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UNIVERSITY OF CALIFORNIA
RIVERSIDE

Regulation of BRCA1/p21 Axis by Prolactin

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Kuan-Hui Chen

December 2011

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ABSTRACT OF THE DISSERTATION

Regulation of BRCA1/p21 Axis by Prolactin

By

Kuan-Hui Chen

Doctor of Philosophy, Graduate Program in
Biomedical Sciences
University of California, Riverside, December 2011
Dr. Ameae Walker, Chairperson

Tumor formation/progression is determined by a wide array of factors including

downregulation of tumor suppressors and upregulation of oncogenes. In breast

cancers, mutation of the tumor suppressor, breast cancer 1 (BRCA1), accounts for the

majority of inherited cases, which themselves account for about 10% of the total.

Given the importance of BRCA1 in the development of breast cancer and the fact that

the majority of breast cancers have wild type BRCA1, the question posed in the

current study was what happens to BRCA1 tumor suppressor activity during the

development of most breast cancers. To examine this issue, we employed two forms

of prolactin that work through the same receptors, differ from one another only by a

single amino acid, and have antagonistic activity in terms of cell proliferation and

survival. Also, since BRCA1 mutations have recently been shown to be important in

ovarian and prostate cancer development, the study additionally included cell lines

representative of these cancers. Results show that wild type BRCA1 could be

functionally silenced by prolactin in terms of its ability to induce transcription of the

cell cycle inhibitor, p21, whereas the prolactin antagonist increased expression of p21

through BRCA1. The functional silencing in response to prolactin occurred as a result

of an interaction with the signaling molecule, Stat5 and could be blocked by a

dominant negative Stat5. In addition, we showed that prolactin also contributes to

tumor progression by regulating p21 at post-transcriptional and post-translational

stages. The post-transcriptional regulation was mediated by miRNA. One candidate

for this activity is miR-106, a miRNA induced by PRL, likely through an estrogen

receptor α (ERα) and c-myc pathway. Prolactin also induced phosphorylation of p21

on threonine 145, causing it to be retained in the cytosol under which circumstances it

inhibits apoptosis. At present, the kinase responsible for prolactin-stimulated

phosphorylation of p21 is unclear, but preliminary data suggest a possible role for

pim-1.

The prolactin antagonist employed in these studies is a molecular mimic of

naturally phosphorylated prolactin. Given its ability to increase expression of p21 and

antagonize the effects of unmodified prolactin, one would predict that the ratio of

unmodified prolactin to phosphorylated prolactin may be important to the

development/progression of cancer. To test this in a clinical setting, a serum-based

assay capable of quantifying phosphorylated prolactin as a separate entity from

unmodified prolactin is required. To develop such an assay, regions of the p21

promoter were deleted to determine a minimal promoter responsive to phosphorylated

prolactin. A 143bp region containing only the BRCA1 response element and TATA

box was determined to be sufficient and specific to the phosphorylated form of the

hormone. Establishment of stable cell lines expressing this minimal promoter linked

to luciferase has shown the resultant bioassay to be sensitive and consistent.

Table of Contents

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 1

General Introduction

<u>Prolactin and the phospho-prolactin mimic, S179DPRL</u>

Prolactin is a peptide hormone produced and released from the pituitary gland (Miyai et al., 2005). It has a wide variety of biological activities, but is best known for its ability to regulate lactation (Cowie et al., 1969). During pregnancy, elevated prolactin in the circulation promotes mammary gland growth and differentiation. Once progesterone levels drop at parturition, prolactin stimulation leads to milk production (Freeman et al., 2000). These activities rely on an interaction between prolactin and multiple prolactin receptors, which in turn activate different signaling pathways leading to different end effects. A number of prolactin receptor isoforms exist (Binart et al., 2010), but of the four major forms in human normal and cancerous tissues (Harris et al., 2004), the two most important forms in terms of growth regulation are the full length prolactin receptor, designated as long form prolactin receptor, and the short form 1b prolactin receptor (Tan and Walker, 2010). This latter form has lost a major portion of the cytoplasmic region on the receptor, but also has a unique region at the very C-terminus (Hu et al., 2001). Upon prolactin binding, the prolactin receptors dimerize. From our earlier studies using bioluminescence

resonance energy transfer, it has been shown that prolactin interacts with both homo- and hetero-dimers of these receptors (Tan et al., 2005). However, the functions of these two receptors differ: the long form prolactin receptor contributes to cell proliferation and the short form 1b prolactin receptor inhibits cell proliferation and promotes apoptosis (Huang and Walker, 2010; Van Coppenolle et al., 2004). In prostate cancer cells (PC3 cells), stable increased expression of the short form 1b prolactin receptor significantly decreased growth and migration/invasion (Huang and Walker, 2010). In breast cancer cells, expression of the short form1b prolactin receptor also downregulated expression of the long isoform (Tan and Walker, 2010).

With its function to promote cell growth and differentiation, a role for prolactin in the development and progression of various cancers has been broadly discussed. In breast cancers, there are several studies showing a correlation between the level of prolactin and the risk of breast cancer (Reynolds et al., 1997). In addition, it was reported in Holtkamp's study that ~44% of patients with metastatic breast disease were hyperprolactinemic (Holtkamp et al., 1984). Other than the pituitary source of prolactin, mammary cells can also synthesize prolactin. It is estimated that ~98% of breast cancers synthesize prolactin and use that prolactin in an autocrine growth loop (Reynolds et al., 1997). MCF-7 cells with an engineered prolactin production

deficiency showed a greater response to exogenous prolactin, and prolactin also

caused a decrease in the cell cycle inhibitor, p21 in this cell line (Carver and Schuler,

2008). Both the pituitary source and autocrine prolactin contribute to the growth of

breast tumors (Manhes et al., 2005; Welsch et al., 1975). Moreover, prolactin induces

expression of the estrogen receptor and also leads to an activation of the estrogen

receptor in a ligand independent manner (Chen et al., 2010).The synergy between

prolactin and estrogen in breast cancer leads to a greater effect on proliferation (Chen

et al., 2010). In addition to cooperating with estrogen, prolactin is also reported to

synergize with epidermal growth factor (EGF) to facilitate breast tumor cell migration

(Maus et al., 1999). The increased motility of breast cancer cells might be mediated

by Nek3 kinase overexpression, which leads to cytoskeletal reorganization, or

Sphingosine kinases which promote cell migration (Döll et al., 2007; Miller et al.,

2007). Furthermore, prolactin also causes phosphorylation of EGFR and ErbB2,

thereby enhancing EGF signaling (Huang et al., 2006).

There is increasing evidence supporting a role for prolactin in promotion of

prostate cancer progression. It was reported that prolactin can increase proliferation

and survival and prevent apoptosis in prostate cancers (Crépin et al., 2007; Ruffion et

al., 2003; Thomas et al., 2011b). In addition, autocrine prolactin is expressed in 54%

of hormone-refractory prostate cancers and 62% of prostate cancer metastases (Dagvadorj et al., 2007) and the level of prolactin goes abnormally high in metastatic prostate cancers (Lissoni et al., 2005). Furthermore, prolactin activated Stat5 signaling is correlated with the histological grade of prostate tumors (Li et al., 2004). Moreover, the activated Stat5 has a synergistic effect with testosterone by enhancing nuclear localization of the androgen receptor (Tan et al., 2008). Prostate cancer cells with her-2 overexpression are highly associated with rapid tumor progression and poor prognosis. Since prolactin activates her-2 in breast cancers (Minner et al., 2010), it may play a similar and important role in prostate disease also.

In ovarian cancers, little has been done so far. It is found that women with a family history of ovarian tumors have a higher level of serum prolactin (Levina et al., 2009). The expression of prolactin receptor is also higher in malignant ovarian tumors (Levina et al., 2009). In addition, ovarian tumor cells produce autocrine prolactin and prolactin treatment does increase proliferation of ovarian cancer cells and prevents apoptosis of ovarian cancer cells in response to stress such as starvation or drug treatment (Asai-Sato et al., 2005; Tan et al., 2011). It is also interesting that prolactin activates the Ras oncogene in ovarian cancers indicating a possible role of prolactin in the malignant transformation process that turns normal ovarian cells to immortal ones

with long term exposure to high levels of prolactin (Asai-Sato et al., 2005; Levina et al., 2009).

As mentioned earlier, when prolactin is produced and secreted from the anterior pituitary gland, it is modified in several ways such as glycosylation and phosphorylation (Markoff et al., 1988; Tuazon et al., 2002). Previous studies showed the phosphorylated prolactin inhibits proliferation and also antagonizes the growth-promoting effect of unmodified prolactin (Coss et al., 1999; Schroeder et al., 2003). The phosphorylation of prolactin is mediated by p21-activated kinase in the pituitary (Tuazon et al., 2002). We therefore made a molecule that mimics naturally phosphorylated prolactin by substituting the normally phosphorylated serine residue at position 179 with an aspartate, thereby producing S179D prolactin (S179DPRL). Our earlier studies with S179DPRL showed that it interacted with the prolactin receptor just as the unmodified prolactin did. Unlike unmodified prolactin that activates Jak2/Stat5 signaling when interacting with the prolactin receptor, S179DPRL does not cause an activation of Jak2/Stat5 signaling. Furthermore, when cells were exposed to both unmodified prolactin and S179DPRL simultaneously, the growth promoting cascade induced by prolactin such as Stat5 activation, and cyclinD1 expression was reduced. S179DPRL has such an effect at about one tenth the

concentration of unmodified PRL (Schroeder et al., 2003). In addition to an inhibitory growth effect, S179DPRL also inhibits tumor cell migration and angiogenesis in several tumors in which prolactin promotes these processes. Therefore, S179DPRL functions in certain way to be as a prolactin antagonist.

Stat 5 (Signal Transducer and Activator of Transcription)

Stat5, earlier known as mammary gland factor, was first discovered in lactating sheep and was reported as an effector of a prolactin stimulus (Schmitt-Ney et al., 1992). It is now known that at least some aspects of both prolactin mediated growth and differentiation rely on the activation of Stat5 signaling (Kazansky et al., 1995). For example, the growth promoting effect of prolactin is mediated partly through upregulation of the cell cycle component, cyclin D, which is transcriptionally regulated by activation of Stat5 (Brockman et al., 2002). In addition, an indicator of mammary gland differentiation, the milk protein β-casein, is also positively regulated by Stat5 signaling. The regulation by Stat5 is through an interaction with a consensus sequence, named interferon gamma activation sequence (GAS) (TTCN2–4GAA) in the promoter region of target genes (Luo and Yu-Lee, 1997).

The growth promoting effect of Stat5 has lead to an investigation of its role in cancer progression. In colon cancers, inhibition of DNA methyltransferase inactivated Jak2/ Stat5 signaling and induced cell cycle arrest through upregulation of p21 (Xiong et al., 2009). In addition, norcantharidin, a commercially available medicine, inhibited breast cancer progression by inducing apoptosis through upregulating p21, which was also an effect of inactivation of MAPK and Jak2/Stat5 signaling (Yang et al., 2011). Furthermore, the evidence from furano-1,2-naphthoquinone treated lung cancer cells, A549 cells, showed the same result. i.e. that upregulation of p21 and increasing apoptosis and cell cycle arrest were accompanied by inactivation of Stat5 signaling (Su et al., 2010). From an in vivo mouse model, mice with hemizygous loss of Stat5 alleles remained normal throughout their entire development, but fewer of them developed tumors and even when they did the tumor size was smaller than those with both copies (Ren et al., 2002). In addition, caveolin-1, a tumor suppressor, expressed abundantly in adipocyte and mammary fat pads, functions in an opposite fashion to Stat5 signaling. Overexpression of caveolin-1 can antagonize cyclin D1 in mammary development and caveolin-1 -/- mice have hyperactivation of Stat5 (Park et al., 2002). Further evidence was produced by Cotarla et al. who analyzed the distribution of Stat5 and its activation status in invasive breast adenocarcinomas. It was found that

Stat5 was constitutively activated and nuclear localized in ~76% of invasive breast adenocarcinomas. Furthermore, the nuclear localized Stat5 was associated with nuclear localized p27, but not p21 (Cotarla et al., 2004). Interestingly, the synergistic interaction between Stat5 and the estrogen receptor further implies an important regulation by Stat5 in estrogen receptor positive breast tumors.

Other than breast tumors, Stat5 also plays essential roles in prostate tumors. It is estimated that 65 ~ 95% (depends on different studies) of prostate tumors showed hyper and constitutive activation of Stat5 and increased activation of Stat5 was correlated with higher histological grade (Li et al., 2004). In vitro studies showed that autocrine prolactin-mediated Stat5 activation supported the proliferation of prostate cancers and inhibition of Stat5 signaling induced cell death (Ahonen et al., 2003; Dagvadorj et al., 2007). In addition, in vivo studies also supported the same conclusion that Stat5 knockdown in mice delayed prostate tumors (Thomas et al., 2011a). Furthermore, results from gene expression profile analysis showed that 21% of Stat5-targeted genes were involved in metastasis of prostate tumors and Stat5 activation induced an 11 fold metastatic rate of prostate tumors in nude mice (Gu et al., 2010). The interaction between active Stat5 and the androgen receptor was also investigated. It is known now that active Stat5 can interact with the androgen receptor

and this interaction enhances the nuclear localization of each and therefore promotes

downstream gene expression (Tan et al., 2008). On the other hand, loss of Stat5 by

knockdown leads to increased degradation of the androgen receptor (Thomas et al.,

2011a).

As for Stat5 in ovarian cancer, little is known so far. It was reported that

activation of Stat5 in ovarian cancers was significantly correlated with the expression

of VEGF and VEGFR1/2 (Chen et al., 2004). Also, in drug resistant ovarian tumor

cells, the expression of Stat5 was upregulated (Jinawath et al., 2010).

BRCA1

BRCA1, a ~220 kDa protein, is an important tumor suppressor in several types

of tumors, including breast, prostate, ovarian, and fallopian tube tumors (Agalliu et al.,

2009; De Leeneer et al., 2011; Gallagher et al., 2010; Levine et al., 2003). Genetic

analysis revealed that women with BRCA1 mutations have a higher incidence of

breast and ovarian tumors (Meindl et al., 2011). In addition, BRCA1 conditional

knockout mice had abnormal mammary ductal morphogenesis and the development of

mammary tumors (Brodie et al., 2001). Furthermore, tumors with mutations in

BRCA1 tend to be more basal like breast tumors, best described as triple negative

breast tumors (ER, EGFR, and PR negative). At the C terminus of BRCA1 is the

BRCT domain (see figure 1.1) which interacts with several phosphoproteins involved

in DNA repair as well as the histone deacetylase complex. It is therefore primarily

responsible for the damage response required to maintain genome stability (Drikos et

al., 2009; Kim et al., 2007; Rodriguez and Songyang, 2008). Closer to the N-terminus

of BRCA1 is a RING finger domain with a function similar to ubiquitin ligase E3

where BARD1 binds and forms a BRCA1-BARD1 ubiquitin complex that mediates

ubiquitination (Wu et al., 2008). Several proteins have been reported to be targets of

ubiquitination led by BRCA1, such as the activated RNA polymerase II, gamma

tubulin, and ER alpha (Heine and Parvin, 2007; Ma et al., 2010; Starita et al., 2005;

Starita et al., 2004). BRCA1 can also function as a transcription activator to positively

regulate expression of genes such as p21 and Gadd45, a DNA damage responsive

gene, or as a transcription repressor to negatively regulate expression of proteins, such

as angiopoietin-1, which is an angiogenesis promoter (Fabbro and Henderson, 2008;

Fan et al., 2002; Furuta et al., 2006; Somasundaram et al., 1997). With its tumor

suppressor function, the expression level of BRCA1 and how it is regulated has been

investigated. It is known that the expression level of BRCA1 is reduced in several

cancers and is believed to be a result of epigenetic modification of BRCA1 genes (Esteller et al., 2000; Miyamoto et al., 2002). It has been reported that stressors like hypoxia can lead to epigenetic modification of the BRCA1 promoter and lower BRCA1 expression (Lu et al., 2011). In addition, transcription factor CtBP-1(Carboxyl-terminal binding protein 1) is recruited to the promoter of BRCA1 and represses its transcription in breast cancers. BRCA1 loss occurred with nuclear localized CtBP-1 and knockdown of CtBP-1 restored the expression of BRCA1 in breast cancers (Deng et al., 2011). Other transcription factors such as NRF-1 containing complex are also linked to reduced expression of BRCA1 in breast cancer (Thompson et al., 2011). Interestingly, the oncogene c-myc was also found to interact with the BRCA1 promoter and increased its expression as a result of feedback regulation (Chen et al., 2011). BRCA1 is also reported to be regulated by miRNAs. miR-335 overexpression promotes expression of active regulators of BRCA1 including sp1, ER α, and IGFR-1 and reduces expression of the repressive regulator ID4, leading to an upregulation of BRCA1 (Heyn et al., 2011). By contrast, miR-146, which is highly expressed in basal like breast tumors was found to interact with the 3' untranslated region of BRCA1 and repressed its expression (Garcia et al., 2011). In addition to miR-146, miR-182 was also reported to downregulate BRCA1 (Moskwa et

al., 2011). Others such as those in miR-17 family, which bind to the 3' untranslated region of BRCA1 in vitro, may all contribute to decreased expression of BRCA1 (Shen et al., 2009).

p21(CDKN1A)

p21, also known as CDKN1A, is a cell cycle inhibitor acting between G1 and S phase. The regulation of cell cycle progression by p21 is through interaction with the Cyclin-CDK complex, inhibiting its function and thereby blocking the cell cycle. The expression of p21 is regulated by multiple molecules and pathways. In response to DNA damage, upregulated p53 transcriptionally drives the expression of p21 (el-Deiry et al., 1994). In addition to p53, another tumor suppressor, BRCA1, also transactivates p21 in both a p53 dependent and independent manner (Somasundaram et al., 1997). Furthermore, other factors such as TGF-β, RAR/RXR and the vitamin D receptor are all reported to regulate its expression (Datto et al., 1995; Freedman, 1999; Liu et al., 1996). Interestingly, a recent study demonstrated that the expression of p21 is negatively regulated by the small RNA, miR-106a which is best known for its oncogenic characteristics (Li et al., 2011; Thapa et al., 2011). In addition to the

expression of p21, it is reported that the function of p21 depends on its cellular distribution. p21 in the nucleus negatively regulates the cell cycle as mentioned above. This may lead to apoptosis (Li et al., 1996). However, cytosolic p21 forms a complex with ASK (apoptosis signal regulating kinase) and procaspase-3, thereby inhibiting the process of apoptosis (Asada et al., 1999). The cellular distribution of p21 is determined by a single phosphorylation on threonine 145 which leads to cytosolic accumulation of p21 when phosphorylated. Kinases that act on this threonine residue include PKA, PKB(Akt) and Pim-1 (Gapter et al., 2006; Scott et al., 2000; Zhou et al., 2001).

How prolactin regulates the expression of p21 is not completely known yet and it may be somewhat tissue specific. In β cells, increased prolactin during pregnancy leads to an upregulation of p21, however, this is contradictory to other studies in tumors (Hughes and Huang, 2011). As described earlier, Stat5 signaling activation is mostly accompanied by a reduction of p21 and vice versa. Although the regulation of p21 by prolactin might be controversial, there is a correlation between Stat5a signaling and p21 cellular distribution (Santos et al., 2010). An interesting experiment was performed by Santos et al. (Santos et al., 2010) who analyzed the effect of Stat5a on p21 expression and distribution. It was found that mice with wild type Stat5a or

Stat5a double knockout (Stat5a -/-) showed no difference in p21 expression after treatment with estrogen and progesterone (E/P). However, the cellular distribution of p21 was altered such that p21 was sequestered in the cytosol after E/P treatment in mice with wild type Stat5a. By contrast, p21 stayed in the nucleus in Stat5a -/- mice after E/P treatment. Another piece of evidence linking Stat5a and p21 location is pim-1 signaling. It has been reported that Stat5a activation leads to an acute transcription of pim-1 (within 1 hour and peaks between 2 and 4 hours) (Buckley et al., 1995; Stout et al., 2004). Since pim-1 is one of the kinases controlling the entry of p21 into nucleus, the greater induction of pim-1 by Stat5a activation, which is also an effect of prolactin treatment, would therefore contribute to an altered distribution of p21.

miRNA

Interfering RNAs are small ribonucleic acids around 18-25 nucleotides in length. Depending on the author, between 60 and 92% of human genes are likely regulated by these small RNAs (Baek et al. 2008, Dai and Ahmed 2011). miRNAs are usually encoded by intergenic or intronic regions of DNA, but may be present in exonic regions of non-protein-coding genes or of protein coding genes subject to alternate

splicing (Rodriguez et al. 2004 , (Kim et al., 2009). In the classical scheme for their

production (Figure 4.1), miRNA regions of the genome are transcribed by RNA

polymerase II as longer sequences including a region that forms a hairpin or stem loop

(pri-miRNA). This is then processed by binding to DGCR8 (DiGeorge Syndrome

Critical Region protein 8) and cleavage by RNASEN (an RNAse III enzyme) to form

a pre-miRNA of about 70 nucleotides in length. The pre-miRNA is exported from the

nucleus by binding to exportin 5, which recognizes its double-stranded hairpin region.

Once in the cytosol, the pre-miRNA is subject to further cleavage by the dicer

complex. This removes the loop portion of the hairpin creating two complementary

strands of miRNAs. These two strands, along with dicer and a binding protein then

interact with Argonaute (Ago) to form RISC (RNA Induced Silencing Complex). One

of the complementary strands is released and degraded. The other, now a

single-stranded miRNA, is able to bind to its target sequence. At this point, the degree

of complementarity between the miRNA and its target sequence determines whether it

functions to inhibit translation or promote the degradation of mRNA. The less the

complementarity, the more likely it will function to inhibit translation without effect

on the level of mRNA. With greater complementarity, miRNAs function more like

siRNAs and promote mRNA degradation (Lee et al. 1993, Bartel 2004, Carthew and

Sontheimer 2009). To accomplish both of these endpoints, the miRNA binds to the 3'

untranslated region (UTR) of mRNAs (Yekta et al. 2004). Interaction with the 3'UTR

relies on a 7 nucleotide "seed sequence" present in the miRNA. An alternate pathway

for miRNA synthesis exists in which splicing of small intronic region (a microRNA

intron region or mirtron region) out of pre-mRNA creates a lasso-like structure (a

pre-mirtron) that subsequently loses its branch to form double-stranded pre-miRNA.

This hairpin double-stranded pre-miRNA is then handled in the same manner as the

RNASEN-processed variety.

