CHAPTER 1

INTRODUCTION

Background and Rationale

Breast cancer is one of the leading causes of cancer death in women worldwide and the commonest cancer among women in Western countries. The age standardized rate (ASR) of American women between 1993 -1997 is 77.98 per 100,000 populations. The ASR in Thailand is 17.2 per 100,000 populations in female, second only to cervical cancer (Parkin et al., 2001). Breast cancer is the most common cancer in females in Bangkok, the second in Songkhla after cervix cancer, the third in Chiang Mai, and Lampang after cervix and lung cancer, and the third in Khon Kaen after liver and cervix cancers (Sriplung, 2003). Although advances in detection and chemotherapy, many women with breast cancer continue to die of this malignancy (WHO, 2003). The breast cancer might result from interactions between genetic elements, various possible introduction environmental factors, and also the difference in ethnicity (Brinton et al., 2002; Hsiao et al., 2004). This disease is currently treated through surgery and/or radiotherapy, and is frequently supported by adjuvant chemo- or hormonotherapies. Unfortunately, these classical treatments are impeded by unwanted side effects and, most importantly, the development of tumor resistance. For example, intensive treatment with radiotherapy or chemotherapy is commonly associated with the range of adverse side effects from nausea to bone marrow failure. The patients who obtain intensive treatment may experience a decline in quality of life (Boik, 1996). Moreover, there is no doubt urgency for novel and effective therapies against breast cancer. Gene therapy becomes an alternative for the patients suffering from cancer. Therefore, the better understanding of the molecular mechanisms involved in breast cancer formation and progression should be helpful in developing more effective treatments for breast cancer. Molecular level disorder is the critical causes of tumorigenesis that effect to regulate the activity of gene which involve the development and breast cell proliferate abnormally as well as decrease the apoptosis of cells. The gene that involves the tumorigenesis can be classified two major

groups; oncogene and tumor suppressor gene in which the activation of proto-oncogene and inactivation of tumor suppressor gene is the critical step in the tumorigenesis.

The WT1 gene was originally identified as a tumor suppressor gene responsible for Wilms' tumor, a kidney neoplasm of childhood (Call et al., 1990; Gessler et al., 1990). WT1 encodes a zinc finger protein which acts as a transcriptional regulator for many genes involved in cell differentiation and growth. Its tumor-suppressive function is supposed to be attributable to transcriptional repression of growth factors and growth factor receptor such as IGF-II, IGFIR, transforming growth factor-β and EGFR (Drummond et al., 1992; Werner et al., 1993; Gashler et al., 1992; Dey et al., 1994 and Hewitt et al., 1995). The role of WT1 in breast cancer has been extensively examined in many studies for the past decade. Recent studies have shown that WT1 plays an important role in the malignant-transformation and progression of leukemia (Inoue et al., 1994, 1998; Chen, 2001), as decreased expression of WT1 protein levels led to growth inhibition and apoptosis in leukemia cells in vitro (Algar et al., 1996; Yamagami et al., 1996) and in vivo (Smith et al., 2000). Therefore, WT1 appears to have an oncogenic role in leukemia. Inoue et al. (1994) reported that the WT1 mRNA expression was significantly up-regulated in leukemia as compared with the normal hematopoietic cells. However, The role of WT1 in breast cancer is largely unknown. Recently, Loeb et al. (2001) have demonstrated that the WT1 mRNA and protein was expressed in nearly 90% of breast cancers, but not in most normal breast tissue. These results suggested that WT1 plays a certain role in the tumorigenesis of breast cancer as an oncogene but not as a tumor suppressor gene in analogy to leukemia. In contrast, Siberstein et al. (1997) studied the WT1 expression by immunohistochemistry and demonstrated that WT1 protein was down-regulated during tumor development. However, in advanced cancer, WT1 expression was more frequently detect in tumors which did not express estrogen receptor (ER) as compared which those ER expressing tumors. The reason for the discrepancy between these studies was not known. For this reason, specific reducing its level by genetic means in established breast cancer cell lines is still helpful for a better understanding of its role in maintaining the malignant phenotype. In this study, we performed to test our hypothesis that the WT1 gene function as oncogene in breast cancer tumorigenesis. We investigated whether specifically decreasing WT1 protein expression in a breast cancer cell line might result in the inhibition of cell growth in vitro.

