THE ROLE OF HUMAN PYGOPUS 2 IN THE PROLIFERATION OF BREAST CANCER CELLS

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By

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Abstract

Pygopus is a recently discovered downstream component of the Wnt signaling pathway required for β–catenin/Tcf dependent transcription. It has been proposed to act as a downstream mediator of transcription through its indirect interaction with β–catenin. Misactivation of Wnt signaling resulting in the overexpression of mitogenic Wnt target genes has been implicated in the progression of human cancer. In fact, aberrant Wnt signaling has been hypothesized to contribute to the formation of breast tumours. However, there are limited studies that conclusively address this question. Therefore, the purpose of this study was to examine the expression and requirement of the newly discovered Wnt signaling component Human Pygopus 2 (hPygo2) in this malignancy.

To assess the expression of hPygo2 in malignant breast cancer cell lines and tumours, I characterized antiserum which specifically recognized hPygo2 protein. hPygo2 was found overexpressed in the nuclei of breast tumour cells and tissue but not in normal breast cells and tissue. Knockdown studies using siRNA or antisense oligonucleotides has demonstrated that hPygo2, but not β–catenin, was required for the growth of MCF-7 and MDA-MB-468 breast cancer cell lines and expression of the Wnt target gene *Cyclin D1*. Furthermore, I found that Pygopus 1 but not Pygopus 2 was required for mediating the Wnt signal through the key mediator, β–catenin. These novel observations suggest that the requirement for nuclear overexpression of hPygo2 can be independent of Wnt/β–catenin in the growth of breast carcinoma cells. Therefore,

hPygo2 may be a more suitable therapeutic target than elements of the canonical Wnt pathway for the treatment of breast cancer.

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On a personal note, I would like to thank my girlfriend, Christine Wells for her encouragement throughout my graduate career. I also want to thank my grandparents for their love and support. Finally, I want to dedicate this work to my mother, whom I love and miss.

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Abbreviations

°C	degrees Celcius
β-TrCP	β-transducin repeat-containing protein
µg	microgram
µl	microliter
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
Bcl-9	B-cell lymphoma-9
CamKII	calmodulin kinase II
CBP	CREB binding protein
CDK	cyclin dependent kinase
cDNA	complementary DNA
CG	cytosine-guanine
CK	casein kinase
CREB	cAMP responsive element binding protein
Dap	dapper
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
Dsh	dishevelled
dsRNA	double stranded RNA
FAP	Familial Adenomatous Polyposis
FBS	fetal bovine serum
g	gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GGGG	guanine quartet
Grg	groucho
GSK3β	glycogen synthase kinase 3β
GST	glutathione S transferase
HDAC	histone deacetylase
ICAT	inhibitor of β–catenin
IP	immunoprecipitation
JNK	c-jun-N-terminal kinase
KDa	kiloDalton
LEF	lymphoid enhancing factor
LRP	low-density lipoprotein related protein
ml	milliliter
mM	milliMolar
MM	mismatch
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
NHD	N-terminal homology domain
NLS	nuclear localization sequence
nM	nanoMolar
NS	non specific
ON	antisense oligodeoxynucleotides
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHD	plant homeodomain
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor γ
pRb	retinoblastoma protein
Pygo	Pygopus
RISC	RNA induced silencing complex
RLU	relative luciferase units
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription- polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	short interfering RNA
TCF	T-cell factor
tPBS	triton-X 100 PBS
UV	ultraviolet
Wg	wingless

CHAPTER 1
INTRODUCTION

1.1 Signal Transduction and Cancer

Signal transduction is a complex process that involves the relaying of messages from outside of the cell, into the cell and ultimately to specific target proteins that can mediate cellular responses. Signaling can be initiated by extracellular molecules known as ligands, or by mechanical stresses encountered by the cell. The signal or message is transduced from the outside of the cell into the cell by the activation or repression of specific intracellular signaling proteins. Inside the cell, further downstream proteins initiate the activation of specific target proteins which can mediate a number of cellular processes, such as: cell metabolism, cell morphology, cell migration, facilitative transport of ions and proteins, gene expression, and so on. Therefore, signal transduction is a mechanism for cells to respond to and interact with their environment.

Signal transduction processes that ultimately result in proliferation, differentiation and survival are often mediated by extracellular messengers known as growth factors or mitogens. The binding of growth factors to their respective cell surface receptors results in the activation or repression of intracellular signal transduction cascades involved in the control of cell numbers. Activation or repression of signal transduction pathways that increase cell proliferation rates and promote cell survival are therefore important for the normal growth and development of an organism. On the other hand, aberrant activation

or repression of the same signaling pathways may cause the uncontrolled growth and proliferation of cells, which may result in cancer.

The loss of the ability for a cell or clone of cells to properly regulate cell growth and division is one hallmark of cancer. Genetic mutations leading to uncontrolled cell growth can arise from a number of different signaling pathways (Vogelstein and Kinzler, 2004). Gain-of-function mutations can occur in signaling pathways that result in the overexpression of proteins involved in the promotion of cell growth. For example, activation of the oncogene *c-myc* through gain-of-function mutations, has been implicated in the progression of human cancer (Pelengaris and Khan, 2003). On the other hand, loss-of-function mutations can occur in signaling pathways that result in the underexpression of proteins which may act to promote the regular death and turnover of cells. For example, inactivation of the tumour suppressor Adenomatous Polyposis Coli (APC), through loss-of-function mutation has been identified as being a cause for familial adenomatous polyposis (Fearnhead *et al.*, 2001).

In the context of cancer, it is clearly very important to understand the molecular mechanism of how signal transduction occurs and to identify mutations that lead to uncontrolled cell proliferation. Such knowledge is required to develop molecular and biological based treatments and therapies. My work presented in this thesis will concentrate on a newly discovered protein of the Wnt signal transduction pathway, known as Pygopus, and the possible role that it may play in cancer.

1.2 The Wnt Signal Transduction Pathway

The central role of Wnt signaling is to promote normal cell proliferation and cell migration in embryogenesis (Cadigan and Nusse, 1997) and in stem cell proliferation in adults (Willert *et al.*, 2003). Since Wnt proteins promote the growth of cells, they can be regarded as growth factors. The Wnt signal transduction pathway is very complex. For example, in humans, there have been 19 Wnt ligands that have been identified and each one likely mediates a different cellular response. In the following sections, I will concentrate on the classical canonical and non-canonical Wnt signaling pathways, as well as the newly discovered protein Pygopus and its role in the canonical Wnt signaling pathway. The regulation of the Wnt pathway is controlled at multiple levels but it is important to remember that Wnt signaling can also be regulated by many different proteins that are intermediates of other cell signal transduction pathways. Together, the cell must carefully orchestrate a variety of signals along with the Wnt signal in the normal growth and development of an organism.

1.2.1 Canonical Wnt Signaling

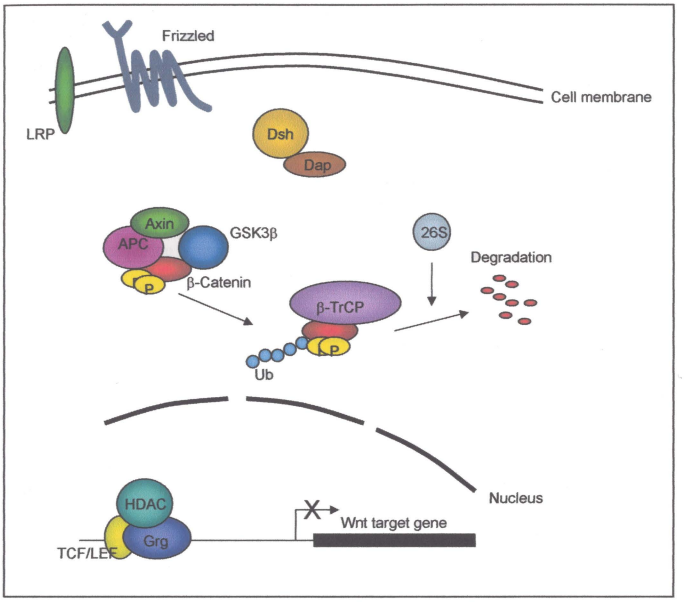
The canonical Wnt signaling pathway refers to the first discovered and most well known signaling pathway mediated by Wnt ligands. It involves the activation of the intracellular signaling intermediate β–catenin and its subsequent transcriptional regulation of Wnt responsive genes. β–catenin was originally identified as a bridging molecule that links E-Cadherin and Actin to mediate cell to cell adhesion (Wheelock and Knudsen, 1991). Since then, it has been found that β–catenin is a key mediator of Wnt

signaling, acting downstream of the ligand receptor complex (Peifer *et al.*, 1993). In the event of a Wnt signal, β–catenin becomes activated and translocates to the nucleus to activate gene transcription. The critical function of Wnt signaling is to regulate the normal growth and proliferation of cells that form complex tissues. Consistent with its role in promoting tissue growth, the canonical Wnt signaling pathway positively regulates genes involved in cell growth and proliferation, such as Cyclin D1 (Tetsu and McCormick, 1999; Shtutman *et al.*, 1999) and c-myc (He *et al.*, 1998). Therefore, the cell must carefully regulate this signaling pathway in the absence of a Wnt ligand to control its growth and proliferation.

In the absence of a Wnt signal or ligand, the pathway is negatively regulated in order to keep it in an inactive or "off" state (Figure 1.1). The binding of Wnt to its receptor causes a derepression of the pathway which attenuates the constitutive degradation of cytoplasmic β–catenin allowing it to accumulate in the nucleus where it displaces (or replaces) transcriptional repressors of Wnt target genes.

The repression of the Wnt pathway in the absence of signaling has been well-documented and can occur at many levels in the pathway. At the cell membrane level, the cytoplasmic Wnt protein Dishevelled (Dsh), which is normally activated by the active Frizzled receptor/Wnt ligand complex, can be repressed by a number of proteins. For example, Frodo/Dapper (Dap) proteins have been shown to bind to Dsh and inhibit its binding to Frizzled (Cheyette *et al.*, 2002; Wong *et al.*, 2003). Naked has also been shown to bind to Dsh, thereby acting as antagonists of Wnt signaling (Rousset *et al.*, 2001; Wharton, Jr. *et al.*, 2001).

Figure 1.1 Regulation of β–catenin in the absence of a Wnt signal. In the absence of a Wnt signal, nuclear and cytoplasmic β–catenin is recruited by APC, resulting in the assembly of the β–catenin destruction complex, consisting of Axin, APC and GSK3β. Specific N-terminal residues of β–catenin are phosphorylated by GSK3β, targeting it for ubiqitination by β-TrCP and subsequent degradation by the 26S proteasome. Wnt target genes are repressed by interacting complexes of TCF/LEF, Groucho (Grg) and HDAC proteins.


Frizzled
Cell membrane
LRP
Dsh
Dap
Axin
APC
GSK3β
P
β-Catenin
26S
Degradation
β-TrCP
P
Ub
Nucleus
HDAC
Grg
TCF/LEF
Wnt target gene

The inhibition or inactivation of Dsh ultimately results in the destruction of free cytoplasmic/nuclear β–catenin. β–catenin turnover is promoted by a protein known as APC, an intracellular scaffolding protein, which binds to free β–catenin in the cytoplasm and the nucleus resulting in its nuclear export (Henderson, 2000; Neufeld *et al.*, 2000). APC acts as a trap for free β–catenin and initiates its degradation by recruiting it to a multiprotein destruction complex, which includes: Axin and Glycogen Synthase Kinase 3β (GSK3β) (Hart *et al.*, 1998). Assembly of the β–catenin destruction complex is essential for the phosphorylation of β–catenin mediated by GSK3β (Ikeda *et al.*, 1998). The phosphorylation at specific N-terminal residues marks β–catenin for ubiquitination by the E3 ubiquitin ligase, β-Transducin repeat-Containing Protein (β-TrCP), therefore targeting it for proteasomal degradation (Hart *et al.*, 1999).

In the nucleus, the expression of Wnt target genes are repressed by a complex including T-Cell Factor/Lymphoid Enhancing Factor (TCF/LEF), whose function is to bind to specific consensus sequences at the promoters of Wnt genes, and the transcriptional repressor Groucho (Grg) (Roose *et al.*, 1998; Cavallo *et al.*, 1998). The interaction between TCF and Grg recruits chromatin remodelling proteins, such as Histone Deacetylase (HDAC) to Wnt target genes resulting in the deacetylation of nearby Histones. This event results in the local condensation of chromatin and therefore, the repression of Wnt target genes (Chen *et al.*, 1999).

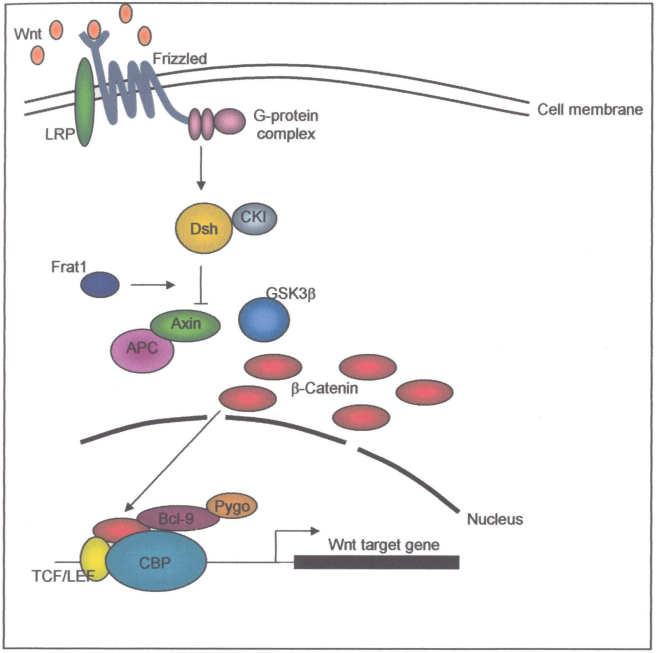
β–catenin activity can also be negatively regulated by a number of other proteins. For example, Inhibitor of β–catenin (ICAT) (Tago *et al.*, 2000), as well as a protein known as Teashirt (Gallet *et al.*, 1999; Waltzer *et al.*, 2001) are among the few proteins

that bind to and regulate β–catenin turnover, and hence its transcriptional activity by promoting its degradation.

Activation of Wnt signaling requires the presence of a Wnt ligand (Figure 1.2). Secreted Wnt ligands bind to seven pass transmembrane receptors, known as Frizzleds (Bhanot *et al.*, 1996). The activation of Wnt signaling is dependent on the ability of the Frizzled receptor to recruit transmembrane proteins of the low-density lipoprotein family, Low-density Lipoprotein Related Protein 5/6 (LRP5/6) upon Wnt ligand binding (Wehrli *et al.*, 2000; Tamai *et al.*, 2000). This assembly of the Wnt activation complex is necessary for the initiation of signal transduction and results in activation or derepression of the cytoplasmic signaling intermediate Dsh, by an unknown mechanism (Klingensmith *et al.*, 1994; Yanagawa *et al.*, 1995). At this point, the Wnt message can be transduced to either the canonical Wnt signaling pathway, or to the non-canonical Wnt signaling pathways (reviewed in section 1.2.2).

In the context of canonical Wnt signaling, Dsh phosphorylation and activation can be mediated by a number of other proteins including Casein Kinase I (CKI) (Peters *et al.*, 1999) and Casein Kinase 2 (CK2) (Willert *et al.*, 1997). Dsh activation ultimately results in the inhibition of β–catenin phosphorylation mediated by the Axin/APC/GSK3β complex (Kishida *et al.*, 1999). More specifically, the interaction of Dsh with Axin and Frat1 promotes the dissociation of Axin and GSK3β (Li *et al.*, 1999). By escaping GSK3β mediated N-terminal phosphorylation, β–catenin is stabilized and accumulates in the cytoplasm. At this point, β–catenin stability is further enhanced by the

Figure 1.2 Activation of the classical canonical Wnt signaling pathway. Wnt ligands bind to Frizzled transmembrane receptors to activate Dishevelled which, in turn, inhibits the formation of the β–catenin destruction complex, responsible for the phosphorylation of β–catenin. β–catenin accumulates in the cytoplasm and is free to translocate into the nucleus to activate transcription. Nuclear β–catenin forms a complex with TCF/LEF, CBP, Bcl-9 and Pygo. This complex interacts with the general transcriptional machinery, which is necessary for the transcription of Wnt target genes.


Wnt
Frizzled
LRP
G-protein complex
Cell membrane
Dsh
CKI
Frat1
GSK3β
Axin
APC
β-Catenin
Nucleus
Bcl-9
Pygo
CBP
TCF/LEF
Wnt target gene

phosphorylation of specific residues distinct from the phosphorylation sites of GSK3β, which is mediated by CK2 (Song *et al.*, 2003).

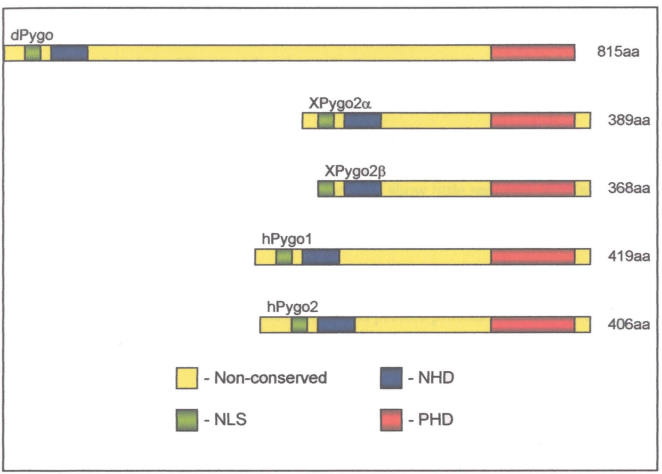
The stabilization of β–catenin, results in its accumulation in the cytoplasm. Subsequently, it can translocate to the nucleus where it interacts with specific nuclear proteins to form an active transcriptional complex. The transcriptionally active β–catenin complex includes many other proteins. TCF/LEF directly binds to Deoxyribonucleic Acid (DNA) enhancer sequences at the sites of Wnt target gene promoters (van De *et al.*, 1997), thought to occur by a mechanism that includes the displacement of Grg/TCF co-repressor complexes. Chromatin remodelling factors, such as the cAMP Responsive Element Binding Protein (CREB) Binding Protein (CBP) play a role in the transcription of target genes by acetylating nearby histone proteins, resulting in the decondensation of DNA to allow TCF/β–catenin complexes and the general transcription machinery to gain access to the Wnt target gene promoter (Waltzer and Bienz, 1998; Takemaru and Moon, 2000) The recently discovered proteins, B-Cell Lymphoma-9/Legless (Bcl-9) and Pygopus (Pygo), have been shown to be involved in the transcription of Wnt target genes. The exact role of Bcl-9 and Pygo in promoting Wnt dependent gene activation is currently unknown, although they have been proposed to play a role in chromatin remodelling as well, allowing access of TCF/β–catenin to the promoters of Wnt target genes (Kramps *et al.*, 2002).

