well in a 96 well plate. A seeding density test was carried out at 0h, while identical plates were stimulated for 120h with either DCT-FBS or FBS. Data represent means ±SEM. Statistical analysis was carried out using standard t-Test (n=10 for each condition).

Overall, the proliferation assay results are inconclusive. The effects of Wnt-4 on GH3 cells were variable, with some experiments showing that Wnt-4 increased the rate of proliferation, and some showing that it decreased proliferation (Figure 3.11). This variability is hard to explain as seeding densities were comparable between conditions (Figure 3.12), though the difference in basal rate of proliferation between cells at different passage numbers may contribute to the variation observed (Figure 3.13).

3.8. Discussion

3.8.1. Oestrogen-induced lactotroph hyperplasia in the Fischer 344 rat

The principal objective of this study was to assess the role of Wnt-4 in E2-induced lactotroph hyperplasia. Although the link between E2 and lactotrophs has been known for more than 40 years, the mechanisms by which it occurs remain elusive.

In this study, Fischer 344 rats were treated for 3 weeks and pituitary tissue was analysed. Rats were treated with 125µg/kg/day for 3 weeks, a dose considered to be towards the lower end of the supraphysiological range, but well above the dose required to replicate physiological E2 levels in ovariectomizsed rats of 25µg/kg/day (Nolan and Levy, 2009a). Pituitary weight and wet uterus weight both increased in accordance with numerous published data. The increase in uterus weight arises from the secretion of uterine luminal fluid which aids sperm motility and maturation and is the standard bioassay for oestrogen effect (Kuo et al., 2009). The increases in pituitary PRL content and the proportion of lactotrophs in oestrogen treated animals indicate lactotroph hyperplasia as the likely cause of increased pituitary weight.

Some animals did not respond to oestrogen treatment as expected. Animals 3B and 2C seemed to only show a slight increase in uterus weight and negligible increase in pituitary weight (Table 4.1). It is unknown why these animals did not respond to E2 treatment, though it is possible that delivery of E2 to the rats was impaired though improper preparation of mini-pumps. Attempts were made to investigate this by testing serum levels of E2. This assay is usually carried out by Prof Alan McNeilly's lab in Edinburgh, though technical difficulties prevented work from being carried out.

3.8.2. Cell proportions in the anterior pituitary

The proportions of cells within the anterior pituitary in this study roughly conform to published data. Table 3.3 shows the findings of the current investigation compared to the predicted proportions of cells in the pituitary (Asa et al., 2002).

Cell type	Asa Published data (%)	Current study Control rats (%)	Current study E2-treated rats (%)
Somatotrophs	40	46	49
Lactotrophs	35	46	65
Corticotrophs	10	5	4
Gonadotrophs	10	3	3
Thyrotrophs	5	6	5

Table 3.3. Cell proportions in the pituitary. The proportions of secretory cell types in control and E2 treated rats from the present study were compared to published data (Asa et al., 2002).

The results published by Asa et al (2002) are based on studies using different protocols to define cell number including immunofluorescence, electron microscopy and flow cytometry which all have drawbacks and limitations (Levy, 2002). The data for control animals in the current study roughly compares to the findings of Asa et al (2002). Clearly the total percentage in our study adds up to more than 100%, however staining for only one hormone at a time (hormone was always co-stained with Wnt-4) means that invariably errors will arise through imaging on different sections and regions within that section. Overall, the rough distribution of cell types observed is as expected.

However, the sum of percentages in E2 treated animals is significantly over 100% which is a slight cause for concern. Immunofluorescence has a number of drawbacks which could explain these errors. Firstly, cells were counted on random images from the pituitary, which does not take into account pituitary regions with high density of a particular cell type. For example, lactotroph cells are more highly expressed in the periphery of the pituitary, with highest expression in lateral regions (Harper et al., 2010). By counting a minimum of 800 cells for each hormone producing cell-type, it was hoped that this intrinsic error could be reduced, but on reflection a higher number of cells should have been counted which would likely allow for different expression patterns of cells. A second drawback is that sometimes it is difficult to make clear distinctions between cells in close proximity to each other, or cells lying on top of each other which can lead to inaccurate counting. Finally, the process does not take into account cells co-expressing hormones, as can be the case of somatolactotroph cells. The errors shown most likely reflect miscounting of somatotroph and lactotroph cells as they are the most common cells in the pituitary, and dual staining with these hormones would most likely reduce the errors to reasonable levels. However, the increase in PRL positive cells in E2-treated animals is reassuring, and fits with the upregulated pituitary PRL content demonstrated and with the well known action of E2 on lactotroph proliferation.

3.8.3. Wnt-4 expression in the pituitary

Our findings indicate that Wnt-4 was highly expressed in all regions of the pituitary. Validation of our antibody was carried out by staining rat kidney sections, and the staining presented here closely resembles Wnt-4 expression patterns in published material (Terada et al., 2003). Furthermore, western blotting with the antibody specifically identified a protein at the correct size for Wnt-4. One validation protocol not presented here is an antibody pre-absorption test on histological sections. Attempts were made to carry out this protocol, but as high concentrations of Wnt-4 antibody were used experimentally, the amount of Wnt-4 protein required to pre-absorb the antibody was extremely large, and hence expensive. Considering the other validation protocols presented, we considered there was adequate evidence for Wnt-4 antibody specificity, and considered it an unnecessary expense to continue with further validation.

A proportion of each secretory cell type in the pituitary expressed Wnt-4. The highest expressing cell type were somatotrophs with roughly 95% of cells expressing Wnt-4, while the lowest expressing cell type were corticotroph cells with only 23% of cells expressing Wnt-4. Only 26% of lactotrophs expressed Wnt-4 in uncontrolled pituitaries, which increased slightly to 33% in E2 treated animals, though the percentage errors render this difference insignificant. However, immunofluorescence techniques cannot quantify the levels of Wnt-4 in any particular cell type. Therefore, although the proportion of cells expressing Wnt-4 did not change in response to E2, the level of expression may have increased which could give rise to the upregulation of Wnt-4 observed in previous published material.

Wnt-4 was upregulated in GH3 cells in response to E2 treatment, however, no increase in Wnt-4 was observed in cultures of dispersed pituitary cells or in intact pituitary tissue. These later models comprise mixed populations of cells and therefore an increase of Wnt-4 production in one particular cell type may get masked by unaltered levels in other cell types.

We have shown that it is possible to generate a lactotroph enriched population using FACS sorting. Although this method could have potentially been used to study Wnt-4 levels in lactotroph cells, the number of rats required to generate enough material for such an experiment rendered it impractical. Also, Wnt-4 was expressed within all the

other cell types in the AL, as well as the marginal zone and IL. To conduct the investigation properly would require suitable protocols to isolate each of the cell types in the pituitary and time was inadequate to take this line of work on. This highlights a key issue in studying the heterogeneous population of cells that comprises the pituitary gland.

The expression of Wnt-4 in the MZ suggests complex regional organisation in the pituitary. It should be noted that the expression of Wnt-4 in the MZ was only detected in the latter stages of the project. Initial focus was placed on the anterior pituitary, and as such the posterior pituitary was always removed during organ processing. When removing the posterior pituitary, the IL and MZ were removed also, and therefore Wnt-4 localisation in the MZ was initially not noted. No alteration in Wnt-4 expression pattern was observed in the MZ, though more detailed analysis of the region is carried out in section 5.4.

In summary, Wnt-4 was expressed in a large number of cells in the pituitary. E2 increased Wnt-4 expression in GH3 cells lines, but no increase in Wnt-4 protein could be detected in heterogeneous pituitary cell populations. It is possible that increases of Wnt-4 in one cell population could be masked by unchanged expression in other cell types resulting in globally unchanged Wnt-4 expression.

3.8.4. Wnt-4 effects on GH3 cell proliferation

The effects of Wnt-4 on GH3 cell proliferation were inconclusive. A number of identical studies were carried out over the process of a few months which gave conflicting results. The reasons for this are unknown but are not thought to be related to the seeding densities of cells as these were measured in later experiments and shown to be equal between conditions. This suggests that the differences in cell population occur after seeding, though why different rates of proliferation then occurred is unknown.

It is interesting that GH3 cells at different passage numbers proliferated at different rates. The reason for this is unknown. Throughout this project, care was taken to maintain GH3 cells between passage numbers 25 and 42, and this result highlights the fact that the characteristics of cell lines can alter through time and should never be considered absolutely constant.

3.8.5. Conclusions

This chapter shows that E2 treatment of Fischer 344 rats induced lactotroph hyperplasia and increased PRL production in the pituitary. Wnt-4 was expressed in GH3 cells and in all secretory cell types in the anterior pituitary, though Wnt-4 upregulation was not demonstrated in heterogeneous pituitary cell populations. However, experimental approaches could not distinguish between Wnt-4 levels in specific cell populations meaning that changes in Wnt-4 expression may have been masked by other cell populations in the pituitary. Wnt-4 was upregulated by E2 in GH3 cells, but the effect of Wnt-4 directly on GH3 cell proliferation is currently unknown.

4.0. Results - Is canonical signalling activated by oestrogen in the pituitary?

4.1. Introduction

The Wnt-canonical pathway is the most studied of the Wnt pathways. The pathways best known action is regulation of cell proliferation which is driven by expression of canonical target genes such as c-myc and cyclin D. Malfunction of the pathway can result in uncontrolled cellular proliferation and has been implicated in the progression of numerous cancers.

The canonical pathway centres around the stability of β-Catenin. In unstimulated cells, β-Catenin is either bound to E-Cadherin at the cell membrane, or free in the cytoplasm. Cytoplasmic β-Catenin is quickly bound by APC and axin, allowing GSK-3β to phosphorylate and degrade β-Catenin. Wnt binding to Fz prevents axin and APC binding to β-Catenin, thus inhibiting its destruction. This allows β-Catenin to accumulate in the cytoplasm, and then translocate into the nucleus where it interacts with transcription factors TCF and LEF to induce transcription of Wnt target genes (Rao and Kuhl, 2010; Widelitz, 2005).

Canonical activation has been demonstrated in 80% of colorectal cancers, 54% of endometriod ovarian cancers, 23% of metaplastic breast carcinomas and several other types of cancer. At the start of this project, evidence in the literature suggested the canonical pathway may be functional in pituitary tumours. A number of canonical pathway molecules were shown to be expressed in the pituitary such as the Frizzled receptor, APC, β-Catenin and TCF (Douglas et al., 2001), and β-Catenin had been shown to play a key role in cell fate determination in the pituitary through interaction with Prop-1 (Olson et al., 2006). Importantly, nuclear β-Catenin was observed in 57% of human pituitary adenomas examined in one study, strongly suggesting the canonical pathway was active in pituitary adenomas (Semba et al., 2001).

However, during the course of this project, conflicting data has arisen confusing these findings. The extracellular canonical inhibitor WIF-1 was down regulated in human non-functioning tumours, and although this was associated with upregulation of the canonical target gene cyclin D2, nuclear β-Catenin was not observed (Elston et al., 2008). Furthermore, in a study of 54 human pituitary adenomas, β-Catenin was found only at the cell membrane and never in the nucleus suggesting the canonical pathway was not activated in these tumours (Miyakoshi et al., 2008b). Taken together, it is currently unknown if the canonical pathway is activated in the pituitary.

Despite classically being considered a non-canonical Wnt, Wnt-4 does activate canonical signalling in a number of circumstances. Wnt-4 activation of β-Catenin controls cell growth and survival in MDCK cells through binding to Fz 6 (Lyons et al., 2004), maintains female germ cells in the fetal mouse ovary (Liu et al., 2010b), and regulates renal nephrogenesis in mice (Park et al., 2007). This suggests that Wnt-4 may activate canonical signalling in the pituitary.

4.2. Aims

The aims of this chapter are as follows:

- To determine whether β-Catenin levels are upregulated in GH3 cells and primary pituitary tissue in response to E2 treatment
- To determine whether β-Catenin translocation to the nucleus can be detected in GH3 cells and primary pituitary tissue in response to E2 treatment
- To determine whether canonical-TCF dependent gene transcription can be induced by E2 or Wnt-4 in GH3 cells
- To determine whether overexpression of β-Catenin in GH3 cells affects cell proliferation

4.3. β-Catenin expression in the pituitary

4.3.1. β-Catenin expression in GH3 cells

Wnt-canonical signalling centres around the translocation of β-Catenin form the cytoplasm into the nucleus where it exerts its effects by activating transcription of canonical target genes. The expression of β-Catenin and its regulation by E2 was assessed in pituitary cells. β-Catenin was expressed at consistent levels in GH3 cells cultured over 120h, and global levels were not changed by treatment with E2 (Figure 4.1).

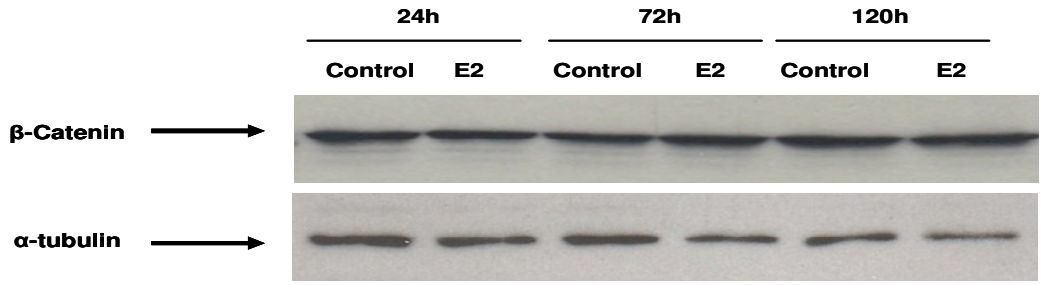


Figure 4.1. β-Catenin expression in GH3 cells is unaffected by oestrogen treatment. Western blot for β-Catenin and α-Tubulin loading control on GH3 cells stimulated for 24, 72 and 120h with either control (BSA) or 10nM E2. Data are representative of 3 repeated experiments.

4.3.2. β-Catenin expression in pituitary tissue

Western blots were also run on lysates generated from cultured pituitary slices. As seen in GH3 cells, β-Catenin was expressed in the pituitary under control conditions, but its regulation was not affected by E2 treatment over the time-courses studied (Figure 4.2).

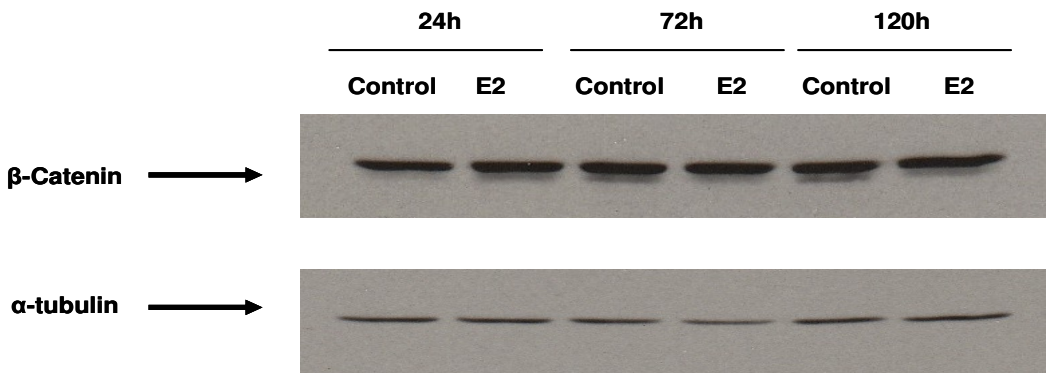


Figure 4.2. β-Catenin levels are unaltered by oestrogen in cultured pituitary slices. Western blot for β-Catenin and α-Tubulin loading control. Whole pituitaries were sliced to a thickness of 300µm and cultured for 24, 72 and 120h with control (DCT-FBS) or 10nM E2. Data are representative of 3 repeated experiments.

Slicing and culturing live pituitaries to maintain pituitary structure is a technique routinely used in our lab, and we are confident that the integrity of the tissue is maintained over the time-courses studied. However, the disruption caused by slicing the pituitary and the exposure of tissue to external growth factors in culture medium renders this an inaccurate, although convenient, model to study. To address this, lysates generated from anterior pituitary tissue of animals treated in vivo for 3 weeks with E2 were analysed for β-Catenin. β-Catenin was expressed in control tissue, and again, its regulation was unaltered by E2 treatment (Figure 4.3).

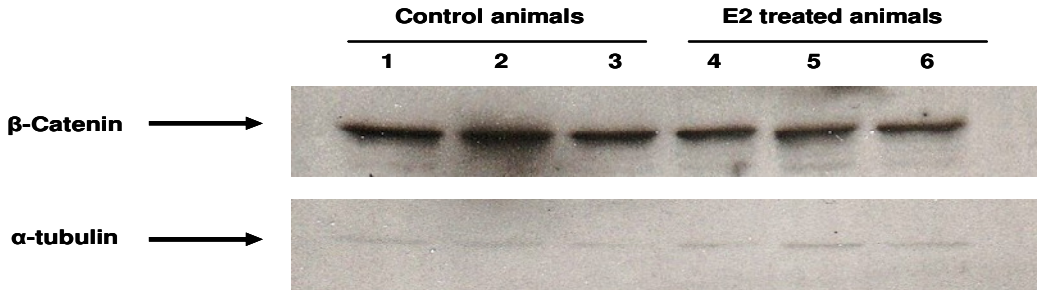


Figure 4.3. β-Catenin expression is unaltered in the anterior pituitary after 3 weeks oestrogen treatment. Fischer 344 rats were treated for 3 weeks with subcutaneously administered vehicle (PEG) or E2 (125µg/kg/day). Posterior pituitaries were removed, lysates were generated from anterior pituitary tissue and tested for β-Catenin and α-Tubulin loading control.

Overall, these results show that global β-Catenin levels are not changed by E2 in GH3 cells or adult pituitary tissue, even when E2-induced pituitary hypertrophy is occurring. This is not particularly surprising as the key component of canonical signalling is translocation of β-Catenin into the nucleus. The actual levels of β-Catenin translocation required to initiate canonical gene expression can be small, and it is likely that western blotting is not a sensitive enough tool to detect this.

4.3.3. β-Catenin distribution in lactotroph cells

A more sensitive way to assess canonical activation is by using immunofluorescence, which visually depicts the subcellular localisation of proteins of interest, and will highlight changes in nuclear β-Catenin more clearly than western blotting. Figure 4.4 shows that β-Catenin was expressed at the cell membrane in GH3 cells, where it is known to interact with E-Cadherin to regulate cell-cell adhesion. Neither treatment with E2 or LiCl altered the distribution pattern of β-Catenin (LiCl is a standard positive control for canonical activation which promotes nuclear translocation of β-Catenin by blocking its degradation by GSK-3β). No nuclear staining for β-Catenin was apparent in any condition.

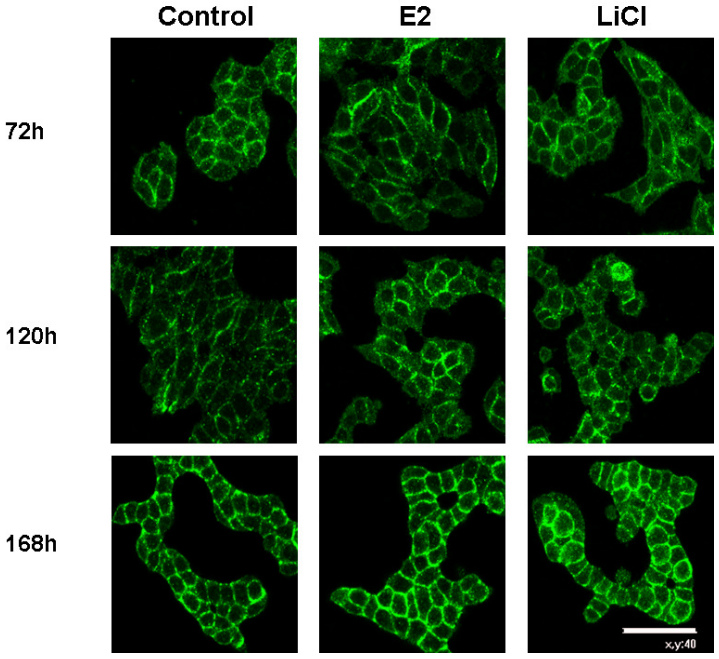


Figure 4.4. Subcellular localisation of β-Catenin is unaltered by E2 or LiCl treatment in GH3 cells. GH3 cells were stimulated for 72, 120 and 168h with control (BSA), 10nM E2 or 10mM LiCl and stained for β-Catenin. White bar represents 40µm.

The same distribution was found in primary lactotroph cells. Pituitaries were dispersed and dual-stained for β-Catenin and PRL to identify lactotroph cells. At 72 hours, expression of PRL was relatively low in control and LiCl treated cells, and much higher in E2 treated cells in line with the known stimulatory effect of E2 on PRL synthesis. At 120h, PRL expression was consistently high between conditions and it was observed that the vast majority of cells at this time point were positive for PRL, most likely reflecting proliferation of lactotrophs in culture.

In all conditions, membrane bound β-Catenin was observed with no nuclear β-Catenin in any condition. At 72h, the intensity of membrane bound β-Catenin appeared to be higher in E2 treated cells than control or LiCl treated cells. At 120h, the intensity of membrane bound β-Catenin was comparable between stimuli, though still appearing more intense than compared to controls at 72h. A major limitation of immunofluorescence is that quantification of data is extremely difficult, and though the images presented are considered representative of the data, these observations cannot be validated. Attempts were made to quantify the data using an external company claiming the ability to quantify the amounts of β-Catenin in distinct subcellular regions. However, attempts were unsuccessful (Figure 4.5).

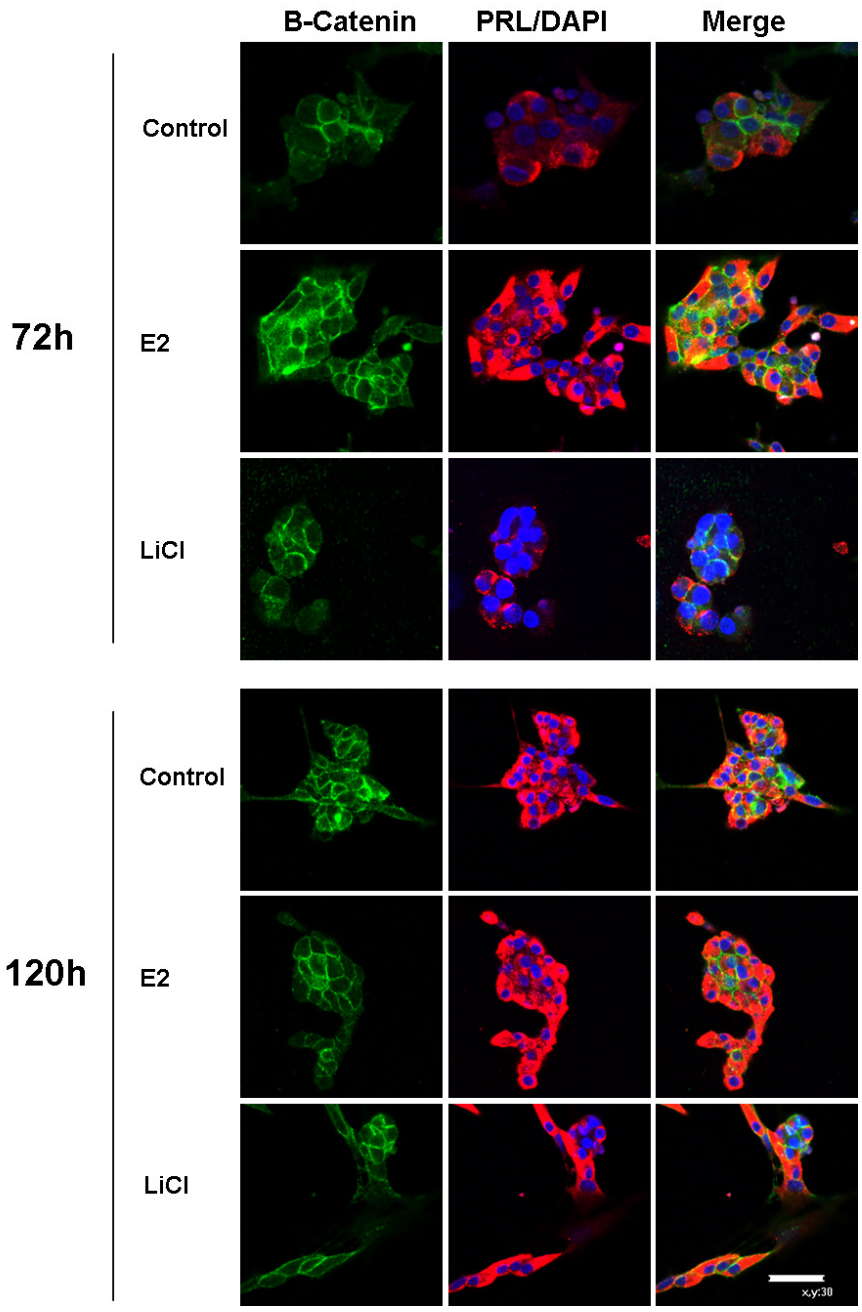


Figure 4.5. Subcellular localisation of β-Catenin is unaltered by treatment with E2 and LiCl in primary lactotroph cells. Pituitaries from female Fischer 344 rats were dispersed and stimulated for 72 and 120h with control (DCT-FBS), 10nM E2 or 10mM LiCl. Cells were stained for β-Catenin (green - left column), PRL and DAPI (red and blue respectively - middle column) with merged images in the right column. White bar represents 30µm.

4.3.4. β-Catenin distribution in the anterior pituitary

As previously described, dispersal of pituitary cells into a monolayer culture may disrupt pituitary networks, altering cell-cell communication and disrupting the natural physiological actions which may occur in vivo. Therefore, staining of β-Catenin was carried out on structurally intact histological pituitary sections from E2 treated rats. As seen in GH3 cells and primary pituitary lactotrophs, a clearly defined β-Catenin membrane staining pattern was observed in the anterior pituitary, which was unaltered by E2 treatment (Figure 4.6). Although only a few images have been presented here, there was not a single instance of nuclear staining found in any pituitary studied.

