PROTEIN TYROSINE PHOSPHATASE PRL-3 EXPRESSION AND TRANSCRIPTIONAL REGULATION IN CANCER AND METASTASIS

By

PATRICK CHUN WEI WONG

B.M.L.Sc., The University of British Columbia, 2003

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

June 2005

ABSTRACT

Elevated expression of protein tyrosine phosphatase PRL-3 is implicated in cell migration, invasion, and metastasis, although the molecular targets of this phosphatase remain unidentified. PRL-3 expression is strikingly upregulated in metastatic colon carcinoma, compared with much lower expression in primary colon tumours and virtually undetectable expression in normal colon epithelia. Increased transcriptional activity is suggested to account for increased PRL-3 expression in 75% of the metastatic samples. We used bioinformatics analyses and cell-based promoter assays to investigate the tumour- and metastasis-specific transcriptional activity of the ~4.3 kb promoter region upstream of the PRL-3 transcription start site.

Through bioinformatics analyses, sequence conservation (human/mouse) and multiple putative transcription factor binding sites were identified in various regions upstream of the PRL-3 transcription start site. A 518bp CpG island (CpG-64) was predicted to have high regulatory potential. The transcriptional activities of the PRL-3 promoter were analyzed by luciferase reporter assays in two cell lines derived from (i) a primary colon adenocarcinoma (SW480) and (ii) a metastasis of the same tumour (SW620). These assays revealed that the full-length promoter region (which contains the CpG-64 island) was 4.5-fold more transcriptionally active in the metastatic colorectal carcinoma cells than in the primary colorectal carcinoma tumour cells. The CpG island itself directed 2.3-fold higher transcriptional activity in the metastatic colorectal carcinoma cells than in the primary colorectal tumour cells. In prostate cancer cells, this CpG-64 island was

8.7-fold more active in the highly invasive androgen-independent DU145 cells compared to the less invasive androgen-dependent LNCaP cells. However, the full length promoter was 3.6-fold more active in the LNCaP cells than in the DU145 cells. Additionally, this CpG-64 island was significantly more active in malignant muscle cells (RD and RMS13) than in normal muscle cells (C2C12). These results suggest that the CpG-64 island contains regulatory elements responsible for the tumour- and metastasis-specific transcriptional upregulation of PRL-3. Identification of these elements and their interacting factors may provide insight into the molecular pathways that are involved in the tumour-to-metastatic transition.

Using tissue-microarray technology, PRL-3 expression was detected in colorectal, uveal melanic, breast, and certain pediatric tumours. Immunohistochemical localization of PRL-3 appeared to be associated with tissue differentiation status. More specifically, nuclear PRL-3 was mainly present in poorly differentiated tissues, whereas cytoplasmic PRL-3 was predominantly found in well differentiated tissues. Also, high levels of PRL-3 in clinical tumour samples correlated with an unfavorable trend in uveal melanic and breast cancer patient prognosis. In conclusion, the evidence from both the basic science and the clinical sides argue strongly for the importance of PRL-3 as a tumourigenic and/or metastatic biomarker. Thus, it is imperative to study this phosphatase at the molecular level to understand the clinical ramifications of its activities.

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LIST OF ABBREVIATIONS

Ab: Antibody

AR: Androgen Receptor

ARMS: Alveolar Rhabdomyosarcoma

CHO: Chinese Hamster Ovary

CRM: *Cis*-Regulatory Modules

EGF: Epidermal Growth Factor

EMT: Epithelial-Mesenchymal Transition

ERMS: Embryonal Rhabdomyosarcoma

EST: Expression Sequence Tags

HEK: Human Embryonic Kidney

IGF-1: Insulin-like Growth Factor 1

IHC: Immunohistochemistry

MEF2: Myocyte Enhancer Factor 2

MMP: Matrix Metalloproteinase

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PRL: Protein in Regenerating Liver

PTK: Protein Tyrosine Kinase

PTP: Protein Tyrosine Phosphatase

RMS: Rhabdomyosarcoma

TFBS: Transcription Factor Binding Sequence

TMA: Tissue Microarray

TSS: Transcriptional Start Site

UCSC: University of California, Santa Cruz

UTR: Untranslated Region

ACKNOWLEDGEMENTS

CHAPTER 1

INTRODUCTION

1.1   THE PROTEIN TYROSINE PHOSPHATASE SUPERFAMILY

Protein tyrosine phosphatases (PTPs) belong to a large superfamily of enzymes observed in eukaryotes, prokaryotes, and viruses. They have been classified into several subfamilies based on their substrate specificity and structural features (Figure 1). In regards to substrate specificity, PTPs fall into two groups. The tyrosine-specific PTPs have an absolute specificity for phosphotyrosine substrates, while the dual specificity PTPs can dephosphorylate both phosphotyrosine and phosphoserine / phosphothreonine residues. In terms of their structures, PTPs fall into three major subfamilies: receptor-like PTPs, non-receptor intracellular PTPs, and the low molecular weight PTPs which share minimal sequence identity with other members of the superfamily. All PTPs are characterized by a highly conserved active site signature motif CX_5R, where the invariant cysteine residue is essential for catalysis. However, no PTPs share any sequence similarity with the protein serine/threonine phosphatases or the alkaline phosphatases.

Protein phosphorylation is a common mechanism that a cell utilizes to control biological processes. At a given time, up to 30% of the total proteins in the cell are phosphorylated and most of these phosphorylated proteins comprise of either serine or threonine phosphorylation (Roach, 1991; Hunter, 1995). Tyrosine phosphorylation occurs to a

much lesser extent but it plays a key role in many cell processes such as cell proliferation and differentiation, cell cycle control, cytoskeletal organization, cell migration, signal transduction, transcriptional regulation, ion-channel activity, and the immune response (Hunter, 1995; Neel, 1997). Increased cellular protein tyrosine phosphorylation is often associated with transformation and oncogenesis (Blume-Jensen and Hunter, 2001). The central role played by a constitutively activated protein tyrosine kinase (PTK) in oncogenesis was first illustrated by the discovery of the transforming v-src PTK (Hunter and Sefton, 1980). Immediately after, a large number of viral and cellular oncogenes that encode PTKs were identified (Blume-Jensen and Hunter, 2001). The observation that many PTKs are proto-oncogenes and growth factor receptors suggests that tyrosine phosphorylation is controlled by PTKs to activate signal transduction pathways. After growth factor stimulation or oncogenic transformation, the phosphotyrosine content of the cell can increase by as much as 100-fold to ~1-2% of the total protein phosphorylation in the cell (Levinson et al., 1980; Sefton et al., 1981.) In resting cells, protein tyrosine phosphatases (PTPs) maintain a basal level of tyrosine phosphorylation on PTKs and also PTK substrates to ensure the proper functioning of the cell (Hunter, 1995; Fischer et al., 1991). In addition to negative regulatory roles, PTPs also play key roles as positive effectors of signal transduction pathways (Tonks and Neel, 1996; Sun and Tonks, 1994).

The tyrosine-specific PTPs include all the receptor-like PTPs and some of the non-receptor PTPs, as well as the low molecular weight PTPs. Both receptor-like PTPs and non-receptor PTPs share a conserved catalytic domain encompassing about 240 amino acids. Among these PTPs, the active site sequence conservation can be extended from

the minimal CX_5R motif to HCXAGXXR[S/T]. In addition, each PTP contains heterogeneous non-catalytic sequences flanking the conserved catalytic domains. These sequences are important for subcellular localization and regulation / interaction with other proteins, such as the formation of a PTP/PTK complex.
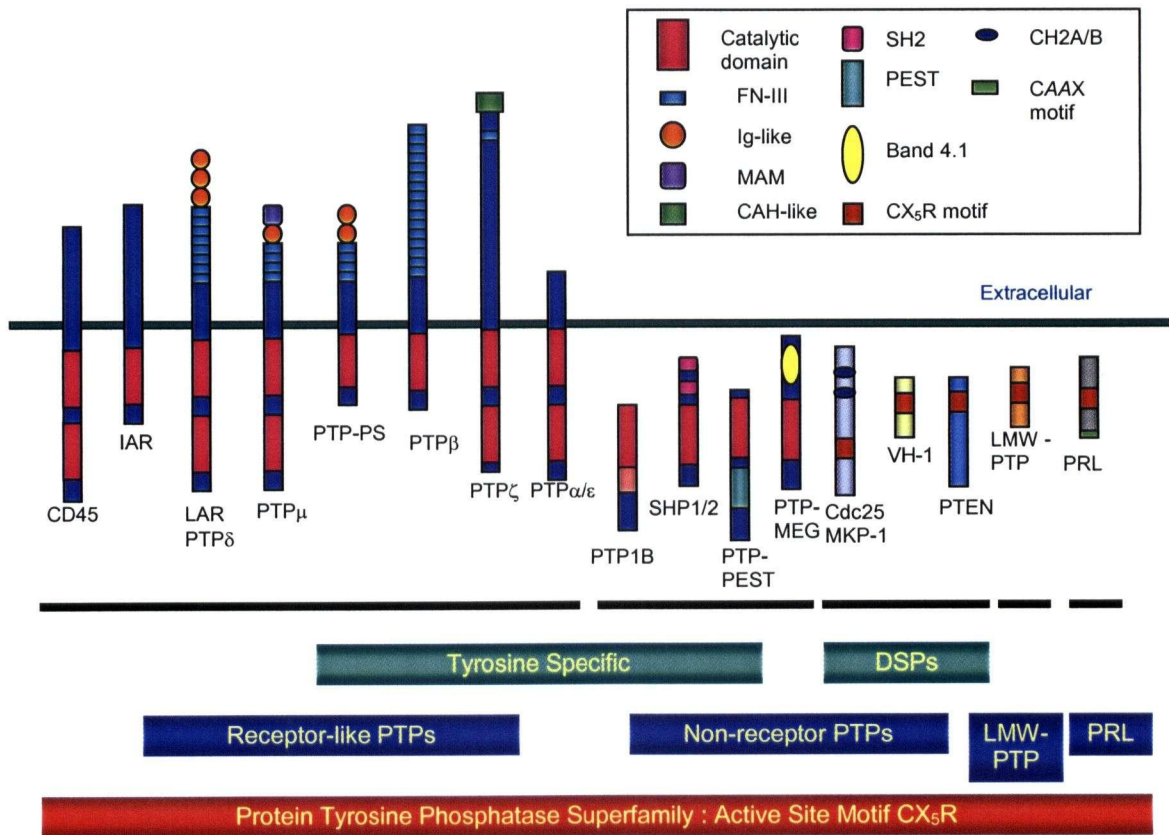
Figure 1. The PTP Superfamily. Schematic diagram of structures of selected members of the PTP superfamily. The inset box shows various structural motifs that are found in the PTPs (FN-III: Fibronectin type-III; MAM: Meprin/A5/PTPµ domain; Ig: Immunoglobulin; CAH-like: Carbonic anhydrase-like; CX_5R: PTP signature motif; Band 4.1: ezrin-band 4.1-merlin-radixin protein; SH2: Src homology 2; PEST: Pro-Glu-Ser-Thr motif; CH2A/B: sequence homology found in Cdc25 and MKP-1; CAAX: prenylation motif).

1.2   THE PROTEIN IN REGENERATING LIVER (PRL) FAMILY

The protein tyrosine phosphatase (PTP) superfamily is made up of a large and diverse family of enzymes, including a subclass of three closely related enzymes called the PRL-PTPs (PRL-1, -2, and -3). PRL-1 was first discovered as an early immediate gene expressed in mitogen-stimulated cells and in regenerating liver, hence the term "**P**rotein in **R**egenerating **L**iver" (PRL) was coined (Mohn *et al.*, 1991). PRL-1 is undetectable in quiescent liver but its expression is elevated after 70% hepatectomy (Diamond *et al.*, 1994). PRL proteins have been identified and cloned from rat, human and mouse (Diamond *et al.*, 1994; Montagna *et al.*, 1996; Cates, *et al.*, 1996; Zhao *et al.*, 1996; Zeng *et al.*, 1998). PRL-1 has little sequence identity with other known PTPs outside its PTP signature motif and is thought to represent a novel group of PTPs.

Three PRL proteins have been reported to date and termed PRL-1 (also known as PTPcaax1), PRL-2 (also known as PTP4A, OV-1 and PTPcaax2) and PRL-3 (also known as PTP4A3). As shown in Figure 2A, mouse PRLs share high sequence identity with one another. As demonstrated in Figure 2B, PRL-2 is 87.4% and 75.4% identical to PRL-1 and PRL-3 respectively, while PRL-3 is 75.7% identical to PRL-1 (Zeng *et al.*, 1998). The PRL-1 amino acid sequence is identical among human, rat and mouse. The PRL-2 amino acid sequence is identical between human and mouse with the substitution of only two residues (Carter, 1998). The PRL-3 amino acid sequence is more diverse between human and mouse with the substitution of six residues (Matter *et al.*, 2001). Interestingly, a PRL has been identified as a putative gene product in *Drosophila* (dPRL-

1) and *C. elegans* (cPRL-1). dPRL-1 and cPRL-1 have over 50% sequence identity with human PRL-1, -2, and -3, which implies that PRL proteins are evolutionarily conserved and that the different isoforms of PRL proteins of human, mouse, and rat may come from the same precursor.

A

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PRL-1   MARMNRPAPVEVTYKNMRFLITHNPTNATLNKFIEELKKYGVTTIVRVCEATYDTTLV
PRL-2   M---NRPAPVEISYENMRFLITHNPTNATLNKFTEELKKYGVTTLVRVCDATYDKAPV
PRL-3   MARMNRPAPVEVSYRHMRFLITHNPSNATLSTFIEDLKKYGATTVVRVCEVTYDKTPL


PRL-1   EKEGIHVLDWPFDDGAPPSNQIVDDWLSLVKIKFREEPGCCIAVHCVAGLGRAPVLVA
PRL-2   EKEGIHVLDWPFDDGAPPPNQIVDDWLNLLKTKFREEPGCCVAVHCVAGLGRAPVLVA
PRL-3   EKDGITVVDWPFDDGAPPPGKVVEDWLSLLKAKFYNDPGSCVLVHCVAGLGRAPVLVA


PRL-1   LALIEGGMKYEDAVQFIRQKRRGAFNSKQLLYLEKYRPKMRLRFKDSNGHRNNCCIQ
PRL-2   LALIECGMKYEDAVQFIRQKRRGAFNSKQLLYLEKYRPKMRLRFRDTNGH---CCVQ
PRL-3   LALIESGMKYEDAIQFIRQKRRGAINSKQLTYLEKYRPKQRLRFKDPHTHKTRCCVM
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B
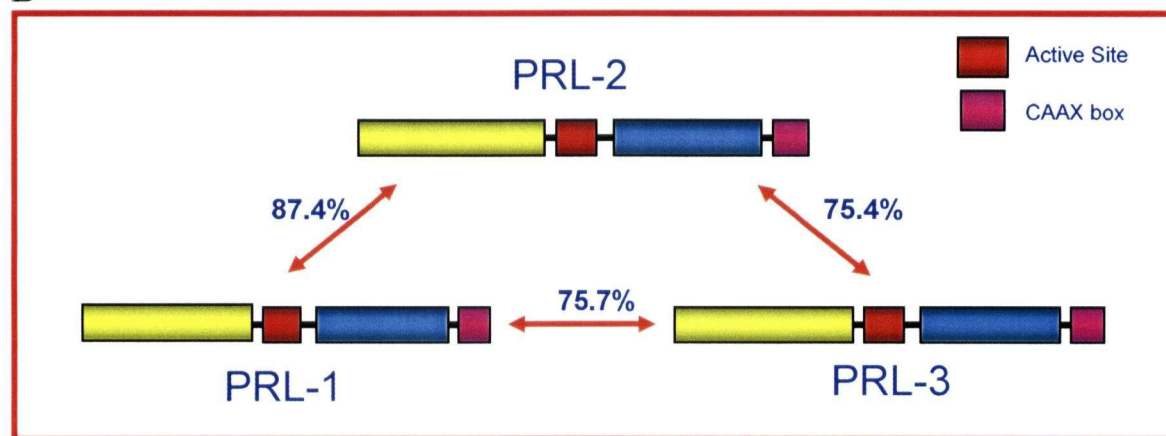
Figure 2. PRL-PTP family member identity. *A*, alignment of mouse PRL-1, -2, and -3 amino acid sequences, identical residues are highlighted in yellow, prenylation motifs are underlined; *B*, percentage identity between the three members of the PRL family.

Expression of PRL-1 is associated with proliferation in liver but with differentiation in intestine, suggesting that PRL-1 may target different substrates in different tissues (Diamond *et al.*, 1996). In addition, over-expression of either PRL-1 or PRL-2 alters cell growth and induces a transformed phenotype in transfected mouse fibroblasts and hamster pancreatic epithelial cells, as well as promoting tumour growth in nude mice (Diamond *et al.*, 1994; Cates *et al.*, 1996). By and large, the cellular substrates and functions of the PRL-PTPs are still unknown.

Many prenylated proteins are important regulators of signal transduction pathways. Protein prenylation is a post-translational modification involving the covalent attachment of a farnesyl (C15) or geranylgeranyl (C20) isoprenoid lipid group to a cysteine residue at or near the C-terminus. Prenylation can be followed by other modifications such as proteolysis, methylation and palmitoylation (Glomset *et al.*, 1990; Zhang and Casey, 1996; Sinensky, 2000). One example of a protein that is post-translationally prenylated is the Ras family GTPase (Casey *et al.*, 1989; Schafer *et al.*, 1989; Hancock *et al.*, 1989) as seen in Figure 3. Protein prenylation is specified by the amino acid motif CAAX, CCXX, CXC or CC at the C-terminus and mediated by CAAX prenyltransferases and geranylgeranyltransferase II (Zhang and Casey, 1996; Sinensky, 2000). Among PTP superfamily members, the PRL-PTPs are unique in possessing a C-terminal prenylation motif (Cates *et al.*, 1996; Zeng *et al.*, 1998), suggesting that the PRL proteins are potentially modified by prenylation. Human PRL-1 and PRL-2 have been shown to be farnesylated by farnesyltransferase *in vitro* (Cates *et al.*, 1996), and PRL-2 has been shown to be farnesylated *in vivo* (Si *et al.*, 2001).
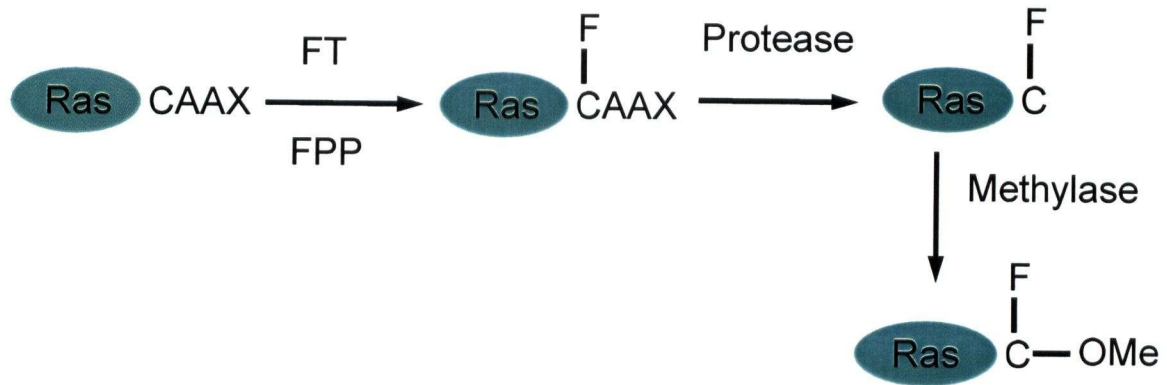
Figure 3. Ras protein prenylation. Newly synthesized Ras is prenylated (FT: farnesyltransferase; FPP: farnesyl-pyrophosphate) and undergoes prenylation-dependent proteolysis and carboxylmethylation in the endoplasmic reticulum.

Protein prenylation can be important for targeting proteins to intracellular membranes and in protein-protein interactions (Zhang and Casey, 1996; Marshall, 1993; and Seabra, 1998). PRL-1 was reported to be a nuclear protein tyrosine phosphatase (Diamond *et al.*, 1994), which is atypical for a prenylated protein because most prenylated proteins are associated with the cytoplasmic side of various intracellular membranes. PRL proteins are prenylated *in vivo* and their prenylation is required for their association with the plasma membrane and the early endosome (Zeng *et al.*, 2000). Inhibition of prenylation, by treatment of cells with a farnesyltransferase inhibitor, results in nuclear localization of newly synthesized PRL proteins (Zeng *et al.*, 2000). The prenylation motifs of the PRLs (CCIQ, CCVQ, and CCVM) conform to either the CAAX motif preferentially recognized by farensyltransferase or to the CCXX motif recognized by geranylgeranyltransferase II, although their relocalization in response to farensyltransferase inhibition indicates that they are likely farnesylated proteins *in vivo*. The prenylation-dependent subcellular localization of the PRLs suggests that regulated prenylation could be a mechanism to control the access of these PTPs to early endosomal or nuclear substrates.

1.3   PRL-3: A METASTASIS-ASSOCIATED PHOSPHATASE

The PRL-PTPs are implicated in cell proliferation and tumourigenesis, and more recently have also been linked to metastasis. Both PRL-1 and PRL-2 have been shown to possess oncogenic activity (Diamond *et al.*, 1994; Cates *et al.*, 1996). PRL-1 is highly expressed in some tumour cell lines, particularly in melanoma cells (Wang *et al.*, 2002). Stable expression of heterologous PRL-1, -2, or -3 increases cell growth rates (Diamond *et al.*, 1994; Cates *et al.*, 1996; Matter *et al.*, 2001). Furthermore, over-expression of PRL-1 in NIH 3T3 mouse fibroblasts, and PRL-1 or -2 in D27 hamster pancreatic ductal epithelial cells caused cell transformation, and in the latter case conferred the ability to form tumours in nude mice (Diamond *et al.*, 1994; Cates *et al.*, 1996).

PRL-2 is among eight genes found to be differentially expressed in benign prostatic fibroblast cells after stimulation with tumourigenic LNCaP cell-conditioned media (Wang *et al.*, 2002). PRL-2 expression is up-regulated 4-fold in benign fibroblast cells and 9-fold in tumour fibroblast cells upon incubation with the conditioned media for 1 day. Among the prostate cancer cell lines examined, androgen-independent cell lines such as PC-3 and DU145 have higher PRL-2 expression than the androgen-dependent LNCaP cell line and normal prostatic epithelial cells. Notably, the clinical transition from the androgen-dependent state to the androgen-independent state is crucial in prostate cancer development (Stearns and McGarvey, 1992; Fossa, 1994; Newling, 1996). Once this transition occurs, the options for effective treatment are substantially reduced. The over-

expression of PRL-2 in two out of the three samples further implicates PRL-2 in advanced prostate cancer.

Saha et al. (2001) found that PRL-3 expression is upregulated in metastatic colorectal carcinoma. Eighteen metastatic colorectal cancer samples were profiled for gene expression analysis. Of the 144 genes that were found to be upregulated, only PRL-3 was consistently over-expressed (50-450 fold) in all 18 metastatic specimens. It was almost undetectable in normal colon tissue, and primary colorectal tumours had intermediate PRL-3 expression levels. Later, Zeng et al. (2003) demonstrated that the over-expression of PRL-3 in Chinese Hamster Ovary (CHO) cells resulted in enhanced motility compared to non-PRL-expressing control CHO cells in a wound-healing assay. Similarly, the PRL-3 expressing CHO cells displayed increased invasiveness in a Matrigel assay. The expression of PRL-3 promoted cell metastasis in vivo as well. This study provided initial evidence for a causal role of PRL-3 in promoting cell motility, invasion, and metastasis, which are all essential characteristic properties of the metastatic process.

PRL-3 is highly expressed in metastatic melanoma B16-BL6 cells but not in the parental B16 cell line with low metastatic ability (Wu et al., 2004). B16 cells transfected with PRL-3 cDNA displayed morphological transformation from epithelial-like to fibroblast-like shaped cells, much like the highly metastatic B16-BL6 melanoma cells. These PRL-3 over-expressing cells showed much higher migratory ability, which was reversed by the presence of specific PRL-3 anti-sense oligodeoxynucleotide. These observations provide convincing evidence that PRL-3 plays a causal role in tumour metastasis.

