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)

Accepted 8 October 2010
Journal of Cell Science 124, 540-547

doi:10.1242/jcs.078642

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

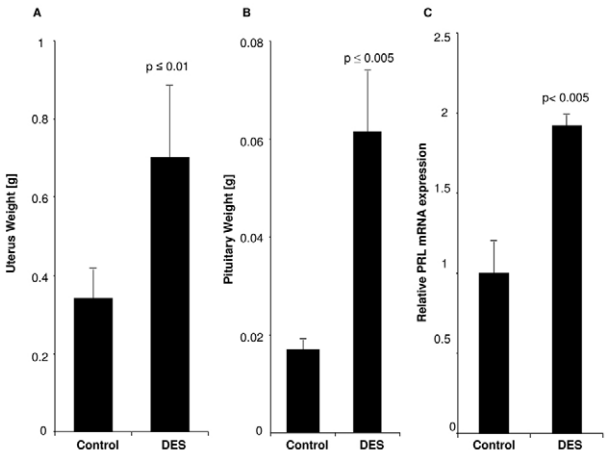

Fig. 1. Three weeks of DES treatment induces uterus growth and lactotroph hyperplasia in Fischer 344 rats. Fischer 344 rats were treated with either placebo or 10 mg of DES in slow-release pellets for 3 weeks. The uterus weight in DES-treated rats increased twofold (**A**), whereas pituitary weight increased 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

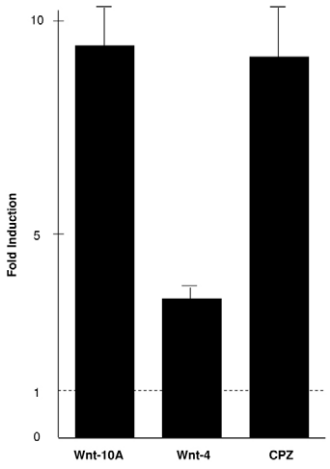

Fig. 2. Quantitative real-time RT–PCR validation of microarray analysis. Q-PCR for genes of interest was performed on pooled RNA extracted from the pituitary glands of Fischer 344 rats. Three weeks of DES treatment induced upregulation of *Wnt4*, *Wnt10a* and *Cpz* (threefold, tenfold and tenfold, respectively)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