RNA interference (RNAi) has become an excellent approach for the specific silencing of gene expression in plant and invertebrates (Hannon, 2002; McManus and Sharp, 2002). In this approach, 21-to 23-nucleotide short interfering RNA (siRNA) complementary to the targeted gene are processed from a long dsRNA precursor by the Dicer enzyme and effectively silence the targeted gene by binding to complementary mRNA and triggering mRNA degradation (McManus and Sharp, 2002; Hammond et al., 2000; Nykanen et al., 2001; Parrish et al., 2000; Zamore et al., 2000). Elbashir et al. (2002) demonstrated that transfection of synthetic 21-nt siRNA duplexes into mammalian cells efficiently inhibited endogenous gene expression in a sequence specific manner.

Here we used RNAi to determine whether this technique could be used for the specific inhibition of WT1 gene expression and whether this inhibition resulted in growth inhibition of the tumor cells. The results showed that specific inhibition of WT1 by siRNA was sufficient to inhibit the growth of MCF-7 cells and suggest that WT1 might serve as a therapeutic target for human breast cancer.

Review of Literatures

1. Breast structure and histology

Mammary glands are highly modified apocrine sweat glands, and in the female undergo further development and function under hormonal influence (Yong and Heath, 2000). With the onset of puberty, the breasts grow under the influence of pituitary and ovarian hormones. During menstrual cycling, the breasts undergo cyclical changes influenced by progressive glandular atrophy and involution. Structurally, the breast consists of three major components: the skin, the subcutaneous adipose tissue, and functional glandular tissue that are composed of parenchyma and stroma. The glandular component of the adult breast comprises 15-25 lobes, separated from each other by dense connective tissue and surrounded with adipose tissue (Junqueira et al., 1995). Each lobe is subdivided into several lobules. The lobules, the secretory unit, consist of a variable number of alveoli or glands (also referred to as acini or

terminal ductules) embedded within loose connective tissue. The alveoli are lined by epithelial cells with myoepithelial cells overlaying the basement membrane. The epithelial cells discharge milk and fluid into the lumen under appropriate hormonal influences. Within this branching duct system, the alveoli connect to intralobular ducts which drain into extralobular ducts. The extralobular ducts drain into interlobular ducts which empty into lactiferous ducts with a sinus opening on the surface of the nipple. The skin at the nipple, the areolar, contains abundant sensory nerves, and sebaceous and apocrine glands. The epithelial lining of the mammary ducts demonstrates a gradual transition from a single layer of columnar or cuboidal cells in the terminal duct to double layers of cuboidal cells in the lactiferous sinus. The epithelial cells of the lactiferous sinus are replaced by stratified squamous epithelium at the external opening.

1.1 Risk factors for breast cancer

Breast cancer is a complex and heterogeneous disease. The etiology of breast cancer is uncertain, with many risk factors being related:

1.1.1 Geographic variation

The rate of incidence and mortality of breast cancer varies between countries. The rate in North America and Northern Europe is significantly higher than in Asia and Africa (Kumar et al., 1997). Environmental factors rather than genetic determinants probably contribute to disease differences, because the risk of disease increased among immigrants moving from low incidence areas to high incidence areas (Buell, 1973).

1.1.2 Family history and genetics

Family history is associated with an increased risk of breast cancer, particularly when disease occurs in first-degree relatives at a young age. The risk is increased 1.5 to 2 times for women having one first-degree relative with breast cancer and up to 4 to 6 times for those having two affected first-degree relatives (Harris et al., 1992). Familial breast cancer is due to

inherited mutations of the tumor suppressor genes p53, BRCA-1 and BRCA-2. Li-Fraumeni syndrome, an autosomal dominant familial syndrome is due to germline mutation of the p53 gene. Sufferers of this syndrome develop a variety of cancers including breast cancer. The breast cancers usually occur in women before the age of 40 and often are bilateral (Malkin et al., 1990). BRCA-1 is a tumor suppressor gene located on chromosome 17q21 (Hall et al., 1990). Women carrying a mutation of BRCA-1 have a high lifetime risk of developing breast cancer (approximately 87%) and a 40-60% lifetime risk of ovarian cancer (Easton et al., 1995). BRCA-2 is located on chromosome 13q12-13 (Wooster et al., 1994). The lifetime risk of breast cancer and ovarian cancer in women with mutations of BRCA-2 is 85% and 10-20% respectively (Easton et al., 1995).