PRL-3 has also been implicated in gastric cancer. Miskad *et al.* (2004) found that 87.5% of Reverse-Transcriptase PCR analyzed gastric cell lines expressed PRL-3. Ninety-four cases of primary gastric and fifty-four cases of matching nodal metastases were also evaluated for immunohistochemical (IHC) PRL-3 expression. It was found that 81.5% of the cases with nodal involvement expressed PRL-3, whereas 50% of the cases without nodal involvement expressed PRL-3. Moreover, 68% of all the primary gastric tumour samples also expressed PRL-3. PRL-3 expression was closely associated with lymphatic invasion, extent of lymph node metastasis, and tumour stage. Furthermore, the distribution pattern of PRL-3 at the cell membrane may be correlated with the metastatic ability of the tumour cells (Miskad *et al.*, 2004). This finding is consistent with previous reports that localization of PRL-3 at the plasma membrane enhanced the migration and invasion properties of cells. These observations strongly suggest that PRL-3 expression plays a significant role in invasion and metastasis of gastric carcinoma.

Since the striking observation was first made that PRL-3 is implicated in colorectal metastasis (Saha *et al.*, 2001), many similar studies have been performed. Peng *et al.* (2004) found, using immunohistochemistry (IHC) to investigate normal, primary, and metastatic colorectal tumour samples, that PRL-3 expression intensity and positivity were not significantly different between normal colorectal epithelium and colorectal cancer, or between metastatic lymph nodes and liver metastasis. However, PRL-3 expression was significantly higher in metastases when compared with normal colorectal epithelia and primary colorectal cancers. Kaplan-Meier survival curve analysis showed that PRL-3

expression was correlated with shorten survival time of colorectal cancer patients. These results suggest that PRL-3 is a predictor of liver metastasis and poor prognosis for patients with colorectal cancer (Peng et al., 2004). Kato et al. (2004) looked at 177 cases of primary colorectal cancer, and 92 cases of metastasis. Of the patients with any types of metastases, ~80% of the primary colorectal cancer tissues displayed increased PRL-3 expression, and of the patients with no lung or liver metastases, ~40% of the primary colorectal cancer tissues had increased PRL-3 expression. However, more than 90% of the liver or lung colorectal metastases had increased PRL-3 expression. This again demonstrates that high expression of PRL-3 is closely correlated with liver and lung metastasis in colorectal cancer. Additionally, PRL-3 siRNA treatment of DLD-1 cells (a colorectal cancer cell line with relatively abundant PRL-3 transcript) caused a 32% decrease in migration of these cells, accompanied with morphological alterations, but had no effect on proliferation (Kato et al., 2004). These findings exemplify the importance of PRL-3 in colorectal cancer progression.

What is the molecular basis underlying the PRL's role in oncogenic transformation and metastasis? In the past three decades, numerous studies have focused on identifying mechanisms of cancer development and progression, developing novel therapeutic strategies, and improving surgical techniques. Cancer progression is a multi-step process from a localized primary tumour mass to an invasive secondary metastasis resulting from the interaction of tumour cells with the organ microenvironment (Kassis et al., 2001; Price and Collard, 2001; Ellenrieder et al., 1999; Portera et al., 1998). This complicated process involves progressive changes from the primary tumour to the distal metastasis.

Cells from the primary tumour must migrate, settle in a remote organ, and grow in the secondary site. The essential steps in the development of metastasis are:

1) neoplastic transformation, proliferation and angiogenesis;

2) detachment of primary tumour cells from the lesion and invasion of the adjacent stroma;

3) aggregation of tumour cells into the circulation;

4) adherence of tumour cells to the subendothelial basement membrane; and

5) invasion into the remote organ, and proliferation and angiogenesis.

In addition, the newly formed metastases can be the source of further metastasis, another characteristic of progressive aggressiveness. Multiple properties have been observed in metastatic cells, including cytoskeletal changes, loss of adhesion, enhanced motility, and expression of matrix metalloproteinases (MMPs) that degrade the basement membrane.

Cytoskeletal remodeling is required for cancer cell mobility (Ridley, 2000). The cytoskeleton controls cell characteristics and processes that are fundamental to carcinogenesis, such as morphology, cell growth, migration, differentiation, and gene expression (Varedi *et al.*, 1997). Phosphorylation and dephosphorylation of cytoskeletal proteins can regulate the shape of cultured cells (Oliver *et al.*, 2002; Mancini *et al.*, 2002; Jackson *et al.*, 1997). Thus, the changes in cell shape of the PRL-3 over-expressing clones might be related to the cytoskeletal rearrangement induced by increased PRL-3 activity. This suggests that PRL-3 could play a key role in the cytoskeletal remodeling that is required for cancer cell motility (Kato *et al.*, 2004). Moreover, protein prenylation is important in targeting proteins to intracellular membranes and in protein-protein

interactions (Seabra, 1998; Si *et al.*, 2001). The metastatic properties of PRL-3 may be dependent on prenyltransferase activity (Zeng *et al.*, 2000 and 2003). Signaling pathways that control the cytoskeletal changes or the expression and activation of proteases and their inhibitors in metastasis are largely unknown.

PTPs were initially thought to have the potential for tumour suppression (Di Cristofano and Pandolfi, 2000; Brown-Shimer *et al.*, 1992). However, further studies have shown that some PTPs might have positive roles in carcinogenesis. For example, PTPα activates Src family kinases by dephosphorylating the inhibitory C-terminal pTyr-527, thereby promoting cell transformation (Su *et al.*, 1999; Ponniah *et al.*, 1999; Zheng *et al.*, 1992). PTPs and PTKs play key roles in the regulation of cellular growth, differentiation, cell cycle, cell-cell communication, and other activities through the dephosphorylation and phosphorylation of tyrosine (Hunter, 2000; Neel and Tonks, 1997; Tan, 1993). Deregulation of phosphorylation is known to result in neoplastic or non-neoplastic diseases (Streuli, 1996). Furthermore, PTPs are as important as the well-studied PTKs because phosphorylation is a dynamic and reversible process (Tonks and Neel, 1996). The study of PRL-3 promises to provide new insights into the biochemical pathways controlling various aspects of metastasis. Stable expression of wild-type active PRL-3 dramatically enhanced cell migration, whereas the catalytically inactive PRL-3 (C104S) mutant greatly reduced this ability (Zeng *et al.*, 2003). These results indicate that the phosphatase activity of PRL-3 is required to promote cell migration.

For patients with solid tumours, the biggest threat to survival is tumour cell metastasis. Metastasis is the main cause of death in cancer patients because of its resistance to conventional chemotherapy or radiotherapy (Susan and Schumacher, 2001). A representative metastasis model is hard to develop because it requires cell-cell and cell-microenvironment interactions. It has become a major challenge, therefore, to identify the underlying molecular switches of the metastatic state. High levels of PRL-3 expression were generally not maintained upon *in vitro* culture of cancer cells (Bardelli *et al.*, 2003). PRL-3 mRNA level in metastatic cancer cell lines was significantly lower than generally observed in metastatic cancer cells *in vivo*. In addition, gene expression patterns in cancers in their natural setting can be considerably different than those of cell lines grown in artificial environments (Zhang *et al.*, 1997). Thus, this truly presents some difficulties in studying the comprehensive relation of PRL-3 and metastasis *in vitro*.

In contrast, there have been many advances in understanding the earlier stages of tumourigenesis revolving around cell autonomous processes that determine the rate of cell division and cell death (Kinzler and Vogelstein, 1998). The earlier phases of tumourigenesis have been associated with cell-specific patterns of gene expression or mutation (Velculescu *et al.*, 1999; Giordano *et al.*, 2001; Dennis *et al.*, 2002). PRL-3 mRNA expression was elevated in nearly all metastatic lesions derived from colorectal cancer, regardless of the site of metastasis (Bardelli *et al.*, 2003). Conversely, the siRNA knock-down of the expression of PRL-3 in DLD-1 cells suppressed metastatic tumour formation *in vivo* (Kato *et al.*, 2004). This indicates that PRL-3 can regulate not only cell motility but also the formation of metastatic lesions. To fully understand the putative role

of PRL-3 in metastasis, PRL-3 needs to be evaluated in relation to known key players of metastasis such as E-cadherin and Snail.

Metastasis is a process determined by cell-cell interactions and specific microenvironments (Weiss, 2000; Fidler, 2001; Chambers, 2002). Epithelial-mesenchymal-transition (EMT) is a process whereby epithelial cells acquire fibroblast-like properties and exhibit reduced cell-cell adhesion and increased motility (Wu *et al.*, 2004). Furthermore, EMT implies a dramatic phenotypic change that includes the loss of epithelial markers (i.e. cytokeratin), the gain of mesenchymal markers (i.e. vimentin), and changes in cell shape (Vega *et al.*, 2004). The process of metastasis is associated with cell type specific gene expression, and EMT has been advocated to be a causative mechanism for the suppression of E-cadherin and tumour progression (Yokoyama *et al.*, 2003). The loss of E-cadherin expression is a major characteristic of highly invasive and metastatic cancers. Moreover, reduced expression of E-cadherin is frequently associated with dedifferentiation, invasion, lymph node or distant metastasis, and poor prognosis in various human malignancies (Jiang, 1996; Jiao *et al.*, 2001; Mukai *et al.*, 2001; Tanaka *et al.*, 2002).

Snail is known to play a vital role in EMT as it suppresses E-cadherin expression and transcriptionally upregulates MMPs. In particular, over-expression of Snail has been shown to induce higher levels of MMP-2 expression and activity. Luciferase reporter assays revealed that the MMP-2 promoter activity was induced by Snail (Yokoyama *et al.*, 2003). MMP-2 is involved in the degradation of the basement membrane and the

extracellular matrix, which further facilitates the EMT process. Proteases that degrade the basement membrane are likely to be essential for invasion (Egeblad and Werb, 2002). This indicates that Snail has a fundamental role in EMT through its suppression of E-cadherin and upregulation of MMP-2. Since EMT is a complex and intricate process, there are possibly many other targets of Snail waiting to be discovered.

Interestingly, PRL-3 signals were also observed in a subset of endothelial cells of some tumours. Since angiogenesis is a major component of the process of establishing a metastasis, PRL-3 expression in tumour vasculature could be relevant to this. Moreover, high PRL-3 expression in primary colorectal cancer significantly correlated with venous invasion (Kato et al., 2004). Neo-angiogenesis is required at the metastatic site allowing micrometastases to grow into macrometastatic lesions. In contrast to other tumour types, invasive breast tumour vasculature exhibited high expression of the Snail transcription factor (Parker et al., 2004). However, aside from its enhanced expression in breast tumour epithelium (Blanco et al., 2002; Cheng et al., 2001), Snail has not been linked to the transcriptional regulation of genes important in angiogenesis. PRL-3 also appears to be expressed predominantly in the vasculature of invasive breast cancers as well. Moreover, adenoviral-expressing PRL-3 human microvascular endothelial cells were also found to have enhanced migratory ability in vitro (Parker et al., 2004).

Does PRL-3 function as one of the key players in the epithelial-mesenchymal-transition? The specific molecular changes that promote the spread of tumour cells from the original site to other parts of the body are still primarily unclear. However, nude mice injected

with PRL-3 expressing cells exhibited tumour formation with metastasis (Zeng *et al.*, 2003; Pathak *et al.*, 2002), and the growth of the tumours was markedly inhibited by the anti-protozoa drug pentamidine [1,5-di(4-amidinophenoxy)pentane] at a tolerable dose (Pathak *et al.*, 2002). Although the specific protein substrate(s) for PRL-3 has not yet been identified, blocking or reducing the functions of PRL-3 in metastasis might be achieved by the inhibition of prenylation and/or the catalytic inactivation of PRL-3. Upregulated PRL-3 in cancer provides an excellent target for developing novel therapeutics, especially for tumours which are intractable due to cancer metastasis.

The mechanism of PRL-3 upregulation in colorectal tumour metastases was investigated by Saha *et al.* (2001). They demonstrated that PRL-3 upregulation was due to gene amplification in 25% of the metastases, and suggested that increased transcriptional activity likely accounted for increased PRL-3 expression in the rest of the cases. Since PRL-3 is upregulated in metastatic cells, it is possible that this protein is important for primary cancer cells to spread. Although many genetic alterations have been attributed to neoplastic development, very few have been associated with metastasis. Thus, PRL-3 could be a potential molecular marker for clinical estimation of tumour aggressiveness and a novel therapeutic target.

1.4   HYPOTHESES AND AIMS

The evidence presented above led to my working hypothesis that the upstream region of the PRL-3 gene contains regulatory elements that mediate its tumour and metastasis-specific transcriptional upregulation. Our first aim is to characterize regulatory elements in the PRL-3 promoter that mediate PRL-3 upregulation in primary and metastatic tumour cell lines using a combination of software predictive and wet-lab based methods. A luciferase reporter assay system and bioinformatics will be used to identify active and recognizable regulatory elements, active and novel elements/regions, and regions responsible for tumour and metastatic-specific regulation of PRL-3. Future identification of the corresponding transcription factors that interact with these elements will provide insight into cellular signals that regulate PRL-3 expression and the processes of tumourigenesis and metastasis.

More than 90% of cancer-related deaths are due to metastatic disease. Since metastasis is often attributed to a poor prognosis and since PRL-3 has been implicated in metastasis, we also hypothesize that over-expression of PRL-3 in clinical tumour samples is correlated with an unfavorable prognosis. Thus, our second aim is to define tumour/metastatic tissues in which PRL-3 is over-expressed, using Tissue Microarray technology and immunohistochemistry, and correlate these findings with clinical data to infer prognostication.

CHAPTER 2

MATERIALS AND METHODS

2.1   BIOINFORMATICS

The PRL-3 gene is located on chromosome 8q24.3 (Bardelli *et al.*, 2003). The sequence (~4.6kb) of the PRL-3 promoter was obtained from the University of California Santa Cruz (UCSC) Genome Browser (Wasserman and Sandelin, 2004; Karolchik *et al.*, 2003) website (Human April 2003 Assembly; chr8:142,111,086-142,115,888; 5'-CGGGCGCCCT.....GAGGCGCCAT-3'). The sequence was downloaded as a FASTA file format for use in primer design, luciferase reporter plasmid construction, and subsequent bioinformatics analyses.

The web-based program *Consite* (Wasserman and Sandelin, 2004; Lenhard *et al.*, 2003) was used to investigate the PRL-3 promoter sequence for orthologue conservation (between mouse and human) and putative transcription factor binding sites (TFBSs). Transcription factors generally have distinct preferences towards specific target sequences. However, TFBS prediction alone is not sufficient as potential binding sites do not necessarily mean functional importance. Therefore, it is imperative to first consider orthologue conservation prior to TFBS analysis. *Consite* eliminates ~90% of predictions while retaining ~70-80% of experimentally validated sites (Wasserman and Sandelin,

2004). The combination of orthologue conservation and putative TFBS searches greatly reduces the chance of false predictions with only a slight reduction in sensitivity.

To analyze the PRL-3 promoter for potential transcript generation and plausible regulatory potential, the appropriate tracks were selected on the UCSC Genome Browser website. Data generated from expression sequence tags (ESTs) were used to align possible transcript positions. Regulatory potential is a new method to investigate promoter regulatory sites. It analyses the patterns of nucleotide identity in subregions of the interspecies alignment and classifies conserved regions as coding or regulatory (Elnitski *et al.*, 2003). This regulatory potential algorithm is based in part on the pattern of observed nucleotide identity.

Another web-based program, CISTER (Frith *et al.*, 2001), was used to examine the possibility of certain trans-activating factors working together in a module to regulate PRL-3 promoter activity. Biochemical specificity of transcription is generated in the cell nucleus by combinatorial interactions between transcription factors (Davidson, 2001). Thus, looking into *cis*-regulatory modules can predict substantially better specificity than analysis of isolated sites.

Analysis	Program	Website Address	References
PRL-3 Promoter Sequence (April 2003 Assembly)	UCSC Genome Browser	http://www.genome.ucsc.edu/cgi-bin/hgGateway	Wasserman and Sandelin, 2004; Karolchik *et al.*, 2003
Transcription Factor Binding Sites	*Consite*	http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/	Wasserman and Sandelin, 2004; Lenhard *et al.*, 2003
Conservation	*Consite*	http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/	Wasserman and Sandelin, 2004; Lenhard *et al.*, 2003
Transcripts (July 2003 Assembly)	UCSC Genome Browser	http://www.genome.ucsc.edu/cgi-bin/hgGateway	Wasserman and Sandelin, 2004
Regulatory Potential (July 2003 Assembly)	UCSC Genome Browser	http://www.genome.ucsc.edu/cgi-bin/hgGateway	Wasserman and Sandelin, 2004; Kolbe *et al.*, 2004
*Cis-*Regulatory Module	CISTER	http://zlab.bu.edu/~mfrith/cister.shtml	Frith *et al.*, 2001

Table 1. Bioinformatics Programs. A list of the web-based programs and corresponding website addresses used for the bioinformatics analyses of the PRL-3 promoter.

2.2   MOLECULAR CLONING

2.2.1   Polymerase Chain Reaction (PCR)

All PRL-3 promoter regions were amplified using normal human genomic DNA
(Clontech) as a template, with a final concentration of 8ng/μl. Primer sequences are
shown in Table 2. All PCR reactions were carried out in a final volume of 25μl.

Long Range (-4173 / +336), full length PRL-3 promoter

The PCR master mix contained 10%DMSO, 1.0mM MgAOc, 160μM dNTP, 160μM
forward and reverse primers each, 0.7 units of *Taq* polymerase (Invitrogen), and 1.25
units of *Accurase* polymerase with the included PCR buffer (BIO/CAN Scientific). The
thermocycling steps were as follows: 94°C-2mins, 40 cycles (94°C-20s, 62°C-30s, 68°C-
3.5mins), and 68°C-7mins.

5'-UTR and Middle Range sequences together (-3656 / +336)

The PCR master mix contained 10%DMSO, 1.5mM MgAOc, 160μM dNTP, 160μM
forward and reverse primers each, 1 unit of *Taq* polymerase (Invitrogen), and 1.25 units
of *Accurase* polymerase with the included PCR buffer (BIO/CAN Scientific). The
thermocycling steps were as follows: 94°C-2mins, 40 cycles (94°C-20s, 60°C-45s, 68°C-
3.5mins), and 68°C-7mins.

5'-UTR (-2027 / +336)

The PCR master mix contained 1.5mM of $MgCl_2$, 200μM dNTP, 160μM forward and reverse primers each, and 1.5 units of *Taq* polymerase with the included PCR buffer (Invitrogen). The thermocycling steps were as follows: 95°C-3mins, 40 cycles (95°C-30s, 70°C-1min, 72°C-1min), and 72°C-10mins.

Middle Range (-3656 / -2027)

The PCR master mix contained 10%DMSO, 1.5mM MgAOc, 160μM dNTP, 160μM forward and reverse primers each, 1 unit of *Taq* polymerase (Invitrogen), and 1.25 units of *Accurase* polymerase with the included PCR buffer (BIO/CAN Scientific). The thermocycling steps were as follows: 94°C-2mins, 40 cycles (94°C-20s, 60°C-30s, 68°C-1.75mins), and 68°C-7mins.

CpG-64 (-4173 / -3656)

The PCR master mix contained 10%DMSO, 1.5mM MgAOc, 160μM dNTP, 160μM forward and reverse primers each, and 1.25 units of *Accurase* polymerase with the included PCR buffer (BIO/CAN Scientific). The thermocycling steps were as follows: 94°C-2mins, 40 cycles (94°C-20s, 60°C-30s, 68°C-1min), and 68°C-7mins.

Region	Coordinates		Primer Sequences	Primer Length / Tm
	From	To		
Long Range (full length promoter)	-4173	+336	Forward: 5'-CCAGGGGGAGCTCCGAGGTTC-3'	21 / 68.79°C
			Reverse: 5'-ATGGCGCCTCCCGACGGG-3'	18 / 70.48°C
Middle Range + 5'-UTR	-3656	+336	Forward: 5'-CGCAGCTTACTTCTCCTGAGACCCG-3'	25 / 69.61°C
			Reverse: 5'-ATGGCGCCTCCCGACGGG-3'	18 / 70.48°C
5'-UTR	-2027	+336	Forward: 5'-GACACCCACCGCCCATTTTAACCTT-3'	25 / 70.14°C
			Reverse: 5'-ATGGCGCCTCCCGACGGG-3'	18 / 70.48°C
Middle Range	-3656	-2027	Forward: 5'-GGGCCTCCAGCCTCCTCC-3'	18 / 63.78°C
			Reverse: 5'-GGTGTCAGGCGCAGTGGCT-3'	19 / 63.97°C
CpG-64	-4173	-3656	Forward: 5'-CTGGCTGGGCTTCCTCTTCC-3'	20 / 63.45°C
			Reverse: 5'-TCTGGGCGACTCGGGGC-3'	17 / 64.62°C

Table 2. PCR Primers. A list of primer sequences used in PCR to amplify specific regions of the PRL-3 promoter for eventual subcloning into the luciferase expression vector. The nucleotide position where transcription starts is designated as +1. Sequences upstream of this transcriptional start site (TSS) have a negative (-) sign. Sequences downstream of this TSS have a positive (+) sign. All primer solutions were made at a stock concentration of 20μM.

2.2.2 TA-vector subcloning

The PCR products were subcloned into either the pCR2.1-TA or the XL-TA vectors using a TOPO-isomerase kit (Invitrogen). The multiple cloning sites in these two vectors are identical. Blue/white selection was used to identify the positive colonies. Insert

orientation was validated using internal restriction cut sites in the PCR insert of non-equivocal lengths. The positive and correctly oriented plasmids were then purified using a mini-prep kit (QIAGEN). The purified plasmids were cut with *Kpn I* and *Xho I* restriction enzymes (New England BioLabs). The digest-released promoter inserts were gel-purified using a gel-extraction kit (QIAGEN).

2.2.3   Luciferase expression vector subcloning

The pGL3-Basic (Promega) vector was transformed into competent DH5α (Invitrogen) cells, and were grown overnight in LB media with the appropriate antibiotics. The pGL3-Basic vector was purified from the bacterial culture using a maxi-prep kit (Sigma). Due to DNA supercoiling, the *Xho I* site is hidden in this vector, therefore a spiking double digest was performed. Briefly, *Kpn I* (New England BioLabs) was incubated with the pGL3-Basic vector at 37°C for 5hrs. Then, *Kpn I* and *Xho I* restriction enzymes (New England BioLabs) were added together to this digest mixture and incubated at 37°C overnight. The next day, the linearized vector was dephosphorylated using calf intestinal phosphatase (Invitrogen) at 37°C for 1hr.

The purified promoter fragments were ligated, using T4 ligase (Invitrogen), with the above linearized and dephosphorylated empty luciferase vector overnight at 4°C. The insert to vector ratio was approximately 3:1 in the ligation mixture. The ligated plasmids were transformed into competent DH5α (Invitrogen) cells, and were grown overnight in LB media with the appropriate antibiotic. The PRL-3 promoter plasmids were purified

from the bacterial culture using a maxi-prep kit (Sigma). Restriction enzyme digests and sequence analyses were carried out to confirm the validity of the promoter constructs.