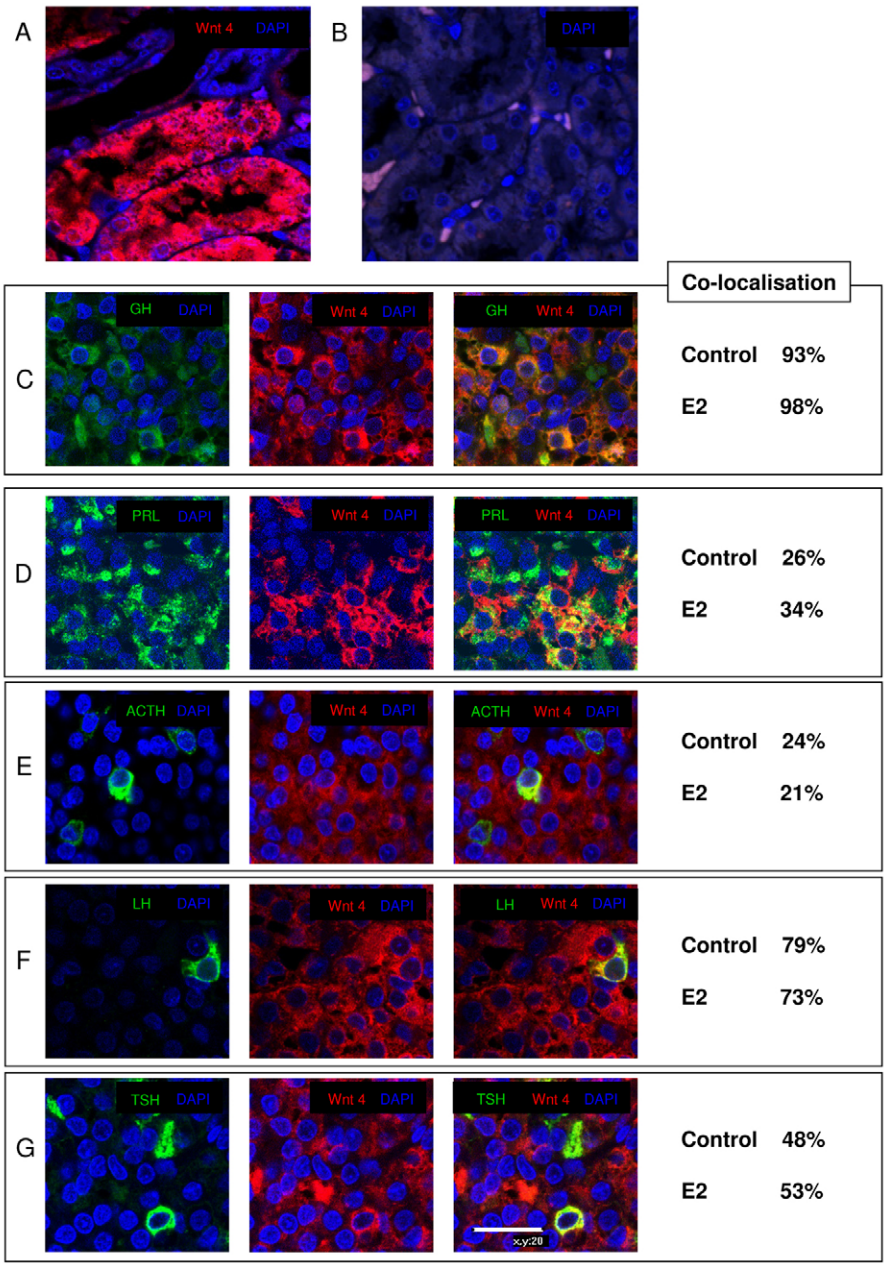

Fig. 3. Wnt4 protein expression in secretory cell types in the rat anterior pituitary. (**A**,**B**) Validation of the anti-Wnt4 antibody staining using rat kidney tissue. Specific staining is seen in cortical renal tubules (A), consistent with previously published material. Panel B is a control with 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