Dual-staining for PRL was not carried out in this instance as lactotroph cells comprise roughly 40% of the cells in the anterior pituitary. Therefore it is assumed that a sizable proportion of the cells present were lactotrophs and it is therefore almost certain that β-Catenin distribution was not altered in lactotroph cells by E2.

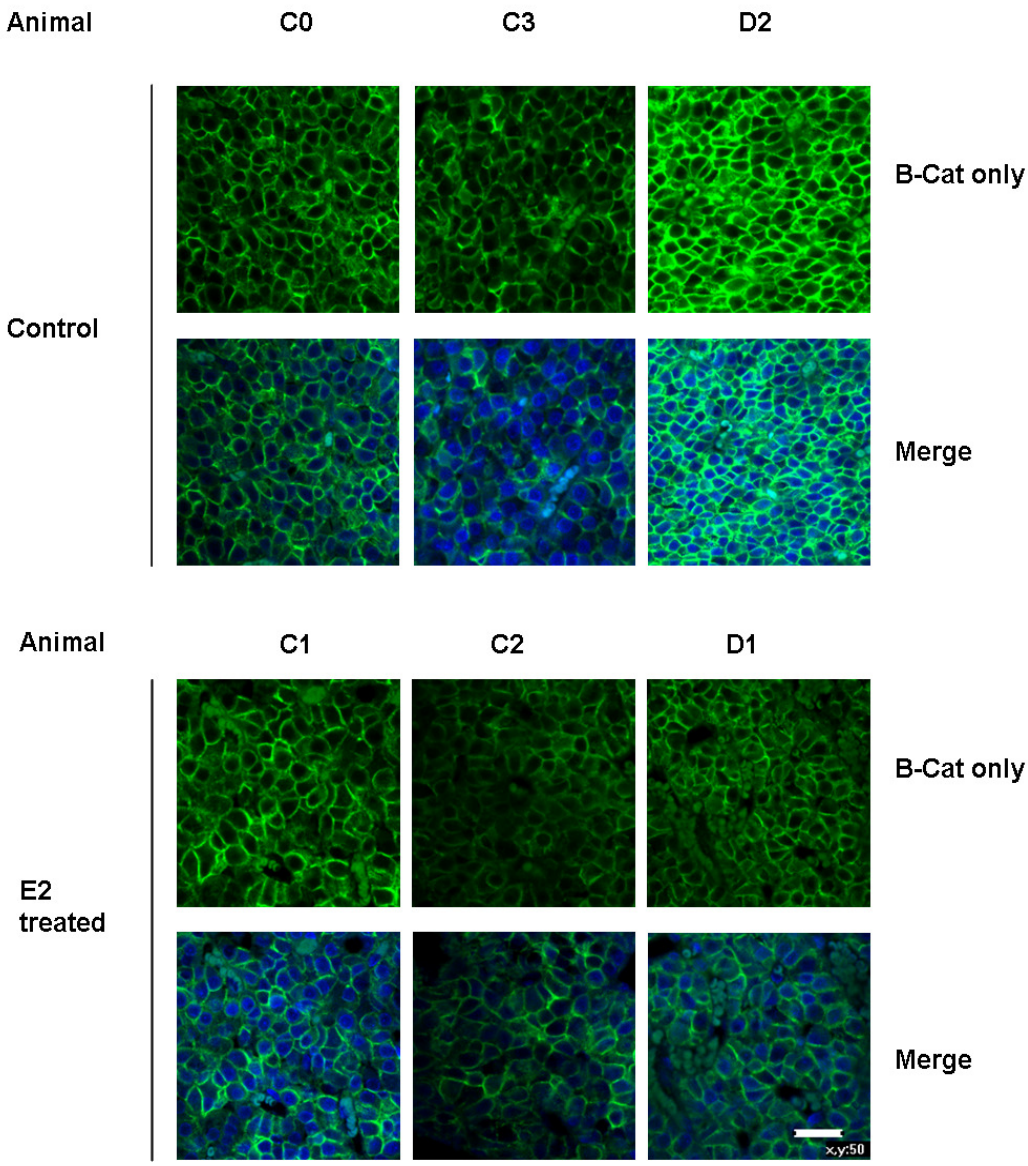


Figure 4.6. β-Catenin distribution is unaltered in the anterior pituitary gland by oestrogen. Fischer 344 rats were treated with vehicle (PEG) or E2 (125µg/kg/day) for 3 weeks. Pituitaries were stained for β-Catenin (green) and DAPI (blue). Animal numbers correspond to Table 3.1. White bar represents 50µm.

4.4. TCF-dependent transcriptional signalling in GH3 cells

Immunofluorescence is a more sensitive tool than western blotting to study the translocation of β-Catenin into the nucleus. However it may still not be sensitive enough to detect low levels of β-Catenin translocation which might induce transcription of canonical target genes. We therefore employed a reporter gene assay technique to measure functional transcriptional output of the canonical pathway in GH3 cells.

The reporter gene Super 8x TopFlash (TopFlash) is a widely used tool in the literature. It contains 8 binding sites for the canonically activated transcription factor TCF, linked to a luciferase expression vector. Activation of the canonical pathway results in binding of TCF to the reporter gene, inducing transcription of luciferase which can subsequently be quantified. A mutated form of the plasmid, FopFlash, was used as a negative control.

4.4.1. Validation of TopFlash/FopFlash

TopFlash and FopFlash were acquired from Addgene, then amplified and purified as described previously. To ensure that the plasmid we obtained was the correct plasmid, a series of restriction enzyme digests was performed.

Two enzymes were used to validate the protocol. Hind III was used alone to determine the size of the plasmid and Hind III and Sal I were used together to fragment the DNA. The size of the plasmid cannot be determined by running the untreated plasmid alone, as the intact plasmid is made of supercoiled DNA which runs faster through the gel than normal DNA. Cutting the plasmid at a single site linearises the DNA which can then be measured accurately.

A band of 5Kb was observed when using Hind III alone, and 2 fragments of 1900bp and 3100bp were detected when using Hind III and Sal I together, in line with predicted fragment lengths. As expected, the undigested plasmid ran relatively faster than linearised DNA, appearing to be 3Kb long, and highlighting the importance of linearising DNA in this type of assay (Figure 4.7).

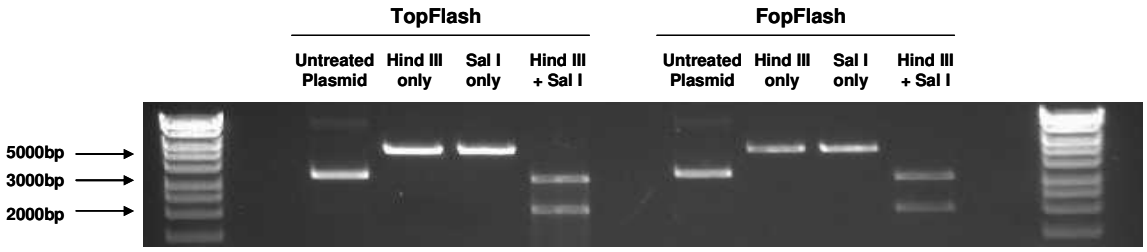


Figure 4.7. Restriction digest of Top/FopFlash plasmids. TopFlash and FopFlash were digested with either Hind III alone, Sal I alone, or Hind III and Sal I together. Untreated and digested plasmids were run on an agarose gel to determine size of DNA.

To check the functionality of the plasmids, TopFlash and FopFlash were transfected in HEK 293 cells and stimulated with LiCl and Wnt-3 conditioned medium. LiCl elicited a 30-fold increase in luciferase expression in cells transfected with TopFlash, while Wnt-3 conditioned medium elicited a smaller, but equally significant expression of luciferase. No response was observed in cells transfected with FopFlash (Figure 4.8).

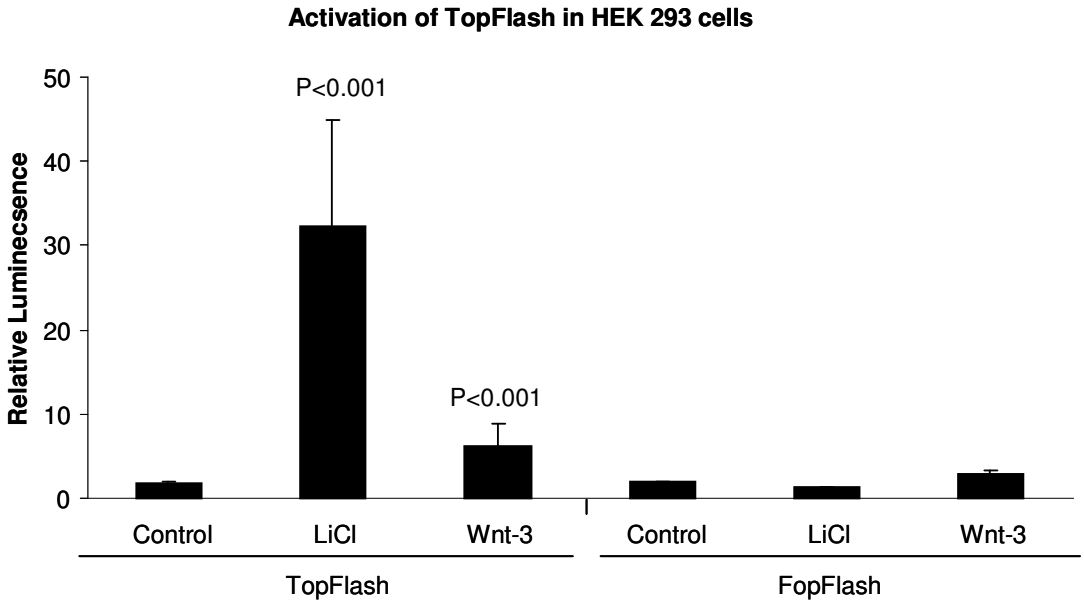


Figure 4.8. Functionality test for Top/FopFlash. HEK 293 cells were transfected with either TopFlash or FopFlash along with Renilla luciferase expression vector. Cells were stimulated for 24h with control (DCT-FBS), 10mM LiCl or Wnt-3 conditioned medium. TCF driven luciferase expression was normalised to Renilla luciferase expression to control for variable transfection efficiency between wells. Data represent means ±SEM. Statistics carried out using the Student t-test (n=10).

4.4.2. Activation of TCF-dependent transcriptional signalling in GH3 cells

GH3 cells were subsequently transfected with TopFlash and stimulated with E2, and Wnt-4 conditioned medium, as well as positive the controls, Wnt-3 conditioned medium and LiCl. Surprisingly, none of the above stimuli were able to induce luciferase expression in GH3 cells, whereas LiCl and Wnt-3 conditioned medium were able to induce luciferase expression in HEK 293 cells in parallel experiments (Figure 4.9).

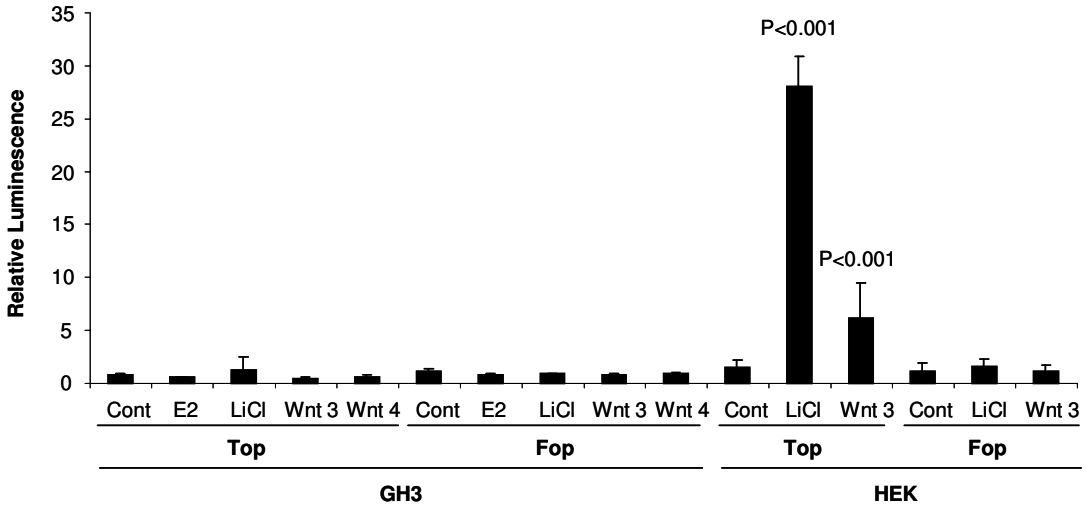


Figure 4.9. The Wnt-canonical pathway is not activated in GH3 cells. GH3 cells were transfected with either TopFlash or FopFlash along with Renilla luciferase expression vector. Cells were stimulated with either control (DCT-FBS), 10nM E2, Wnt-4 conditioned medium, 10mM LiCl or Wnt-3 conditioned medium for 24h, and TCF driven luciferase expression was measured and normalised to Renilla luciferase expression. Data represent means ±SEM. Statistics carried out using the Student t-test (n=10).

The lack of transcriptional output in GH3 cells is not due to unsuccessful transfection of GH3 cells. Cells were co-transfected with a Renilla-luciferase expression plasmid, and higher levels of Renilla luciferase expression were detected in GH3 cells than HEK 293 cells indicating GH3 cells were successfully transfected. Therefore it appears that some aspect of the canonical pathway is inhibited or non-functional in GH3 cells.

4.5. Overexpression of mutant β-Catenin in GH3 cells

To further address this issue, we acquired a constitutively active mutant β-Catenin expression plasmid termed mβ-Cat. This plasmid contains a C^{98}→A missense mutation in the normal mouse β-Catenin sequence which changes Ser^{33}→Tyrosine. This mutation prevents GSK-3β binding to β-Catenin and targeting it for degradation at the proteasome, rendering in constitutively active (Morin et al., 1997).

4.5.1. Validation of mβ-Cat plasmid

Validation of mβ-Cat was carried out using restriction digestion. Sma I was used to make a single cut in the plasmid to check overall plasmid length, while Hind III was used to make multiple cuts to further validate the plasmid. The restriction sites have been shown on the plasmid map in Figure 4.10.

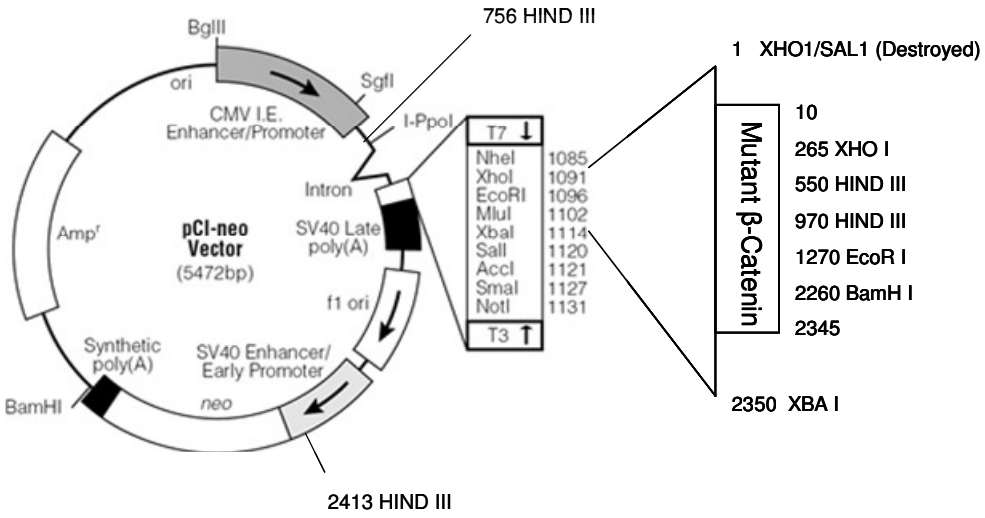


Figure 4.10. Plasmid map of mβ-Cat. mβ-Cat was cloned into a pCI-neo backbone. Map shows a number of restriction sites including all 4 Hind III restriction sites.

Using Sma I, a single band at 8Kb was observed. Using Hind III, 4 bands were observed at 4000bp, 2500bp, 900bp and 400bp. These values correspond closely with the predicted fragment lengths of 3815bp, 2662bp, 879bp and 420bp validating the plasmid. As expected, the undigested plasmid ran faster than the Sma I digested plasmid, giving an inaccurate measure of plasmid length (Figure 4.11).

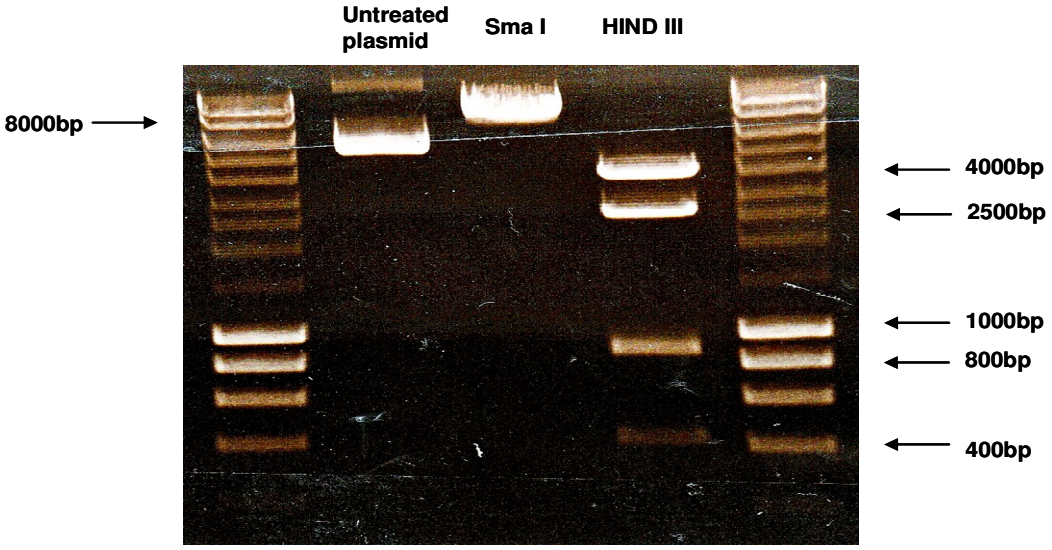


Figure 4.11. Restriction digest of mβ-Cat plasmid. Untreated plasmid, and plasmid digested with either Sma I or Hind III were run on an agarose gel, showing bands at lengths corresponding to predicted lengths.

The mβ-Cat plasmid should harbour a single C^{98}→A point mutation. To identify if this nucleotide was indeed mutated in mβ-Cat, primers were designed to amplify a 621bp length of DNA containing C^{98}. PCR was run on the plasmid, as well as on cDNA generated from GH3 cells, and a band of the correct size was amplified in both cases (Figure 4.12).

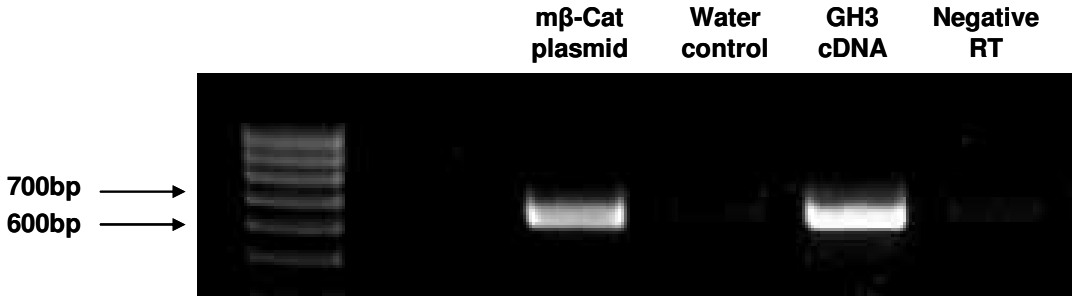
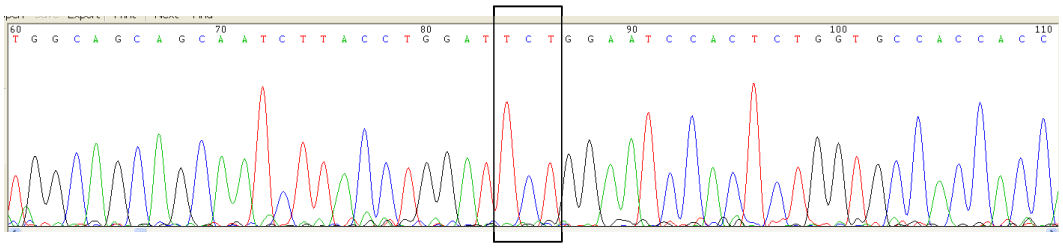


Figure 4.12. PCR amplification of region encoding the C^{98} mutation. PCR was run on mβ-cat plasmid and cDNA obtained from GH3 cells. Negative control for mβ-Cat replaced the plasmid with water, negative control for cDNA was cDNA generated without RT.

The two sequences should be identical apart from the point mutation. PCR products were purified and sequenced using the reverse primer and as expected, the two products showed over 99% homology. After reversing and complementing the sequences obtained, the mutation should be present at bp 85 in the sequence. Figure 4.13 shows that in GH3 cells, bp 85 was a cytosine residue, and in the mβ-Cat sequence it was an adenine residue. This changes the codon translated from TCT (serine) to TAT (tyrosine) as predicted.

GH3 sequence



mβ-Cat sequence

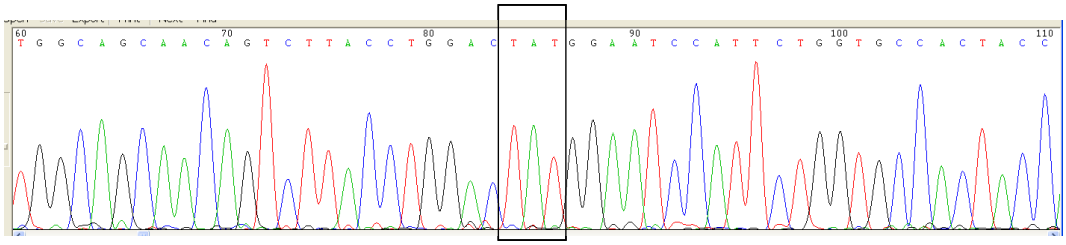


Figure 4.13. C^{98}→A point mutation is present in mβ-Cat. DNA sequencing was carried out on PCR products from Figure 4.12. The sequences were reversed and complemented and a region spanning the mutation site is presented. The C^{98}→A point mutation at bp 85 is shown, and a box is drawn around the altered codon.

The functionality of mβ-Cat was tested by co-transfecting HEK 293 cells with mβ-Cat and TopFlash. As expected, this spontaneously induced a robust luciferase output which was not observed in cells not co-transfected with mβ-Cat. Therefore the mβ-Cat plasmid functions as predicted (Figure 4.14).

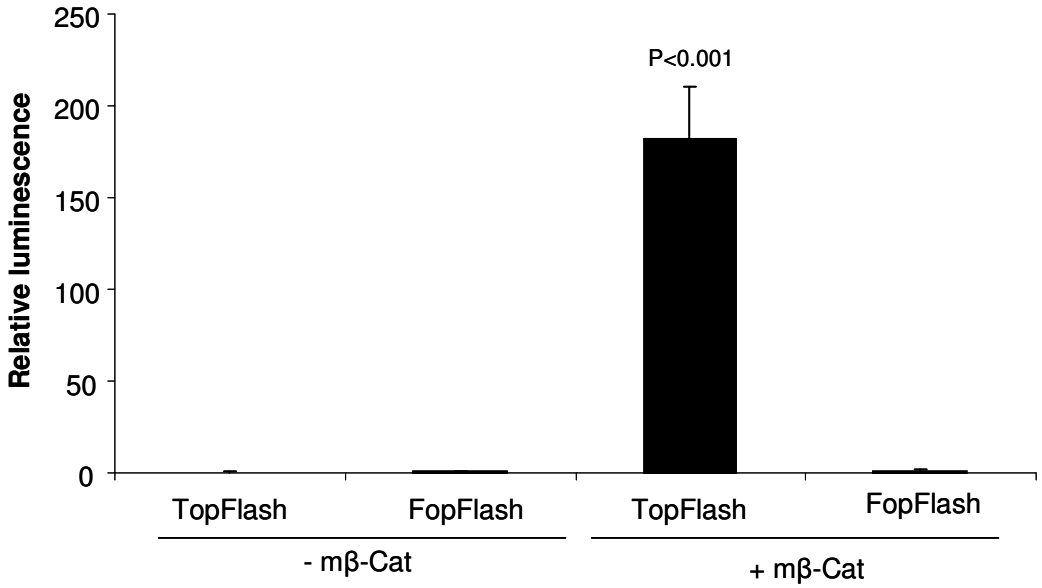


Figure 4.14. mβ-Cat plasmid spontaneously activates TopFlash reporter gene expression. HEK 293 cells were transfected with either TopFlash or FopFlash, with or without mβ-Cat, and luciferase expression was measured after 24h. Data represent means ±SEM. Statistics carried out using the Student t-test (n=10).

4.5.2. Effect of mβ-Cat expression in GH3 cells

The effect of β-Catenin overexpression was studied in GH3 cells by generating GH3 cell lines stably expressing mβ-Cat. Three viable clonal cell lines were generated, termed mβ-Cat 2, 3 and 4. The cell lines were continuously cultured in medium supplemented with G-418 antibiotic, and the continued viability of the cells in this medium ensured the cells maintained the integrated plasmid.

mβ-Cat cell lines were transfected with TopFlash and spontaneous luciferase expression was measured. None of the cell lines exhibited spontaneous activation of TopFlash. Normal GH3 cells were co-transfected with mβ-Cat and TopFlash, and these cells also did not exhibit any activation of TopFlash, while HEK 293 cells co-transfected with mβ-cat and TopFlash did exhibit spontaneous activation of TopFlash (Figure 4.15) These data indicate that constitutively active β-Catenin was unable to activate TCF-dependent transcription in GH3 cells.