2.2.4 PRL-3 promoter constructs

Reporter assays were conducted to assess PRL-3 promoter activity. This method involves measuring the ability of a DNA sequence (putative promoter) to stimulate transcription of a 3' gene, usually luciferase, when a plasmid containing these sequences is introduced into cultured cells. The pGL3 vector series (Promega) contains the Firefly luciferase gene. All of the promoter regions (Figure 4) were cloned into the multiple cloning site of the pGL3-Basic vector, which contains the Firefly luciferase gene and lacks any promoter elements. To normalize transfection efficiency, the phRL-TK plasmid, which contains the *Renilla* luciferase gene, was co-transfected with the pGL3 plasmids. The Firefly and *Renilla* luciferases have dissimilar enzyme structures and substrate requirements because of their distinct evolutionary origins. These differences make it possible to selectively discriminate between their respective bioluminescent reactions. With the Dual-Luciferase Reporter Assay kit (Promega), the luminescence from the Firefly luciferase reaction can be measured, and then quenched while simultaneously activating the luminescent reaction of *Renilla* luciferase.
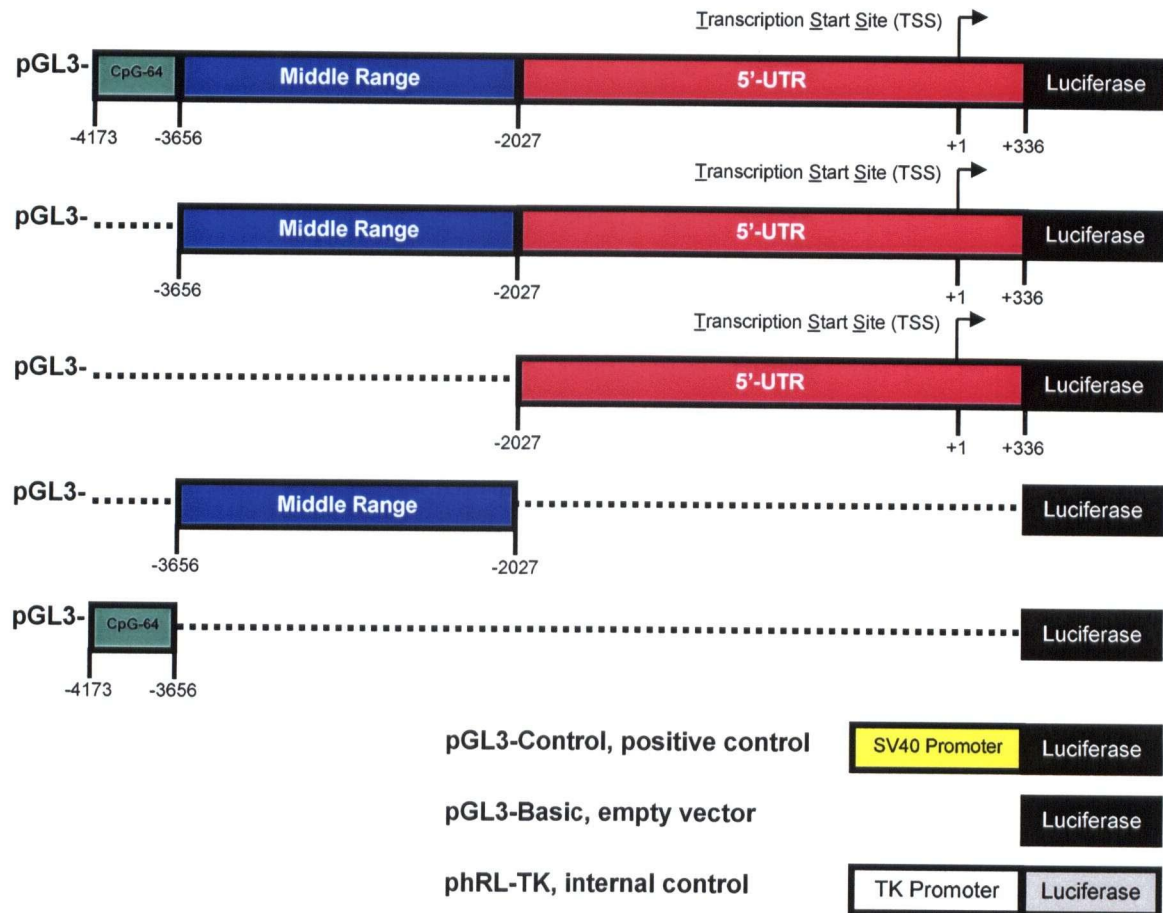
Figure 4. PRL-3 promoter constructs. Various regions of the PRL-3 promoter were cloned into the pGL3-Basic luciferase reporter plasmid. The full-length PRL-3 promoter was divided into three discrete regions (top). The CpG-64 island, Middle Range, and the 5'-UTR sequences are represented by green, blue, and red boxes, respectively. The black box represents the Firefly luciferase gene. These plasmids were transfected into selected cancer cell lines to evaluate their activity and subsequently define a region(s) of regulatory importance. The phRL-TK vector was used as an internal control to normalize transfection efficiency. The grey box represents the *Renilla* luciferase gene.

2.3   TRANSFECTION AND LUCIFERASE ASSAY

2.3.1   Cell line propagation

The **HEK293T** (derived from human embryonic kidney cells transformed with adenovirus 5 DNA, ATCC#: CRL-1573), **PRL-3-HEK293T** (PRL-3 stably expressing HEK293T cells created by D. Bessette in the Pallen lab), **C2C12** (derived from mouse normal myoblastic cells, ATCC#: CRL-1772), **RD** (derived from human embryonal rhabdomyosarcoma cells, ATCC#: CCL-136), and **RMS13** (derived from human alveolar rhabdomyosarcoma cells isolated from metastatic bone marrow, ATCC#: CRL-2061) cell lines were maintained in DMEM media, supplemented with 10% FBS. The **SW480** (derived from human colorectal adenocarcinoma cells, ATCC#: CCL-228) and **SW620** (derived from human colorectal adenocarcinoma cells isolated from metastatic lymph node, ATCC#: CCL-227) cell lines were maintained in DMEM media with 1.5g/L sodium bicarbonate, supplemented with 10% FBS. The **LNCaP** (derived from human prostate carcinoma cells isolated from metastatic left supraclavicular lymph node, ATCC#: CRL-1740) cells were grown in RPMI-1640 media with 2.0mM L-glutamine and phenol red, supplemented with 15% FBS. Lastly, the **DU145** (derived from human prostate carcinoma cells isolated from metastatic brain, ATCC#: HTB-81) cells were grown in MEM media with Earle's salts (BSS), 2.0mM L-glutamine, 0.1mM non-essential amino acids, and 1.0mM sodium pyruvate, supplemented with 10% FBS. Propagation methods were employed as described by the American Type Culture Collection (ATCC). All cell lines were grown in a 5%CO_2 incubator at 37°C.

2.3.2 Transfection

Cells were plated onto 6-well plates (Falcon) 24hrs prior to transfection. Each well contained 3.0mL of growth media and 2.0×10^5 or 4.0×10^5 cells per well. Cells were transfected by the cation-lipid method. All transfections were done according to the manufacturer's (Invitrogen) protocol. Briefly, $1.5 \mu g$ of the Firefly luciferase pGL3-plasmids (pGL3-Control, pGL3-Basic, or pGL3-promoter-constructs) and $0.5 \mu g$ of the *Renilla* phRL-TK plasmid (Promega) were mixed with $5 \mu L$ of Lipofectamine reagent (Invitrogen). The DNA-lipid complexes were then incubated with the selected cell lines in serum-free media for 5-6hrs. The cells were then switched to full growth media and allowed to grow for 24, 48, or 72hrs.

2.3.3 Luciferase Assay

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's protocol. At confluency, transfected cells were washed with room temperature phosphate buffered saline (PBS) prior to addition of Passive Lysis Buffer (Promega). Cells were lysed at room temperature for 30-40mins with orbital shaking. After addition of the appropriate luminescent substrates, the signal was detected directly in the 6-well plates using a PerkinElmer Victor-3 Multiplate reader.

2.4   WESTERN BLOT

2.4.1   Antibodies

A 9-amino-acid peptide (DPHTHKTRC) that is unique to PRL-3 was used to immunize a

rabbit for antibody production. This unique region is found near the C-terminal CAAX

box of PRL-3. After initial injection, three more injections of the antigen were

administered at 14-day intervals. Antibodies specific for PRL-3 were purified from the

immunized rabbit serum using a recombinant PRL-3 affinity column. The final

polyclonal anti-PRL-3 antibody concentration was about 1mg/ml, and was used at a

1:5000 dilution. This antibody was produced by my colleague Dr. Jing Wang. In

addition, a monoclonal anti-PRL-3 antibody was generously donated to us by Dr. Qi Zeng

(Institute of Molecular and Cell Biology, Singapore), and was used at a 1:2000 dilution.

The anti-Snail antibody was purchased from Santa Cruz Biotechnology, Inc., SNAI 1 (E-

18): sc-10432, and was used at a 1:200 dilution. The anti-FLAG antibody was purchased

from Sigma, and was used at a 1:1000 dilution. The anti-c-myc antibody was purchased

from Santa Cruz, and was used at a 1:1000 dilution.

2.4.2   Cell line treatments

All cells were harvested in RIPA lysis buffer (10mM sodium phosphate, 0.15M sodium

chloride, 1mM EDTA, 50mM sodium floride, 1% NP-40, 0.1% SDS, 10μg/ml aprotinin,

1mM sodium orthovanadate, and 0.1mM PMSF).

PRL-3-Stable-HEK293T cells:

A PRL-3 stably expressing HEK293T cell line was created by my colleague Mr. Darrell

Bessette. PRL-3 expression can be induced upon appropriate antibiotic treatment.

Briefly, at ~80% confluency, these cells were treated with $1\mu g/ml$ of Doxycycline in full

growth media, and harvested after 24hrs.

MDA-MB-435 cells:

These cells were derived from human ductal breast carcinoma cells isolated from

metastatic pleural effusion, and were maintained in DMEM media with 1.5g/L sodium

bicarbonate, supplemented with 10% FBS. These cells were used to visualize

endogenous Snail protein as described in Zhou *et al.* (2004). Briefly, at ~80% confluency

and immediately after a media change (full growth media), the cells were treated with

40mM LiCl (Sigma) together with $10\mu M$ MG132 (Sigma) for 5hrs prior to harvest.

HEK293T cells:

Western Blot analysis determined that the affinity-purified anti-PRL-3 antibody

specifically recognized PRL-3 and not PRL-1 or -2 (Dr. Jing Wang, personal

communication). Moreover, the ability of the immunizing PRL-3 peptide to block

recognition of PRL-3 protein by the antibody was tested. First, HEK293T cells were

transfected with a plasmid encoding *myc*-tagged-PRL-3, and incubated in full growth

media for 24hrs prior to harvest. The polyclonal anti-PRL-3 antibody was pre-incubated

with the unique PRL-3 peptide that was used to immunize the rabbit for antibody

production. The antibody-peptide solution was left to rotate at 4°C for 2hrs prior to use for Western Blot analysis of PRL-3 detection in the cell lysate.

2.4.3 SDS-PAGE

All whole cell lysates were resolved on a 12.5% polyacrylamide gel and transferred onto a PVDF membrane at room temperature for 1hr using an electrotransfer apparatus. The membrane was then blocked with 5%(w/v)-skim milk powder for 1hr at room temperature. The primary antibody solution (in 1%(w/v)-skim milk powder) was incubated with, shaking, the membrane at 4°C overnight. The next day, the primary antibody solution was removed and the membrane was washed with PBS at room temperature (shaking), 3X 10mins. The membrane was then probed with the corresponding secondary antibody (also in 1%(w/v)-skim milk powder) at room temperature for 1hr, shaking. After 3X 10mins PBS washes (shaking), the membrane was visualized with ECL solution on exposable film.

2.5   IMMUNOHISTOCHEMISTRY

2.5.1   Polyclonal anti-PRL-3 antibody specificity

To further confirm specificity, the polyclonal anti-PRL-3 antibody was pre-incubated with the same unique PRL-3 peptide that was used to immunize the rabbit for antibody production. The antibody-peptide solution was left to rotate at 4°C for 2hrs prior to use for immunohistochemistry.

2.5.2   Immunohistochemistry staining

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized and hydrated via conventional methods. After a brief tap water rinse, the slides were placed in hot (70°C) 10mM sodium citrate buffer at pH 6.0 for 40mins for antigen retrieval/pre-treatment. After another tap water rinse, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 30mins at room temperature. The slides were then rinsed with phosphate-buffered-saline (PBS) at pH 7.4. Endogenous proteins were blocked with avidin and biotin blocking solutions (Vector) for 15mins each at room temperature with PBS washes in between. Excessive hydrophobicity in the tissues was blocked by a 3% BSA solution (ID Labs) incubated for 30mins at room temperature. The solution was then drained, and without washing the slides, the primary antibody solution (1:200 dilution) was added to the sections for 30mins at room temperature. After a quick PBS wash, the slides were incubated with the secondary antibody (ID Labs) for 30mins at

room temperature. After another PBS wash, the labeling solution (Streptavidin-horse-radish peroxidase, ID Labs) was added to the slides and incubated for 30mins at room temperature. After another PBS wash, 0.1M sodium acetate solution at pH 5.2 was incubated with the sections for 1min. The solution was drained and without washing, the chromagenic solutions were added to the sections. At the desired staining intensity, the slides were placed in tap water to stop the colour change reaction and were later counter-stained with hematoxylin. Slides were then finally mounted using an aqueous mounting media.

2.5.3 Tissue Microarray

The analysis of many sample core sections mounted on a single slide, known as tissue microarray (TMA), is efficient and reduces staining variability compared to analysis of single sections on many slides. In addition, multiple-tumour tissue arrays are an efficient method of assessing the sensitivity and specificity of antibodies used in determining origin or cell type of human tumours (Hsu et al., 2002). PRL-3 expression in various TMAs was evaluated as follows.

After immunohistochemistry (IHC) staining, the staining intensity of each core was assigned a score. A score of 0 corresponded to 0-5% staining, a score of 1 corresponded to 5-50% staining, and a score of 2 corresponded to >50% staining. Generally, a score of 0, 1, and 2 represented negative, weak, and strong staining, respectively. Uninterpretable cases were assigned an X.

CHAPTER 3

RESULTS

3.1   TRANSCRIPTIONAL ANALYSIS OF THE PRL-3 PROMOTER

3.1.1   PRL-3 promoter activity in human embryonic kidney 293T cells

HEK293T cells exhibit high transfection efficiency and are suitable cells to be used as a model system to initially confirm plasmid expression and to optimize cell splitting, transfection, and luciferase assay techniques. Thus, HEK293T cells were transfected with the reporter constructs to evaluate the functionality of the PRL-3 promoter plasmids.

Luciferase activities measured in the panel of transfected cells are shown in Figure 5. As expected, the pGL3-Control luciferase activity was significantly greater than pGL3-Basic and thus validated the luciferase assay. Cells transfected with the 5'-UTR (-2027 / +336), Middle Range (-3656 / -2027), and 5'-UTR + Middle Range (-3656 / +336) all exhibited no significant luciferase activity. However, the full-length promoter (-4137 / +336) resulted in ~6.8-fold ($p < 0.031$) higher luciferase activity than observed with the empty vector (pGL3-Basic). Moreover the CpG-64 island (-4137 / -3656) alone induced ~131.5-fold ($p < 0.01$) higher luciferase activity than did the empty vector. When comparing the fold difference in activity between the full-length promoter (-4137 / +336) and the CpG-64 island (-4137 / -3656), the latter sequence was ~19.3-fold ($p < 0.03$) more

effective in promoting luciferase expression. The CpG-64 island thus appeared to be an

important region for PRL-3 transcriptional regulation. A significant level of promoter

activity is only seen with the presence of this CpG-64 island (alone or in the full-length

promoter).

Figure 5. PRL-3 promoter activity in HEK293T cells. 1μg of the pGL3-Control
(Firefly), pGL3-Basic (empty vector), or pGL3-PRL-3 promoter plasmids were co-
transfected with 1μg of the phRL-TK (*Renilla*) vector. After 24hrs in full media, the cells
were lysed and luciferase activity was measured. Three independent duplicate
experiments were performed. The fold-increase in luciferase activity of certain plasmids
relative to pGL3-Basic was calculated, and the asterisk (*) depicts p-values <0.05
(student's *t-test*). The CpG-64 island (green box) and the full-length promoter showed a
significant induction of luciferase activity in HEK293T cells. The 5′-UTR (red box),
Middle Range (blue box), and 5′-UTR + Middle Range sequences (red + blue boxes)
exhibited no significant induction of luciferase activity.

3.1.2 Auto-transcriptional regulation of the PRL-3 promoter in human embryonic kidney 293T cells

To investigate the possibility that PRL-3 expression might be regulated via auto-activated transcription, the promoter activity of the pGL3-PRL-3 promoter constructs was assessed in HEK293T cells with inducible expression of heterologous PRL-3 protein. These cells were stably transfected (Mr. Darrell Bessette, Pallen Lab) with a plasmid that expresses PRL-3 protein in response to doxycycline treatment (Figure 6). Thus, the ability of PRL-3 protein to interact with PRL-3 promoter elements and thus regulate transcription was tested using HEK-*FLAG*-PRL-3 cells treated with or without doxycycline.

Western blot analysis was performed to demonstrate that PRL-3 is expressed in this inducible system. As shown in Figure 6, PRL-3 expression is evident upon antibiotic treatment (+). Conversely, no PRL-3 can be detected without antibiotic treatment (-). The induced PRL-3 protein has an N-terminal *FLAG*-tag; therefore, the cell lysates were also probed with anti-*FLAG* antibody. A *FLAG*-tagged protein was only detected in the antibiotic treated cells (Figure 6), and thus further substantiating this PRL-3 inducible system. This experiment was performed in conjunction with the luciferase reporter analysis of the auto-transcriptional role of PRL-3 (Figure 7).
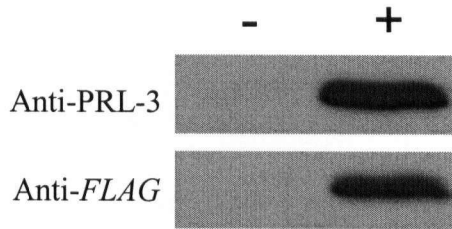
Figure 6. Inducible expression of PRL-3 in stably transfected HEK-*FLAG*-PRL-3 cells. Cells were untreated (-) or treated (+) with 1μg/ml of Doxycycline for 24hrs and harvested. Cell lysates were probed for PRL-3 expression using anti-PRL-3 (top panel) and anti-*FLAG* (bottom panel) antibodies.

Luciferase activities measured in the panel of transfected cells that were induced to express PRL-3 or were left un-induced are shown in Figure 7. The pGL3-Control luciferase activity was significantly greater than that expressed by the pGL3-Basic plasmid in both PRL-3 expressing and non-expressing cells, and thus validates the luciferase assay. Cells transfected with the 5′-UTR (-2027 / +336), Middle Range (-3656 / -2027), and 5′-UTR + Middle Range (-3656 / +336) plasmids exhibited no significant luciferase activity either with or without induced PRL-3 expression. However, in the un-induced cells, the full-length promoter (-4137 / +336) stimulated ~3.7-fold (p<0.003) higher luciferase expression than did the empty vector. In the PRL-3 induced cells, the full-length promoter (-4137 / +336) had ~4.6-fold (p<0.001) higher transcriptional activity than the empty vector. There was no statistical difference when comparing luciferase activities induced by the full-length promoter (-4137 / +336) in the un-induced cells and the cells induced to express the PRL-3 protein. This suggests that PRL-3 has no direct auto-transcriptional role.
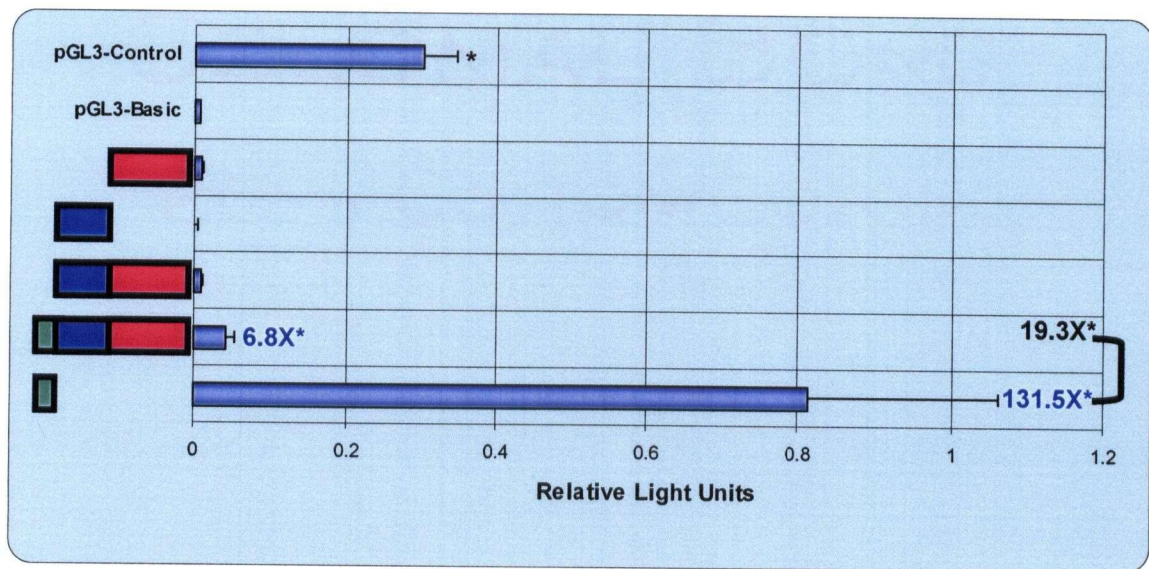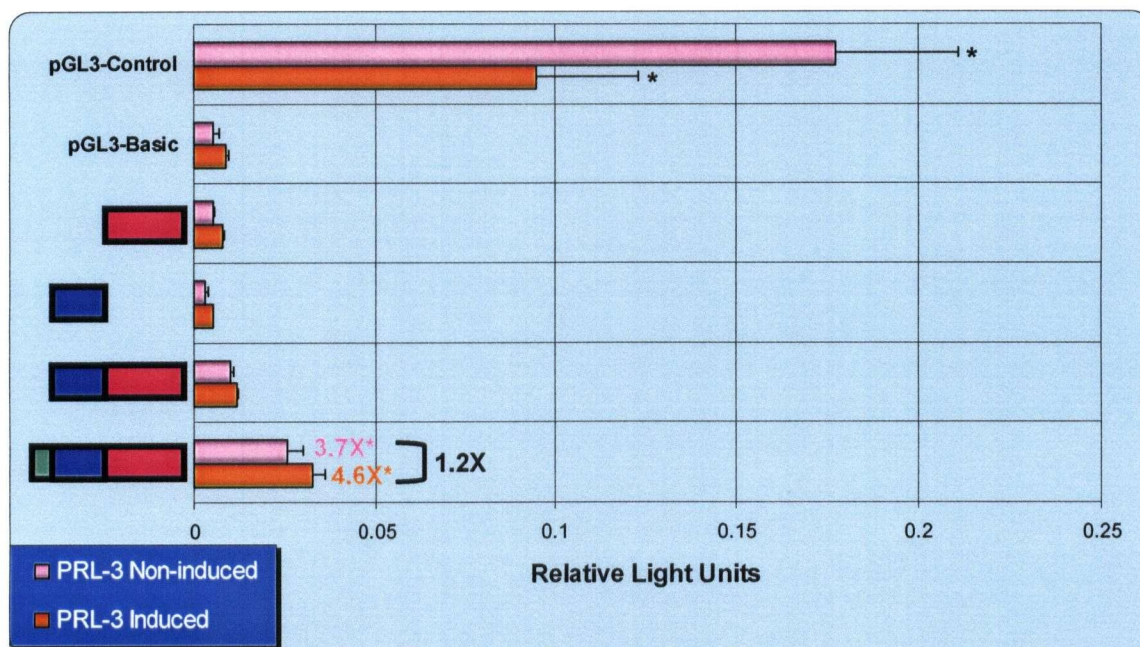
Figure 7. PRL-3 auto-transcriptional role in HEK293T cells. 1µg of the pGL3-Control (Firefly), pGL3-Basic (empty vector), or pGL3-PRL-3 promoter plasmids were co-transfected with 1µg of the phRL-TK (*Renilla*) vector. After 24hrs in full media with and without 1µg/ml of Doxycycline, the cells were lysed and luciferase activity was measured. Two independent duplicate experiments were performed. The fold-increase in luciferase activity of the plasmids relative to pGL3-Basic was calculated, and the asterisk (*) depicts p-values <0.05 (student's *t-test*). The 5′-UTR (red box), Middle Range (blue box), and 5′-UTR + Middle Range sequences (red + blue boxes) induced no significant level of luciferase activity. A significant level of activity from the full-length promoter (which contains the CpG-64 island) is observed in both PRL-3 induced and non-induced conditions. However, there is no significant difference between PRL-3 induction and non-induction on the promoter construct activities, suggesting that PRL-3 has no direct auto-transcriptional role.

3.1.3 PRL-3 promoter activity in primary and metastatic colorectal cancer cell lines

PRL-3 expression is upregulated in metastatic colorectal and gastric carcinomas (Saha *et al.*, 2001; Bardelli *et al.*, 2003; Peng *et al.*, 2004; Kato *et al.*, 2004; Miskad *et al.*, 2004), as well as in melanoma (Wu *et al.*, 2004). To investigate whether the PRL-3 promoter activates transcription in response to metastasis-specific factors, promoter activity was determined in a matched pair of tumour and metastatic cancer cell lines. The SW480 cell line was derived from the primary tumour of a colorectal cancer patient. The SW620 cell line was derived from a metastatic site (lymph node) of the same patient.