1.1.3 Hormonal status

Early onset of menstrual cycling, late menopause, delayed child brearing, and mulliparity all prolong exposure to estrogens, which predisposed breast epithelium to proliferation rather than differentiation (Dupont et al., 1993).

1.1.4 Preexisting proliferative breast disease

A proliferative epithelial lesion, eg atypical hyperplasia, within the breast results in a doubling of the risk of breast cancer compared with women without such lesions (London et al., 1992, Dupont et al., 1993). The above risk factors are well-established, predisposing influences for breast cancer. In addition, there are many less-well established risk factors including estrogen replacement therapy (ERT), oral contraceptives, obesity, alcohol consumption, cigarette smoking, and high-fat diet (Harris et al., 1992).

1.2 Pathogenesis and classification of breast cancer

1.2.1 Pathogenesis

There are three factors influencing the development of breast cancer: genetic changes, hormonal influences, and environmental factors. Several studies support the concept that breast carcinogenesis is a multistep process (Dawson et al., 1996, Dupont et al., 1993, Page et al., 1985). Breast cancer is considered to arise from a benign lesion at the terminal duct lobular unit (TDLU), which progresses through epithelial hyperplasia without atypia to atypical hyperplasia, subsequently developing into in situ and invasive cancer. Similarly, atypical lobular hyperplasia (ALH) is considered to progress to lobular carcinoma in situ (LCIS) and then invasive breast carcinoma (IBC) (Dawson et al., 1996).

1.2.2 Classification

Most breast cancers arise from ductal epithelium, and the various histological types are classified using the World Health Organization Classification:

A. Non invasive (in situ)

1. Intraductal carcinoma
2. Intraductal carcinoma with Paget's disease
3. Lobular carcinoma in situ

B. Invasive (infiltrating)

1. Invasive ductal carcinoma not otherwise specified (NOS)
2. Invasive ductal carcinoma with Paget's disease
3. Invasive lobular carcinoma
4. Medullary carcinoma
5. Colloid carcinoma (mucinous carcinoma)

2. Wilms' tumor 1 gene (WT1)

The Wilms' tumor suppressor gene (*WT1*) was first identified as a tumor suppressor gene, which played a critical role in the development of Wilms' tumor. Expression of the wild type (wt) gene is crucial for normal urogenital development (Pritchard-Jones et al., 1990; Schedl and Hastie, 1998). Losses of function germline mutations (e.g. deletions) in humans are associated with Wilms' tumor, undescened testis and hypospadias (Bruening et al., 1993). Missense and nonsense mutations (Pelletier et al., 1991; Bruening et al., 1993; Bardeesy et al., 1994) are associated with more severe development disorders, such as those found in the Danys-Drash syndrome (DDS). Other malignancies such as mesotheliomas (Park et al., 1993) and leukemias (King-Underwood et al., 1996) have also been found to possess *WT1* point mutaions. High levels of *WT1* expression have been shown to be associated with aggressiveness of acute leukemias (Inoue et al., 1994). However, the role of *WT1* in breast cancer is unclear.

2.1 Structure of Wilms' Tumor 1 gene

The Wilms tumor 1 (*WT1*) gene spans about 50 Kb at chromosome 11p13. It comprises ten exons and transcribes a 3.1 Kb mRNA (Call et al., 1990; Gessler et al., 1992). The gene encodes a transcriptional regulatory protein that binds DNA via four zinc fingers in C-terminal domain that consists of four (Cys) 2-(His) 2 zinc fingers (Madden et al., 1991; Madden et al., 1993). The N-terminus of WT1 contains two major functional domains; repression domain and activation domain (Fig. 1).

There are at least 24 isoforms of *WT1* that result from a combination of alternative splicing, alternative translational start sites and RNA editing. The *WT1* gene product may activate or repress target gene transcription, depending on the type of cells, ratio of WT1 isoform and the status of interactive proteins (Wang et al., 1993, Menke et al., 1997). In mammals, exons 5 and 9 of *WT1* are alternatively spliced, giving rise to four different splice isoforms (Gessler et al., 1992; Haber et al., 1993). Depending on the absence or presence of the two splice inserts, the WT1 proteins have molecular masses of 52-54 KDa (Morris et al., 1991).