1.2.2 Pygopus, a Novel Component of the Wnt Signaling Pathway

As briefly mentioned in section 1.2.1, Pygopus is a novel component of Wnt signaling proposed to be required for Wnt dependent transcription. Pygopus was originally discovered in *Drosophila* by yeast two-hybrid analysis of proteins that were able to interact with another novel Wingless (Wg)/Wnt component, Legless/Bcl-9 (Kramps *et al.*, 2002). Pygopus was also discovered by another group by a genetic screen of suppressors of an activated Armadillo (*Drosophila* β–catenin homologue) phenotype in the *Drosophila* eye (Thompson *et al.*, 2002). The name "Pygopus" is also the name of a class of legless lizards with scaly skin found in Australia. It was used to describe the phenotype of mutant Pygo flies, which lacked legs and antennae. Pygo mutants were strikingly similar to other Wg signaling mutants, which lead both groups to believe that Pygopus might be involved in transducing the Wg signal. Further characterization of Pygo revealed that it was indeed a component of Wg signaling in *Drosophila*. Mutants of Pygo were able to suppress direct Wg target genes, such as *Ultrabithorax B* and *Distal-less*. In the Kao lab, *Xenopus* Pygopus was initially discovered to be involved in neural patterning.(Lake and Kao, 2003). Since then, *Xenopus* Pygopus has been shown to be required for body axis formation (Belenkaya *et al.*, 2002). Finally, in human colorectal carcinoma cells, Pygo was shown to be required for TCF-dependent transcription (Thompson *et al.*, 2002; Kramps *et al.*, 2002). Therefore, there is much evidence implicating a role for Pygopus proteins in vertebrates, as in *Drosophila*, as downstream mediators of Wnt signaling.

Pygopus proteins (Figure 1.3) in general, are rich in proline and glycine residues and share several conserved amino acid sequences. At the N-terminus, there are two

Figure 1.3 Structure and conserved domains of Pygopus proteins. Pygopus proteins from *Drosophila* (dPygo), *Xenopus* (Xpygo) and human (hPygo) share several conserved domains. At the C-terminus, Pygo family members share a conserved PHD domain, responsible for the protein-protein interaction with Bcl-9. At the N-terminus, Pygo family members share two conserved protein motifs. The NLS is responsible for mediating the nuclear localization of Pygo proteins. The NHD domain shares no homology to any known protein motifs. The NHD is conserved amongst Pygo protein family members and is hypothesized to be required for the activation of Wnt-dependent transcription.


dPygo
815aa
XPygo2α
389aa
XPygo2β
368aa
hPygo1
419aa
hPygo2
406aa
- Non-conserved
- NHD
- NLS
- PHD

conserved amino acid sequences present in all known Pygo family members: a nuclear localization sequence (NLS), as well as a conserved sequence known as the N-terminal Homology Domain (NHD) (Thompson *et al.*, 2002; Kramps *et al.*, 2002). The NHD shows no sequence similarity to any known protein domains or conserved sequences. The proposed function of the NHD domain is to act as a transcriptional co-activator, since the N-terminal region of Pygopus fused to a dominant negative form of TCF can restore Wg signaling in *Drosophila* (Thompson, 2004). Further analysis of this domain including the proteins that bind to it, may reveal its exact function. The intervening regions of amino acids are rich in proline and glycine residues and show little sequence similarity between Pygo family members. At the C-terminus, Pygopus contains a highly conserved C4-H-C3 zinc-binding domain. This domain is known as the Plant Homeodomain (PHD) (Aasland *et al.*, 1995), and it is thought to be involved in protein-protein interactions. The PHD domain has been implicated in the remodelling of chromatin, since it has been found in proteins that have known chromatin remodelling activity, including CBP (Bordoli *et al.*, 2001; Kalkhoven *et al.*, 2002). The only known function of the PHD domain in Pygopus is to mediate protein-protein interaction with Legless/Bcl-9, forming a bridge to the β–catenin/TCF complex (Kramps *et al.*, 2002). Specific residues of the PHD domain of Pygo have been shown to be required for its binding to Bcl-9 and to relay the Wnt signal in normal development (Lin *et al.*, 2000; Townsley *et al.*, 2004). Given the fact that PHD domains are usually in proteins that have chromatin remodelling activity, Pygopus has been proposed to mediate the remodelling of chromatin to allow the TCF/β–catenin complex to gain access to, and activate Wnt responsive promoters

(Thompson *et al.*, 2002; Kramps *et al.*, 2002), although there is no direct evidence to support this role.

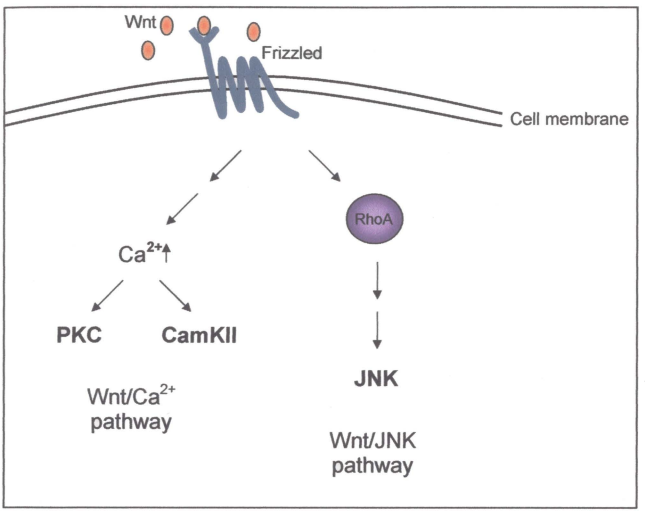
Limited studies to date outline the role of Pygopus proteins in Wnt signaling and normal development (Thompson *et al.*, 2002; Kramps *et al.*, 2002; Belenkaya *et al.*, 2002; Parker *et al.*, 2002; Lake and Kao, 2003; Townsley *et al.*, 2004), but the exact roles of Pygopus proteins are still unknown. As well, the role of Pygopus in human cancer has not been directly addressed. While Pygo plays a role in Wnt signaling, aberrant Wnt signaling can contribute to formation of many types of tumours, as I will discuss in Section 1.2.4.

1.2.3 Non-canonical Wnt Signaling

As I have discussed in section 1.2.1, the function of classical canonical Wnt signaling is to activate the key signaling intermediate, β–catenin, and downstream TCF/LEF responsive target genes. Non-canonical Wnt signaling (Figure 1.4), on the other hand, refers to other Wnt signaling pathways that have been identified after the initial discovery of the classical Wnt/β–catenin pathway. Non-canonical Wnt signaling can be divided into at least two distinct signaling pathways, the Wnt/Jun-N-terminal Kinase (JNK) and the Wnt/Ca^{2+} pathways, both of which are mediated by a Wnt ligand binding to its frizzled receptor.

The Wnt/JNK pathway, also known as the planar cell polarity pathway in *Drosophila*, involves the arrangement of cuticular cells that are lined up or polarized with respect to the body axis. It has been shown that the Frizzled receptor is required for this

Figure 1.4 Non-canonical Wnt signaling pathways. The binding of Wnt ligands to Frizzled receptors can also activate non-canonical Wnt signaling pathways responsible for planar cell polarity and convergent extension movements. The binding of Wnt to Frizzled can result in the intracellular increase in Ca^{2+} concentration, leading to the activation of PKC, CamKII as well as a number of other proteins. The binding of Wnt to Frizzled can also result in the activation of the small GTPase, RhoA. Activation of RhoA results in the activation of the c-Jun N-Terminal Kinase (JNK) pathway.


Wnt
Frizzled
Cell membrane
RhoA
Ca2+↑
PKC
CamKII
Wnt/Ca2+
pathway
JNK
Wnt/JNK
pathway

pathway, but the Wnt ligand that mediates this signal is currently unknown. The Frizzled receptor was found to be required for the polarity of wing hairs, or trichomes, in *Drosophila* (Vinson and Adler, 1987) and for the polarity of photoreceptor clusters, or ommatidia, in the *Drosophila* compound eye (Zheng *et al.*, 1995). This same pathway exists in the vertebrate *Xenopus* and was shown to regulate morphogenetic or convergent extension movements during gastrulation (Heisenberg *et al.*, 2000; Yamanaka *et al.*, 2002). Non-canonical activation of Frizzled proteins can in turn, activate small GTPases, such as RhoA, which is a potent activator of the JNK pathway (Shulman *et al.*, 1998). Therefore, the activation of this non-canonical Wnt pathway appears to be very important for cell morphology, movements and adhesion during normal development.

Activation of the non-canonical Wnt/Ca^{2+} signaling pathway ultimately results in an increase of intracellular Ca^{2+} ions, which can mediate a number of cellular responses. Frizzled protein receptors have a very similar structure to G-protein coupled receptors and it has been shown that activated Frizzled receptors can activate G-proteins resulting in the stimulation of phosphatidylinositol signaling and the subsequent intracellular release of Ca^{2+} ions (Slusarski *et al.*, 1997). Further study of Frizzled proteins and G-protein signaling revealed that Frizzled receptors closely resemble G-protein coupled receptors (Barnes *et al.*, 1998).The increase in cytoplasmic Ca^{2+} concentration results in the activation of Protein Kinase C (PKC) and Calmodulin Kinase II (CamKII) (Malbon *et al.*, 2001; Malbon, 2004). It appears that the most important role of Ca^{2+} signaling in *Xenopus* embryonic development, for example, is in the formation of ventral structures and in its inhibitory effect on convergent extension movements mediated by canonical Wnt signaling, which is required for the development of dorsal structures (Kuhl, 2002).

1.2.4 Wnt Signaling and Cancer

It is clear that Wnt signaling is important for the normal growth and development of an organism. Misexpression of Wnt signaling components in the early stages of normal development results in the formation of developmental defects, and in later stages of the growing adult, Wnt signaling is important for the normal growth and proliferation of cells and is therefore strictly regulated (Giles *et al.*, 2003). If this pathway becomes aberrantly deregulated in adult tissues, it may contribute to the uncontrolled growth and proliferation of cells, a primary characteristic of cancer. The normal function of Wnt signaling is to stabilize the key Wnt transducer, β-catenin. Therefore, nearly all cancers that have identified mutations in Wnt signaling display an active nuclear form of β–catenin, whose role is to promote the transcription of Wnt target genes. Overexpression of Wnt target genes, especially those involved in promoting cell cycle progression such as Cyclin D1 and c-myc (He *et al.*, 1998; Tetsu and McCormick, 1999), can indeed contribute to the uncontrolled proliferation of cells. As well, there are many other documented Wnt target genes involved in cell movements and migration, such as several Matrix Metalloproteinases (MMP), such as MMP-7 (Brabletz *et al.*, 1999; Crawford *et al.*, 1999), and MMP-26 (Marchenko *et al.*, 2002) . Overexpression of these genes may result in the ability of a cell to modify its extracellular environment and downregulate cell-to-cell adhesion, characteristic of the malignant phenotype.

Mutations in the Wnt signaling pathway have been identified and are thought to contribute to the malignancy of many tissue types. For example, Wnt mutations are most commonly identified in colorectal tissue. Loss-of-function mutations identified in APC are thought to be one of the main causes of a condition known as Familial Adenomatous

Polyposis (FAP). FAP is a cancer characterized by the formation of hundreds to thousands of colorectal polyps which are caused by the inactivation of the tumour suppressor APC (Groden *et al.*, 1991; Nishisho *et al.*, 1991), which then results in the accumulation of β–catenin and overexpression of Wnt target genes. Gain-of-function mutations have been identified in β–catenin in colorectal cancer cell lines (Morin *et al.*, 1997), and it has been hypothesized to be mutated in approximately 10% of all colon cancers. On the other hand, the frequency of Wnt mutation in other cancers is relatively low. For example, mutations in Wnt pathway components are extremely rare in breast tumours. Yet surprisingly, some breast tumours display nuclear β–catenin and Wnt target gene expression.

1.2.5 Wnt Signaling and Breast Cancer

Earlier work implicated a role for the oncogene Int1 (renamed to Wnt-1 after the discovery of *Wg* in *Drosophila*) in promoting mammary tumours. Retroviral infection of mice with the mouse mammary tumour virus results in its integration into the *Int1* locus of the host genome, resulting in the overexpression of the Int1 gene. This induced overexpression of Int1 was hypothesized to contribute to the formation of mammary tumours (Nusse and Varmus, 1982). These initial studies drew much attention to the role of Wnt/β–catenin signaling in breast cancer, and despite all of the subsequent studies, there is still little known about the causes of the deregulation of β–catenin in breast cancer (Howe and Brown, 2004). Therefore, there is a need to identify the molecular causes of aberrant β–catenin expression and nuclear localization in breast cancer.

Presently, there is strong evidence implicating a role for Wnt signaling in breast cancer due to the expression of nuclear localized β–catenin. Significantly, it has been shown that the overexpression of a constitutively active form of β–catenin in transgenic mice results in the formation of mammary tumours (Michaelson and Leder, 2001). In humans, cytoplasmic and nuclear overexpression of β–catenin staining was observed in approximately 60% of breast tumours and also correlated with poor patient prognosis (Lin *et al.*, 2000; Ryo *et al.*, 2001). But studies demonstrating the frequency of genetic β–catenin mutation in breast cancer was very low (van De *et al.*, 2001), therefore the nuclear localization of β–catenin observed in breast tumours is unlikely due to a direct mutation of the β–catenin gene. Therefore, proteins that directly interact with and regulate β–catenin are likely involved in its aberrant expression and nuclear localization. I will classify these proteins into two groups for further discussion. The first group are proteins that are known to be directly associated with the Wnt signaling pathway, and the second group are proteins which are indirectly involved in the Wnt signaling pathway.

There are many examples of Wnt signaling components whose aberrant expression may be correlated with the overexpression and nuclear localization of β–catenin. Wnt ligands have been found to be overexpressed in breast cancers. For example, Wnt1 overexpression in transgenic mice results in the overexpression of target genes and the formation of mammary tumours (Li *et al.*, 2003) and Wnt5a expression is upregulated in primary breast cancers (Lejeune *et al.*, 1995). The upstream β–catenin regulator, Dsh was also shown to be overexpressed in primary breast tumours (Nagahata *et al.*, 2003), but it has not been correlated with the expression and localization

of β–catenin. The overexpression of CK2 resulting in the phosphorylation and increased stability of β–catenin, is suspected of contributing to aberrant Wnt signaling in breast cancer (Landesman-Bollag *et al.*, 2001). Uncommonly, there have been few studies in breast cancer that have identified genetic mutations in Wnt signaling intermediates resulting in pathway activation. For example, the key β–catenin regulators, Axin (Webster *et al.*, 2000) and APC (Furuuchi *et al.*, 2000) have been shown to be mutated in a low percent of breast cancers. Therefore, there are many examples of how the overexpression or activation of Wnt pathway components may contribute to the stabilization and nuclear localization of β–catenin and furthermore, the development of breast cancer.

On the other hand, it is possible that β–catenin nuclear localization may be due to misexpression of β–catenin regulators indirectly involved in Wnt signaling. For example, it has been shown that β–catenin expression and activity is downregulated by p53 (Sadot *et al.*, 2001). This finding may help to explain the presence of nuclear β–catenin in breast cancers that harbour p53 mutations. Also, hypermethylation of the APC promoter has been described in a significant number of breast cancers (Jin *et al.*, 2001). Promoter hypermethylation due to aberrant regulation of DNA methyl-transferases, may possibly result in decreased APC expression. This evidence therefore implies that the misexpression of proteins indirectly involved in Wnt signaling may result in the increased stability or activation of β–catenin in breast cancer.

Nuclear β–catenin participates in the expression of Wnt target genes by the formation of transcriptionally active complexes at Wnt/TCF consensus sites and there are

several studies of Wnt target gene expression in breast cancer. For example, the expression of Wnt target gene Cyclin D1 has been shown to be upregulated by approximately 50% in breast tumours, both at the messenger Ribonucleic acid (mRNA) level (Buckley *et al.*, 1993) and also at the protein level (Bartkova *et al.*, 1994). Overexpression of Cyclin D1 in the mammary gland of transgenic mice results in abnormal cell proliferation and the formation of mammary adenocarcinomas (Wang *et al.*, 1994). It has also been shown that a number of breast cancer cell lines exhibited high levels of Wnt dependent transcription correlating with the overexpression of the target gene Cyclin D1 (Lin *et al.*, 2000). It appears that not only Cyclin D1 is required for tumourigenesis mediated by aberrant Wnt signaling, since Cyclin D1 knockout mice are susceptible to the formation of tumours mediated by the overexpression of Wnt1 (Yu *et al.*, 2001). Together, these results suggest that the activation of Wnt target genes may be necessary for the formation of mammary tumours. Further studies will be needed to demonstrate what other downstream Wnt targets alone or in combination, are necessary for mammary tumourigenesis.