The luciferase activities measured in panels of pGL3-Control, pGL3-Basic, and pGL3-PRL-3 promoter plasmid transfected cells are shown in Figure 8. The luciferase activity in the SW480 and SW620 cells transfected with the pGL3-Control plasmid was significantly greater than in the cells transfected with the pGL3-Basic plasmid, thus validating the luciferase assay. As expected, both pGL3-Control transfected SW480 cells and SW620 cells exhibited similar luciferase activities, since both of these cell lines were derived from the same patient. This further validates the reliability of this luciferase assay. Luciferase expression was not significantly affected by the 5'-UTR (-2027 / +336) or the Middle Range (-3656 / -2027) sequences in either cell lines. The Middle Range and 5'-UTR sequences together (-3656 / +336) increased luciferase expression by ~3-fold (p<0.04) compared to pGL3-Basic (empty vector) in the SW480 cells, but no difference was detected in the SW620 cells. However, the additional presence of the CpG-64 (-4137

/ -3656) sequence with the Middle Range (-3656 / -2027) and the 5'-UTR (-2027 / +336)

sequences (full-length promoter) caused a dramatic and significant ~18-fold (p<0.002)

increase in luciferase activity in the metastatic SW620 cells. Also, the full-length

promoter induced a ~4-fold (p<0.01) elevation in luciferase activity in the SW480 cells.

Together, these results suggest that the CpG-64 island in the PRL-3 promoter is active in

colorectal carcinoma cells. The full-length promoter activity was ~4.5-fold (p<0.007)

higher in the SW620 cells than in the SW480 cells. The CpG-64 (-4137 / -3656)

sequence alone induced a ~13-fold (p<0.004) increase in luciferase expression in the

SW480 cells and a ~30-fold (p<0.0001) increase in the SW620 cells compared to that

observed in these cells transfected with the pGL3-Basic vector. The CpG-64 promoter

activity was ~2.3-fold (p<0.01) higher in the SW620 cells than in the SW480 cells. This

strongly argues for the transcriptional importance of the CpG-64 island in the PRL-3

promoter, and suggests that PRL-3 may potentially play a vital role in the metastatic

process.

Using interphase Florescent *In-Situ* Hybridization analysis, Bardelli *et al.* (2003)

concluded that classic gene amplification was not invariably (only 25% of cases) the

cause of PRL-3 over-expression in colorectal cancer metastasis. Since gene amplification

is not the major cause of increased PRL-3 expression, then increased transcriptional

activity is most likely responsible for PRL-3 upregulation. This further substantiates the

merits of our investigation of the PRL-3 promoter.
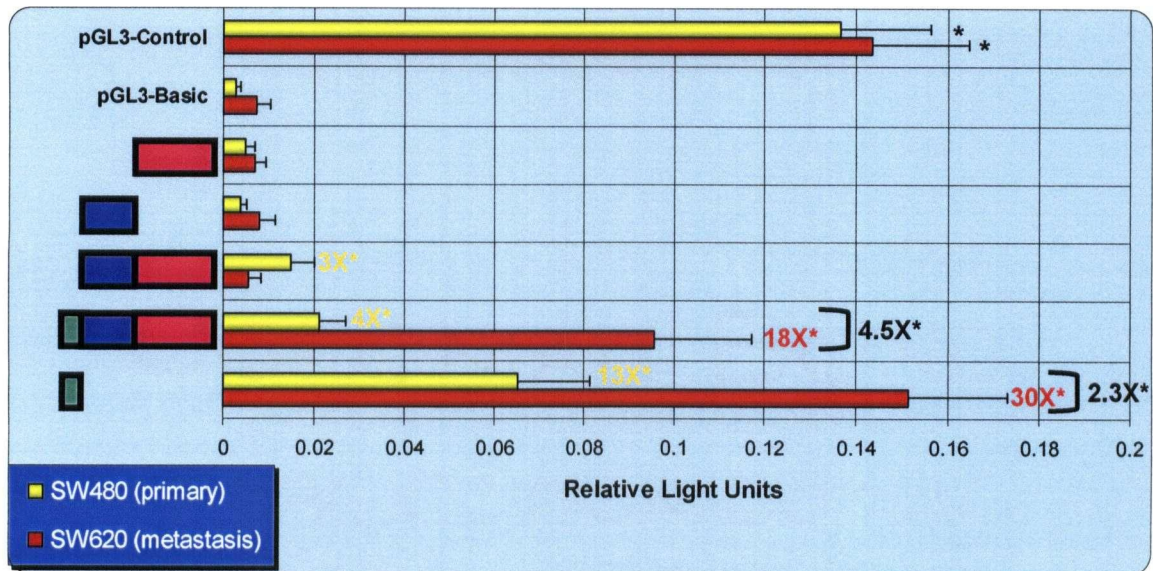
Figure 8. PRL-3 promoter activity in SW480 primary and SW620 metastatic colorectal carcinoma cell lines. 1.5µg of the pGL3-Control (Firefly), pGL3-Basic (empty vector), or pGL3-PRL-3 promoter plasmids were co-transfected with 0.5µg of the phRL-TK (*Renilla*) vector. After 72hrs in full media, the cells were lysed and luciferase activity was measured. Three independent duplicate experiments were performed. The fold-increase in luciferase activity induced by various plasmids relative to pGL3-Basic was calculated, and the asterisk (*) depicts p-values <0.05 (student's *t-test*). The 5′-UTR (red box) and the Middle Range (blue box) did not induce significant levels of luciferase activity in either cell line. However, the 5′-UTR + Middle Range sequences (red + blue boxes) induced a slight elevation in luciferase activity in the SW480 cells, but not in the SW620 cells. Moreover, a significant level of activity from the full-length promoter (which contains the CpG-64 island) is observed in both cell lines with the full-length promoter sequence being more (~4.5-fold) active in the SW620 cells than in the SW480 cells. Also, a significant level of activity from the CpG-64 island (green box) alone is observed in both cell lines with the CpG-64 sequence being more (~2.3-fold) active in the SW620 cells than in the SW480 cells.

3.1.4 PRL-3 promoter activity in metastatic prostate cancer cell lines

To compare PRL-3 promoter activity in cell lines with different invasive properties, a less invasive androgen-dependent LNCaP cell line and a more invasive androgen-independent DU145 cell line (Ghosh *et al.*, 2005) were used. These cell lines are of prostate carcinoma origin, and LNCaP cells were derived from a nodal metastasis of a patient, whereas the DU145 cells were derived from the brain metastasis of another patient.

The luciferase activities measured in the panels of transfected LNCaP and DU145 cells are shown in Figure 9. The 5'-UTR (-2027 / +336) or 5'-UTR and Middle Range together (-3656 / +336) did not induce statistically significant increases in luciferase activity in either cell lines, but a slight elevation is apparent. However, transfection of the full-length promoter (-4173 / +336) containing the additional CpG-64 sequence (-4173 / -3656) resulted in a dramatic ~30.6-fold ($p < 0.003$) increase in luciferase expression in LNCaP cells compared to that observed upon transfection with the empty vector. In the DU145 cells, the full-length PRL-3 promoter (-4137 / +336) stimulated a lesser ~8.5-fold ($p < 0.003$) increase in luciferase activity. Thus, the full-length PRL-3 promoter is ~3.6-fold ($p < 0.02$) more active in the LNCaP cells than in the DU145 cells. Although not statistically significant ($p > 0.05$), the CpG-64 island alone induced a ~6-fold increase (compared to pGL3-Basic) in luciferase activity in the LNCaP cells. This is quite surprising as the CpG-64 island is usually very active. Comparatively, the same CpG-64 island in DU145 cells exhibited a ~52.3-fold ($p < 0.001$) higher promoter activity

compared to pGL3-Basic. Thus, the CpG-64 promoter region is about ~8.7-fold

(p<0.0004) more active in DU145 cells than in LNCaP cells.
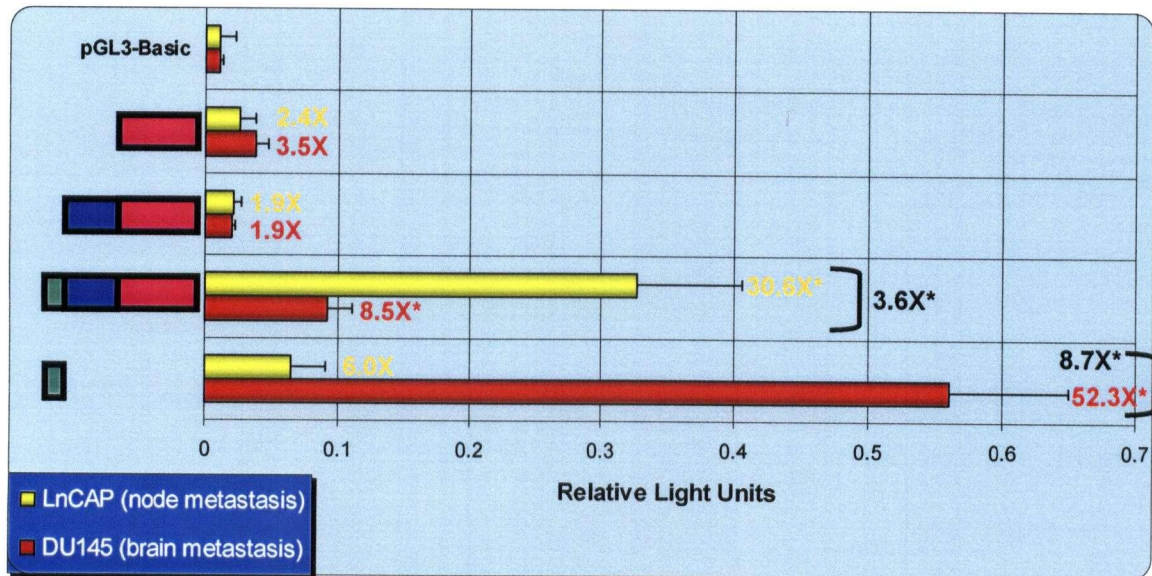
Figure 9. PRL-3 promoter activity in less invasive androgen-dependent LNCaP cells and in more invasive androgen-independent DU145 cells. 1.5µg of the pGL3-Control (Firefly), pGL3-Basic (empty vector), or pGL3-PRL-3 promoter plasmids were co-transfected with 0.5µg of the phRL-TK (*Renilla*) vector. After 48hrs in full media, the cells were lysed and luciferase activity was measured. Three independent duplicate experiments were performed. The fold-increase in luciferase activity induced by the various plasmids relative to pGL3-Basic was calculated, and the asterisk (*) depicts p-values <0.05 (student's *t-test*). The 5′-UTR (red box) or the 5′-UTR + Middle Range (red + box boxes) sequences did not induce significant levels of luciferase activity in either cell lines. However, a significant level of activity stimulated by the full-length promoter (which contains the CpG-64 island) was observed in both cell lines with the full-length promoter sequence being more (~3.6-fold) active in the LNCaP cells than in the DU145 cells. Also, a statistically significant level of activity from the CpG-64 island (green box) alone was observed in the DU145 cells but not in the LNCaP cells with the CpG-64 sequence being more (~8.7-fold) active in the DU145 cells than in the LNCaP cells.

3.1.5 PRL-3 promoter activity in normal muscle and rhabdomyosarcoma cell lines

PRL-3 is naturally expressed in striated muscle cells (Matter *et al.*, 2001). Thus, the C2C12 (normal mouse myoblast) cell line was used to examine PRL-3 promoter activity. In addition, to evaluate the effect of malignant transformation of muscle cells on PRL-3 promoter activity, the pGL3-PRL-3 promoter plasmids were transfected into cancerous muscle cell lines as well. RD cells were derived from a well-differentiated embryonal rhabdomyosarcoma (ERMS) tumour, whereas RMS13 cells were derived from a poorly-differentiated alveolar rhabdomyosarcoma (ARMS) tumour.

The luciferase activities measured in transfected C2C12, RD, and RMS13 cells are shown in Figure 10. Minimal luciferase activity was induced by the 5'-UTR (-2027 / +336), 5'-UTR and Middle Range together (-3656 / +336), or the full-length promoter (-4173 / +336) in C2C12 cells. Although not statistically significant, these same three PRL-3 promoter constructs induced a higher luciferase activity in the rhabdomyosarcoma cell lines than in the C2C12 cells. More measurements may confirm whether the possible trends in the observations are due to chance or real tendencies. The levels of promoter activity observed in the RD and RMS13 cells from each of these three constructs were similar. Moreover, the 5'-UTR and the Middle Range sequences together (-3656 / +336) promoted slightly lower luciferase expression than did the 5'-UTR region alone in RD and RMS13 cells. Luciferase activity induced by the full-length PRL-3 promoter in the RD cells was about ~9.4-fold ($p<0.02$) higher than the empty vector, whereas it was about ~13.4-fold ($p<0.002$) higher in the RMS13 cells. The CpG-64 island (-4173 / -3656) was

the only sequence that showed a significant level (~14-fold, p<0.003) of promoter activity in the C2C12 cells. Since PRL-3 is normally expressed in striated muscle cells, and since no other promoter regions show a significant elevation in promoter activity, this strongly suggests that this CpG-64 island is essential for PRL-3 transcriptional regulation. This same region exhibited ~91-fold (p<0.002) and ~55-fold (p<0.0003) higher activity in the RD cells and RMS13 cells, respectively. The CpG-64 sequence was ~6.5-fold (p<0.004) more active in RD cells than in C2C12 cells, and ~3.9-fold (p<0.003) more active in RMS13 cells than in C2C12 cells. Although not statistically significant, the CpG-64 promoter region was more active in the RD cells than in the RMS13 cells. More measurements are needed to validate this trend.
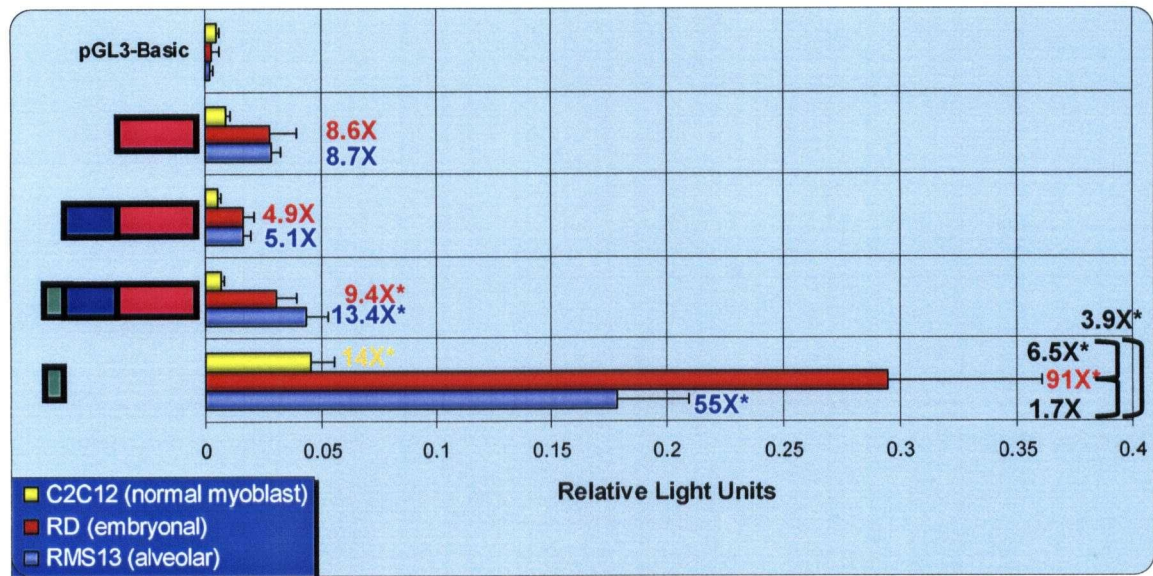
Figure 10. PRL-3 promoter activity in normal muscle and rhabdomyosarcoma cell lines. 1.5μg of the pGL3-Control (Firefly), pGL3-Basic (empty vector), or pGL3-PRL-3 promoter plasmids were co-transfected with 0.5μg of the phRL-TK (*Renilla*) vector. After 48hrs in full media, the cells were lysed and luciferase activity was measured. Three independent duplicate experiments were performed. The fold-increase in luciferase activity induced by various plasmids relative to pGL3-Basic was calculated, and the asterisk (*) depicts p-values <0.05 (student's *t-test*). The 5′-UTR (red box) or the 5′-UTR + Middle Range (red + box boxes) exhibited no significant induction of luciferase activity in C2C12 cells, but a slight elevation in luciferase activity was effected by these promoter regions in both RD and RMS13 cells. However, a significant level of activity induced by the full-length promoter (which contains the CpG-64 island) was observed in RD and RMS13 cells, but not in C2C12 cells. Also, a significant level of activity from the CpG-64 island (green box) alone was observed in all three cell lines with the CpG-64 sequence being more active in the RD (~6.5-fold) and RMS13 (~3.9-fold) cells than in the C2C12 cells.

3.2   BIOINFORMATICS ANALYSES OF THE PRL-3 PROMOTER SEQUENCE

Orthologue conservation analysis between mouse and human PRL-3 promoters indicated

that the CpG-64 island contains regions that are most highly conserved (Figure 11). As

an assumption, mutations within functional regions of genes will accumulate more slowly

than mutations within non-functional regions. So from an evolutionary perspective, the

comparison of sequences from orthologous genes can indicate segments that might direct

transcription (Wasserman and Sandelin, 2004). Sequence similarity that results from

selective pressure during evolution is the foundation for many bioinformatics methods

(Ureta-Vidal *et al.*, 2003; Frazer *et al.*, 2003). One key assumption in the application of

phylogenetic footprinting is that the orthologous genes are regulated by the same

mechanisms in different species. Although the definition of orthology does not include

retained functionality, for the purpose of phylogenetic footprinting one assumes that

orthologous genes are under common evolutionary pressures.

Trans-activating factors generally have distinct preferences towards specific *cis* target

sequences. However, identification of putative transcription factor binding sites (TFBSs)

does not necessarily translate into functional importance. Potential functionality is

greatly enhanced by analyzing TFBSs in highly conserved regions. To more accurately

reflect the characteristics at each position of a TFBS, a matrix that contains the number of

observed nucleotide at each position is created, called the position frequency matrix.

After conversion of this frequency matrix to a log-scale, a position weight matrix (PWM)

is created. A quantitative score for any DNA sequence can now be generated by

summing the values that correspond to the observed nucleotide at each position. *Consite* eliminates ~90% of predictions while retaining ~70-80% of experimentally validated sites (Wasserman and Sandelin, 2004). Therefore, the combination of phylogenetic footprinting and PWM searches applied to orthologous human and mouse gene sequences reduces the rate of false predictions by an order of magnitude with only modest reduction in sensitivity. This analysis of the PRL-3 promoter region found four discrete regions, arbitrarily designated regions A-D, in which all *cis*-elements were located (Figure 11). Region A, in which the CpG-64 island is located, contains multiple cancer-associated *cis*-elements, such as *n-myc*, Max, Mef-2, and Snail (Table 3). There are also many other types of *cis*-elements in regions B, C, and D (Table 4). However, region A possesses the most abundance of cancer-associated *cis*-elements. Moreover, the CpG-64 island also exhibits high orthologue conservation as indicated by the peaks in Figure 11.
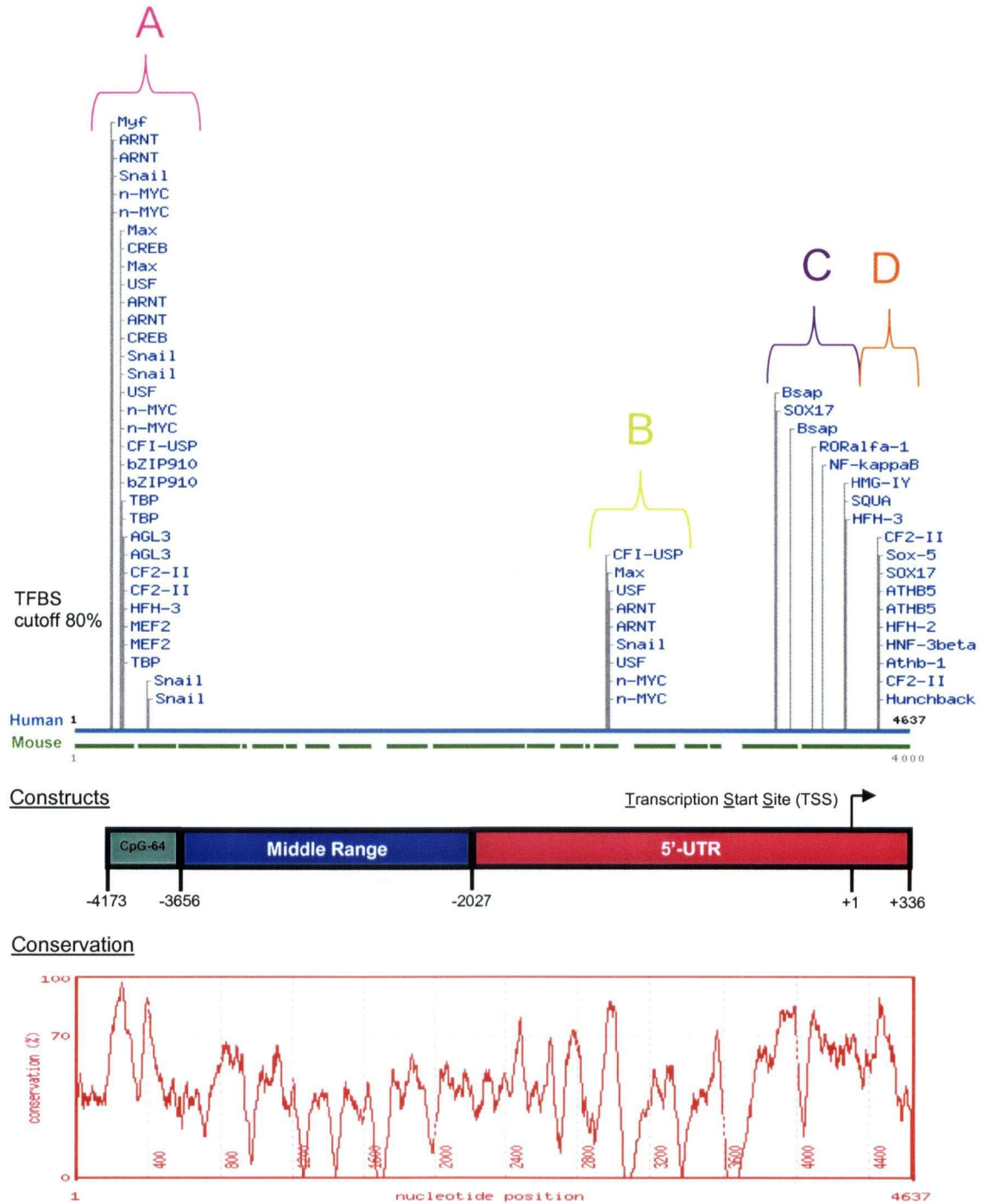
Figure 11. Putative TFBSs and orthologue conservation alignment of the human protein tyrosine phosphatase PRL-3 promoter. The human and mouse PRL-3 promoter sequences were submitted to *Consite* for orthologue conservation analysis and alignment. A window size of 50 nucleotides and a conservation cut-off of 70% were chosen. The sequences with 70% or more conservation were analyzed for putative transcription factor binding sites (TFBSs) with a cut-off of 80%. There are many cancer-associated *cis*-elements and high orthologue conservation within the PRL-3 promoter, especially in the CpG-64 island.

Region A (-4099 to -3886), 213bp			
Transcription Factor	Number of *cis*-elements	Coordinates	Strand
Myf	1	-4099 to -4088	+
ARNT	4	-4093 to -4088	+/-
		-4045 to -4040	+/-
Snail	5	-4093 to -4088	+
		-4045 to -4040	+/-
		-3902 to -3897	-
		-3891 to -3886	+
n-myc	4	-4093 to -4088	+/-
		-4045 to -4040	+/-
Max	2	-4048 to -4039	+
		-4046 to -4037	-
CREB	2	-4046 to -4035	+
		-4045 to -4034	-
USF	2	-4046 to -4040	-
		-4045 to -4039	+
CFI-USP	1	-4044 to -4035	-
bZIP910	2	-4044 to -4038	-
		-4042 to -4036	+
TBP	3	-4035 to -4021	-
		-4033 to -4019	-
		-4028 to -4014	+
AGL3	2	-4028 to -4019	+/-
CF2-II	2	-4028 to -4019	+/-
HFH-3	1	-4028 to -4017	-
MEF2	2	-4028 to -4019	+/-

Table 3. Putative *cis*-elements identified within the CpG-64 island of the PRL-3 promoter. Sequences upstream of the transcriptional start site (TSS) are designated by a negative (-) sign. Region A is within the CpG-64 island (-4173 / -3656) of the PRL-3 promoter. The PRL-3 promoter sequences identified as TFBSs have at least 70% orthologue conservation (human / mouse) and 80% matching homology to known *cis*-sequences.