Alternative splice I results in the inclusion or exclusion of exon 5 encoding a 17 amino acid which is located between the proline-glutamine rich amino-terminus and the zinc-finger domain of WT1. The proteins which contain and do not contain this segment are designed as WT1+17 and WT1-17, respectively. The alternative splice II donor site at the end of exon 9 results in incorporation of three amino acids; lysine, threonine and serine (KTS), between the third and fourth zinc fingers. The proteins which contain or do not contain the KTS insert are designated WT1+KTS and WT1-KTS, respectively. Moreover, WT1-KTS and +KTS behave differently in terms of their ability to bind to DNA. The WT1-KTS isoform recognizes the nucleotide core sequence 5'GCGGGGGCG'3 and $(TCC)_n$ that are present in the promoter regions of several growth factor and growth factor receptor genes such as IGFIR, IGFII, PDGF-A chain and EGFR. However, the WT1+KTS isoform recognizes more extended CG DNA sequence within the PDGF-A chain promoter (Wang et al., 1992). Therefore, due to the splicing events, four major WT1 isoforms can be generated: WT1 (+17aa/-KTS), WT1 (-17aa/-KTS), WT1 (-17aa/+KTS) and WT1 (+17aa/+KTS) (Fig. 2). Additional variants result from RNA editing event that changes Leu to Pro in the proline rich region (Sharma et al., 1994) and the use of an alternative start site (CGT) that results in the addition of 73 amino acids at the N-terminus of WT1 (Bruening and Pelletier, 1996). By these three mechanisms, 24 isoforms of WT1 have been generated.

2.2 The biological functions of WT1

2.2.1 WT1 as a transcription factor

WT1 has been well studied for its role in the regulation of several genes. Using transient transfection assay, WT1 has been found to control several target genes which contain the Egr-1 consensus sequence in their promoter. However the results from those studies were variable and have been proposed to be influenced by the cell type utilized in the studies, the type of expression vector, the architecture of the promoter and status of other WT1 interacting, especially p53. For example, WT1-KTS functions as a transcriptional repressor of the Egr-1 promoter in NIH3T3 cells (Maheswaran et al., 1993) whereas it behaves as a transcriptional activator for the same promoter in both Saos-2 (Maheswaran et al., 1993) and U2OS (Englert et al., 1995). The

role of different WT1 isoforms in the regulation of gene expression has been examined in several recent studies. The earlier studies attempted to identify the WT1 DNA binding sites of different WT1 isoforms by using binding site selection with oligonucleotides, whole genome PCR and Dnase I footprinting analyses (Rauscher et al., 1990; Bickmore et al., 1992; Nakagama et al., 1995; Hamilton et al., 1995; Wang et al., 1993). Although the DNA sequence that can bind to WT1 -KTS has been well defined as a Egr-1 core sequence, the DNA binding sequence of WT1 +KTS has only recently been identified. Using transient transfection studies, WT1 -KTS and +KTS have been shown to regulate the promoter of IGFII gene (Drummon et al., 1994), PDGF-A chain (Wang et al., 1995), the *WT1* gene (Rupprecht et al., 1994) and the PAX-2 gene (Ryan et al., 1995). These findings support the idea that both splice variants may differentially regulate the same target gene. Thus far, several putative target genes for *WT1* have been identified mainly by transient transfection assays in which the consensus sequences responsible for that interaction were clearly identified by in vitro binding assays. Most of these genes are involved in cellular proliferation, differentiation and apoptosis. The list of the putative *WT1* target genes is shown in the table 1. The biological significance of modulation of these target genes by *WT1* has been clearly shown in tumor models and in vivo experiments. In Wilms' tumor, loss of *WT1* regulatory function in tumors harboring *WT1* mutation has been implicated in up regulation of IGFIR which enhances IGFIR-IGFII mediated growth signal which may promote tumor development and progression (Werner et al., 1993).