1.3 Expressional Knockdown Strategies using an Antisense Approach

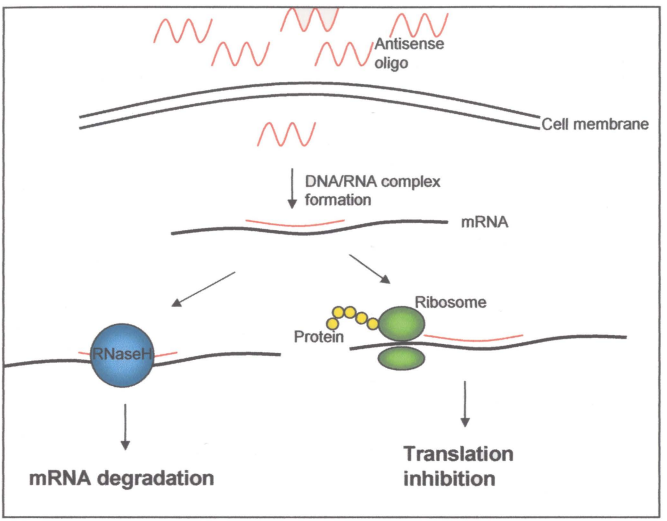
As I have discussed in section 1.1, cancers can arise from single cells that lose their ability to properly regulate cell growth and proliferation. This may result from genetic mutations in a cell either causing the underexpression of genes which are normally expressed at high levels, or from the overexpression of genes which are not normally expressed at high levels. A relatively new and important therapeutic approach to specifically target genes that are overexpressed in a disease state is to reduce or block

their expression. Many cancer cells and tumours have been shown to overexpress genes that lead to uncontrolled growth and proliferation, so reduction in their activities may be an important molecular therapeutic approach.

Single genes and the information that they carry are stored in the form of DNA. In the living cell, the flow of genetic information from DNA to messenger ribonucleic acid (mRNA) and then to protein has been coined as the "central dogma" of molecular biology. The "knocking" down or reduction of the expression of a particular gene using an antisense approach usually involves targeting its mRNA and therefore inhibiting protein production. Antisense molecules specifically bind to the message or mRNA of a particular gene resulting in its degradation or the blocking of translation of that mRNA. There are a number of different ways to reduce the expression of a gene. In this thesis, I concentrated on the use of antisense oligonucleotides and short interfering RNA (siRNA) as tools for reducing the expression of a protein.

Antisense oligodeoxynucleotides (ON) are usually short (15-25 bases), single stranded DNA molecules that are complementary to a given mRNA sequence. ON bind in a sequence specific manner by Watson and Crick base pairing to the complementary mRNA, resulting in the formation of an RNA-DNA duplex. There are several well known processes by which antisense ON can interfere with the production of protein from an mRNA (Figure 1.5). The binding of an antisense ON to its target mRNA sequence can result in ribosomal stalling during translation of mRNA into protein. Another well known mechanism by which the antisense ON can interfere with protein production is mediated by the nuclease, RNase H (Green *et al.*, 2000; Baker *et al.*, 2001; Crooke, 2004). RNase H is a ubiquitously expressed enzyme in prokaryotic and

Figure 1.5 Knockdown of gene expression by antisense oligonucleotides. When introduced into the cell, antisense oligonucleotides base pair to the complementary sequence of a target mRNA, resulting in a decrease in protein production. The formation of a DNA/RNA complex is recognized and degraded by the intracellular nuclease, RNaseH. Inhibition of translation can also occur due to ribosomal stalling at the site of the DNA/RNA complex.

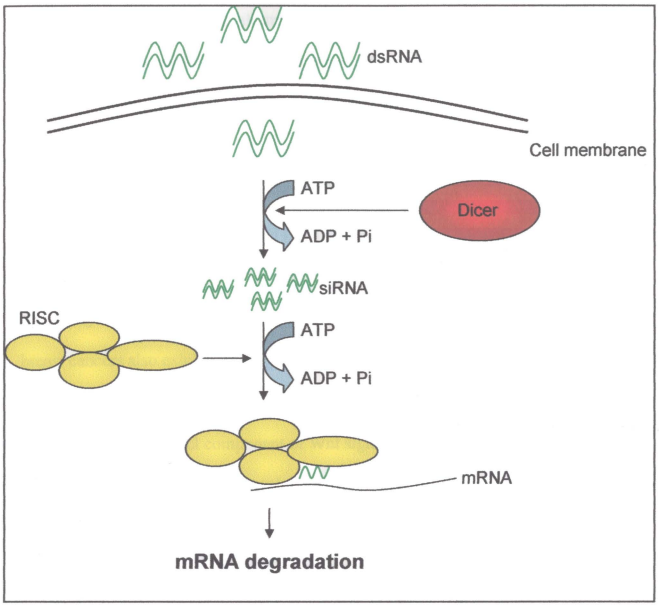

Antisense
oligo
Cell membrane
DNA/RNA complex
formation
mRNA
Ribosome
Protein
RNaseH
mRNA degradation
Translation
inhibition

eukaryotic cells. In humans, RNase H1 recognizes and cleaves the RNA strand of an RNA-DNA duplex, resulting in degradation of the mRNA species (Wu et al., 1999). Therefore, the use of antisense ON can exploit known mechanisms of RNA regulation to experimentally manipulate the down regulation of gene expression.

The discovery that double stranded RNAs have inhibitory effects on gene expression, or RNA interference (RNAi), was first demonstrated in *Caenorhabditis elegans* (Fire et al., 1998). In fact, RNAi has been found to be a useful tool and mechanism in mammalian cells to knock down gene expression. In the cell, double stranded RNA (dsRNA), that either occurs naturally or is introduced, is specifically recognized by an RNase III ribonuclease, known as Dicer (Bernstein et al., 2001) (Figure 1.6). This enzyme is responsible for the Adenosine Triphosphate (ATP)-dependent cleavage or processing of dsRNA into siRNAs, which are 21-23 nucleotide duplexes of dsRNA. siRNAs are then incorporated into an RNA induced silencing complex (RISC), where they are unwound in an ATP-dependent manner. Target mRNAs are then recruited to the antisense siRNA and are cleaved by the RISC, which initiates the degradation of the RNA (Denli and Hannon, 2003; Dykxhoorn *et al.*, 2003; Shi, 2003).

The use of RNAi has evolved as a useful method to knock down gene expression in mammalian cells. For example, it has been shown that this may be particularly useful for the study of overexpressed genes. siRNAs directed against β–catenin are effective at inhibiting the growth of human colon cancer cells which display activating mutations in Wnt signaling and overexpression of β–catenin (Verma *et al.*, 2003). Therefore, the use of siRNA to reduce gene expression in humans likely represents a new therapy based future treatment of disease.

Figure 1.6 Mechanism of RNA degradation by RNA interference. The generation of siRNA can result in the degradation of a target mRNA. Double stranded RNA (dsRNA) molecules are cleaved into short interfering RNA (siRNA) by the RNaseIII enzyme known as Dicer, in an ATP dependent manner. ATP is also necessary for the unwinding of the siRNA by the RNA induced silencing complex (RISC). The antisense strand of the siRNA is displayed by the RISC, binds to the target mRNA and is cleaved by the RISC. This process results in the degradation of mRNA and the inhibition of protein production.


dsRNA
Cell membrane
ATP
Dicer
ADP + Pi
siRNA
RISC
ATP
ADP + Pi
mRNA
mRNA degradation

1.4 Thesis Rationale

Misexpression of Wnt signaling has been implicated in the progression of human cancer. Misexpression or mutations in Wnt signaling components results in the activation and nuclear localization of the key signaling intermediate, β–catenin. Nuclear β–catenin then interacts with a multiprotein complex to activate the expression of Wnt target genes. Indeed, the overexpression of Wnt target genes is associated with the malignant phenotype as was discussed in Sections 1.2.4 and 1.2.5. Many cancers display the active nuclear localized form of β–catenin, as well as Wnt target gene overexpression. This has been most commonly identified in colorectal cancers that frequently harbour activating mutations in Wnt signaling intermediates, although a significant number of breast cancers also exhibit Wnt signaling activation by nuclear β–catenin expression (Lin *et al.*, 2000; Ryo *et al.*, 2001) by an unknown molecular mechanism.

Pygopus, a novel component of Wnt signaling, has been shown to be a downstream mediator of Wnt signaling (Thompson *et al.*, 2002; Kramps *et al.*, 2002). To date, a limited number of studies have outlined the role of Pygopus proteins in Wnt signaling and normal development (Thompson *et al.*, 2002; Kramps *et al.*, 2002; Belenkaya *et al.*, 2002; Parker *et al.*, 2002; Lake and Kao, 2003; Townsley *et al.*, 2004), but there have been no studies that directly implicate a role for Pygopus in human cancer. It has been previously shown that knockdown of Pygopus in colorectal carcinoma cells by RNAi resulted in a decrease of TCF/β–catenin driven transcription (Thompson *et al.*, 2002), therefore confirming its role in Wnt signaling. Other than this requirement, it is not clear whether or not the cells required Pygo for their growth or survival.

It has been hypothesized that aberrant Wnt signaling contributes to the malignancy of breast cancer and that nuclear β–catenin has been observed in a large number of breast cancers. The purpose of this thesis was to determine the expression and requirement of Pygopus in breast cancer, given the central role of the canonical Wnt signaling pathway in this malignancy. My hypothesis is that the overexpression of Pygopus may contribute to the growth of breast cancer cells. Therefore, the objectives of my study were:

1. To design a protein antigen against the non-conserved regions of the human Pygopus2 (hPygo2) protein and use it to immunize rabbits. The resulting antiserum will then be collected and characterized.

2. To examine the expression of human Pygopus family members as well as the expression of other downstream Wnt components which have been shown to interact with Pygopus in a variety of normal and cancer cells, including breast cell lines and tumours

3. To address the requirement of hPygo2 in the growth of human cancer cells including breast cancer cells by designing antisense ONs to specifically target hPygo2, but not the closely related family member, human Pygopus 1 (hPygo1).

4. To address the role of hPygo2 in the regulation of Wnt dependent transcription in breast cancer cells.

My results are the first to demonstrate the expression of Pygopus in breast cancer. I have found that hPygo2 mRNA and protein are expressed at high levels in the nuclei of malignant breast cancer cells and at low levels in normal breast cells and tissue. This expression of hPygo2 was strongly correlated with the expression of the key Wnt signaling mediator β–catenin, but not with other newly discovered components of the pathway, including hPygo1 and Bcl-9. I have also demonstrated that hPygo2 but not

β–catenin was required for the growth of human cancer cells, including the two breast cancer cell lines MCF-7 and MDA-MB-468. Furthermore, the reduction of cell growth due to hPygo2 knockdown was accompanied by a reduction in the cell cycle regulatory protein and Wnt target gene, Cyclin D1. Finally, I have demonstrated that hPygo1 but not hPygo2 functions as an activator of the Wnt signaling, indicating an alternative role of hPygo2 outside of the canonical Wnt signaling pathway in the mediation of breast cancer cell growth.

CHAPTER 2
MATERIALS AND METHODS

2.1 Production of hPygo2 Antiserum

2.1.1 Production of Purified GST fusion hPygo2 Proteins

Regions encoding amino acids 1-45 and 74-312 of hPygo2 (accession number NM_138300) that were selected for antibody production lacked the conserved NHD and PHD regions, therefore reducing the potential for cross reactivity with other proteins that contain these protein domains. The regions were also tested for antigenic sites using the online program "Antigenic" (http://bioweb.pasteur.fr/seqanal/interfaces/antigenic.html), which can be used to predict antigenic sites within a protein, based on the hypothesis that regions of hydrophobic residues that are located on the surface of a protein are more likely to be antigenic (Kolaskar and Tongaonkar, 1990). hPygo2 inserts were PCR amplified with the following primers: hPygo2 (1-45) (F: 5'-GTCCCCCACTCCATGGCCGCCTCG; R: 5'-TCATCGCTTCTTTTCTGGACTCTTC) hPygo2 (74-312) (F: 5'-GCATCCAACCCTTTTGAAGATGAC; R: 5'-TCAGCCAGGGGGTGCCAAGCTGTTG) from I.M.A.G.E. Consortium (LLNL) hPygo2 complementary DNA (cDNA) clones (CloneIDs: 41570072 and 3627860) obtained from Incyte Genomics Inc. PCR products were ligated in frame with the Glutathione S Transferase (GST) tag in the *EcoRI* and *XhoI* restriction sites of pGEX-4T1 (Amersham) using T4 DNA Ligase (Invitrogen). The resulting plasmids were then

midi-prepped (grown in 150 ml Luria-Bertani media containing 50 μg/ml ampicillin at 37°C overnight, and harvested by the alkali-lysis method (Birnboim, 1983)) using the Qiagen HiSpeed Plasmid Midi Kit. DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using the USB Sequenase Version 2.0 kit (Amersham) to confirm specificity. The resulting purified GST fusion proteins were synthesized and isolated from BL-21 Codon Plus RP, competent *E.coli* cells (Stratagene) (a gift from Dr. G. Paterno) and purified over Glutathione Sepharose 4B beads (Amersham) as per the GST Gene Fusion System (Amersham) protocol. Proteins were then concentrated using Centricon-10 protein concentrators (Amicon), resuspended in Phosphate buffered saline (PBS) and stored at -70°C. Purified proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then staining gels with Coomassie blue (Sigma) or by western blotting with an anti-GST antibody (Santa Cruz). Approximate protein concentration was determined using the BioRad reagent as per the manufacturers' instructions.

2.1.2 Production of hPygo2 Antiserum

Preimmune serum was collected prior to immunization of New Zealand White rabbits (Charles River Laboratories). The purified GST-hPygo2 fusion proteins were reconstituted using the Ribi Adjuvant System (Corixa), as per manufacturer's protocol. Two rabbits per protein construct were injected with approximately 400 μg of the appropriate GST fusion protein. Injections were performed in three week intervals, the

first two injections were intramuscular and subsequent injections were subcutaneous. After the third boost, serum was collected as described (Ryan and Gillespie, 1994). Briefly, approximately 20-30 ml of blood was collected into a 50ml sterile Falcon Tube by ear artery catheterization. Sodium azide was added to a final concentration of 0.01% to prevent microbial growth and the blood was incubated at room temperature for 6-8 hours to allow a clot to form. The resulting serum was isolated by centrifugation on a clinical centrifuge and stored at -20°C.

2.1.3 Immunoprecipitation of *in vitro* Translated hPygo2 Protein

^{35}S labeled hPygo2 protein was prepared using the coupled transcription-translation cell free system (Promega), as per manufacturer's instructions, using pCS2+/hPygo2 (see plasmids section 2.4.1) as a template. For immunoprecipitation (IP), *in vitro* translated hPygo2 protein was combined with 10-20 µl of preimmune or immune serum in 1X Triton IP buffer (10 mM Tris pH 7.5, 1% Triton-X-100, 10mM EDTA, 0.002% sodium-azide, 20mM Methionine, 1mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 50 µg/ml Nor-P-tosyl-L-lysine chloromethyl ketone) and rotated overnight at 4°C. After overnight incubation, 50 µl of a 50% slurry of Protein A Sepharose (Amersham), prepared as per manufacturer's protocol, was added and further incubated for 1hr at 4°C. Finally, beads were washed three times with ice cold 1X Triton IP buffer and three times with an ice cold 150 mM solution of NaCl. Proteins were eluted in protein sample buffer and analyzed by SDS-PAGE. Exactly one half of *in vitro* translated hPygo2 protein was used as a positive control.

2.2 Cell Culture

All cell lines, except normal endocervical (HEN) and normal ectocervical (HEC) cell lines (Tsutsumi *et al.*, 1992), were purchased from the American Type Culture Collection. T98G and Sk-N-Sh cells were maintained in Minimal Essential Media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). MDA-MB-231 cells were maintained in Leibovitz's L-15 Medium (Gibco) supplemented with 10% FBS. HEN and HEC cells were maintained in Keratinocyte Serum Free Media (Gibco). Sk-Ov-3, Es-2, HeLa, Caski, Hs-574, BT-20, Bt-474, MCF-7, MDA-MB-157 and MDA-MB-468 cells were all maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS. Hs-578Bst and Hs-578T were both maintained in DMEM supplemented with 10% FBS with 10μg/ml insulin. Hs-578Bst cells were further supplemented with 30ng/ml Epidermal Growth Factor. All cells were maintained in a humidified atmosphere with 5% CO_2 at a temperature of 37°C, except MDA-MB-231, which were maintained in a humidified atmosphere with 0% CO_2 at a temperature of 37°C.

2.3 Expression Analysis

2.3.1 Northern Analysis

Total RNA was extracted from cell lines using the Nucleospin RNA II Kit (Clontech Laboratories Inc.). Approximately 5μg of total cellular RNA from all cell lines was run on a formaldehyde gel and transferred to GeneScreen™ hybridization

membranes (Dupont). RNA was then UV crosslinked to the membranes and dried. A PCR product of hPygo2 was generated with hpygo2 (74-312) primers described in section 2.1.1, and was purified with a DNA gel extraction kit (Millipore). The purified PCR template was then radioactively labeled with α^{32}P-dATP by random priming (Prime-a-Gene; Promega), according to the manufacturers protocol. Blots were hybridized at 60° C using ExpressHyb (Clontech Laboratories Inc.), washed at high stringency (60°C in 0.1%SDS and 0.1XSSC) and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (pTRI-GAPDH; Ambion) under the same hybridization conditions to ensure equal loading and transfer of RNA.

2.3.2 RT-PCR Analysis

Cells in culture were harvested by trypsinization and briefly washed with ice cold 1XPBS. Total RNA was extracted from cell lines using the Nucleospin RNA II Kit (Clontech Laboratories Inc.). Reverse Transcription-PCR (RT-PCR) was performed essentially as described (Lake and Kao, 2003). Briefly, RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen). PCR cycling parameters included: denaturation at 94°C for 4 minutes, hybridization of primers to target sequences for 45-60 seconds, at temperatures specified in Table 2.1, and elongation at 72°C for 45-60 seconds. During the last cycle instead of 45-60 seconds, elongation occurred for 7-10 minutes. PCR products were electrophoresed on 1-1.5% agarose gels and photographed under UV light. Equal levels of cDNA used in the PCR reactions were achieved by normalization to *GAPDH* levels. Primers used in the analysis included those complementary to hpygo2

(74-312), hPygo1 (accession number NM_015617) F: 5'-GCCACGACAACCAAGAGGTG; R: 5'-CCAGTACAGATCCGATGAAACC, Bcl-9 (accession number NM_182557) F: 5'-GATGTTGTCCTGGTGTCTTG; R: 5'-GGTCACGACACTGCAGTGCTC and GAPDH (Ju *et al.*, 1995) All primers for RT-PCR were synthesized by Invitrogen.