Region B (-1341 to -1317), 24bp			
Transcription Factor	**Number of *cis*-elements**	**Coordinates**	**Strand**
CFI-USP	1	-1341 to -1332	-
Max	1	-1325 to -1316	+
USF	2	-1323 to -1317	-
		-1322 to -1316	+
ARNT	2	-1322 to -1317	+/-
Snail	1	-1322 to -1317	-
n-myc	2	-1322 to -1317	+/-

Region C (-407 to -13), 394bp			
Transcription Factor	**Number of *cis*-elements**	**Coordinates**	**Strand**
Bsap	2	-407 to -388	-
		-331 to -312	-
SOX17	1	-398 to -390	+
RORalfa-1	1	-205 to -196	+
NF-kB	1	-149 to -140	+
HMG-IY	1	-28 to -13	-
SQUA	1	-26 to -13	-
HFH-3	1	-24 to -13	+

Region D (+159 to +177), 18bp			
Transcription Factor	**Number of *cis*-elements**	**Coordinates**	**Strand**
CF2-II	2	+159 to +166	+
		+168 to +177	+
Sox-5	1	+163 to +169	+
SOX17	1	+164 to +172	-
ATHB5	2	+166 to +174	+/-
HFH-2	1	+166 to +177	+
HNF-3beta	1	+166 to +177	+
Athb-1	1	+167 to +174	-
Hunchback	1	+168 to +177	-

Table 4. Putative *cis*-elements within ~1.5kb of the transcription start site of the PRL-3 promoter. Sequences upstream of the transcriptional start site (TSS) are designated by a negative (-) sign, and those downstream of the TSS are designated by a positive (+) sign. The PRL-3 promoter sequences identified as TFBSs have at least 70% orthologue conservation (human / mouse) and 80% matching homology to known *cis*-sequences.

Transcript data were obtained from alignments between human expressed sequence tags (ESTs) in GenBank and the genome. ESTs are single-read sequences, typically about 500 bases in length that usually represent fragments of transcribed genes. In general, the 3'-ESTs mark the end of transcription reasonably well, but the 5'-ESTs may end at any point within the transcript. To be considered spliced, an EST must show evidence of at least one canonical intron (i.e. one that is at least 32 bases in length and has GT/AG ends). Evidence of transcripts potentially starting at the CpG-64 island (spliced and unspliced human ESTs) is apparent and suggests that the first PRL-3 exon may originate from this region (Figure 12).

A new method analyses the patterns of nucleotide identity in subregions of the alignment and classifies conserved regions as coding or regulatory (Elnitski *et al.*, 2003). This 'regulatory potential' algorithm is based on the pattern of observed identical nucleotides between species. Coding regions tend to vary at the third codon position and have insertion/deletion lengths that are multiples of three. However, non-coding regulatory sequences tend to have more frequent insertions/deletions and variations occur in distinct blocks that are separated by segments of high similarity (Wasserman and Sandelin, 2004). When homologous sequences from more than two species are available, blocks of strongly conserved sequences can effectively predict binding sites for transcription factors (Gumucio *et al.*, 1992; Hardison *et al.*, 1997b). A three-way (human / mouse / rat) regulatory potential analysis was used to evaluate the PRL-3 promoter (Figure 12). A broad peak is present only within the CpG-64 island. This signifies this region as having

transcriptional importance. Although there are sharp discrete peaks in the Middle Range (-3656 / -2027) region, this area lacks significant nucleotide identity; thus, the functional implication is limited.

Biochemical specificity of transcription is generated in the cell nucleus by combinatorial interactions between transcription factors (Davidson, 2001). Thus, gene regulation that is mediated by cooperative interactions between transcription factors that bind to clusters of sites within *cis*-regulatory modules (CRMs) can be defined using computational software to improve performance. Compared with the rate of predictions of individual TFBSs, the focus on CRMs eliminates ~99% of false TFBS predictions while retaining 60% functional regions (Wasserman and Sandelin, 2004). Analyzing groups of *cis*-elements in a module substantially improves the prediction specificity over analyzing isolated sites. Analysis of CRMs within the PRL-3 promoter reveals that only the CpG-64 island contains such modules. Mef-2 has the highest posterior probability, on either strand, for cooperative binding. Mef-2 is a trans-activating factor responsible for myogenesis and is a muscle-specific marker (Chen *et al.*, 2001; Molkentin *et al.*, 1995; Naidu *et al.*, 1995; Black and Olson, 1998). Since PRL-3 is naturally expressed in striated muscle, then it follows that muscle-specific *cis*-elements exist within the PRL-3 promoter. Moreover, only the CpG-64 island contains such myogenic *cis*-sequences, suggesting that this promoter region is transcriptionally essential for PRL-3 expression.
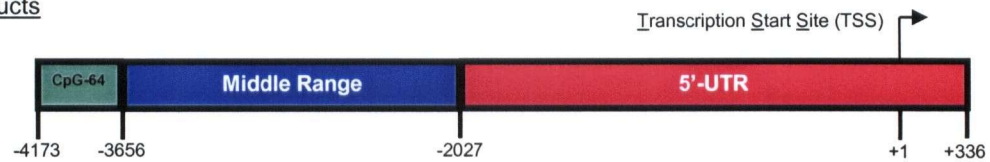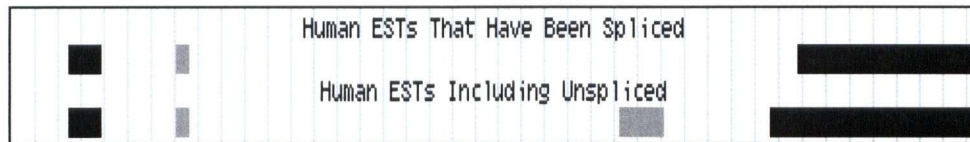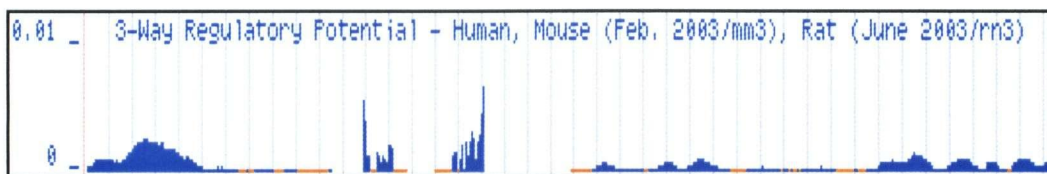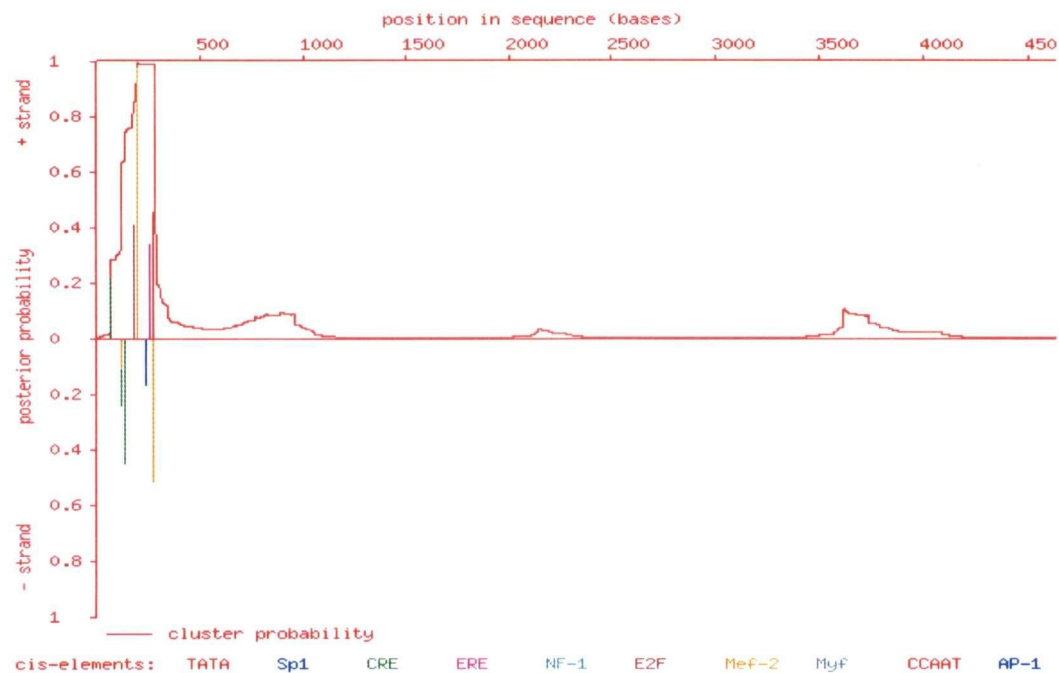
Figure 12. Transcript generation, regulatory potential, and *cis*-regulatory module analyses of the human protein tyrosine phosphatase PRL-3 promoter. This figure displays spliced and unspliced human ESTs starting from the CpG-64 island. Regulatory potential analysis defined a broad band over the CpG-64 island. Moreover, many *cis*-modulators exist within the CpG-64 island of the PRL-3 promoter. Mef-2 and TATA binding proteins appear to have the highest probability of cooperative binding.

3.3   ENDOGENOUS SNAIL AND PRL-3 EXPRESSION

Bioinformatics analyses of the PRL-3 promoter revealed that putative Snail binding sites were the most abundant predicted *cis*-element (Figure 11 and Table 3). All these sites were located within the CpG-64 island. In addition, Snail transcription factor activity is involved in tumourigenesis and metastasis (Vega *et al.*, 2004), suggesting an interesting potential link to PRL-3. Thus, I wished to determine if Snail expression induced PRL-3 expression. In cell lines, Snail expression is usually low as it is highly unstable, with a short half-life of about 25min (Zhou *et al.*, 2004). GSK-3β binds to and phosphorylates Snail at two consensus motifs to dually regulate the subcellular localization and function of this protein (Zhou *et al.*, 2004). In the hypo-phosphorylated state, Snail is active and is present in the nucleus where it functions as a transcription factor. Upon phosphorylation by GSK-3β, phospho-Snail is relocated into the cytoplasm. An additional phosphorylation of Snail by GSK-3β tags this hyper-phosphorylated Snail for proteosomal degradation. Thus, in order to study the effects of endogenous Snail, its degradation needs to be inhibited. As previously reported by Zhou *et al.* (2004), LiCl and MG132 were used to treat MDA435 invasive breast ductal carcinoma cells to inhibit GSK-3β and proteosomal activities, respectively. Western Blot analysis demonstrated that these treatments enhance Snail protein level (Figure 13). Notably, probing the same cell lysates for PRL-3 revealed that increased PRL-3 expression is seen with endogenous Snail accumulation (Figure 13). In the absence of the LiCl and MG132 inhibitory treatments, no Snail was seen and very little PRL-3 was detectable. This could reflect a positive regulatory effect of Snail on PRL-3 transcription. By inhibiting

(hyper)phosphorylation of Snail, active Snail accumulates in the nucleus. At the same time, PRL-3 protein level also increases as Snail protein is stabilized. Thus, this suggests a correlation between Snail and PRL-3.
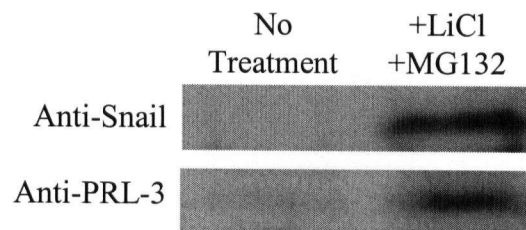
Figure 13. Enhanced PRL-3 protein level is seen with inhibition of endogenous Snail degradation in breast cancer cells. Invasive ductal breast carcinoma cells (MDA-MB-435) at ~80% confluency were either left untreated or treated with 40mM LiCl (lithium chloride) and 10μM MG132 for 5hrs prior to harvesting in RIPA lysis buffer. Untreated cells have no Snail expression and extremely low PRL-3 expression. When Snail degradation is inhibited by LiCl and MG132, PRL-3 protein increases drastically. I thank my colleague, Ms. Hoa Le, for her assistance with the PRL-3 Western blot.

3.4   PRL-3 EXPRESSION IN CLINICAL TUMOUR SAMPLES

3.4.1   Polyclonal anti-PRL-3 antibody specificity

The following studies of PRL-3 protein expression in patient tumours relied upon recognition of PRL-3 by PRL-3 polyclonal antibody. It was thus important to demonstrate antibody specificity for PRL-3 to ensure that signal detected upon probing tumour samples really represented PRL-3. Specificity was initially confirmed to heterologous PRL-3 expression in cultured cells. The polyclonal anti-PRL-3 antibody was pre-incubated with the same unique PRL-3 peptide that was used to immunize the rabbit for antibody production. The antibody-peptide solution was left to rotate at 4°C for 2hrs prior to use for immunohistochemistry. If this antibody is specific only to this peptide, then pre-incubating the antibody and peptide together before use will neutralize the function of this antibody. This was tested by probing lysates of HEK293T cells that had been transfected with a *myc*-tagged PRL-3 expression plasmid. Western blot using the polyclonal anti-PRL-3 antibody showed a tight band at the expected weight of PRL-3 (22KDa) and no other non-specific bands (Figure 14). Pre-incubating the PRL-3 antibody with the competitive peptide at 5X the concentration of the antibody considerably reduces the signal to a faint band. At 10X blocking peptide concentration, the PRL-3 band is completely abolished. This demonstrates the specificity of the antibody to the epitope that it was raised against. In addition, probing with the *c-myc* antibody confirms that the PRL-3 band was produced from the transfected construct.
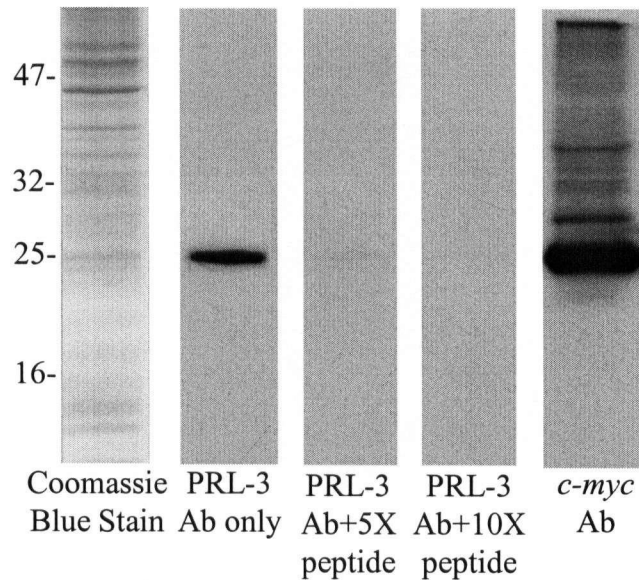
Figure 14. PRL-3 antibody specificity analysis using immunoblot. HEK293T cells were transfected with a *myc*-tagged PRL-3 expression plasmid. Transfected cells were harvested after growth in full media (DMEM, 10%FBS) for 24hrs. The whole cell lysate was resolved by SDS-PAGE and stained with Coomassie Blue to visualize total protein, or transferred to a membrane and probed with the polyclonal anti-PRL-3 antibody alone or antibody pre-incubated with different blocking peptide concentrations. The anti-*c-myc* antibody was used to verify *myc*-PRL-3 expression and detection. The positions of protein standards are shown to the left (KDa).

Immunohistochemical detection of PRL-3 using the polyclonal anti-PRL-3 antibody was initially performed and optimized by staining cardiac tissue, because PRL-3 is naturally expressed in striated muscle (Matter *et al.*, 2001). PRL-3 expression (red) was detectable in the cytoplasm as well as in the nucleus of cardiac muscle cells (Figure 15). Pre-incubation of the PRL-3 antibody with the antigenic peptide used to raise this antibody abolished antibody recognition of PRL-3 protein (Figure 15). This demonstrates that the immunohistochemical signal detected in these tissue sections is not due to non-specific binding of the anti-PRL-3 antibody.
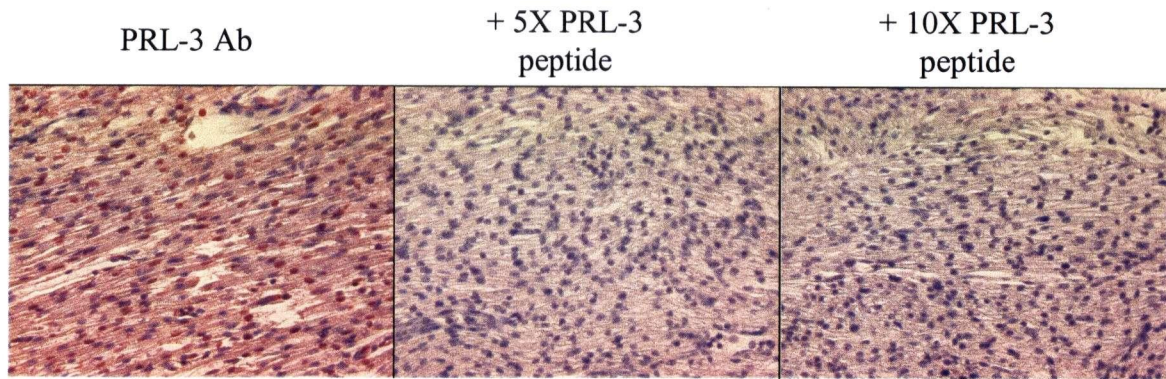
Figure 15. PRL-3 immunohistochemical staining specificity in cardiac muscle. 128.5X. Human cardiac tissues were stained with the polyclonal anti-PRL-3 antibody alone or after pre-incubation with the blocking peptide at either 5X or 10X the concentration of the antibody. The AEC chromagenic substrate was used to visualize PRL-3 expression (red), and the sections were counterstained with hematoxylin (blue).

To rule out the possibility of non-specific serum antibody contamination during the affinity purification process, the cardiac sections were also probed with pre-immune rabbit serum (Figure 16). Some faint background staining was observed with the pre-immune serum but this was less intense than that observed using the PRL-3 antibody. This is generally expected with polyclonal antibodies, but more so with serum. However, upon addition of the PRL-3 peptide, the staining pattern or intensity did not change. This suggests that the red staining pattern was not due to PRL-3 expression and represents a non-specific reactivity that is not present in the purified anti-PRL-3 antibody preparation.

| Pre-immune serum | + 5X PRL-3 peptide | + 10X PRL-3 peptide |
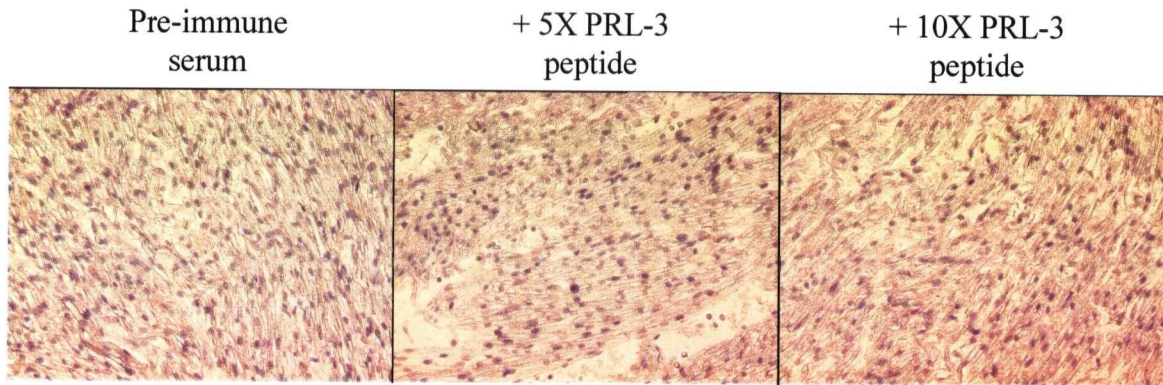
Figure 16. Cardiac muscle immunohistochemical staining with pre-immune rabbit serum. 128.5X. Human cardiac tissues were stained with pre-immune rabbit serum before or after pre-incubation with 5X or 10X PRL-3 blocking peptide. The AEC chromagenic substrate was used to visualize staining (red), and the sections were counterstained with hematoxylin (blue).

3.4.2   PRL-3 expression in Colorectal Carcinoma

Colorectal cancer is the second leading cause of cancer-related death in both men and women in the United States. The incidence of colorectal cancer has remained stable in the Western world for the last 100 years (Damjanov, 2000). Colorectal cancers are twice as common in men as in women, but the incidence does not reflect gender discrimination.

The study conducted by Saha *et al.* (2001) showed that only the PRL-3 gene was consistently over-expressed in all metastatic colorectal carcinoma samples analyzed. As an initial test and a proof-of-principle, a colorectal cancer TMA (Imgenex) was used to initially optimize PRL-3 IHC staining conditions. Ten normal colon epithelia, forty primary carcinomas, and ten matching metastatic cases were examined. Figure 17 shows that the normal colon tissues showed no IHC staining for PRL-3. In contrast, more than

half of the primary carcinoma cases exhibited strong PRL-3 staining, and roughly a quarter of the cases showed weak PRL-3 expression, with the remaining cases either being negative for PRL-3 expression or deemed uninterpretable (Table 5). In accordance with other studies (Saha *et al.*, 2001; Bardelli *et al.*, 2003; Peng *et al.*, 2004; Kato *et al.*, 2004), 70% of colorectal metastatic cases showed greatly elevated expression of PRL-3 (Table 5). These findings are consistent with others as Bardelli *et al.* (2003) reported that over 80% of colorectal cancer metastatic cases over-express PRL-3. In addition, Peng *et al.* (2004) discovered that over 60% of colorectal cancer metastases display over-expression of PRL-3, with detectable PRL-3 expression in primary carcinomas and minimal detection in normal colon epithelium. Similarly, Kato *et al.* (2004) found that more than 90% of colorectal metastatic cases over-express PRL-3. As seen here, our findings are consistent with previous observations, thus confirming that PRL-3 is indeed over-expressed in colorectal metastases, and validating our method of investigation.
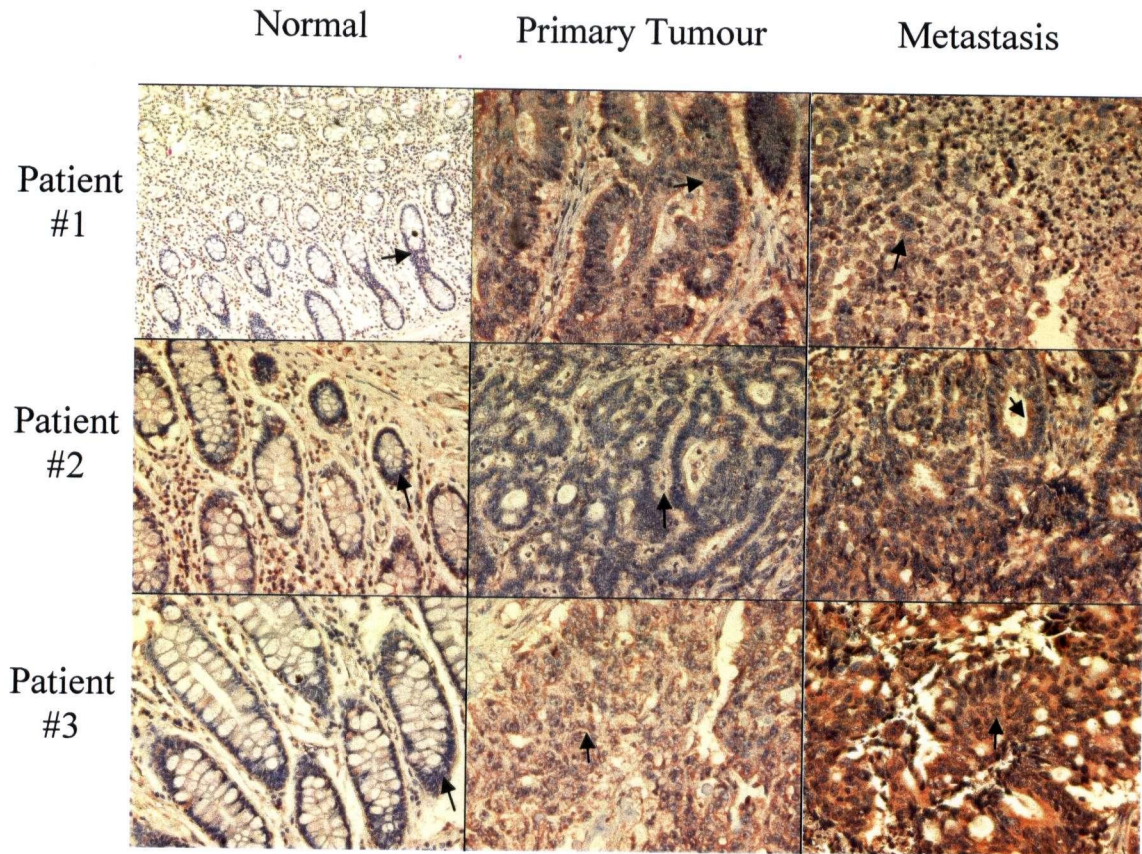
Figure 17. Immunohistochemical detection of PRL-3 in a colorectal carcinoma tissue-microarray. 88.5X. The basophilic appearance of the crypt cells suggests that PRL-3 is not expressed in normal colon epithelium (arrows: normal colon). Prominent PRL-3 staining is seen in the primary tumours of patients #1 and #3, but only weakly in patient #2. The metastatic lesions are generally more intense in staining compared to their matching primary tumours, especially in patients #2 and #3. PRL-3 localization appears to be mainly in the cytoplasm (arrows: cancerous lesions).