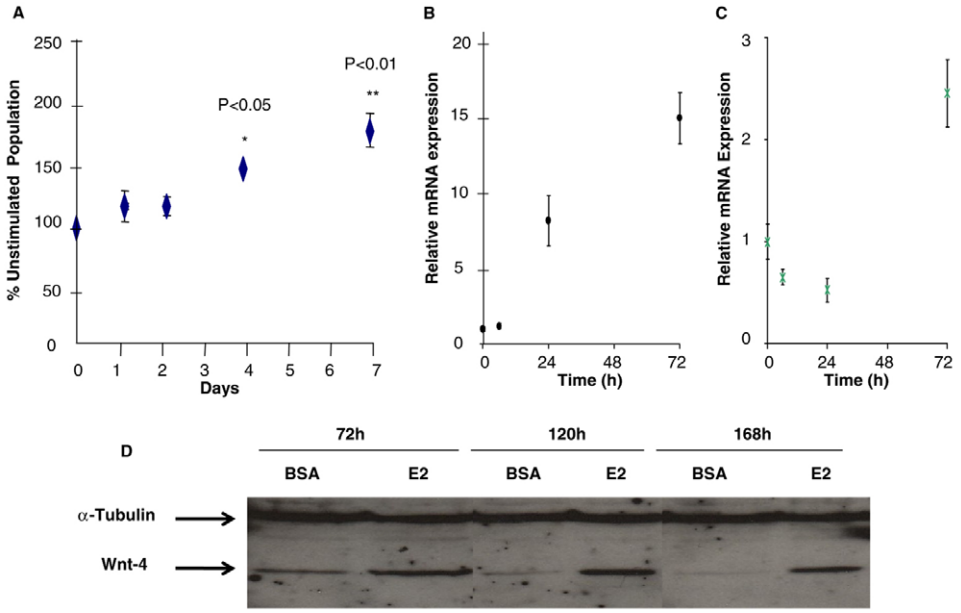

Fig. 4. Effects of estradiol in the GH3 somatolactotroph cell line. (**A**) GH3 cell proliferation assay. GH3 cells were treated for 1, 2, 4 and 7 days with 10 nM E2, inducing a proliferative response. Q-PCR analysis of *PRL* (**B**) and *Wnt4* (**C**) expression treatment with 10 nM E2 for 6, 24 and 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

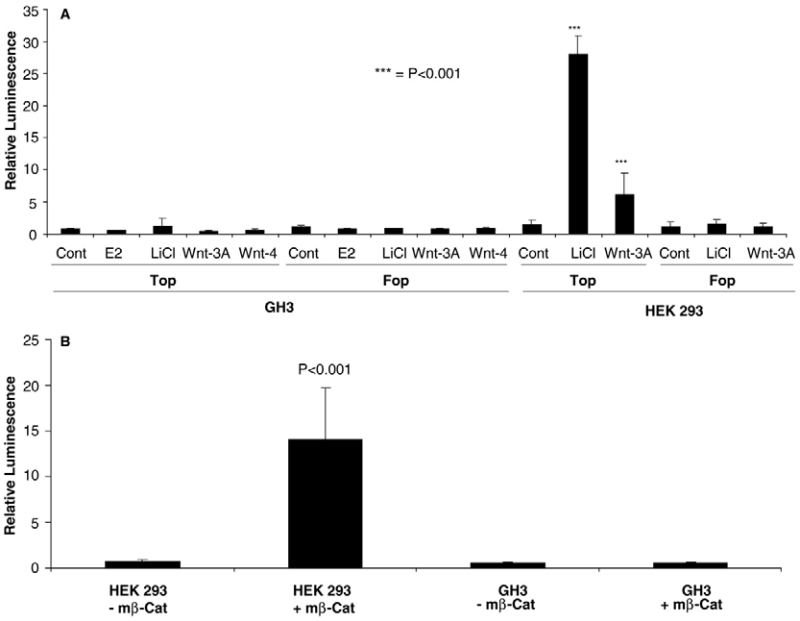

Fig. 5. Canonical Wnt signaling is not functional in pituitary GH3 cells. (**A**) TopFlash reporter gene assay in GH3 and HEK-293 cells. Cells were transiently transfected with either TopFlash or the FopFlash control and then stimulated for 24 hours with control medium (DCT-FBS), E2 (10 nM), LiCl (10 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).