2.2.2 WT1 and apoptosis

Several lines of evidence from both in vivo and in vitro models imply a role of *WT1* in the apoptosis. In transgenic *wt1* null mice that lack *WT1* expression, the metanephric mesenchymal cells failed to differentiate but became degenerated by apoptosis. In this cell type, WT1 has been proposed to function as a survival factor. This data is supported by transient transfection studies that demonstrate that *WT1* can suppress p53-induce apoptosis (Maheswaran et al., 1995). The antisense experiment in K562 also recapitulated this phenomenon in which the suppression of WT1 expression induced the cells to apoptosis. In contrast, overexpression of each WT1 isoform in Saos-2 and U2OS cells resulted in apoptosis (Englert et al., 1995) while the

induced expression of WT1 -KTS in HepG2 and Hep3B cells resulted in apoptosis (Menke et al., 1997). The underlying mechanism for WT1-regulated apoptosis has been studied by identifying the target genes of *WT1*, which are involved in that process. There are several studies showing that WT1 controls apoptosis by modulating genes, involved in both apoptotic and growth stimulating pathways. *WT1* has been shown in transient transfection studies with WT1 +KTS and -KTS isoforms, to equally repress the reporter constructs contain promoters of Bcl2 and c-myc (Hewitt et al., 1995). Due to the fact that Bcl2 is an antiapoptotic protein and c-myc can induce cellular proliferation of apoptosis depending on the presence of growth signal, the absence of wild type WT1 can lead to derepression of Bcl2 and c-myc and induce cellular proliferation (Hewitt et al., 1995).

To determine whether the cells will go into apoptosis, several survival signals have been studied. Due to the fact that WT1 controls several growth factors and growth factor receptors, the contribution of *WT1* in this process is understandable. For example, down regulation of IGFIR induces apoptosis in C6 glioblastoma cells (Resnicoff et a., 1995) and overexpression of EGFR rescues both U2OS (Englert et al., 1995) and Hep3B (Menke et al., 1997) from *WT1* induced apoptosis. Overexpression of insulin receptor also rescues Hep3B cells from WT1 induced apoptosis (Menke et al., 1997). The other mechanism of *WT1*-mediated apoptosis is related to the ability of WT1 to interact with the proteins that are involved in apoptosis. As mentioned before, WT1 can interact with p53 and inhibit p53-induced apoptosis (Maheswaran et al., 1995). Par-4 (prostate apoptosis response protein) has been shown to interact with the zinc finger domain of W1 via leucine zipper domain and can inhibit transcriptional activation and increase the transcriptional activation by WT1 (Johnstone et al., 1996; Sells et al., 1997). Moreover, Par-4 also rescues the growth suppression by WT1. Interestingly, overexpression of WT1 can attenuate the apoptosis mediated by Par-4. So these protein-protein interactions clearly play a role in apoptosis and cell proliferation (Sells et al., 1997).

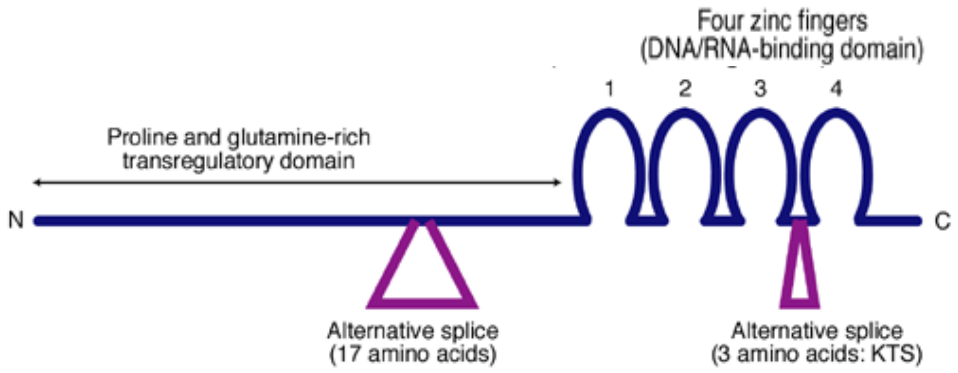

Figure 1. Schematic structure of WT1 protein (Expert Reviews in Molecular Medicine @2001 Cambridge University Press)