IHC Score (Staining)	Normal	Primary Tumour	Metastasis
0 (Negative)	100%	10%	0
1 (Weak)	0	22.5%	0
2 (Strong)	0	52.5%	70%
X (uninterpretable)	0	15%	30%

Table 5. Frequency table of PRL-3 expression in colorectal carcinoma TMA. A tissue microarray containing cores of 10 normal colon samples, 40 cases of primary colorectal carcinoma, and 10 cases of colorectal metastasis was analyzed for PRL-3 expression.

3.4.3 PRL-3 expression in Uveal Melanoma

Intraocular melanomas are the most common primary ocular malignancy in Caucasians

(Griffin *et al.*, 1998). Compared to cutaneous melanoma with a 1:90 to 1:100 chance of

developing disease over a lifetime for the Caucasian population, uveal melanoma has an

annual expected frequency of 6 cases per million in the adult Caucasian population

(Osterlind, 1987). In 1998, White *et al.* (along with Horsman and White, 1993; Sisley *et*

al., 1990) reported that certain cytogenetic findings were consistent among uveal

melanoma samples. In particular, isochromosome 8q was associated with a poor

prognosis due to death by metastatic disease (White *et al.*, 1998). Moreover, the gain of

8q was significantly associated with poor overall survival (Aalto *et al*, 2001). The PRL-3

gene is situated on chromosome 8q24.3, and PRL-3 over-expression has been associated

with metastatic disease (Saha *et al.*, 2001; Bardelli *et al.*, 2003; Peng *et al.*, 2004; Kato *et*

al., 2004; Miskad *et al.*, 2004; Wu *et al.*, 2004). PRL-3 expression in uveal melanoma

was explored to elucidate the potential of PRL-3 as a prognostic marker for uveal

melanoma.

The uveal melanoma TMA was obtained from Dr. Valerie White. It contained duplicate

cores of 184 formalin-fixed, paraffin embedded samples from eyes enucleated for large

primary non-irradiated uveal melanoma. Diameter cores of 0.6mm were consecutively

taken from tumour tissue and implanted into an empty paraffin block (in duplicates), with

1.0mm between each core. Figure 18 shows representative IHC detection of PRL-3 in

uveal melanoma. As seen in Table 6, a majority of the cases had weak PRL-3 expression.

The distribution of PRL-3 localization between cytoplasm and nucleus was similar. Only a small portion of the cases on this uveal melanoma TMA had strong PRL-3 staining. Kaplan-Meier survival curve analysis showed a positive trend (Figure 19). Although not statistically significant, patient samples that were positive for PRL-3 had a poorer survival prediction than those patient samples that were negative for PRL-3. Nonetheless, more patient samples need to be evaluated before drawing a statistically relevant conclusion.
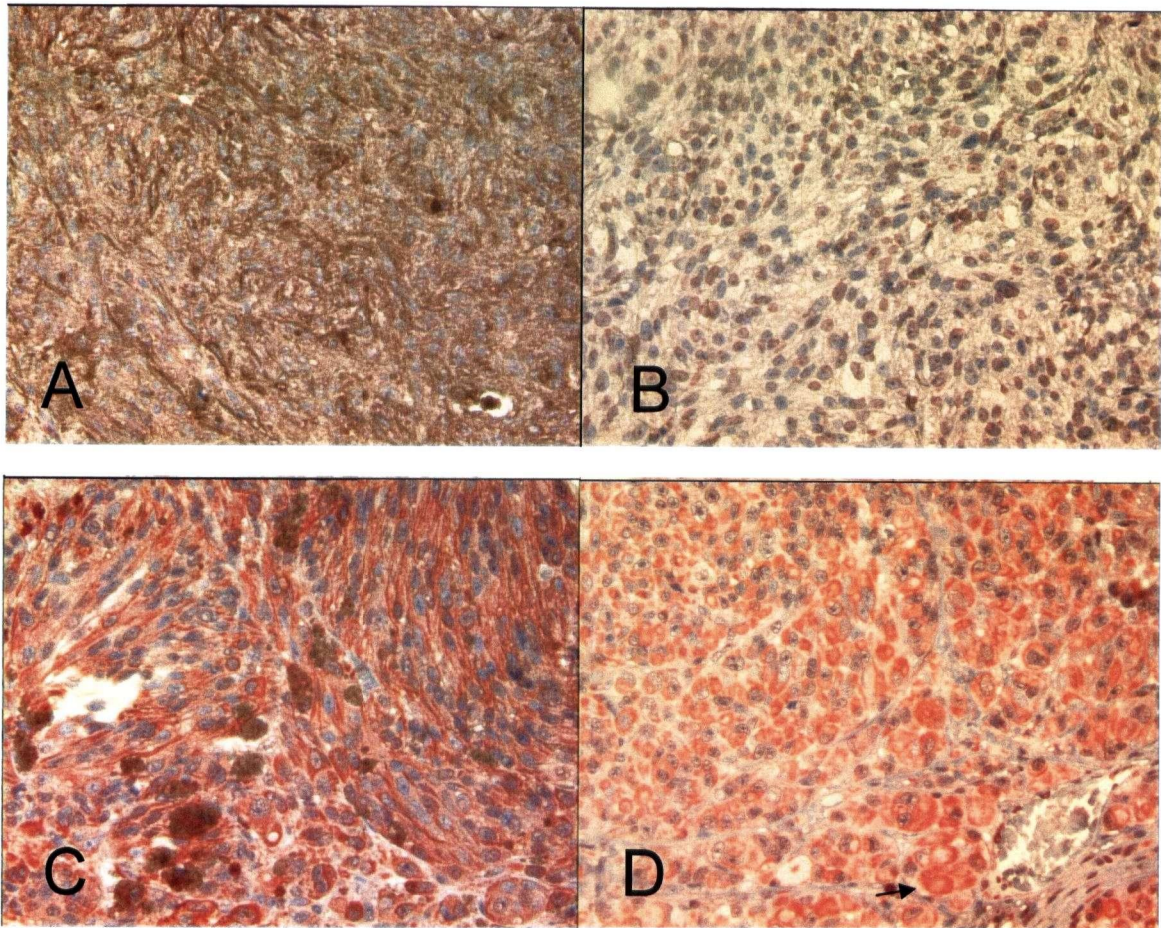
Figure 18. Immunohistochemical detection of PRL-3 in a uveal melanoma tissue-microarray. 191.5X. *A*, the case seen here was heavily pigmented with melanin (brown) and showed little PRL-3 staining (red), it was thus assigned a score of 0. Although this tumour contained a lot of melanin, the basophilic nuclei were still seen; *B*, this case had many positively staining nuclei, but not more than 50% of these nuclei were red, hence the score of 1; *C*, this case is a Spindle type B lesion and the localization of PRL-3 stain was mainly in the cytoplasm and not in the nucleus, a score of 2 was assigned to this patient due to evidently strong stain; *D*, this case is an epitheliodal tumour, PRL-3 staining was predominantly localized in the cytoplasm as seen by the circumferential staining pattern (arrow), a score of 2 was also assigned to this patient.

IHC Score (Staining)	Percentage of Cases
0 (Negative)	18.5%
1 (Weak)	60.9%
2 (Strong)	13.0%
X (uninterpretable)	7.6%

Table 6. Frequency table of PRL-3 expression in a uveal melanoma TMA.
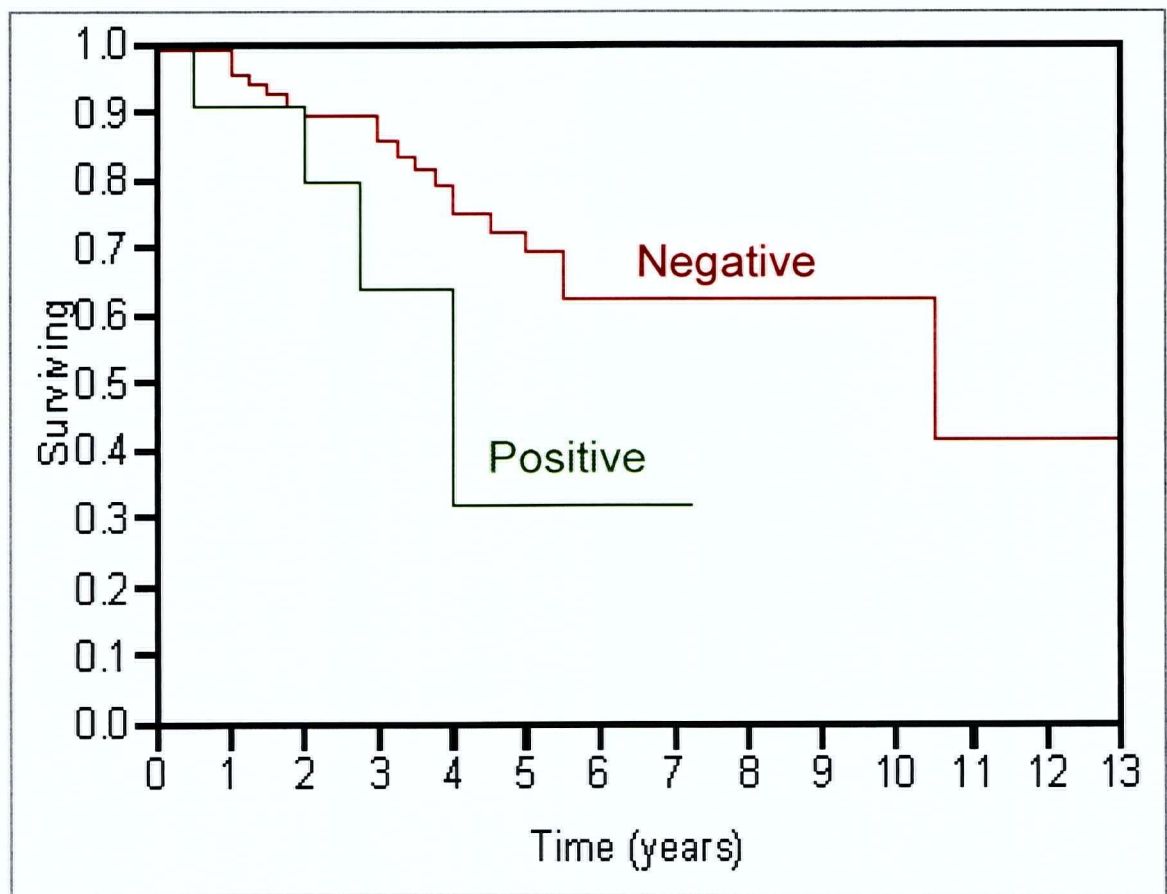
Figure 19. Kaplan-Meier survival curve for uveal melanoma patient samples with negative and positive IHC staining for PRL-3. This is a disease-specific survival plot with 107 patients analyzed. Scores of 0 and 1 were grouped as 'negative', whereas a score of 2 was considered as 'positive'. This plot shows no statistically significant correlation between IHC of PRL-3 and survival (p=0.11). However, a positive trend is observed as patients with positive staining cores have poorer survival outcome compared to those with negative staining scores.

3.4.4 PRL-3 expression in Breast Carcinoma

Breast cancer is the most common cancer in women, surpassed in lethality only by lung cancer. An estimated 1 in 14 woman will develop breast cancer during their life span (Damjanov, 2000). At least 180,000 new cases of breast cancer are diagnosed each year. Unfortunately, the number of new cases is increasing steadily. Approximately 46,000 women die of breast cancer every year in the United States.

A TMA with 495 cases of breast carcinoma was obtained from Dr. Blake Gilks and stained for PRL-3. Localization of PRL-3 IHC staining is found to be cytoplasmic as well as nuclear (Figure 20). The majority of the cases were negative for cytoplasmic PRL-3, with only a small group of cases having high levels of cytoplasmic PRL-3 staining (Table 7). Cases with high cytoplasmic PRL-3 expression had a poor clinical prognosis compared to cases with no / low cytoplasmic PRL-3 expression (Figure 21). Conversely, majority of the cases were positive for nuclear PRL-3 IHC staining (Table 8). Cases with high nuclear PRL-3 expression had no correlation with clinical outcome of the patients (Figure 22). Carcinoma *in-situ* lesions have 100% of the epithelial cells classified as neoplastic, exhibit prominent features of dysplastic changes, and typically have basement membrane involvement. Stromal invasion is not seen in carcinoma *in-situ*. Invasive / infiltrating lesions have all the characteristics of carcinoma *in-situ* but with the additional presences of typical 'Indian files'. This single-file cell arrangement pattern is found in the surrounding stroma, and is a good indicator of disease aggressiveness.
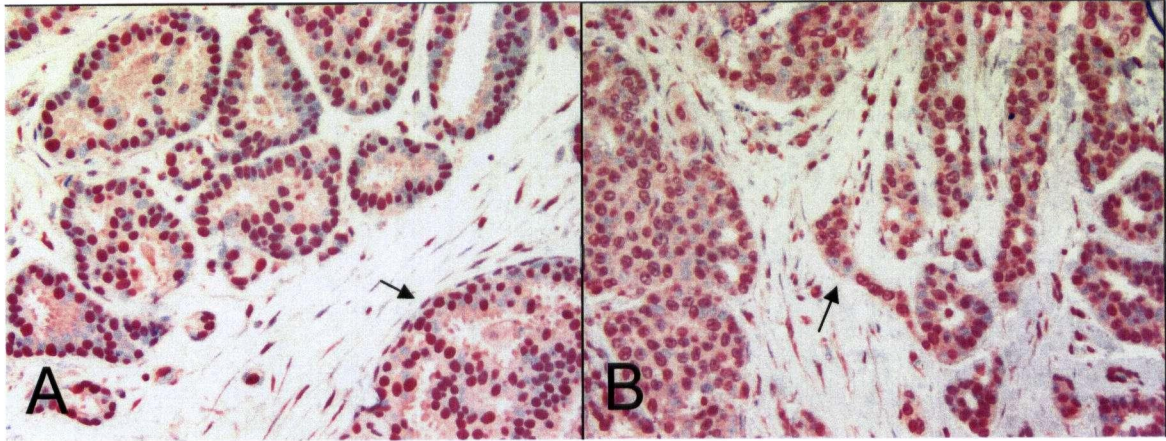
Figure 20. Immunohistochemical detection of PRL-3 in a breast carcinoma tissue-microarray. 191.5X. This figure shows the commonest form of breast cancer, ductal carcinoma, which accounts for roughly 75% of all carcinomas of the breast. Nests of cells and retained tube-like structures are seen in ductal carcinomas. A high level of PRL-3 staining (red) was observed in both of these cases. Strong PRL-3 staining was localized to the cytoplasm as well as in the nucleus of these cancerous cells. *A*, the panel on the left depicts a carcinoma *in-situ* lesion (arrow), the neoplastic cells that are surrounded / encompassed by the myoepithelial cells exhibit typical dysplastic features of a high-grade carcinoma lesion, and stromal involvement is not seen this is case; *B*, the panel on the right shows a high-grade invasive ductal carcinoma lesion. The metastatic / invasive cells appearing as a single file are seen wandering in the stroma (arrow). Stromal involvement is a typical feature of invasive carcinoma.

IHC Score (Staining)	Percentage of Cases
0 (Negative)	53.1%
1 (Weak)	26.3%
2 (Strong)	2.63%
X (uninterpretable)	17.97%

Table 7. Frequency table of cytoplasmic PRL-3 expression in breast carcinoma TMA. PRL-3 expression was analyzed by immunohistochemical staining of 495 carcinomas. The staining intensity was scored from 0-2 as shown.
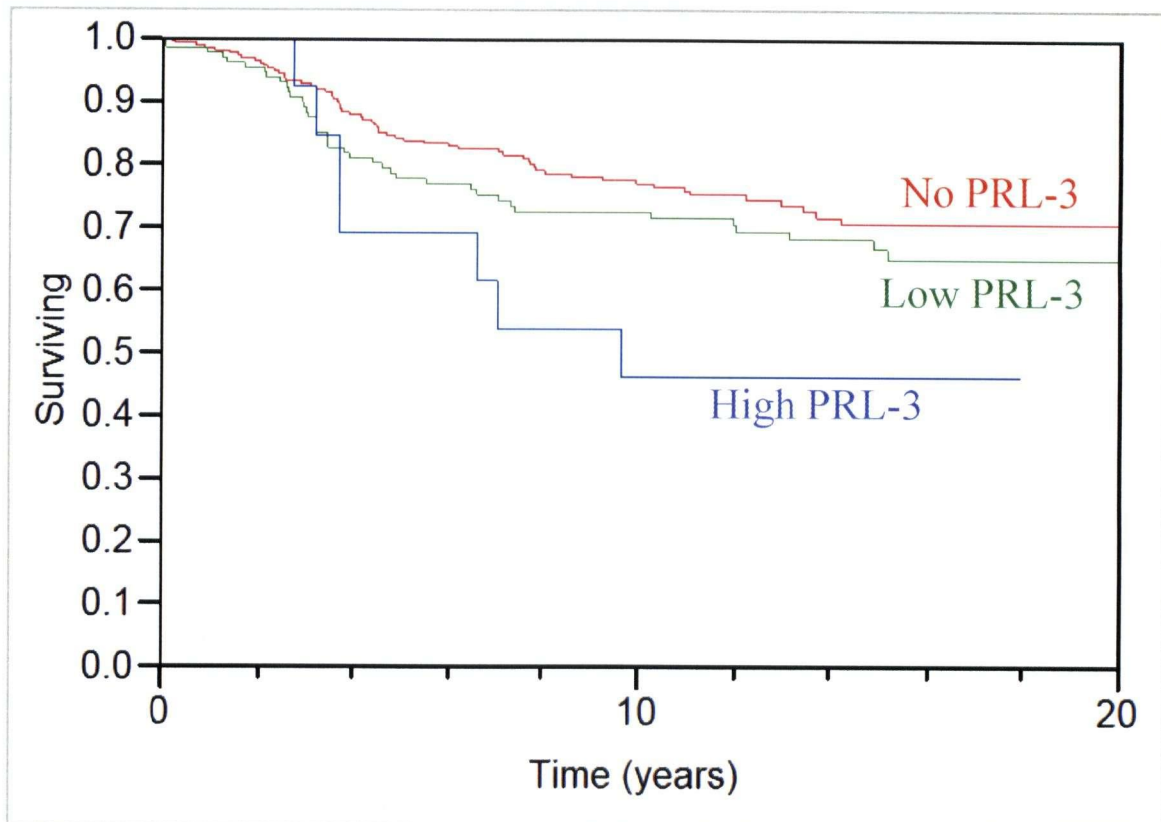
Figure 21. Kaplan-Meier survival curve of cytoplasmic PRL-3 expression in breast carcinoma. No, low, and high IHC staining for cytoplasmic PRL-3 expression was plotted against patient outcome data (n=405). This is a disease-specific survival plot. A score of 0 was considered to be null for PRL-3 expression. Scores of 1 or 2 represented low and high staining intensity, respectively. This plot shows a significant correlation (p=0.0441) between IHC expression of cytoplasmic PRL-3 and survival. Patients with high levels of PRL-3 have a poorer prognosis than those with no / low levels of PRL-3.

IHC Score (Staining)	Percentage of Cases
0 (Negative)	13.54%
1 (Weak)	21.21%
2 (Strong)	47.07%
X (uninterpretable)	18.18%

Table 8. Frequency table of nuclear PRL-3 expression in breast carcinoma TMA. PRL-3 expression was analyzed by immunohistochemical staining of 495 carcinomas. The staining intensity was scored from 0-2 as shown.
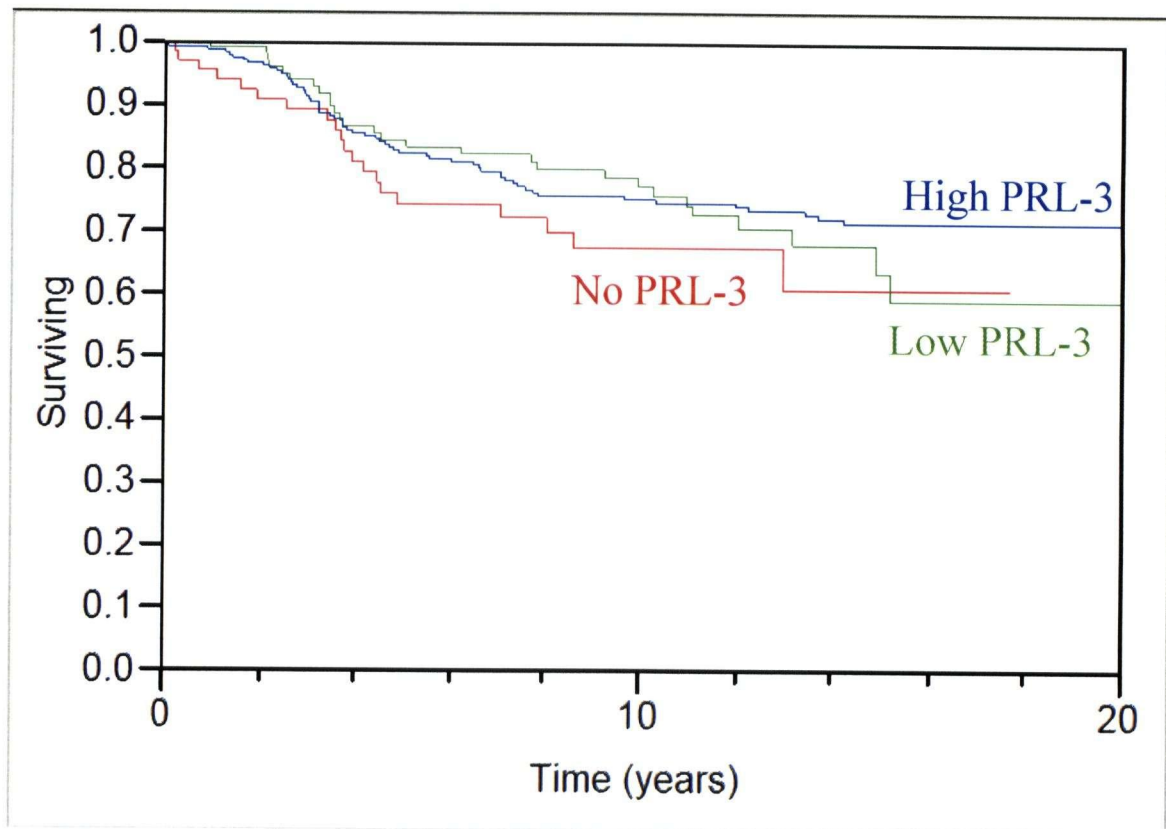
Figure 22. Kaplan-Meier survival curve of nuclear PRL-3 expression in breast carcinoma. No, low, and high IHC staining for nuclear PRL-3 expression was plotted against patient outcome data (n=406). This is a disease-specific survival plot. A score of 0 was considered to be null for PRL-3 expression. Scores of 1 or 2 represented low and high staining intensity, respectively. This plot shows no significant correlation (p=0.16) between IHC expression of nuclear PRL-3 and survival.

3.4.5 PRL-3 expression in Pediatric Tumours

Cancer in childhood and adolescents represents 2-3% of all cancers, but is the leading cause of non-violent deaths in the 1-15 year age group in developed countries. However, of the 200,000 new cases of cancer that arise every year in this age group, 85% occur in developing countries, with the expectation that this proportion will soon rise to 90%. Childhood cancers differ markedly from adult cancers in type, biology, genetic features, and diagnostic requirements.