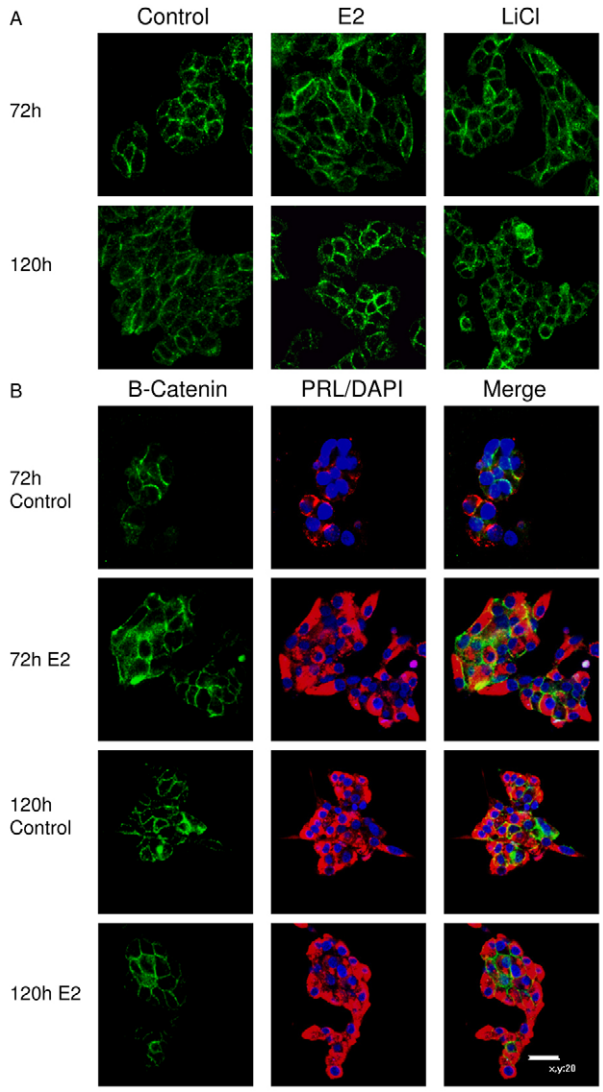

Fig. 6. Subcellular β-catenin localization is unchanged in lactotrophs upon estrogen treatment. GH3 cells (**A**) or cultures of dispersed primary pituitary cells (**B**) were treated with control (0.5% BSA), E2 (10 nM) or LiCl (10 mM) for 72 or 120 hours, followed by immunofluorescence analysis of β-catenin 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

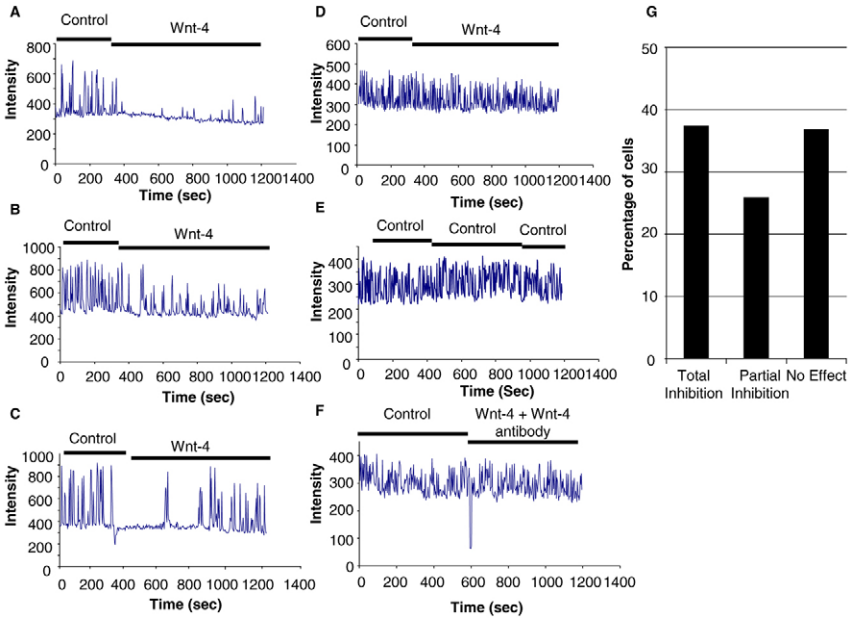

Fig. 7. Ca^{2+} oscillations in GH3 cells. GH3 cells were loaded with Fluo-4 and images were taken every 3 seconds. Areas of interest were drawn around cells and the fluorescence in each cell was quantified over the course of the experiment. Cells were treated with control (**E**) (DCT-FBS; no effect 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).

We thank the Wellcome Trust, UK for funding this work (project grant 082794, to J.R.E.D. and G.B.). A.G. was supported by a PhD studentship from the Biotechnology and Biological Sciences Research Council (BBSRC), UK. C.V.H. was supported by the BBSRC (BBF0059381). The Centre for Cell Imaging has been supported through BBSRC REI (grant BBE0129651), and Hamamatsu Photonics and Carl Zeiss Limited provided technical support. We are grateful to Dave Spiller for assistance in the Centre for Cell Imaging, University of Liverpool, and to Leo Zeef, University of Manchester Genomic Technology Facility, for valuable help in the analysis of the array data. The Developmental Biomedicine Research Group is supported by the Manchester Academic Health Sciences Centre (MAHSC) and the NIHR Manchester Biomedical Research Centre. Deposited in PMC for release after 6 months.