Table 2.1 Primer Hybridization Conditions

Primer	TA* (°C)	N**	Reference
hPygo2	60	30	---
hPygo1	60	30	---
Bcl-9	55	30	---
GAPDH	55	23	Ju et al., 1995

*Annealing temperature

**Number of annealing/elongation cycles

2.3.3 Immunoblot Analysis

Total protein from tissue culture cells was extracted in protein sample buffer. Approximate concentration of protein was determined using the Biorad assay reagent. Approximately 50 μg of total cell lysate was separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL™; Amersham). Blots were probed with the following antibodies: monoclonal GST (Santa Cruz), monoclonal and polyclonal β–catenin (Santa Cruz), monoclonal β-Actin (Sigma), monoclonal Cyclin D1 (BD

Biosciences), and polyclonal hPygo2. As a positive control for hPygo2, *in vitro* translated hPygo2 protein was prepared using the coupled transcription-translation cell-free system (Promega). Blots were then visualized by enhanced chemiluminescence (Amersham). To confirm equal loading of protein, blots were reprobed with β-Actin.

2.3.4 Immunocytochemistry

For immunofluorescent analysis Hs-574-mg, Bt-474 and MCF-7 cells were fixed in 4% paraformaldehyde (30 minutes) rinsed in PBS twice and 0.2% triton-X 100/PBS (tPBS) for 10 minutes. Cells were blocked in 10% normal donkey/goat serum prior to an overnight incubation with primary antibodies in 1.5% normal sera/PBS. After a 30 to 40 minute wash in 0.2% tPBS, cells were incubated 30 minutes with secondary antibodies in 1.5% normal sera. For hPygo-2, biotinylated donkey anti-rabbit (Amersham) and for β-Catenin, Cy3 donkey anti-mouse (Jackson ImmunoResearch Laboratories, Inc.) was used. After a 30 to 40 minute wash in 0.1 – 0.2% tPBS, cells were incubated in streptavidin fluorescein (Amersham) in 1.5% normal sera/PBS for 30 minutes. Cells were washed in 0.1-0.2% tPBS 30 to 40 minutes before mounting in 10% glycerol/PBS or Vectashield (Vector Laboratories, Inc.). Images were collected using confocal microscopy (Olympus).

2.3.5 Immunohistochemistry

Breast tumour sections were obtained from the Memorial University Division of Laboratory Medicine. Immunohistochemistry was carried out as previously described

(Rorke *et al.*, 2001). Briefly, tumour sections were deparaffinized in Xylene and then rehydrated in a graded ethanol series to distilled water. Endogenous peroxidase activity was blocked in a 3% solution of H_2O_2 and then washed briefly in distilled water. To retrieve/unmask the antigen, sections were incubated in a boiling solution of 10 mM Citrate buffer, pH 6.0 and then cooled and washed with a running tap water bath. Slides were then washed with PBS and placed in a humid chamber. Slides were blocked in a 10% normal goat serum/PBS. All antibody dilutions were made up in a solution of 1% bovine serum albumin in PBS. Pre-immune and hPygo2 primary antibodies were added at a predetermined dilution and incubated overnight at 4°C. Slides were then rinsed with PBS and incubated with a biotinylated goat anti-rabbit IgG (Zymed) secondary antibody, rinsed again with PBS and incubated with an HRP-streptavidin antibody (Zymed). Slides were rinsed in PBS and incubated with a solution of DAB (Sigma) and H_2O_2, until a brown colour developed. Slides were again, rinsed with PBS and counterstained with Ehrlich's hematoxylin (Sigma) and destained in water, acid alcohol (70% ethanol, 1% concentrated HCl), tap water, and Scott's Tap Water Substitute (2-3.5g/L Sodium bicarbonate, 20 g/L Magnesuim sulfate). Slides were then dehydrated in a graded ethanol series to Xylene, then mounted with Permount (Sigma) and allowed to dry overnight.

2.4 Functional Analyses

2.4.1 Plasmids

Mammalian hPygo2 expression constructs were produced by subcloning hPygo2 from pOTB7/hPygo2 (engineered by Dr. B. Lake) and insertion into pCS2+ (a gift from

Dave Turner). The full length hPygo2 construct (pCS2+/hPygo2) was prepared by releasing the hPygo2 insert from pOTB7/hPygo2 and ligating it into the *EcoRI* and *XhoI* restriction sites of pCS2+. pCS2+/hPygo2 was sequenced with the USB Sequencing Kit (Amersham) to confirm specificity.

Gal-4-hPygo2 fusion proteins were constructed by PCR amplification of different regions of hPygo2 (primers given in Table 2.2) followed by the insertion into the *EcoRI* and *XhoI* restriction sites of pCMV-Tag2b (Stratagene). hPygo2 inserts were then subcloned from pCMV-Tag2b into the *EcoRI* and *HindIII* restriction sites of pMG4 to make N-terminal Gal-4-hPygo2 fusion proteins. All plasmids were sequenced with the USB Sequencing Kit (Amersham) to confirm correct insert sequences. hPygo1 and Bcl-9 expression constructs were prepared by PCR amplification from a human mammary carcinoma cDNA (Clontech). hPygo1 was then ligated into the *BamHI* and *EcoRI* restriction sites of pCMV-Tag2b and Bcl-9 was ligated into the *HindIII* and *SalI* restriction sites of pCMV-Tag2c. A full length expression construct of β–catenin S37A was prepared by RT-PCR amplification of β–catenin from the ovarian cancer cell line TOV-112D, which harbors a homozygous β–catenin missense mutation resulting in an amino acid substitution of a Serine residue to an Alanine residue at amino acid position 37 (Wu *et al.*, 2001) and inserted into the *BamHI* and *ClaI* restriction sites of pCS2+. Primers used included hPygo1 (accession number NM_015617) F: 5'-GCCACGACAACCAAGAGGTG; R: 5'-CCAGTACAGATCCGATGAAACC,

Table 2.2 Primer Sequences and PCR Conditions for Gal-4-hPygo2 Constructs

Construct	Upstream primer (5'-3')	Downstream primer (5'-3')	TA*	N**
Gal-4-hPygo2-1	GTCCCCCACTCCAT-GGCCGCCTCG	TCAGCCAGGGGGTG-CCAAGCTGTTG	60	35
Gal-4-hPygo2-2	**ATGGCTCCAAAGAAG-AAGCGTAAGGTA**CAG-CCTCCCCCAGGCTTGG	TCACCCATCGTTAGC-AGCC	60	35
Gal-4-hPygo2-3	**ATGGCTCCAAAGAAGA-AGCGTAAGGTA**GCATCC-AACCCTTTTGAAGATGAC	TCAGCCAGGGGGTG-CCAAGCTGTTG	60	35
Gal-4-hPygo2-4	GTCCCCCACTCCATG-GCCGCCTCG	CCAAGGAATGGAGG-GGCTGCAAC	65	35
Gal-4-hPygo2-5	ATGAAGAGTCCAGAA-AAGAAGC	CCAAGGAATGGAGG-GGCTGCAAC	65	35
Gal-4-hPygo2-6	ATGAAGAGTCCAGAAA-AGAAGC	TCACCCATCGTTAGC-AGCC	60	35

* Primer annealing temperature

**Number of annealing/elongation cycles

Synthetic nuclear localization sequence is in **bold**

Synthetic stop codon is underlined

Bcl-9 (accession number NM_182557) F: 5'-GATGTTGTCCTGGTGTCTTG; R: 5'-GGTCACGACACTGCAGTGCTC and β–catenin (accession number NM_001904) F: 5'-ATGGCTACTCAAGCTGATTTGATGG; R: 5'-CCTAAAGGATGATTTACAGGTCAG. All plasmids were sequenced with the USB Sequencing Kit (Amersham) to confirm correct insert sequences.

2.4.2 Transfection of Plasmid Constructs

All transfections with plasmid constructs were performed with Lipofectamine/Plus (Invitrogen), as per manufacturer's protocol. Growth media was replaced every 24 hours.

For western analysis of Gal-4-hPygo2 protein constructs, HeLa cells were seeded at a density of 7.5 x 10^4 cells/well in twelve well plates. 0.5 μg of plasmid were transfected and total cell protein was extracted 48 hours after transfection. Approximately 50 μg of the protein extracted was used for western analysis.

2.4.3 Antisense Oligonucleotides and siRNA

Antisense oligonucleotides (Table 2.3) (Invitrogen) against hPygo2 were designed to contain three phosphorothioate bonds at each terminus to enhance nuclease resistance. All oligonucleotides were designed avoiding Guanine quartets (GGGG) and repeated Cytosine Guanine (CG) sequences which may result in non-specific antisense effects (Green *et al.*, 2000; Baker *et al.*, 2001).

β–catenin siRNA and non-specific control siRNA were purchased as a β–catenin siRNA/siAB™ Assay Kit (Upstate).

Table 2.3 Antisense Oligonucleotide Sequences and Binding Positions

Oligonucleotide	Sequence (5'-3')	Binds RNA (nt position on cDNA)
Non-specific	T*T*T*GCGCCGTTTCTT*C*T*C	---
Mismatch 5	G*C*C*TGAGCTAATCATT*G*G*T	---
1	G*A*G*CTGCAGCAACCACA*A*A*G	55-74
2	G*G*A*CCCGGGTTAGCGGCA*G*C*G	144-164
3	C*C*A*CCTCCCTCCAGCTTG*T*C*C	198-219
4	G*G*A*GGACTAAAGTTTT*G*A*C	687-705
5	G*G*C*TGAGCAAATCGTT*G*G*G	807-825
6	G*A*A*AAGCAGTAGAAGCA*G*G*T	967-986
7	C*T*C*ACGGATGTAGAC*A*G*A	1340-1357
8	C*C*T*CTGGCCAGAAAC*T*T*T	1817-1835
9	C*T*C*TTCTACCTTTGAG*T*A*C	2434-2452
10	C*A*C*TGTATCTTGAGC*T*G*G	2720-2737

Mismatches are underlined

* indicates a phosphorothioate bond

2.4.4 Transfection of Oligonucleotides and siRNA

All transfections with oligonucleotides and siRNA were performed with Oligofectamine (Invitrogen) as per the manufacturer's instructions, replacing the growth

media every 24 hours. For HeLa and MCF-7 cells, hPygo2 antisense/control oligonucleotides were transfected to a final concentration of 250 nM and all siRNAs were transfected to a final concentration of 100nM. For MDA-MB-468 cells, hPygo2 antisense/control oligonucleotides were transfected to a final concentration of 67.5 nM and all siRNAs were transfected to a final concentration of 25nM. For RT-PCR analysis, HeLa cells were seeded at a density of 1.5×10^5 cells/well in six-well plates and were harvested 24 hours after transfection for RNA extraction. For Western analysis, MCF-7 and MDA-MB-468 cells were seeded at a density of 10^5 cells/well in twelve-well plates. Protein was extracted at 48 and 72 hours respectively. HeLa cells were seeded at 7.5×10^4 cells/well in six-well plates, and were harvested 48 hours after transfection. Finally, for cell growth analysis cells were seeded in triplicate at a density of 7.5×10^4 cells/well for MCF-7 and MDA-MB-468 cells and 5×10^4 cells/well for HeLa in twelve-well plates. Cells were counted 48 and 72 hours after transfection using trypan blue exclusion (Sigma) with a hemacytometer.

2.4.5 Luciferase (TOPFLASH) Wnt Reporter Assay

Approximately 18 hours prior to transfection, MCF-7 cells were seeded in triplicate at a density of 1.5×10^5 cells per well in 12-well plates. Cells were transfected using the Lipofectamine Plus reagent (Invitrogen). 0.5 μg of either pTOPFLASH-Luc, a positive control reporter plasmid containing TCF/LEF-1-binding sites, or with pFOPFLASH-Luc, a negative control plasmid having mutant binding sites, were transiently transfected along with molar equivalents of pCS2+ or pCS2+/

β–catenin S37A, in combination with pCMVTag2b, pCMVTag2b/hPygo1, pCMVTag2b/hPygo2 or pCMVTag2c/hBcl-9.

Cells were harvested 48 hours after transfection by trypsinization, followed by two washes in 1X PBS. Luciferase assays were performed on cell lysates using a Monolight 2010 Luminometer (Analytical Luminescense Laboratory) with the Luciferase assay reagent (Promega), according to the manufacturers' instructions. Briefly, cell pellets were resuspended in 50-100 µl of 1X reporter lysis buffer, incubated on ice for 30 minutes and then centrifuged at 16000 x g for 2 minutes at 4 °C. Soluble supernatant was collected and stored at -70°C until needed. 10-20 µl of cell lysate was then added to 100 µl of luciferase assay reagent and then luciferase activity was determined immediately. The values obtained in relative luciferase units (RLU), were normalized to the total amount of protein in the sample. Each experiment was performed in triplicate and was repeated three times.

CHAPTER 3
RESULTS

There are a limited number of studies to date outlining the role of human Pygopus 2 in human cancer. Therefore, the main objectives of my experiments were to: (1) generate and characterize an antibody directed against hPygo2, (2) examine the expression of hPygo2 and other Wnt signaling components in a number of cancer cell lines and breast tumours, (3) address the requirement and functional role of hPygo2 in cancer cell growth, and finally to (4) address the role of hPygo2 in the transcription of Wnt target genes.

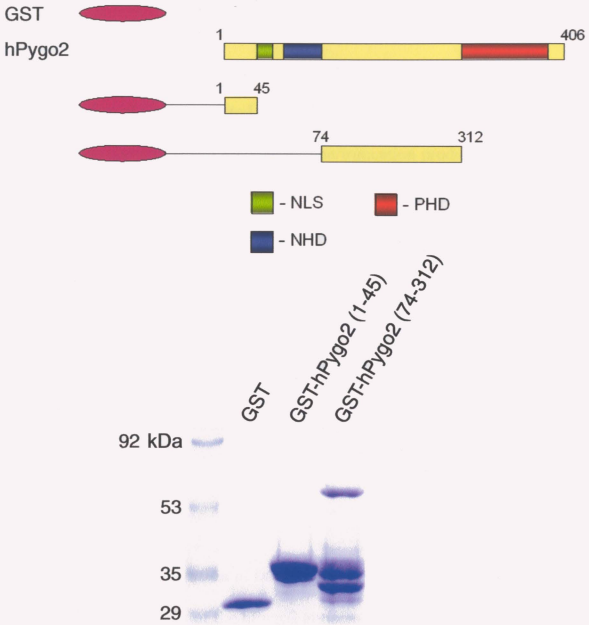
3.1 Production and characterization of hPygo2 antibodies

For the production of an hPygo2 antibody, purified hPygo2 proteins were made which could be used as antigens to generate polyclonal antibodies when injected into rabbits.

3.1.1 Production of purified GST-fusion hPygo2 proteins

In order to purify different antigenic regions of hPygo2, GST-fusion proteins of hPygo2 were prepared. The GST epitope was then used to affinity purify the resulting GST-hPygo2 fusion proteins. Following the purification and concentration steps, the GST-hPygo2 fusion proteins were then analyzed by SDS-PAGE (Figure 3.1). Purified GST migrated to its expected size of approximately 30 kDa. The GST-hPygo2 (1-45)

Figure 3.1 Production of purified GST-hPygo2 fusion proteins. The protein maps of the GST-hPygo2 fusion proteins, GST-hPygo2 (1-45) and GST-hPygo2 (74-312) are as indicated. Purified proteins were separated by SDS-PAGE and stained with Coomassie blue. GST migrated to approximately 30 kDa and GST-hPygo2 (1-45) migrated to 37 kDa. GST-hPygo2 (74-312), on the other hand migrated to 60 kDa, with protein degradation/truncation products migrating to approximately 32-37 kDa. Sizes of molecular weight markers are indicated.


GST
hPygo2
1
406
1
45
74
312
- NLS
- PHD
- NHD
GST
GST-hPygo2 (1-45)
GST-hPygo2 (74-312)
92 kDa
53
35
29

fusion protein migrated as a single band to approximately 35 kDa. The full GST-hPygo2 (74-312) fusion protein on the other hand, migrated to approximately 60 kDa, although protein degradation/truncation products were detected and were between 30-35 kDa in size. To confirm that all proteins that were produced were indeed GST-fusion proteins, western analysis was performed with an antibody against GST (Figure 3.2). All GST-hPygo2 fusion proteins migrated to the same sizes as determined in Figure 3.1. This analysis also confirmed that the degradation/truncation products in the GST-hPygo2 (74-312) lane were indeed GST fusion protein products and not due to contaminating bacterial proteins. The absence of other bands indicated that both GST-hPygo2 fusion proteins were indeed pure and therefore could then be used for injection into two rabbits. GST-hPygo2 (1-45) was used for injection into two rabbits (named: Puddin and Thumper) and GST-hPygo2 (74-312) was injected into two different rabbits (named: Hausenpfeffer and Smidgen).

3.1.2 Characterization of hPygo2 antibodies

3.1.2.1 Immunoprecipitation of *in vitro* transcribed/translated hPygo2 protein

To test the ability of the hPygo2 antisera to bind to hPygo2 protein, immunoprecipitation was performed using *in vitro* transcribed/translated ^{35}S-labeled hPygo2 protein. As can be seen, immune sera from all four rabbits was able to bind to and pull down approximately 50% of labeled hPygo2 protein (IP: first bleed; Figure 3.3 a). In contrast, pre-immune serum (IP: pre-immune) used at the same dilution as the

Figure 3.2 Western analysis of GST-hPygo2 fusion proteins. Western analysis was performed on the purified GST-hPygo2 fusion proteins using an antibody that recognizes GST. All purified proteins migrated to their expected sizes. The degradation products of GST-hPygo2 (74-312) were also recognized by the anti-GST antibody, indicating that they contain GST and are likely degradation/truncation products of the GST-hPygo2 (74-312) protein. Relative positions of molecular weight markers are indicated.