As a preliminary survey, IHC was performed on 60 cases of the commonest types of pediatric tumours on a TMA (14 cases of Primitive Neuroectodermal Tumour (PNET) / Ewing's sarcoma, 13 cases of neuroblastoma, 13 cases of rhabdomyosarcoma, 7 cases of fibromatosis, 6 cases of congenital fibrosarcoma, and 7 cases of miscellaneous rare tumours). The majority of the cases exhibited high levels of PRL-3 expression compared to control tissue (fetal muscle).

Neuroblastoma accounts for 6% of all pediatric cancers and is the most common cancer in children under 2 years of age. Neuroblastoma is a peripheral nervous system tumour and typically arises from the sympathetic nervous system, mainly from the adrenal medulla. The poorly differentiated form of this tumour is the blastic neuroblastoma, and the well differentiated form is called ganglioneuroma. Patients with ganglioneuromas typically have a better clinical picture compared to patients with neuroblastomas. Rhabdomyosarcoma (RMS) accounts for 4% of all pediatric cancers and is the most

common soft tissue sarcoma in childhood. Rhabdomyosarcoma arises from skeletal muscle and is analogous to fetal muscle at 10-12 weeks gestation. The more primitive form of RMS with little muscle differentiation is called alveolar RMS, and the typically more differentiated form is called embryonal RMS. Alveolar RMS is usually more aggressive than embryonal RMS (Kouraklis *et al.*, 1999). In general, poorly differentiated neuroblastomas and rhabdomyosarcomas are classically more prognostically unfavorable. Due to the associations of *n-myc* and Mef-2 with neuroblastoma and rhabdomyosarcoma respectively, and the presence of these *cis*-elements within the PRL-3 promoter, IHC expression for PRL-3 in neuroblastoma and rhabdomyosarcoma was considered (Figure 23). Although the cohort was small, strong PRL-3 IHC staining is evident in more than 60% of the neuroblastoma and rhabdomyosarcoma cases (Tables 9 and 10). One interesting observation is that PRL-3 tends to be more localized in the nucleus of cells in the poorly differentiated tumours, such as blastic neuroblastoma and alveolar rhabdomyosarcoma, and localized in the cytoplasm of cells in the well differentiated tumours, such as ganglioneuroma and embryonal rhabdomyosarcoma. For neuroblastoma, this observation is statistically valid as all of the poorly differentiated (blastic) neuroblastoma tumours exhibit nuclear PRL-3 IHC staining and all of the well differentiated (gangliocytic) neuroblastoma tumours exhibit cytoplasmic PRL-3 IHC staining (Table 11). As for rhabdomyosarcoma, there was no significant statistical correlation between tissue type and PRL-3 localization (Table 12). More cases of both neuroblastoma and rhabdomyosarcoma need to be investigated to further confirm the relationship between PRL-3 IHC localization and differentiation type in pediatric tumours.
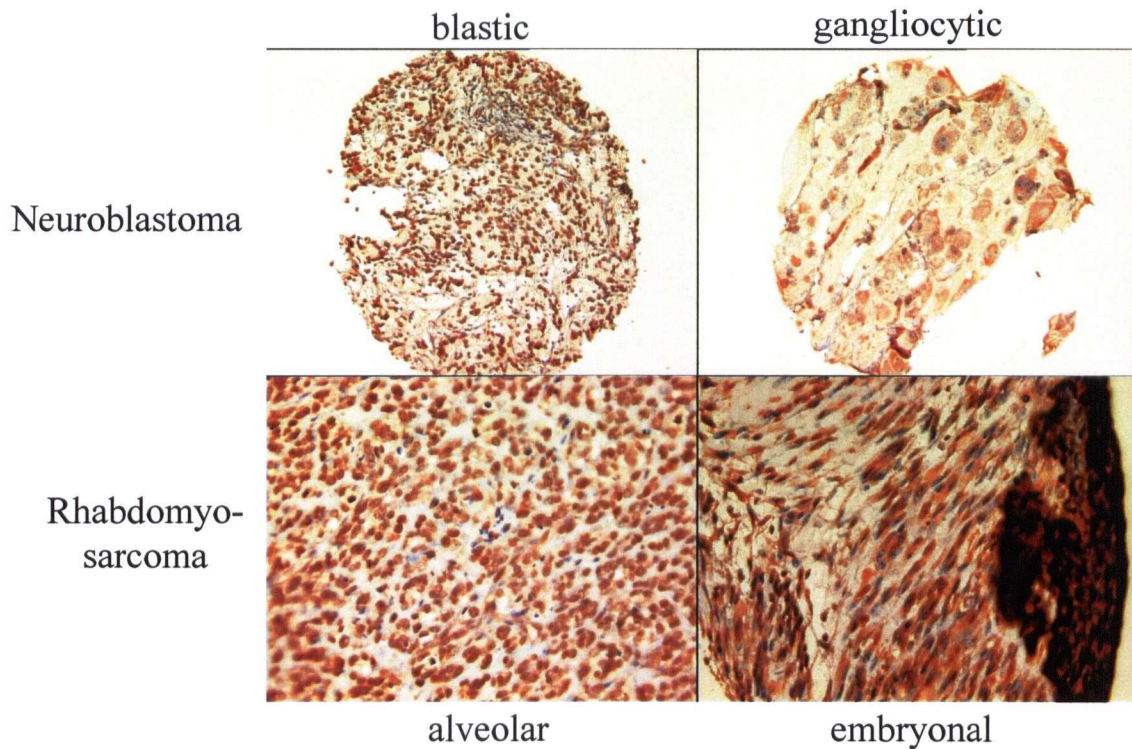
Figure 23. Immunohistochemical detection of PRL-3 in a pediatric tumour tissue-microarray. Neuroblastoma, 71.8X. Rhabdomyosarcoma, 143.6X. This figure shows two fairly common forms of solid tumours found in children. Neuroblastoma is typically a poorly-differentiated tumour with a predominant blastic appearance. Ganglioneuroma is a well-differentiated form of neuroblastoma. Rhabdomyosarcoma can be generally divided into two categories; alveolar (typically less differentiated) and embryonal (typically more differentiated). This figure shows that PRL-3 is generally localized in the nucleus of poorly differentiated tumour cells and is predominantly localized in the cytoplasm of well-differentiated tumour cells.

IHC Score (Staining)	Percentage of Cases
0 (Negative)	7.7%
1 (Weak)	23.0%
2 (Strong)	61.5%
X (uninterpretable)	7.8%

Table 9. Frequency table of PRL-3 expression in pediatric neuroblastoma. The TMA contained 13 cases of neuroblastoma.

IHC Score (Staining)	Percentage of Cases
0 (Negative)	15.5%
1 (Weak)	23.0%
2 (Strong)	61.5%
X (uninterpretable)	0

Table 10. Frequency table of PRL-3 expression in pediatric rhabdomyosarcoma. The TMA contained 13 cases of rhabdomyosarcoma.

Fisher's Exact 2-Tail Test p<0.01		Differentiation / Type		Total
		Poor / Blastic	Well / Gangliocytic	
Localization	Cytoplasmic	0	3	3
	Nuclear	8	0	8
Total		8	3	11

Table 11. Pediatric Neuroblastoma 2X2 Contingency Table. χ^2- statistical analysis was used to test for independence between IHC of PRL-3 localization and tumour differentiation status of neuroblastoma. This table shows a significant correlation between the two variables (p<0.01) as all of the poorly differentiated (blastic) neuroblastoma tumours exhibit nuclear PRL-3 IHC staining and all of the well differentiated (gangliocytic) neuroblastoma tumours exhibit cytoplasmic PRL-3 IHC staining.

Fisher's Exact 2-Tail Test p>0.05		Differentiation / Type		Total
		Poor / Alveolar	Well / Embryonal	
Localization	Cytoplasmic	0	1	1
	Nuclear	4	2	6
	Cytoplasmic/Nuclear	3	1	4
Total		7	4	11

Table 12. Pediatric Rhabdomyosarcoma 2X2 Contingency Table. χ^2- statistical analysis was used to test for independence between IHC of PRL-3 localization and tumour differentiation status of rhabdomyosarcoma. This table shows no significant correlation between the two variables (p=0.43). Cases with both cytoplasmic and nuclear stains were excluded from statistical analysis on this table.

CHAPTER 4

DISCUSSION

4.1   IMPORTANCE OF THE CPG-64 ISLAND IN THE PRL-3 PROMOTER

PRL-3 is a relatively new phosphatase that has received much attention over the past few years. PRL-3 has been shown to be significantly over-expressed in colorectal (Saha *et al.*, 2001; Bardelli *et al.*, 2003; Peng *et al.*, 2004; Kato *et al.*, 2004), gastric (Miskad *et al.*, 2004), and melanoma metastasis (Wu *et al.*, 2004). PRL-3 over-expressing CHO cells exhibit enhanced metastatic properties such as migratory abilities and invasiveness (Zeng *et al.*, 2003). However, very little is known about the molecular actions of this protein, let alone the genetic properties that regulate its transcription. Since PRL-3 is over-expressed in metastatic colorectal lesions, and increased gene copy number of PRL-3 is not the primary cause of this over-expression (Bardelli *et al.*, 2003), I investigated the transcriptional regulation of PRL-3 expression by characterizing the PRL-3 promoter. The PRL-3 upstream sequence (-4173 / +336) was evaluated for its overall and region-specific promoter activity, with the particular aim of identifying sequences active in promoting transcription in metastatic cells or cells with metastatic properties. Of the promoter regions evaluated via reporter assays and bioinformatics, the CpG-64 sequence is generally the most active, and most probable as a promoter of PRL-3 expression.

I established that the PRL-3 promoter has differential transcriptional activities in various cell lines exhibiting assorted levels of invasiveness, metastatic state, malignancy, and cellular differentiation. Luciferase reporter assays confirm that the CpG-64 island is an important region for PRL-3 transcriptional regulation. The CpG-64 island is more active in colorectal metastatic cells than in primary tumour cells, more active in invasive prostate androgen-independent tumour cells than in androgen-dependent tumour cells, and more active in malignant muscle cells than in normal muscle cells. Bioinformatics analyses revealed that this CpG-64 island has multiple cancer-associated *cis*-elements, high orthologue conservation, potential transcript generation, and detectable regulatory potential. Other regions in the PRL-3 promoter have some, but not all, of these above characteristics. Together, these findings suggest that the CpG-64 island is a candidate alternative promoter region that is differentially utilized in various stages of cancer development and progression.

4.1.1 CpG-64 island: The only or alternative promoter to PRL-3?

CpG islands are short DNA sequences / regions that are typically a few hundred base pairs in length and are highly enriched with the nucleotides cytosine and guanine. Methylation on cytosine silences transcription. Within regulatory sequences, CpGs remain unmethylated, whereas up to 80% of CpGs in other regions are methylated on a cytosine (Wasserman and Sandelin, 2004). Approximately 60% of all human promoters are situated proximally to CpG islands (Gardiner-Garden and Frommer, 1987). Functional regulatory regions that control transcription rates are usually proximal to the

transcription start site(s). The current set of laboratory-annotated regulatory sequences indicates that sequences near a TSS are more likely to contain functionally important regulatory controls than those that are more distal; however, the exact position of a TSS can be difficult to locate due to alternative start sites (Wasserman and Sandelin, 2004). Computationally, the CG dinucleotide imbalance can be a powerful tool for finding regions in genes that are likely to contain promoters (Hannenhalli and Levy, 2001).

In addition, CRM analysis also showed that only the CpG-64 island of the PRL-3 promoter contains a TATA box. The posterior probability of this TATA box is the second highest of the positive strand binding trans-activating factors. This again argues for the fact that the CpG-64 island can be a promoter for PRL-3. TATA/GATA regions are typically used for induction of genetic expression, whereas GC-rich regions are mainly utilized for maintenance and housekeeping genetic expression (Mura *et al.*, 2004). Having a TATA box within a CG-rich region (CpG-64 island) suggests that PRL-3 has differential levels of expression. The CG-rich regions cause low / housekeeping level expression of PRL-3, whereas the TATA box causes a high level of PRL-3 expression when the appropriate stimuli are present for induction. CG-rich regions are typically located in the vicinity of the transcription start site and are sufficient to direct transcription initiation in TATA-less promoters. In addition, they usually display multiple transcription start sites and a lower level of RNA synthesis than transcripts directed by TATA promoters (Blake *et al.*, 1990; Lu *et al.*, 1994; Pugh and Tjian, 1990).

With respect to potential PRL-3 transcript generation, spliced and unspliced human ESTs can be found within the full-length PRL-3 promoter and appear to begin at the CpG-64 island (Figure 12). This suggests that the CpG-64 island can be a promoter of PRL-3 transcription. The 3-way regulatory potential was investigated between human, mouse, and rat to determine the level of possible functional importance of specific regions of the PRL-3 promoter. A broad peak was predicted that extended across the CpG-64 island (Figure 12), indicating that this promoter region can potentially regulate the transcription of the PRL-3 gene. There are also two sharp peaks within the Middle Range (-3656 / -2027) region. Regulatory potential analysis is based on the principle of interspecies nucleotide identity correlating with functional importance. The Middle Range region exhibits minimal orthologue conservation and no putative TFBSs. Thus, these two sharp peaks in the Middle Range regions may have little or no functional significance. Additionally, cis-regulatory modules can only be found within the CpG-64 island and nowhere else in the PRL-3 promoter (Figure 12). Trans-activating factors mainly work in a combinatorial fashion, or module, for promoter regulation. The lack of any CRMs within the PRL-3 promoter other than the CpG-64 island demonstrates the likely negligible transcriptional importance of the rest of the PRL-3 promoter regions. This again validates the CpG-64 island as acting as a promoter for PRL-3 gene expression.

In accordance with these bioinformatics analyses, luciferase reporter assays also indicate that the CpG-64 island has high promoter activity. It is typically the strongest transcriptional director among the various PRL-3 promoter regions tested, especially in the more invasive / metastatic cell lines. In HEK293T cells, the CpG-64 island alone

induced a ~131.5-fold increase in luciferase activity compared to the empty vector

(Figure 5). Other promoter regions lacking the CpG-64 island induced no significant

luciferase activity. The full-length promoter sequence containing the CpG-64 island also

stimulated significant luciferase activity, whereas removal of the CpG-64 island resulted

in negligible induction of luciferase expression. This suggests that the CpG-64 island

indeed contains transcriptionally important regions whereas other PRL-3 promoter

sequences lack such activity, at least in the cell lines and under the conditions examined.

In colorectal cancer cell lines (Figure 8), the CpG-64 island exhibited ~2.3-fold higher

promoter activity in the metastatic cells (SW620) than in the less invasive primary tumour

cells (SW480). These results, obtained by conducting luciferase reporter assays in a

metastatic colorectal carcinoma cell line and a matching cell line derived from the

primary tumour of the same patient, indicate that the CpG-64 island can potentially be

involved in the metastatic transition of colorectal carcinoma cells. This same CpG-64

island has high luciferase activity even in the SW480 primary tumour cells, providing

further evidence of the transcriptional importance of this promoter region. Moreover, the

full-length PRL-3 promoter containing the CpG-64 sequence is also more active in the

SW620 cells than in the SW480 cells, while this enhanced activity was lost upon deletion

of the CpG-64 region. Again, this supports a metastasis-associated promoter function of

this CG-rich region in regulating PRL-3 expression.

Consistent with the above findings in HEK293T and colorectal cancer cell lines, the

CpG-64 island exhibited ~8.7-fold higher promoter activity in the more invasive (DU145)

metastatic prostate cancer cells than in the less invasive (LNCaP) metastatic prostate

cancer cells (Figure 9). Interestingly, the CpG-64 island promoter activity was unusually

low in the LNCaP cells. Perhaps this is due to the androgen-dependent and less invasive

nature of LNCaP cells, while PRL-3 has been associated with invasive properties. If the

CpG-64 island is indeed the sole promoter of PRL-3 transcription, then it is reasonable

that this promoter region would be less active in a less invasive cell line. Since the CpG-

64 island activity is higher in the DU145 cells than in the LNCaP cells, it would be

expected that the same relationship is maintained when the CpG-64 is placed in

conjunction with other promoter regions (i.e. the full-length promoter). Surprisingly

however, the full-length promoter stimulated ~3.6-fold higher luciferase expression in the

less invasive LNCaP cells than in the more invasive DU145 cells. The basis of the

inverse promoter activities of the full-length and the CpG-64 PRL-3 upstream sequences

in LNCaP versus DU145 cells is unclear. However, since both cell lines were derived

from prostate tumour metastases, this might reflect activities regulated in an unknown

manner by the differential androgen-dependence or invasive abilities of these cell lines.

Lastly, since PRL-3 is normally expressed in striated muscle, a normal skeletal muscle

cell line (C2C12) was used to investigate PRL-3 promoter activity. Surprisingly in

C2C12 cells, none of the PRL-3 promoter constructs induced any significant levels of

luciferase activity except the CpG-64 island. This again argues for the function of the

CpG-64 island as the major, if not only, regulator of normal PRL-3 expression. To

investigate which PRL-3 promoter region(s) is operative in malignant transformation,

rhabdomyosarcoma (RMS) cell lines were used. All promoter regions (full-length and

truncations) were comparatively more active in the RMS cell lines than in the C2C12 cell

line. The CpG-64 island was ~6.5-fold more active as a promoter in the RD (embryonal RMS) and ~3.9-fold more active in the RMS13 (alveolar RMS) cell lines compared to in the C2C12 cells (Figure 10). This again supports the promoter function of the CpG-64 island in transcriptional control and also demonstrates its potential role in up-regulating PRL-3 expression in the malignant phenotype. Other promoter regions do not exhibit these drastic differences in activity between normal and malignant muscle cell lines.

The luciferase reporter assay results in various cell lines indicate that the CpG-64 island functions as the most important (if not the only) region of the PRL-3 promoter. In the absence of the CpG-64 island, very little promoter activity is typically seen. This suggests that the CpG-64 island could possibly be the only PRL-3 promoter. In malignant / metastatic transformation however, other promoter regions may participate in transcriptional regulation, as observed in the prostate and muscle cancer cell lines.

4.1.2   CpG-64 island and cancer-associated *cis*-elements

The CpG-64 island is potentially a promoter for PRL-3 gene transcription. Thus, putative TFBSs that appear within the CpG-64 island may exert significant transcriptional control on PRL-3 expression. In addition, orthologue conservation (human / mouse) shows that this promoter region is highly conserved, further illustrating the functional importance of the CpG-64 island. Although there were many *cis*-elements identified within the CpG-64 island of the PRL-3 promoter (Figure 11 and Table 3), I have chosen four transcription factors (Max, *n-myc*, Mef-2, and Snail) for further discussion. These transcription factors

have demonstrated associations with muscle gene expression and / or the regulation of

gene expression in processes such as proliferation and cancer that are potentially relevant

to PRL-3 transcriptional regulation.

4.1.2A   Max

The Myc/Max/Mad network comprises a group of transcription factors whose distinct

interactions result in gene-specific transcriptional activation or repression. A great deal

of research indicates that this protein network plays roles in cell proliferation,

differentiation, and death. Both Myc and Mad form heterodimers with Max. These DNA-

bound heterodimers recruit coactivator or corepressor complexes that generate alterations

in chromatin structure, which in turn modulate transcription (Grandori *et al.*, 2000). In

their biochemical behavior, the Mad proteins are similar to the Myc family members in

that they homodimerize poorly but form specific heterodimers with Max which can be

detected *in vitro* and *in vivo*. Furthermore, Mad-Max dimers recognize the same

CACGTG E-box sequence as do Myc-Max dimers (Ayer *et al.*, 1993; Zervos *et al.*, 1993;

Hurlin *et al.*, 1995b). Myc proteins are prominent oncogenic transcription factors. On

the other hand, the correlation between Mad gene expression and differentiation has been

confirmed by biochemical studies using cells that can be induced to undergo terminal

differentiation *in vitro*. The increase in Mad protein levels is accompanied by a switch

from Myc-Max complexes to Mad-Max complexes following induction of differentiation

(Ayer and Eisenman, 1993; Hurlin *et al.*, 1995a). Thus, the Myc-Max complex is

responsible for cellular proliferation, whereas the Mad-Max complex is responsible for

cellular differentiation. There are two Max binding sites within the CpG-64 island of the PRL-3 promoter (Figure 11 and Table 3). Stable expression of heterologous PRL-1, -2, or -3 increases cell growth rates (Diamond *et al.*, 1994; Cates *et al.*, 1996; Matter *et al.*, 2001). Thus, it is plausible that the Myc-Max complex binds to the CpG-64 island and activates transcription of PRL-3, and this in turn enhances proliferation. Moreover, it is also interesting to note that *n-myc* binding sequences are in proximity to Max binding sequences within the CpG-64 island. This suggests a possible relationship between Myc-Max interaction and PRL-3 expression in proliferating cells. On the other hand, the Mad-Max complex may also bind to these Max sites. Mef-2 binding sequences are also found within the CpG-64 island, and Mef-2 is an activator and marker for muscle differentiation (Chen *et al.*, 2001; Molkentin *et al.*, 1995; Naidu *et al.*, 1995; Black and Olson, 1998). Since PRL-3 is naturally expressed in mature striated muscle, then it is plausible that the Mad-Max complex can also bind to these sites and, together with Mef-2, activate PRL-3 expression in differentiating muscle cells.

4.1.2B   N-myc

The Myc family proteins function in the integrated coregulation of both proliferative and apoptotic signal transduction pathways (Cole and McMahon, 1999). More specifically, the *n-myc* protein appears to act as a transcriptional regulator and is thought to govern the transcription of critical genes involved in mitogenesis and multidrug resistance. A close correlation between the expression of *n-myc* and the multidrug resistance-associated protein gene has been reported (Norris *et al.*, 1996; Haber *et al.*, 1999). The childhood

tumour neuroblastoma is characterized by *n-myc* amplification in aggressive and highly

proliferative tumours that occur in a subset of patients (Kim and Carroll, 2004). *N-myc*

gene amplification is found in ~20-30% of all neuroblastoma cases (Misawa *et al.*, 2000).

Direct evidence that *n-myc* contributes to tumourigenesis has been obtained from

transgenic mice over-expressing *n-myc*, that showed a high incidence of neuroblastoma

(Weiss *et al.*, 1997). Chronic upregulation of *n-myc* in the SH-EP neuroblastoma cell line

accelerates progression into S-phase early after mitogenic stimulation of quiescent cells

(Lutz *et al.*, 1996) and enhances the malignant phenotype of neuroblastoma cells

(Schweigerer *et al.*, 1990). Since the *n-myc* protein is a well known transcription factor

and is one of the most powerful prognostic factors in predicting poor survival for

neuroblastoma patients (Seeger *et al.*, 1985), *n-myc* may also be involved in PRL-3

expression as there are four *n-myc* binding sequences within the CpG-64 island of the

PRL-3 promoter. The *n-myc* binding sequence is the second most abundant *cis*-element

within the CpG-64 island. Thus, *n-myc* may be one of perhaps several trans-activating

factors that cause PRL-3 over-expression in highly metastatic and aggressive cancers.