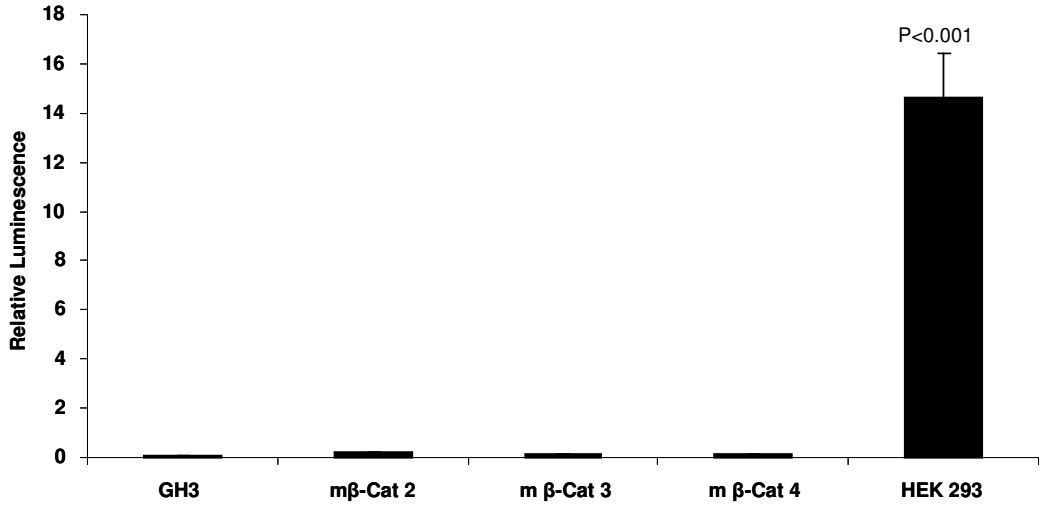


Figure 4.15. Constitutively active β-Catenin does not activate TopFlash in GH3 cells. mβ-Cat cell lines were transfected with TopFlash reporter gene and Renilla expression vector, GH3 and HEK 293 cells were co-transfected with Renilla expression vector, mβ-Cat plasmid and TopFlash. Spontaneous activation of TopFlash was recorded after 24h and normalised to Renilla luciferase expression. Data represent means ±SEM. Statistics carried out using the Student t-test (n=10).

To assess whether linearisation of the mβ-Cat plasmid may effect its ability to activate TopFlash in mβ-Cat cell lines, intact and linearised plasmid were co-transfected with TopFlash into HEK 293 cells, and expression of luciferase was measured. Both forms of the plasmid elicited robust expression of luciferase indicating that the linearised form of the plasmid transfected into mβ-Cat lines functioned normally (Figure 4.16 A). Finally, endogenous TCF levels were measured in GH3 cells and mβ-Cat cell lines to ensure that the negative result observed was not related to absence of TCF. TCF-4 was expressed in all cell lines indicating the machinery required for luciferase expression was present. (Figure 4.16 B). TCF western was carried out by Julia Resch.

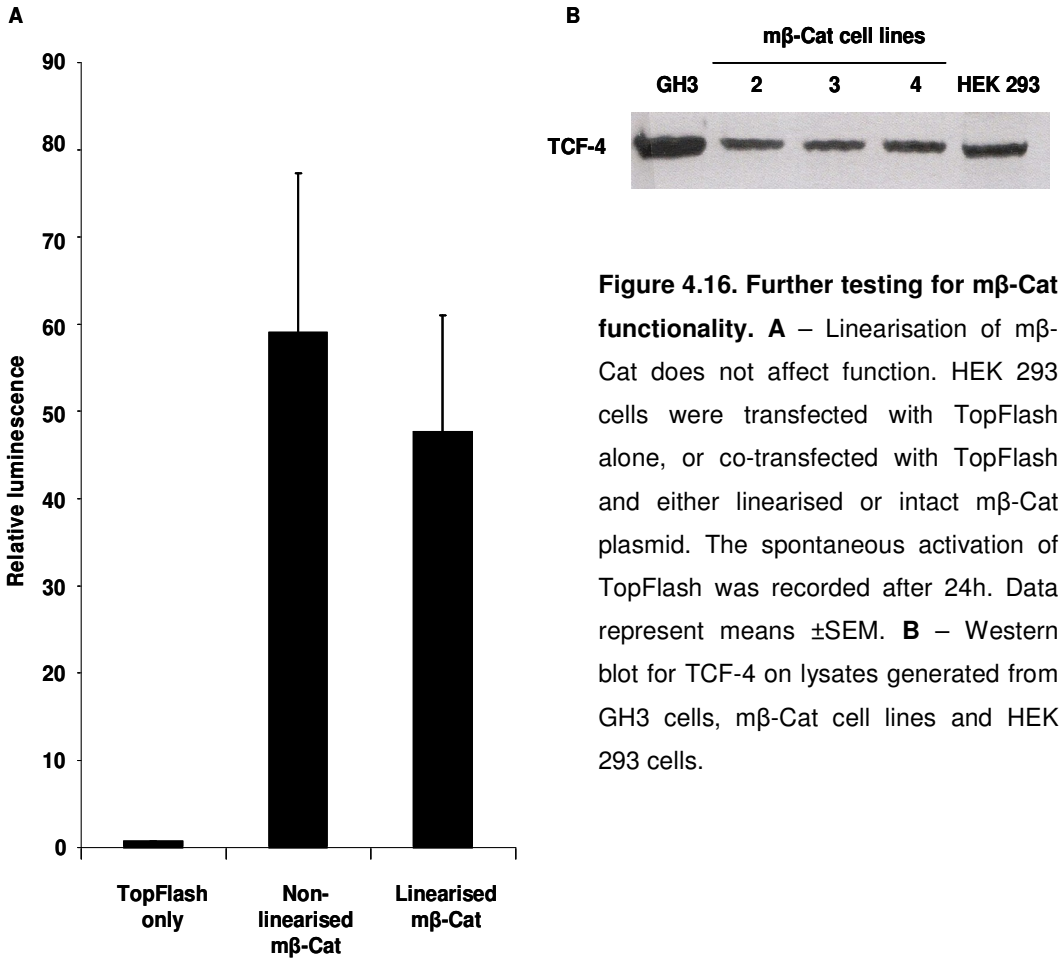


Figure 4.16. Further testing for mβ-Cat functionality. A – Linearisation of mβ-Cat does not affect function. HEK 293 cells were transfected with TopFlash alone, or co-transfected with TopFlash and either linearised or intact mβ-Cat plasmid. The spontaneous activation of TopFlash was recorded after 24h. Data represent means ±SEM. **B** – Western blot for TCF-4 on lysates generated from GH3 cells, mβ-Cat cell lines and HEK 293 cells.

The rate of proliferation of GH3 cells and mβ-Cat cell lines was compared. Cells were treated with either normal FBS or DCT-FBS for 5 days to test their basal rate of proliferation. In both cases, mβ-Cat cell line 4 proliferated at a higher rate than GH3 cells. No difference in proliferation was observed between GH3 cells and mβ-Cat lines 2 and 3 when treated with DCT-FBS, but both mβ-Cat lines 2 and 3 proliferated more slowly than GH3 cells when treated with normal FBS (Figure 4.17).

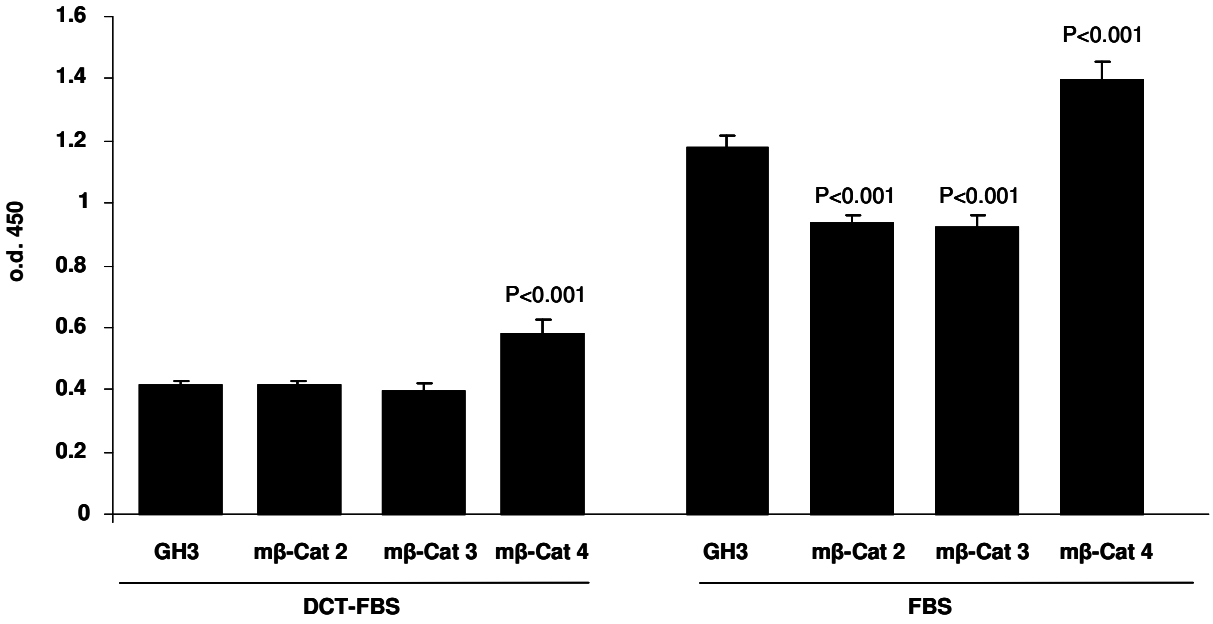


Figure 4.17. mβ-Cat cell lines proliferate at different rates. GH3 cells and mβ-Cat cell lines were seeded at $1x10^4$ cells per well in a 96 well plate and stimulated for 120h with either DCT-FBS or normal FBS. Cell number was quantified using MTS assay. Data represent means ±SEM. Statistics carried out using the Student t-test (n=10).

4.6. Discussion

Canonical activation is generally associated with cellular proliferation which is brought upon by activation of cell cycle drivers such as cyclin D and c-myc, and growth factors like VEGF. Aberrant canonical signalling has been implicated in the development of numerous tumours and as such, it would seem a likely pathway by which Wnt-4 may contribute to prolactinoma development.

Virtually all research on the canonical pathway focuses on the activation and downstream signalling events of β-Catenin. Experimentally, β-Catenin is relatively easy to study. It is easily purified and a number of good antibodies have been designed against it which are now standard within the field. The extensive research into its function has led to the development of a number of validated molecular tools such as reporter genes and loss/gain-of-function plasmids, some of which have been used in this report.

Unlike other Wnt pathways which act on generic intracellular signalling networks, the actions of β-Catenin are fairly well confined to Wnt signalling. Other than translocation to the nucleus in response to Wnt signalling, its only other well documented role is in cell-cell adhesion at the cell membrane. As discussed previously, even this may be part of a Wnt-signalling network by sequestering β-Catenin away from the nucleus. Importantly, its translocation into the nucleus is thought to be specific to Wnt-canonical signalling (Chien et al., 2009), meaning any nuclear expression of β-Catenin, or β-Catenin driven gene expression can be ascribed to activation of the canonical pathway.

β-Catenin was highly expressed in GH3 cells and in other cell types in the pituitary. Global levels of β-Catenin did not increase in response to E2 treatment in the pituitary as judged by western blotting. Activation of the pathway is defined by the translocation of β-Catenin from the cytoplasm to the nucleus, rather than upregulation of protein levels, therefore this is not particularly surprising. Attempts were made to fractionate anterior pituitary tissue into cytoplasmic and nuclear compartments to compare nuclear β-Catenin levels between control and E2 treated rats, though attempts were unsuccessful. However, as only small amounts of β-Catenin may be required to translocate to the nucleus to elicit canonical gene transcription, this method may still not have been sensitive enough to identify activation of the pathway.

Immunofluorescence techniques are a more accurate measure of nuclear localisation of β-Catenin, and they are widely used in the literature to study canonical activation. In all models studied, β-Catenin was only ever observed at the cell membrane. Immunocytochemistry on GH3 cells and dispersed cultures of primary pituitary cells showed membrane localised β-Catenin within the lactotroph population as well as other uncharacterised pituitary cells. Immunohistochemical analysis on histological pituitary sections showed that this distribution was the same in all cell types in the anterior pituitary. Not a single example of nuclear localised β-Catenin was observed in any model in this project, suggesting the canonical pathway was not activated in response to E2 in the pituitary. This directly contradicts findings by Semba et al (2001) who did find nuclear β-Catenin in human pituitary tumours (Semba et al., 2001). However, our work is in line with more recent findings that suggest that β-Catenin does not localise to the nucleus in the pituitary (Miyakoshi et al., 2008b).

Once again, the levels of β-Catenin translocation required to activate gene transcription may be extremely low, and the negative result observed could be related to technical limitations in detection. However, the antibody used in this project is the most commonly used and validated antibody in literature, while the microscope setup used was extremely sensitive. Therefore, the systems employed in this study are likely to have detected nuclear β-Catenin if it had been present.

A still more accurate measure of canonical activation is using the TCF-dependent reporter gene TopFlash. This has the benefit over previous methods that we are not measuring the absolute level of β-Catenin in the nucleus, but the functional transcriptional output initiated by β-Catenin translocation. Interestingly, no luciferase expression was detected in GH3 cells in any condition. Wnt-3A and LiCl are standard positive controls in the literature, and efficiently activated canonical signalling in HEK 293 cells in this study. The reporter gene was validated both structurally by restriction enzyme digest, and functionally in HEK 293 cells. One possible issue could be transfection of GH3 cells, though this is unlikely. Cells were co-transfected with Renilla luciferase expression plasmid to normalise transfection efficiencies between wells. In all cases Renilla luciferase expression was comparable between GH3 cells and HEK 293 cells indicating successful transfection. This suggests that some aspect of canonical signalling is inhibited in GH3 cells.

This concept is strengthened by data using the constitutively active mβ-Cat plasmid. The plasmid was validated structurally by restriction digest, the mutated nucleotide was identified in the correct location, and the plasmid spontaneously induced expression of luciferase in HEK 293 cells when co-transfected with TopFlash. However, no luciferase induction could be detected when TopFlash and mβ-Cat were co-transfected into GH3 cells, nor could luciferase expression be detected in GH3 cells stably transfected with mβ-Cat. The stable cell lines were continuously cultured in medium containing G-418 antibiotic. Each new bottle of medium used was tested for its ability to kill normal GH3 cells, and the retained ability for transfected cell lines to survive in G-418 medium indicates the plasmid remained integrated into the host DNA. Furthermore, the linearised version of the plasmid used to generate the stable cell lines was just as effective at activating TopFlash in HEK 293 cells as the non-linearised plasmid. Finally, GH3 cells and mβ-Cat cell lines all expressed TCF-4 demonstrating the necessary signalling machinery was present to activate TopFlash.

The proliferation assays provide some interesting results, if only to highlight the necessity for repeating experiments. One of the mβ-Cat cell lines consistently proliferated at a greater rate than GH3 cells, while the other two proliferated slower than GH3 cells. Had only one cell line been used for this study, conclusions would have been drawn about the effect of mutant β-Catenin overexpression on GH3 cell proliferation, but as such, this data is inconclusive. The reason for this discrepancy cannot be explained, but is likely due to differences in integration site of the plasmid into the host DNA rather than from any direct action of the plasmid which we have shown does not function in our cell line.

Taken together, our data strongly suggest that canonical signalling is not activated in the pituitary in response to E2, and does not function in GH3 cells. This data is in line with other work that had been published since the start of the project from other groups suggesting canonical pathway activation was not involved in the pituitary (Elston et al., 2008; Miyakoshi et al., 2008b). Therefore, although it would have been interesting to find out why canonical signalling is non-functional in GH3 cells, it was decided not to investigate this pathway further, but instead to focus on other potential Wnt signalling pathways in the pituitary.

5.0. Results 3 - Wnt-4 signalling via non-canonical pathways in the pituitary

5.1. Introduction

Wnt-4 is widely expressed in the pituitary. It is expressed in all the secretory cell types in the anterior pituitary, though the proportions of each secretory cell type expressing Wnt-4 vary. Wnt-4 is expressed in primary lactotroph cells, and is upregulated by E2 in GH3 cells. Wnt-4 is also highly expressed in the marginal zone (MZ) a region of the pituitary suspected to harbour progenitor stem cells which may contribute to cell renewal and restructuring of the pituitary during pituitary plasticity. Data from Results Section 4.0 strongly suggest that the canonical pathway is not activated in the pituitary and that it is non-functional in the GH3 cell line. Wnt molecules signal via two other signalling pathways, collectively termed the non-canonical pathways: the Wnt-calcium pathway, and the Wnt-planar cell polarity (PCP) pathway. Currently there are no data in the literature regarding the activation of either pathway in the pituitary.

5.1.1. The Wnt-calcium pathway

Calcium is a vital regulator of intracellular signalling in virtually all cells in multicellular organisms. Wnt binding to Fz increases intracellular levels of calcium through activation of IP3 channels on the endoplasmic reticulum. Newly released calcium is quickly absorbed either by calcium buffers, or calcium dependent enzymes such as PKC or calmodulin (CaM). These in turn activate secondary messengers such as calmodulin dependent kinases (CaMK I-IV) and calcineurin which regulate downstream calcium signalling events. Calcineurin has subsequently been shown to activate the transcription factor NFAT which may mediate some Wnt-calcium pathway actions.

The mechanism of rapid calcium release and protein binding results in oscillating levels of intracellular calcium, and alterations in the amplitude and frequency of these transients can determine downstream cellular responses. GH3 cells have been shown to exhibit spontaneous calcium oscillations which may regulate PRL release (Wozniak et al., 2005) indicating that calcium signalling operates in GH3 cells. The eventual effects of the Wnt-calcium pathway are thought to include altered cytoskeletal remodeling and cell motility, differentiation, apoptosis and morphogenesis. The Wnt-calcium pathway has been implicated in tumour progression in prostate cancer, breast cancer and human cutaneous melanoma cells.

5.1.2. The Wnt-planar cell polarity pathway

Planar Cell Polarity (PCP) is the generation of a uniform orientation of a population of cells along a single epithelial plane and is found throughout the animal kingdom. The most studied example of PCP is the arrangement of bristles on the wing of Drosophila which is controlled by the polar distribution of Fz and a number of other cytoplasmic proteins along the proximal-distal axis.

This polar organisation is also evident during convergent extension during gastrulation in Xenopus, where polarised mesenchymal cells derived from the mesoderm interact to lengthen the embryo along the anterior-posterior axis. In this circumstance, activation of the PCP pathway induces transition of epithelial cells into mesanchymal cells. Epithelial cells are held in place by adherens junctions which are composed of membrane bound E-Cadherin and β-Catenin. Induction of PCP signalling deconstructs these adherens junctions, transforming them into mesenchymal cells and allowing them to migrate past each other. This transition is highlighted by the loss of E-Cadherin at the cell membrane and increased N-Cadherin expression in the cytoplasm.

Epithelial to mesenchymal transition is a vital step in the progression of a number of malignant tumours. The loss of cell to cell contact allows tumour cells to migrate away from their original location to proliferate and invade surrounding tissues. The mechanism by which PCP signalling induces this in mammals is poorly understood. Binding of Wnt to Fz activates small GTPases such as Rho A and Rac which then activate downstream kinases such as JNK and ROCK. Alterations in gene transcription can be induced by the activation of transcription factors like Jun, or kinases can act directly on the actin cytoskeleton. Both pathways are thought to alter cytoskeletal organisation which can lead to modulation of adherens junctions. Clearly defined instances of PCP signalling in mammals and detailed analysis of the signalling molecules involved in the pathway have been poorly documented to date.

The pituitary displays a region which suggests the participation of PCP signalling. The marginal zone (MZ) is a single cell planar line of cells which acts as a border between the anterior lobe (AL) and the intermediate lobe (IL) of the pituitary. The function of MZ cells is currently unknown, though they have been shown to express stem cell markers such as Sox 2, Sox 9 and nestin which may play a role in populating the anterior

pituitary during tissue plasticity. Wnt molecules have been demonstrated to maintain stem cell niches in the body. It is possible that the planar organization of the MZ is a result of Wnt-PCP signalling, and that Wnt-4 may be acting via this pathway to maintain the cells in this region of the gland.

5.2. Aims

5.2.1. The Wnt-calcium pathway

The effects of Wnt-4 on the Wnt-calcium pathway in lactotrophs will be studied as follows:

- To assess whether spontaneous oscillations in intracellular calcium can be observed in GH3 cells
- To assess whether Wnt-4 has any effect on GH3 cell calcium oscillations
- To determine which, if any, NFAT isoforms are expressed in GH3 cells
- To assess whether NFAT signalling is activated in GH3 cells by Wnt-4

5.2.2. The Wnt-planar cell polarity pathway

The effects of Wnt-4 on the Wnt-planar cell polarity pathway in lactotrophs will be studied as follows:

- To assess the expression pattern of key PCP signalling proteins in control pituitaries and pituitaries undergoing E2-induced lactotroph hyperplasia
    - Focus will be placed on cells in and around the MZ, as well as in the AL
- Proteins to be studied include:
    - Developmental proteins and potential stem cell markers: Wnt-4, Sox 9
    - Proteins whose expression pattern may be altered in PCP signalling: E-Cadherin, N-Cadherin, β-Catenin

5.3. The Wnt-calcium pathway

5.3.1. Wnt-4 inhibits calcium oscillations in GH3 cells

GH3 cells are reported to undergo spontaneous oscillations of intracellular calcium. Live cell calcium imaging was performed on GH3 cells to see if Wnt-4 affects these oscillations. Calcium oscillations are extremely sensitive and can be affected by changes in temperature or movement. The effect of adding new medium onto cells may be enough to affect oscillations. In order to ensure that our protocol did no affect calcium oscillations, sequential control medium to control medium transitions were carried out. GH3 cells did spontaneously oscillate in culture, and oscillations were unaffected by this transition demonstrating a protocol where any effect on oscillations can be deemed specific to addition of stimulus. The trace also demonstrates oscillations were maintained for the duration of the experiment. This experiment lasted 20 minutes and generally experimental duration did not exceed this time (Figure 5.1 A)

As a positive control for calcium signalling, cells were treated with Bay-K-8644 (Bay K), an L-type calcium channel agonist, which causes calcium influx into cells through activated voltage-dependent calcium channels. Addition of Bay K brought about a classical increase in baseline of intracellular calcium indicating cells were viable (Figure 5.1 B).

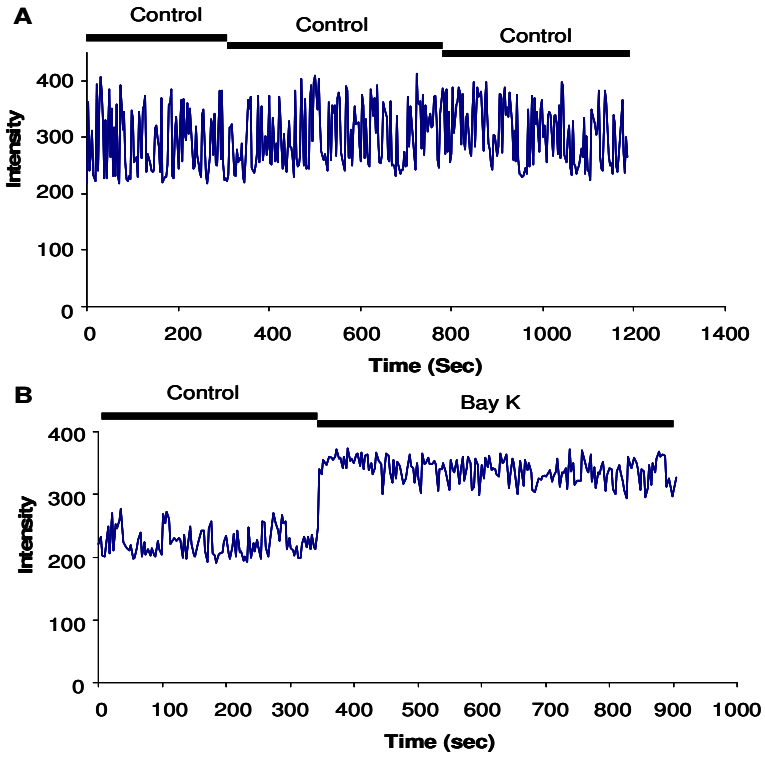


Figure 5.1. GH3 cell calcium oscillation control experiments. GH3 cells were loaded with Fluo-4 and images were taken every 3 seconds. Areas of interest were drawn around cells and fluorescence for each cell was quantified over the course of the experiment. Cells were stimulated at time points indicated by bars with either control (A – DCT-FBS, no effect seen in 35/35 cells) or Bay K (B – 0.5µM, effect observed in 16 out of 16 cells).

Wnt-4 produced a variable effect on GH3 cell calcium transients (Figure 5.2). In some cells, Wnt-4 induced total inhibition of calcium transients for long periods (Figure 5.2 A – 37% of cells studied) while partial inhibition of transients was observed in other cells (Figure 5.2 B/C – 26% of cells studied). Partial inhibition of transients could be further subcategorised into 2 types, where Wnt-4 induced a reduction in frequency and amplitude of oscillations for the duration of the experiment (Figure 5.2 B), or gave a temporary inhibition of oscillations (Figure 5.2 C). In some cells, addition of Wnt-4 had no effect on calcium transients (Figure 5.2 D – 37% of cells studied). Overall, Wnt-4 inhibited calcium oscillations in 63% of cells. To test the specificity of our protein, Wnt-4 recombinant protein was immunoneutralised by prior incubation with Wnt-4 antibody before addition to cells. This abrogated the inhibitory effect of Wnt-4 in 19 out of 19 cells studied over 2 separate experiments, indicating the inhibitory effect observed was specific to Wnt-4 (Figure 5.2 E).