To date, the best studied miRNAs implicated in carcinogenesis are in the

miR-17-92 family. This family consists of six members : miR-17, miR-18a, miR-19a,

miR-20a, miR-19b, and miR-92a. They are all transcribed from the same

polycistronic cluster, the miR-17-92 cluster on chromosome 13. In addition in

mammals, there are two paralogs, the miR-106b-25 cluster on chromosome 7, and the

miR-106a-363 cluster on the X chromosome. These resulted from gene duplications

of the miR-17-92 cluster during evolution. As mentioned earlier, miRNAs interact

with the 3'UTR of target mRNAs through their seed sequence; hence miRNAs with

the same seed sequence may share the same targets. Based on homology of the seed

sequences, miRNAs in these paralogous clusters can be grouped into four different

families, miR-17,miR-18, miR-19 and miR-92, as shown in table 4.1.

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Fig. 1.1 Functional motifs in BRCA1.
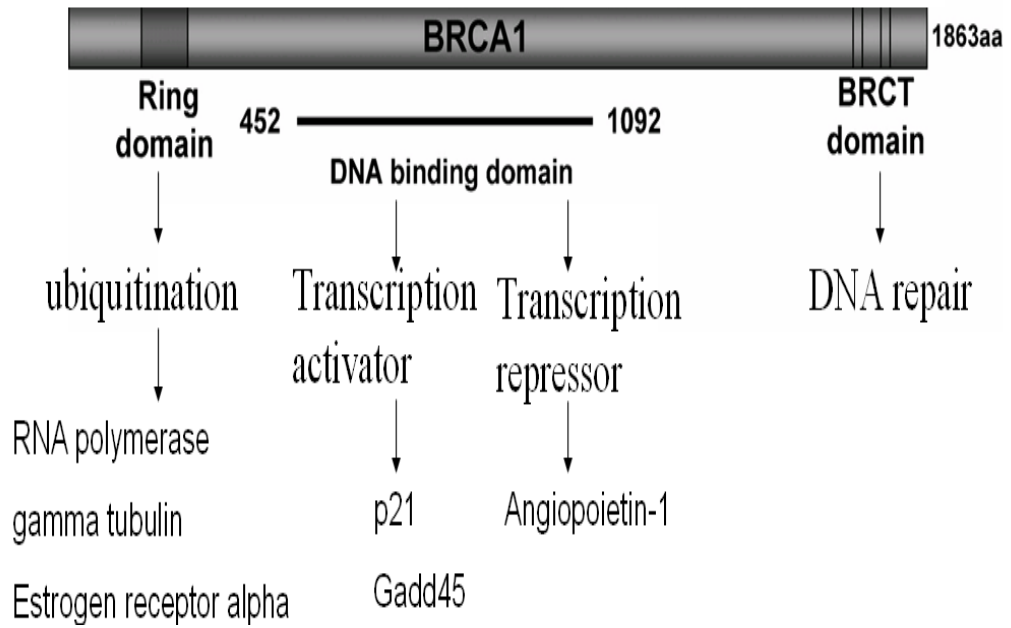
Chapter 2

Introduction

The tumor suppressor BRCA1 has been linked to breast, prostate and ovarian cancers (Agalliu et al., 2009; De Leeneer et al., 2011; Gallagher et al., 2010; Levine et al., 2003). In vivo studies showed BRCA1 knockout caused tumor formation (Brodie et al., 2001). In addition, the fact that people with BRCA1 mutations have a higher incidence of these tumors further claimed its importance as a tumor suppressor (Meindl et al., 2011). However, breast and ovarian tumors with wild type BRCA1 occur (Elledge and Amon, 2002). Thus, the functionality of wild type BRCA1 in tumors remained an interesting question. The tumor suppressive function of BRCA1 is mediated in several ways, of which the transactivation of p21 was reported to negatively regulate the cell cycle, leading to growth inhibition (Somasundaram et al., 1997). p21, also known as CDKN1A, causes cell cycle arrest between the G1/S phases through an interaction between p21 and cyclin/CDK complex. However, this happens in the nucleus. Thus, the function of p21 is also determined by its subcellular distribution (Li et al., 1996). It has been reported that accumulation of cytoplasmic p21 promoted cell growth, while nuclear p21 is a growth inhibitor (Asada et al., 1999;

Li et al., 1996). The distribution of p21 is determined by phosphorylation on threonine 145 (Zhou et al., 2001).

The peptide hormone prolactin is best known for its ability to promote milk production (Cowie et al., 1969). However, it is also an important growth factor for most tissues. In addition, most cancer cells produce prolactin and use it as an autocrine growth factor (Reynolds et al., 1997). Interestingly, we have shown that the prolactin antagonist, S179DPRL, inhibited cell growth through upregulation of p21, while unmodified prolactin promoted cell growth without causing an effect on p21. With the opposite function in growth and p21 regulation, we therefore ask whether prolactin and its antagonist, S179DPRL, regulate p21 expression through BRCA1.

In this study, we will examine the connection between prolactin, the antagonist, S179DPRL, and the functionality of BRCA1 in terms of p21 transactivation in multiple tumor cell lines with wild type BRCA1. In addition to the expression of p21, the function of p21 will be examined, as will its cellular location and post-translational phosphorylation. Furthermore, we will be looking at p21 regulation at other stages. Since BRCA1 is an important suppressor in different tumors, our study will include breast, prostate and ovarian tumor cells.

Methods and Materials

<u>Cell Culture and treatment</u>

All cell lines were originally purchased from ATCC and were routinely cultured in RPMI 1640 medium supplemented with 10% FBS (No penicillin/streptomycin added). The information of all 6 cell lines is listed in the table below.

Cell Line Name	Tissue Type	ATCC Number	Some Characteristics
T-47D	Breast cancer	HTB-133	p53 mutant, BRCA1 wt
MCF-7	Breast cancer	HTB-22	Both p53 and BRCA1 wt
PC3	Prostate cancer	CRL-1435	p53 mutant, BRCA1 wt
TOV-112D	Ovarian cancer	CRL-11731	p53 mutant, BRCA1 NHS
OV-90	Ovarian cancer	CRL-11732	p53 mutant, BRCA1 NHS
TOV-21G	Ovarian cancer	CRL-11730	p53 wt, BRCA1 NHS

TOV-112D, OV-90 and TOV-21G were all isolated from French Canadians.

NHS(No hot spots) represents the negative results for screening the Founder mutation sites (hot spot mutation sites in French Canadians) on BRCA1 in these tumors (Samouëlian et al., 2004).

For experiments, the density for seeding most cells was 5×10^5 cells/well in a 6 well plate and 1×10^6 cells/dish in a 10cm dish. The dose of Prolactin or S179DPRL was originally set at 500ng/mL but was repeated again in all cases with 100ng/mL dosage. For both MCF-7 and TOV-21G (as far as is known with both p53 and BRCA1 tumor suppressors wild type), the cells grow much slower than the others (between 34-40 hours for doubling), and so they are plated at higher density in order to coordinately transfect with the others. The seeding density was $7.5\sim8\times10^5$ cells/well and 1.5×10^6 cells/dish.

Molecular Cloning

Competent cell preparation

Competent cell strain, E. coli DH5α (New England Biolabs cat # C2987H) was cultured in 5mL LB medium (without antibiotics) with vigorous shaking (~200 to 250 rpm) at 37℃ overnight. The next day, 300μl of the overnight culture was transferred into 50mL fresh LB medium (without antibiotics) and incubated at 37℃ with vigorous shaking for 2 hours or until the OD. 595 was approximately 0.4. The competent cells were placed on ice for 10 min followed by a 10 min cold centrifugation at 5000 g. The cell pellet was resuspended in 10mL 0.1M $CaCl_2$

solution and incubated on ice for 30min. After pelleting again for 5 min at 3000 rpm, 4°C, the supernant was discarded and resuspended in 1mL 0.1M $CaCl_2$ solution. Competent cells were then aliquoted (100μl) into sterile 1.5mL microcentrifuge tubes and stored at -80℃ for up to 6 months.

p21 promoter construction

The different lengths of the p21 promoter region were amplified by PCR, ligated to TA vector, digested with restriction enzymes and cloned into pGL4.17vector (Promega cat#E6721). All information is included in the table below.

The 1390bp p21 promoter construct was digested from the 1439bp p21 promoter construct using DraI digestion and then self-ligated to form the 1390bp p21 promoter construct. This construct was missing the fragment from -84 to -126 (cut off by DraI) where the BRCA1 interacting element was present. The 1224bp plasmid was constructed by generating a XhoI cutting site at 1227 bp through PCR mutagenesis and then digesting with XhoI to get rid of the fragment between 2400bp and 1224bp.

Construct Name	Sequence of primer pairs used	5' cutting enzyme	3' cutting enzyme
2400bp	R : GCAGCTGCTCACACCTCAGC		
1439bp	F : AGGAGAAAGAAGCCTGTCCT R : GCAGCTGCTCACACCTCAGC		
1224bp	F : GTTTCAGGCACAGACTCGAGGCAAAGGTGAAGTCCAGG R :CCTGGACTTCACCTTTGCCTCGAGTCTGTGCCTGAAAC		
840bp	F : TCCTGGCCAACAAAGCTGCT R : GCAGCTGCTCACACCTCAGC	XhoI	HindⅢ
143bp	F : CGCTGGGCTAGCCAGG R : CCCAAGCTTAGCTGCTCACACCTCAGC TGGCGCAGCTCAGCGCGGCCCTGATATA	NheI	HindⅢ
1390bp	Digest with DraI from 1439bp plasmid		

Dominant negative Stat5a Y694F and Stat5a 740bp truncation construction

The Stat5a Y694F construct was produced using a Quickchange site-directed mutagenesis PCR kit (Stratagene, cat#200517-4). The Stat5a 740bp truncation construct was characterized by Wang and Yamashita et al. (Wang et al., 1996;

Yamashita et al., 2003) and shown to act as dominant negative. Only the amino acids

after 740 were deleted and therefore this DN Stat5a is still able to be phosphorylated

by Jak2 but fails to function as a transcription factor. The primer information to make

these two constructs is listed below.

Construct	Primer sequence
Stat5a Y694F	Forward : GTTGATGGATTTGTGAAACCA Reverse : TGGTTTCACAAATCCATCAAC
Stat5a 740bp	Forward : GGGGATCCATGGCGGGCTGGATCCAG Kpn I Reverse : CCGCTCGAGTCACTGTGGGTACATGT Xho I

p21-3'UTR luciferase reporter plasmid construction

The 3'UTR fragment of p21 was first amplified using the primer pair listed

below. The amplified fragment and the pMIR reporter vector (Applied Biosystem,

Cat# AM5795) were then digested with MluI and Hind Ⅲ. These 2 digests were then

gel purified and ligated together at 4℃ O/N.

Ligation information (New England Biolabs, cat# M0202S, including the 10x

ligation buffer and DNA ligase):1μl ligation buffer (10x) + 2μl pMIR reporter vector

digest + 6μl amplified p21-3'UTR digest + 1μl DNA ligase.

Construct	Primer sequence	
P21 3'UTR	Forward : CGACGCGTCCGCCCACAGGAAG	MluI
	Reverse : CCAAGCTTGAGCACCTGCTGTA	HindⅢ

Transformation

One vial of home-made competent cells was removed from storage at -80℃ and 1~10μL DNA (usually plasmid or ligation products) was added and gently mixed by patting the vial softly. This was incubated on ice for 30 min and then the cells were heat shocked at 42℃ for 2min followed by cooling on ice for 1 min. 800μl LB medium without any antibiotics were added and incubation proceded at 37℃ with vigorous shaking for 1~2 hours. 100μl of now transformed competent cells were then transferred onto an Amp-LB agar plate (100μg/mL ampicillin) and incubated in Warm Room overnight.

Plasmid isolation

Plasmid isolation was performed as per the guidelines of the Pureyield plasmid miniprep system (Promega cat# A1223).

Immunoprecipitation

Cells were seeded in 6 well plates and incubated until confluent. Medium was then replaced with serum free RPMI1640 for 24 hours to synchronize the cells and lower background signals. Cells were then treated with 100ng/mL PRL or S179DPRL for 24 hours and harvested using 100μl RIPA lysis buffer (see buffers in table below). Protein concentration was quantified using Biorad protein assay (Biorad, cat#500-0006). 200μg total protein lysate were immunoprecipitated with 5μl rabbit polyclonal BRCA1 antibody O/N at 4 ℃. The next day 100μl protein A/G beads (Pierce #20421) were added and smoothly agitated 2 hours at RT. The beads were then washed with 0.5mL IP buffer and pelleted 2-3 min at 2500 g. This wash step was repeated 5 times before eluting with 50μl Elution buffer by incubation for 5min at RT. Repelleting of the beads was then followed by collection of the supernant. This elution step was repeated once and the supernantants from both were combined (total volume 100μl). The eluate was adjusted to physiological pH by adding 10μl Neutralization buffer per 100μl eluate. The eluate was now ready for Western blot analysis.

Materials needed for IP :

RIPA lysis buffer	IP buffer	Elution buffer	Neutralization buffer
50mM Tris base	25mM Tris,	0.1M glycine·HCl	1M Tris
150mM NaCl	150mM NaCl,	pH 2.5	pH 9
1mM NaF	pH 7.2		
1% NP40			
0.25% Na-deoxycholate			
1mM EGTA			
pH 7.4			
add protease inhibitor prior use			

Nuclear and cytoplasmic extraction

Cells were seeded in 10 cm dish as previously described and grown until 70-80%
confluent. They were then synchronized in serum free RPMI 1640 medium for 24
hours and treated with 100ng/mL prolactin or S179DPRL for 30 min. The medium
was discarded and the cells rinsed with ice cold PBS. After removal of the PBS by
aspiration, 1mL cytoplasmic extraction buffer (see recipe below) was added and the

cells scraped into this solution before transfer to a 1.5mL microcentrifuge tube. After centrifugation, 1 min at 14000 rpm, 4℃, the supernantant was collected (this is the cytoplasmic fraction). The pellet was resuspended in 100μl ice cold cytoplasmic extraction buffer, mixed by pipetting up and down and incubated on ice for 10 min before centrifugation for 10 min at 13000g at 4℃. This second supernant containing more of the cytoplasmic fraction, was then combined with the 1st extraction. 80μl Nuclear extraction buffer was then added to the pellet and the pellet was resuspended by pipetting up and down several times (the pellet was sticky and gel like). This suspension was shaken for 10min at 4℃, then centrifuged for 10 min at 13000g at 4℃. The pellet was separated (This contained DNA, which was used for other purposes) from the supernant (Nuclear fraction). Both the cytoplasmic and nuclear extracts were flash frozen at -80℃.

Protein concentration was quantified using the Biorad protein assay and 50μg cytoplasmic protein and 20μg nuclear protein were used for Western blot analysis. For nuclear co-immunoprecipitation, 200μg protein was used in the same general immunoprecipitation process described above.

Recipe for Cytoplasmic and nuclear extraction buffer :

Cytoplasmic fraction extraction buffer	Nuclear extraction buffer
1M Hepes (2ml)	1M Hepes (4ml)
3M KCl (666.67ul)	3M NaCl (26.66ml)
0.25M EDTA (80ul)	0.25M EDTA (800ul)
10% NP40 (8ml)	dd H20 (168.54ml)
dd H20 (189.25ml)	

Western Blot Analysis

For p21 or molecules with molecular weight smaller than 30 kDa, 12% SDS-PAGE was prepared and for molecules larger than 30 kDa, 8% SDS-PAGE was preferred. Recipe for SDS-PAGE preparation is indicated below. Protein samples, mixed with protein loading buffer, were boiled 5 min and then placed on ice for 10min and then centrifuged to recover all fluid to maintain an accurate concentration. Gels were run at a constant voltage of 70 for 1 hour (stacking gel) and then switched to a constant voltage of 100 for the resolving gel for another 2-3 hours. The proteins on the gel were then transferred to nitrocellulose (NC) membrane (Whatman, 0.2μm, Cat# NBA083C) using a semidry transfer system for 1 hour with constant voltage at 7

V. For BRCA1 transfer, a longer time was required due to its large molecular weight

(1hour and 30min).

Recipe for SDS-PAGE preparation

	12% SDS PAGE		8% SDS PAGE	
	Resolving	Stacking	Resolving	Stacking
H2O	3.87mL	2.92mL	4.77mL	2.92mL
40:1 Acrylamide	2.7mL	0.5mL	2.25mL	0.5mL
1.5M Tris pH8.8	2.25mL		1.8mL	
1M Tris pH 6.8		0.5mL		0.5mL
SDS	90μl	40μl	90μl	40μl
APS	90μl	40μl	90μl	40μl
TEMED	4μl	4μl	4μl	4μl

After transfer, the NC membrane was then blocked in 5% BSA for 1hour at RT

followed by incubation with 1st antibody at 4℃ O/N. The dilution for all first

antibodies used is given below. The membrane was then washed in TBST three times

(10mL/10min/per wash) and incubated in 2nd antibody for 1 hour at RT with shaking.

The information for 2nd antibody preparation is described below. After incubating with

2nd antibody, the membrane was washed again in TBST three times (10mL/10min/per

wash), drained and developed using a chemiluminescent substrate (Denville Scientific

Inc., Hyglo Quick Spray, Cat.# E2400) for 2min at RT prior to film exposure.

1st antibody preparation

Name	Supplier and Cat.#	Dilution used
p21	Santa Cruz sc-397	1:1000 in 5%BSA with 0.02% Sodium Azide
BRCA1	Santa Cruz sc-646	1:200 in 5%BSA with 0.02% Sodium Azide
Stat5a	Santa Cruz sc-1081	1:1000 in 5%BSA with 0.02% Sodium Azide
pStat5a	Santa Cruz sc-101806	1:1000 in 5%BSA with 0.02% Sodium Azide
p-p21	Santa Cruz sc-20220-R	1:1000 in 5%BSA with 0.02% Sodium Azide
Myc	Santa Cruz sc-40	1:1000 in 5%BSA with 0.02% Sodium Azide
ER alpha	Santa Cruz sc-7207	1:1000 in 5%BSA with 0.02% Sodium Azide
p-ERK	Santa Cruz sc-7383	1:1000 in 5%BSA with 0.02% Sodium Azide
ERK 1/2	Santa Cruz sc-94	1:1000 in 5%BSA with 0.02% Sodium Azide
pAkt	Cell signaling #4060X	1:1000 in 5%BSA with 0.02% Sodium Azide
Akt	Santa Cruz sc-5298	1:1000 in 5%BSA with 0.02% Sodium Azide
Actin	Santa Cruz sc-1016	1:10000 in 5%BSA with 0.02% Sodium Azide
pER α	Upstate 07-487	1:1000 in 5%BSA with 0.02% Sodium Azide

2nd antibody preparation

Goat anti-rabbit HRP	Sigma A0545	1:40000 in 10mL TBST buffer
Goat anti-mouse HRP	Santa Cruz sc-2062	1:10000 in 10 mL TBST buffer

miRNA reporter assay (pMIR reporter)/Luciferase assay and beta-gal assay

Cells were seeded in 6 well plates and incubated until 80% confluent (~24hours).

Cells were then transfected with 500ng p21-3'UTR-luciferase reporter plasmid and

100ng beta-gal plasmid (Details of transfection with different reagents are given

below). After transfection, medium was changed (RPMI 1640 +10%FBS) with

100ng/mL prolactin or S179DPRL for 20hours. The medium was then removed and

the cells were washed with 2mL PBS once before lysis using 100μl 1x reporter lysis

buffer (Promega, cat# E397A, 5x stock included in luciferase substrate pack, make

dilution to 1x and use 100μl for each well). The lysate was then removed from the

dish and snap frozen in liquid nitrogen. After thawing at 37℃ (or RT). The lysate was

subjected to centrifugation at 13000g for 1min. 50μl of supernatant were transferred

into a 96 black well plate (Corning, code 3603) and 50μl luciferase substrate

(Promega, cat# E4030) was added into each well and mixed by pipetting up and down

6 times. The luminance was read on a luminoreader (Berthold Technology, Tristar LB

941). For the beta gal assay, 30μl of supernatant was transferred to a regular 96well plate and 200ul 125mg/mL ONPG solution (RPI, cat# N81000-1.0 Lot# 28798) was added into each well and incubated at 37℃ for 1-3hours (Yellow color development was checked every 30min). Absorbance at 414nm was determined. The reading from the luciferase assay was normalized to the reading from beta-gal assay and was analyzed by at least 3 repeats.

Transfection With lipofectamine 2000

The reporter plasmid (0.5μg p21-3'UTR-luciferase reporter) and the control plasmid (0.1μg β-gal plasmid) were combined in 100μl serum free RPMI 1640 medium. To this was added 2μl lipofectamine 2000 diluted in another 100μl RPMI1640 and the mix (200μl) was incubated at RT for 20 min. The cell growth medium was then replaced with serum free RPMI 1640 medium (1.8mL/well) and 200μl of the transfection complex was added directly into each well. Cells were then incubated at 37℃ for 4hours after which time the medium was changed to 10% FBS RPMI 1640 containing either 100ng/mL PRL or S179DPRL. Cell lysates were collected 20 hours later.

Transfection with Fugene 6

The same quantities of reporter and control plasmids were combined as before in 100μl serum free RPMI 1640 medium, but in this case 2μl fugene 6 were added. The mix was then incubated at RT for 15 min and 100μl transfection complex was added directly into each well (no medium change). Cells were then incubated at 37℃ O/N. The next day, the medium was replaced with fresh 10% FBS RPMI 1640 containing either 100ng/mL PRL or S179DPRL and cell lysates were prepared after 20 hours.

Transfection With Transfectene

In this instance, the same amounts of plasmid were combined in 100μl EC buffer and 3.2μl of enhancer was added, vortex mixed for 1 sec, and incubated. Transfectene (10μl) was then added, vortex mixed for 10sec and incubated for 10 min. 600μl serum free RPMI 1640 was added and mixed by pipetting up and down. 700μl of this was added directly to cells (no medium change required) to a total volume of 1.8mL. Cells were incubated O/N and the medium (10% FBS RPMI 1640) containing either 100ng/mL PRL or S179DPRL was replaced the next morning. Cell lysates were collected after 20 hours incubation with PRL or S179DPRL.

Chromatin Immunoprecipitation

Cells were seeded in 10cm dishes and incubated at 37℃ until 80% confluent. Medium was changed to serum free and cells were incubated for 24hour before prolactin treatment. Then either 100ng/mL prolactin or S179DPRL were added for 20 hours. Cells were then washed with 10mL PBS twice, the PBS was aspirated and the cells fixed by adding 10mL PBS containing 270μl 37% formaldehyde for 10 min at room temp. Cross-linking was stopped by adding 1mL 1.25M Glycine for 5 min at Room Temp. After removal of this, the cells were washed with 7.5mL cold PBS twice and the final wash was completely aspirated before scraping the cells into 1250μl cold PBS and centrifugation at 2000 rpm for 2 min at 4℃. The supernatant was discarded and the pellet resuspended in 1mL CHIP sonication buffer plus protease inhibitors (see recipe below), and then snap frozen at -80℃.