4.1.2C   Mef-2

The capability to escape the differentiation program and undergo deregulated

proliferation is a hallmark of tumour cells. This may be achieved by several mechanisms,

such as disrupting cell growth regulatory pathways and inactivating functional proteins

that stimulate the differentiation program (Puri *et al.*, 2000). Rhabdomyosarcoma (RMS)

is a malignant tumour arising from skeletal muscle. It is the most common soft tissue

sarcoma in children under the age of 15, accounting for 5-8% of all cases of childhood cancers (Miller *et al.*, 1995; Pappo *et al.*, 1995), and is the third most common extracranial solid tumour after neuroblastoma and Wilm's Tumour. RMS arises from muscle precursor cells that fail to complete the differentiation program despite the expression of muscle regulatory proteins (Dias *et al.*, 1992; Tapscott *et al.*, 1993). RMS can be divided into two main categories, embryonal (ERMS) and alveolar (ARMS). ERMS mainly occurs in young children and accounts for 60% of all RMS cases. Histologically, malignant spindle and round cells that contain skeletal muscle cross-striations are located in specific sites; such as the head/neck, genitourinary tract, and orbit. ARMS mainly occurs in adolescents as primary tumours of the extremities or trunk. Histologically, fibrovascular septa form alveolar-like spaces filled with monomorphous malignant cells (Pappo *et al.*, 1995; Pappo and Shapiro, 1997; Maurer *et al.*, 1977). Mef-2 is a transcription factor involved in skeletal muscle differentiation and activation of muscle-specific gene expression (Chen *et al.*, 2001; Molkentin *et al.*, 1995; Naidu *et al.*, 1995; Black and Olson, 1998). Mef-2 localizes to the nucleus during muscle differentiation. Trafficking of Mef-2 into the nucleus of ARMS cells is impaired and thus, the transcriptional activity of myogenic factors is impaired in ARMS cells. Moreover, Mef-2 function is significantly compromised in ARMS cells (Chen *et al.*, 2001). Mef-2 is expressed in ARMS and ERMS, but accumulates in the cytoplasm of ARMS cells. In contrast, ERMS cells accumulate large amounts of Mef-2 in the nucleus upon differentiation stimuli. Inappropriate dysfunctional trafficking of Mef-2 in ARMS cells relative to ERMS cells may correlate with the poor prognosis for individuals with ARMS (Chen *et al.*, 2001). Since PRL-3 is naturally expressed in striated muscle and

Mef-2 binding sites (identified via bioinformatics analysis of putative TFBS) were found within the CpG-64 island, it is possible that PRL-3 is also implicated in malignant muscle transformation. CRMs analysis indicates that Mef-2 has the highest probability of cooperative binding within the CpG-64 island of the PRL-3 promoter, thus PRL-3 may be involved in myogenesis as well. Furthermore, Mef-2 binding sites are only found within this CpG-64 island. Luciferase reporter assays carried out in normal and malignant muscle cell lines (Figure 10) demonstrated that the PRL-3 promoter is in general more active in the RMS cell lines than in the normal muscle cell line (C2C12), suggesting a relationship between myocytic malignant transformation and PRL-3 promoter activity. Although not statistically different (borderline), the luciferase activity induced by the CpG-64 island is higher in the RD cells (ERMS) than in the RMS13 cells (ARMS). The slight increase in the CpG-64 island promoter activity may be attributed to the fact that Mef-2 is functional and localizes more in the nucleus in ERMS cells than in ARMS cells. In addition to other trans-activating factors contributing to the CpG-64 promoter activity, Mef-2 may play an additive role in ERMS; hence, the observed increase in promoter activity of the CpG-64 island in RD (ERMS) cells compared to RMS13 (ARMS) cells.

4.1.2D   Snail

Epithelial-mesenchymal transition (EMT) is the process whereby epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. It is typically associated with the loss of E-cadherin expression (Thiery, 2002; and Nieto, 2002). In cancer, down-regulation of E-cadherin is a key step towards the invasive /

metastatic phase of carcinoma, and dominant transcriptional repression is largely responsible for the loss of E-cadherin expression (Behrens *et al.*, 1991; Birchmeier *et al.*, 1991, Hajra *et al.*, 1999; Ji *et al.*, 1997; Giroldi *et al.*, 1997). Snail is a zinc-finger transcription factor known to repress E-cadherin expression and induce EMT (Batlle *et al.*, 2000; Guaita *et al.*, 2002; Cano *et al.*, 2000). Snail is expressed in the invasive cells of tumours induced in the skin of mice (Cano *et al.*, 2000), biopsies from patients with ductal breast carcinomas (Cheng *et al.*, 2001; Blanco *et al.*, 2002), gastric cancer (Rosivatz *et al.*, 2002), and hepatocellular carcinoma (Sugimachi *et al.*, 2003). Snail appears as an early marker of the malignant phenotype and has implications as a prognostic factor (Blanco *et al.*, 2002). The E-box sequence that binds Snail is the most abundant *cis*-element within the CpG-64 island of the PRL-3 promoter. Since both PRL-3 and Snail have been implicated in cancerous metastasis and invasion, PRL-3 and Snail may have a functional relationship. Nevertheless, since Snail itself is a key regulator of EMT, it likely has many other targets, in addition to E-cadherin. I have demonstrated a possible relationship between endogenous active Snail accumulation and PRL-3 expression in invasive breast carcinoma cells (Figure 13). A mechanism whereby Snail induces PRL-3 expression would be consistent with the known actions / effects of these proteins in EMT.

Parker *et al.* (2004) identified Snail as being induced in breast tumour vasculature. Surprisingly in the same study, PRL-3 was also found to be highly induced in breast tumour vasculature (endothelium). Breast cancer is one of the most studied cancers in relation to neovascularization and has provided a paradigm for the role of angiogenesis in

cancer (Leek, 2001). There is a lack of molecular markers that allow accurate prediction of responses to specific therapies, or more precisely determine whether a tumour is likely to metastasize to distant organs (Dexter *et al.*, 1978; Klein *et al.*, 2002). Enhanced angiogenesis is associated with an increased risk of metastasis and poor prognosis in breast cancer (Linderholm *et al.*, 2000; Olewniczak *et al.*, 2002). Snail was found to be expressed in both endothelium and epithelium of invasive breast cancer (Parker *et al.*, 2004). Expression of Snail in tumour epithelium was previously reported (Fujita *et al.*, 2003; Blanco *et al.*, 2002), but its expression in breast cancer endothelium is a novel finding. PRL-3 expression was absent in normal mammary epithelium and showed little expression in invasive breast epithelium, but was highly expressed in invasive breast endothelium. This is the first suggestion of a proportional relationship between Snail and PRL-3 expression.

An inverse correlation of Snail and E-cadherin expression has been reported in many types of human cancers, including squamous cell carcinoma. Yokoyama *et al.* (2003) compared three E-cadherin-negative squamous cell carcinoma cell lines with strong Snail expression to three other squamous cell carcinoma cell lines that express E-cadherin with weak Snail expression, and found that the cell lines with strong Snail expression were more invasive and had a higher ability to express matrix metalloproteinase-2 (MMP-2). MMPs are a group of matrix degrading enzymes which are highly expressed in invasive cancer cells. Luciferase reporter assays revealed that Snail induces MMP-2 promoter activity (Yokoyama *et al.*, 2003). In addition, the MMP family is known to play a key role in the invasion of various human cancers including hepatocellular carcinoma

(Nebeshima *et al.*, 2002). Reverse-Transcriptase PCR showed that MMP-1, -2, and -7 expressions were strongly upregulated by Snail (Miyoshi *et al.*, 2004). The downregulation of E-cadherin and upregulation of MMPs by Snail further facilitates the EMT process. However, the precise mechanism of how Snail promotes cancer invasion still remains unknown.

Prostate cancer is the most common malignancy detected in men in Western countries (Gronberg, 2003; Amanatullah *et al.*, 2000). The progression from androgen-dependence to independence (which occurs in patients treated with endocrine therapies) is associated with a higher invasive potential and malignant phenotype (Bonaccorsi *et al.*, 2000; Cinar *et al.*, 2001). This can be explained, at least in part, by the loss of androgen control of genes involved in limiting invasion (Baldi *et al.*, 2003). Recent evidence indicates that androgen-sensitive prostate cancer cells have a less malignant phenotype characterized by reduced migration and invasion (Bonaccorsi *et al.*, 2004). Moreover, upon EGF treatment, androgen-independent prostate cancer cells become more migratory (Bonaccorsi *et al.*, 2004). However, transfection of the androgen receptor (AR) cDNA into invasive androgen-independent cells confers a less malignant phenotype by interfering with EGFR autophosphorylation and signaling that leads to invasion in response to EGF (Bonaccorsi *et al.*, 2004). Thus, androgens may disrupt EGF-mediated signaling pathways that lead to invasive properties. Interestingly, prominent Snail mRNA expression was evident after EGF treatment of DU145 cells, the highly invasive androgen-independent metastatic prostate cancer cell line (Lu *et al.*, 2003). As shown in Figure 9, it is evident that the usually highly active CpG-64 island has minimal activity in

the androgen-dependent LNCaP cells, yet exhibits very high promoter activity in the androgen-independent DU145 cells. If EGF stimulation does increase Snail expression, and if Snail is involved in the positive transcriptional upregulation of PRL-3, then PRL-3 expression may be a downstream effect of EGF signaling. Although this proposed mechanism has not yet been experimentally validated in this study, it leaves room for further work. Figure 9 indicates that the CpG-64 island induces higher luciferase activity in the cell line without AR (DU145) than in the cell line with AR (LNCaP). This suggests that the androgen receptor may be disrupting signaling pathways leading to PRL-3 transcriptional activity. This inverse relationship between AR and PRL-3 is consistent with the proposed model as the loss of AR and the over-expression of PRL-3 has been both attributed to increased invasiveness, migration, and metastatic capacity.

Saha *et al.* (2001) were the first to characterize PRL-3 as being an important component of colorectal cancer metastasis. Although we have shown that increased PRL-3 promoter activity is evident in metastatic and invasive cell lines, the molecular actions of PRL-3 that can induce metastasis and invasion are still not clear. Although many gene changes have been attributed to cancer development, very few have been associated with metastasis. PRL-3 is potentially a valuable marker to predict or detect metastasis and / or aggressive cancers.

4.2   POOR PROGNOSIS AND FUNCTIONAL LOCALIZATION OF PRL-3

PRL-3 expression is observed in clinical colorectal, uveal melanic, breast, and certain

pediatric tumour samples. The localization of the PRL-3 IHC staining pattern is

associated with the differentiation status of the cells. More specifically, PRL-3 tends to

be localized in the cell nuclei of poorly differentiated pediatric tumours and localized in

the cell cytoplasm of well differentiated pediatric tumours (neuroblastoma and

rhabdomyosarcoma). In addition, high levels of PRL-3 in patient tumour samples are

correlated with an unfavorable prognosis, as seen in uveal melanoma and breast

carcinoma. These findings demonstrate the clinical significance of PRL-3 as a tumour

aggressiveness / metastasis marker. However, further clinical studies are still needed to

confirm the vital role of PRL-3 in the metastatic process. Once the clinical role of PRL-3

has been validated, therapeutic development and testing can take place and in so doing,

patients may then have a chance of reducing the aggressiveness of their disease and the

risk of relapse.

4.2.1   Colorectal Carcinoma

To confirm previous observations of PRL-3 over-expression in colorectal metastatic

samples and as a proof-of-principle experiment testing the utility of the PRL-3 antibody

in detecting endogenous PRL-3 in tumour sections, a tissue microarray (TMA) containing

normal, primary, and matching metastatic tissues was purchased from Imgenex. Our

findings were consistent with previous observations, confirming that PRL-3 is indeed over-expressed in colorectal metastases, and validating our method of investigation.

It is interesting to note that the IHC staining of PRL-3 was only detected in the cell cytoplasm from our investigation of the colorectal cancer TMA. Virtually no nuclear IHC PRL-3 staining was observed. Similarly, the IHC data from others (Saha et al., 2001; Bardelli et al., 2003; Peng et al., 2004; Kato et al., 2004) also showed that PRL-3 staining was predominantly cytoplasmic in the colorectal cancer samples analyzed. Thus, virtually all IHC staining done by myself and others demonstrates predominant cytoplasmic localization of PRL-3. The site of action of PRL-3 therefore appears to be in the cytoplasm, more specifically, at the plasma membrane, at least with respect to its role in tumour progression in metastatic colorectal cancer.

4.2.2   Uveal Melanoma

Isochromosome 8q was associated with a poor prognosis for uveal melanoma patients due to death by metastatic disease (White et al., 1998). Moreover, the gain of 8q was significantly associated with poor overall survival (Aalto et al, 2001). The PRL-3 gene is situated on chromosome 8q24.3, and PRL-3 over-expression has been associated with metastatic disease (Saha et al., 2001; Bardelli et al., 2003; Peng et al., 2004; Kato et al., 2004; Miskad et al., 2004; Wu et al., 2004). PRL-3 expression in uveal melanoma was explored in order to elucidate the potential of PRL-3 as a prognostic marker for uveal melanoma.

A TMA containing 184 cases was immunohistochemically stained for PRL-3. Weak

PRL-3 expression was detected in majority (~60%) of cases, and only ~13% had strong

staining. Notably, the localization of PRL-3 staining was observed to vary from

cytoplasmic to nuclear. Prenylation status determines PRL-3 localization, with the

prenylated phosphatase localized at the plasma membrane and the unprenylated PRL-3

found in the nucleus (Zeng *et al.*, 2000). This has led to the suggestion that PRL-3

substrates and / or function may be localization-dependent. What these are, and how they

relate to tumour progression and metastasis, are still unknown. A specific correlation

between PRL-3 localization and various histological parameters in this TMA has not yet

been determined. However, although not statistically significant, a prognostic trend

between PRL-3 nuclear expression and clinical outcome is apparent. Cases with no or

weak nuclear PRL-3 IHC staining were grouped as 'negative' and cases with strong

nuclear PRL-3 IHC staining were termed 'positive'. As seen in the Kaplan-Meier

survival curve for uveal melanoma (Figure 19), patients with positively staining samples

have a poorer survival outcome than patients with negative staining samples. This

reinforces the clinical ramifications of PRL-3 as being a key component of an aggressive

malignant phenotype and as a prognostic marker. To determine whether the subcellular

localization of PRL-3 was linked to prognostic outcome, a survival curve was plotted for

cytoplasmic PRL-3 staining (data not shown). However, this revealed less statistical

difference between the negative and positive cytoplasmic staining samples, although the

survival linkage between positive PRL-3 expression and poor outcome remains the same.

This suggests that PRL-3 may also have a nuclear role in the progression of certain melanomas.

PRL-3 expression has been implicated in mouse B16 melanoma invasiveness and metastatic potential (Wu et al., 2004). Heterologous expression of PRL-3 in melanoma cells with low invasive properties resulted in altered extracellular matrix adhesion and up-regulated integrin-mediated cell spreading efficiency (Wu et al., 2004). This study also confirmed that PRL-3 facilitated lung and liver metastasis of the melanoma cells with intrinsically low metastatic ability in an experimental metastasis model in mice, consistent with the observed accelerated proliferation (MTT assay) and growth rate of the cells both in vitro and in vivo. Since PRL-3 is involved in metastatic progression in mouse melanoma models, and is implicated as a prognostic indicator of poor clinical survival, then PRL-3 may also be involved in melanoma progression as well.

4.2.3   Breast Carcinoma

There have been a number of publications recently regarding the role of the Snail family of zinc-finger transcription factors in tumourigenesis. The relationship between Snail and EMT is clear, and Snail promotes the invasive capacity of the epithelium in breast cancer (Parker et al., 2004). The transition from epithelial to mesenchymal cells promotes enhanced cellular migratory and invasive properties; hence such a transition may contribute to tumour progression. Since Snail has been shown to be over-expressed in invasive breast epithelium and endothelium (Fujita et al., 2003; Blanco et al., 2002;

Parker *et al.*, 2004), and PRL-3 expression increased under conditions that permitted accumulation of Snail (Figure 13), the expression of PRL-3 in breast cancer was explored to elucidate the potential of PRL-3 as a prognostic marker for breast cancer.

The staining localization of PRL-3 was again observed to be quite variable in the 495 cases of breast carcinoma examined. Although the majority of the breast carcinoma cases on the TMA scored positive for nuclear PRL-3 expression, a significant portion exhibited strictly or concomitant cytoplasmic PRL-3 staining. Others have reported that PRL-3 is not typically expressed in normal breast epithelial tissue, but when it is expressed, PRL-3 mainly resides in the nucleus (Miskad *et al.*, 2004). The significance of PRL-3 localization in breast cancer has yet to be determined. Of the 495 breast carcinoma cases examined, over 50% of the tissues had undetectable cytoplasmic PRL-3 expression. About a quarter of the cases had weak cytoplasmic PRL-3 expression, and only a fraction (<3%) of the cases displayed strong cytoplasmic PRL-3 staining. Interestingly however, the patient samples that expressed high levels of cytoplasmic PRL-3 had a noticeable and statistically significant reduction in survival outcome (Figure 21). Patients with no or low cytoplasmic PRL-3 expression tended to do better than those with high PRL-3 expression. This further exemplifies the clinical relevance of PRL-3 as a biomarker for cancerous progression and as a prognostic indicator. Since PRL-3 is not naturally found in breast epithelia, the presence of PRL-3 in breast cancer may also indicate that PRL-3 could be involved in tumourigenesis and / or progression of breast carcinoma.

By the same token, nuclear PRL-3 expression was also evaluated relative to patient survival. The majority of the cases on this breast cancer TMA had strong nuclear PRL-3 staining (Table 8). However, when survival outcomes of patients with or without nuclear PRL-3 expression were compared, there were no differences between the groups (Figure 22). This may suggest that nuclear PRL-3 has no role in disease progression in breast cancer, and that cytoplasmic (prenylated) PRL-3 does have an affect on the clinical picture of breast cancer patients.

Her-2 is a well known receptor tyrosine kinase that is activated by a paracrine secretion mechanism and has a role in both early proliferative and late invasive / metastatic changes in breast cancer. Over-expression of the Her-2 receptor has been associated with poor prognosis (Winters *et al.*, 2003) in breast cancer. Heterodimerization of Her-2 with other members of its family of receptor kinases have been shown to either activate the MAP-kinase or PI3-kinase pathways (Nahta *et al.*, 2004). Her-2 heterodimers are very stable, and hence can stimulate prolonged signaling. Like Her-2, prenylated PRL-3 is localized at the plasma membrane and can activate various growth, angiogenic, anti-apoptotic, or migratory signaling pathways. Nonetheless, cytoplasmic PRL-3 is associated with poor clinical survival for breast cancer patients, and signifies a functional localization of PRL-3.

4.2.4 Pediatric Tumours: Neuroblastoma and Rhabdomyosarcoma

The functional localization of the PRLs is an issue of interest and not much is known of their possibly regulatable localization and cytoplasmic versus nuclear substrates and functions. However, PRL-1 has been found to be localized in the nucleus during proliferation of liver cells and during differentiation of intestinal cells (Diamond *et al.*, 1996; Kong *et al.*, 2000).

In general, poorly differentiated neuroblastomas and rhabdomyosarcomas are more prognostically unfavorable than the well differentiated tumours. IHC staining of PRL-3 in neuroblastoma and rhabdomyosarcoma tissues revealed that the less differentiated tumours have predominantly nuclear PRL-3, whereas the well differentiated tumours display mainly cytoplasmic PRL-3 (Figure 23). Although the functional localization of PRL-3 in pediatric tumours has yet to be determined, a relationship between tissue differentiation and PRL-3 localization can be inferred. It is not clear whether PRL-3 expression / localization causes differentiation or if differentiation causes specific PRL-3 expression / localization, but PRL-3 localization is associated with differentiation status of the tissue. A statistical correlation between PRL-3 IHC localization and tissue differentiation is seen in neuroblastoma (Table 11), but not in rhabdomyosarcoma (Table 12). More clinical samples need to be analyzed before verifying whether the possible trend in the observations is due to chance or a real trend.

It is also interesting to note that PRL-3 and Mef-2 localization appears to have an inverse correlation. Mef-2 accumulates in the nucleus of well differentiated (embryonal) RMS and in the cytoplasm of poorly differentiated (alveolar) RMS (Chen *et al.*, 2001). Conversely, PRL-3 staining is localized in the cytoplasm of well differentiated (embryonal) RMS and in the nucleus of poorly differentiated (alveolar) RMS. This is quite fascinating and proposes a potential role PRL-3 may have on cellular differentiation. However, a specific relationship between PRL-3 and Mef-2 localization has not been determined.

CHAPTER 5

SUMMARY

5.1   CONCLUSIONS

Metastasis is the leading cause of death in cancer patients and involves a complex,

multistep process including detachment of tumour cells from the primary cancer, invasion

of surrounding tissue, entry into the circulatory system, reinvasion, and proliferation at a

distant secondary site (Boyd, 1996; Bashyam, 2002). The process of metastasis is highly

complex, and is the result of accumulating genetic mutations that cause changes in

different places and times, rather than just the alteration of a single gene. PRL-3 may act

as a key player to initiate and maintain tumour cell growth in the distant organs by

transforming the tumour cell characteristics and subsequently enhancing their metastatic

ability.

Here, I demonstrated that the PRL-3 promoter has differential transcriptional activities in

various cell lines exhibiting assorted levels of invasiveness, metastatic state, malignancy,

and cellular differentiation. More specifically, reporter assays confirm that the CpG-64

island is an important region for PRL-3 transcriptional regulation. The CpG-64 island is

more active in colorectal metastatic cells than in primary tumour cells, more active in

invasive prostate androgen-independent tumour cells than in androgen-dependent tumour

cells, and more active in malignant muscle cells than in normal muscle cells.

Bioinformatics analyses revealed that this CpG-64 island has multiple cancer-associated *cis*-elements, high orthologue conservation, potential transcript generation, detectable regulatory potential, and high *cis*-regulatory module binding probability. Other regions in the PRL-3 promoter have some, but not all, of these above characteristics. Together, our findings suggest that the CpG-64 island is a candidate alternative promoter region that is differentially utilized in various stages of cancerous development.

Investigating PRL-3 expression in clinical tumour samples, I confirmed that PRL-3 expression is observed in colorectal tumours, and discovered that it is also expressed in uveal melanic, breast, and certain pediatric tumours. The localization of the PRL-3 IHC staining pattern is associated with the differentiation status of the cells in some tumour types. PRL-3 tended to have a nuclear localization in poorly differentiated pediatric tumours and was localized in the cytoplasm of well differentiated pediatric tumours (neuroblastoma and rhabdomyosarcoma). Furthermore, high levels of PRL-3 in clinical tumour samples were correlated with an unfavorable prognosis, as seen in uveal melanoma and significantly so in breast carcinoma. These findings demonstrate the clinical relevance of PRL-3 in screening, monitoring, and prognosis of cancer patients. Furthermore, they suggest that PRL-3 could be a valid therapeutic target. Knowledge of the substrates and functional localization of PRL-3 will be invaluable to the development of PRL-3 directed inhibitors for anti-cancer treatment.

5.2 FUTURE DIRECTIONS

With the remaining time left in the lab (prior to September 2005), I propose to carry out the following investigations. Since specific growth factors have been shown to exacerbate cancer progression, I intend to analyze the PRL-3 promoter plasmids in selected cancer cell lines under growth factor stimulation. IGF-1 stimulation has been shown to cause plasma membrane ripples, motile actin microspike formation, and cell separation in non-motile lowly invasive breast cancer MCF-7 cells (Guvakova *et al.*, 2002). Thus, IGF-1 stimulation of MCF-7 cells may stimulate, through specific PRL-3 promoter regions, the upregulated expression of PRL-3 that plays a role in enhanced tumour cell invasive properties. On another note, EGF stimulation of DU145 cells has been shown to upregulate Snail expression. EGF stimulation of DU145 cells could provide cellular conditions that allow testing of whether Snail causes a positive transcriptional upregulation of PRL-3. Moreover, IGF-1 has been shown to cause *n-myc* induction (Misawa *et al.*, 2000) and increased motility (Meyer *et al.*, 2001) in neuroblastoma cell lines. Thus, IGF-1 stimulation of San-2 cells (neuroblastoma cell line with no *n-myc* gene amplification) may provide appropriate conditions to determine if a specific region of the PRL-3 promoter is responsible for increased PRL-3 expression that may be linked to neuroblastoma cell motility, and if *n-myc* is responsible for this increased transcriptional activity. IGF-1 has also been shown to induce differentiation in primitive muscle cells (Xu and Wu, 2000). Therefore, IGF-1 stimulation in normal (C2C12) and malignant (RD and RMS13) muscle cells may shed light on the role of PRL-3 in muscle differentiation.

To determine whether the CpG-64 island is an authentic promoter of PRL-3, I will use the Rapid Amplification of cDNA Ends (5'-RACE) method. 5'-RACE determines the coding sequence at the 5'-end of the transcript; hence, it identifies the first transcribed exon. On a different note, all PRL-3 promoter regions investigated using reporter assays, in this study, contain non-methylated cytosines. Since cytosine-methylation is a prominent epigenetic transcriptional regulatory process, and genomic cytosines may or may not be methylated in the PRL-3 promoter, I intend to investigate whether methylation is responsible for PRL-3 transcriptional regulation in tumourigenesis and metastasis by investigating the methylation status of genomic DNA from various cancer cell lines.

In recent years, upregulated PRL-3 expression has been correlated with metastasis and poor clinical outcome of several adult cancers (Saha *et al.*, 2001; Bardelli *et al.*, 2003; Peng *et al.*, 2004; Kato *et al.*, 2004; Miskad *et al.*, 2004; Wu *et al.*, 2004). No similar studies have been conducted to determine if PRL-3 expression is a marker of similar clinical trends in pediatric malignancies. I will construct a 168 case pediatric tumour tissue microarray consisting of the most common types of pediatric malignancies with, where available, matching metastases. The sections from this array will be IHC stained for PRL-3 to evaluate PRL-3 protein levels and localization. These sections will also be probed for PRL-3 mRNA levels using Chromagenic *In-Situ* Hybridization. The IHC and Chromagenic *In-Situ* Hybridization data collected on this array for PRL-3 will subsequently be correlated with clinical outcome in pediatric cases to infer prognostication.

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