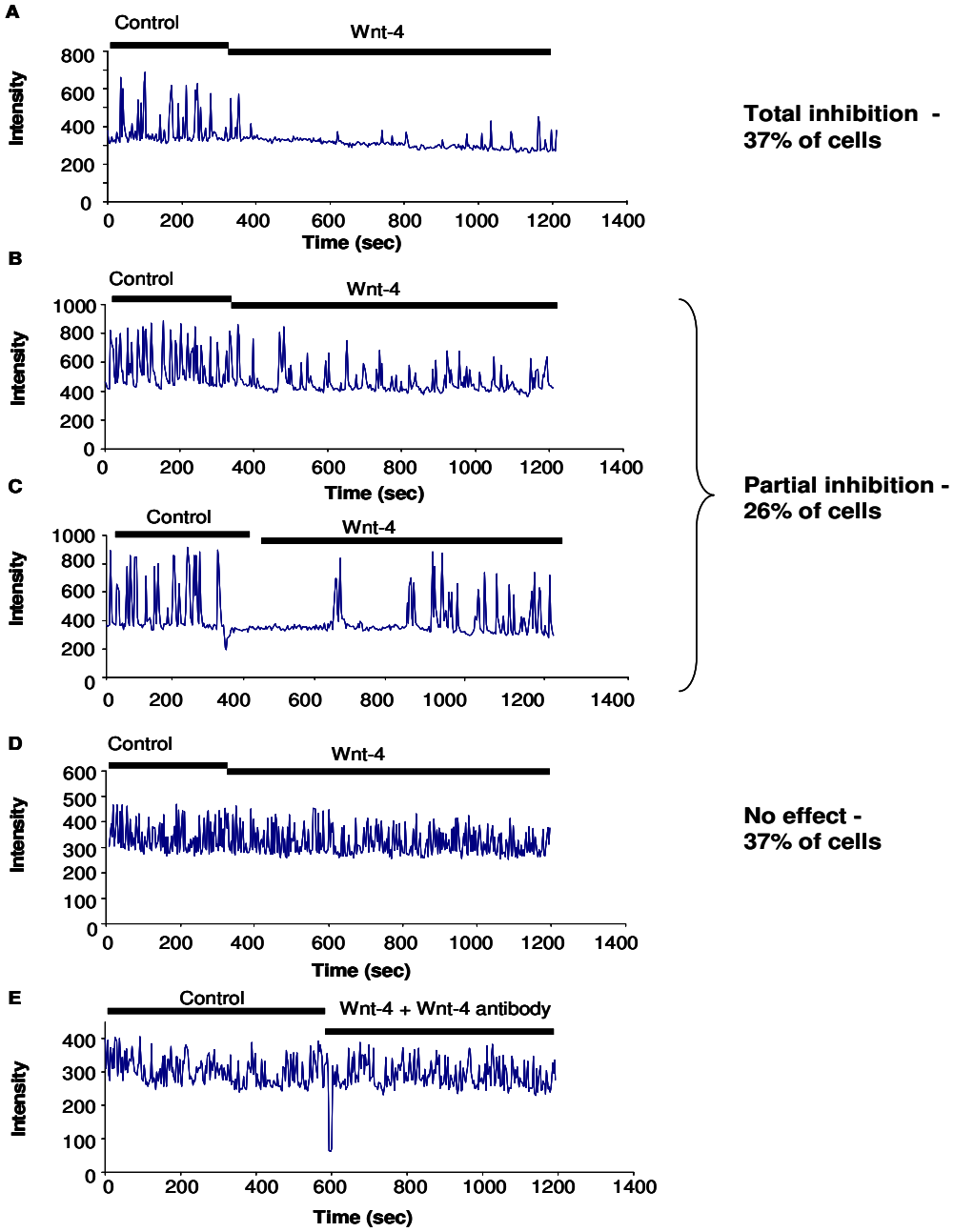


Figure 5.2. Wnt-4 inhibits calcium oscillations in GH3 cells. GH3 cells were loaded with Fluo-4 and images were taken every 3 seconds. Cells were treated with either control (DCT-FBS) (D), recombinant Wnt-4 protein (200ng/ml, examples A-C) or Wnt-4 protein which had been immunoneutralised by prior incubation with Wnt-4 antibody (E, no effect observed in 19/19 cells). Total cell number studied for Wnt-4 only effect = 209 from 12 separate experiments, and the percentage of cells in each subtype of Wnt-4 effect is indicated.

Attempts were made to rescue calcium transients after Wnt-4 inhibition. Replacing Wnt-4 medium with control medium was unable to rescue calcium transients (Figure 5.3 A), but addition of Bay K did bring about an increase in intracellular calcium and induced calcium transients (Figure 5.3 B) indicating viability of GH3 cells after Wnt-4 treatment.

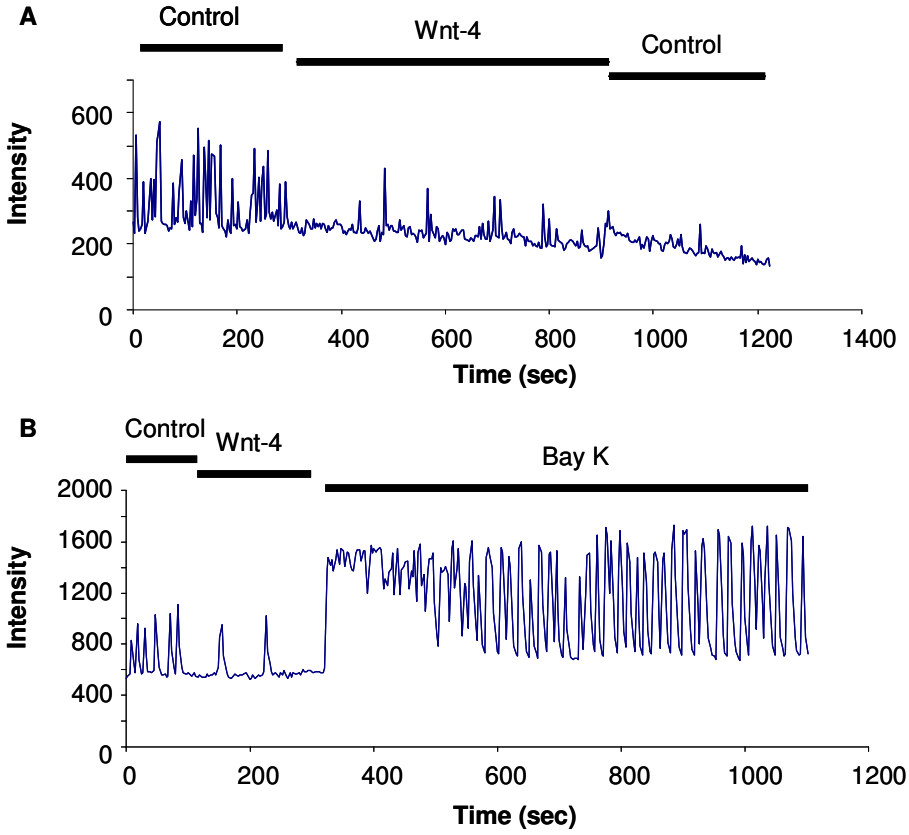


Figure 5.3. Calcium transient rescue experiments. GH3 cells were loaded with Fluo-4 and images were taken every 3 seconds. Cells were treated with Wnt-4 recombinant protein (200ng/ml), before medium was removed and replaced with either control medium (DCT-FBS) or Bay K (0.5µM).

5.3.2. Cell cycle analysis

Flow cytometry was carried out on GH3 cells to assess whether the variable response of Wnt-4 on calcium oscillations was related to the cell cycle. Analysis indicated that 63% of GH3 cells were in G1 phase of the cell cycle, 36% were in S phase and 1% were in G2/M phase, suggesting a potential link between calcium response to Wnt-4 and the cell cycle (Figure 5.4).

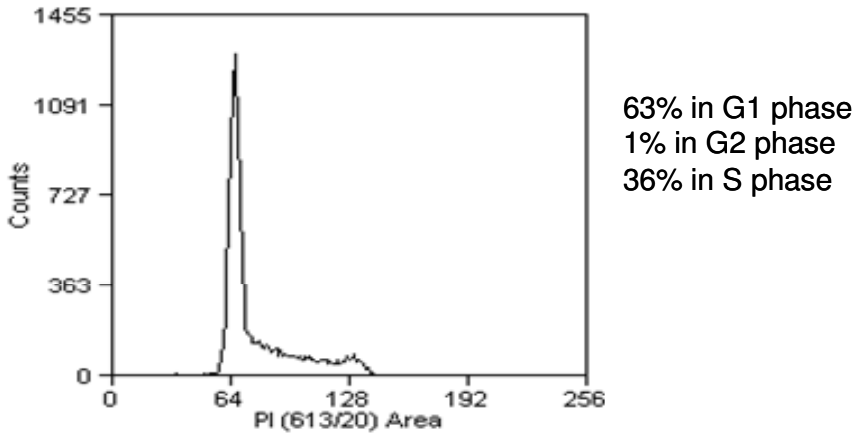


Figure 5.4. Cell cycle analysis of GH3 cells. GH3 cells were loaded with propidium iodide and analysed by flow cytometry at a wavelength of 488nm. The proportion of cells in each phase of the cell cycle is indicated. Data represent means between 2 repeated experiments

5.3.3. NFAT signalling in lactotroph cells

The Wnt-calcium pathway is proposed to signal through nuclear factor of activated T-cells (NFAT). There are 4 isoforms of NFAT, termed NFAT1-4, which are phosphorylated by calcineurin upon calcium influx into the cell. Phosphorylation of NFAT results in its translocation into the nucleus where it functions as a transcription factor to elicit downstream effects of calcium. The expression of NFAT isoforms and calcineurin was assessed in GH3 cells and FACS sorted lactotrophs (FACS sorting was carried out by Frederic Madec). Figure 5.5 shows that all 5 genes were expressed in both cell populations.

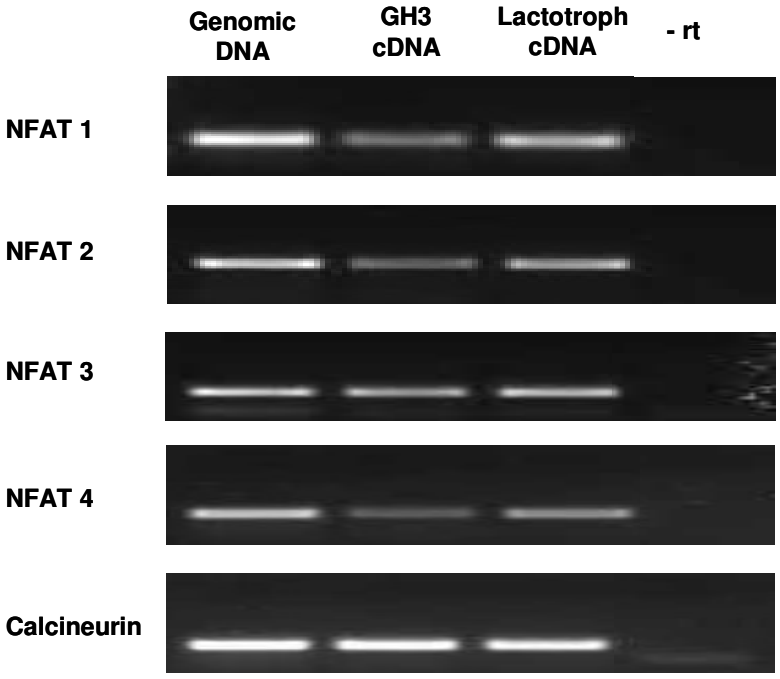


Figure 5.5. Expression of NFAT isoforms and calcineurin in GH3 cells. PCR was run for NFAT 1-4 and calcineurin on rat genomic DNA (positive control) and cDNA generated from GH3 cells and FACS sorted lactotrophs. Negative control was run on sample generated without reverse transcriptase.

An NFAT reporter gene (pNFAT) was used to assess the effect of Wnt-4 calcium signalling in GH3 cells. Its negative control, pTA, contains the minimal TATA box promoter linked luciferase. pNFAT has the same TATA box promoter linked to luciferase, but with 3 additional NFAT binding sites. This region is conserved across all NFAT isoforms, so activation of any isoform should elicit luciferase transcription. Functionality of the plasmid was tested in HEK 293 cells which were stimulated with the calcium ionophore ionomycin, and the PKC activator 12-O-tetradecanoylphorbol-13-acetate (PMA), which together induced a dose dependent expression of luciferase (Figure 5.6). Luciferase output for pTA was significantly lower in both cases, though there was some luciferase output with this plasmid.

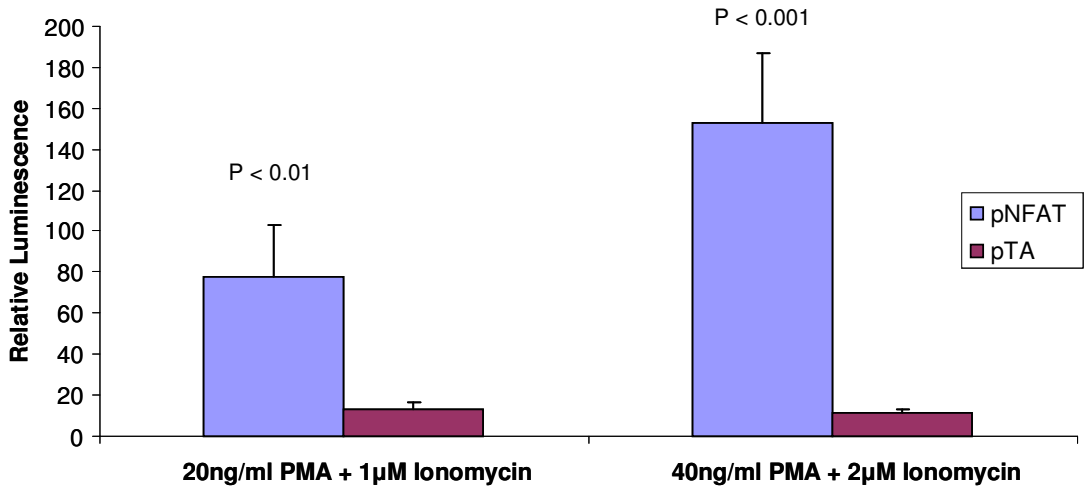


Figure 5.6. Functional validation of pNFAT and pTA. HEK 293 cells were transiently transfected with Renilla luciferase and either pNFAT or pTA, and stimulated for 24h with either 20ng/ml PMA and 1µM ionomycin, or 40ng/ml PMA and 2µM ionomycin, and luciferase output was quantified relative to Renilla luciferase expression. Data represent means ±SEM. Statistics carried out using Student T-Test (n=5).

Wnt-4 effects on NFAT transcriptional output were assessed in GH3 cells and HEK 293 cells. No luciferase expression was induced in GH3 cells with either Wnt-4 or positive controls. In HEK 293 cells, Wnt-4 had no effect on luciferase expression, though high levels of luciferase expression were observed after stimulation with PMA and ionomycin. In all conditions, cells transfected with pTA negative control elicited a degree of spontaneous luciferase expression, though this is much smaller than the large signal evoked upon stimulation of HEK 293 cells transfected with pNFAT with PMA and ionomycin (Figure 5.7).

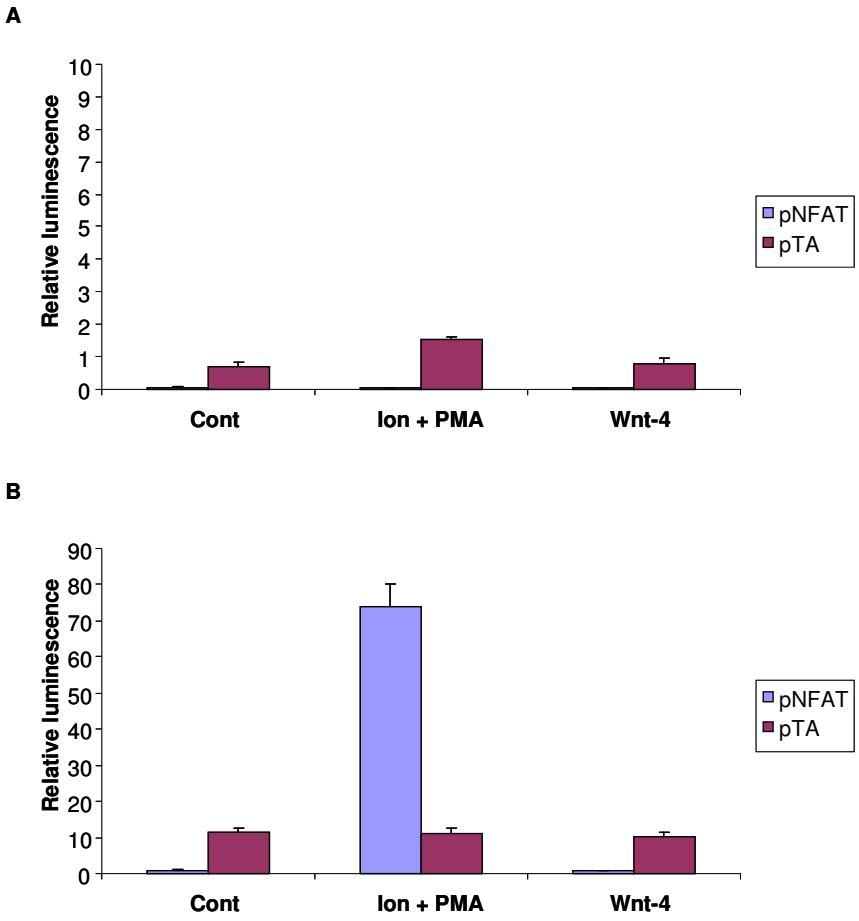


Figure 5.7. Wnt-4 does not activate NFAT in GH3 or HEK 293 cells. GH3 (**A**) cells and HEK 293 cells (**B**) were transfected with either pNFAT or pTA along with Renilla luciferase expression vector. Cells were stimulated for 24h with either control (DCT-FBS), 20ng/ml PMA and 1µM ionomycin, or Wnt-4 recombinant protein (200ng/ml) and luciferase expression was quantified relative to Renilla luciferase expression. Data represent means ±SEM.

This is troubling as the calcium-NFAT pathways are common to many cell types, and the stimuli administered induce large influxes of calcium which would be expected to induce an NFAT response in most cell types. As with the TopFlash reporter gene studies, the lack of functional output is not likely due to an inability to transfect GH3 cells. The spontaneous expression of Renilla and pTA demonstrate that cells were transfected and that luciferase expression could be detected at levels comparable to HEK 293 cells. Furthermore, the huge transcriptional output observed from pNFAT in HEK 293 cells demonstrates the functionality of the plasmid and would suggest that

only a small amount of plasmid transfected into cells would be able to elicit a detectable luciferase output in GH3 cells. Lack of signalling is therefore more likely related to the absence of a critical signalling component within the pathway, or inhibition from cross talk with other signalling pathways, however the reasons remain unknown.

To test this further, GH3 cells were transfected with increasing amounts of pNFAT plasmid. No matter how much pNFAT was transfected into cells, the luciferase output was still negligible, while transfection efficiency as judged by Renilla luciferase expression was maintained. Conversely, low levels of pNFAT were transfected into HEK 293 cells and elicited a 35-fold increase in luciferase output when stimulated with PMA and ionomycin (Figure 5.8).

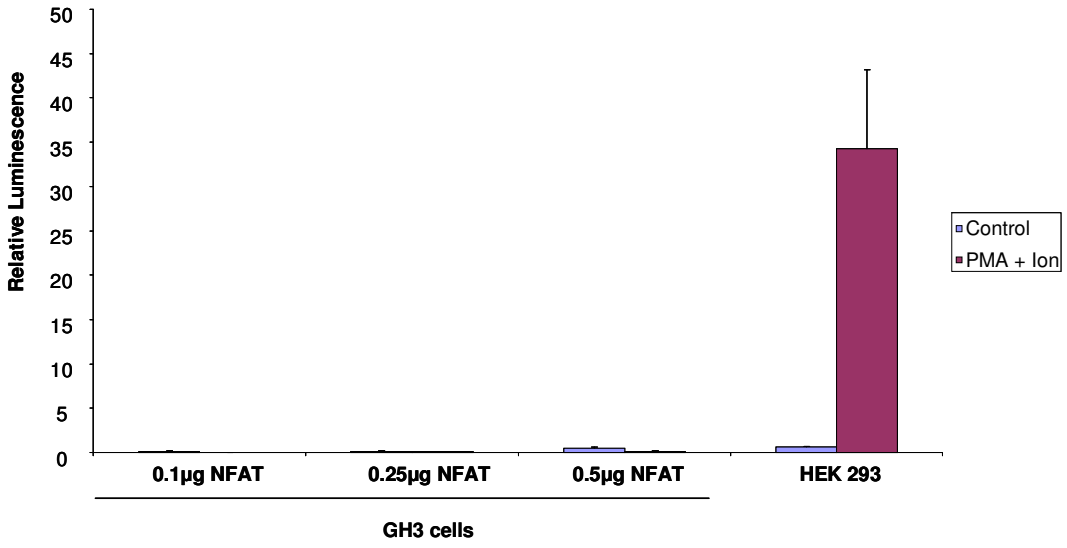


Figure 5.8. pNFAT is not activated in GH3 cells. GH3 and HEK 293 cells were transfected with pNFAT and Renilla expression plasmid. The amount of pNFAT transfected into GH3 cells was varied and is indicated. HEK 293 cells were transfected with 0.05µg pNFAT. Cells were stimulated for 24h with either control (DCT-FBS) or 20ng/ml PMA and 1µM ionomycin and luciferase expression was quantified. Data represent means ±SEM.

5.3.3.1. Activation of NFκB signalling pathways in the pituitary

It is slightly concerning that no transcriptional output was detected in GH3 cells using two separate reporter genes (TopFlash and pNFAT). Both these plasmids function as expected in HEK 293 cells, and Renilla expression indicated successful transfection protocol during co-transfection studies. However, using well established positive controls, no expression of luciferase was detected in any circumstance in GH3 cells.

A poster at the ENDO 2010 conference last year reported a novel Wnt signalling pathway where Wnt-10A was shown to activate NFkB in osteoblast proliferation (Moedder et al, poster communication ENDO Conference, 2010). Our group has experience working with NFκB, and we have an NFκB reporter gene which has been used successfully in GH3 cells (Adamson et al., 2008).

GH3 cells were co-transfected with an NFκB reporter construct (NFκB-Luc) and Renilla luciferase expression vector, and stimulated with increasing amounts of recombinant Wnt-4 protein and positive control tumour necrosis factor α (TNFα). Wnt-4 did not elicit transcription of luciferase at any concentration, however a robust response was observed when cells were treated with TNFα indicating luciferase transcriptional output could be induced and detected in GH3 cells (Figure 5.9).

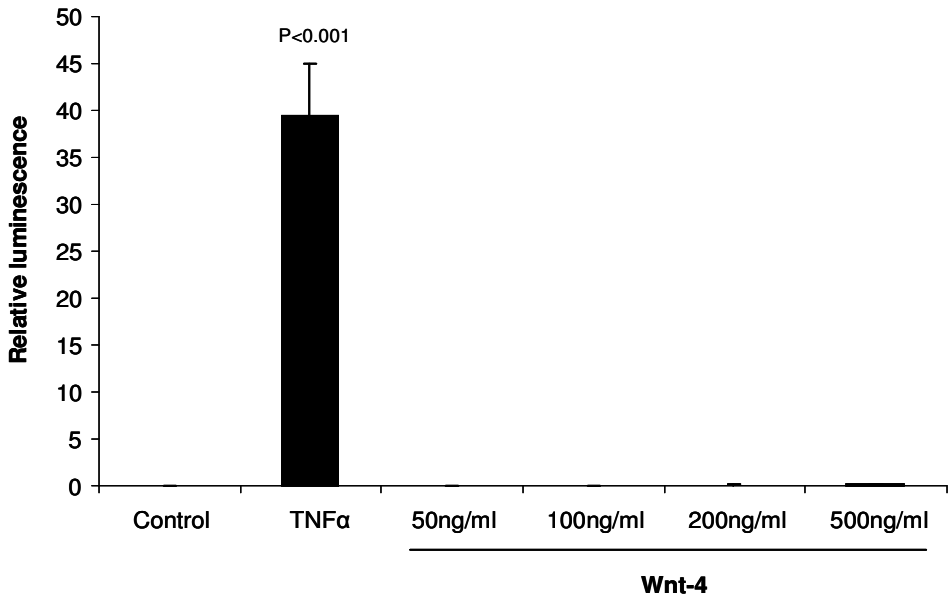


Figure 5.9. Wnt-4 does not activate NFκB signalling in GH3 cells. GH3 cells were co-transfected with NFκB-Luc reporter construct and Renilla luciferase expression vector and stimulated for 24h with control (DCT-FBS), 10ng/ml TNFα, or Wnt-4 recombinant protein at specified concentrations. NFκB driven luciferase expression was measured and normalised to Renilla luciferase expression. Data represent means ±SEM. Statistics carried out using the Student t-test (n=10).

5.4. The Wnt-planar cell polarity pathway

In this section, the expression of proteins which may be involved in PCP-induced lactotroph hyperplasia were studied. Proteins of interest included Wnt-4, cell-cell adhesion proteins (E-Cadherin, N-Cadherin, β-Catenin) and the transcription factor Sox 9.

Focus was initially placed on cells in and around the MZ. Progenitor stem cells have been proposed to exist in the MZ which may proliferate to increase the population of endocrine cells in response to external stimuli. Attention then shifted to the AL to assess whether oestrogen induced changes in cell-cell adhesion throughout the pituitary during E2-induced lactotroph hyperplasia.

5.4.1. Protein expression in the marginal zone and surrounding pituitary regions

5.4.1.1. Wnt-4 expression around the marginal zone

The effect of E2 treatment on Wnt-4 expression in the MZ was assessed. In both conditions, Wnt-4 was expressed at higher levels in the MZ than surrounding regions, however, no difference in Wnt-4 staining intensity could be detected between control and oestrogen treated animals (Figure 5.10).