ChIP sonication Buffer (100mL solution)	
Just before use, add 10 ul Aprotinin, 10 ul Leupeptin, and 5 ul PMSF to each 10 ml.	
1% Triton X-100	10ml 10% Triton X-100
0.1% Deoxycholate,	1ml 10% Deoxycholate
50mM Tris 8.1	5ml 1M Tris-Cl pH 8.1
150mM NaCl	3ml 5M NaCl
5mM EDTA	1ml 0.5M EDTA

Samples were sonicated according to the following schedule: Maximum power on

Sonic dismembranator 60 (Fisher) for 20 sec, place on ice for 10sec. Repeat this

process for 40 min or more). The sonicates were centrifuged at 13000g for 15 min at 4

℃ to remove debris and the supernatant was retained. Am aliquot (40μl) was used to

check for sufficient DNA fragmentation (a smear region between 300-800bp). The

remaining sample was precleared using 1-5μg IgG plus 40μl protein A/G beads for

30-60 min. ChIP buffer (see below) was added to make a final of 1mL for each

sample. To this was added 10μl 100x BSA with thorough mixing.

10x ChIP buffer		
10% Triton X-100	1.9% EDTA, disodium dehydrate	1 % SDS

Each sample was then divided into 4 tubes as follows :

      a)   40μl Input   → snap freeze and store at -80℃

      b) 400μl for anti BRCA1

      c) 400μl for anti rabbit IgG or nothing

      d) 160μl leftover → freeze

ChIP buffer without Triton (800μl) and 5μg antibody were added to tubes b,c of

each sample (BRCA1 / rabbit IgG). The tubes were then nutated in the cold room for

2 hrs or O/N. Protein A/G beads were washed before use and then 40 μl were added to

each tube along with 2μl of 10 mg/mL herring sperm DNA to avoid nonspecific DNA

binding and nutated in the cold room for 2hrs or O/N. Samples were centrifuged at

400g 2.5min at 4℃ and the supernatants stored at -20 ℃. The beads were washed

with 1mL cold ChIP buffer w/o protease inhibitors by inverting the sample to suspend

the resin then pelleting again, as before and removal of supernatant. Following 2

further washes, this time in cold PBS, the pelleted and drained beads were

resuspended in 250μl of Elution buffer (see below) and placed on a nutator at RT for

15-20 min.

Elution Buffer (10mL recipe)			
1mL 10% SDS	2mL 0.5M NaHCO$_3$	10μl sperm DNA	6.99mL ddH$_2$O

The beads were pelleted and the supernatant saved. The elution step was repeated,

but the tubes were placed in a 100°C heat block for 60s followed by nutation 20 min

at RT. After pelleting again, the supernatants were combined. 500μl of elution buffer

were added to the input sample and this was processed along with the antibody

precipitated samples. To each were added 40μl of 2.5M NaCl followed by placement

in a 65℃ bath for 4 h. The precipitated DNA fragment was purified using gel

extraction (using Sigma Genelute Gel extraction NA1111-1KT). The eluate was then used for PCR.

The primers used for the BRCA1 response element were the same as those used for cloning.

RNA stability assay (DRB assay)

Cells were seeded in 10cm dishes and incubated for 24hours. The next day, the medium was changed to serum free RPMI 1640 and the cells were incubated for another 24hours. DRB was added to a final concentration of 40nM 2hours prior to prolactin or S179DPRL treatment (100ng/mL). Incubation continued for 0, 2, 4, and 6 hours. Total RNA was extracted using Ribozol (ISC-Bioexpress N-580) following the manual protocol. RNA was then reverse transcribed into cDNA and p21 mRNA stability was then assessed by real time PCR.

Primer pair for p21 stability real time PCR
Forward : CGACTGTGATGCGCTAATGG
Reverse : GGCGTTTGGAGTGGTAGAAATC

Protein stability assay (MG132 assay)

Cells were seeded in 10cm dishes and incubated for 24hours. The next day the medium was changed to serum free RPMI 1640 and the cells were incubated for

another 24hours. MG132 was added to a final concentration of 25 μM for 2 hours prior to prolactin or S179DPRL(100 ng/mL) treatment for another 30min. Protein was then extracted using RIPA lysis buffer and quantified. A 50ug protein sample was analyzed by Western blot.

miRNA microarray

Analysis of miRNA was by commercial microarray (Signosis cat# AP-0002) following the protocol in the user manual.

Results

BRCA1 expression was increased in response to both prolactin and S179DPRL

Earlier studies defined prolactin as a growth promoting hormone and S179DPRL as an antagonist to the growth promoting effects of prolactin. We therefore sought to examine the individual effects of these two molecules on the tumor suppressor, BRCA1. A panel of 6 human cancer cell lines, including two breast cancer cell lines, MCF-7 and T-47D, one prostate cancer cell line, PC3, and three ovarian cancer cell lines, TOV-112D, TOV-21G and OV-90, were analyzed for expression of BRCA1 after prolactin or S179DPRL treatment. Contrary to what was expected, an increase in BRCA1 expression was detected in response to either prolactin or S179DPRL treatment in all cancer cell lines tested although the degree of induction and the time the amount peaked were different in the different cell types (Fig. 2.1). Previous studies indicated that different prolactin receptors activate different signaling and therefore different end effects. In order to determine whether the time course and degree of induction of BRCA1 in the different cell lines was a result due to differential expression of different prolactin receptors in these cell lines, we

overexpressed one or other of the two major forms of prolactin receptor. PC3 cells

express extremely few prolactin receptors, but do respond to prolactin by activating

all signaling pathways. They serve as a good model to test increased expression of the

prolactin receptor isoform since all necessary components are present and the

transfected receptors overwhelm the endogenous. As shown in Fig. 2.2,

overexpression of each form of prolactin receptor led to an increase in BRCA1

expression in response to either prolactin or S179DPRL, indicating that even the short

1b receptor that lacks a large portion of the intracellular domain of the receptor could

mediate the effect of prolactin on BRCA1 expression. Furthermore, because there was

no difference between the two types of receptor, differential ratios of long form to

short form 1b receptor expression does not explain the differential time course or

degree of response among the cell lines.

The function of BRCA1 to transactivate p21 was altered by prolactin

To further evaluate the impact of prolactin or S179DPRL on the induction of

BRCA1, p21 expression was examined. As expected, BRCA1 induced by S179DPRL

was able to transactivate its downstream target p21 leading to an elevation of p21. In

contrast, BRCA1 induced by prolactin was not capable of transactivating p21 (Fig. 2.3). The induction of p21 in response to S179DPRL was dose dependent (OV90 and PC3 cells shown), whereas no cell line showed any effect of prolactin at any dose (Fig. 2.4, PC3 cells shown).

Expression of p21 is regulated by a variety of factors, including p53, BRCA1 and other transcription factors. To confirm that induction of p21 by S179DPRL was mediated by BRCA1, we constructed the full length p21 promoter and connected it upstream of a luciferase reporter in a pGL 4.17 vector. A series of constructs containing different regions of the p21 promoter were also created to determine the involvement of different transcription factor elements (Fig. 2.5). PC3 cells transiently transfected with different constructs and short form 1b prolactin receptor were then analyzed upon treatment with prolactin or S179DPRL. With the full length p21 promoter or other constructs containing the BRCA1 response element (ranging from -93~-133bp on p21 promoter) (Somasundaram et al., 1997), a S179DPRL stimulus caused higher luciferase expression. In addition, removal of one p53 element had no effect, but removal of both p53 elements showed a slightly decreased expression of luciferase, suggesting a possible role of p53 in BRCA1- mediated p21 expression, although the difference was not statistically significant. Most interestingly, the

minimal promoter containing only the BRCA1 response element and a basic TATA

element was still able to drive luciferase expression in response to S179DPRL. On the

other hand, when the BRCA1 response element was taken out while keeping all others

intact, the expression of luciferase was decreased, indicating an involvement of

BRCA1 in p21 induction in response to S179DPRL (Fig. 2.6). The responses to

S179DPRL are not large in magnitude, although highly reproducible, primarily

because PC3 cells are hard to transfect and expression of the receptor is low. However,

as shown in a later chapter the degree of induction is much improved upon

development of a stable cell line. In comparison to the results with S179DPRL, the

prolactin- induced BRCA1 was not able to stimulate p21 induction in cells transfected

with the same constructs; the luciferase values were the same as with no addition of

prolactin. This implies a loss of function or functional interference with BRCA1

under these circumstances.

The activating signaling molecule, p-Stat5, formed a complex with BRCA1 and

transcriptionally interfered with its p21 transactivation function

We next investigated how prolactin treatment led to the disability of BRCA1 to

transactivate p21. We first analyzed the difference in signaling activation upon

treatment with prolactin or S179DPRL. Among the signaling molecules examined,

activation of Stat5 was completely different with prolactin versus S179DPRL;

prolactin activated Stat5, whereas S179DPRL did not (Fig. 2.7). To test whether there

was an interaction between Stat5 and BRCA1, co-immunoprecipitation was

performed using a BRCA1 antibody and Western blotting with anti-p-Stat5. Fig. 2.8

shows the result with the human breast cancer cell line, T-47D. This cell line (as do

all of the lines used in this study) expresses autocrine prolactin. As seen in Fig. 2.8,

both prolactin treatment (cells exposed to both exogenous and autocrine) and control

(cells exposed to autocrine) showed a complex formation between p-Stat5 and

BRCA1 while this did not occur with S179DPRL treatment. Thus, S179DPRL not

only did not activate Stat5, but also inhibited the ability of autocrine prolactin to

activate Stat5.

The inability of BRCA1 to increase p21 expression in response to prolactin could

be explained in three ways: (1) The cellular distribution of BRCA1 was changed

when complexing with Stat5, (2) The binding efficacy of BRCA1 to the p21 promoter

was modulated after forming a complex with Stat5 or (3) The BRCA1-Stat5 complex

was not able to drive transcription of p21. To determine which of these was the case,

T-47D cells were treated with prolactin and the cytosolic and nuclear fractions were

isolated and co- immunoprecipitated with anti-BRCA1 followed by immunoblotting

with anti-p-Stat5. As shown in Fig. 2.9, the BRCA1-p-Stat5 complex was located in

the nucleus indicating there was no effect on the entry into the nucleus when forming

a complex with p-Stat5. The interaction with the p21 promoter was examined by

chromatin IP (Fig. 2.10), which showed there was no difference in the binding of

BRCA1 to the p21 promoter, implying that this complex formation may interfere with

the transcription of the downstream p21. To further prove that complex formation

affected p21 transcription, we constructed a dominant negative form of Stat5a. With

overexpression of this dominant negative form of Stat5a, treatment with prolactin was

now able to drive p21 expression, demonstrating an important role of Stat5a in

interference with BRCA1 function (Fig. 2.11).

MicroRNA regulation of p21

Given that prolactin inhibits BRCA1's ability to increase p21 expression

transcriptionally through an interruption of BRCA1 function, we asked whether there

was also an inhibitory effect of prolactin on p21 expression at any other level. miRNA

regulates the expression of lots of genes in mammals. We therefore tested whether

there was miRNA regulation of p21 expression in response to prolactin. To do this,

we constructed the 3' untranslated region (UTR) of p21 mRNA downstream of a

luciferase reporter. In this assay, production of miRNA in response to prolactin and its

interaction with the 3'UTR of p21 mRNA would lead to a decrease in luciferase

reporter production. As shown in Fig. 2.12, a slightly decreased luciferase expression

was seen when MCF-7 cells were incubated with prolactin. However, luciferase

expression was increased when stimulation was with S179DPRL, indicating that one

or some miRNAs acting on the 3'UTR of p21 was induced by prolactin and

downregulated by S179DPRL. Using an online miRNA search engine, pictar, six

possible miRNAs are predicted to be able to interact with the 3'UTR on p21. These

miRNAs include miR-17, miR-106a, miR-106b, miR-363 and miR-20. All of them

are in the same seed sequence family. To determine which miRNA(s) was involved in

p21 regulation, a miR-microarray was performed. This revealed that 12 miRNAs were

downregulated in response to S179DPRL (table 1). Among these miRNAs, it was not

surprising to see miR-106, a known p21 silencer (Ivanovska et al., 2008). Another

miRNA that drew our attention was miR-214, which was downregulated by

S179DPRL and upregulated by prolactin. Though miR-214 can not directly target p21

or BRCA1 mRNA , it has been shown by other groups that individuals with a BRCA1

deficiency have a higher level of miR-214, indicating some possible indirect

relationship (Sempere et al., 2007).

miRNA regulates its target primarily through translational repression, however,

it was also reported that the cleavage of target mRNA occurs (for review see chapter

4). To address how miR-106 regulated p21 mRNA after prolactin treatment, mRNA

stability was assessed with a DRB assay. In Fig. 2.13, one can appreciate that the

stability of p21 mRNA was not significantly affected when prolactin or S179DPRL

treatment occurred; therefore, the action of miR-106 on p21 mRNA could be through

translational repression (see below). Before examining this, we next sought to

determine how prolactin upregulated the expression of miR-106. Others have reported

that myc overexpression in several cancers led to upregulation of members of the

miR-106 cluster. We therefore examined myc involvement. With overexpressed myc

in MCF-7 cells, there was a great decrease in luciferase expression, indicating a huge

amount of miRNA(s) produced and targeted to the 3' UTR of p21 (Fig. 2.14). To

establish a connection between prolactin and miR-106 production, we sought forward

to an upstream myc regulator, the estrogen receptor alpha. We have previously

reported that prolactin could activate the estrogen receptor in a ligand-independent

manner (Chen et al., 2010). This is also shown in (Fig. 2.15). When the effect of

prolactin versus S179DPRL on expression of myc protein was followed short term,

we found a decrease in myc expression occurred within an hour with S179DPRL

treatment. This correlated with the decreased miRNA expression in response to

S179DPRL (Fig. 2.16). Therefore, the maintenance of myc expression in prolactin

treatment (either autocrine or exogenous addition) could be crucial for miR-106

expression and suppressed p21 translation.

Posttranslational phosphorylation of p21 on threonine 145

The function of p21 partly depends on its location in cells. Cytosolic p21 is

responsible for an anti-apoptosis effect and nuclear p21 functions as a cell cycle

inhibitor (el-Deiry et al., 1994). To address the potential differential location of p21 in

response to prolactin and S179DPRL, we examined the phosphorylation status of p21

on threonine 145, the site that was crucial for its cellular distribution (Zhou et al.,

2001). Prolactin caused a peak in p-thr145-p21 at 15 min, whereas S179DPRL

showed a pattern with a gradual decrease in p-thr145-p21 (Fig. 2.17). Further

examination of the cellular distribution of p21 revealed cytosolic accumulation of p21

occurred with prolactin treatment and nuclear retention of p21 was seen in response to

S179DPRL (Fig. 2.18). Akt, pim-1 and PKA have all been reported to phosphorylate

p21 on threonine 145 (Gapter et al., 2006; Scott et al., 2000; Zhou et al., 2001).

Interestingly, pim-1 has been reported to be upregulated by prolactin through Stat5a in

a short time, peaking at 2~3 hours and lasting up to 24 hours which would therefore

contribute to a long term effect (Buckley et al., 1995; Stout et al., 2004). However, the

observed phosphorylation of p21 could be from the endogenous pim-1 activated by

treatment within minutes. Since there was not much difference in Akt activation in

response to prolactin or S179DPRL (Fig. 2.7), this was considered an unlikely

candidate. To determine whether prolactin caused p21 phosphorylation at theonine

145 through PKA, cells were pretreated with 100nM PKI, a PKA inhibitor, for 1.5

hours followed by prolactin treatment. The p21 phosphorylation is shown in Fig. 2.19.

As seen here, incubation with PKI did not interfere with the phosphorylation of p21

by prolactin.

Prolactin did not affect p21 protein stability

We have already shown that prolactin can regulate p21 expression at

transcriptional and post transcriptional stages. We next sought to determine whether

prolactin affected p21 protein stability. MCF-7 cells were treated with the proteasome inhibitor, MG132, for 2 hours prior to prolactin or S179DPRL treatment and the expression of p21 was assessed 30 min after the treatment. As shown in Fig. 1.20, there was no short term effect on p21 protein stability after treatment.

From the results presented here, we propose that prolactin activates Stat5a, which in turn formed a complex with BRCA1 leading to a downstream p21 transcription failure. Phosphorylation of p21, perhaps by pim-1, leads to a cellular redistribution of p21. Furthermore, the activation of ERα by prolactin maintains the expression of myc, which is crucial for the expression of miR-106 and therefore further repressed the translation of p21 mRNA. The action of miR-106 did not include p21 mRNA degradation. The proposed model is shown in Fig. 2.21.

Discussion

In the current study, we have shown that both prolactin and its antagonist, S179DPRL, could induce the expression of the tumor suppressor BRCA1. The amount of BRCA1 induction differed from cells to cells. We therefore investigated whether this was an effect of differential expression of the amount or type of prolactin receptors in different tissue cell lines. With overexpression of either major form of prolactin receptor in PC3 cells, we were able to determine that both prolactin receptors when interacting with prolactin or S179DPRL could lead to the induction of BRCA1. The induction of BRCA1 is therefore a product of some signaling pathway common to both prolactin and S179DPRL and both receptor types. BRCA1 expression and protein stability have been linked to Akt signaling, which is one of the common signaling pathways activated by both prolactin and S179DPRL (Nelson et al., 2010). Although both prolactin and S179DPRL induced BRCA1 expression, we tested whether this in turn could lead to an increase in the downstream molecule, p21. Results show that prolactin was not able to drive p21 expression, which was consistent with results from others (Schroeder et al., 2003). Furthermore, we also confirmed the involvement of BRCA1 in S179DPRL driven p21 expression using

promoter chopdown analysis. Interestingly, with removal of one p53 element from the reporter construct, there was no difference from the full length p21 promoter. Removal of both p53 response elements perhaps showed a slight decrease in luciferase expression. Our earlier study indicated that S179DPRL led to an increase in VDR expression in human prostate and mouse mammary cells, which in turn interacted with the p21 promoter leading to an elevation of p21 (Wu et al., 2006; Wu et al., 2005; Xie et al., 2010). However, removal of VDR response element from p21 promoter caused loss of response of luciferase activity implying an important role of VDR in p21 induction in response to S79DPRL (Xie et al., 2010). In opposite to this finding, results presenting in this study showed the removal of BRCA1 response element while keeping the VDR response element was also irresponsive to S179DPRL. Interestingly, in osteosacoma, we have shown that prolactin blocks nuclear translocation of the VDR through its interaction with BRCA1, suggesting a functional cooperation between BRCA1 and VDR (Deng et al., 2009). Therefore, BRCA1 and VDR may both contribute to p21 upregulation in response to S179DPRL but this could be interrupted when removal of either VDR or BRCA1 response element that caused the structural change of the promoter preventing an interaction of this complex to either response element.

The minimal p21 promoter contains the BRCA1 response element and basic core promoter region such as TATA box and few sp1 sites which are considered as basal transcription factor for gene transcription. The removal of BRCA1 response element may also remove part of these essential elements leading to a huge decrease in response. Since the BRCA1 response element overlaps with part of the sp1 binding sites, a future study with an application of mithramycin A, sp1 inhibitor, would therefore help to answer the question that the loss of response is due to the deletion of BRCA1 or part of the sp1 sites.

We also found that the function of wild type BRCA1 could be hindered by the signaling molecule, Stat5, in tumor cells. This was consistent with the fact that hyperactivation of Stat5 was found in tumors (Cotarla et al., 2004) and could explain well why the absence of BRCA1 mutation was found in most tumor cells. The functional interference of BRCA1 may be temporary in normal tissues, but constitutive activation of Stat5 signaling in cells may confer a longer term inactivation of BRCA1 and therefore may contribute to malignant transformation and cancer development.

Stat5 signaling is initiated by several growth factors including interleukins, growth hormone and prolactin. Our previous results showed that prolactin was

produced in several modified forms, one of which, the phosphorylated form, was able to block the activation of Stat5 signaling. In this study, S179DPRL as a mimic of naturally phosphorylated hormone, also blocks Stat5 signaling and treatment with either S179DPRL or a dominant negative form of Stat5 lead to an elevation of p21 in response to autocrine or exogenous prolactin.

We also presented evidence of further effects of prolactin and S179DPRL on p21 expression by performing miRNA analysis. Our data indicate that miR-106 was a candidate for the regulation of p21 expression in MCF-7 cells. Since all members in the miR-17 family, including miR-17, miR-20, miR-106 and miR-93, are expressed differentially in different tissues, the question is whether miR-106 is responsible for p21 regulation in response to S179DPRL or whether there is just a tissue specific expression profile of this family in response to S179DPRL. With the current data, this question cannot be answered. However, it is consistent with the results as follows: CARM-1 is a potential target regulated by miR-106. The induced miR-106 by prolactin would therefore target CARM1 leading to lower methylation levels of p300 (Lee et al., 2011) which is an essential event to recruit BRCA1 to the p21 promoter. Instead, the repression of miR-106 by S179DPRL would therefore cause a higher level of CARM1, high levels of p300 methylation and therefore recruit more BRCA1

to the p21 promoter (Lee et al., 2011). In addition, the results presented here did not show a correlation between the p21 mRNA stability and prolactin or S179DPRL treatment using DRB assay. However, it was showed by Wood and Shilatifard that DRB treatment would lead to p21 production through adopting a stress-specific transcriptional program for stress related gene expression such as p21 (Wood and Shilatifard, 2006). However, the induction of miRNA by prolactin stimulus could still illustrate a regulation at the post-transcriptional stage.

The function of p21 is dependent on its cellular location. In this study, we showed that prolactin treatment caused p21 phosphorylation at threonine 145 which led to an accumulation of p21 in the cytosol. This result was consistent with an earlier study in which Stat5 activation sequestered p21 in the cytosol (Zhou et al., 2001). In conclusion, we've shown that prolactin interferes with the tumor suppressors BRCA1 and p21 at several stages, including transcriptional regulation through complex formation with activated Stat5, post-transcriptional regulation through a possible candidate miR-106, and post-translational modification on threonine This study offers potential therapeutic targets and suggests the use of a prolactin antagonist would benefit patients at an early stage of tumor development.

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Fig. 2.1 Three day trial of BRCA1 induction in cells after treatment with either 100ng/mL prolactin or S179DPRL.