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

References

Asa, S. L., Penz, G., Kovacs, K. and Ezrin, C. (1982). Prolactin cells in the human pituitary. A quantitative immunocytochemical analysis. *Arch. Pathol. Lab Med.* **106**, 360-363.

Bahar, A., Simpson, D. J., Cutty, S. J., Bicknell, J. E., Hoban, P. R., Holley, S., Mourtada-Maarabouni, M., Williams, G. T., Clayton, R. N. and Farrell, W. E. (2004). Isolation and characterization of a novel pituitary tumor apoptosis gene. *Mol. Endocrinol.* **18**, 1827-1839.

Benjamin, J. M. and Nelson, W. J. (2008). Bench to bedside and back again: molecular mechanisms of alpha-catenin function and roles in tumorigenesis. *Semin. Cancer Biol.* **18**, 53-64.

Bilodeau, S., Vallette-Kasic, S., Gauthier, Y., Figarella-Branger, D., Brue, T., Berthelet, F., Lacroix, A., Batista, D., Stratakis, C., Hanson, J. et al. (2006). Role of Brg1 and HDAC2 in GR trans-repression of the pituitary POMC gene and misexpression in Cushing disease. *Genes Dev.* **20**, 2871-2886.

Bolstad, B. M., Irizarry, R. A., Astrand, M. and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185-193.

Brinkmeier, M. L., Davis, S. W., Carninci, P., MacDonald, J. W., Kawai, J., Ghosh, D., Hayashizaki, Y., Lyons, R. H. and Camper, S. A. (2009). Discovery of transcriptional regulators and signaling pathways in the developing pituitary gland by bioinformatic and genomic approaches. *Genomics* **93**, 449-460.

Chang, J., Sonoyama, W., Wang, Z., Jin, Q., Zhang, C., Krebsbach, P. H., Giannobile, W., Shi, S. and Wang, C. Y. (2007). Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. *J. Biol. Chem.* **282**, 30938-30948.

Chauvet, N., El-Yandouzi, T., Mathieu, M. N., Schlernitzauer, A., Galibert, E., Lafont, C., Le, T. P., Robinson, I. C., Mollard, P. and Coutry, N. (2009). Characterization of adherens junction protein expression and localization in pituitary cell networks. *J. Endocrinol.* **202**, 375-387.

Douglas, K. R., Brinkmeier, M. L., Kennell, J. A., Eswara, P., Harrison, T. A., Patrianakos, A. I., Sprecher, B. S., Potok, M. A., Lyons, R. H., Jr, MacDougald, O. A. et al. (2001). Identification of members of the Wnt signaling pathway in the embryonic pituitary gland. *Mamm. Genome* **12**, 843-851.

Elston, M. S., Gill, A. J., Conaglen, J. V., Clarkson, A., Shaw, J. M., Law, A. J., Cook, R. J., Little, N. S., Clifton-Bligh, R. J., Robinson, B. G. et al. (2008). Wnt pathway inhibitors are strongly down-regulated in pituitary tumors. *Endocrinology* **149**, 1235-1242.

Fanto, M. and McNeill, H. (2004). Planar polarity from flies to vertebrates. *J. Cell Sci.* **117**, 527-533.

Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim. Biophys. Acta* **1653**, 1-24.

Goluboff, L. G. and Ezrin, C. (1969). Effect of pregnancy on the somatotroph and the prolactin cell of the human adenohypophysis. *J. Clin. Endocrinol. Metab.* **29**, 1533-1538.

Gurlek, A., Karavitaki, N., Ansorge, O. and Wass, J. A. (2007). What are the markers of aggressiveness in prolactinomas? Changes in cell biology, extracellular matrix components, angiogenesis and genetics. *Eur. J. Endocrinol.* **156**, 143-153.