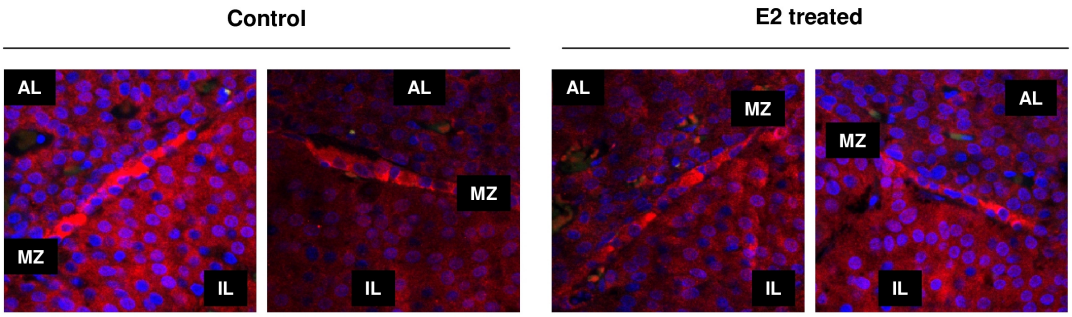


Figure 5.10. Wnt-4 expression in the marginal zone is unaltered by E2 treatment. Pituitary sections from control and E2 treated rats (n=3 for each condition) containing the MZ were stained for Wnt-4 (red) and DAPI (blue). Regions of the pituitary are indicated; AL – Anterior lobe, IL – Intermediate lobe, MZ – Marginal zone. Images were taken along the length of the MZ and images displayed are considered representative of the experiment.

5.4.1.2. Sox 9 and E-Cadherin expression around the marginal zone

Cells in the marginal zone are reported to express stem cell markers including Sox 9. We assessed the expression of Sox 9 across the marginal zone to see if E2 treatment resulted in Sox 9 upregulation. To easily identify MZ cells, sections were co-stained for E-Cadherin which we have previously shown is expressed specifically in cells along the MZ (Figure 3.6). E-Cadherin was specifically expressed in all MZ cells, was weakly expressed in a small number of cells in the AL and was not expressed in the IL. Sox 9 was expressed in a proportion of cells along the MZ, and a small number of cells in the AL in keeping with previously published material (Fauquier et al., 2008). No change in E-Cadherin expression pattern was found between control and E2 treated rats (Figure 5.11 A). The percentage of cells in the MZ expressing Sox 9 was quantified. Roughly 50% of cells in the MZ express Sox 9, and this was unaltered by treatment with E2 (Figure 5.11 B).

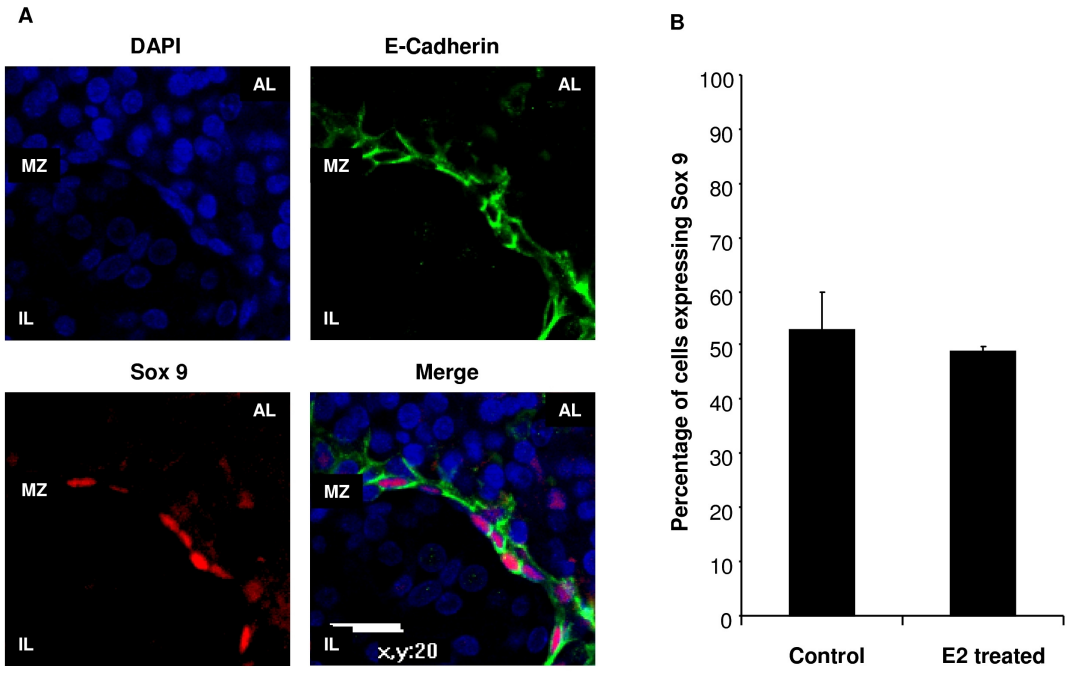


Figure 5.11. Sox 9 and E-Cadherin expression in the marginal zone. A – IHC staining of the MZ and adjacent pituitary regions for DAPI (blue), E-Cadherin (green), and Sox 9 (red) showing individual staining and merged image. Image was taken from a control rat pituitary, but is considered representative of both control and E2 treated rats (n=5 from each condition). MZ – Marginal zone, IL – Intermediate lobe, AL – Anterior lobe. White bar represents 20µm. **B** – Percentage of cells in the marginal zone expressing Sox 9 in control and E2 treated rats (n=5). Data represent means ±SEM.

5.4.1.3. N-Cadherin expression around the marginal zone

During epithelial to mesenchymal transition, cells are able to move across each other due to loss of E-Cadherin at the membrane. This can be visualised by decreased E-Cadherin expression at the cell membrane which is often accompanied by increased expression of N-Cadherin. If Wnt-4 is acting via the PCP pathway to induce lactotroph hyperplasia, the expression of N-Cadherin may be increased around the MZ. N-Cadherin was strongly expressed within the IL and along cells of the marginal zone in both control and E2 treated rats. The MZ was not highlighted by co-staining with E-Cadherin in this experiment, though the boundary between the IL and MZ could be seen from differences in nuclei distribution as previously described. In the images

presented below, the difference in cellular distribution between the AL and IL is not as clear in E2 treated rats as in control animals. However, when viewed under the microscope from a wider field of view, this difference was still clear. No change in N-Cadherin expression was detected between control and E2 treated rats (Figure 5.12). N-Cadherin staining was carried out by Frederic Madec.

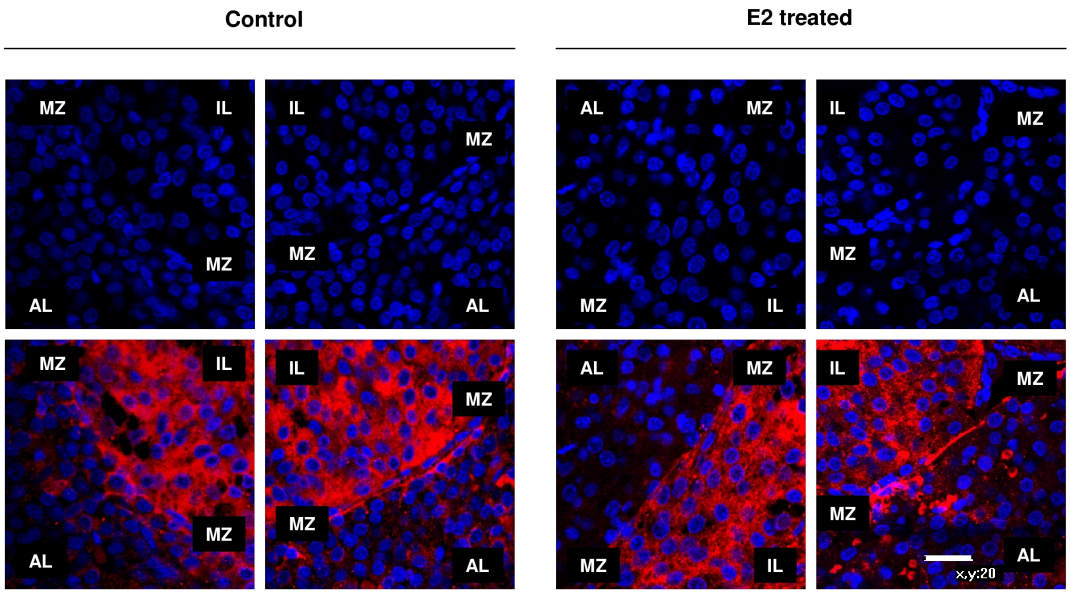


Figure 5.12. N-Cadherin expression in the marginal zone. IHC staining of the MZ and adjacent pituitary regions in control and E2 treated rats showing DAPI (blue) and N-Cadherin (red). Top row shows DAPI staining alone, bottom row shows DAPI and N-Cadherin merged images. MZ – Marginal zone, IL – Intermediate lobe, AL – Anterior lobe. White bar represents 20µm. Images in each column were taken from different animals and are considered representative of the experiment.

5.4.1.4. β-Catenin expression around the marginal zone

β-Catenin plays an intrinsic role at the cell membrane as a functional component of adherens junctions. Its expression was assessed in the MZ and anterior, posterior and intermediate lobes in control and E2 treated rats. Membrane bound β-Catenin was strongly expressed in the AL (as previously shown in Results chapter 4.0), the IL and the PL. Expression was consistently stronger in the IL than the AL or PL, and no specific expression was observed in the MZ as seen with E-Cadherin expression.

There was not a single example of nuclear localised β-Catenin in any animal in any area of the pituitary, and no difference in the distribution of β-Catenin was observed between control and E2 treated rats (Figure 5.13).

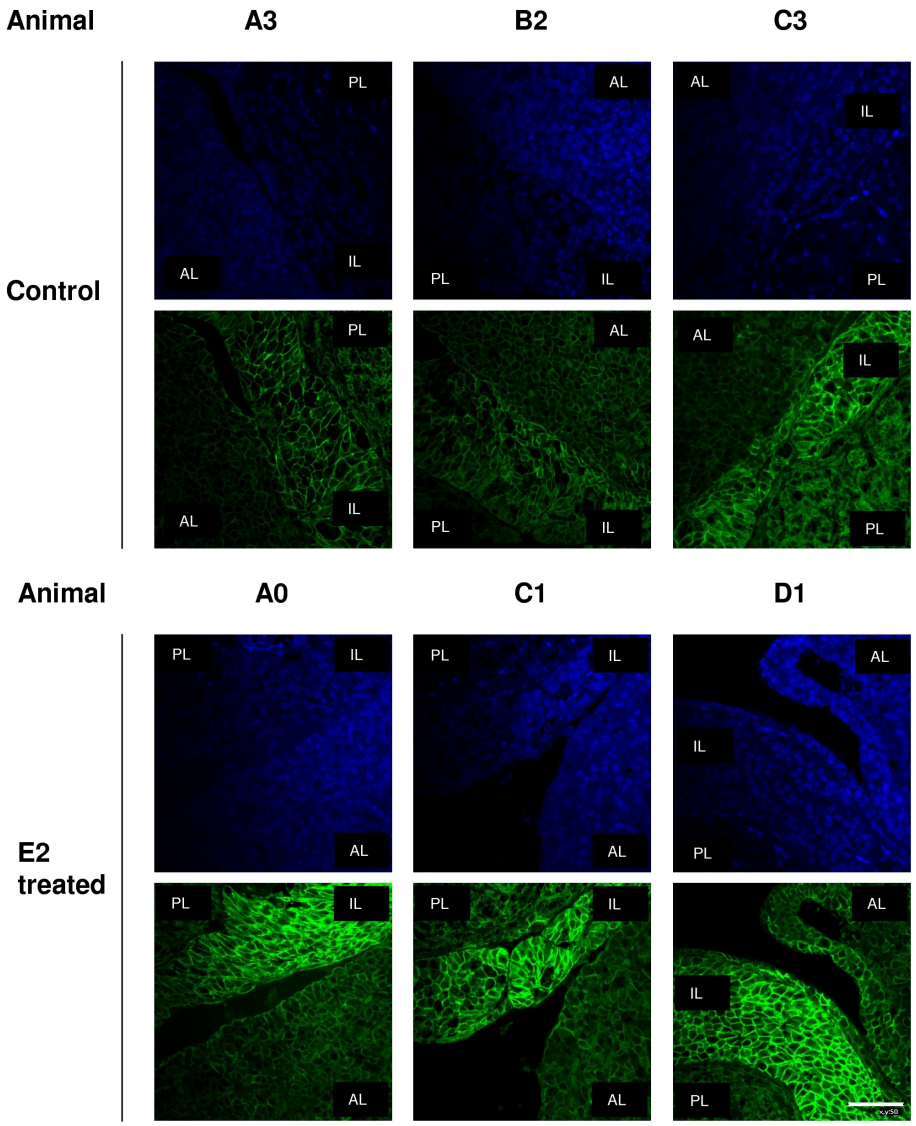


Figure 5.13. β-Catenin expression in posterior and anterior pituitary lobes. Pituitaries from control and E2 treated rats containing the posterior, intermediate and anterior lobes were stained for DAPI (blue) and β-Catenin (green). The animal number is indicated and relates to Table 3.1. Regions of the pituitary are indicated; AL – Anterior lobe, IL – Intermediate lobe, PL – posterior lobe. White bar represents 50µm. The microscopic fields of vision are considered to be representative of the whole study.

5.4.2. Proteins of interest in the anterior lobe

The expression patterns of Wnt-4 and β-Catenin in the AL of the pituitary have already been discussed in Results Chapters 3.0 and 4.0 respectively and were both unaffected by E2 treatment. The expression of N-Cadherin, E-Cadherin and Sox 9 was assessed in the AL to see whether the expression patterns of possible PCP pathway mediators were altered by E2 treatment during pituitary remodelling.

5.4.2.1. N-Cadherin expression in the anterior lobe

N-Cadherin was widely expressed in control pituitaries, and staining intensity appeared to decrease after E2 treatment (Figure 5.14 A). Images were quantified by assigning values to the intensity of staining for individual cells. Counting cells revealed that in control animals, 10% of cells did not stain for N-Cadherin, 77% of stained with low intensity and 13% of cells stained with high intensity. In E2 treated rats, 84% of cells did not stain for N-Cadherin, 15% stained with low intensity, while less than 1% stained with high intensity. Statistical analysis shows that the proportion of cells not expressing N-Cadherin increased significantly with E2 treatment ($P < 0.001$), while the proportion of cells expressing N-Cadherin (i.e. +1 and +2 intensity cells added together) decreased significantly with E2 treatment ($P < 0.001$ - Figure 5.14 B). This was further demonstrated by western blotting as faint expression for N-Cadherin was detected in 2 out of the 3 control animals tested, while no band was present in E2 treated animals (Figure 5.14 C).

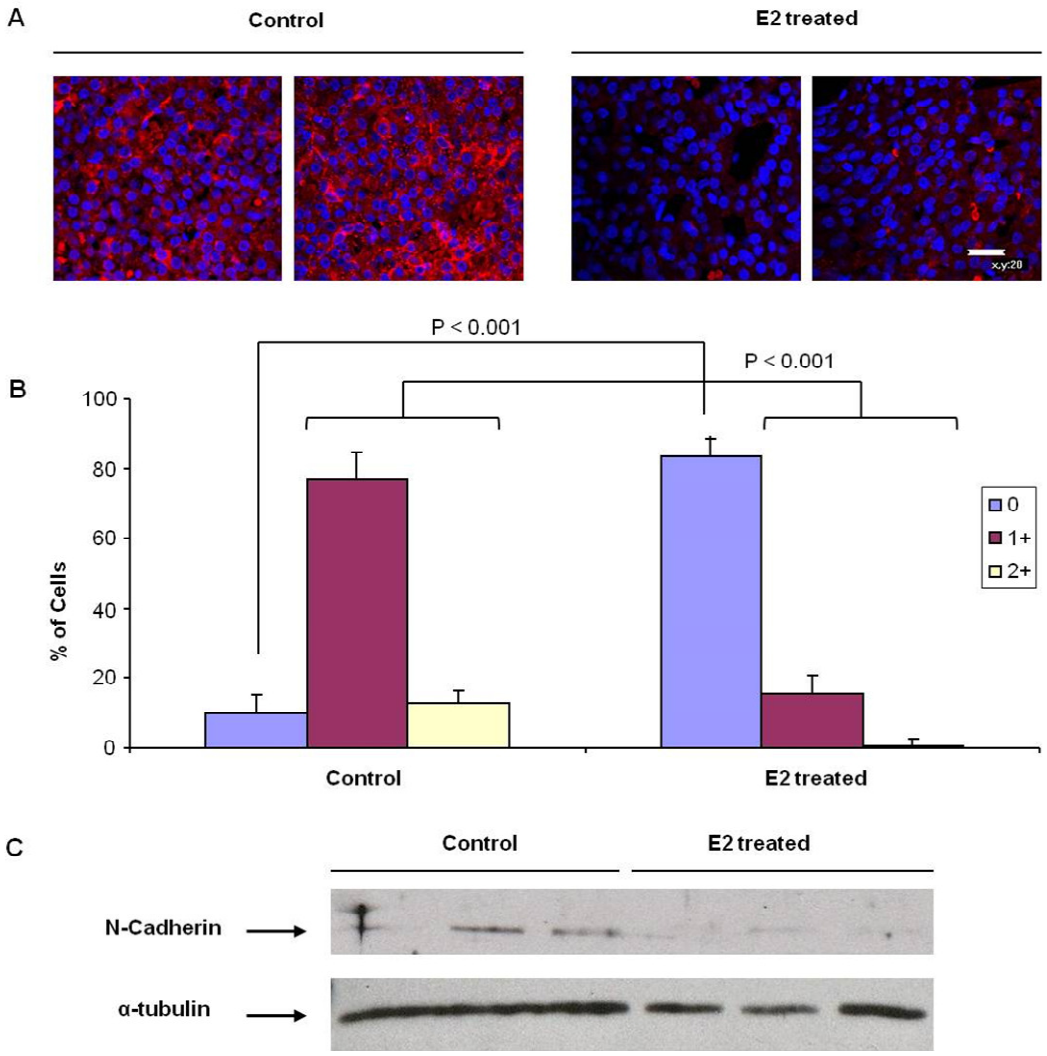


Figure 5.14. N-Cadherin expression in the anterior lobe. A – IHC staining of histological pituitary sections from control and E2 treated rats. Sections were stained for N-Cadherin (red) and DAPI (blue). White bar represents 20µm. **B** – Quantification of IHC images. Cells were rated according to staining intensity as follows - 0 – no staining, +1 – low staining, +2 – high staining. For counting, 2 random images of the AL were selected from each animal (n=3) and at least 800 cells counted per animal according to the above criteria. Statistical analysis was carried out on cells not expressing N-Cadherin, and cells expressing N-Cadherin (+1 and +2 cells combined) using Student T-Test (n= 6). **C** – Western blot for N-Cadherin on AL lysates**.** Fischer 344 rats were treated for 3 weeks with subcutaneously administered vehicle (PEG) or E2 (125µg/kg/day). Posterior pituitaries were removed, lysates were generated from anterior pituitary tissue and tested for N-Cadherin and α-Tubulin loading control.

5.4.2.2. E-Cadherin expression in the anterior lobe

In contrast to the MZ, E-Cadherin was expressed at low intensity in the cell membrane in a relatively small proportion of cells which were scattered through the AL. The phenotype of these cells was not determined by co-staining hormones with E-Cadherin due to species cross-reactivity between antibodies. No difference in E-Cadherin expression pattern could be determined visually (Figure 5.15 A). Quantification of the number of cells expressing E-Cadherin in the AL shows that there is a slight, though non-significant, reduction in the number of E-Cadherin expressing cells in E2 treated rats (Figure 5.15 B). However, western blots show that global levels of E-Cadherin were reduced in the AL by E2 treatment (Figure 5.15 C).

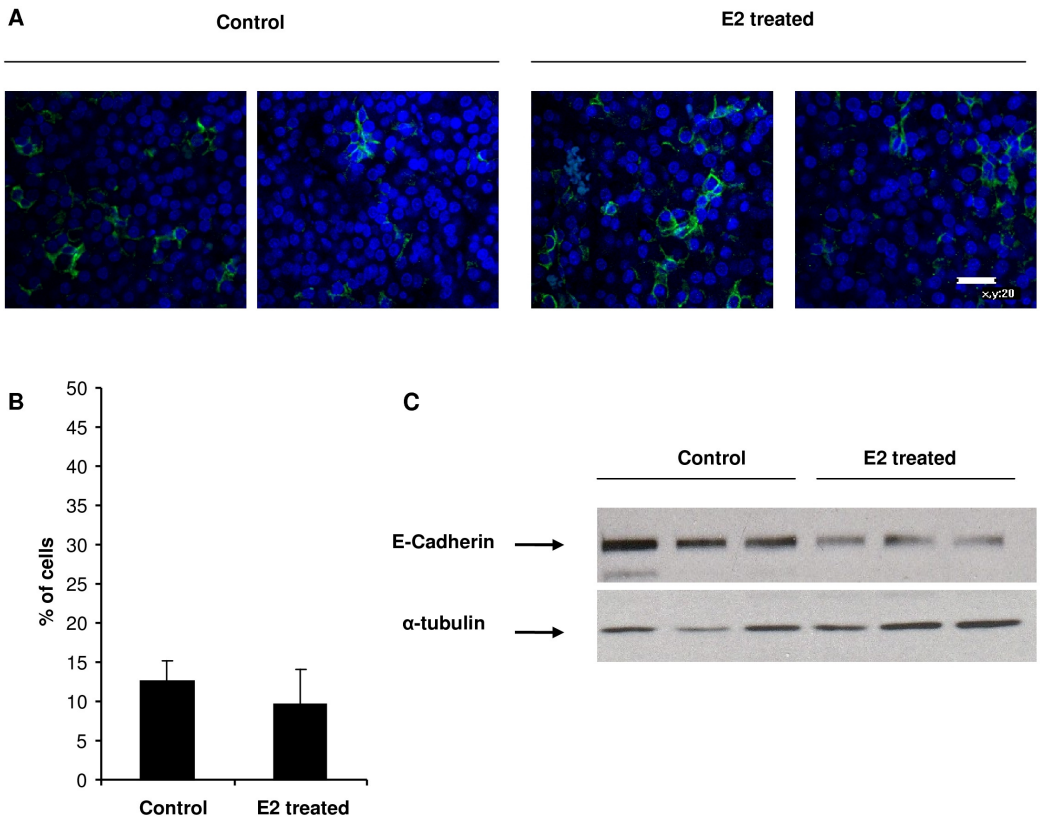


Figure 5.15. E-Cadherin expression in the anterior lobe. A – IHC staining of histological pituitary sections from control and E2 treated rats. Sections were stained for E-Cadherin (green) and DAPI (blue). White bar represents 20µm. **B** – Quantification of the proportion of cells in the AL expressing E-Cadherin. 2 random images of the AL were selected from each animal (n=3) and at least 800 cells counted per animal. **C** – Western blot for N-Cadherin on AL lysates**.** Fischer 344 rats were treated for 3 weeks with subcutaneously administered vehicle (PEG) or E2 (125µg/kg/day). Posterior pituitaries were removed, lysates were generated from anterior pituitary tissue and tested for E-Cadherin and α-Tubulin loading control. Each lane represents a lysate generated from a single animal.

5.4.2.3. Sox 9 expression in the anterior lobe

Sox 9 was expressed in the nuclei of a small number of cells in the AL (Figure 5.16 A). Dual expression studies were not carried out in order to identify which cell types in the pituitary expressed Sox 9. Roughly 5% of cells expressed Sox 9 in the AL, and this was not altered by E2 treatment (Figure 5.16 B).

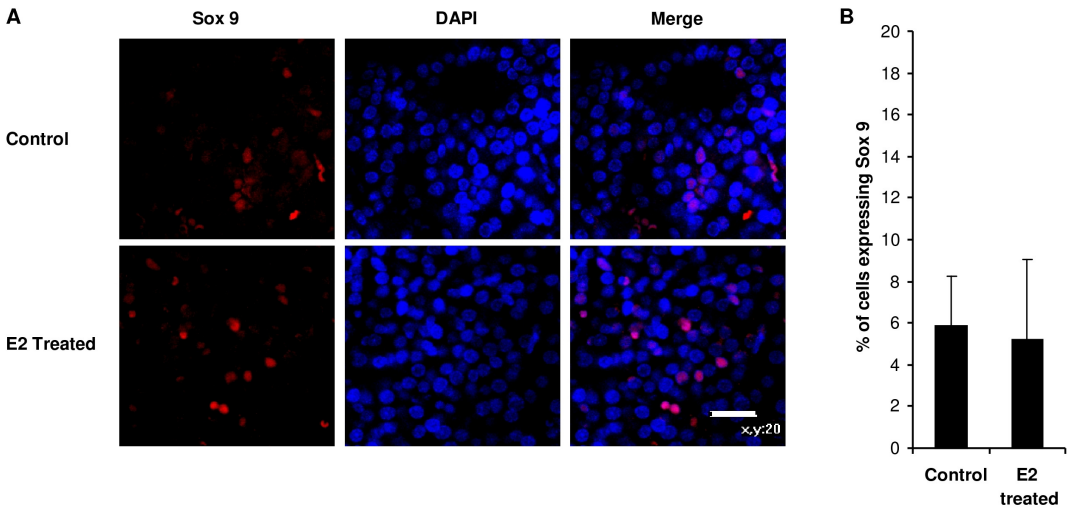


Figure 5.16. Sox 9 expression in the anterior lobe. A – IHC staining histological pituitary sections from control and E2 treated rats. Sections were stained for Sox 9 (red, left column) and DAPI (blue, middle column) with merged images in the right column. White bar represents 20µm. **B** - Quantification of the proportion of cells in the AL expressing Sox 9. 2 random images of the AL were selected from each animal (n=3) and at least 800 cells counted per animal. Data represent means ±SEM.