Cells were seeded in 6 well plates and treated with 500ng/mL (100ng/mL showed the same results) prolactin or S179DPRL for 24, 48, 72 hours. Control cells were obtained without any treatment at 0 hour time point. (controls at 24, 48, 72 hour time point were also checked and there was not much difference in BRCA1 expression.) Results show that both prolactin and S179DPRL induce BRCA1 expression though the time and the amount peaked were different from cells to cells.
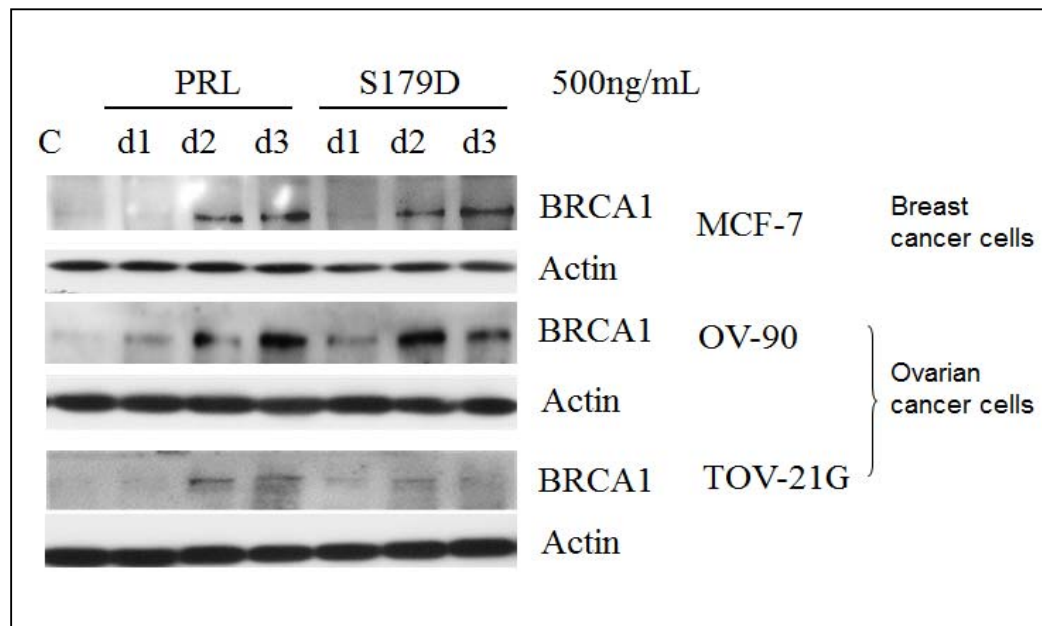
Fig. 2.2 Overexpression of a particular prolactin receptor(either short form 1b prolactin receptor (SF1b) and long form prolactin receptor(LF)) did not alter the ability of both prolactin and S179DPRL to induce BRCA1 expression.

PC3 cells were seeded in 6 well plates and transfected with either long form prolactin or short form 1b prolactin receptor. Cells were then treated with 500ng/mL (100ng/mL worked later) for 72 hours and BRCA1 expression was examined by western blot. Results indicate that the induction of BRCA1 by prolactin or S179DPRL was not an effect through a particular receptor isotype signaling.
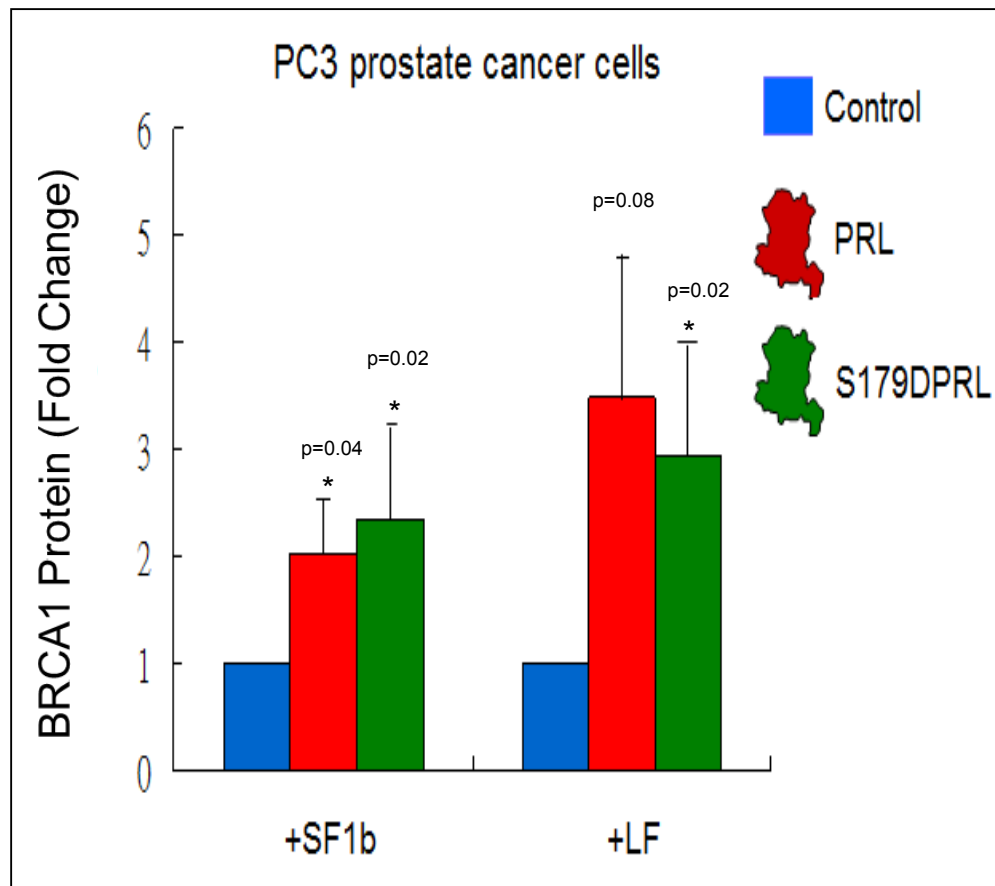
Fig. 2.3 Prolactin did not upregulate p21 expression as a result of BRCA1 induction, however, S179DPRL did increased p21 expression in cells.

Cells were seeded in 6 well plates and treated with 500ng/mL (100ng/mL worked) prolactin or S179DPRL for 72 hours. P21 expression was then analyzed by western blot. PC3, T-47D and TOV-112D are representatives of prostate, breast and ovarian tumors.
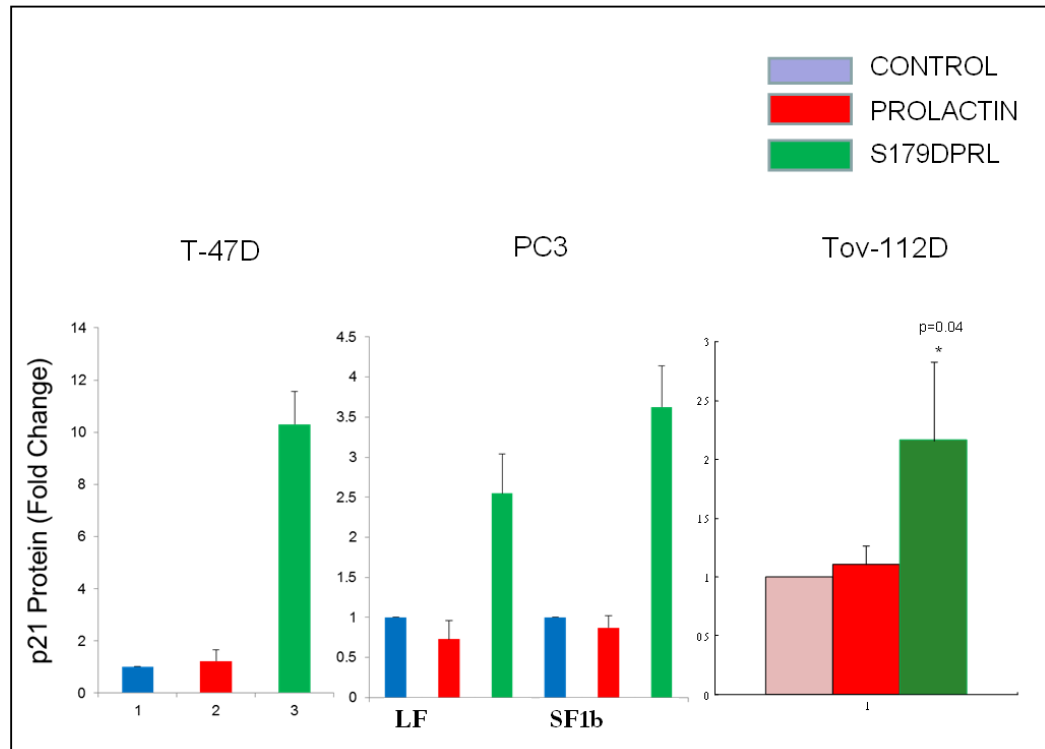
Fig. 2.4 p21 expression was related to dose of S179DPRL in different cancer cell lines.

Cells were seeded in 6 well plates and treated with 100ng/mL for 24 hours. p21 expression was analyzed by western blot. p21 expression was higher when the concentration of S179DPRL increased and as little as 50ng/mL showed a response.. The PC3 cells used in this test were not transfected with any forms of prolactin receptors. p21 expression was not increased by any   concentration of prolactin.
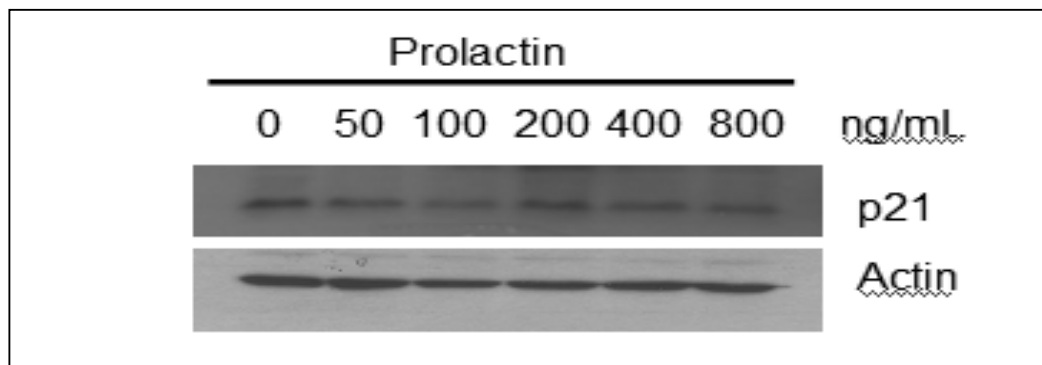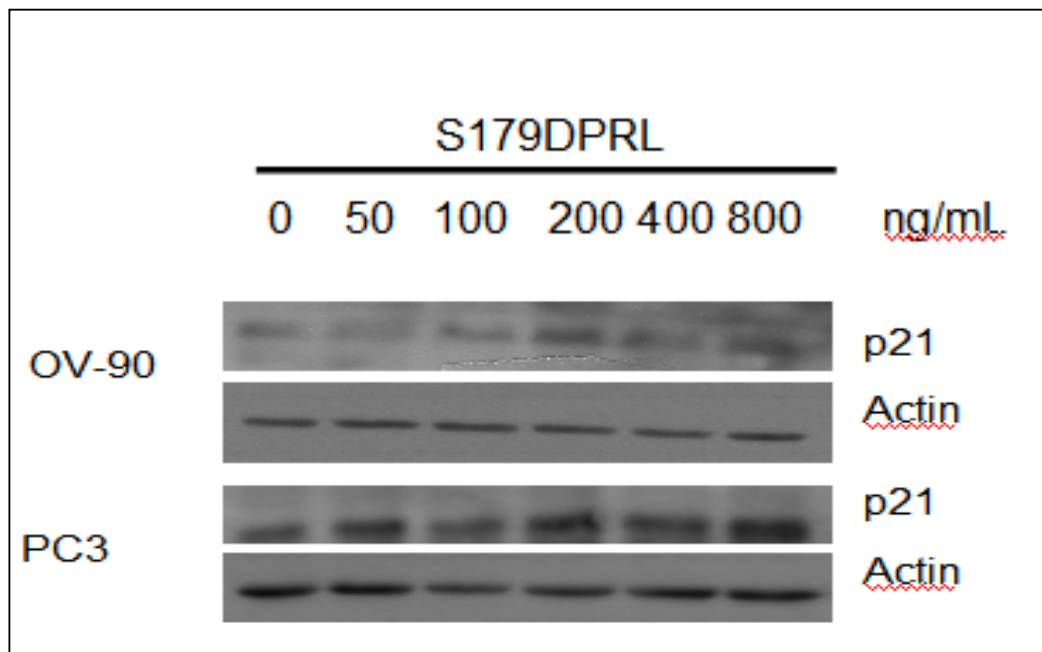
Fig. 2.5 Diagram of all p21 promoter constructs as well as possible transcription factor interacting sites.
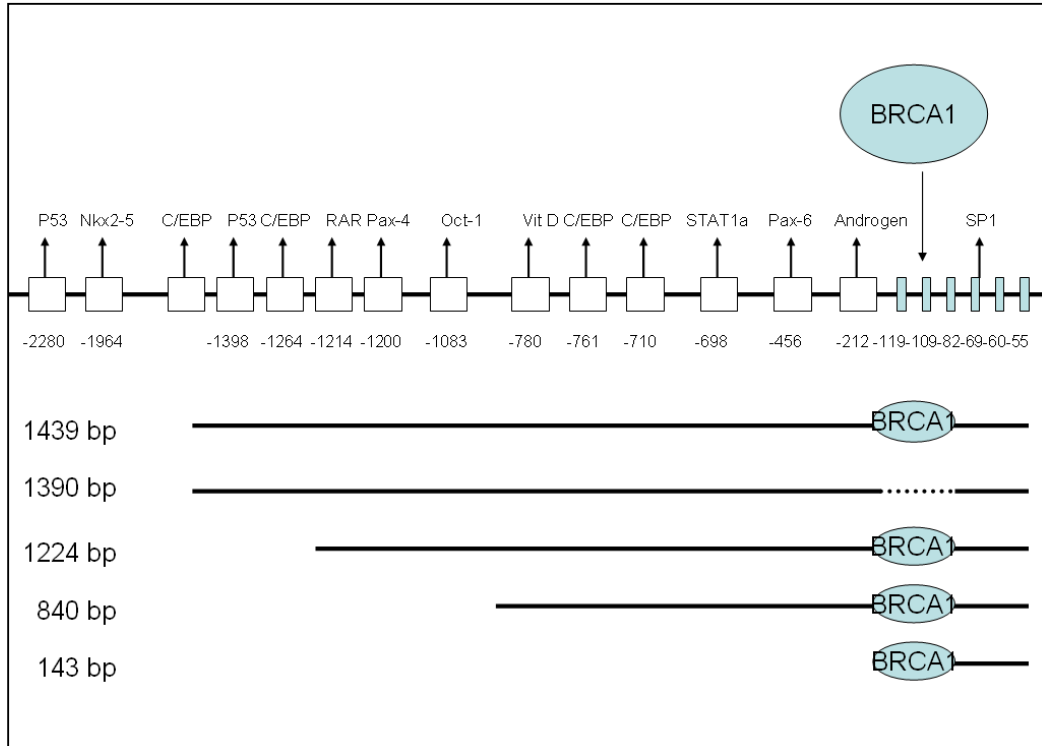
Fig. 2.6 BRCA1 is involved in the p21 induction by S179DPRL treatment.

PC3 cells were seeded in 6 well plates and transfected with short form 1b prolactin receptor, a plasmid containing the different lengths of p21 promoter, and a plasmid expressing β-galactosidase. After 4 hours transfection, cells were treated with 500ng/mL prolactin, S179DPRL or without any treatment for 20 hours. The data presented are normalized to the group   transfected with all the same constructs but without any treatment. Cells treated with prolactin showed a very similar result to w/o any treatment.
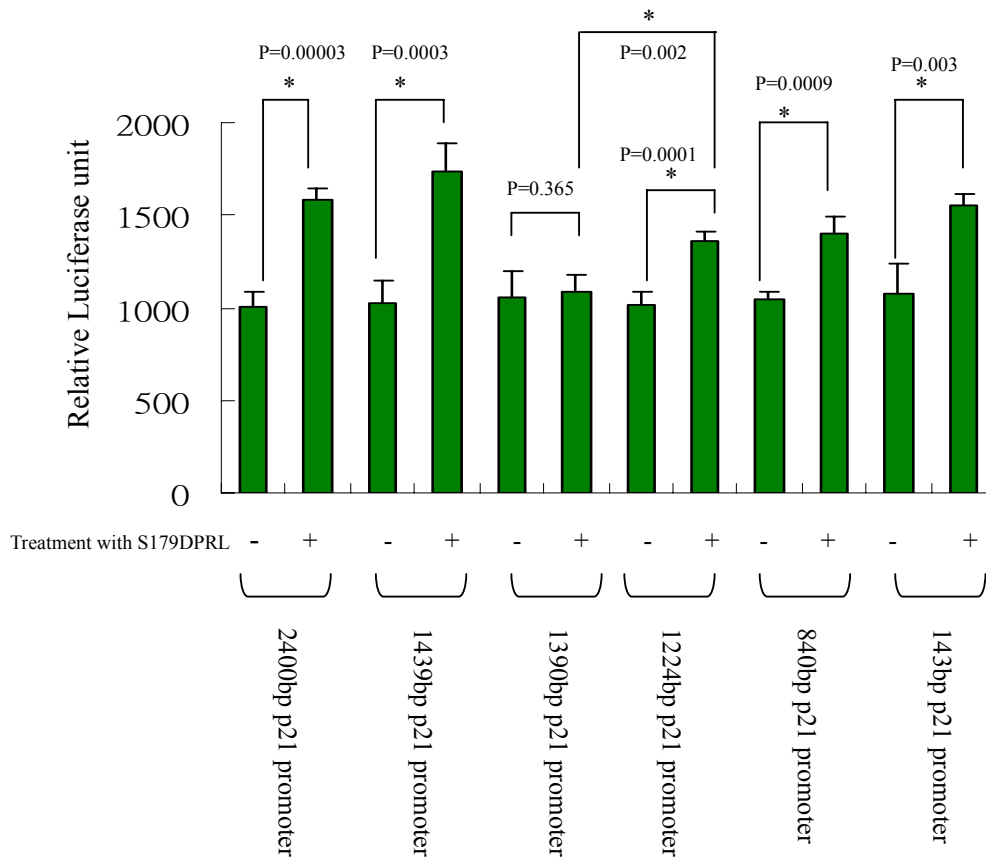
Fig. 2.7 Difference in signaling pathways that were activated by prolactin or S179DPRL.

Cells were seeded in 3cm dishes and treated with 100ng/mL prolactin or S179DPRL for different time periods. The signaling molecules were examined by western blot analysis.
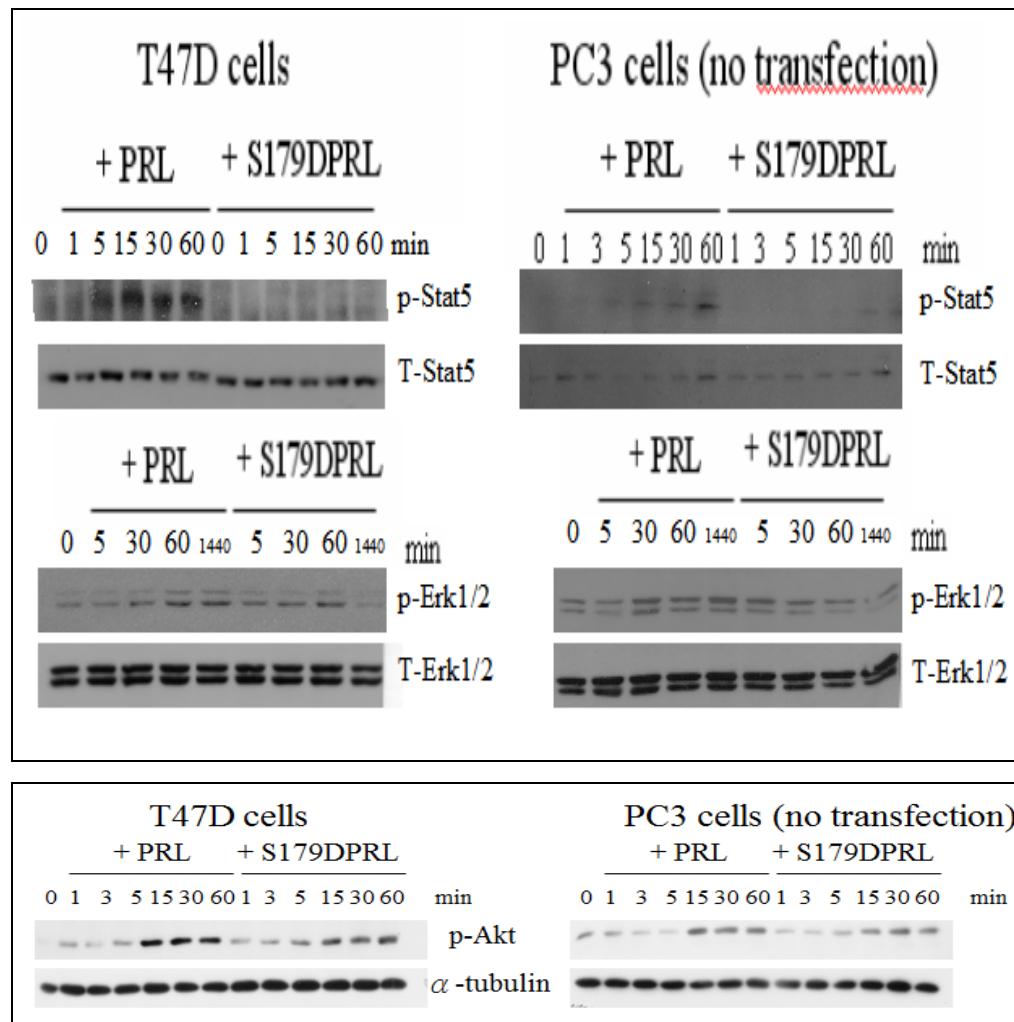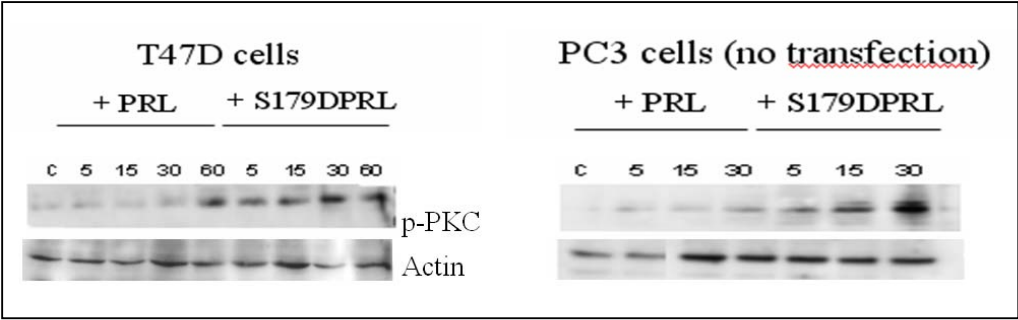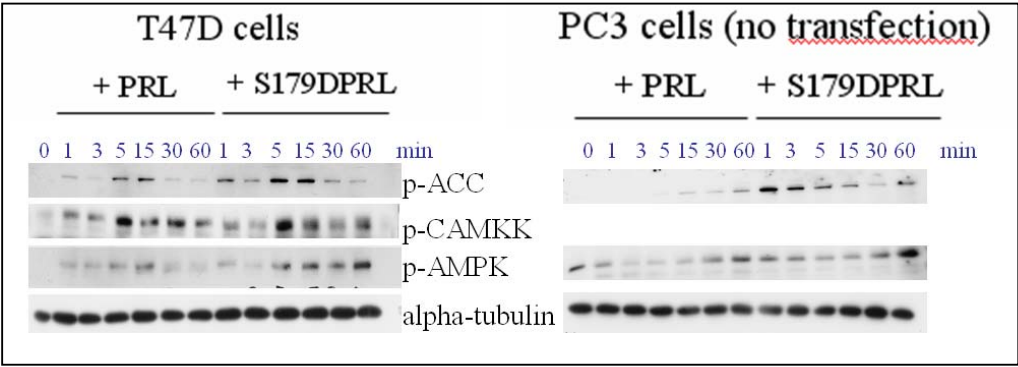
Fig. 2.8 pStat5 formed a complex with endogenous BRCA1.