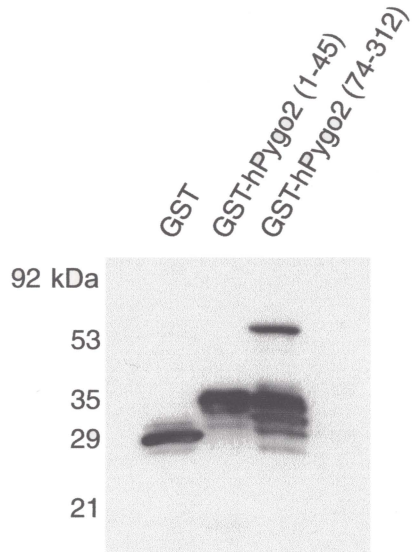
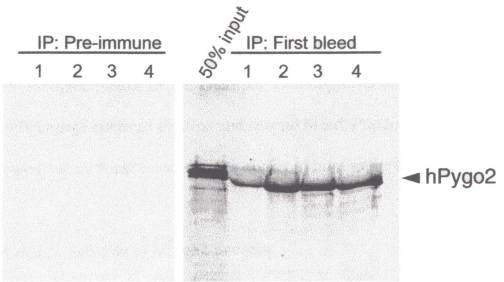
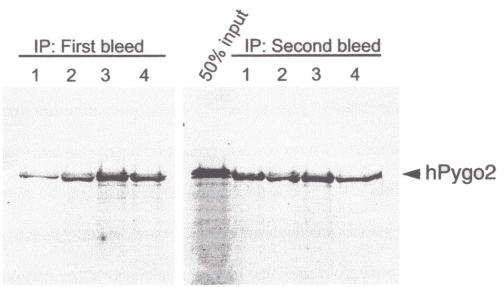

GST
GST-hPygo2 (1-45)
GST-hPygo2 (74-312)
92 kDa
53
35
29
21

Figure 3.3 Confirmation of the ability of the hPygo2 antiserum to bind to hPygo2 protein. *In vitro* transcribed/translated hPygo2 was immunoprecipitated with the hPygo2 antiserum collected. 50% of the labeled hPygo2 protein was loaded on the gel as a positive control. Rabbit serum used is as follows: 1 Puddin, 2 Thumper, 3 Hausenpfeffer, 4 Smidgen (a) Immunoprecipitation of hPygo2 with first bleed immune serum. Preimmune serum was used as a negative control. (b) Immunoprecipitation using first and second bleed immune serum.

a


IP: Pre-immune
1 2 3 4
50% input
IP: First bleed
1 2 3 4
hPygo2

b


IP: First bleed
1 2 3 4
50% input
IP: Second bleed
1 2 3 4
hPygo2

immune sera did not bind to the hPygo2 protein. These results indicate the immune sera contains antibodies that specifically interact with the labeled hPygo2 protein.

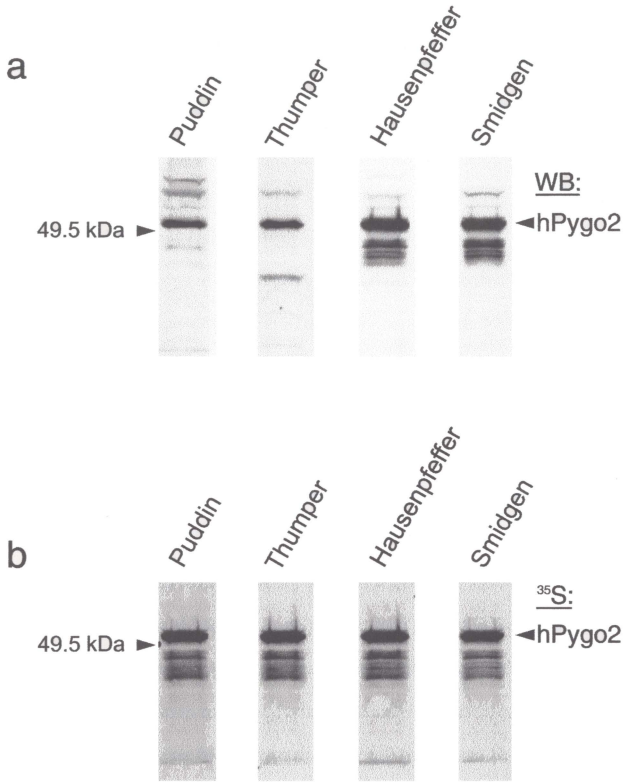
I also compared the ability of first and second bleed hPygo2 antisera to bind to and immunoprecipitate hPygo2 protein (Figure 3.3 b). As can be seen, levels of hPygo2 protein immunoprecipitated by the Thumper, Hausenpfeffer and Smidgen antisera did not significantly change between the first and second bleed. Puddin antiserum, on the other hand, showed a significant increase in its ability to bind to the labeled hPygo2 protein.

3.1.2.2 Western analysis of hPygo2 protein

The hPygo2 antiserum was previously shown to bind to non-denatured hPygo2 protein (Section 3.1.2.1). I next wanted to determine if the antiserum against hPygo2 was able to interact with denatured protein using western analysis to be used for future experiments.

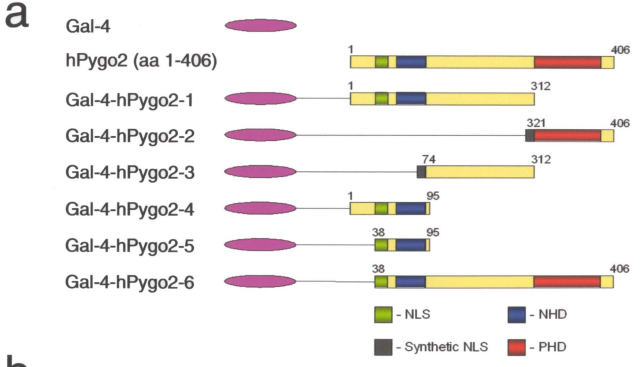
The hPygo2 antisera from all four rabbits were able to detect the denatured 50 kDa *in vitro* transcribed/translated hPygo2 protein by western analysis (Figure 3.4 a). There was some non-specific banding that occurred at higher and lower molecular weights than hPygo2 in all of the immunoblots. The lower molecular weight banding in the blots probed with Hausenpfeffer and Smidgen antisera are likely truncations of the hPygo2 protein, as they were also present when the blots were exposed to reveal the relative position of ^{35}S-labeled hPygo2 protein (Figure 3.4 b). Hausenpfeffer antiserum gave the lowest amount of non-specific banding and was used for further experiments.

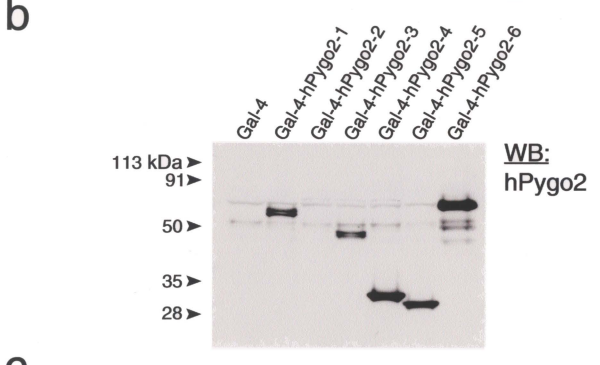
Figure 3.4 Test of immune serum for western analysis. *In vitro* transcribed/translated ^{35}S labeled hPygo2 protein was separated by SDS-PAGE and immunoblotted with immune serum that was collected from all four rabbits. (a) Western analysis of in vitro translated hPygo2 protein with Puddin, Thumper, Hausenpfeffer and Smidgen immune serum. All antibodies recognize a band that corresponds to hPygo2 at approximately 50 kDa. (b) After the chemiluminescent signal had been reduced, the nitrocellulose membranes from (a) were dried and exposed to film to reveal the ^{35}S labeled hPygo2 protein. hPygo2 appears as a band at approximately 50 kDa. The relative position of the 49.5 kDa molecular weight marker is indicated.


a
Puddin
Thumper
Hausenpfeffer
Smidgen
WB:
49.5 kDa
hPygo2
b
Puddin
Thumper
Hausenpfeffer
Smidgen
35S:
49.5 kDa
hPygo2

To confirm the ability of the Hausenpfeffer antiserum to detect different portions of the hPygo2 protein, HeLa cells were transiently transfected with different regions of the hPygo2 protein fused to Gal-4 (Figure 3.5 a). The transfected Gal-4-hPygo2 constructs were detected in HeLa cell lysate by immunoblotting with the Hausenpfeffer antiserum, targeting the hPygo2 protein (Figure 3.5 b). The hPygo2 antibodies present in the Hausenpfeffer antiserum detected all Gal-4-hPygo2 tagged proteins, except for the transfected Gal-4 tagged PHD region of hPygo2 (Gal-4-hPygo2-2). This result was consistent with the design of the antigen which did not include the PHD region. The Hausenpfeffer antiserum, designed to interact with amino acids 74-312 of hPygo2, strongly recognized hPygo2 mutants that were truncated at amino acid number 95 (Gal-4-hPygo2-4, Gal-4-hPygo2-5). This result indicated that the antiserum strongly interacts with a region of hPygo2 containing amino acids 74-95. Expression of the Gal-4-hPygo2 constructs was confirmed by immunoblotting for the Gal-4 epitope (Figure 3.5 c), using an antibody against the Gal-4 protein. The Gal-4 antibody detected all transfected Gal-4 tagged hPygo2 fusion proteins including the PHD region of hPygo2 (Gal-4-hPygo2-2), confirming that this tagged hPygo2 protein was indeed present in the HeLa cell lysate. The Hausenpfeffer antibody was also tested for its ability to interact with endogenous hPygo2 protein by western analysis of total whole cell lysate from cultured breast cancer cell lines. Immunoblotting freshly extracted total cell lysate from Bt-474 and MCF-7 breast carcinoma cells with Hausenpfeffer antiserum showed little or no non-specific antibody binding (Figure 3.6), but with strong binding to a band at approximately

Figure 3.5 Ability of Hausenpfeffer immune serum to recognize different regions of hPygo2. Different regions of hPygo2 fused to Gal-4 and were transiently transfected into HeLa cells. Protein was extracted and analyzed for the expression of the transfected constructs. The same amount of protein was loaded onto each gel. (a) Gal-4-hPygo2 fusion protein constructs that were transfected into HeLa cells. (b) Western analysis of Gal-4-hPygo2 constructs using Hausenpfeffer antiserum. (c) Western analysis of Gal-4-hPygo2 constructs using an anti-Gal-4 antibody. The Gal-4 antibody recognizes all Gal-4-hPygo2 protein constructs.


a
Gal-4
hPygo2 (aa 1-406)
1
406
Gal-4-hPygo2-1
1
312
Gal-4-hPygo2-2
321
406
Gal-4-hPygo2-3
74
312
Gal-4-hPygo2-4
1
95
Gal-4-hPygo2-5
38
95
Gal-4-hPygo2-6
38
406
- NLS
- NHD
- Synthetic NLS
- PHD


b
Gal-4
Gal-4-hPygo2-1
Gal-4-hPygo2-2
Gal-4-hPygo2-3
Gal-4-hPygo2-4
Gal-4-hPygo2-5
Gal-4-hPygo2-6
113 kDa
91
50
35
28
WB:
hPygo2

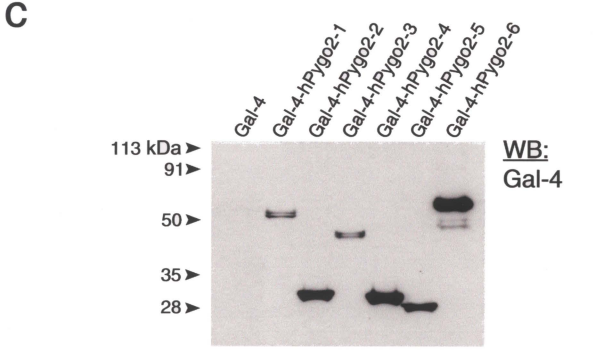
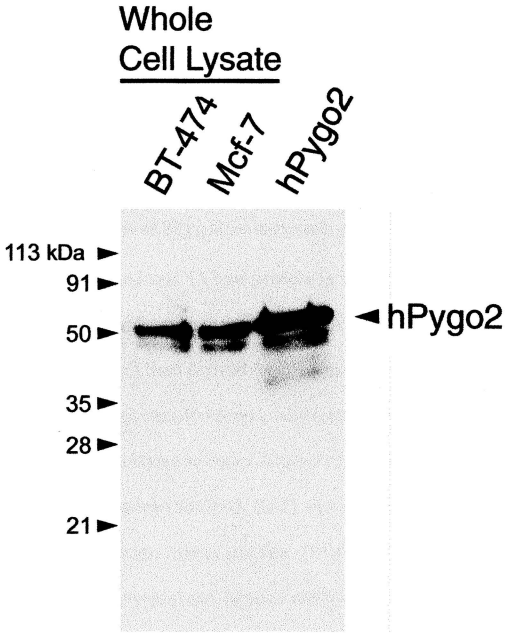

c
Gal-4
Gal-4-hPygo2-1
Gal-4-hPygo2-2
Gal-4-hPygo2-3
Gal-4-hPygo2-4
Gal-4-hPygo2-5
Gal-4-hPygo2-6
113 kDa
91
50
35
28
WB:
Gal-4

Figure 3.6 Hausenpfeffer immune serum recognizes endogenous cellular hPygo2. Whole cell lysate from Bt-474 and Mcf-7 cells was extracted and analyzed for the endogenous expression of hPygo2. *In vitro* transcribed/translated hPygo2 (hPygo2) was used as a positive control. A 50 kDa hPygo2 band was detected in both cell lines, with little or no non-specific binding.


Whole
Cell Lysate
BT-474
Mcf-7
hPygo2
113 kDa
91
50
35
28
21
hPygo2

50 kDa. This band most likely represented hPygo2 since it co-migrated with *in vitro* transcribed/translated hPygo2 protein used as a positive control.

These results indicate that the antibodies present in the Hausenpfeffer immune serum specifically interact with denatured synthetically produced as well as endogenously produced hPygo2 protein by western analysis, with little non-specific antibody binding.

3.2 Expressional analysis of hPygo2 in cancer

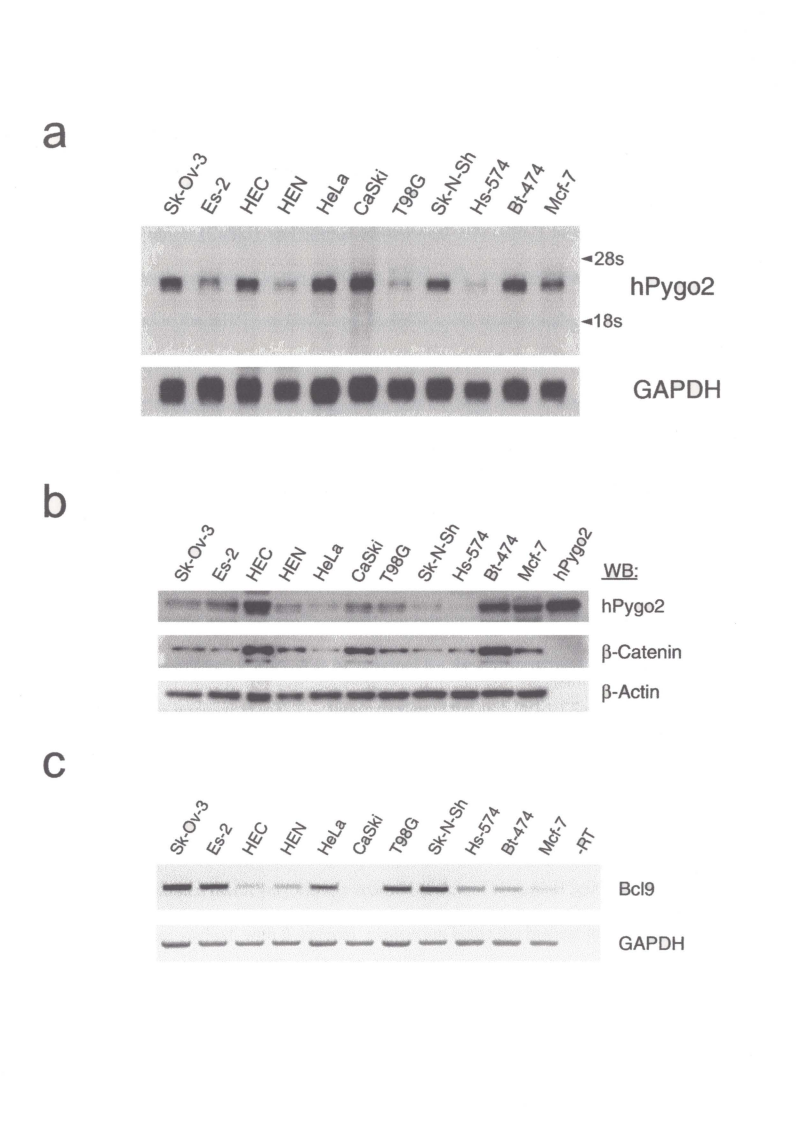
3.2.1 Expressional analysis of hPygo2 in various cancer cell lines

3.2.1.1 Expression of hPygo2 mRNA and protein in various cancer cell lines

To study the role of *hPygo2* in cancer, the expression of its mRNA was determined in a variety of cell lines derived from normal and malignant tissues. A specific probe against hPygo2 was developed, which excluded conserved regions, and was used for northern blot analyses to detect *hPygo2* mRNA (Figure 3.7 a). Messages were highly expressed in ovarian (Sk-Ov-3, Es-2), cervical (HeLa, CaSki), neuroblastoma (Sk-N-Sh), breast cancer cell lines (Bt-474, MCF-7), and normal ectocervical (HEC) cells. Alternatively, *hPygo2* mRNA expression was very low in neuroblastoma (T98G), normal endocervical (HEN) and a "normal" breast (Hs-574) cell line. To confirm equal loading and transfer of RNA the same blot was reprobed for the "housekeeping" gene *GAPDH*.