5.5. Discussion

Unlike the canonical pathway which has been extensively defined, non-canonical pathways are very poorly understood. Both pathways are thought to interact with generic kinases and cell regulators such as Jnk kinases, cytoskeletal regulators and modulators of calcium signalling, which integrate signals from numerous inputs to control diverse aspects of cell behaviour. As such, it is hard to isolate aspects of these pathways experimentally, and tools to study non-canonical Wnt pathways are not yet commercially available. The effects of modulating generic signalling pathways are clearly variable depending on cell type and tissue context, and so far there is no consensus in the literature regarding the best way to study non-canonical Wnt signalling pathways. In this section, attempts were made to examine both these pathways in the pituitary.

5.5.1. The Wnt-calcium pathway

The effects of Wnt-4 on the calcium pathway were assessed by studying calcium transients in GH3 cells in response to Wnt-4 treatment. In initial studies characterising the Wnt-calcium pathway, it was shown that injection of Wnt-5A RNA into zebrafish embryos doubled the frequency of calcium transients in the subsequently formed blastocyst (Slusarski et al., 1997a; Slusarski et al., 1997b). GH3 cells are known to exhibit spontaneous calcium oscillations (Wozniak et al., 2005), so the effects of recombinant Wnt-4 protein on these oscillations was studied.

In our study, intracellular calcium levels in GH3 cells did spontaneously oscillate in culture. Calcium transients can be very easily influenced by physical changes in movement and temperature, and as such it was vital that addition of new stimuli under the microscope did not affect calcium transients. We demonstrated that transients were unaffected by transition between different media, and that GH3 cells responded appropriately to administration of Bay K, an L-Type calcium channel agonist which generated an increase in levels of intracellular calcium (Figure 5.1).

Addition of recombinant Wnt-4 protein resulted in inhibition of transients in 63% of cells and had no effect in 37% of cells (Figure 5.2). In retrospect it may have been useful to carry out statistical analysis in the calcium oscillation data. Small spikes present in the

blots most likely reflect noise, and an amplitude threshold could have been made under which spikes could have been discounted. Quantification of the number, amplitude and frequency of spikes above this threshold could have then been carried out which would have given more information on the inhibitory effect of Wnt-4 on calcium transients in GH3 cells. Overall, these data show that the number of cells undergoing inhibition of calcium oscillations correlates closely with cell cycle analysis, which showed that under these experimental conditions 63% of GH3 cells were in the G1 phase, 36% were in the S phase and 1% were in the G2 phase. This suggests that calcium oscillations in cells in the G1 phase could be inhibited, while there is no effect on transients of cells in the S phase, though this was not confirmed.

The Wnt-calcium pathway may signal through NFAT, a cytoplasmic transcription factor which when activated by calcineurin, translocates to the nucleus to elicit gene transcription. Calcineurin and all 4 isoforms of NFAT were expressed in GH3 cells and FACS enriched lactotroph cells, however using an NFAT-dependent reporter gene, no expression of NFAT could be detected in GH3 cells. As observed with TopFlash reporter gene studies, this is likely not to result from inability to transfect cells with the plasmid as Renilla expression levels were comparable between GH3 cells and control HEK 293 cells. Furthermore, then negative control vector pTA did elicit some transcriptional output which was consistent between GH3 cells and HEK 293 cells. The plasmids are structurally very similar and the same protocol was used to transfect both plasmids, therefore it is highly unlikely that the negative control pTA was transfected, while the positive plasmid, pNFAT, was not. However, the result is slightly disconcerting as the NFAT pathway is a common pathway in mammalian cells, the necessary signalling components were expressed in GH3 cells, the positive stimuli (PMA and ionomycin) are well documented to induce large changes in intracellular calcium, and calcium machinery is clearly functional in GH3 cells as observed through live cell calcium imaging data presented here. Furthermore, parallel experiments in HEK 293 cells show that the plasmid is clearly functional, eliciting huge transcriptional output in response to positive controls.

In order to validate the transfection protocol in GH3 cells, the effect of Wnt-4 on NFκB activity was assessed. Wnt-10A may activate NFκB in osteoblast cells, and our group has previously studied NFκB signalling with reporter constructs in GH3 cells. Using the same transfection protocol as was used throughout this thesis, the NFκB reporter construct was transfected into GH3 cells and stimulated with Wnt-4 and positive control TNFα. Although Wnt-4 elicited no effect on NFκB, TNFα induced expression of

luciferase indicating the successful transfection and activation of the reporter construct in GH3 cells (Figure 5.9). Therefore, it is likely that the negative results for NFAT signalling in GH3 cells do reflect a lack of NFAT signalling, though this cannot be explained at present. However, NFAT is only one pathway through which the Wnt-calcium pathway may signal. Other possibilities not studied here include PKC, PLC and CAMKII signalling pathways providing further scope for this pathway in the pituitary.

5.5.2. The Wnt-planar cell polarity pathway

The PCP pathway is even more difficult to study than the calcium pathway. As a cell signalling molecule, calcium itself can be isolated and followed experimentally in terms of its oscillatory action. Though the downstream signalling effects of calcium inhibition are far more difficult to understand, it was relatively easy to ascertain a clear effect on calcium signalling by Wnt-4, which warrants further investigation.

However, no such easily identifiable molecule exists in the PCP pathway. The pathway is so poorly understood in vertebrates that only a few instances of signalling have been attributed to the PCP pathway which may involve GTPases and kinases with numerous possible cellular outcomes. In an attempt to study the pathway in a more functional manner, immunofluorescence techniques were used to study the distribution of key developmental proteins and cadherin molecules throughout the pituitary, which may be downstream targets of PCP signalling. Different cell types in the pituitary express different cadherin molecules which are thought to link cells to each other in networks (Chauvet et al., 2009; Fauquier et al., 2001), and alterations in the distribution of cadherins has been shown to contribute to pituitary dysmorphology (Ezzat et al., 2004b; Moran et al., 2010).

The expression patterns of the following proteins were compared between control and E2 treated rats: Sox 9, Wnt-4, β-Catenin, E-Cadherin, N-Cadherin. Research focused on 2 different areas: the AL, and the MZ.

5.5.2.1. Planar cell polarity signalling in the anterior lobe

The expression patterns of Wnt-4 and β-Catenin in the AL have been discussed at length already in Results Chapters 3.0 and 4.0 respectively, and will not be discussed further here.

The most striking difference between control and E2-treated rats was the marked reduction in N-Cadherin expression. This was shown by western blot where global levels of N-Cadherin were decreased, and by immunofluorescence where the staining intensity for N-Cadherin was markedly decreased. This was coupled with a decrease in E-Cadherin levels, though the expression of E-Cadherin was already relatively low in control pituitaries. Dual staining with hormones would have been useful to see whether the downregulation of cadherins observed was specific to a particular cell type, or whether it reflects a global morphological change in response to pituitary enlargement and lactotroph hyperplasia.

Sox 9 was expressed sporadically throughout the pituitary, though once again dual staining for hormones was not performed to ascertain which cell types expressed Sox 9. It has been reported that FS cells specifically express Sox 9 in the adult mouse pituitary (Fauquier et al., 2008), and the proportion of FS cells reported in the rat is comparable to the proportion of cells expressing Sox 9 in this study (Jin et al., 2001). The proportion of Sox 9 expressing cells decreased slightly with E2 treatment, though no significant change was observed. The expression of Sox molecules in the pituitary has been implicated by a number of different groups as a possible marker for progenitor stem cells (Chen et al., 2009; Fauquier et al., 2008; Garcia-Lavandeira et al., 2009; Moran et al., 2010), though the downstream effects of Sox signalling has not been studied yet.

5.5.2.2. Planar cell polarity signalling in the marginal zone

All proteins of interest were expressed in and around the MZ, though no change in the expression of any of the proteins was observed with E2 treatment. Wnt-4, β-Catenin and N-Cadherin were all expressed in cells along the MZ and in adjacent IL and AL regions. Wnt-4 staining intensity was higher in the MZ than surrounding areas, though

β-Catenin and N-Cadherin staining was more intense in the IL than the AL, and no specific difference could be deduced between IL and MZ staining for these proteins.

Cells in the MZ expressed E-Cadherin at high intensity. Some cells in the AL did express E-Cadherin as previously discussed, though the intensity of staining was considerably higher in the MZ. Sox 9 was expressed in roughly 50% of cells in the MZ, and was unaltered by E2 treatment. The incidence of Sox 9 expression in the MZ was considerably higher than in the AL (50% of cells in the MZ against 5% in the AL) suggesting it may play an important role in this region.

Although no changes in the expression of these proteins was observed with E2 treatment, the expression of stem cell markers and the clear structural characteristics of the MZ certainly warrant further investigation.

5.6. Summary

Overall this chapter shows the novel finding that Wnt-4 impacts on a non-canonical pathway in the pituitary by inhibiting calcium oscillations in lactotroph cells. Although attempts to study the downstream effects of this inhibition were unsuccessful, it does open new possible avenues of research for the scientific community which has thus far concentrated solely on the canonical pathway. Attempts to understand the role of PCP signalling in the pituitary were less successful. However the high expression of Wnt-4 in this region, the planar organisation of the MZ, and alterations in cell to cell adhesion molecules during oestrogen-induced lactotroph hyperplasia do suggest a possible role for the pathway in pituitary remodelling.

6.0. Discussion

6.1. Introduction

The pituitary gland is a fascinating organ in the manner in which multiple secretory cell phenotypes exist in close proximity to each other. The origin of these cell types has been extensively studied and has given key insight into the mechanisms by which networks of transcription factors work simultaneously to develop the heterogeneous pool of cells that constitutes the mature gland. More recently it has emerged that the adult pituitary is plastic, with the relative proportions of secretory cells changing in response to the physiological demands of the body. The mechanisms by which this occurs are unknown but are likely to involve locally produced transcription and growth factors which selectively induce proliferation of a particular cell type. This process would appear to be hijacked during the development of pituitary adenomas, where the low rate of proliferation of a particular cell type results in a slowly expanding adenoma which can take over 20 years to reach clinical significance depending on the cell type involved. To date, little information is present in the literature regarding control over adult tissue plasticity, with researchers focusing on the causes of pituitary adenomas to explain proliferative effects in plastic pituitary response.

6.2. Oestrogen effect on lactotroph proliferation

The basis of this thesis is the well known proliferative effect of oestrogen on the lactotroph population. In this study, Fischer 344 rats were treated with E2 for 3 weeks which induced lactotroph hyperplasia. The Fischer 344 rat is oestrogen sensitive, and the rate of lactotroph proliferation and prolactinoma development is higher in Fischer 344 rats than other rat models. However, E2 treatment of other rat strains does induce lactotroph hyperplasia, albeit less marked.

In a direct comparison between Fischer 344 rats and Sprague Dawley rats, 8 weeks E2 treatment doubled pituitary weight in Fischer 344 rats (as in the present study) but pituitary weight in Sprague Dawley rats only increased from 22mg to 30mg. Pituitary PRL content was comparable between the two strains, and although plasma PRL levels were increased in Sprague-Dawley rats, they were significantly lower than in Fischer 344 rats (Lawson and Parker, 1992). In a different study, treatment of Sprague

Dawley rats with high levels of E2 (500µg/kg/day compared to 125µg/kg/day in our study) for 7 weeks induced pituitary tumours and increased PRL secretion (Diaz-Torga et al., 1998). In two further studies, chronic E2 treatment of Fischer 344 and Sprague Dawley rats did result in lactotroph hyperplasia, although the degree of proliferation was less in Sprague Dawley rats than in Fisher 344 rats and the duration of E2 exposure required to induce comparable hyperplasia was longer in Sprague Dawley rats (Lawson and Parker, 1992; Suarez et al., 2002).

Male Wistar rats were treated with 2mg/kg/day estradiol benzoate for 3 weeks which doubled pituitary weight (Nedvidkova et al., 2001) though it was not stated if prolonged treatment resulted in tumour development, and the dose used in this study is considered extremely high. Not all rats undergo lactotroph hyperplasia in response to E2 treatment. In a direct comparison between comparison between Fischer 344 and Holtzman rats, chronic E2 treatment induced tumours in Fischer 344 rats but not in Holtzman rats (Lieberman et al., 1981).

Overall, the system employed in this study can be considered somewhat reflective of normal rat physiology. One question that does become apparent when considering other studies is that the dose of E2 used in this project was relatively low and of short duration (125µg/kg/day for 3 weeks). Other studies treated rats with up to 2mg/kg/day and in some studies treatment lasted for up to 8 weeks. In our model, lactotroph hyperplasia was induced, but prolactinoma formation was not, though would be expected to if the duration of treatment was extended. It is therefore important when considering results obtained from this model that further treatment may induce more chronic and detectable changes than observed in this thesis.

When compared to humans, the link between chronic oestrogen treatment and prolactinoma development is not so clear. An example of chronic oestrogen treatment to humans is in the treatment of transsexuals with high doses of oestrogen to suppress testosterone production and promote femininity. Prolactinomas are not more prevalent in these people than normal people. However, the numbers of transsexuals studied in this context are extremely low, and therefore increased rates of prolactinoma development may have not been detected. It has been shown that women taking an oestrogen-containing contraception pill may have a slight increase in serum PRL levels and a slight increase in incidence of prolactinoma development (Luciano et al., 1985), though other studies show no difference in tumour incidence (Davis et al., 1984). Considered together, the rat models used in this study may be relevant to humans,

though direct comparisons between the models cannot be made at without further investigation.

6.3. Wnt-4 upregulation in the pituitary

Over the last 10 years, a number of studies have implicated Wnt-4 as being a key regulator of pituitary development, as well as playing a role in pituitary adenoma formation; Wnt-4 knock out resulted in decreased pituitary cell number in mice (Potok et al., 2008; Treier et al., 1998), Wnt-4 was expressed in the adult rat pituitary and oestrogen treatment to GH producing MtT/S cells increased Wnt-4 expression (Miyakoshi et al., 2009), and Wnt-4 was upregulated in human pituitary tumours (Miyakoshi et al., 2008b). Pervious work from our group also showed that Wnt-4 mRNA was upregulated in pituitaries undergoing lactotroph hyperplasia (Giles et al., 2011). In the present study, Wnt-4 protein was widely expressed throughout the pituitary, was upregulated in GH3 cells in response to oestrogen treatment, and was also expressed in the MZ, a poorly understood region of the pituitary which may contain progenitor cells.

However, upregulation of Wnt-4 protein could not be detected in primary tissue. The reasons for this are not understood though may relate to RNA message for some reason not being translated into protein expression, upregulation of Wnt-4 in only a subset of cells which may go undetected when measuring global levels of Wnt-4 in the pituitary, or too short a duration of E2 treatment to elicit a measurable increase in Wnt-4 protein level.

6.4. Site of Wnt-4 expression in the pituitary

The wide expression of Wnt-4 poses a question as to which cells Wnt-4 may be exerting its effects on in the pituitary. The initial premise of this project was that Wnt-4 was expressed specifically in lactotroph cells, and that upregulation of Wnt-4 would result in proliferation of the lactotroph population through paracrine action of Wnt-4. If true, numerous aspects of Wnt signalling could have been, and were, studied using the

somatolactotroph GH3 cell line. Clearly data gathered using the GH3 cell line cannot be considered representative of other cell types in the anterior pituitary.

Our data clearly show that Wnt-4 is expressed in all cell types studied in the pituitary. This is supported from data demonstrating that Wnt-4 is upregulated in a number of different pituitary adenoma subtypes, and not restricted to prolactinomas (Miyakoshi et al., 2008b). Ideally, the downstream signalling events of Wnt-4 would be studied in all pituitary cell types, though the lack of well characterised cell lines for other pituitary cell phenotypes limits experimental scope.

The heterogeneous nature of the pituitary renders isolation of a specific cell type from primary tissue difficult. In this thesis, FACS sorting was used to isolate a population of cells 80% positive for PRL. However, this is still far from ideal as the identity of the other 20% of cells in this population is unknown and furthermore, the amount of rats required to obtain enough material to study these cells would be expensive and unsuitable for most experimental approaches. Another possibility would be to use transgenic rats generated in our group which express green fluorescent protein (GFP) driven by the human PRL promoter. Using the GFP signal, our group is able to isolate a pure population of lactotroph cells through FACS sorting. A separate group has used a similar protocol to isolate somatotrophs by fusing GFP to the N-terminus of the hGH gene enabling isolation of a pure GH producing population (Magoulas et al., 2000). However, the same limitations apply regarding the amount of useful experimental material that can be attained through these procedures.

Though protocols are available to isolate lactotroph and somatotroph cells, we are still unable to isolate pure populations of other cell types to study. This problem is especially evident when trying to study the single layer of cells which constitutes the MZ. The only way in which we, and others, have studied this region is though immunofluorescence techniques, which have been extremely useful in identifying the expression of proteins which may regulate tissue plasticity. The high levels of Wnt-4 expression in the MZ suggest a role for Wnt-4 in this region, and the planar organisation suggests a possible action for PCP signalling in regulating the region.

6.5. Downstream Wnt signalling in the pituitary

6.5.1. Canonical signalling in the pituitary

Despite numerous data reporting the involvement of Wnt molecules in the pituitary, few groups have tried to fully examine the downstream signalling events Wnts may employ. Predictably, most groups have focused on whether or not the canonical pathway is activated in the pituitary due to the relative ease with which this pathway can be studied.

Although initial evidence suggested that the canonical pathway was activated in pituitary adenomas (Semba et al., 2001), more recent evidence suggests that the canonical pathway is not activated in the pituitary (Miyakoshi et al., 2008b). Our data clearly shows that canonical signalling could not be induced in GH3 cells, even when a constitutively active mutant form of β-Catenin was introduced into cells. IHC analysis shows that no examples of nuclear β-Catenin were found in the project strongly suggesting that canonical signalling is not active in the pituitary.

Craniopharyngiomas, a distinct subset of pituitary tumours originating from the remnants of the Rathke's pouch, are thought to be caused by aberrant β-Catenin signalling (Pettorini et al., 2010). They exhibit a much more aggressive proliferation profile than pituitary adenomas which usually requires medical intervention in early youth. The difference in proliferation profiles of pituitary adenomas and craniopharyngiomas suggests that canonical signalling plays no role in pituitary adenoma growth.

This concept is strengthened when studying other cancers caused by aberrant canonical signalling. In colorectal cancer, a mutation in APC renders the canonical pathway constitutively active resulting in uncontrolled cell proliferation and malignancy. Tumours in the pituitary are almost never malignant, once again arguing that the proliferative effect initiated by the canonical pathway would likely cause more aggressive tumour growth than observed in pituitary adenomas.

Finally, unpublished data presented in an oral communication last year shows an interesting effect of upregulated canonical signalling in the pituitary. Constitutively

active mutant β-Catenin (not confirmed if it was the same mutant β-Catenin plasmid used in this thesis) was selectively expressed under two different promoters in the mouse pituitary. In the first case, mutant β-Catenin expression was driven by Hesx-1, which is expressed in the Rathke's pouch and precursors to all secretory cell types in the AL between e9.5 and e15.5, and is essential for normal pituitary development (Mantovani et al., 2006). Secondly, mutant β-Catenin expression was driven by the PRL promoter. In Hesx-1 driven expression, mice developed pituitary tumours similar to craniopharyngiomas, while no effect was observed in mice with PRL driven mutant β-Catenin expression (Martinez-Barbera, personal oral communication, 2010).

Taken together, with the single exception of immunohistochemical analysis presented by Semba et al (2001), all data in the literature, and in this report, suggest that canonical signalling is not involved in pituitary adenoma pathogenesis.

6.5.2. Non-canonical signalling in the pituitary

Attempts were made to study the non-canonical pathways in the pituitary. As previously described, no specific tools are currently available to study non-canonical pathways due to the poor understanding of both pathways.

6.5.2.1. Planar cell polarity signalling in the pituitary

The PCP pathway was studied by assessing the expression of Cadherin molecules in different regions of the pituitary. The PCP pathway is thought to alter cytoskeletal organisation and cell to cell adhesion in order to generate planes of cells from which the pathway derives its name. Alterations in cadherin expression are important in tumour progression. Loss of cell to cell adhesion through downregulated E-Cadherin expression allows cells to migrate past each other and invade surrounding tissue (Huber et al., 2005). Recent data shows that disruption of cadherin structures by Notch upregulation results in disorganised cellular organization at the IL/PL border and altered progenitor cell (defined by Sox 2 expression) localisation (Moran et al., 2010).

In the present study, both E-Cadherin and N-Cadherin levels were decreased in E2 treated pituitaries, suggesting some form of structural reorganisation. However, E-Cadherin was only expressed in a small number of cells in the AL and furthermore was

only expressed at low levels when compared to the high expression observed in the MZ. It is therefore hard to ascribe too great an importance to this decrease. However, N-Cadherin was strongly expressed throughout the AL in control animals, and its expression was markedly decreased after E2 treatment.

It has been shown that specific cell types in the pituitary express different cadherin molecules which are likely to form networks between cells (Chauvet et al., 2009). It is likely that reorganisation of these structural networks takes place during pituitary remodelling to allow for cellular proliferation, but whether this reorganisation is directly caused by PCP signalling in the pituitary or secondary to reorganisation induced by proliferation is unknown.

In contrast to the low expression of E-Cadherin in the AL, E-Cadherin was highly expressed in the MZ where it was specifically expressed along the MZ, though its expression in this site is unaltered by E2 treatment. The planar organisation of the MZ suggests a possible role for the PCP pathway in regulating this region. The MZ may contain stem cells which may regulate the plasticity of the adult gland by regulating proliferation of new cells from existing progenitor cells. Wnt molecules have been proposed to maintain stem cell niches (Reya et al., 2003; Willert et al., 2003) and it is possible that the function of Wnt-4 in the pituitary is to maintain cells in the MZ to allow them to regulate tissue plasticity.

For some time the origin of new lactotroph cells during lactotroph hyperplasia was debated. Possible origins include proliferation of already existing lactotroph cells, trans-differentiation of somatotrophs into lactotrophs, or emergence of newly differentiated cells from a stem cell niche. Recent findings suggest that trans-differentiation is unlikely to be the cause as less than 1% of cells were shown to trans-differentiate from lactotrophs to somatotrophs after weaning. This period is thought to reflect a reversal of E2-induced lactotroph proliferation, returning the pituitary to a state where somatotrophs are more prevalent than lactotrophs.

Recent data shows that in control animals, 31% of lactotrophs incorporated BrdU compared to 36-39% of lactotrophs in E2 treated animals, suggesting only a small increase in the number of lactotrophs undergoing mitosis induced by E2 treatment (Nolan and Levy, 2009c). The same group also showed that although the mitotic index of lactotrophs was increased for a week after E2 stimulation, it decreased upon prolonged treatment of up to 28 days (Nolan and Levy, 2009b). Together, these data

suggest that the increase in lactotroph cell number is partially bought about by proliferation of already existing lactotroph cells, though it is likely that this would not be sufficient to induce the reported increase in lactotroph content in the pituitary. It is therefore likely that proliferation of progenitor cells also contributes to the increase in lactotroph cell number during E2-induced lactotroph hyperplasia.

Overall, the MZ is a subset of cells which expresses a number of key pituitary regulators and markers for stem cells. In culture, MC's can form spheres and differentiate into all hormone producing cell types strongly suggesting they play a role in regulating pituitary plasticity (Fauquier et al., 2008). The defined planar organisation of the MZ suggests that the PCP pathway may play a role in regulating the region, and the high expression of Wnt-4, and the known action of Wnt-4 in maintaining other stem cell populations suggests this could be a key site for Wnt-4 action in the pituitary. However, studying this region is currently difficult, as the lack of specific markers for MC cells makes isolation of a pure population difficult. One possibility for isolating the MZ is by using laser capture microdissection where groups of cells can be selected under a microscope and then isolated using a laser. This technique has been used successfully to obtain a pure population of FS cells from Wistar rats (Jin et al., 2001), and could provide a way of specifically studying the MZ which may provide important information regarding the regulation of adult pituitary plasticity.

6.5.2.2. Calcium signalling in the pituitary

The Wnt-calcium pathway was the least studied of the Wnt pathways in this thesis. Experimental limitations prevented study on primary tissue and as such all data regarding the calcium pathway were collected from GH3 cells, the drawbacks of which have already been discussed.

Our data show that Wnt-4 has a clear inhibitory effect on the frequency and amplitude of calcium transients in GH3 cells, and is the first example of non-canonical Wnt signalling in the pituitary. Interestingly, we also demonstrated an effect where Wnt-4 inhibited calcium transients, where the Wnt-calcium pathway is usually related to an increase in frequency and amplitude of oscillations. Whether this represents a new action of Wnt, or simply that other groups have not observed inhibition of calcium signalling is unknown, but considering the relatively poor understanding of the pathway

this should not be cause for concern. The key finding is that Wnt-4 impacts strongly on calcium signalling in GH3 cells.

The downstream function of this inhibition however is far from understood. As described previously, calcium signalling impacts on virtually all cells in the body in a hugely variable manner. Despite much interest over the last 20 years, very little information has arisen regarding the function of calcium signalling. Numerous regulators and effectors have been identified, and clearly some effect cellular actions such as transcription and structural reorganisation, but the later responses, and mechanisms of selectively altering these effects have not been elucidated.

The same problem was encountered here. Calcium signalling is known to play a role in PRL release (Wozniak et al., 2005) and is likely to impact on other lactotroph actions. However, as the mechanisms by which calcium functions are unknown, the effects of inhibition are also unknown. This data does however reveal a novel input into GH3 cells which clearly regulated calcium signalling, and may well encourage further investigation into this pathway in the pituitary.

6.6. Possible roles for Wnt-4 in the pituitary

The role of Wnt-4 in the pituitary has been examined but no conclusive results regarding Wnt-4 function have been determined. However, the initial hypothesis of this thesis that activation of the canonical pathway by Wnt-4 in lactotroph cells would result in lactotroph proliferation is clearly not correct. Presented here are three possible roles for Wnt-4 in the adult that warrant further investigation:

1. Wnt-4 acts as a tumour suppressor in the AL. All cell types in the AL express Wnt-4, which may act as a break over cell proliferation in response to proliferative inputs to control overall cell number.
2. Wnt-4 acts via the PCP pathway to maintain the MZ, which then regulates the cellular content of the AL by acting as a progenitor stem cell niche.
3. Wnt-4 acts to control Cadherin expression in different cell types in the AL, and thus control cellular networks in the pituitary which may regulate tissue plasticity. This may involve the calcium pathway which is known to have effects on cell migration via alterations and cell-cell adhesion characteristics.