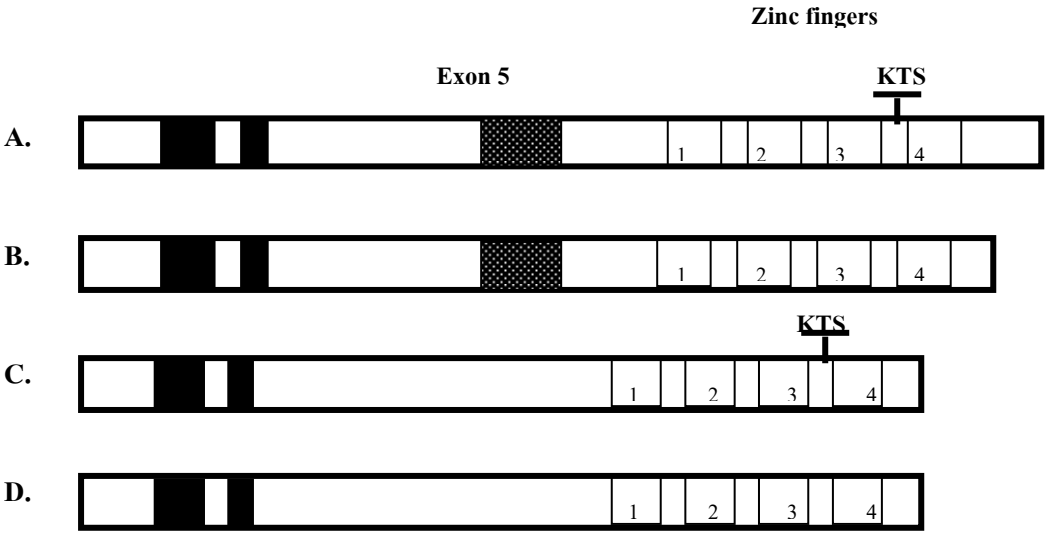

Figure 2. Schematic 4 isoforms of *WT1* result from alternative splicing.

(A = WT1 +17aa/+KTS, B = WT1+17aa/-KTS, C = WT1 -17aa/+KTS and D = -17aa/-KTS) (Harber et al., 1993).

Table 1. Potential target genes of the Wilms' tumor gene products (Scharnhorst et al., 2001.)

Gene	References
PDGF-A	Ryan et al., 1995; Wang et al., 1992, 1993, 1995
IGFII	Drummon et al., 1992
TGF-β	Harington et al., 1993; Dey et al., 1994
Inhibin α	Hsu et al., 1995
Midkine	Adachi et al., 1996
Amphiregulin	Huang et al., 1999
IGFIR	Tajinda et al., 1999; Werner et al., 1993, 1994
EGFR	Englert et al., 1995
Insulin receptor	Hewitt et al., 1995; Wang et al., 1993
RAR α	Goodver et al., 1995
Egr-1	Madden et al., 1991
WT1	Hofmann et al., 1993; Rupprecht et al., 1994
c-myc	Malik et al., 1994; Hewitt et al., 1996
c-myb	Hewitt et al., 1995; Wang et al., 1993
p53	Werner et al., 1998
PAX2	McCann et al., 1995
Syndecan 1	Cook et al., 1996
Nov H	Martmerie et al., 1996
Ecadherin	Hosono et al., 2000
CSF-1	Harington et al., 1993
ODC	Moshier et al., 1996
G protein -α1	Kinane et al., 1996
Bcl2	Gashler et al., 1992
K-ras	Wang et al., 1993
HTERT	Oh et al., 1999

2.3 WT1 and Cancer

Significant roles for WT1 in human cancer are supported by the fact that alterations in WT1 and its expression have been detected in several types of tumor specimens. Whereas WT1 mutations were detected in a small percentage of human malignancies, aberrant expression of WT1 has been detected in the majority of several types of tumors. Overexpression of WT1 was also detected in other tumors; 75% in ovarian tumors (Bruening et al., 1993), and about 80% of acute leukemias. In contrast, WT1 expression cannot be detected in normal peripheral blood and bone marrow (Inoue et al., 1994; Menssen et al., 1995). A correlation between WT1 expression and prognosis of acute leukemia has been clearly shown (Inuoe et al., 1994) in that lower WT1 expression was correlated with a higher rate of complete remission, disease free and overall survival. However, the role of WT1 in breast cancer remains unclear. Previous Immunohistochemical study showed that WT1 expression was down regulated in the majority of breast cancerous tissue as compared to the benign counterpart (Silberstein et al., 1997). Furthermore, the ratio between WT1 splice isoforms was also changed in those cancerous tissues. However, the recent study showed a different result in which by using RT-PCR and western blot, WT1 protein and transcript were detected in the majority of breast cancer tissue but not in benign counterpart (Loeb et al., 2001). Furthermore, Miyoshi Y. and member in 2002 showed that high expression of WT1 mRNA detected by real-time RT-PCR was associated with poor prognosis in breast cancer patient. The summary of the potential roles of WT1 in human cancer based on the pattern of expression and the functions of WT1 in these cells is shown in the table 2 .