Gwack, Y., Feske, S., Srikanth, S., Hogan, P. G. and Rao, A. (2007). Signalling to transcription: store-operated Ca2+ entry and NFAT activation in lymphocytes. *Cell Calcium* **42**, 145-156.

Haymes, A. A. and Hinkle, P. M. (1993). Activation of protein kinase C increases Ca2+ sensitivity of secretory response of GH3 pituitary cells. *Am. J. Physiol.* **264**, C1020-C1028.

Heaney, A. P., Horwitz, G. A., Wang, Z., Singson, R. and Melmed, S. (1999). Early involvement of estrogen-induced pituitary tumor transforming gene and fibroblast growth factor expression in prolactinoma pathogenesis. *Nat. Med.* **5**, 1317-1321.

Horvath, E. and Kovacs, K. (1988). Fine structural cytology of the adenohypophysis in rat and man. *J. Electron Microsc. Tech.* **8**, 401-432.

Hou, X., Tan, Y., Li, M., Dey, S. K. and Das, S. K. (2004). Canonical Wnt signaling is critical to estrogen-mediated uterine growth. *Mol. Endocrinol.* **18**, 3035-3049.

Kansra, S., Yamagata, S., Sneade, L., Foster, L. and Ben-Jonathan, N. (2005). Differential effects of estrogen receptor antagonists on pituitary lactotroph proliferation and prolactin release. *Mol. Cell. Endocrinol.* **239**, 27-36.

Kim, D. S., Fong, J., Read, M. L. and McCabe, C. J. (2007). The emerging role of pituitary tumour transforming gene (PTTG) in endocrine tumourigenesis. *Mol. Cell. Endocrinol.* **278**, 1-6.

Labeur, M., Paez-Pereda, M., Haedo, M., Arzt, E. and Stalla, G. K. (2010). Pituitary tumors: cell type-specific roles for BMP-4. *Mol. Cell. Endocrinol.* **326**, 85-88.

Levy, A. (2008). Molecular and trophic mechanisms of tumorigenesis. *Endocrinol. Metab. Clin. North Am.* **37**, 23-50.

Lieberman, M. E., Maurer, R. A., Claude, P. and Gorski, J. (1982). Prolactin synthesis in primary cultures of pituitary cells: regulation by estradiol. *Mol. Cell. Endocrinol.* **25**, 277-294.

Lloyd, R. V., Cano, M. and Landefeld, T. D. (1988). The effects of estrogens on tumor growth and on prolactin and growth hormone mRNA expression in rat pituitary tissues. *Am. J. Pathol.* **133**, 397-406.

McCabe, C. J., Boelaert, K., Tannahill, L. A., Heaney, A. P., Stratford, A. L., Khaira, J. S., Hussain, S., Sheppard, M. C., Franklyn, J. A. and Gittoes, N. J. (2002). Vascular endothelial growth factor, its receptor KDR/Flk-1, and pituitary tumor transforming gene in pituitary tumors. *J. Clin. Endocrinol. Metab.* **87**, 4238-4244.

McDonald, S. L. and Silver, A. (2009). The opposing roles of Wnt-5a in cancer. *Br. J. Cancer* **101**, 209-214.

Medyouf, H. and Ghysdael, J. (2008). The calcineurin/NFAT signaling pathway: a novel therapeutic target in leukemia and solid tumors. *Cell Cycle* **7**, 297-303.

Miyakoshi, T., Takei, M., Kajiya, H., Egashira, N., Takekoshi, S., Teramoto, A. and Osamura, R. Y. (2008). Expression of Wnt4 in human pituitary adenomas regulates activation of the beta-catenin-independent pathway. *Endocr. Pathol.* **19**, 261-273.

Miyakoshi, T., Kajiya, H., Miyajima, K., Takei, M., Tobita, M., Takekoshi, S. and Osamura, R. Y. (2009). The expression of Wnt4 is regulated by estrogen via an estrogen receptor alpha-dependent pathway in rat pituitary growth hormone-producing cells. *Acta Histochem. Cytochem.* **42**, 205-213.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790.