6.7. Future work

A number of experimental options are available to carry on this work. The use of transgenic animals is likely to shed useful light on the function of Wnt-4 in the pituitary. Overexpression of Wnt-4 or conditional expression of siRNA to knock down Wnt-4 could be targeted either to lactotroph cells or precursor cells such as Hesx-1 expressing cells. Current transgenic Wnt-4 KO models die before birth, so conditional knock down would allow study of Wnt-4 function in adult life after pituitary development has correctly occurred.

The inhibition of calcium signalling, the planar organisation of the MZ and the clear lack of canonical signalling in the pituitary strongly suggests that Wnt-4 impacts on non-canonical signalling pathways. Unfortunately, most data regarding these pathways focuses on activation of generic kinases and signalling molecules such as Jnk, Erk, PKC and PLC. Proteomic analysis of these pathways could be carried out on purified populations of GH or PRL cells, and could even be done in conjunction with conditional siRNA knock down of Wnt-4 in the pituitary.

Furthermore, it is likely that more than one Wnt molecule is functioning in the pituitary. In the original microarray study published by our group, Wnt-10A was also upregulated during oestrogen induced lactotroph hyperplasia (Giles et al., 2011). Focus in this thesis was given to Wnt-4 as more information had been published regarding Wnt-4 in the pituitary, and better antibodies were available for Wnt-4. In addition, Wnt-11, Wnt-5A and Wnt-6 have all been shown to be expressed in the pituitary during development and others may well be expressed in the pituitary. It will be useful to characterise fully which Wnt molecules are expressed in the pituitary, and attempt to answer which Wnts bind to which Fz receptors in the pituitary.

6.8. Implications of research

In the context of the pituitary, our published work should go on to encourage researchers within the field to examine non-canonical Wnt pathways which is likely to shed light on the development and maintenance of the most important endocrine regulator in the body. From a clinical context, understanding of these pathways may

well contribute to the development of therapeutic techniques to regulate pituitary adenoma growth and reduce the necessity for surgery as is currently required in all tumours except prolactinomas.

However, this research is also important in developing our understanding of Wnt signalling in a more general context. Wnt signalling is found in virtually every organ and system in the body, and in those where it has not been demonstrated, it most likely will be soon. However our knowledge of how Wnts function is relatively poor. Since the discovery of Wnt signalling and its involvement in cancer, huge amounts of research have been poured into the canonical pathway. Though our understanding of this pathway is considerable, it has come at the cost of the other signalling pathways. With growing evidence to suggest that Wnts signal as a complex network, it is becoming clear that the canonical pathway cannot be studied in isolation.

An overall change in approach to studying Wnt signalling is needed from researchers. To date most research has simply focused on whether canonical signalling is activated in a given context or not. Most “non-canonical” actions of Wnts are simply ascribed in a given context based on initial findings in Wnt research, which showed that certain Wnts were unable to transform C57 MG mammary epithelial cells, and were therefore characterised as “non-canonical” (Wong et al., 1994). Since then, numerous papers have shown that a “non-canonical Wnt” is expressed in a given context and defined its actions as non-canonical, often without examining any downstream signalling events. Furthermore, virtually all the downstream non-canonical events published have been studied using Wnt-5A in Xenopus and Drosophila embryogenesis. As the effects of Wnts are so varied and dependent on tissue and cell-type context, the effects of non-canonical signalling cannot simply be defined using these models. For example, this thesis (and our recently published paper) reports for the first time inhibition of calcium signalling by a Wnt molecule. This is not likely to be an exception to Wnt signalling, just an aspect that was not documented in the effects of Wnt-5A in Xenopus development where Wnt-5A resulted in upregulation of calcium transients.

It is important that researchers begin to examine all downstream signalling events relative to a particular Wnt molecule in an experimental context. It will also be important to fully characterise which Wnts are present in a given context, and which ligands interact with which receptors to produce which effects. The Wnt network will only become understood through consensus from published data in this context. Already arrays are available which will make this kind of screening commonplace within the lab.

This thesis provides a basic framework with which Wnt signalling could be studied. It focuses on a region where Wnt signalling is known to function, isolates a Wnt molecule to study, then attempts to study the downstream signalling events in all common pathways (and novel pathways e.g. NFκB signalling) which take place relative to that Wnt. Though the research into non-canonical aspects was not a detailed as canonical aspects, attempts were made to imagine functional outputs of non-canonical pathways which fall outside the limited current knowledge in the literature. There has been too easy a tendency to ignore non-canonical pathways, though efforts must be made to examine them if the physiology of Wnt signalling is to be understood. Understanding the intricacies of its function will considerably advance our knowledge of mammalian physiology.

7.0. Bibliography

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8.0. Appendix

8.1. List of publications

8.1.1. Poster presentations

Wnt signalling in oestrogen-induced lactotroph proliferation

- Poster presented at British Endocrine Society, 2009

Wnt-4 and Wnt-10A induction during estrogen-stimulated lactotroph proliferation

- Poster presented at ENDO, 2010

Wnt signaling in estrogen-induced lactotroph proliferation

- Poster presented at ENDO, 2010

Wnt signalling in oestrogen-induced lactotroph proliferation

- Poster presented at Faculty of Medical and Human Sciences showcase, 2010

8.1.2. Papers

Wnt signaling in estrogen-induced lactotroph proliferation

- This paper was recently published in Journal of Cell Science and a copy is inserted overleaf

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Wnt signaling in estrogen-induced lactotroph proliferation

Adam Giles, Frederic Madec, Sönke Friedrichsen, Karen Featherstone, Tom Chambers, Claire V. Harper, Julia Resch, Georg Brabant and Julian R. E. Davis*

Endocrinology & Diabetes Group, Faculty of Medical & Human Sciences, AV Hill Building, Oxford Road, University of Manchester, Manchester M13 9PT, UK

*Author for correspondence (julian.davis@manchester.ac.uk)

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Summary

Prolactinomas are the most common type of functioning pituitary adenoma in humans, but the control of lactotroph proliferation remains unclear. Here, using microarray analysis, we show that estrogen treatment increased expression of *Wnt4* mRNA in adult Fischer rat pituitary tissue. Dual immunofluorescence analysis revealed that Wnt4 expression was not confined to lactotrophs, but that it was expressed in all anterior pituitary cell types. Estradiol induced proliferation in the somatolactotroph GH3 cell line, in parallel with Wnt4 mRNA and protein induction. A reporter gene assay for TCF- and LEF-dependent transcription revealed that there was no activation of the canonical Wnt pathway in GH3 cells upon stimulation with Wnt-conditioned culture medium or coexpression of constitutively active mutant β-catenin. Expression of β-catenin in both GH3 cells and normal rat anterior pituitary cells was restricted to the cell membrane and was unaltered by treatment with estradiol, with no nuclear β-catenin being detected under any of the conditions tested. We show for the first time that Wnt4 affects non-canonical signaling in the pituitary by inhibiting Ca^{2+} oscillations in GH3 cells, although the downstream effects are as yet unknown. In summary, Wnt4 is expressed in the adult pituitary gland, and its expression is increased by estrogen exposure, suggesting that its involvement in adult tissue plasticity is likely to involve β-catenin-independent signaling pathways.

Key words: Wnt, Lactotroph, Pituitary, Prolactinoma, β-Catenin

Introduction

Prolactinomas represent approximately 60% of all pituitary tumors in humans (Gurlek et al., 2007). They result from abnormal lactotroph cell proliferation and usually display only very slow growth, but the pathogenesis of prolactinoma formation and progression has remained elusive. Classical oncogenic mechanisms seem unlikely to be involved in most cases, and none of the common genetic mutations causing cancer has so far been found to operate in prolactinomas (Levy, 2008). A number of proteins have been implicated in pituitary adenoma development; pituitary tumor transforming gene (PTTG) (Kim et al., 2007), basic fibroblast growth factor (bFGF) (Zhang et al., 1999), vascular endothelial growth factor (VEGF) (McCabe et al., 2002), bone morphogenetic protein 4 (BMP4) (Labeur et al., 2010), pituitary tumor apoptosis gene (PTAG) (Bahar et al., 2004) and histone deacetylase 2 (HDAC2) (Bilodeau et al., 2006), among several others, have been demonstrated to play a role in pituitary tumorigenesis but the origins of many tumors are still unknown.

Estrogen has long been known to exert a proliferative effect on lactotroph cells. High circulating estrogen levels during pregnancy result in lactotroph hyperplasia, pituitary enlargement and increased circulating prolactin (PRL) levels (Asa et al., 1982; Goluboff and Ezrin, 1969; Lloyd et al., 1988). In vitro, estradiol (E2) induces proliferation of the somatolactotroph GH3 cell line (Horvath and Kovacs, 1988; Kansra et al., 2005; Lieberman et al., 1982; Song et al., 1989) and the effects of E2 on lactotroph proliferation can be studied in vivo using the estrogen-sensitive Fischer 344 rat. In this model, lactotroph hyperplasia, and eventual prolactinoma formation, can be induced by treatment with estradiol or the synthetic estrogen diethylstilbestrol (DES) (Heaney et al., 1999; Mucha et al., 2007; Phelps and Hymer, 1983; Wiklund et al., 1981).

Wnt molecules, a family of 19 secreted signaling proteins in humans, are expressed in overlapping temporal and spatial patterns during development (Yavropoulou and Yovos, 2007). They regulate diverse cellular processes, such as proliferation, differentiation, apoptosis and cell survival (Willert and Jones, 2006), and are crucially involved in embryonic development. In this capacity, they are thought to interact with other traditional signaling pathways regulating development, including the BMP, sonic hedgehog (Shh), sox and notch pathways, although the interactions are complex. Abnormalities in Wnt signaling pathways have been associated with numerous cancers (Giles et al., 2003) and, in most cases, activation of canonical Wnt signaling is involved in cancer progression (Reya and Clevers, 2005; Willert and Jones, 2006).

The canonical Wnt signaling pathway centers on activation of β-catenin. In unstimulated cells, β-catenin is either bound to E-cadherin, at the cell membrane, or resides unbound in the cytoplasm (Benjamin and Nelson, 2008). Cytoplasmic β-catenin is bound rapidly by adenomatous polyposis coli (APC) and axin, allowing glycogen synthase kinase (GSK)-3β to phosphorylate β-catenin, which promotes its degradation (Price, 2006). Wnt binding to its frizzled (Fzd) receptor prevents axin and APC binding to β-catenin, thus inhibiting its destruction (Rao and Kuhl, 2010). This allows β-catenin to accumulate in the cytoplasm and then translocate into the nucleus, where it interacts with the transcription factors T-cell-specific transcription factor (TCF) and lymphoid-enhancer-binding

factor (LEF) to induce transcription of Wnt target genes (Widelitz, 2005).

Wnt molecules also signal through two non-canonical pathways; in the Wnt–Ca^{2+} pathway, binding of Wnt to Fzd induces Ca^{2+} influx through calcium-release-activated calcium (CRAC) channels to regulate downstream effectors, such as calcineurin and nuclear factor of activated T-cells (NFAT) (Medyouf and Ghysdael, 2008). The Wnt–planar cell polarity (PCP) pathway regulates the polar orientation of a cell using small GTPases, such as Cdc42 and Rho-A, to alter cell–cell adhesion through cadherin molecules (Widelitz, 2005). To date, these two pathways have been poorly defined, and their downstream effects appear to be tissue- and cell-type-specific. Wnt4 has been shown to activate both canonical and non-canonical pathways (Wang et al., 2007; Chang et al., 2007); however, there is presently no information in the literature regarding regulation of non-canonical Wnt pathways in the pituitary.

Wnt signaling might be involved in pituitary pathophysiology: Wnt4 affects expansion of specific cell types in the normal developing mouse pituitary. It is expressed from embryonic day 9.5 (E9.5) to E14.5, and *Wnt4*$^{-/-}$ mice have diminished cell numbers in the anterior pituitary (Treier et al., 1998; Potok et al., 2008). Wnt4 is rapidly upregulated by estrogen during uterine growth in mice and this is associated with activation of the canonical signaling pathway (Hou et al., 2004). Molecules associated with Wnt signaling, such as the frizzled receptor, APC, β-catenin and TCF, are expressed in the developing mouse pituitary (Douglas et al., 2001), and β-catenin has been shown to interact with Prop-1 to control key stages in cell fate determination in the developing pituitary (Olson et al., 2006). The evidence regarding the downstream effects of Wnt molecules in the adult pituitary is contentious. Semba and colleagues (Semba et al., 2001) found frequent nuclear accumulation of β-catenin in 57% of the human pituitary adenomas that they studied. However, in a similar study using 54 human pituitary adenomas, Miyakoshi and colleagues (Miyakoshi et al., 2008) found that, although Wnt4 expression was increased in adenomas producing growth hormone (GH), thyroid-stimulating hormone (TSH) and PRL, β-catenin was restricted to the cell membrane and was never found in the nucleus, suggesting a non-canonical action of Wnt4 (Miyakoshi et al., 2008). The same group also reported that Wnt4 was specifically expressed in the majority of somatotrophs and in a few thyrotrophs in the untreated rat pituitary, and that estrogen increased Wnt4 expression in these cell types (Miyakoshi et al., 2009). Finally, downregulation of Wnt inhibitory factor 1 (WIF1) has been reported in a series of human pituitary tumors, and this was associated with increased nuclear β-catenin accumulation, and transfection of GH3 cells with WIF1 decreased cell proliferation (Elston et al., 2008).

In the present study we conducted a microarray analysis on pituitary tissue obtained from estrogen-treated Fischer 344 rats in order to identify novel genes and pathways involved in lactotroph hyperplasia. Among numerous genes upregulated by estrogen, we noted induction of *Wnt4* and *Wnt10a*. The induction of Wnt4 mRNA and protein was confirmed both in vivo, in the rat pituitary gland after estrogen treatment, and in vitro, in the somatolactotroph GH3 cell line. We sought to clarify whether Wnt4 acts via the canonical pathway in the pituitary gland, and found no evidence for activation of canonical Wnt signaling in either GH3 cells or primary Fischer 344 rat pituitary cells. However, Wnt4 did inhibit Ca^{2+} oscillations in GH3 cells, suggesting that non-canonical signaling pathways might be involved in the pituitary remodeling response to estrogen.

Results

Wnt signaling components are upregulated in estrogen-induced pituitary hyperplasia

Treatment of female Fischer 344 rats with DES for three weeks resulted in a twofold increase in uterus weight, a threefold increase in pituitary weight and a twofold increase in *PRL* mRNA expression (Fig. 1). Microarray analysis was conducted on the estrogen-treated pituitary tissue using an Affymetrix rat genome 2.0 array. Analysis of the array data revealed significant increases in a series of mRNAs that we expected to identify, including those encoding galanin, Pttg1 and transforming growth factor alpha (TGFα) (see Table 1 for selected examples, a more complete data set is shown in supplementary material Table S1). A number of genes involved in cell proliferation were also upregulated, including those encoding calpain 8, calbindin 3, cyclin A2, cyclin B2 and Ki67 (Mki67). *Wnt4* and *Wnt10a*, which are both known to play roles in the developing pituitary, were both markedly increased (75- and 6.6-fold respectively). Other genes related to Wnt signaling pathways were also upregulated, including those encoding carboxypeptidase Z (Cpz), protein kinase C (PKC)-β1 and Wif1. Jagged 2 and delta-like 3, two members of the notch signaling pathway, which is known to interact with the Wnt pathway, were also increased (fourfold and sevenfold, respectively). To validate the microarray analysis, quantitative RT–PCR analysis confirmed significant increases in *Wnt10a*, *Wnt4* and *Cpz* expression in rat pituitary tissue (Fig. 2).

Wnt4 protein expression in rat anterior pituitary tissue

In order to identify the Wnt4-expressing endocrine cell types in the pituitary, adult female rat pituitary tissue was analyzed by dual immunofluorescence staining for Wnt4 with GH, PRL, adrenocorticotropic hormone (ACTH), luteinizing hormone (LH) and TSH-β (Fig. 3C–G). The specificity of the anti-Wnt4 antibody was assessed using adult Fischer 344 rat kidney tissue, and we confirmed expression of Wnt4 in cortical renal tubules and absence of expression in adjacent cells (Fig. 3A), in keeping with the

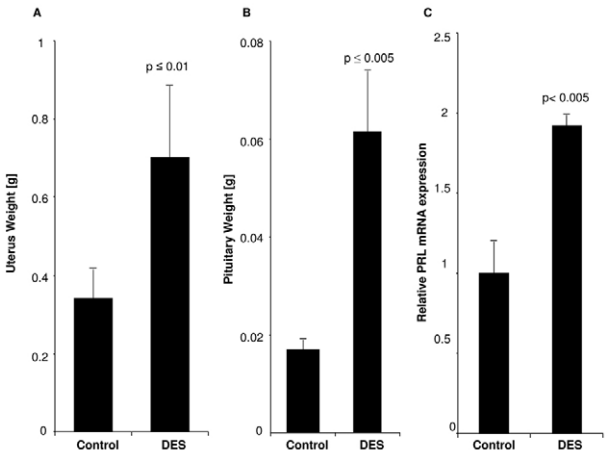

Fig. 1. Three weeks of DES treatment induces uterus growth and lactotroph hyperplasia in Fischer 344 rats. Fischer 344 rats were treated with either placebo or 10 mg of DES in slow-release pellets for 3 weeks. The uterus weight in DES-treated rats increased twofold (**A**), whereas pituitary weight increased threefold (**B**) in comparison with the weight in controls. *PRL* mRNA was increased twofold by DES treatment (**C**). Data are means+s.e.m. (*n*=5).

Table 1. Selected examples of induced genes from microarray analysis of DES-treated pituitary tissue

Gene name	GenBank accession number	Mean expression level		
		Control	DES-treated	Fold change
Galanin	NM_033237	38.6	10800	279
Dopamine receptor 4	BI284462	36.1	589	16.3
Pituitary tumor transforming gene 1	NM_022391	9.64	272	28.2
Transforming growth factor alpha	BG670310	0.58	66.7	114
Calpain 8	D14480	14.0	705	50.3
Calbindin 3	NM_012521	25.7	5010	195
Cyclin A2	AA998516	0.14	103	718
Cyclin B2 (predicted)	AW253821	5.23	258	49.3
Ki-67	AI714002	1.85	185	100
Wnt4	NM_053402	5.35	403	75.3
Wnt10a	AI029140	101	670	6.60
Carboxypeptidase Z	NM_031766	10.7	353	33.1
Protein kinase C beta 1	M13706	7.26	216	29.7
Wnt inhibitory factor 1	NM_053738	5.30	20.9	3.94
Jagged 2	AI715578	36.5	151	4.12
Delta-like 3	BE107343	1.27	9.11	7.15

Genes have been split into three functional groups. The top section refers to genes previously known to be upregulated in the pituitary in response to estrogen, showing the expected increases in a hyperplastic response. The middle section refers to genes involved in cell proliferation. The bottom section shows increases in Wnt ligands and Wnt-associated genes expressed in the pituitary. All gene expression changes in the table have a probability of positive log-ratio (PPLR) value of close to 1 indicating a high degree of significance (Bolstad et al., 2003).

previously observed expression patterns in this tissue (Terada et al., 2003). All of the endocrine cell types in the anterior pituitary were found to express Wnt4, although the prevalence was highest in somatotrophic cells. No major alterations in coexpression patterns were seen in animals treated with estrogen.

Estradiol induces Wnt4 expression in somatolactotroph GH3 cells

To investigate the effect of estrogen on Wnt signaling in the pituitary, we used the somatolactotroph GH3 cell line as a model system. Estradiol induced a slow proliferative response in the rat pituitary GH3 cell line; cell number increased by 50% after stimulation for 4 days, and was almost doubled after 7 days (Fig. 4A). Quantitative RT–PCR (Q-PCR) analysis confirmed induction of *PRL* mRNA in GH3 cells, by eightfold at 24 hours and 14-fold at 72 hours (Fig. 4B). The level of *Wnt4* mRNA was unchanged at 24 hours, but was increased 2.5-fold at 72 hours (Fig. 4C), and the level of Wnt4 protein was increased at 72, 120 and 168 hours (Fig. 4D).

Estradiol does not induce canonical Wnt signaling in GH3 cells

Activation of the canonical Wnt signaling pathway was assessed using the TopFlash reporter gene, which displays a transcriptional response to activation of TCF and LEF by β-catenin, inducing luciferase expression. No induction of luciferase activity occurred in GH3 cells after treatment with either estradiol or Wnt4-conditioned medium. Lithium chloride and Wnt3a-conditioned medium, two well-characterized inducers of canonical Wnt signaling in many cell lines, were also unable to induce TCF-mediated gene expression in GH3 cells, but gave a robust induction in human embryonic kidney HEK-293 cells (27-fold and sevenfold, respectively; Fig. 5A). RT–PCR analysis of Fzd receptor expression confirmed that Fzd2, 4, 5 and 6 were all expressed in GH3 cells (data not shown), indicating that their lack of response to Wnt ligands was not related to the absence of receptor expression. Furthermore, GH3 cells cotransfected with plasmids encoding a constitutively active mutant β-catenin (mβ-Cat) and the TopFlash reporter showed no induction of luciferase, whereas HEK-293 cells treated under the same conditions exhibit a 14-fold induction of luciferase (Fig. 5B). Taken together, these results indicate that canonical Wnt signaling is not inducible in pituitary GH3 cells.

Translocation of β-catenin from the cytoplasm to the nucleus is a key feature of activation of the canonical Wnt pathway. Immunocytochemical analysis of GH3 cells and primary pituitary cells showed that β-catenin was predominantly at the cell membrane in unstimulated cells, with no apparent nuclear staining. Neither treatment with estradiol nor LiCl was able to induce nuclear

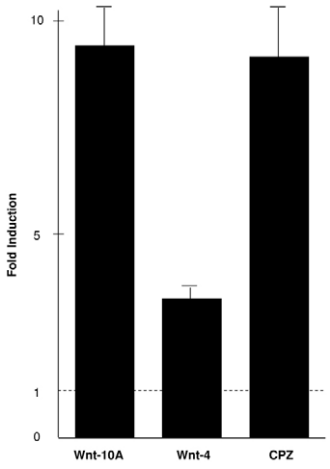

Fig. 2. Quantitative real-time RT–PCR validation of microarray analysis. Q-PCR for genes of interest was performed on pooled RNA extracted from the pituitary glands of Fischer 344 rats. Three weeks of DES treatment induced upregulation of *Wnt4*, *Wnt10a* and *Cpz* (threefold, tenfold and tenfold, respectively) mRNA in comparison with the level in controls. Data are means+s.e.m. (*n*=5).

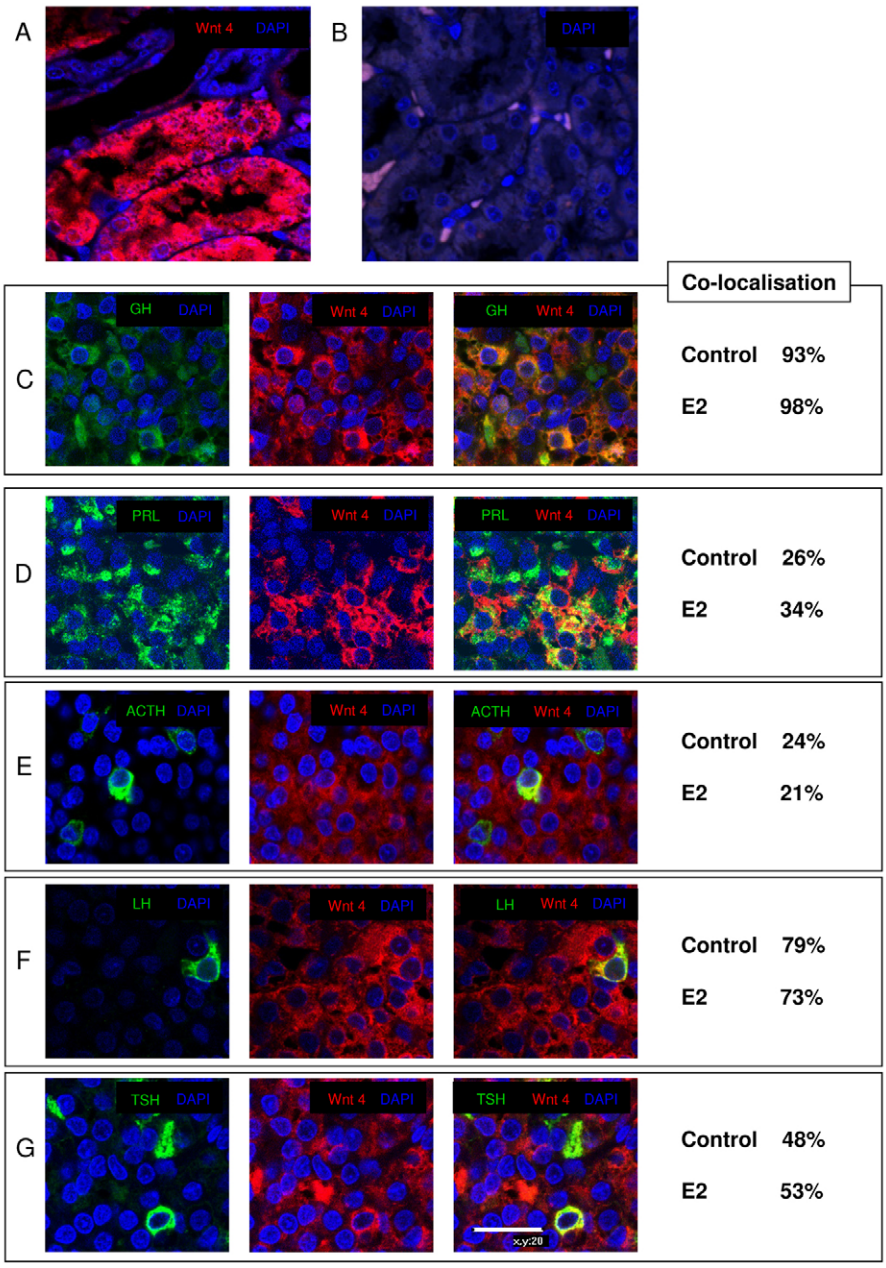

Fig. 3. Wnt4 protein expression in secretory cell types in the rat anterior pituitary. (**A**,**B**) Validation of the anti-Wnt4 antibody staining using rat kidney tissue. Specific staining is seen in cortical renal tubules (A), consistent with previously published material. Panel B is a control with secondary antibody only. (**C**–**G**) Pituitary sections from untreated adult female rats were co-stained for Wnt4 (red) with GH (C), PRL (D), ACTH (E), LH (F) and TSH-β (G). The immunostaining of the hormone is shown in the left-hand column, Wnt4 staining is shown in the central column and merged images in the right-hand column. Examples of Wnt4 colocalization with each hormone are shown, and the percentage of each endocrine cell type coexpressing Wnt4 in untreated (Control) or estrogen (E2)-treated rats is indicated.

translocation of β-catenin, and membrane staining remained unchanged, further indicating that canonical signaling was not activated in GH3 cells or in primary rat pituitary cell cultures (Fig. 6).