To confirm the expression of hPygo2 at the protein level, western analysis was performed on total cell lysate that was extracted from the cell lines that were used for the

Figure 3.7 Expression of hPygo2 and downstream Wnt components in various cancer cell lines. (a) Expression of *hPygo2* mRNA determined by northern analysis, using a probe that specifically recognizes the *hPygo2* message. Loading levels of mRNA were assessed by reprobing blots with a probe that specifically recognizes *GAPDH* mRNA. The relative positions of the *28s* and *18s* ribosomal RNAs are indicated. (b) Expression of hPygo2 and β–catenin protein levels determined by western analysis of total cell lysate. *In vitro* transcribed and translated full length hPygo2 protein (hPygo2) was used as a positive control. Loading levels of protein were assessed by reprobing blots for β-Actin. (c) Expression of Pygo binding partner Bcl-9. Total RNA was analyzed by RT-PCR using primers specific to Bcl-9. Levels of RNA were standardized by RT-PCR analysis for GAPDH. -RT, (negative control), without reverse transcriptase.


a
Sk-Ov-3
Es-2
HEC
HEN
HeLa
CaSki
T98G
Sk-N-Sh
Hs-574
Bt-474
Mcf-7
28s
18s
hPygo2
GAPDH
b
Sk-Ov-3
Es-2
HEC
HEN
HeLa
CaSki
T98G
Sk-N-Sh
Hs-574
Bt-474
Mcf-7
hPygo2
WB:
hPygo2
β-Catenin
β-Actin
c
Sk-Ov-3
Es-2
HEC
HEN
HeLa
CaSki
T98G
Sk-N-Sh
Hs-574
Bt-474
Mcf-7
-RT
Bcl9
GAPDH

analysis of *hPygo2* mRNA (Figure 3.7 b). *In vitro* transcribed/translated hPygo2 was used as a positive control for hPygo2 protein expression. The 50 KDa hPygo2 protein was expressed at high levels in the ovarian cancer (Es-2), breast cancer (Bt-474, MCF-7), cervical cancer (CaSki), and normal ectocervical cells (HEC). hPygo2 was expressed at higher levels compared to the mRNA in T98G cells, and expressed at lower levels compared to the mRNA in HeLa and Sk-N-Sh cells. Interestingly, hPygo2 was expressed at the highest levels in breast cancer cells (Bt-474, MCF-7) and at very low levels in the "normal" breast Hs-574 cells, suggesting that the high level of hPygo2 expression may play an important role in the breast tumour cells. Finally, the analysis of endogenous hPygo2 mRNA and protein shows that its expression was consistently higher in CaSki, Bt-474 and MCF-7 and consistently lower in Hs-574 (Figure 3.7 a, 3.7 b).

3.2.1.2 Expression of hPygo2 strongly correlates with β–catenin but not Bcl-9

Pygopus was proposed to be required for the transcription from Wnt responsive genes by an interaction with β–catenin mediated through Bcl-9 (Thompson *et al.*, 2002; Kramps *et al.*, 2002). Therefore, in an attempt to correlate the expression of known Pygo binding partners, β–catenin protein expression was examined and compared to the expression of hPygo2 protein. Examination of β–catenin protein expression (Figure 3.7 b), demonstrated that it very closely resembled that of hPygo2 (Figure 3.7 a) in most of the cell lines studied, with β–catenin expression being the highest in Sk-Ov-3, Es-2, HEC, CaSki, T98G, Bt-474 and MCF-7 cells. β–catenin was expressed at lower levels in HeLa and Sk-N-Sh cells. Again, the expression of β–catenin was lower in the normal Hs-

574 breast cells compared to the breast cancer cells. This result indicates that the expression of β–catenin strongly correlates with the expression of hPygo2, and it may be related to the co-operative function shared by these two proteins.

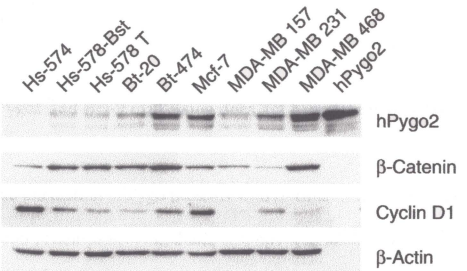
Given that Pygopus interacts with the β–catenin complex through Bcl-9, the relative expression levels of Bcl-9 were assessed by RT-PCR in a variety of cell lines (Figure 3.7 c). Bcl-9 mRNA was expressed highly in one cervical (HeLa), two ovarian (Sk-Ov-3, Es-2), and two neuroblastoma (T98G, Sk-N-Sh) cell lines, while it was expressed at lower levels in all of the other cell lines examined. Surprisingly, there was little correlation in the expression of Bcl-9 with the expression of hPygo2 and β–catenin.

3.2.2 Expressional analysis of hPygo2 in breast cancer

3.2.2.1 hPygo2 is overexpressed in breast cancer cells

To identify a potential requirement in the malignancy of breast cancer, hPygo2 expression was determined in a variety of cell lines derived from both normal and malignant tissues. hPygo2 protein levels were highest in the breast cancer cells Bt-474, MCF-7, MDA-MB 231 and MDA-MB 468, levels were lower in the breast cancer cells Hs-578 T, Bt-20 and MDA-MB 157 as well as the normal breast cells Hs-574 and Hs-578 Bst (Figure 3.8). hPygo2 protein levels were consistent with β–catenin protein expression in all cancer cell lines tested, except MDA-MB-231 which showed reduced β–catenin and higher levels of hPygo2, as well as the normal breast cell lines Hs-574 and Hs-578 Bst, which showed higher levels of β–catenin than hPygo2 (Figure 3.8 a). Therefore, this

Figure 3.8 Expression of hPygo2 and downstream Wnt proteins in various breast cancer cell lines. Expression of: hPygo2, β–catenin and Cyclin D1 by western blot analysis of total cell lysate of the various breast cancer cell lines. *In vitro* transcribed and translated full length hPygo2 protein (hPygo2) was used as a positive control. Loading levels of protein were assessed by reprobing for β-Actin.


Hs-574
Hs-578-Bst
Hs-578 T
Bt-20
Bt-474
Mcf-7
MDA-MB 157
MDA-MB 231
MDA-MB 468
hPygo2
hPygo2
β-Catenin
Cyclin D1
β-Actin

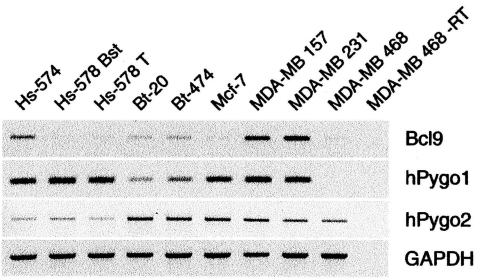
result supports my hypothesis that the upregulation of hPygo2 may play an important role in breast cancer.

Finally, the average expression of the cell cycle regulatory protein and Wnt target gene, Cyclin D1 was also examined in the breast cancer cell lines by western analysis (Figure 3.8 a). Cyclin D1 protein was expressed at the highest levels in the normal breast cell lines Hs-574, Cyclin D1 was also highly expressed in Bt-474 and MCF-7 malignant breast cell lines. Surprisingly, there was little correlation of Cyclin D1 with hPygo2 and β–catenin.

3.2.2.2 hPygo1 and Bcl-9 expression does not correlate with hPygo2 in breast cancer cells

The interaction of Pygo proteins with β–catenin was shown to be mediated by Legless/Bcl-9 (Thompson *et al.*, 2002; Kramps *et al.*, 2002). Therefore, I assessed the relative expression levels of *hPygo1, hPygo2* and *Bcl-9* in the breast cancer cell lines examined by RT-PCR (Figure 3.9). *hPygo2* expression was highest in the breast cancer cells Bt-20, Bt-474, MCF-7, MDA-MB 157, MDA-MB 231 and MDA-MB 468. *hPygo2* was expressed at lower levels in Hs-578 T and the normal cells Hs-574 and Hs-578. *hPygo1* expression was highest in Hs-574 and Hs-578 normal breast cells and MCF-7, MDA-MB-157 and MDA-MB-231 breast cancer cells, while *Bcl-9* expression was highest in MDA-MB-157 and MDA-MB-231 breast cancer cells. My results demonstrate an upregulation of hPygo2 in 6 of the 7 breast cancer cell lines analyzed. This trend was not observed with hPygo1, nor Bcl-9. These results suggest that a functional role of

Figure 3.9 Expression of human Pygo and Bcl-9 RNAs in breast cancer cell lines. Expression of *hPygo1*, *hPygo2* and *Bcl-9* was analyzed by RT-PCR in breast cancer cell lines. Levels of RNA were standardized by RT-PCR analysis for GAPDH. –RT, negative control, without reverse transcriptase.

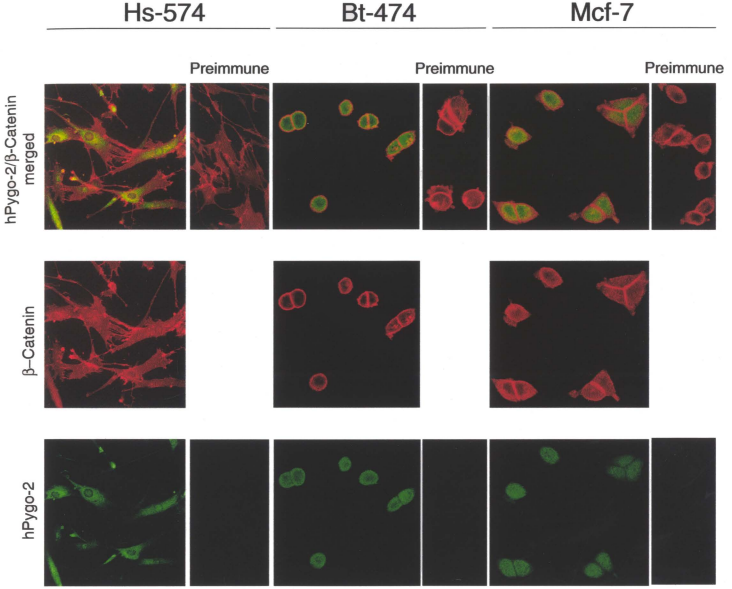

Hs-574
Hs-578 Bst
Hs-578 T
Bt-20
Bt-474
Mcf-7
MDA-MB 157
MDA-MB 231
MDA-MB 468
MDA-MB 468 -RT
Bcl9
hPygo1
hPygo2
GAPDH

hPygo2 in breast cancer may not require its interaction with the β–catenin transcription complex through Bcl-9.

3.2.2.3 β–catenin and hPygo2 do not co-localize in MCF-7 and Bt-474 cells

Since the expression of hPygo2 in breast cancer cell lines correlated with the expression of β–catenin. It is possible, however, that the β–catenin in these cells localizes to a different subcellular compartment than hPygo2. To address this possibility, I used indirect immunofluorescence and confocal microscopy to determine the subcellular localization of hPygo2 and β–catenin in the normal and malignant breast cell lines (Figure 3.10). As a negative control, preimmune serum was used at the same dilution as the hPygo2 immune serum, and showed no significant staining. Endogenous hPygo2 protein was predominantly localized to the nuclei of both breast cancer cell lines that I analyzed (Bt-474 and MCF-7) but to the cytoplasm and weakly to the nuclei of the normal breast cell line (Hs-574). Unlike hPygo2, the majority of β–catenin was associated with the plasma membrane of the normal and cancer cell lines. Some weak perinuclear staining of hPygo2 and β–catenin was observed in all cell lines, which may indicate a possible hPygo2 and β–catenin interaction. These observations indicate that the majority of hPygo2 and β–catenin were localized to different compartments in the breast cancer cells.

Figure 3.10 Expression and subcellular localization of hPygo2 and β-catenin in breast cell lines. The expression of hPygo2 (green) and β–catenin (red) were determined in normal breast (Hs-574) cells and malignant breast cancer (Bt-474, Mcf-7) cells using immunoflourescence and confocal microscopy. Preimmune serum was used at the same dilution as hPygo2 immune serum for a negative control. (Magnification = 200X)


Hs-574
Bt-474
Mcf-7
Preimmune
Preimmune
Preimmune
hPygo-2/β-Catenin merged
β–Catenin
hPygo-2

3.2.2.4 hPygo2 is expressed in malignant breast tumours

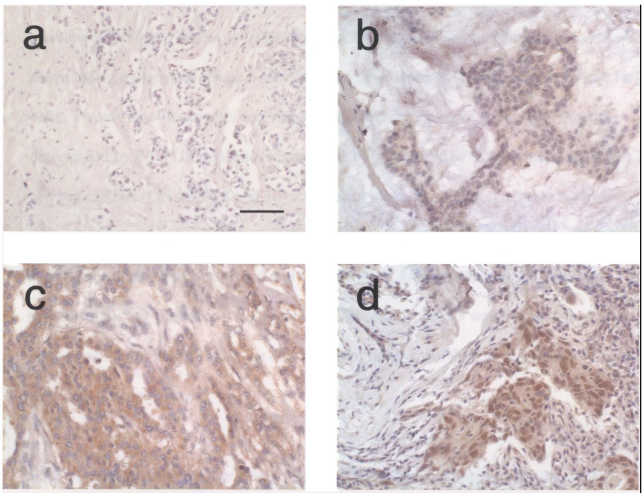
The expression of hPygo2 in archived surgical breast tumour specimens was determined by immunohistochemical analysis. I obtained 22 archived breast tumour sections, most of which were invasive ductal carcinomas, and 1 normal breast section and stained them for hPygo2 protein (Figure 3.11). Tumor cells were differentiated from normal cells based on irregularities in cell morphology (Kumar *et al.*, 2003) and were examined by a clinical pathologist (Dr. D. Robb). In 14 (64%) of tumours, there was staining of hPygo2 in malignant cells, but not in the surrounding non-tumour cells. Of the 14 positively stained specimens 6 (43%) had distinct nuclear and cytoplasmic hPygo2 staining (Figure 3.10 d), whereas the remaining 8 (57%) tumours had only cytoplasmic hPygo2 staining (Figure 3.10 b, c). hPygo2 could not be detected in a normal breast tissue section (Figure 3.10 a). I also obtained 4 lymph node sections from breast tumour patients. Two out of four contained metastatic tumour cells which stained positive for hPygo2 in the nucleus and cytoplasm, the remaining two did not contain tumour cells and did not stain with hPygo2. Thus, the expression of hPygo2 in the malignant cells of these tumours confirms the results found in established breast cancer cell lines and further supports the hypothesis that hPygo2 may play an important role in breast cancer.

3.3 Functional Analysis of hPygo2

3.3.1 Design of an antisense oligonucleotide capable of hPygo2 knockdown

It is possible that full length antisense constructs cause non-specific antisense

Figure 3.11 Immunohistochemical analysis of hPygo2 in breast tumours. (a) Normal breast tissue negatively stained for hPygo2 (brown). (b-d) Infiltrating ductal carcinomas stained with hPygo2 immune serum used at the same dilution which the pre-immune serum showed no staining. (b)Weak cytoplasmic hPygo2 staining, (c) strong cytoplasmic hPygo2 staining, (d) Strong nuclear and moderate cytoplasmic hPygo2 staining. Tumour sections were counterstained with hematoxylin (blue). Scale=100 micrometers.


a
b
c
d

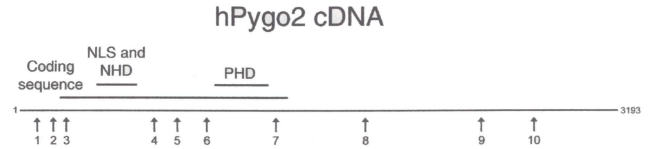
effects in cell culture by forming double stranded RNA or by containing G-quartet sequences (Burgess *et al.*, 1995). To more specifically target the expression of hPygo2, I therefore designed smaller antisense molecules avoiding sequences that may cause non-specific effects. I designed ten antisense ONs that bind to non-conserved regions of the *hPygo2* mRNA (Figure 3.12), the relative positions to which they bind the hPygo2 cDNA are shown in Figure 3.12 a. In order to determine which ON would be useful to knock down the expression of *hPygo2*, all ONs were first tested by transfecting them into HeLa cells. Twenty four hours after transfection, the relative levels of *hPygo2* mRNA was determined by RT-PCR analysis (Figure 3.12 b). A number of ONs reduced the levels of hPygo2 mRNA (ON #3, 5, 8 and 10), compared to the transfection reagent control (Oligofectamine) and an ON designed against *Xenopus Pygopus 2* (Non-specific oligo). Spot densitometry was performed and expression of *hPygo2* was normalized to the expression of the "housekeeping" gene *GAPDH*. Since ON #5 was found to have the greatest ability to knock down the expression of *hPygo2* mRNA, it was therefore used in subsequent experiments.

3.3.2 hPygo2 is required for the growth of HeLa cervical cancer cells by specific knockdown of hPygo2 using antisense ON

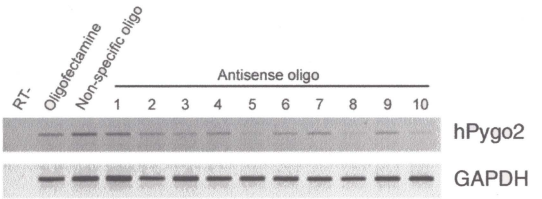
A single oligonucleotide (ON#5, hereon referred to as α-hpy2), which had the greatest ability to knock down hPygo2 mRNA expression, was chosen for further analysis in HeLa cells. α-hpy2 was transfected along with two control ON, one

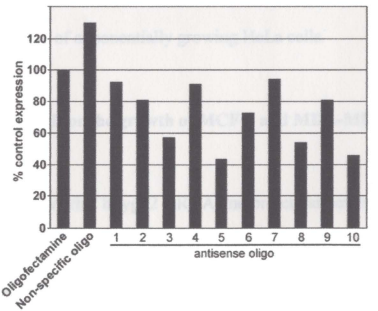
Figure 3.12 Identification of an antisense oligonucleotide capable of knocking down *hPygo2* expression. (a) Antisense oligonucleotides were designed against the full hPygo2 cDNA sequence, avoiding conserved sequences such as the NHD and PHD domains. (b) Antisense oligonucleotides were transfected into HeLa cells at a concentration of 250nM. RNA was extracted 24 hours later and RT-PCR analysis was performed to access the relative knockdown of *hPygo2* RNA levels. Densitometry was performed, standardizing the relative *hPygo2* levels to the relative *GAPDH* levels. RT-, (negative control), without reverse transcriptase.

a


hPygo2 cDNA
Coding sequence
NLS and NHD
PHD
1
3193
1 2 3
4 5 6
7
8
9
10

b


RT-
Oligofectamine
Non-specific oligo
Antisense oligo
1 2 3 4 5 6 7 8 9 10
hPygo2
GAPDH


% control expression
120
100
80
60
40
20
0
Oligofectamine
Non-specific oligo
1 2 3 4 5 6 7 8 9 10
antisense oligo

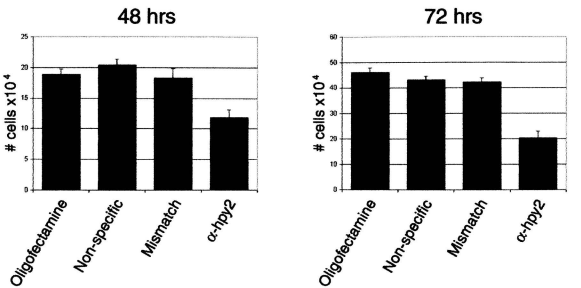
complementary to *Xenopus Pygopus 2* (Non-specific) and another ON complementary to the α-hpy2 ON with a 4 base-pair sequence substitution (Mismatch). Knock down of hPygo2 using the α-hpy2 ON, was accompanied by a significant decrease in cell numbers at 48 and 72 hours after transfection compared to reagent alone (Oligofectamine) and the control ON (Figure 3.13 a). Most of the reduction in cell growth occurred at 48 hours after transfection, while cell number doubled by 72 hours after transfection, most likely due to the normal proliferation of untransfected cells. In order to confirm the knockdown of hPygo2 mRNA and protein by the α-hpy2 ON, RT-PCR as well as western analysis were used (Figure 3.13 b, c). Specific knock down of hPygo2 mRNA expression was achieved 24hrs after transfection of α-hpy2 compared to the control ON, without affecting the expression of the alternate, but related, Pygo family member hPygo1 (Figure 3.13 b). The introduction of α-hpy2 into HeLa cells also resulted in a knock down of endogenous hPygo2 protein 48 hours after transfection with the α-hpy2 ON (Figure 3.13 c). These results suggest that specific knockdown of hPygo2, but not hPygo1 results in a decrease in cell numbers of exponentially growing HeLa cells.