Table 2. The role of WT1 in tumors

Tumor cell type	Possible role of WT1		References
	Oncogenic	Tumor suppressor	
Wilms' tumor	+	+	Haber et al., 1993
Ovarian cancer	+	_	Bruening et al., 1993
Granulosa-leydig cell tumor	+	_	Coppes et al., 1993
Malignant melanoma	+	_	Rodeck et al., 1994
Acute leukemia	+	_	King-Underwood et al., 1996
Mesothelioma	+	_	Amin et al., 1995
Breast cancer	+	+	Silberstein et al., 1997; Loeb et al., 2001

2. Gene silencing: RNA interference

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing (PTGS) in animals and plants, and initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene (Elbashir et al., 2001a; 2001b). PTGS/RNAi involves the targeted elimination of mRNA by a homologous dsRNA that is processed into short interfering RNAs (siRNAs) approximately 21-23 nucleotides in length (~2 nucleotides of 3' overhang on each strand) by an RNase III type enzyme termed Dicer. The siRNAs are thought to guide an RNA-Induced Silencing Complex (RISC) to the complementary mRNA, which is subsequently degraded (Figure 2) (Bernstein et al., 2001; Chicas and Macino, 2001; Cogoni, 2001; Tuschl, 2001; Vance and Vaucheret, 2001; Kurreck, 2003). RISC is a multiple turnover RNA endonuclease complex and one of its components is a member of the Argonaute protein family (Hutvagner and Zamore, 2002; Martinez et al., 2002). The target mRNA is cleaved within the region recognized by the anti-sense siRNA strand and the cleavage site is located opposite to the phosphodiester bond between 10th and 11th nt of the anti-sense strand counting from its 5' end. The mRNA fragments deprived of either cap structure or polyA tail, both required for mRNA stability, are subsequently degraded by cellular nucleases. Consequently, the specific depletion of mRNA from the cell leads to a reduction of the corresponding protein, resulting in a knock down phenotype (Elbashir et al., 2001a; 2001b; Martinez et al., 2002).

RNA silencing mechanisms were first recognized as antiviral mechanisms that protect organisms from RNA viruses, or which prevent the random integration of transposable elements. First discovered in plants, the phenomenon of homology dependent gene silencing came into the scientific world through serendipity. Two seminal studies were those of Napoli and Jorgensen (1990) and Van der Krol (1990), each attempting to make purple petunias an even darker purple by introducing a transgene designed to overproduce the chalcone synthase enzyme. However, overexpression of chalcone synthase transgenes did not increase coloration. Instead, some flowers were completely colorless (white), and others showed interesting patterns such as loss of pigmentation along the veins, but full pigmentation in other areas. It was the complete loss of color in some or all flower cells that indicated a new phenomenon had been stumbled upon,

for not only was the transgene silent in these cells but also the endogenous copy of the chalcone synthase gene was silenced as well. Napoli and Jorgensen (1990) thus introduced the term "co-suppression" to describe the coordinate silencing of a transgene and its endogenous homologs.