Cells were seeded in 10cm dishes and treated with 500ng/mL prolactin or S179DPRL for 30min. The total protein lysates were then immunoprecipitated with anti-BRCA1 and immunoblotted with anti-p-Stat5. As shown in Fig. 2.8, either control conditions (autocrine prolactin exists) or exogenous prolactin showed a complex formation between BRCA1 and Stat5.
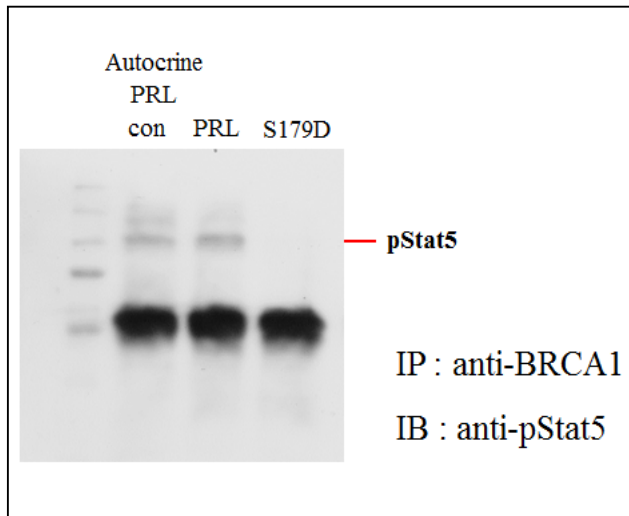
Fig. 2.9 The BRCA1-pStat5 complex mostly appeared in the nucleus.

Cells were seeded in 10cm dishes and treated with 500ng/mL prolactin or S179DPRL for 30min. Cytosolic and nuclear fractions were then isolated and precipitated with BRCA1 antibody followed by immunoblotting with anti-p-Stat5. Most of the complex was sequestered in the nucleus.
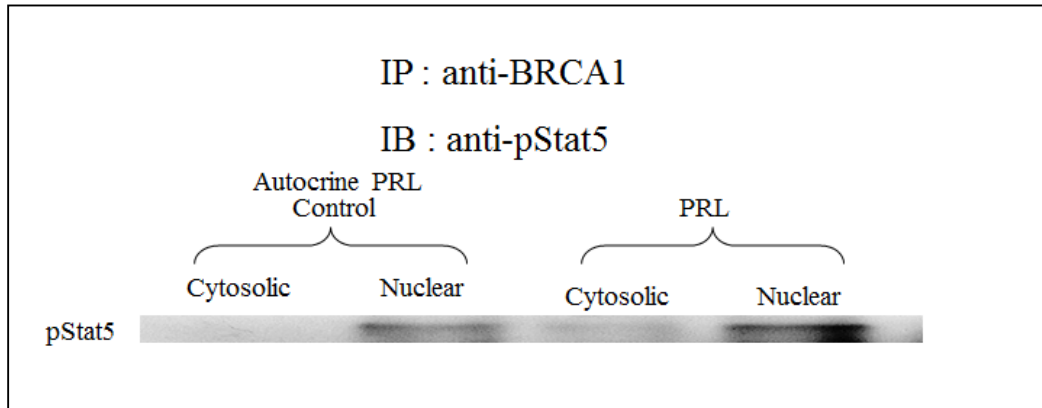
Fig. 2.10 BRCA1 was still capable of interacting with the p21 promoter after complexing with p-Stat5a.

Cells were seeded in 10cm dishes and treated with 500ng/mL prolactin or S179DPRL for 20 hours. Chromatin IP was performed and there was no difference in the interaction between BRCA1 and the p21 promoter with either S179DPRL or prolactin.
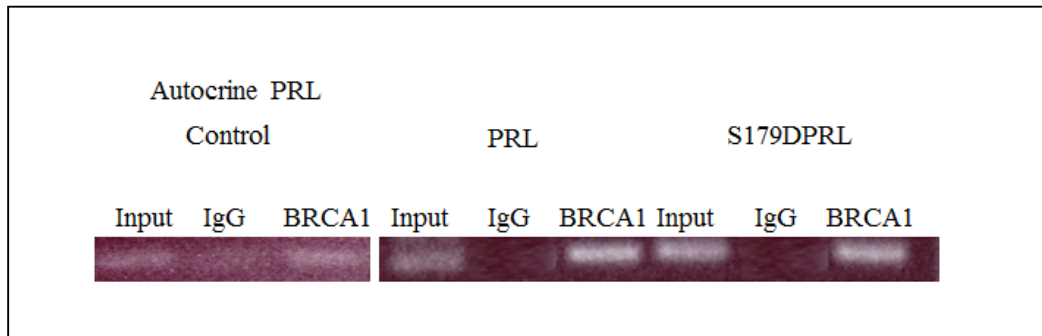
Fig. 2.11 Prolactin was able to induce p21 in the presence of a dominant negative form of Stat5a.

Cells were seeded in 6 well plates and transfected with the minimal (143bp) p21 promoter luciferase construct, different forms of Stat5a and the β gal plasmid. Cells were then treated with 100ng/mL prolactin or S179DPRL for 20 hours and luciferase and β-gal readings were recorded. In this figure, prolactin alone could not drive the p21 promoter, but S179DPRL showed an increase in p21 promoter activity. In opposite, when overexpression of dominant negative form of Stat5a , the ability of prolactin to drive p21 promoter was restored.
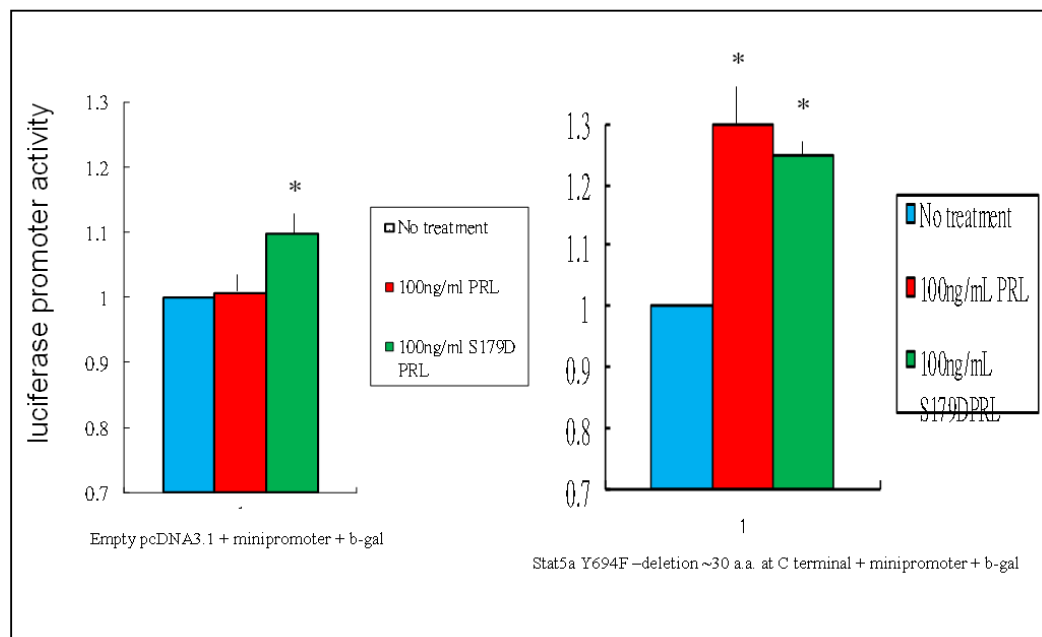
Fig. 2.12 Induction of miRNA that acted on the 3'UTR of p21 mRNA.

Cells were seeded in 6 well plates and transfected with p21-3'UTR reporter plasmid. Cells were then treated with 500ng/mL (100ng/mL worked) prolactin or S179DPRL for 20 hours. Both luciferase and β-gal readings were recorded. Results shown here indicated that miRNA(s) was produced and targeted to the 3'-UTR of p21 by prolactin treatment and that there was less miRNA produced in response to S179DPRL.
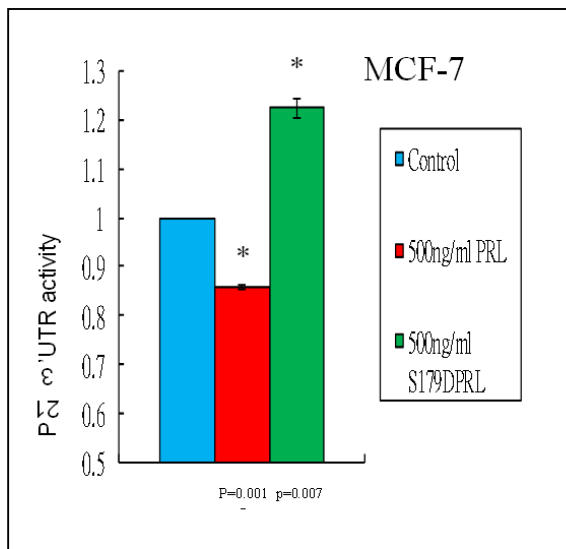
Table 1   Summary of miRNA microarray.

Legend:
- 🟩 Decreased expression (at least 1.5 fold)
- 🟨 Increased expression (at least 1.5 fold)
- ⬜ Increased expression (less than 1.5 fold)

miRNA Array

miRNA	P	S	miRNA	P	S	miRNA	P	S	miRNA	P	S	miRNA	P	S
mir-9-1			mir-30b			mir-135b			mir-188			mir-219		
mir-10b			mir-30c			mir-136			mir-190			mir-221		
mir-17-3			mir-92			mir-137			mir-191			mir-222		
mir-22			mir-92b			mir-140			mir-196a			mir-296		
mir-23a			mir93			mir-141			mir-196b			mir-372		
mir-24			mir-95			mir-142			mir-197			mir-373		
mir-26a			mir-101			mir-149			mir-198			mir-488		
mir-26b			mir-103			mir-150			mir-200b			mir-100		
mir-27a			mir-106a			mir-151			mir-202			mir-127		
mir-27b			mir-106b			mir-153			mir-203			mir-142		
mir-29a			mir-107			mir-154			mir-205			mir-31		
mir-29b			mir-128a			mir-181d			mir-210			mir-213		
mir-29c			mir-128b			mir-183			mir-214					
mir-30a-3			mir-132			mir-185			mir-215					
mir-30a-5			mir-134			mir-186			mir-218					

|	Potential Targets
miR 24	p16, ALK4, DHFR
mir-92a	ER β
mir-92b	ER β
mir-106b	p21
mir-107	HIF-1β
mir-141	FGFR2
mir-142	
mir-149	Akt1, E2F1, b-myb
mir-214	plexin-B1
mir-215	thymidylate synthase
mir-100	mTOR signaling
mir-127	BCL6

Fig. 2.13 DRB assay to determine the stability of p21 mRNA.

Both PC3 and MCF-7 cells were seeded in 6 well plates. The next day, cells were treated with 100ng/mL prolactin or S179DPRL in the presence of 40μM transcriptional inhibitor, 5,6-dichlorobenzimidazole riboside(DRB) and total RNA was isolated at 2, 4, 6 hours. As shown in this figure, no difference between prolactin and S179DPRL treatment on the stability of p21 mRNA was seen.
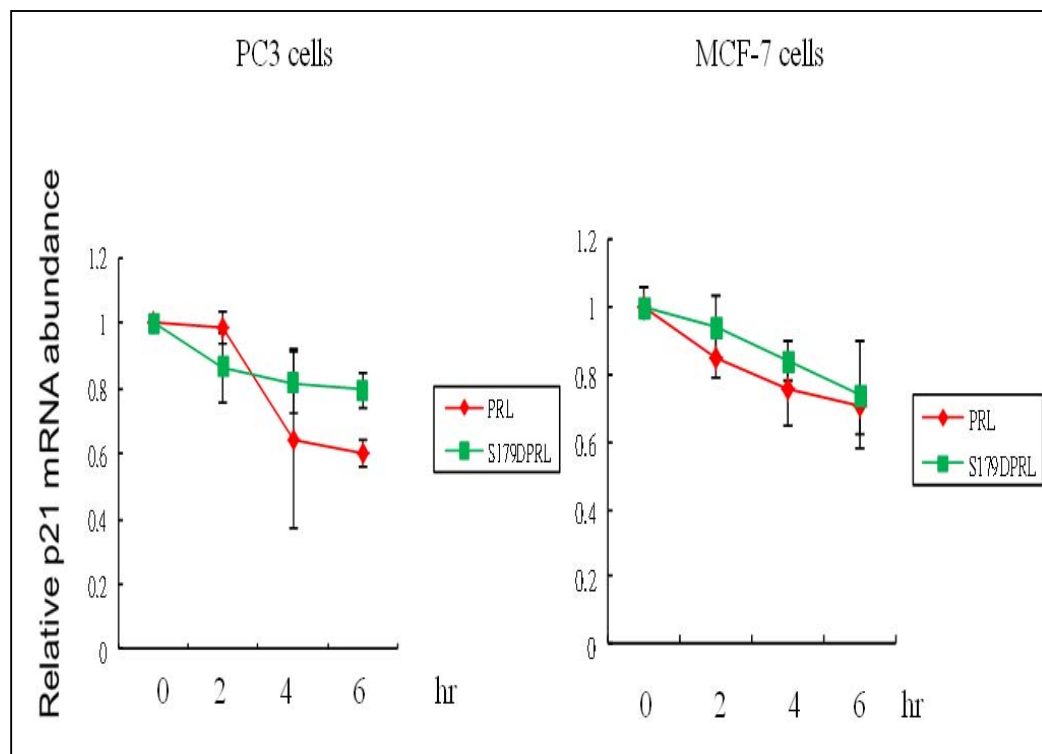
Fig. 2.14 miRNA(s) acting on the 3'UTR of p21 was regulated by the expression of myc.

Cells were seeded in 6 well plates and transfected with the p21-3'UTR reporter plasmid, β-gal plasmid with or without a myc expression plasmid. With overexpressed myc, there was a great decrease in luciferase expression, indicating a huge amount of miRNA(s) produced targeted to the 3' UTR of p21.
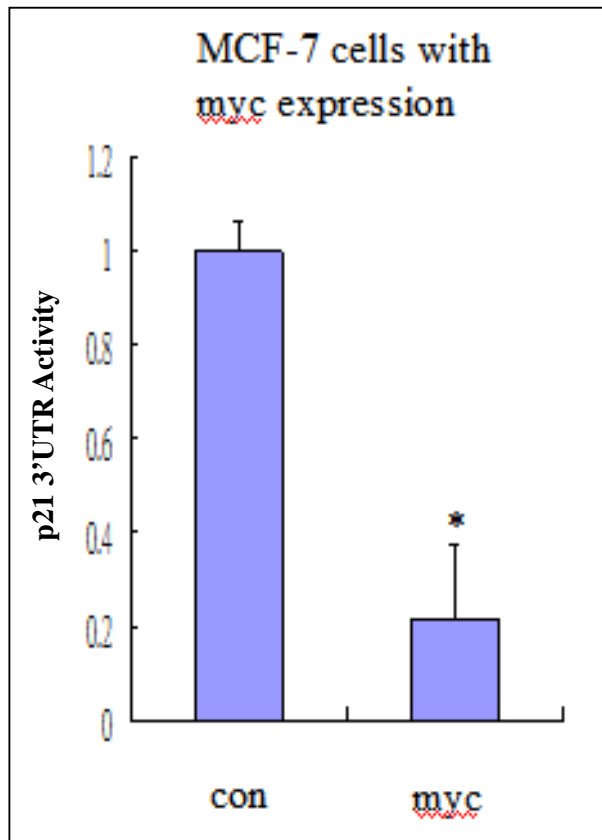
**Fig. 2.15 Prolactin activates estrogen receptor in a ligand independent manner.**

Cells were seeded in 6 well plates and treated with 100ng/mL prolactin or S179DPRL for 1 hour. Cells lysates were then collected using RIPA lysis buffer and the phosphorylation of estrogen receptor α (ERα) on Serine 118 was analyzed by western blot. Result below showed prolactin caused a phosphorylation on serine 118 of ERα but S179DPRL did not.
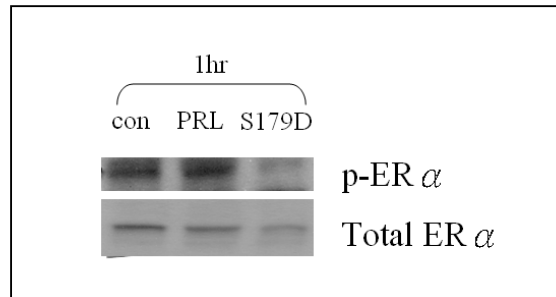
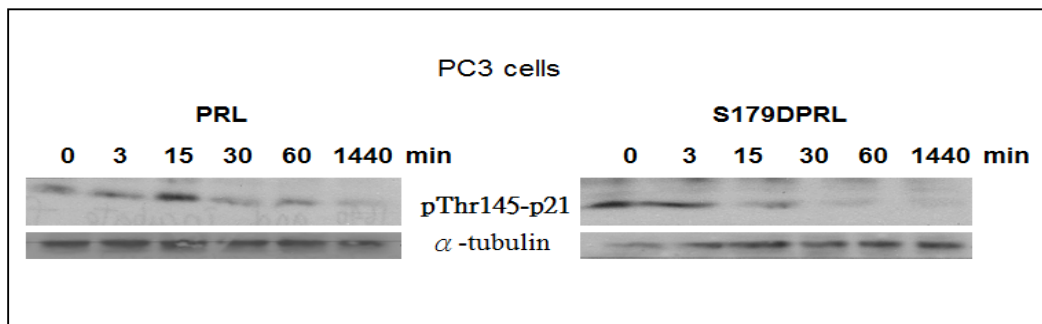**Fig. 2.16 S179DPRL caused a rapid decrease in c-myc within 1 hour.**

Cells were seeded in 6 well plates and treated with 100ng/mL prolactin or S179DPRL for 1 hour. C-myc expression was analyzed using western blot analysis. Data shown here indicated that prolactin (autocrine or exogenous) could maintain a certain level of myc expression which maintained miRNA expression acting on the 3'-UTR of p21.
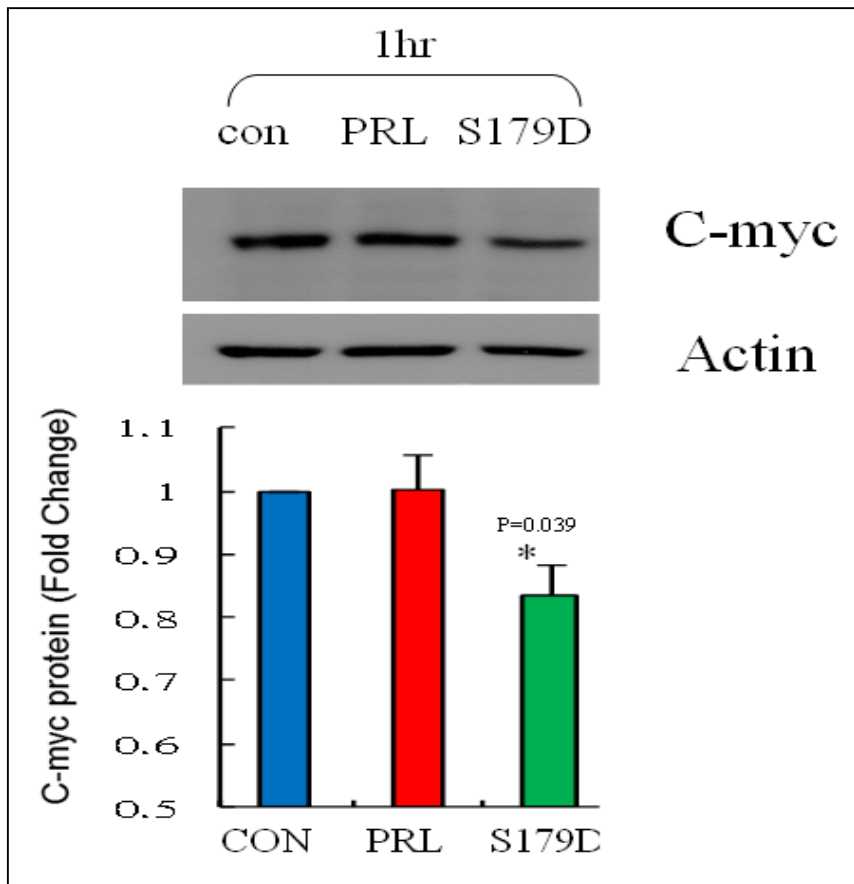
Fig. 2.17 Prolactin led to p21 phosphorylation at threonine 145, however, S179DPRL did not share this ability.

Cells were seeded in 6 well plates and treated with 100ng/mL prolactin or S179DPRL for 30min. The p-p21 expression was analyzed by western blot analysis. Prolactin caused a phosphorylation of p21 at threonine 145, peaked at 15min and could also last for 24 hr, in the other hand, S179DPRL showed a pattern with gradually decrease in p-thr145-p21 which was almost disappeared as fast as 30min.

Fig. 2.18 Prolactin caused p21 retention in the cytosol while S179DPRL caused nuclear retention.