3.3.3 hPygo2 is required for the growth of MCF-7 and MDA-MB-468 breast cancer cells

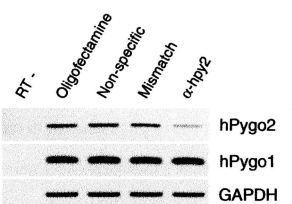
Since I have shown that hPygo2 mRNA and protein are expressed at relatively high levels in breast cancer cells compared to normal breast cell lines (Figure 3.8, 3.9), I hypothesized that the high levels of expression of hPygo2 in breast cancer cells may be required for their growth.

Figure 3.13 Knockdown of endogenous hPygo2 using antisense ON in HeLa cervical cancer cells. Reagent control (Oligofectamine), antisense *Xenopus* Pygopus2 (non-specific), and four base mismatch (mismatch) controls are indicated. (a) Knockdown of hPygo2 by antisense ON results in a decrease of HeLa cell numbers 48 and 72 hours after transfection. Cell number was assayed by direct counting with a hemacytometer using trypan blue exclusion. (b) RT-PCR analysis showing specific knockdown of *hPygo2* mRNA without affecting expression of the related Pygo family member, *hPygo1*. RT-, (negative control), without reverse transcriptase. (c) Western blot analysis showing knockdown of endogenous hPygo2 protein. Levels of cDNA and protein were standardized using GAPDH and β-Actin. Experiments were performed in triplicate.

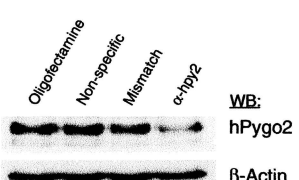
a


48 hrs
72 hrs
cells x10^4
Oligofectamine
Non-specific
Mismatch
α-hpy2

b


RT -
Oligofectamine
Non-specific
Mismatch
α-hpy2
hPygo2
hPygo1
GAPDH

c


Oligofectamine
Non-specific
Mismatch
α-hpy2
WB:
hPygo2
β-Actin

Transfection of the α-hpy2 ON into MCF-7 cells resulted in a considerable reduction of cell numbers (Figure 3.14 a) 48 and 72 hours after transfection with the hPygo2-specific α-hpy2 ON, compared to the cells transfected with the NS and MM control ONs. At 72 hours after transfection, cell numbers were reduced to 52%, compared to 93% with the NS control or 83% for the MM control. The reduction in cell numbers was preceded by a significant downregulation of hPygo2 protein 48 hours after transfection, compared to the non-specific and mismatch control ONs while β–catenin levels remained unaltered (Figure 3.14 b). Further analysis of the Wnt target gene and cell cycle regulatory protein Cyclin D1 (Figure 3.14 b), showed that its levels were decreased after transfection of the α-hpy2 oligonucleotide suggesting that the reduction of MCF-7 cell number may be due to a reduction of cell growth due to cell cycle arrest.

Similarly, transfection of the breast cancer cell line, MDA-MB-468 with the hPygo2 specific ON and control ONs resulted in a significant reduction in cell growth 72 hours post-transfection. Cell numbers were reduced to less than 50% of controls after transfection of the α-hpy2 ON (Figure 3.15 a). hPygo2 protein levels, and to a lesser extent β–catenin, were found to be reduced 72 hours after transfection, (Figure 3.15 b).

3.3.4 β–catenin is not required for growth of MCF-7, nor MDA-MB-468 cells

It was previously shown that MCF-7 cells exhibit Wnt dependent transcription and expression of the Wnt target gene, Cyclin D1 (Lin *et al.*, 2000). Therefore, to confirm the involvement of hPygo2 in the canonical Wnt pathway in these cells, I analyzed the

Figure 3.14 Knockdown of hPygo2 in Mcf-7 breast cancer cells using antisense ONs. Reagent control (Oligofectamine), antisense Xenopus Pygopus2 (non-specific), and four base mismatch (mismatch) controls are indicated. Cells were counted with a hemacytometer using trypan blue exclusion (a) Growth of Mcf-7 cells 48 and 72 hours after initial treatment with antisense ON. Growth is given as the % control transfected with transfection reagent alone. (b) Western analysis of hPygo2, Cyclin D1 and β–catenin 48 hours after hPygo2 knockdown by antisense ON. Results indicated are based on three experiments performed in triplicate.

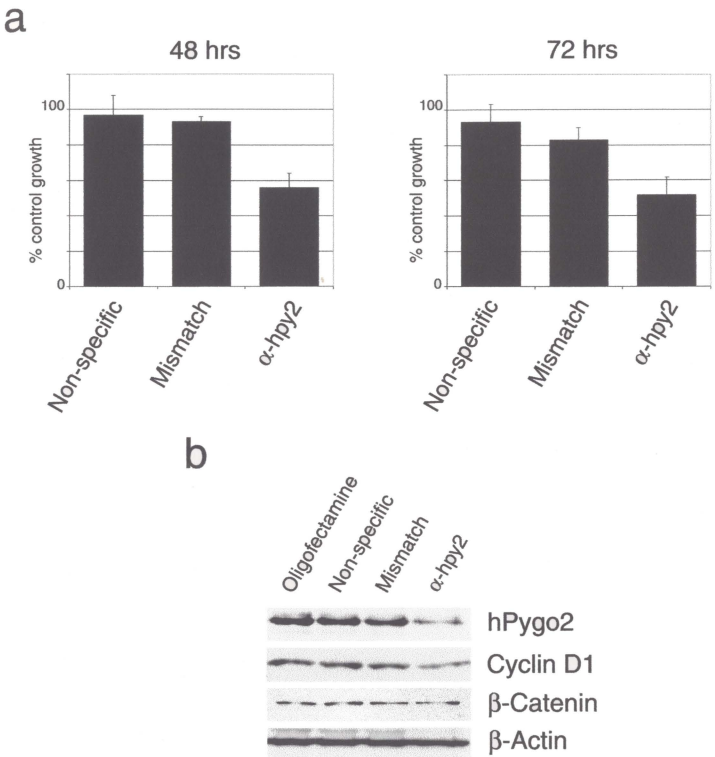
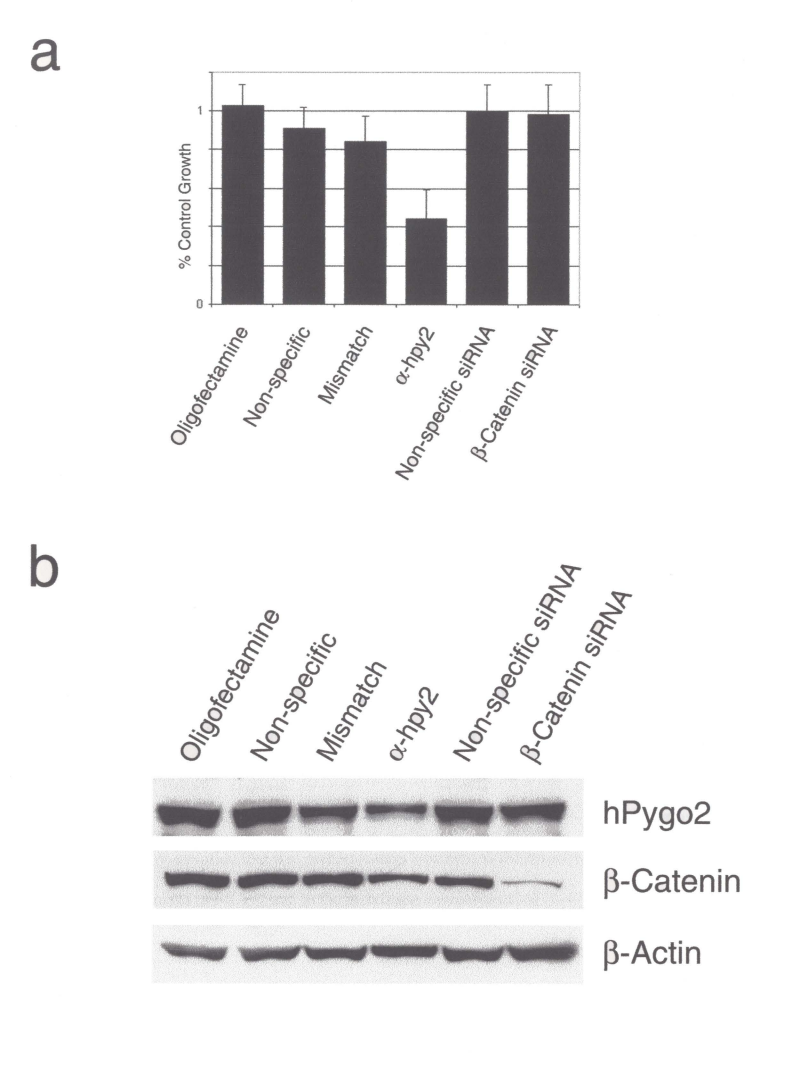

a
48 hrs
72 hrs
100
0
% control growth
Non-specific
Mismatch
α-hpy2
b
Oligofectamine
Non-specific
Mismatch
α-hpy2
hPygo2
Cyclin D1
β-Catenin
β-Actin

Figure 3.15 Knockdown of hPygo2 and β-catenin in MDA-MB 468 cells using antisense ONs and siRNA. Reagent control (Oligofectamine), antisense Xenopus Pygopus2 (non-specific), and four base mismatch (mismatch) control ONs, along with Non-specific control siRNA and β–catenin siRNA are indicated. (a) MDA-MB-468 cell growth was assayed at 72 hrs after transfection. (b) Western analysis of hPygo2 and β–catenin 72 hours after knockdown of hPygo2 and β–catenin. Protein levels were standardized by reprobing blots with β-Actin. Results indicated are based on three experiments performed in triplicate.


a
% Control Growth
1
0
Oligofectamine
Non-specific
Mismatch
α-hpy2
Non-specific siRNA
β-Catenin siRNA
b
Oligofectamine
Non-specific
Mismatch
α-hpy2
Non-specific siRNA
β-Catenin siRNA
hPygo2
β-Catenin
β-Actin

requirement of β–catenin for cell growth in MCF-7 cells by knocking down its expression with commercially available siRNA. Since β–catenin and hPygo2 proteins were expressed at high levels in these cells, I hypothesized that growth would be reduced following knockdown of β-Catenin, as was the case for the knockdown of hPygo2.

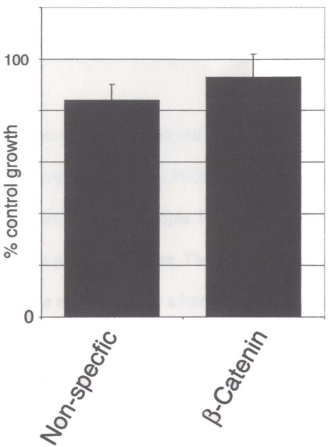
Surprisingly, the depletion of β-Catenin, was not accompanied by a reduction in cell numbers when compared to non-specific siRNA controls 72 hours after transfection (Figure 3.16 a). Knockdown of approximately 100% of β–catenin protein levels with β–catenin siRNA was confirmed by western analysis (Figure 3.16 b). Expression levels of hPygo2 and Cyclin D1 remained unchanged after β–catenin knockdown when compared to reagent alone (Oligofectamine) or a non-specific siRNA (Figure 3.16 b). Thus, β–catenin is not required for cell growth, nor for the expression of the Wnt target gene Cyclin D1 in MCF-7 cells.

The knockdown of β–catenin in MDA-MB-468 cells similarly showed no reduction in cell numbers 72 hours after transfection compared to the reagent and non-specific controls (Figure 3.15 a). A reduction in β–catenin protein levels was confirmed by immunoblotting siRNA treated MDA-MB-468 cell lysate harvested 72 hours post-transfection (Figure 3.15 b).

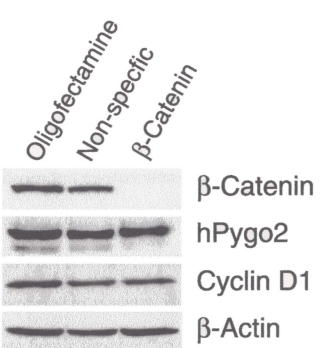
The lack of requirement for β–catenin in the growth of MCF-7 cells is consistent with our observation that it is predominantly localized to the cytoplasm and membrane compartments of MCF-7 and to the membrane compartment of Bt-474 cells (Figure 3.10)

Figure 3.16 Knockdown of β-catenin in Mcf-7 cells using RNAi. Reagent control (Oligofectamine) and non-specific siRNA controls are indicated. (a) Growth of Mcf-7 cells 72 hours after initial treatment with β–catenin siRNA. (b) Western analysis of hPygo2, Cyclin D1 and β-Catenin 48 hours after β–catenin knockdown by RNAi. Protein levels were standardized by reprobing blots with β-Actin. Results indicated are based on three experiments performed in triplicate.

a


100
0
% control growth
Non-specfic
β-Catenin

b


Oligofectamine
Non-specfic
β-Catenin
β-Catenin
hPygo2
Cyclin D1
β-Actin

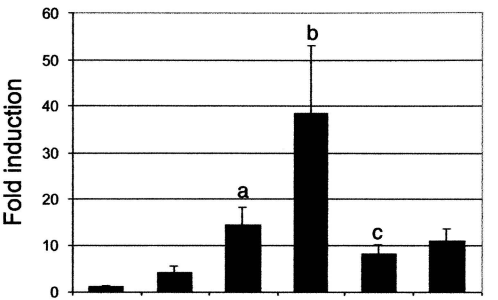
and therefore, is not present in a transcriptionally active Wnt complex to promote cell growth.

3.3.5 hPygo1 but not hPygo2 can induce the activation of Wnt-dependent transcription

To directly address the role of Pygopus proteins in the transcription of Wnt target genes, I performed transcription assays in MCF-7 cells using the TOPFLASH vector (Figure 3.17). TOPFLASH contains multiple TCF/LEF binding sites which control the expression of the reporter gene, Luciferase. The amount of Luciferase protein transcribed and translated can then be measured with a luminometer. As a positive control, a constitutively active mutant form of β–catenin (S37A) was used which contained a missense mutation resulting in an amino acid substitution replacing a Serine residue with Alanine. This mutant has been shown to be resistant to proteasomal degradation due to lack of phosphorylation at this residue which normally targets β–catenin for degradation (Wu *et al.*, 2001).

Transfection of S37A into MCF-7 cells resulted in an activation of Wnt dependent transcription greater than three-fold, while co-transfection of hPygo2 or Bcl-9 did not show an activation of Wnt dependent transcription above this level. In fact, transfection of hPygo2 and S37A resulted in a slight decrease of Wnt dependent transcription. In contrast, co-transfection of hPygo1 and S37A resulted in an approximate 9 fold increase in the Luciferase reporter compared to an empty vector control. These results confirm that hPygo1 is likely the Pygo family member that is involved in the transcription of Wnt target genes.

Figure 3.17 hPygo1 but not hPygo2 mediates canonical Wnt signaling transcription in Mcf-7 cells. Mcf-7 cells were transiently transfected with molar equivlents of either FOPFLASH (Mutant Tcf binding sites) or TOPFLASH (Tcf binding sites) Wnt reporter luciferase constructs, in conjunction with constitutively active β–catenin S37A, hPygo1, hPygo2 or Bcl-9. Relative luciferase units were determined and normalized to total cellular protein. Means and standard deviations indicated are based on three experiments performed in triplicate. [a and b, $p < 0.005$ ($p = 0.0025$); a and c, $p < 0.005$ ($p = 0.0018$)]



Fopflash	+	-	-	-	-	-
Topflash	-	+	+	+	+	+
β-Catenin S37A	+	-	+	+	+	+
hPygo1	-	-	-	+	-	-
hPygo2	-	-	-	-	+	-
Bcl-9	-	-	-	-	-	+

CHAPTER 4
DISCUSSION

Although poorly understood, aberrant Wnt signaling has been implicated in the progression of breast cancer (Michaelson and Leder, 2001; Li *et al.*, 2003; Hatsell *et al.*, 2003; Howe and Brown, 2004) . Therefore, the main objectives of this thesis were to evaluate the expression and requirement of the novel Wnt signaling family member, hPygo2, in breast cancer. With an antibody raised against non-conserved regions of hPygo2, I was able to determine its expression in normal and malignant cell lines and tumours. To address a possible functional requirement for hPygo2, I designed antisense oligonucleotides that specifically knocked down its expression. Interestingly, my results indicate a role for hPygo2 in the growth of breast cancer cells, independent of β-catenin. This finding may help provide insight into novel hPygo2 functions. These results also suggest that hPygo2 may be a more suitable therapeutic target than β–catenin for the treatment of breast cancer.