Wnt4 inhibits Ca^{2+} oscillations in GH3 cells

GH3 cells are known to have spontaneous oscillations in intracellular Ca^{2+} concentrations (Haymes and Hinkle, 1993). To assess whether Wnt4 signals through the Wnt–Ca^{2+} pathway, we carried out live-cell Ca^{2+} imaging on GH3 cells loaded with Fluo-4 and measured whether treatment with Wnt4 recombinant protein modulated the spontaneous Ca^{2+} oscillations (Fig. 7). The addition of Wnt4 partially or completely suppressed oscillations in intracellular Ca^{2+} in 63% of cells. By comparison, no change was observed in the oscillations following the addition of a control medium (Fig. 7E). Wnt4 induced the total inhibition of oscillations for long periods in some cells (Fig. 7A), whereas in other cells it induced a reduction in frequency and amplitude of oscillations (Fig. 7B), gave a temporary inhibition of oscillations (Fig. 7C) or had no effect (Fig. 7D). Immunoneutralization, by prior incubation with an anti-Wnt4 antibody, completely prevented the inhibition of the Ca^{2+} transient currents by Wnt4 (Fig. 7F). The proportion of the cells displaying complete suppression, partial inhibition or no effect is shown in Fig. 7G. Flow cytometry analysis indicated that 63% of GH3 cells in these conditions were in the G1 phase of the cell cycle, 36% were in S phase and 1% were in G2 or M phase (data not shown), suggesting a potential link between the Ca^{2+} response to Wnt4 and the cell cycle.

Discussion

Here, we have provided evidence for the involvement of Wnt4 in the proliferative response of the pituitary gland to estrogen. We

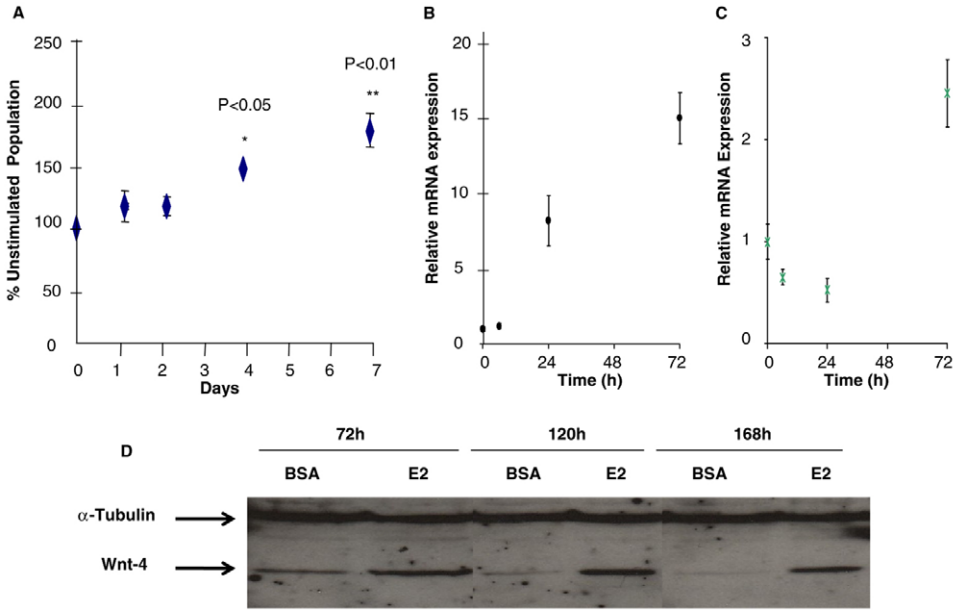

Fig. 4. Effects of estradiol in the GH3 somatolactotroph cell line. (**A**) GH3 cell proliferation assay. GH3 cells were treated for 1, 2, 4 and 7 days with 10 nM E2, inducing a proliferative response. Q-PCR analysis of *PRL* (**B**) and *Wnt4* (**C**) expression treatment with 10 nM E2 for 6, 24 and 72 hours. (**D**) Western blotting analysis of Wnt4 protein expression following treatment with 10 nM estrogen for 72, 120 and 168 hours. Staining of α-tubulin was used as a loading control. Data are means±s.e.m. (*n*=3).

have shown that Wnt4 expression was increased upon estrogen treatment in pituitary tissue in vivo and that its expression is also induced by estrogen in the somatolactotroph GH3 cell line. Expression of Wnt4 in the pituitary was not restricted to lactotrophic cells, and Wnt4 action in pituitary cells did not involve the canonical β-catenin signaling pathway, implying that other pathways are likely to be involved.

Wnt4 is a growth factor involved in many developmental processes and is thought to have a role in fetal pituitary development (Treier et al., 1998; Potok et al., 2008; Brinkmeier et al., 2009). Wnt4 is expressed in the developing mouse pituitary between e9.5 and e14.5 and might regulate differentiation of ventral cell types (Potok et al., 2008; Treier et al., 1998), perhaps through interaction with Prop1 (Olson et al., 2006). Wnt4-deficient mice display pituitary hypoplasia, at least affecting the somatotroph and thyrotroph lineages (Potok et al., 2008); however, until now, very little information regarding Wnt4 in the adult rat pituitary has been available. In the present study, we found that the expression of Wnt4 in the adult rat was not confined to the lactotroph population but was readily detectable in all of the endocrine cell types in the pituitary. Estrogen treatment increased the proportion of lactotrophic cells in the pituitary (data not shown), as expected, but did not markedly alter the proportions of cell types that expressed Wnt4. Previous data from Miyakoshi and colleagues (Miyakoshi et al., 2009) suggested that Wnt4 expression was confined to the GH-producing cells and a minority of the TSH-producing cells in the rat pituitary. The reasons for the discrepancy between the Miyakoshi study and the present study are not clear, but we found that Wnt4 expression was seen in over 90% of somatotrophic cells. Other reasons for this difference could relate to the different rat strains used (Fischer 344 in the present study and Sprague Dawley in the Miyakoshi study), or the antibodies used for

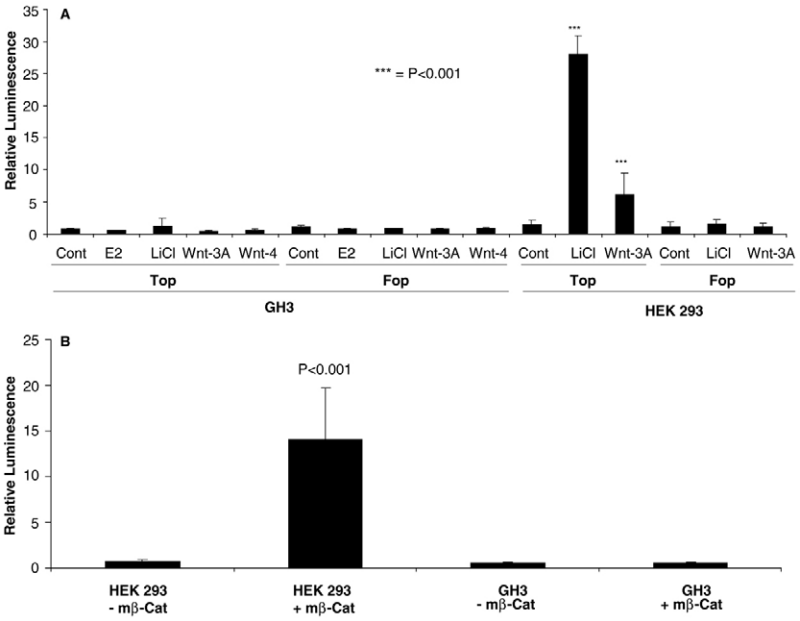

Fig. 5. Canonical Wnt signaling is not functional in pituitary GH3 cells. (**A**) TopFlash reporter gene assay in GH3 and HEK-293 cells. Cells were transiently transfected with either TopFlash or the FopFlash control and then stimulated for 24 hours with control medium (DCT-FBS), E2 (10 nM), LiCl (10 mM), Wnt3a-conditioned medium or Wnt4-conditioned medium. (**B**) HEK-293 cells and GH3 cells were transfected for 24 hours with TopFlash with (+) or without (–) a constitutively active mutant β-catenin expression vector (mβ-Cat). Spontaneous activity of mβ-Cat was measured in HEK-293 cells and GH3 cells. Data are means+s.e.m. (*n*=3).

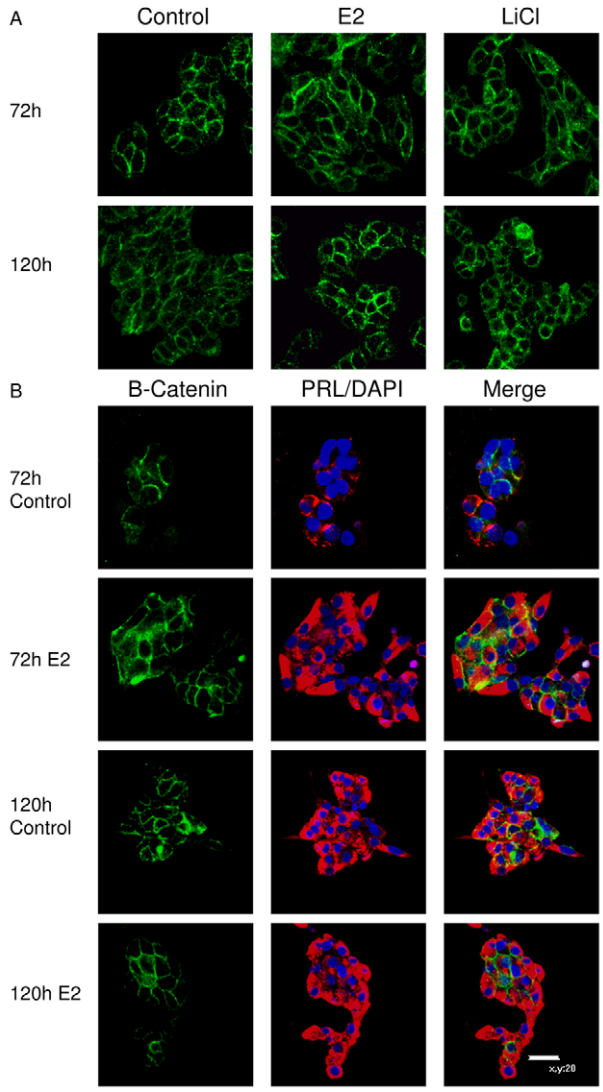

Fig. 6. Subcellular β-catenin localization is unchanged in lactotrophs upon estrogen treatment. GH3 cells (**A**) or cultures of dispersed primary pituitary cells (**B**) were treated with control (0.5% BSA), E2 (10 nM) or LiCl (10 mM) for 72 or 120 hours, followed by immunofluorescence analysis of β-catenin and PRL protein localization. β-Catenin staining is shown in green (A, and left-hand column in B). In B, the middle column shows PRL (red) and DAPI (blue) staining, and the right-hand column shows merged images.

immunohistochemistry; the antibody used in the previous report was raised against a 14-amino-acid oligopeptide, whereas our antibody was produced against a 100-residue peptide. Antibody specificity was confirmed in the present study by checking for the specific staining in renal tubules, the lack of non-specific staining in the absence of primary antibody and by establishing that a protein of the appropriate size was detected by western blotting.

Wnt4 was recently found to be highly expressed in several human pituitary adenoma types, including lactotroph, somatotroph and thyrotroph adenomas (Miyakoshi et al., 2008). The authors of that report speculated that Wnt4 might be involved in proliferation of those cell lineages, and the present findings support the general hypothesis that Wnt4 is involved in plasticity of function and structure in the adult pituitary gland. In addition, a recent study found that expression of WIF1, an extracellular inhibitor of Wnt signaling, was significantly reduced in pituitary adenomas, and that its overexpression reduced GH3 cell proliferation, further implicating Wnt signaling in pituitary growth (Elston et al., 2008).

The signaling pathway involved in Wnt action in the pituitary was previously unclear, but here we have shown that canonical signaling was not activated in the rat pituitary GH3 cell line, either by estrogen or by Wnt4 or Wnt3a. Furthermore, a constitutively active mutant β-catenin protein, which markedly activated TCF- and LEF-dependent transcriptional signaling in HEK-293 cells, had no effect in pituitary GH3 cells. We have found no evidence for nuclear localization of β-catenin in GH3 cells, in primary cultures of rat pituitary cells or in intact rat pituitary tissue (data not shown), and similarly no evidence has been found for nuclear expression of β-catenin in those pituitary adenomas that displayed Wnt4 overexpression (Miyakoshi et al., 2008).

Non-canonical Wnt signaling pathways remain less well defined than the canonical cascade, but in different systems have been found to target Ca^{2+} signaling or kinase pathways that might affect PCP (Rao and Kuhl, 2010). One target of the Wnt–PCP pathway is E-cadherin, through which Wnt ligands might alter cell–cell adhesion in order to control the orientation and development of a number of organs (Fanto and McNeill, 2004). The cellular patterns of cadherin expression in the pituitary have recently been identified (Chauvet et al., 2009), and the interaction of β-catenin and E-cadherin at the cell membrane might play an important role in the development and plasticity of cell networks. We show here, for the first time, that Wnt4 has an impact upon non-canonical signaling in pituitary cells by inhibiting spontaneous Ca^{2+} oscillations. The mechanism by which this inhibition occurs has not been studied but might well involve inactivation of CRAC channels (Gwack et al., 2007), although further study will need to be performed to elucidate the details of this signaling pathway in the pituitary. The Wnt–Ca^{2+} pathway has recently been implicated in the progression of a number of cancers, where it has both suppressive and inductive properties (McDonald and Silver, 2009), and, therefore, study into the effects of the non-canonical actions of Wnt4 in the pituitary is likely to throw new light on the nature of adult pituitary remodeling and adenoma formation.

Materials and Methods

Microarray analysis

Female Fischer 344 rats were implanted with slow-release subcutaneous pellets (containing 10 mg of DES, *n*=5, or placebo, *n*=5; Innovation Research, Novi, MI) for 3 weeks. Animal experiments were performed according to UK Home Office guidelines and rats were killed by a schedule 1 method. Pituitary glands were harvested, then washed with ice-cold PBS and snap-frozen on dry ice. RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's instructions. Expression profiling was performed using an Affymetrix gene chip rat genome 2.0 array (no. 230; three chips per group with individual animals for each chip). Background correction, quantile normalization and gene expression analysis were performed using the robust multichip average (RMA) function in Bioconductor (Bolstad et al., 2003).

Cell culture

GH3 cells and HEK-293 cells (ATCC, Rockville, MD) were grown at 37°C under a 5% CO_2 atmosphere, in Phenol-Red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g of glucose/l, 10% fetal bovine serum (FBS) and 1% Glutamax (all Gibco). Two stably transfected cell lines were used to obtain conditioned media: LM (TK–) cells transfected with a Wnt3a expression vector (ATCC) and NIH-3T3 cells transfected with a Wnt4 expression vector (kindly donated by Andreas Kispert, Institute of Molecular Biology, Hannover Medical School, Hannover, Germany). The conditioned media were generated as previously described (Willert et al., 2003).

PCR

For the verification of microarray results, RNA from all five animals from each group was pooled for quantitative real-time PCR (Q-PCR) analysis. For cell culture

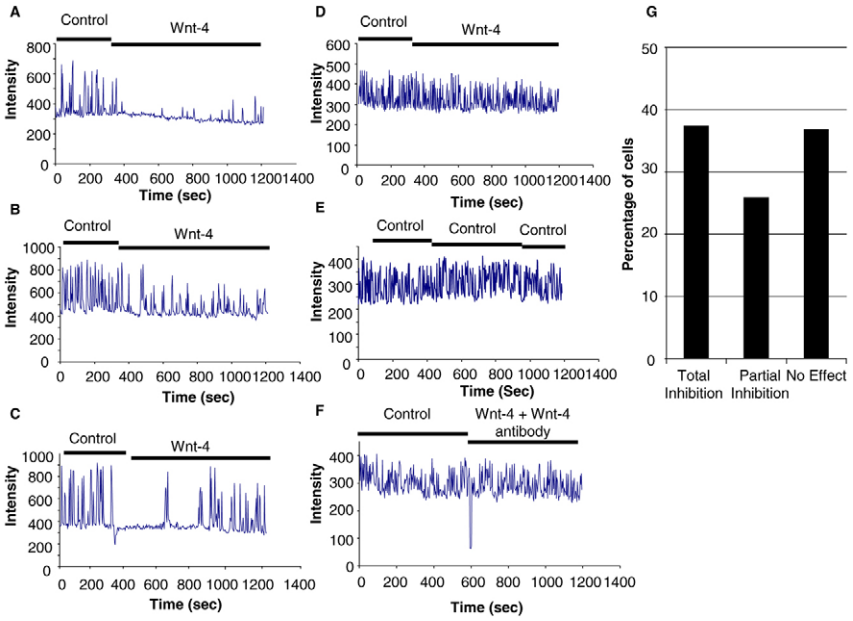

Fig. 7. Ca^{2+} oscillations in GH3 cells. GH3 cells were loaded with Fluo-4 and images were taken every 3 seconds. Areas of interest were drawn around cells and the fluorescence in each cell was quantified over the course of the experiment. Cells were treated with control (**E**) (DCT-FBS; no effect seen in 35/35 cells), Wnt4 recombinant protein (200 ng/ml) (**A–D**) or Wnt4 protein that had been immunoneutralized by incubation with the anti-Wnt4 antibody (**F**) (no effect observed in 19/19 cells). (**G**) A bar graph showing the percentage of cells exhibiting total inhibition, partial inhibition and no effect upon Wnt4 treatment. The total cell number studied was 209.

timecourse experiments, GH3 cells were grown in 25 cm^2 flasks (~5×10^6 cells), serum-starved for 24 hours and then stimulated with 10 nM E2 (Sigma) for the indicated times. Cells were harvested by trypsinization and washed twice in ice-cold PBS. Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's instructions.

cDNA was generated with the Omniscript RT system (Qiagen). Q-PCR was performed using the Stratagene Mx3000 P thermocycler (Stratagene) and the SYBRgreen Jump Start Taq ready mix (Sigma). Cyclophilin was used as the housekeeping gene for normalization. The following primers were used: cyclophilin, 5′-TTTTCGCCGCTTGCTGCAGAC-3′ and 5′-CACCCTGGCACATGAATCCTGGA-3′; PRL, 5′-AGCCAAGTGTCAGCCCGGAAAG-3′ and 5′-TGGCCTTGGCAATAAACTCACGA-3′; Wnt4, 5′-acgaggggccgcacttgcaaca-3′ and 5′-ggcacaggcgggcacggtcac-3′; Cpz, 5′-ccccagggcgcgtaggcagc-3′ and 5′-cccgggccgtggaggtggacat-3′; and Wnt10a, 5′-ccagcttcagtgcattgcccaaca-3′ and 5′-agctcaggcaggtgggggtggtag-3′.

GH3 cell proliferation assay

GH3 cells were plated at a density of 1×10^4 cells per well in 96-well plates. They were starved for 24 hours in 0.25% BSA, before stimulation at defined timepoints. The cell number was measured using the CellTiter 96 AQ_{ueous} one solution cell proliferation assay (MTS; Promega) according to the manufacturer's instructions.

Antibodies

Primary antibodies used were against: Wnt4 (rabbit Ig, 1:1000 for western blot and 1:50 for immunohistochemistry; SDI, Newark, Germany), α-tubulin (mouse Ig, 1:25000; Abcam, Cambridge UK), GH (goat Ig, 1:50; R&D Systems, Abingdon, UK), PRL (mouse Ig, 1:4000, Pierce, Rockford, IL), TSH (guinea-pig Ig, 1:100; NIDDK, Bethesda, MD), ACTH (mouse Ig, 1:200; Novocastra, Milton Keynes, UK) or LH (mouse Ig, 1:1000; kindly provided by Janet Roser, University of California-Davis, Davis, CA), R51 PRL (rabbit Ig, 1:500, kindly donated by Alan McNeilly, MRC Human Reproductive Sciences Unit, Edinburgh, UK) and β-catenin (mouse Ig, 1:400; BD Transduction Laboratories, Oxford, UK).

Secondary antibodies were: anti-(rabbit Ig)–HRP-conjugated (donkey Ig, 1:2000) and anti-(mouse Ig)–HRP (donkey Ig, 1:25000) (both from Santa Cruz Biotechnology), anti-(rabbit Ig)–Alexa-Fluor-546 (donkey Ig, 1:500, Invitrogen), anti-(mouse Ig)–Alexa-Fluor-488 (donkey Ig, 1:1000, Invitrogen), anti-(guinea-pig Ig)–FITC (goat Ig, 1:64, Sigma), anti-(goat Ig)–FITC (donkey Ig, 1:500, Santa Cruz Biotechnology) and anti-(rabbit Ig)–Texas-Red (donkey 1:500, Santa Cruz Biotechnology).

Western blotting analysis

GH3 cells were plated at ~7.5×10^5 cells per well in six-well plates and serum-starved in 10% dextran-charcoal-treated FBS (DCT-FBS) (Perbio Scientific, Cramlington, UK) for 24 hours before stimulation with either control (DCT-FBS) or E2 (10 nM, Sigma). Cells were washed twice with ice-cold PBS and lysed using RIPA buffer with Complete mini EDTA-free protease inhibitors (Roche). Cells were scraped, agitated for 30 minutes on a rocker and centrifuged for 10 minutes at 12,000 ***g*** at 4°C, and the supernatant was collected and stored at –80°C. Samples were subjected to SDS-PAGE (10% gels), before transfer onto nitrocellulose membrane. Primary antibodies were applied overnight at 4°C, then secondary antibodies were applied for 1 hour at room temperature. Staining was detected with EZ-ECL (Pierce) using Kodak Biomax XAR film.

Immunofluorescence

Female Fischer 344 rats were killed by a schedule 1 method and pituitary glands were removed for either wax-embedding or dissociation. Pituitary glands were fixed for 2 hours in 4% PFA before wax-embedding and sectioning at a thickness of 5 µm. Deparaffinization of wax-embedded slices was conducted in xylene, and sections were subsequently re-hydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by boiling in 10 mM sodium citrate for 20 minutes. Pituitary cells were dissociated as previously described (Sartor et al., 2004), and cultures of primary pituitary cells or GH3 cells were plated on poly-(L-lysine)-coated glass coverslips and stimulated as indicated, before fixation in 4% PFA.

Cells or tissue were blocked in 5% donkey serum in PBS for 1 hour, and incubated with the first primary antibody overnight at 4°C followed by the secondary antibody for 2 hours at room temperature. Samples were blocked again in 5% donkey serum, before an overnight incubation with the second primary antibody at 4°C, followed by the secondary antibody for 2 hours at room temperature. Samples were treated with DAPI (0.1 µg/ml, Sigma) for 20 minutes at room temperature, and then mounted for analysis in Permafluor (Thermo Scientific). Images were collected using a Nikon C1 confocal microscope (Bioimaging Facility, Faculty of Life Sciences, University of Manchester, Manchester, UK).

Reporter gene assays

GH3 and HEK-293 cells were plated in sterile white 96-well plates at ~1×10^4 cells per well and ~5×10^3 cells per well, respectively. Cells were transfected using Fugene 6 transfection reagent (Roche) according to the manufacturer's instructions. Cells were transfected with either Super 8× TopFlash, Super 8× FopFlash negative control (both at 0.1 µg per well; Addgene, Cambridge, MA) or a vector encoding mβ-Cat [0.1 µg per well; a kind gift from Hans Clevers (Hubrecht Institute, Utrecht, The Netherlands) (Morin et al., 1997)] with pRL-TK Renilla (Promega, 0.1 µg per well for GH3 cells and 0.01 µg per well for HEK-293 cells). Fugene:DNA ratios were 3:1 for GH3 cells and 6:1 for HEK-293 cells. Cells were left for 24 hours, then stimulated as specified. Luminescence was measured using the Dual-Glo luciferase assay system (Promega) according to the manufacturer's instructions.

Live-cell Ca^{2+} imaging

GH3 cells were seeded in glass-bottomed dishes (Iwaki) and left to settle for 24 hours. Cells were loaded with Fluo-4 (Invitrogen) for 30 minutes and then the dish was transferred onto the stage of a Zeiss Axiovert 200 microscope with an attached XL incubator (at 37°C and under a humid 5% CO_2 atmosphere). Cells were either stimulated with vehicle (DCT-FBS) or Wnt4 recombinant protein (Novus Biologicals, Littleton, CO). For the immunoneutralization experiment, Wnt4 protein was incubated with Wnt4 antibody for 1 hour at room temperature, before addition to cells in a 10:1 antibody:protein molar ratio. Images were taken every 3 seconds, areas of interest were drawn around cells and mean intensity throughout the experiment was calculated using Kinetic Imaging AQM6 software (Andor, Belfast, UK).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/4/540/DC1

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