Mucha, S. A., Melen-Mucha, G., Godlewski, A. and Stepien, H. (2007). Inhibition of estrogen-induced pituitary tumor growth and angiogenesis in Fischer 344 rats by the matrix metalloproteinase inhibitor batimastat. *Virchows Arch.* **450**, 335-341.

Olson, L. E., Tollkuhn, J., Scafoglio, C., Krones, A., Zhang, J., Ohgi, K. A., Wu, W., Taketo, M. M., Kemler, R., Grosschedl, R. et al. (2006). Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. *Cell* **125**, 593-605.

Phelps, C. and Hymer, W. C. (1983). Characterization of estrogen-induced adenohypophyseal tumors in the Fischer 344 rat. *Neuroendocrinology* **37**, 23-31.

Potok, M. A., Cha, K. B., Hunt, A., Brinkmeier, M. L., Leitges, M., Kispert, A. and Camper, S. A. (2008). WNT signaling affects gene expression in the ventral diencephalon and pituitary gland growth. *Dev. Dyn.* **237**, 1006-1020.

Price, M. A. (2006). CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev.* **20**, 399-410.

Rao, T. P. and Kuhl, M. (2010). An updated overview on Wnt signaling pathways: a prelude for more. *Circ. Res.* **106**, 1798-1806.

Reya, T. and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature* **434**, 843-850.

Sartor, P., Garcia, L., Madec, F., Dufy-Barbe, L., Rigoulet, M. and Dufy, B. (2004). Regulation of intracellular chloride concentration in rat lactotrope cells and its relation to the membrane resting potential. *Gen. Physiol. Biophys.* **23**, 173-193.

Semba, S., Han, S. Y., Ikeda, H. and Horii, A. (2001). Frequent nuclear accumulation of beta-catenin in pituitary adenoma. *Cancer* **91**, 42-48.

Song, J. Y., Jin, L. and Lloyd, R. V. (1989). Effects of estradiol on prolactin and growth hormone messenger RNAs in cultured normal and neoplastic (MtT/W15 and GH3) rat pituitary cells. *Cancer Res.* **49**, 1247-1253.

Terada, Y., Tanaka, H., Okado, T., Shimamura, H., Inoshita, S., Kuwahara, M. and Sasaki, S. (2003). Expression and function of the developmental gene Wnt-4 during experimental acute renal failure in rats. *J. Am. Soc. Nephrol.* **14**, 1223-1233.

Treier, M., Gleiberman, A. S., O'Connell, S. M., Szeto, D. P., McMahon, J. A., McMahon, A. P. and Rosenfeld, M. G. (1998). Multistep signaling requirements for pituitary organogenesis in vivo. *Genes Dev.* **12**, 1691-1704.

Wang, L., Shao, Y. Y. and Ballock, R. T. (2007). Thyroid hormone interacts with the Wnt/beta-catenin signaling pathway in the terminal differentiation of growth plate chondrocytes. *J. Bone Miner. Res.* **22**, 1988-1995.

Widelitz, R. (2005). Wnt signaling through canonical and non-canonical pathways: recent progress. *Growth Factors* **23**, 111-116.

Wiklund, J., Wertz, N. and Gorski, J. (1981). A comparison of estrogen effects on uterine and pituitary growth and prolactin synthesis in F344 and Holtzman rats. *Endocrinology* **109**, 1700-1707.

Willert, K. and Jones, K. A. (2006). Wnt signaling: is the party in the nucleus? *Genes Dev.* **20**, 1394-1404.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., III and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452.

Yavropoulou, M. P. and Yovos, J. G. (2007). The role of the Wnt signaling pathway in osteoblast commitment and differentiation. *Hormones (Athens)* **6**, 279-294.

Zhang, X., Horwitz, G. A., Heaney, A. P., Nakashima, M., Prezant, T. R., Bronstein, M. D. and Melmed, S. (1999). Pituitary tumor transforming gene (PTTG) expression in pituitary adenomas. *J. Clin. Endocrinol. Metab* **84**, 761-767.