3.1 Experimental applications

3.1.1 Invertebrate

The term RNA interference (RNAi) was coined after the discovery that injection of dsRNA into the nematode C. elegans leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was also observed subsequently in insects (Kennerdell and Carthew, 1998), and frog (Oelgeschlager et al., 2000). The natural function of RNAi and cosuppression appears to the protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when the become active (Jensen et al., 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999; Malinsky et al., 2000). Specific mRNA degradation prevents transposon and virus replication, although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Anondalakshmi et al., 2000; Lucy et al., 2000; Voinnet et al., 2000). dsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA. The dsRNA is processed to 21-23-nt RNA fragments (Zamore et al., 2000). These short fragments were also detected in extracts prepared from *Drosophila melanogaster* Schneider 2 cells that were transfected with dsRNA before cell lysis (Hammond et al., 2000) or after injection of radiolabeled dsRNA into D. *melanogaster* embryos (Yang et al., 2000) or C.*elegans* adults (Parrish et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hammilton and Baulcombe, 1999). It has been suggested that the 21-23-nt fragments are the guide RNAs for target recognition (Hamilton and Baulcombe, 1999; Hammond et al., 2000), which is supported by the finding that the target mRNA is cleaved in 21-23-nt intervals (Zamore et al., 2000). The systems are commonly used in plants such as *Arabidopsis* (Dalmay et al., 2000; Dalmay et al., 2001; Park et al., 2002) and

invertebrates such as *Caenrhabditis* (Kamath et al., 2001; Timmons et al., 2001; Alder et al., 2003; Lee et al., 2003), and *Drosophila* (Dzitoyeva et al., 2001; Kalidas and Smith, 2002; Wei et al., 2003).

3.1.2 Mammalian system

Practical use of RNAi in mammalian experiments was delayed because the long dsRNA used in plants and invertebrates triggered cell death pathway. In most types of mammalian cells, dsRNA longer than 30 nt triggers dsRNA-activated protein kinase (PKR) and 2', 5'-oligoadenylate synthetase (2', 5'-AS). These two responses lead to blockage of protein synthesis. The protein synthesis is blocked by phosphorylation and inactivation of the translation factor eIF2a as well RNase L mediated RNA degradation, respectively (Kumar and Carmichael, 1998). These pathways are common to the interferon response which is ubiquitous in mammalian cell with the exception of oocytes (Svoboda et al., 2000) and early embryonic cells (Billy et al., 2001). Elbashir et al (2001a, 2001b) demonstrated that short RNA duplexes (siRNAs) could be used to mediate a gene silencing in mammalian cells. This finding triggered many studies using RNAi in mammalian cells either in cultured cell (Elbashir et al., 2001a; 2001b; Elbashir et al., 2002; Bridge et al., 2003; Czauderna et al., 2003), or whole animals (Hasuwa et atl., 2002; Shinagawa and Ishii, 2003; Stein et al.,2003).

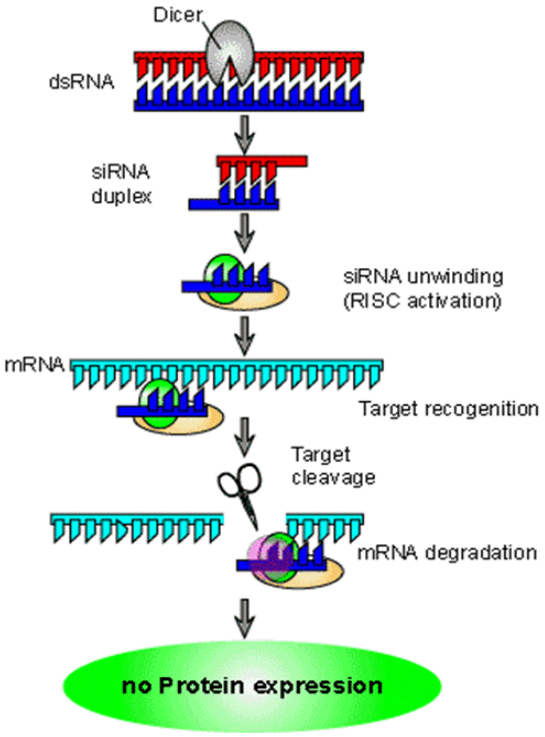

Figure 3 . Schematic representation of gene silencing pathway.

dsRNA, double-stranded RNA; siRNA, small interfering RNA; RISC, RNA-induced silencing complex. (http://www.bioteach.ubc.ca/MolecularBiology/AntisenseRNA/)

Objectives

1. To develop the WT1 gene silencing method by using siRNA in breast cancer cells.
2. To investigate the role of WT1 gene in oncogenesis of breast cancer cells by studying the effect of WT1 gene silencing on tumorigenic property of breast cancer (the proliferation rate).