4.1 Development of hPygo2 antisera

One of the initial objectives of this thesis was to produce an antibody against hPygo2. The antibody could then be used as a molecular tool to study the expression of hPygo2. In chapter 3.1 of the results section, I have shown the successful production and characterization of an antibody that specifically recognizes hPygo2. In general, all antibodies were able to bind specifically to the native hPygo2 protein compared to the preimmune sera used as a negative control as shown by immunoprecipitation and by

immunoflourescence. The preimmune sera, however, was not used as a negative control for the western blots that were performed. This would have been a good negative control to confirm the specificity of the antibodies used to identify denatured hPygo2 protein.

The Hausenpfeffer immune serum was raised to avoid cross reactivity with conserved protein motifs present in the Pygo2 protein which may be present in other cellular proteins. For example, the PHD domain was proposed to mediate protein-protein interactions (Aasland *et al.*, 1995), and has since been shown to be present in many other proteins. It was important to avoid raising antibodies that would cross react with this domain since I wanted them to specifically interact with hPygo2. Therefore, I designed the Pygopus protein antigen to exclude all conserved amino acid sequences.

4.2 Antisense oligonucleotide and siRNA design

The ability of antisense ONs to inhibit protein production was first discovered in 1978, when a short 13 nucleotide antisense ON was used to inhibit the translation of viral proteins and therefore inhibit the replication of Rous sarcoma virus (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). Since then, the use of antisense ONs has become a common method to knock down the expression of a protein. This is a particularly useful method to specifically target overexpressed gene products that may play roles in the malignancy of a given cancer.

There are a number of potential problems associated with the use of antisense ON and a number of considerations must be taken into account in the design and use of antisense ON with regard to the mRNA structure, ON sequence and modifications.

The three dimensional structure resulting from folding and post transcriptional modification of an mRNA is complex. Therefore, the efficacy of an antisense ON is mostly dependent on its accessibility to the target sequence. Complex algorithms are useful in predicting efficient antisense ON sequences, but they cannot exactly predict how mRNA is folded or arranged in the cell, so effective antisense ON design is typically done stochastically.

When choosing the sequence for an antisense ON, it is important to carefully consider the antisense ON sequence. This will have a direct impact on the formation of secondary structures within the antisense ON. The less secondary structure that there is present the less likely that the ON will interact with itself instead of interacting with its target sequence. Other considerations must also be made with regard to the antisense ON sequence. For example, it is also important to avoid repeated CG sequences as well as GGGG sequences. In eukaryotes the cytosine adjacent to a guanine is usually methylated. Therefore, unmethylated CG sequences in vivo can initiate the activation of an immune response, as it has been shown that unmethylated CG sequences in mice result in B-cell activation and the secretion of antibodies (Krieg *et al.*, 1995). Also, antisense ON containing GGGG may also result in non-specific effects. For example, it was found that antiproliferative effects of antisense ON for c-myb and c-myc in smooth muscle cells was caused by a non-antisense mechanism likely due to the presence of a GGGG sequence (Burgess *et al.*, 1995). These examples illustrate the importance of the antisense ON sequence chosen for experimentation.

Lastly, it is possible to chemically modify ON. This is an important consideration to take into account when designing an antisense ON. The half life of the ON will have direct consequences on its efficiency to reduce levels of gene expression. In the cell, the nuclease family of enzymes function to degrade DNA molecules. Therefore, many antisense ONs that are used today contain modifications that enhance their nuclease resistance. Although many modifications of antisense ON exist, one of the most popular modification of antisense ON is the replacement of phosphodiester bonds with phosphorothioate bonds (Green *et al.*, 2000). In an antisense ON, phosphorothioate bonds greatly enhance the half life of an antisense ON to resist nuclease mediated degradation More recently, antisense ON containing 2'-O-methyl groups have been shown to have greater stability and higher affinity for target RNA molecules (McKay *et al.*, 1996). Therefore, 2'-O-methyl modified antisense ON directed against hPygo2 may have improved antisense effects compared to phosphorothioate ON used in this study. Indeed, the use of antisense ON is an efficient way to down regulate certain gene products in a disease state. There are many examples studying the effects of antisense ON and there are some antisense ON which have entered clinical trials, such as an antisense ON targeting Protein Kinase Cα (McGraw *et al.*, 1997).

4.3 Role of hPygo2 in breast cancer

To date, canonical Wnt signaling has a poorly understood role in breast cancer. While β–catenin has been reported to have nuclear/cytoplasmic staining in approximately 60% of breast tumours in two independent studies (Lin *et al.*, 2000; Ryo *et*

al., 2001), mutations in Wnt pathway components that activate β–catenin occur at relatively low frequencies (Polakis, 2000; Smalley and Dale, 2001; Giles *et al.*, 2003). It has been suggested that overexpression of Wnt ligands or reduced expression of Wnt antagonists may lead to the nuclear stabilization of β–catenin in mammary carcinogenesis (Howe and Brown, 2004). The absence of a conclusive answer to this apparent discrepancy necessitates an understanding of the true requirement for Wnt and β–catenin nuclear complexes in breast epithelial malignancy.

My results are the first demonstration of the potential requirement for the protein, hPygo2, in cancer. I have shown that hPygo2 protein, which is normally present at lower levels in the cytoplasm of normal adult cells, is found at higher levels in the nuclei of certain cancer cells, potentially representing a key step in the progression of this disease. Nuclear localization of Pygopus is important for its proposed function as a transcriptional activator (Thompson *et al.*, 2002; Kramps *et al.*, 2002). It is possible that hPygo2 may be sequestered in the cytoplasm in normal cells by another cytoplasmic anchoring protein, as is the case for the cytoplasmic retention of β–catenin in the absence of a Wnt signal. On the other hand, when cells are actively dividing, hPygo2 may be recruited to the nucleus, to function as a transcriptional regulator thereby activating the expression of genes involved in promoting cell cycle progression or repressing genes involved in apoptosis.

More work is required to address the functional significance of hPygo2 in normal breast cells. This could be done by constructing a nuclear localization deficient hPygo2 protein, which could be used as "bait" in order to examine cytoplasmic interacting proteins. The identification of key cytoplasmic interacting proteins may help to elucidate

the role of hPygo2 in normal breast cells. Also, knockdown of hPygo2 with antisense ON could also be performed in normal breast cells. Thereby confirming, whether or not, that the growth reduction of the breast cancer cell lines examined was specific to a cancer cell and not to a normal cell.

My data also indicate that hPygo2 was required for the growth of mammary carcinoma cells (MCF-7, MDA-MB 468) and for the expression of the Wnt target, and cell cycle promoting gene *Cyclin D1* in Mcf-7 cells. The Cyclin D1 protein is a positive regulator of cell cycle and required for G1 to S phase transition (Coqueret, 2002; Stacey, 2003). The increased expression of Cyclin D1 in actively dividing cells promotes cell cycle progression by forming a complex with Cyclin Dependent Kinase 4 (CDK4). This complex has been shown to increase phosphorylation of the Retinoblastoma protein (pRb), therefore promoting cell cycle progression through the G1 phase (Kato *et al.*, 1993; Ewen *et al.*, 1993). Therefore, the reduction of Cyclin D1 by hPygo2 antisense suggests that the cells may be arresting in the G1 phase of the cell cycle and may be due to a decrease in the phosphorylation of pRb.

Along with growth reduction and a reduction of Cyclin D1 protein, hPygo2 siRNA treatment of MCF-7 cells, resulted in a decrease in the expression of Cyclin A (K. Kao, unpublished observations). Cyclin A, which is involved promoting in S phase of the cell cycle by binding to CDK 2, thereby activating proteins involved in DNA replication, may also result in growth arrest (Yam *et al.*, 2002). It is possible that the reduction in Cyclin A, a gene that is not directly regulated by Wnt signaling, is due to a cascade effect involving the downregulation of Cyclin D1. For example, a decrease in the levels of

Cyclin D1 results in a decrease in E2F transcription factor activity (Johnson, 1995). A reduction in the E2F transcription factor can then lead to a decrease in the expression of Cyclin A (Schulze *et al.*, 1995). Therefore, the decrease in Cyclin A after hPygo2 siRNA treatment may be a secondary effect resulting from the knock down of Cyclin D1. This could be addressed by a knockdown of Cyclin D1 with antisense ON or siRNA to determine if levels of Cyclin A are affected. Also, it is possible that hPygo2 may be directly or indirectly regulating the transcription of Cyclin A by an undetermined mechanism. Therefore, these results suggest that the knockdown of hPygo2 may be resulting in the growth arrest of the cancer cells examined. Although, more work is required to confirm that the reduction in cell numbers was not due to apoptosis.

4.4 hPygo2 and β–catenin may function independently in breast cancer cells

Pygo proteins overexpressed in breast cancer may play an additional role than the proposed mediation of canonical Wnt signals. This hypothesis is consistent with studies demonstrating transcriptional activity of DNA tethered Gal4-Pygo2 fusion proteins (Belenkaya *et al.*, 2002; Townsley *et al.*, 2004) and of Pygo2 constructs lacking β–catenin binding sequences that are either fused to dominant activating TCF (Thompson, 2004) or expressed on their own (Lake and Kao, 2003) . In fact, a Gal-4-Pygo2 fusion protein tethered to a Gal-4 binding site can activate transcription in ovarian cancer cells that do not express β–catenin (K. Kao, unpublished observations). Therefore, hPygo2 may be recruiting a number of other proteins to activate transcription in the absence of β–catenin, indicating another possible functional role of hPygo2. Also I have shown that

hPygo1 mediates the Wnt signal through β–catenin, while hPygo2 does not (Figure 3.17). These results, therefore, support a model in which human Pygo2 probably acts as a transcription factor, by recruiting other proteins in a β–catenin independent manner, and that human Pygo1, like *Drosophila Pygo*, is likely dedicated to the Wnt signaling pathway through β–catenin.

In further support of this model, my results demonstrated that the expression levels of hPygo2 correlated with the expression of β–catenin but not its only known binding partner, Bcl-9. The breast cancer cell line MCF-7, used in this study, was previously shown to exhibit Wnt-dependent transcription, Cyclin D1 expression, as well as β–catenin/TCF complex formation (Lin *et al.*, 2000). The Wnt-dependent transcriptional activity of this cell line has also been compared to a number of other breast cancer cell lines, including one that contains a mutation in APC (DU4475) which results in constitutive Wnt activation and transcription. Compared to these APC-deficient cells, the MCF-7 cells have a relatively low, almost basal level of Wnt activity (van De *et al.*, 2001), in contradiction to that reported by Lin. My results confirm those obtained in the former study (van *De et. al.*, 2001) and it is therefore not surprising that Bcl-9 expression does not correlate with that of β–catenin and hPygo2.

These findings, as well as the lack of Bcl-9 expression in MCF-7 cells, supports my hypothesis that hPygo2 may have an independent function from β–catenin. If Wnt signaling were actively occurring in these cells, which is unlikely, I would have expected Bcl-9 to be present to tether Pygo to the β–catenin/TCF complex (Kramps *et al.*, 2002).

Therefore, I suspect that canonical Wnt signaling may be absent or occur at low levels in these cells, given that the expression of Bcl-9 is limiting.

In further support of my hypothesis, inhibition of β–catenin by ICAT overexpression had no effect on HeLa cell growth (Sekiya *et al.*, 2002), consistent with my observations using β–catenin siRNA in MCF-7 cells. These data suggest that at least in two cancer cell lines, canonical Wnt signaling does not appear to be important for cell growth. Indeed, it has been found that hPygo2, but not β–catenin was required for survival of at least one other epithelial ovarian cancer cell line, TOV-21G (K. Kao, Manuscript in preparation). It has also been previously reported a requirement for Pygo2 in the transcription of the non-Wnt responsive genes in *Xenopus* embryos (Lake and Kao, 2003). These data further imply an additional functional role of hPygo2, independent of β–catenin.

The proposed function of Pygo in mediating the remodeling of chromatin may be too simplistic, since DNA tethered Pygo shows transcriptional activity (Belenkaya *et al.*, 2002; Townsley *et al.*, 2004). It is also well known that the histone acetyltransferase, p300/CBP is associated with the β–catenin/TCF complex and likely functions to regulate chromatin structure at Wnt promoter sites (Hecht *et al.*, 2000). In fact, p300/CBP also contains a PHD domain which has been shown to be an essential component of the acetyltransferase domain of p300/CBP (Kalkhoven *et al.*, 2002). Therefore, the requirement of the PHD domain of Pygopus as well as its putative chromatin remodeling function in the transcription of Wnt target genes seems to be redundant in the transcription of Wnt target genes.

It has been previously shown that levels of Wnt signaling determined by transcription assays showed a strong correlation with the expression of Cyclin D1 in breast cancer cells including MCF-7 (Lin *et al.*, 2000). Because, as I have shown, β–catenin is not required for the expression of Cyclin D1 in these cells, the expression of Cyclin D1 may not be a true indication of canonical Wnt signaling. This result is consistent with immunohistochemical studies of infiltrating ductal carcinomas that were shown to exhibit a divergence of Cyclin D1 and β–catenin expression (Lim and Lee, 2002). Indeed, Cyclin D1 expression has been shown to be required for cell growth and is regulated by other non-Wnt dependent proteins in MCF-7 cells, such as estrogen receptor (Watts *et al.*, 1994) and Peroxisome Proliferator-activated Receptor γ (PPARγ) (Yin *et al.*, 2001; Qin *et al.*, 2003). My results suggest that Cyclin D1 may not be strictly regulated by β–catenin in MCF-7 cells, as Cyclin D1 is likely regulated by many other factors in these cells.

Reduction of hPygo2 on the other hand, did result in a decrease of Cyclin D1 (Figure 3.14), which leads one to question the role of hPygo2 in its expression. Since Cyclin D1 is a known Wnt target gene, it is possible that hPygo2 may directly regulate its expression independently of β-Catenin. Since Cyclin D1 expression is regulated by factors (estrogen receptor and PPARγ) other than β–catenin, I hypothesize that hPygo2 might be either directly or indirectly involved in the regulation of these factors in the transcriptional regulation of the *Cyclin D1* gene. Futhermore, hPygo2 may regulate the turnover of Cyclin D1 by a post translational mechanism independent of transcriptional regulation.

4.5 Conclusions

In conclusion, I have confirmed my hypothesis that the overexpression of hPygo2 may contribute to the growth of breast cancer cells, which I have demonstrated through expressional knockdown of hPygo2. Wnt-dependent transcription was shown to require Pygopus in colorectal cancer cells (Thompson *et al.*, 2002), and, while not assayed, growth of these cells is predicted to be inhibited by Pygo knockdown consistent with that shown for β–catenin (Roh *et al.*, 2001; Sekiya *et al.*, 2002; Verma *et al.*, 2003). Not all cancers likely involve deregulation of the β–catenin/hPygo2 complex. Divergence of these components, however, suggests the potential for a more global involvement of Pygo proteins than β–catenin in cancer. This would make Pygo a more suitable therapeutic target, and necessitate a greater understanding of Pygo functional protein associations.

4.6 Future directions

The expression of Pygopus in malignant cell lines and tumours, suggests that there may be a correlation between hPygo2 expression and disease. To confirm this finding, a study utilizing a larger patient population would be needed. Factors such as patient survival, disease-free survival as well as information on how the patient was treated for their disease could be correlated with hPygo2 expression to determine if hPygo2 could be used as a prognostic marker.

There has been no work demonstrating the transcriptional regulation of hPygo2. This is an important question to consider since hPygo2 expression was upregulated in

cancer. Is the overexpression of hPygo2 in breast cancer due to its constitutive transcriptional up-regulation? To address this question, I analyzed the promoter region of hPygo2 and found that there are consensus sites for the transcription factor E2F (Data not shown). Many cancers downregulate the pRB pathway resulting in E2F activation and the transcription of genes involved in cell cycle progression (Yamasaki, 2003), this may partially explain the high levels of expression of Pygpus in cancer. Experiments to further explore this finding could include cloning the promoter region of hPygo2 into a promoter-less expression vector to regulate the expression of a reporter gene such as Luciferase. To further characterize a possible transcription factor-DNA interaction, chromatin immunoprecipitation could be used, alone or in conjunction with electrophoretic mobility shift assay to determine which regions of the promoter that the hypothesized transcription factors bind. A functional demonstration of the transcriptional regulation of hPygo2 by E2F could be performed by knockdown or overexpression of E2F followed by western blotting for hPygo2 to monitor protein expression changes.

Legless/Bcl-9 is hypothesized to interact with Pygopus in *Drosophila* (Kramps *et al.*, 2002), and I have shown that hPygo1, but not hPygo2, is required for Wnt dependent transcription. It would therefore be interesting to study the interaction of Bcl-9 with both human Pygo family members. Since I have shown that Bcl-9 does not activate Wnt dependent transcription, I hypothesize that its interaction with Pygopus may be restricted to hPygo2. This could be easily confirmed by co-immunoprecipitation experiments to identify the protein interactions.

Finally, in order to directly address my hypothesis that hPygo2 may act independently of β–catenin, proteomics could be used as a tool to identify hPygo2 interacting proteins. Using the antibodies that I have produced to immunoprecipitate hPygo2 from MCF-7 cells followed by proteomic analysis would be a great method to identify proteins that interact with hPygo2. Candidate hPygo2 interacting proteins would have to be further confirmed to interact, and analyzed to determine the functional significance of the protein-protein interaction.

Identification of known proteins that interact with hPygo2 could be identified by immunoprecipitation of hPygo2 from cells followed by separation of proteins by high resolution, two dimensional gel electrophoresis. Proteins spots stained with Coomassie Blue could then be isolated and digested with a specific protease, such as trypsin. Trypsin digestion yields a peptide "fingerprint" which contains specific peptide fragments of a larger protein. The peptide "fingerprint" of the mass/charge ratios of the peptide fragments could then be determined by matrix-assisted laser desorption/ionization time of flight, also known as MALDI-TOF mass spectroscopy. Database analysis of the mass/charge ratios of peptide fragments could then be used to identify the interacting protein.

Identification of unknown proteins that interact with hPygo2 could be identified in the same way as stated above, except that tandem mass spectroscopy could be used. Tandem mass spectroscopy ultimately can lead to the determination of amino acid sequence present within the mixture of hPygo2 interacting proteins. Amino acid

sequences could then be used to identify predicted proteins present within the human genome and hence, identify unknown predicted proteins that interact with hPygo2.

CHAPTER 5
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