Cells were seeded in 6 well plates and treated with 100ng/mL prolactin or S179DPRL for 30min. The cytosolic and nuclear fractions were isolated and p-p21 distribution was analyzed by western blot analysis. In this figure, the total p21 (combined the cytosolic and nuclear part) was equal among the three treatments. With control (the autocrine prolactin) or Prolactin treatment (exogenous prolactin), the p21 was phosphorylated and retained in the cytosol. As a result, most p21 in the cytosol upon prolactin treatment was p-p21. By contrast to what was seen in the prolactin treated group, S179DPRL did not cause a phosphorylation and therefore most p21 in the nucleus with S179DPRL treatment was non-phosphorylated.
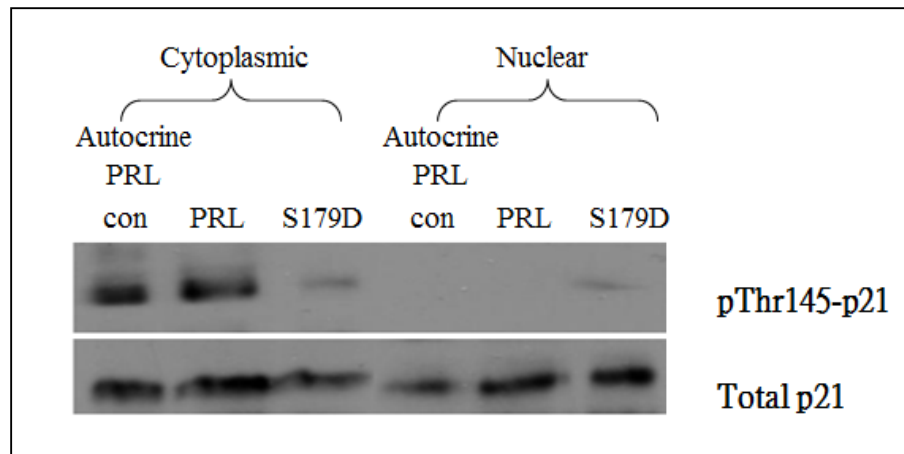
Fig. 2.19 PKA did not involve in p21 phosphorylation in response to prolactin.

Cells were seeded in 6 well plates and treated with 100nM PKI, inhibitor peptide of PKA, for 90 min followed by 100ng/mL prolactin treatment for different time course. Results here show prolactin caused p21 phosphorylation at threonine 145 and peaked at 15 min which was not affected when pretreated cells with PKI.
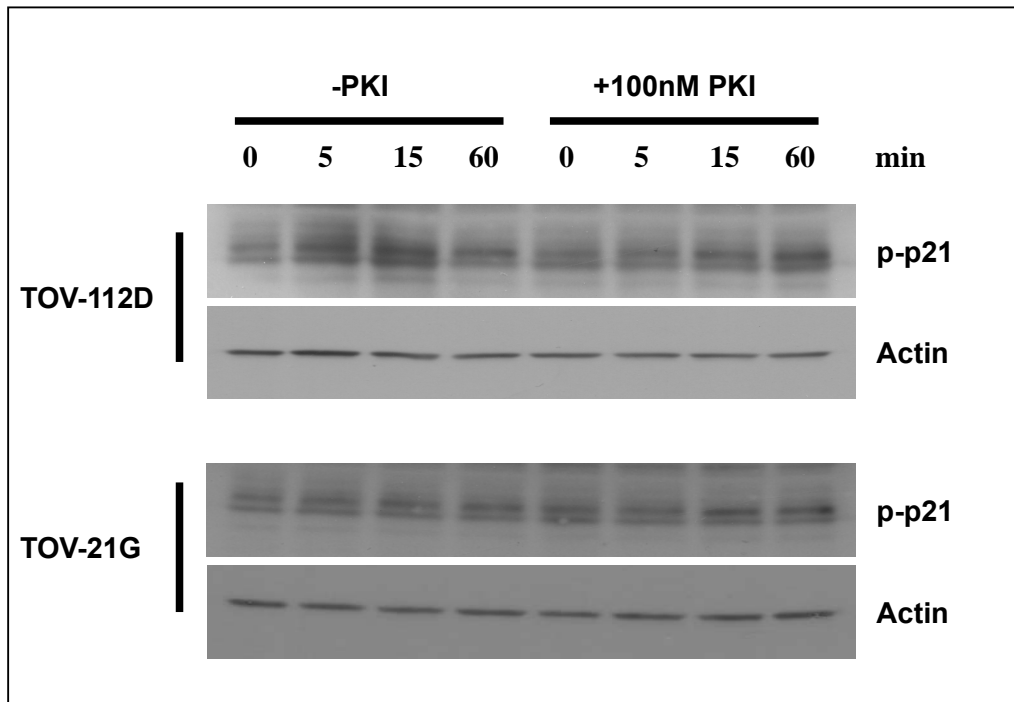
Fig. 2.20 Neither prolactin nor S179DPRL had an effect on p21 protein stability in the short term.

Cells were seeded in 6 well plates and treated with MG132 for 2 hours followed by 30min of treatment with 100ng/mL prolactin or S179DPRL. The short time frame was preferred since S179DPRL-driven p21 expression in the longer period might interfere with observation of protein stability. Data presented here showed no difference in p21 protein stability after 30min treatment with either prolactin or S179DPRL.
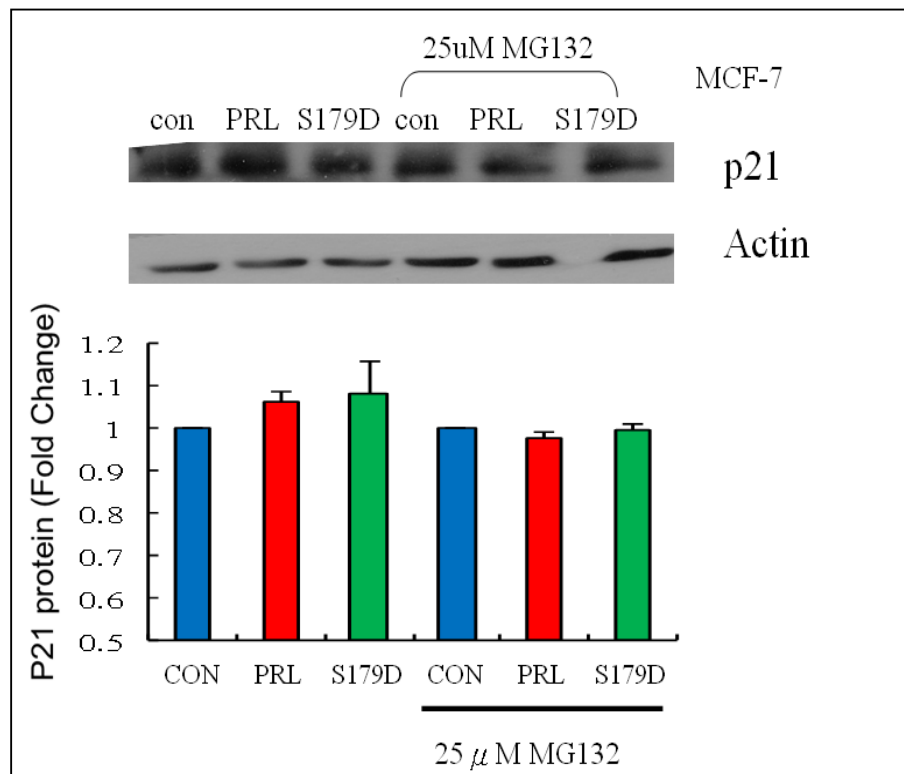
Fig. 2.21 proposed model

Prolactin activates Stat5a which in turn forms a complex with BRCA1 leading to a downstream p21 transcription failure. Prolactin also causes p21 phosphorylation leading to a cellular redistribution of p21 to the cytosol. Furthermore, the activation of ERα by prolactin maintains the expression of myc which is crucial for the expression of miR-106 and therefore further represses the translation of p21 mRNA. The action of miR-106 was not involved in p21 mRNA degradation but translational repression. Lastly, prolactin has no influence on short term p21 protein stability.
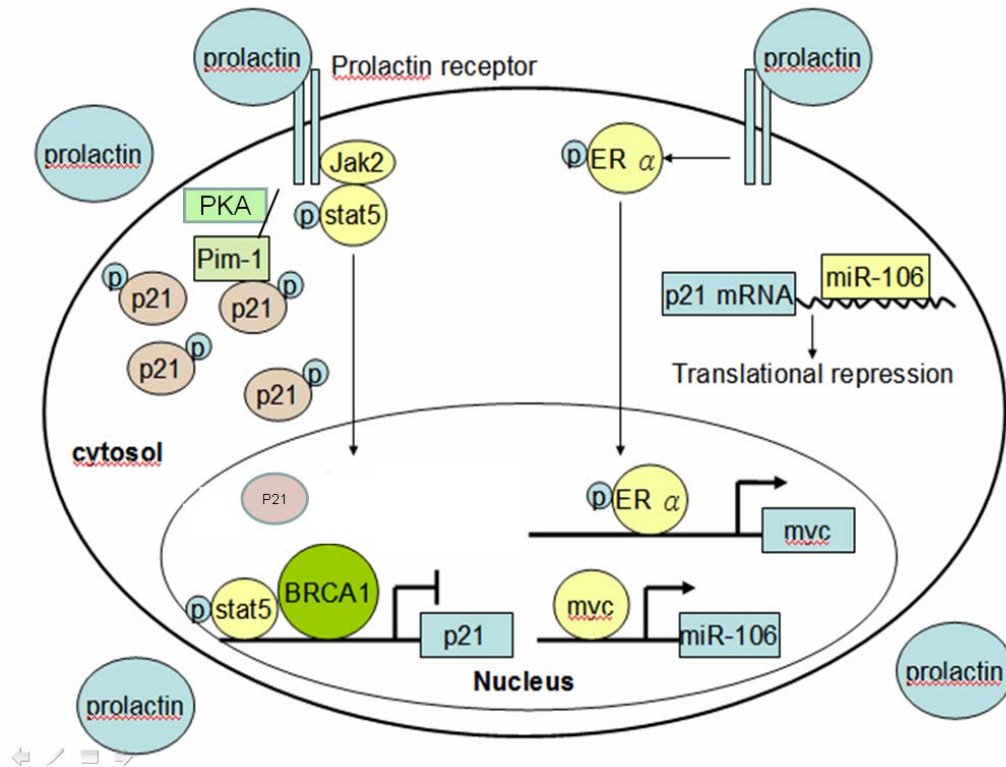
Chapter 3

A bioassay to quantify serum phosphorylated prolactin.

Introduction

Cancer progression has been linked to many hormones. Among these, prolactin has been shown to be related to at least tumors of the breast, prostate and ovary (Jacobson et al., 2011; Levina et al., 2009; Vonderhaar, 1999). In breast cancers, there are several studies showing a correlation between the level of prolactin and the risk of breast cancer (Hankinson et al., 1999; Holtkamp et al., 1984; Reynolds et al., 1997). In addition, Holtkamp's study showed that ~44% of patients with metastatic breast disease were hyperprolactinemic (Holtkamp et al., 1984). In prostate cancers, the level of prolactin also rises to abnormally high levels with metastasis (Lissoni et al., 2005). In ovarian tumors, it is found that women with a family history of ovarian tumors have a higher level of serum prolactin (Levina et al., 2009; Mor et al., 2005). Thus, the level of serum prolactin may play an important role in cancer progression. Prolactin is produced by the pituitary in a variety of forms, with the most important and abundant forms being unmodified and monophosphorylated forms (Oetting and Walker, 1986; Tuazon et al., 2002). Unmodified prolactin stimulates cell growth and proliferation, while phosphorylated prolactin inhibits cell growth and promotes differentiation (Wu et al., 2003). Since phosphorylated prolactin feeds back on the

pituitary to limit further secretion and states of elevated prolactin are characterized by larger proportions of unmodified prolactin (Johnson et al., 2003; Walker, 2007), we hypothesize the ratio of unmodified prolactin to phosphorylated prolactin is important in cancers and might serve as a predictor of likely progression.

Human prolactin is primarily phosphorylated at the serine 179 position (Tuazon et al., 2002). The region around the phosphorylated serine 179 is absolutely conserved in most species, and therefore is not antigenic. This has precluded the development of antibody-based methods of quantification of unmodified versus phosphorylated based on the peptide sequence that is modified. The peptide sequence in the chicken is slightly different (Luck et al., 1989) and this difference was enhanced by the addition of two more amino acids to the human sequence peptide before using as an antigen. This did produce an antibody that recognized phosphorylated prolactin somewhat differently from the unmodified form, but insufficiently differently to use as a clinical assay. In addition, earlier efforts using chromatography and mass spectrum methods did not produce a reliable assay. It has therefore remained difficult to measure the amount of phosphorylated prolactin. However, earlier studies showed that a molecular mimic of naturally phosphorylated prolactin, S179DPRL, induced the cell cycle inhibitor protein, p21 (Wu et al., 2005), while the unmodified version had no effect.

Results in an earlier chapter indicated that the induction of p21 by S179DPRL is BRCA1 dependent. BRCA1 is a tumor suppressor that transactivates p21 expression when cells are damaged. When the BRCA1 response element was removed from the p21 promoter, there was no response to S179DPRL. We therefore determined the minimal promoter containing BRCA1 and a basic TATA element derived from the p21 promoter that was responsive to phosphorylated/S179DPRL (see chapter 2). This minimal promoter was linked to a luciferase reporter with the idea that there would be luciferase activity with phosphorylated/S179DPRL, but no activity with unmodified prolactin.

Methods and Materials

Cell Culture , Stable cell lines and Treatment (For details, see methods and materials in chapter 2)

Transient transfection: PC3 and MCF-7 cells were cultured in RPMI1640 supplemented with 10% FBS. Cells were seeded in 6 well plates (see seeding information in chapter 2) and were transfected with the minimal p21 promoter construct (143 bp p21 promoter) and β-galactosidase (β-gal) plasmid as control the next day. Medium was changed to 10% FBS RPMI1640 with different concentrations of S179DPRL after 4 hours of transfection and then incubated for a further 20 hours. Cell lysates were then collected and both luciferase and βgal assays were performed. Results obtained from the luciferase assay were normalized to the β-gal reading.

Stable cell line establishment: On the minimal p21 promoter plasmid, there is a gene encoding for aminoglycoside phosphotransferase that confers resistance to aminoglycoside antibiotics, such as geneticin (G418). Taking advantage of this gene included in the plasmid, PC3 and MCF-7 cells were selected in 10% FBS RPMI1640 containing 800 ng/mL and 400ng/mL G418 (Geneticin), respectively. Medium was

changed every 2 days during the selection process. After selection, cells were maintained in medium with half the selection concentration of G418 (i.e. 400ng/mL and 200ng/mL for PC3 and MCF-7, respectively).

Dose Response and sensitivity

Transiently transfected cells or stable cell lines were incubated in 10% FBS RPMI1640 containing 0, 0.01, 0.1, 1, 10 , 100 ng/mL S179DPRL for 20 hours. The cell lysates were collected then and luciferase activity was analyzed.

Assay Precision

Inter- and intra-assay variation was assessed and the coefficient of variation was calculated. For intra-assay variation, 6 duplicated samples of each concentration of S179DPRL were analyzed on the same day and the results compared. For inter-assay variation, replicate assays were performed on different days and the coefficient of inter-assay variation was calculated.

The coefficient of variation was calculated as follows:

Standard Deviation of the means of the duplicates   X 100%

Grand Mean of the Duplicates

A figure equal to or less than 10% is considered satisfactory (Murray and

Lawrence, 1993).

Results

<u>Sensitivity of the minimal promoter assay</u>

As described in chapter 2, the minimal p21 promoter of 143bp is responsive to S179DPRL, but not unmodified prolactin at a dose of 100ng/mL. Since the general concentration of phosphorylated prolactin in human serum is often only several ng/mL, we sought to determine the actual sensitivity to S179DPRL. PC3 cells were transiently transfected with the minimal p21 promoter and treated with different doses of S179DPRL. As shown in Fig. 3.1, the transiently transfected PC3 cells responded to S179DPRL at concentrations as low as 1ng/mL. The dose-response curve was best between 1ng/mL and 10ng/mL, the response slowed between 10ng/mL and 100ng/mL and became saturated when the concentration of S179DPRL was higher than 100ng/mL. We then established the stable PC3- and MCF-7-derived cell lines. The dose response curves of the stably transfected cell lines are shown in Fig. 3.2. In these cell lines, the overall response was greater and the lowest concentration of S179DPRL that produced a response went down further to 100 pg/mL with both cell lines.

Standard Curve

Based on the dose response represented in Fig. 3.1 and 3.2, a sharp response was found between the concentrations of 1ng/mL and 10ng/mL. We therefore narrowed down the dose region to see whether an ideal standard curve could be generated in PC3 cells in this dose range (Fig. 3.3).

Examination of the precision of this assay

In order to confirm that this assay is reliable and consistent, the intra-assay and inter-assay coefficients were determined. Fig. 3.4A shows the actual variation within one assay, while Fig 3.4B shows the actual variation among assays performed on different occasions.

Table 3.1 shows the intra-assay coefficient of variation under different concentrations of S179DPRL and table 3.2 shows the inter-assay coefficient of variation. The CV values demonstrate that the assay is reliable and consistent.

Discussion

The purpose of this study was to develop a method to quantifiy phosphorylated prolactin. Knowing that phosphorylated prolactin inhibits cell growth through induction of the cell cycle inhibitor protein, p21, and unmodified prolactin has no effect on p21, we aimed to utilize this knowledge to produce a bioassay that specifically recognized phosphorylated prolactin. In chapter 2 of this dissertation, we showed analysis of the p21 promoter and demonstrated that a 143bp region of the promoter was sufficient to generate a response to S179DPRL. The results shown in this chapter indicate that this promoter reporter-based assay is sensitive to S179DPRL as low as 100pg/mL. Extension of the incubation time with S179DPRL from 20 hours to 72 hours might increase the degree of response and/or the sensitivity of the assay. A sensitivity of 100pg/ml is more than sufficient for our needs, but the advantage of increased sensitivity would be the ability to dilute serum samples and hence lower the risk of interfering substances. Having removed most response elements from the p21 promoter, we have eliminated almost all possibility that there will be other substances in serum that will drive luciferase expression. However, the possibility remains that there is another entity in serum for which these cells have a receptor, activation of

which will elevate BRCA1. This can be tested by several rounds of immunoprecipitation of all prolactin out of serum and testing for any residual activity in the assay, and also by adding serum to known quantities of S179DPRL at the low end of the dose response curve to see if there is any change in the measured amount.

The stable cell lines were not derived from a single cell and so it remains possible that clones with even further increased responsivity are present in the population. This will be analyzed in future experiments, and a clonal cell line developed such as to reduce drift in the assay over time.

The purpose of this study was to develop a method to quantify serum phosphorylated prolactin and then to calculate the ratio of serum unmodified prolactin versus phosphorylated prolactin. This could be done by using ELISA to determine total prolactin and subtracting the amount of phosphorylated prolactin. The real concentration of unmodified prolactin would therefore be the difference.

This promoter-based assay method is not a direct measurement of the physical amount present in serum, but is better inasmuch as it measures biological activity. By quantifying the amount of total and phosphorylated prolactin we can determine whether the ratio of unmodified to phosphorylated prolactin is predictive of disease progression. In addition, we can learn more about the biological function of

phosphorylated prolactin. We look forward to see whether there is any correlation between the ratio of unmodified prolactin to phosphorylated prolactin and different stages of human breast or other cancers.

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Fig. 3.1 Dose response of transiently transfected PC3 cells to S179DPRL.

PC3 cells were transiently transfected with the minimal p21 promoter and the control β-galactosidase plasmid. Cells were then treated with different doses of S179DPRL and cell lysates were collected after 20 hours incubation with S179DPRL. The results shown here were normalized to the β-gal values. In this figure, the sensitivity was as low as 1ng/mL.
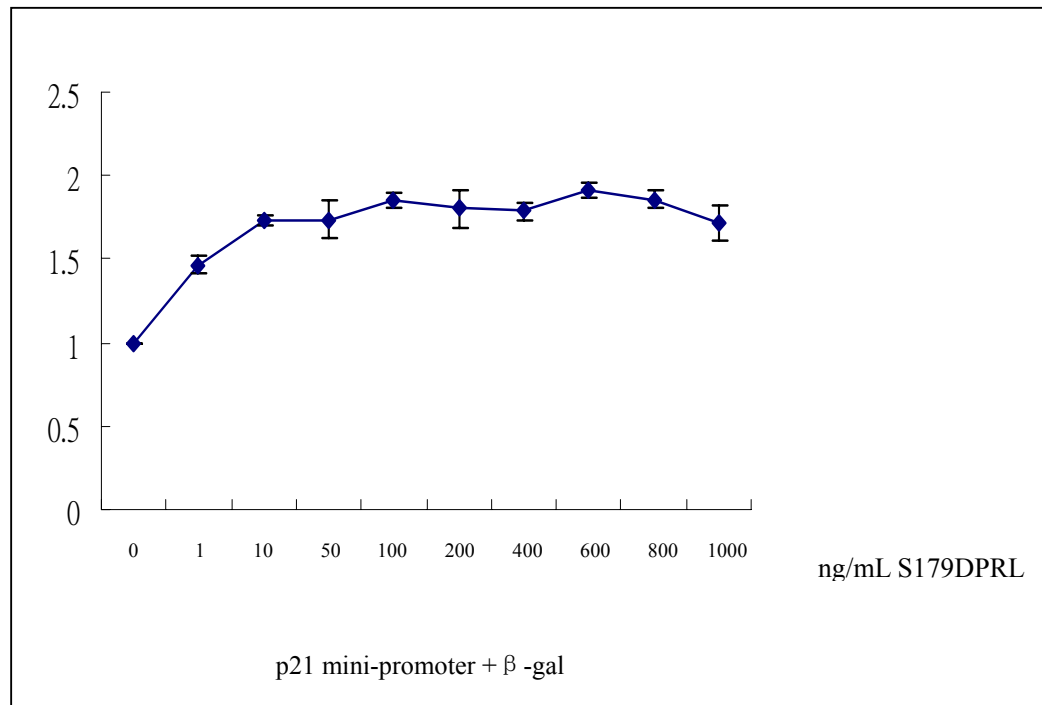
Fig. 3.2 The dose response curve of stably transfected PC3 (A) and MCF-7 (B) cells.

The stable cell lines, (A) PC3 cells and (B) MCF-7 cells treated with different doses of S179DPRL and cell lysates were collected after 20 hours incubation with S179DPRL. The magnitude and sensitivity of the stable cell lines was much improved than the transiently transfected cells.
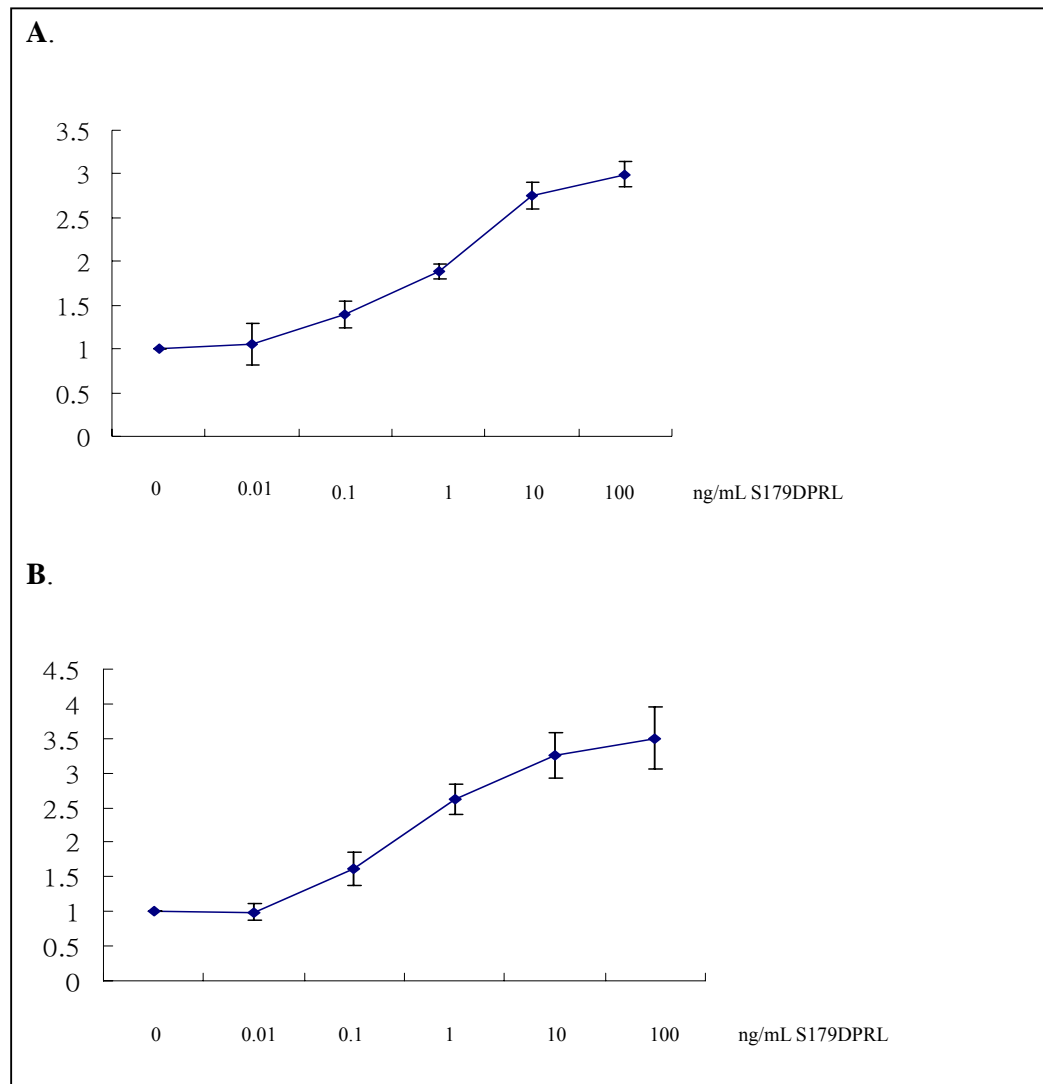
Fig. 3.3 Standard curve in PC3 cells.
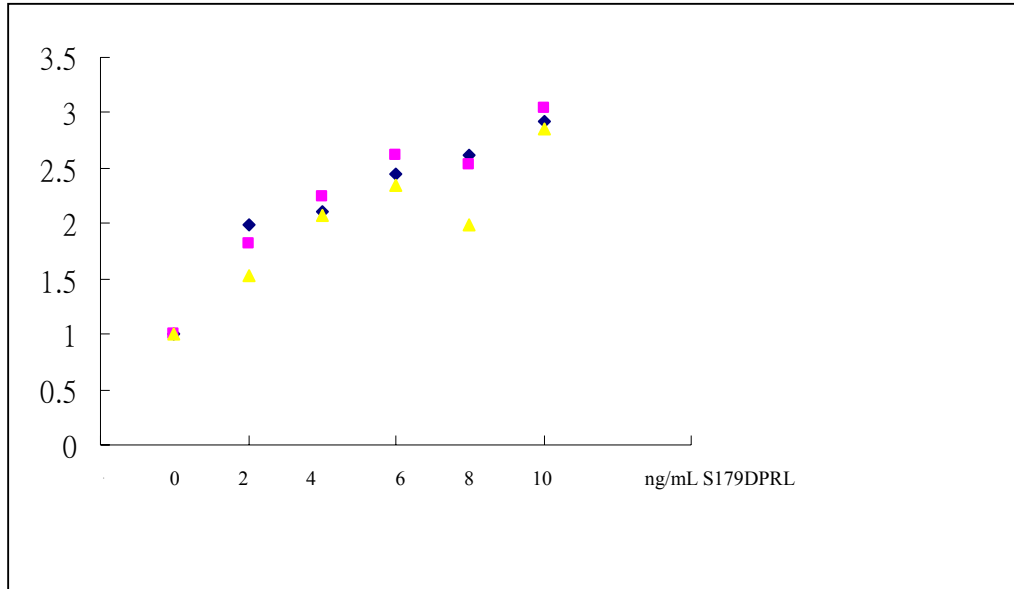
Fig. 3.4 Intra-assay (A) and inter-assay (B) in PC3 cells.

(A) Six replicates were performed and the luciferase induction with different doses of S179DPRL stimulation was determined on the same day. (B) The inter-assay presented here were results generated from trials on different days.
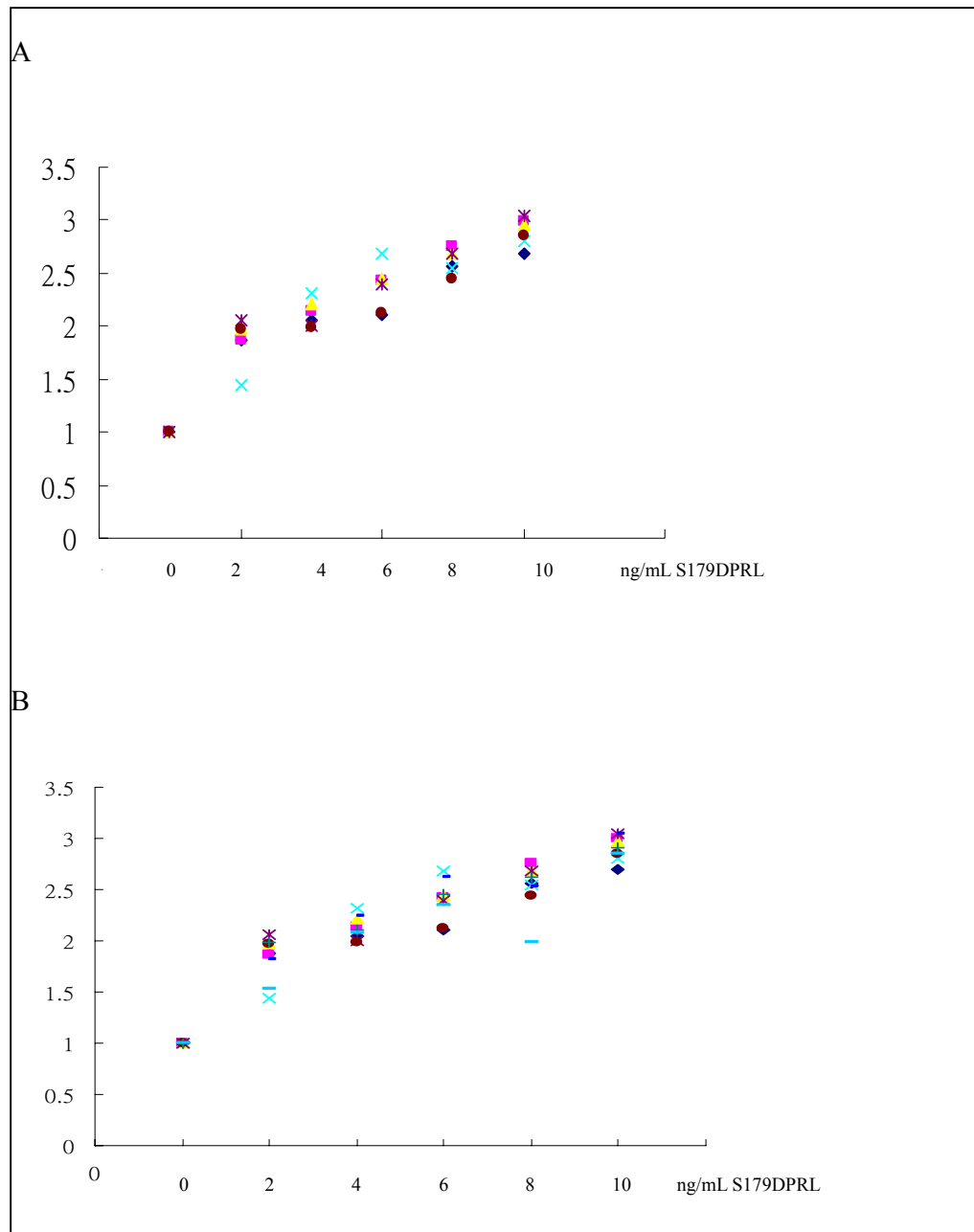
Table 3.1 Intra-assay Coefficient of variation at different doses of S179DPRL in PC3 cells.

Intra-assay Coefficient of variation in response to different doses of S179DPRL.						
Concentration of S179DPRL (ng/mL)	0	2	4	6	8	10
Coefficient of Variation	N/A	11.61	6.02	9.37	4.44	4.57
Average of Coefficient of Variation for intra-assay : 7.202						

Table 3.2 Inter-assay Coefficient of variation in response to different doses of S179DPRL in PC3 cells.

Inter-assay Coefficient of variation in response to different doses of S179DPRL.						
Concentration of S179DPRL (ng/mL)	0	2	4	6	8	10
Coefficient of Variation	N/A	12.23	5.04	7.57	9.05	3.93
Average of Coefficient of Variation for inter-assay : 7.564						

Chapter 4

Potential Roles of miR-106a in Breast Cancer

1.Introduction

The discovery of interfering RNAs uncovered a new level of regulation of gene expression. It is now believed that as much as 92% of gene expression may be regulated by interfering RNAs. Interfering RNAs may be micro RNAs (miRNAs) or small interfering RNAs (siRNAs). Our focus is on miRNAs. These are mostly coded in intronic or intergenic regions of DNA and are grouped into families on the basis that they likely evolved from a common ancestral gene. Among the miRNA families, the miR17-92 family has attracted attention because of its oncogenic activity. miRNAs in this family include the miR17-92 cluster and two paralogs, the miR-106a and miR-106b clusters. Expression of these miRNAs is markedly upregulated in several types of cancer, and they are considered oncomirs. The two paralogs derive from an ancient gene duplication event involving the miR17-92 cluster. They therefore share highly similar sequences with miR17-92 family members and each other. As a result, they also work on very similar targets, primarily inhibiting the translation of target mRNAs by binding to the 3' untranslated region. The miR-106 paralogs are located on different chromosomes from the miR17-92 cluster: miR-106a is intriguingly located on the X chromosome, miR-106b on chromosome 7, and

miR17-92 on chromosome 13. Regulation of expression of any of the paralogs can therefore occur without concomitant regulation of the other two. This review examines the thesis that miR-106a in particular may play an important role in the development and progression of breast cancer. Because relatively little attention has yet to be given to miR-106a, the potential role of miR-106a is often suggested on the basis of a known role of a related family member. Similarly, defined roles of miR-106a and family members in other neoplasms are used to suggest a role in breast cancer.

2. Small Interfering RNAs

Interfering RNAs are small ribonucleic acids around 18-25 nucleotides in length. Depending on the author, between 60 and 92% of human genes are likely regulated by these small RNAs (Baek et al. 2008, Dai and Ahmed 2011). Interfering RNAs may be microRNAs (miRNAs) or small interfering RNAs (siRNAs). Both share a similar mechanism of action, but differ in their initial cellular processing. miRNAs are usually encoded by intergenic or intronic regions of DNA, but may be present in exonic regions of non-protein-coding genes or of protein coding genes subject to

alternate splicing (Rodriguez et al. 2004 , Kim et al. 2009). In the classical scheme for

their production (Figure 4.1), miRNA regions of the genome are transcribed by RNA

polymerase II as longer sequences including a region that forms a hairpin or stem loop

(pri-miRNA). This is then processed by binding to DGCR8 (DiGeorge Syndrome

Critical Region protein 8) and cleavage by RNASEN (an RNAse III enzyme) to form

a pre-miRNA of about 70 nucleotides in length. The pre-miRNA is exported from the

nucleus by binding to exportin 5, which recognizes its double-stranded hairpin region.

Once in the cytosol, the pre-miRNA is subject to further cleavage by the dicer

complex. This removes the loop portion of the hairpin creating two complementary

strands of miRNAs. These two strands, along with dicer and a binding protein then

interact with Argonaute (Ago) to form RISC (RNA Induced Silencing Complex). One

of the complementary strands is released and degraded. The other, now a

single-stranded miRNA, is able to bind to its target sequence. At this point, the degree

of complementarity between the miRNA and its target sequence determines whether it

functions to inhibit translation or promote the degradation of mRNA. The less the

complementarity, the more likely it will function to inhibit translation without effect

on the level of mRNA. With greater complementarity, miRNAs function more like

siRNAs and promote mRNA degradation (Lee et al. 1993, Bartel 2004, Carthew and

Sontheimer 2009). To accomplish both of these endpoints, the miRNA binds to the 3'

untranslated region (UTR) of mRNAs (Yekta et al. 2004). Interaction with the 3'UTR

relies on a 7 nucleotide "seed sequence" present in the miRNA (see table 4.1).

An alternate pathway for miRNA synthesis exists in which splicing of small

intronic region (a microRNA intron region or mirtron region) out of pre-mRNA

creates a lasso-like structure (a pre-mirtron) that subsequently loses its branch to form

double-stranded pre-miRNA. This hairpin double-stranded pre-miRNA is then

handled in the same manner as the RNASEN-processed variety.

SiRNAs, by contrast, originate via viral infection or are introduced into a cell

experimentally. Either way, the cell gains long stretches of double-stranded RNA.

These are recognized and bound by specific binding proteins which initiate cleavage

by dicer into short 18-25 nucleotide lengths of double-stranded RNA that can interact

with Ago. This interaction results in the release and degradation of one strand and the

targeting of the specific complementary strand. Since SiRNAs have perfect

complementarity, they result in mRNA degradation rather than inhibition of

translation.

Having discussed the differences and similarities between these two forms of

interfering RNA, focus is now on miRNAs. Although several miRNAs have been

proposed to be of importance in breast cancer, the purpose of this review is to draw

attention to the potential role of miR-106a.

3. The miR-106a Cluster (paralog to miR-106b and miR-17-92 clusters)

To date, the best studied miRNAs implicated in carcinogenesis are in the

miR-17-92 family. This family consists of six members : miR-17, miR-18a, miR-19a,

miR-20a, miR-19b, and miR-92a. They are all transcribed from the same

polycistronic cluster, the miR-17-92 cluster on chromosome 13. In addition in

mammals, there are two paralogs, the miR-106b-25 cluster on chromosome 7, and the

miR-106a-363 cluster on the X chromosome. These resulted from gene duplications

of the miR-17-92 cluster during evolution. As mentioned earlier, miRNAs interact

with the 3'UTR of target mRNAs through their seed sequence; hence miRNAs with

the same seed sequence may share the same targets. Based on homology of the seed

sequences, miRNAs in these paralogous clusters can be grouped into four different

families, miR-17,miR-18, miR-19 and miR-92, as shown in table 4.1.

According to this grouping, miR-106a, for example, may target the same

mRNAs as miR-17,miR-20a, miR-20b, miR-106b and miR-93. Tanzer et al.(2004)

analyzed the evolutionary history of these miRNAs, a history based on the seed sequence. Interestingly, while an ortholog of the miR-17-92 seed sequence family occurs in Drosophila and C. elegans, both the miR-17 and miR-19 seed sequence families seem to be vertebrate innovations. Moreover, miR-106a seems to exist only in mammals; it was found in mouse, rat, human, and chimp, but not in any non-mammalian vertebrates tested. This raises the possibility of a specific role for miR-106a in mammals where one defining feature is the presence of mammae.

4. Regulation of miRNAs

4.1 Regulation of miRNA by Methylation

In addition to protein expression being regulated by miRNAs, formation of miRNAs can be regulated by hypermethylation. Thus, hypermethylation of CpG islands that encompass or are adjacent to miRNA regions can inhibit transcription, as can histone modification (Lehmann et al, 2008). In fact, the frequency of epigenetic regulation of miRNA regions on the genome is estimated to be about an order of magnitude greater than for protein-coding regions. The regions of miRs-124-1, 124-2, 124-3, 126, 141, 148a, 152, 199a-1, 199a-2, 200c, 34a, 663, and 9-1, previously

associated with breast cancer, are epigenetically modified, showing an established role for regulation of miRNAs by methylation in breast cancer. The miR-106a region has also been reported to be epigenetically modified in colon cancer (Kunej et al, 2011). Although not yet specifically examined, it is possible therefore that miR-106a is also epigenetically modified in breast cancer, becoming either hypo- or hyper-methylated.

4.2 Regulation of miR-106a by myc and Estrogen

In several cancers, upregulation of the oncogene, myc, is accompanied by the induction of many miRNAs, including several members from the miR-17-92, miR-106a-363, and miR-106b-25 clusters (O'Donnell et al. 2005). Evidence that myc directly regulated the expression of these miRNAs was produced by chromatin immunoprecipitation (ChIP). This showed that myc could interact with a fragment upstream of the miR-17-92 cluster. Though there were seven putative myc binding sites (CACGTG) upstream of the miR-106a-363 cluster, no interaction was found in the ChIP assay. However, the expression of miR-106a-363 was undetectable in their tested cell line, P493-6 B lymphoma cells. Castellano et al. (2009) expanded this

study to breast cancer cells and included upstream regulation by estrogen. With estrogen stimulation, expression of myc, and both miR-17-92 and miR-106a-363 clusters was upregulated. There is an estrogen receptor response element 70 bp upstream of the c-myc binding site on the miR-17-92 promoter. However, no detectable interaction between the estrogen receptor and this DNA region was observed. Expression levels of miR106a were too low to make this determination. For miR-17-92, this suggests that estrogen induction of myc preceeds myc induction of the miR-17-92 cluster. Although an indirect induction, it is nevertheless an important link between estrogen, a known oncogene, and the miR-17-92 cluster. miR-106a expression can also be negatively regulated in some cancers. As reported in monocytopoiesis, the transcription factor, acute myeloid leukaemia-1 (AML-1), also known as Runt-related transcription factor 1 (Runx1) can bind to the promoter region of the miR-106a-363 cluster and repress the expression of miR-106a (Fontana et al. 2007).

5. The Expression Pattern of miR-106a Correlates with Breast Tumor Development and Other Tumor Development

Table 4.2 illustrates the relative expression of miR-106a in tumors versus normal

tissue and then in metastasized versus non-metastasized tumors. As can be appreciated, as breast cancer progresses, expression of miR-106a increases. This is also true for several other tumors in which the analysis was carried through to the metastatic stage. Wang et al. (2010), for example, examined breast tumors, matching serum and adjacent normal tissue from patients and showed that miR-106a was consistently and significantly overexpressed in both breast tumors and matching serum samples. The expression was gradually increased as the stage of breast cancer progressed. In addition, the expression was higher in progesterone receptor negative versus positive cancers, as well as in estrogen receptor negative versus ER positive cancers (Wang et al. 2010). An interesting experiment was performed by Fassan et al. (2009) during which they compared the miRNA expression profiles in male and female breast cancer patients. When compared to female breast tumors, the expression of miR-106a in male tumor samples was downregulated, indicating there might exist a different regulation mechanism between male and female breast cancer, perhaps resulting from a different X chromosome complement (see below).

Macrophages play a dual role in tumor development, acting first to present tumor antigens to T cells that kill transformed cells, and later contributing to tumor progression in a number of different ways (Lamagna et al, 2006). miR-106a inhibits

monocyte and therefore macrophage development (Fontana et al 2007). This might be predicted to reduce initial clearing responses to transformed cells and therefore to increase the incidence of breast cancer.

6. Potential Significance of X Chromosome Location of miR-106a

Group B retroviruses, like the mouse mammary tumor, share a common integration site on the X chromosome (Mueller et al. 1992). This is close to the promoter region for the miR-106a cluster. As a result,there is elevated expression of miR-106a.

Irregardless of virus involvement, there are multiple studies indicating reactivation of the silenced X chromosome in breast cancer, particularly basal-like breast cancers (Richardson et al. 2006). Such reactivation could elevate expression of the miR-106a cluster. Some features of the inactive X chromosome (Xi) have been identified. These include hypermethylation of DNA and hypoacetylation of Histones 3 and 4. Reactivation of Xi would therefore have to reverse these features. As we will discuss later, it is interesting to note that miR-106a may target SUV420H1, a DNA methyltransferase, and BRMS1-L, a component of the histone deacetylase complex (HDAC). Downregulation of these two proteins by targeting their mRNA by

miR-106a would result in DNA hypomethylation and histone acetylation, thereby linking elevated miR106a to the possibility of X chromosome reactivation.

There is also another potential link between breast cancer and X reactivation, in this case related to BRCA1 functionality. Thus, BRCA1 has been reported to regulate Xist transcription from the X chromosome that should be inactive. When transcribed, BRCA1 then guides Xist to reinteract with and therefore re-silence the same chromosome (Ganesan et al., 2004; Ganesan et al., 2002; Silver et al., 2007). However, this is not a universal finding (Pageau et al., 2007; Xiao et al., 2007).

7. Potential Targets of miR-106a

Although miR-106a has not been extensively investigated, there are several ways in which reports connect it to an influence on tumor progression. From results derived from a miRNA target search, for example, over 700 potential targets for miR-106a were identified (Sinha et al., 2008). These include cell cycle regulatory proteins, and proteins that regulate apoptosis, angiogenesis, autophagy, metastasis, and drug resistance.

7.1 Involvement in Cell Cycle Regulation and Apoptosis

Using a miRNA target search engine, Sinha et al.(2008) proposed that miR-106a had up to 40 targets involved in the regulation of cell proliferation, and up to 44 targets involved in the regulation of apoptosis (Table 4.3). Among these targets, the best studied example to date is the tumor suppressor protein, retinoblastoma 1(RB1). RB is a tumor suppressor whose inactivation is involved at some stage in many cancers. Phosphorylation of the Rb protein blocks progression of the cell cycle from G1 to S phase. Inactivation of RB therefore has a proliferative effect. Several studies have shown upregulation of miR-106a was accompanied by downregulation of Rb in a number of different cancers (Zhou et al. 2010, Xiao et al. 2009, Volinia et al. 2006). In addition, RB attenuation also appears to be important in the development of resistance to anti-estrogens, including Tamoxifen (Boscoe et al. 2007, Lehn et al., 2011, Thangavel et al. 2011). Moreover, therapeutically activating RB has been shown to reestablish cell cycle control in endocrine therapy-resistant breast cancer (Thangavel et al. 2011).

Another important tumor suppressor is p21, also known as cyclin-dependent kinase inhibitor 1 (gene is CDKN1A on table 4.3). This also regulates cell cycle

progression between the G1 and S phase and contains several putative miR-106a sites

in its 3'-UTR. The importance of p21 specifically in breast cancer is currently unclear.

However, it is widely accepted that loss of function of p21, caused by mutations,

reduced expression, or abnormal cellular translocation, would promote breast cancer

progression (Trimis et al. 2008, Winters et al. 2003, Balbín et al. 1996). Also,

upregulation of miR-106a downregulates p21 expression, and transfection with an

antimir of miR-106a restores expression (Ivanovska et al. 2008). Thus, p21 expression

is clearly regulated by miR-106a even though direct demonstration of the use of the

putative 3' UTR sites has yet to be reported.

There is a complicated and highly regulated interplay among the many pro- and

anti-apoptotic proteins in a cell. Bim (gene called BCL2L11 in table 4.3) is a

pro-apoptotic molecule, involved in regulating anoikis in the normal developing

mammary gland to create a duct lumen (Whelan et al., 2010), as well as responses of

breast cancer cells to chemotherapeutics such as paclitaxel (Kutuk and Letai, 2010).

Early breast cancer is in many instances characterized by a duct lumen filled with

cells that have not undergone normal anoikis. Caspase 6 is the direct activator of

caspase 8 in the intrinsic pathway for initiation of apoptosis (Cowling and Downward,

2002). A reduction in expression of Bim, caspase 6 and caspase 8 brought about by

elevations of miR-106a would therefore be expected to reduce anoikis/apoptosis leading to increased cell number. Increased proliferation and decreased apoptosis also predict poor prognosis in recurrent breast cancers (Vakkala et al. 1999).

The activation of oncogenes usually induces cellular apoptosis or senescence as a protective mechanism (Li et al. 2009a, Maes et al. 2008b). In an activated ras oncogene model, it was shown that overexpression of the miR-106a-363 cluster abolished ras-induced senescence. With further deletion analysis, only miR-106a and miR-20b were essential for this function (Hong et al. 2010). The upregulation of miR-106a in cancer therefore might play an important role in inhibition of oncogene-induced senescence, allowing cancer cells to escape this anti-tumor defensive pathway.

7.2 Involvement in Metastasis /Differentiation of Tumors

As shown earlier in table 4.2, the expression of miR-106a increases with metastasis in breast cancer. This is also true of a number of other cancers and suggests a potential role for miR-106a in the metastatic process. Laminin 5 is a component of the basement membrane that mediates attachment of epithelial cells. Laminin 5 is a

direct target of the tumor suppressor, smad4, and increased laminin 5 increases cell

adhesion and reduces cancer cell migration (Zapatka et al. 2007). Moreover, epithelial

cell interaction with the basement membrane promotes mammary differentiation

(McCave et al. 2010). Overexpression of miR-106a down-regulates laminin 5 in the

breast cancer cell line, MCF-7, and with an antimir to miR-106a expression is

normalized (Wenrich et al. 2007). Thus, reduced laminin 5 is associated with reduced

differentiation and reduced cell adhesion to the basement membrane. However, if

laminin 5 is cleaved by matrix metalloproteases it becomes a tumor-promoting factor

that stimulates cell motility (Carpenter et al. 2009). Thus, the end effect of miR-106a

via laminin 5 will depend on the level of matrix metalloprotease activity.

BRMS1L (Breast Cancer Metastasis 1 Like) suppresses metastasis of human

breast cancer. It is a component of the mSin3a family of histone deacetylase

complexes (HDAC) and therefore suppresses transcription of genes (Meehan et al.

2004). As for the other examples, this protein has a potential binding site for

miR-106a on its 3'-UTR. Edmonds et al. (2009) investigated the miRNA expression

profile related to expression of the related protein, BRMS1, in breast cancer.

Unfortunately, miR-106a was not within their tested array. Given the binding site,

however, miR-106a may promote breast cancer metastasis through downregulation of

BRMS1-L. Other than this function to suppress metastasis, the related protein, BRMS1, has also been reported to be involved in maintaining sensitivity of breast cancer to chemotherapy (Vaidya et al. 2009).

The protein product of the ARID4A (AT Rich Interactive Domain 4A) gene has been reported to interact with the tumor suppressor proteins, BRMS1 and RB, and therefore to participate in tumor suppression (Hurst et al. 2008). As a predicted target of miR-106a, downregulation of this protein would be expected to promote breast cancer progression.

7.3 Involvement in Angiogenesis

The role of miR-106a in angiogenesis is hard to predict from the amount of information currently available. On the one hand, thrombospondin-1 (TSP-1) and connective tissue growth factor (CTGF/CCN2), both anti-angiogenic factors, are targeted by members of the same seed family and therefore would be predicted to be targeted by miR-106a. Downregulation of both contributes to endothelial cell migration and therefore tumor progression (Dews et al. 2006, Chien et al. 2011). On

the other hand, vascular endothelial growth factor (VEGF), one of the most important pro-angiogenic factors (Delli Carpini et al., 2010) also has putative binding sites for miR-106a on the 3'UTR. Hua et al. (2006) made a reporter construct by connecting the 3'UTR of VEGF downstream of a luciferase reporter and then co-transfected this construct into cells with different miRNAs reported to act on this 3'UTR. Among the miRNAs examined (miR-106a, miR-106b, miR-17, miR-20a, miR-20b, miR-150, miR-29b), miR-106a showed the greatest inhibition of luciferase expression (Hua et al. 2006). Further analysis will therefore be required to identify all counterbalancing activities in regard to miR-106a, angiogenesis and breast cancer. All that can be said at present is that both miR-106a and VEGF are increased as a function of breast cancer progression and hence that other factors must influence the interaction between miR-106a and the 3'UTR of VEGF mRNA. PRDM6 (PR/SET Domain Protein 6) is another angiogenesis-related potential target protein. High expression of this protein inhibits endothelial cell proliferation and differentiation (Wu et al. 2008). Down regulation of this protein by miR-106a may initiate breast cancer metastasis through promotion of both endothelial cell differentiation and proliferation.

7.4 Other Potential Targets in Breast Cancer

7.4.1 SUV420H1, a DNA Methyltransferase

DNA methylation governs the expression of genes and an abnormal epigenetic pattern may contribute to disease. DNA hypomethylation is associated with the worst stages of breast cancer (Soares et al. 1999), and the DNA methyltransferase, SUV420H1, is severely downregulated in human breast cancers (Tryndyak et al. 2006). As mentioned eariler, RB, which forms a complex with this methyltransferase, is also a target of miR-106a. Thus, an elevation of miR-106a would concurrently reduce expression of both RB and the methyltransferase, thereby enhancing hypomethylation.

7.4.2 Atg7 (Autophagy-related protein 7)

Autophagy, or self eating, is a lysosomal process that occurs in all cells in order to recycle the components of worn out organelles, to reduce unecessary organelles or cytoplasmic constituents when physiological demands change, or upon

cellular stress. Autophagy can serve as a tumor suppressor since defective autophagy provides an oncogenic stimulus, resulting in malignant transformation and spontaneous tumors (Dalby et al. 2010). At the same time, autophagy can function as a cell survival mechanism (Dalby et al. 2010). Atg7 (Autophagy-related protein 7) is a potential target of miR-106a. The effect of reduction in expression of Atg7, as assessed in a knockout mouse model, is increased cell survival (Xue et al. 2010), an effect that would be predicted to contribute to tumor progression.

7.5 Targets Related to Chemotherapy Resistance

Xia et al. (2008) investigated the correlation between miRNA expression and the development of drug resistance in gastric cancers. The data showed that miR-106a was downregulated in the vincristine (VCR)-resistant gastric cancer cell line, SGC7901/VCR (Xia et al. 2008). However, in human breast cancer doxorubicin-resistant MCF-7 cells, there was an upregulation of miR-106a (Kovalchuk et al. 2008). There were no further experiments performed regarding the functional role of this altered expression of miR-106a in either cancer in these papers. Much drug resistance develops through increased expression of multidrug resistance

transporter proteins such as MDR-1. In B cell lymphomas, Fu et al. (2009) examined

the relationship between miRNAs and drug resistance. Based on the observation that

patients with mantle cell lymphomas (MCL) express higher miR-17-92, he

overexpressed miR-17-92 in MCL cells and exposed them to the chemotherapy drug,

topotecan. The miR-17-92 overexpressing cells were more resistant to drug treatment.

Interestingly, David et al. (2004) found an association between DNA hypomethylation

in breast cancer and drug resistance that occurred through regulation of the multidrug

resistance protein, MDR-1.

8. miR-106a in Development

There are many correlates between early embryogenesis and tumor formation and

progression. We therefore sought information concerning the role of miR-106a in

development. Foshay et al. (2009) examined the expression of miR-17, miR-20a,

miR-106a, and miR-93 (all members of the same seed sequence family) during mouse

development. At an early stage of development (E 4.0), both miR-17 and miR-20a

were expressed more in the trophectoderm. By contrast, miR-106a was expressed

primarily in the inner cell mass, a region considered as the source of stem cells with

the potential to differentiate into most cell types. The expression of miR-93 was seen in both the trophectoderm and primitive endoderm. As development progressed (E 6.5), the visceral endoderm had low expression of all four miRNAs, however, the expression of miR-106a and miR-20 was relatively higher. One might speculate therefore that miR-106a expression may be related to stem cell function and differentiation in endoderm-derived tissues. However, in regard to the latter none of the members of the miR-106a-363 cluster, including miR-106a, miR-18b, miR-20b and miR-363, was expressed in early embryonic lung (Lu et al. 2007). The role of miR-106a in development was best described by Ventura et al. who analyzed the consequences of miR-17-92, miR-106a-363 and miR-106b-25 cluster deletion, separately or in combination (Ventura et al. 2008). miR-17-92 deficient mice cannot survive due to severe lung failure. Furthermore, deletion of the miR-17-92 cluster caused defects in B-cell development. However, neither deletion of miR-106b-25 nor miR-106a-363 had any obvious effects. The combined deletion of miR-106b-25 and miR-106a-363 also showed no effect, but the double knockout of miR-106b-25 and miR-17-92 caused more serious problems than deletion of miR-17-92 alone. This analysis either implies a straightforward lack of importance of miR-106a-363 in development or perhaps a degree of subtlety of its effects not easily appreciated. If

miR-106a is important to stem cell function, one might predict early tissue aging. Concordant with this suggestion is downregulated expression in human aging (Hackl et al. 2010).

9. Potential Roles of miR-106a in Other Cancers

As shown in table 4.2, the expression of miR-106a was upregulated in gastric cancer. This was accompanied by low expression of RB1, mentioned previously as a direct target of miR-106a (Zhou et al. 2010, Xiao et al. 2009). Further analysis revealed a positive correlation between miR-106a expression and the stage of tumor-node-metastasis. Higher expression of miR-106a was associated with increasing gastric tumor size, and lymphatic and distant metastasis (Xiao et al. 2009), implying an important role of miR-106a in gastric tumor progression.

In colorectal cancer, miR-106a was overexpressed at both stages I and II, but was decreased at stages Ⅲ and Ⅳ. In addition, high expression of miR-106a was inversely correlated with the cell proliferation-associated target, E2F1 (table 4.3) (Schetter et al. 2008, Guo et al. 2008). Late stage downregulation of miR-106a predicted shortened disease-free survival. (Díaz et al. 2008).

Slaby et al. (2010) studied miRNA expression in renal cell carcinoma (RCC) versus renal parenchyma from disease-free areas. They found a similar pattern as that described for colorectal cancer i.e. higher levels initially, followed by lower levels when metastasized.

In pancreatic and hepatocellular cancer, miR-106a was upregulated, but no further analysis has yet been performed (Volinia et al. 2006, Kutay et al. 2006).

Primary lung cancer can be classified into 2 types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC is usually diagnosed when the cancer has already spread. The expression of miR-106a is higher in lung cancer compared to non-cancerous regions and higher still in SCLC than NSCLC (Navarro et al. 2009). In addition, it was also shown that patients with higher miR-106a expression had a significantly worse prognosis (Yanaihara et al. 2006).

In vitro analyses have shown that miRNAs in the miR-106a-363 cluster are overexpressed in both Hodgkins lymphoma cells and T cell leukemia (Gibcus et al. 2011, Landais et al. 2007). Targets in leukemia were also identified : myosin regulatory light chain–interacting protein, which regulates actin stress fibers and motility in non-muscle cells, and RB1-like protein, a known tumor suppressor (Landais et al. 2007). p27^{kip1}-deficient mice that are highly susceptible to viral

infections and develop lymphomas were used to analyze effects in vivo. Among the miRNAs tested (188) that were overexpressed were members of the miR-106a-363 cluster. Their expression was even higher when there was a MMuLV integration at the Xpcl1 locus, the locus responsible for expression of the miR-106a-363 cluster on chromosome X (Kuppers et al. 2011).

In prostate cancer, expression of miR-106a was not merely increased but there was also in incremental increase that correlated with increasing cancer risk. Furthermore, there was a positive correlation between the expression of miR-106a and metastatic status (Moltzahn et al. 2011).

Schulte et al. (2008) examined the expression pattern of miRNAs at different stages of neuroblastoma. However, there was no correlation with the presence or absence of disease or stage of neuroblastoma. In contrast to neuroblastoma, when surgical samples of astrocytoma were compared to adjacent non-astrocytoma tissue, miR-106a was downregulated in astrocytomas when compared to normal tissue. In addition, patients with reduced miR-106a had a lower survival rate. These results imply a rather different and possibly protective role of miR-106a in the brain (Zhi et al. 2010).

10. Conclusion

In this review we have presented experimental, bioinformatic and correlative data and our speculations supporting a role for overexpression of miR-106a in breast cancer. We have discussed the potential role of miR-106a in cell proliferation, apoptosis, metastasis, angiogenesis, gene repression through DNA hypomethylation, and the development of resistance to therapies. From this perspective, we propose that knockdown of miR-106a may be therapeutically beneficial.

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Fig. 4.1. Classical and alternate pathways of miRNA generation and the mechanisms of inhibition of

target gene expression. Figure modified from one by Dai and Ahmed (2011).
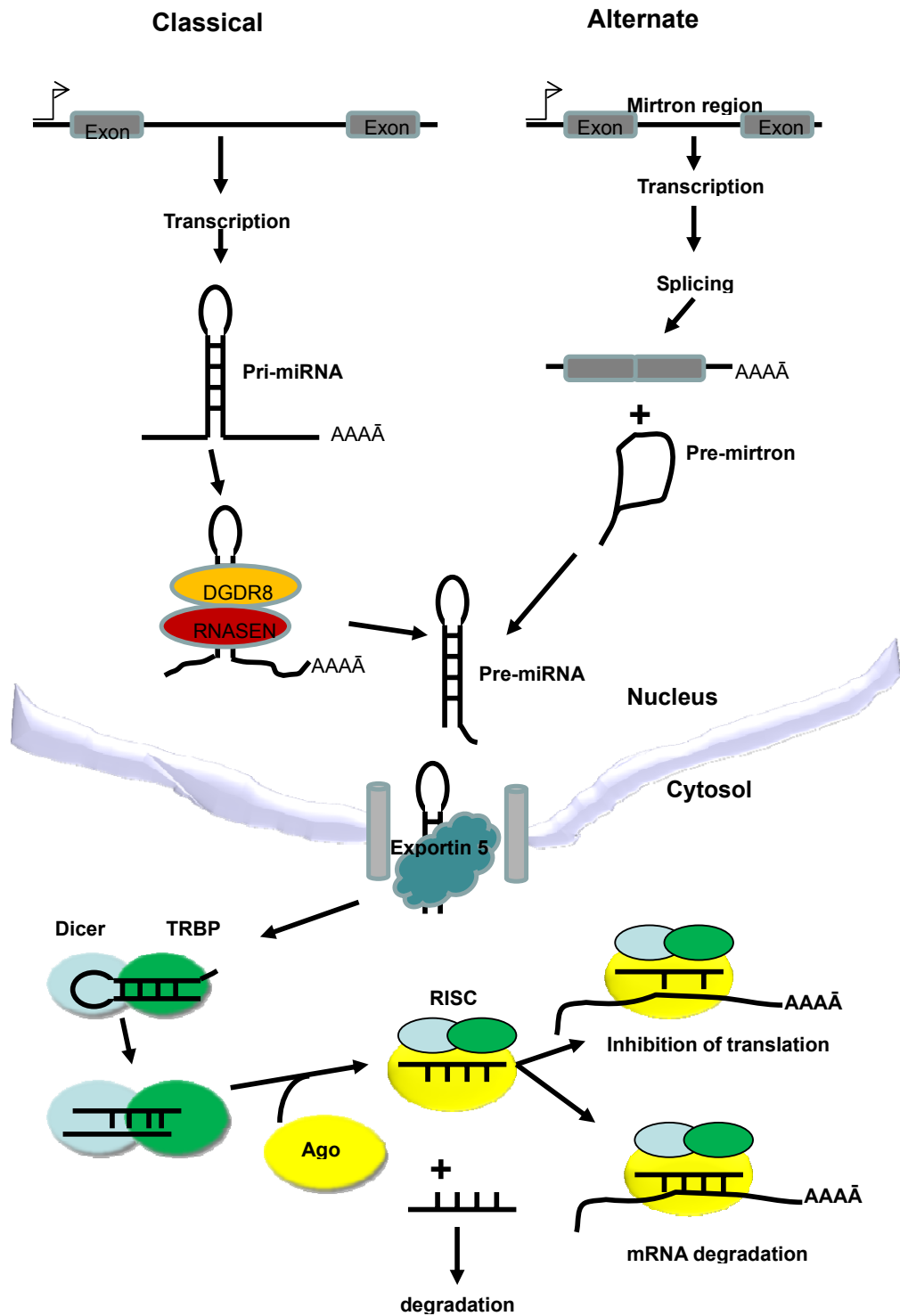
Table 4.1. miRNAs from miR-17-92, miR-106a-363 and miR-106b-25 clusters were

grouped into 4 different families based on their seed sequences. Table adapted from

Van Haaften et al. (2010)

Seed Sequence	Members in miR-17-92 cluster	Members in miR-106a-363 cluster	Members in miR-106b-25 cluster
AAAGUG (miR-17 family)	miR-17, miR-20a	miR-20b, miR-106a	miR-106b, miR-93
AAGGUG (miR-18 family)	miR-18a	miR-18b	
GUGCAA (miR-19 family)	miR-19a, miR-19b-1	miR-19b-2	
AUUGCA (miR-92 family)	miR-92a-1	miR-92a-2, miR-363	miR-25

Table 4.2. Summary of expression pattern of miR-106a in different tissues and in metastasized tumors. ND, not determined

Tissue	Expression of miR-106a in tumor compared to non-tumor tissue	Expression of miR-106a in metastasized tumor to non-metastasized tumor
Gastric	Up-regulated	Increased
Colon	Up-regulated	decreased
Renal	Up-regulated	decreased
Pancreas/Liver	Up-regulated	ND
Lung	Up-regulated	Increased
Nervous system	Down-regulated	ND
Prostate	Up-regulated	Increased
Immune	Up-regulated	ND
Breast	Up-regulated	Increased

Table 4.3. Predicted targets of miR-106a involved in cell proliferation and apoptosis. Data

from Sinha et al. (2008). Genes in bold type are those chosen as examples in the text.

Predicted targets of miR-106a associated with cell proliferation	Predicted targets of miR-106a associated with apoptosis
BCL11B, BCL6, BHLHB3, BMPR2, BTG1,BTG2, BTG3, **CDKN1A,** *COL4A3, CSF1,DERL2, E2F1, EBI3, EDD1, EDG1, EFNB1,EREG, FLT1, FZD3, GAB1, HDAC4, KLF11,LIF, MAP3K11, MAPRE1, PAFAH1B1, PCAF,PDGFRA, PPARD, PTEN, PTHLH, PURB,* **RB1,** *RBBP7, TAL1, TBX3, TGFB1, TOPORS,TSG101, TUSC2*	*ACIN1, ACVR1B, APBB2, APP,* **BCL2L11,** *BCL2L2, BCL6, BIRC4, BNIP2, BTG1,* **CASP6,CASP8,** *CDKN1A, CFLAR, COL4A3, DAPK2,DEDD, DNASE2, DNM2, E2F1, EGLN3,EP300, FASTK, FOXL2, HIF1A, INHBA,LALBA, MAP3K5, PAK7, PIK3R1, PLAGL2,PPARD, PPP2CA, PTEN, PURB, SQSTM1,STK17B, TAOK2, TAX1BP1, TIMP3,TMEM23, TNFRSF21, TOPORS, TP53INP1*

Conclusion

S179DPRL and prolactin regulate cell growth differently. We've shown that the signaling molecule Stat5 that is activated by prolactin but not S179DPRL plays an important role in regulating the BRCA1/p21 axis therefore leading to different effects on growth. We further showed p21 expression can be regulated post-transcrionally by miR-106 and post-translationally by pim-1 kinase. The regulation by miR-106 causes unstable p21 mRNA or reduced translation of p21 mRNA resulting in fewer p21 expression and therefore promote cell growth. The post-translational phophorylation of p21 by prolactin stimulus further relocates p21 in the cytosol where the p21 behaves as an apoptosis inhibitor. Taken all together, the study indicated a potential role of prolactin in tumor progression through regulating the expression of p21 and also revealed a potential therapeutic effect using a prolactin antagonist.

The minimal p21 promoter reporter construct was further applied to quantify the concentration of S179DPRL or the naturally phosphorylated prolactin. This developing assay would help to know the ratio of phosphorylated prolactin to unmodified prolactin and determine whether the distribution of each form of prolactin would contribute to tumor formation.