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**IDENTIFICATION AND CHARACTERIZATION OF
TETRATRICOPEPTIDE REPEAT PROTEIN 9**

CAO SHENGLAN

**SCHOOL OF BIOLOGICAL SCIENCES
NANYANG TECHNOLOGICAL UNIVERSITY**

2007

**Identification and Characterization of
Tetratricopeptide Repeat Protein 9**

Cao Shenglan

School of Biological Sciences

This thesis submitted to the Nanyang Technological University
in fulfilment of the requirement for the degree of
Doctor of Biological Sciences

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LIST OF ABBREVIATIONS

36B4	human acidic ribosomal phosphoprotein
aa	amino acids
Ab	antibody
AF	activation factor
AP	alkaline phosphatase
AR	androgen receptor
ATCC	American Type Culture Collection
ATM	ataxia telangiectasia mutated
bp	base pair
BRCA1	breast cancer 1 gene
BRCA2	breast cancer 2 gene
BSA	bovine serum albumin
BUS	B-upstream segment
cAMP	cyclic adenosine monophosphate
CD	circular dichroism
cDNA	complementary DNA
COX IV	cytochrome c oxidase subunit IV
CT	computerized tomography

List of abbreviations

DBD	DNA binding domain
DCC	dextran-coated charcoal
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
E2	17 β -estradiol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ER α	estrogen receptor subtype α
ERE	estrogen-response element
ERK	extracellular signal-regulated protein kinase
ERR	estrogen-related receptor
EST	expressed sequence tag
FBS	fetal bovine serum

List of abbreviations

FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FPLC	fast-performance liquid chromatography
FT-IR	Fourier Transform Infrared
FXR	Farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)piperazine-1- ethanesulfonic acid
HMW	high-molecular-weight
hr	hour
HRE	hormone-response element
HRP	horseradish peroxidase
Hsp	heat-shock protein
IDC	invasive ductal carcinoma
IgG	immunoglobulin G
ILC	invasive lobular carcinoma

List of abbreviations

IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	kilobase
kD	kilo Dalton
KLH	keyhole limpet hemocyanin
LacZ	β -galactosidase gene
LB	Luria-Bertani (medium)
LBD	ligand-binding domain
LMW	low-molecular weight
MAPK	mitogen-activated protein kinase
MCS	multi-cloning site
min	minute
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger RNA
MWCO	molecular weight cut-off
NCBI	The National Center for Biotechnology Information
NCC	National Cancer Center
N-CoR	nuclear receptor corepressor
NF	nuclear factor

List of abbreviations

NLS	nuclear localization signals
NMR	nuclear magnetic resonance
NR	nuclear receptor
NRID	nuclear-receptor-interaction domain
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	positron emission tomography
PKC	protein kinase C
PMSF	phenylmethanesulphonylfluoride
PNPP	p-nitrophenyl phosphate
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PR-A	progesterone receptor isoform A
PR-B	progesterone receptor isoform B
PRE	progesterone-response element
PRKCBP1	protein kinase C binding protein 1
PTS	peroxisomal targeting signal

List of abbreviations

RAR	retinoic acid receptor
Ras	Retrovirus-associated DNA sequences oncogene
RT	reverse transcription
SD	synthetic dropout/defined (medium)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMRT	silencing mediator for retinoid and thyroid hormone receptor
SR	steroid receptor
SRC-1	steroid receptor coactivator-1
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPR	tetratricopeptide repeat
TR	thyroid hormone receptor
Tris	2-amino-2-(hydroxymethyl)-1,3- propanediol
Trx	thioredoxin
TTC9	tetratricopeptide repeat protein 9

List of abbreviations

UTR	untranslated region
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

LIST OF PUBLICATIONS

Article

1. Cao, S., Iyer, J. K., and Lin, V. (2006). Identification of tetratricopeptide repeat domain 9, a hormonally regulated protein. *Biochem Biophys Res Commun* 345, 310-317.

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1. Cao, S., Ho, G. H., and Lin, V. (2005). A novel hormonally-regulated protein with a tetratricopeptide repeat that is over-expressed in breast tumor. In 96th Annual Meeting of American Association for Cancer Research, Anaheim, April 16-20
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SUMMARY

Breast cancer is second only to lung cancer as the leading cause of cancer death among women. Steroid hormones, such as estrogen and progesterone, are important factors in the regulation of normal female reproductive function and the tumor development. Studies on steroid hormone regulated genes will provide information on the functioning mechanism of steroid hormone action, and will assist in understanding the mechanism of breast cancer development.

Tetratricopeptide repeat protein 9 (TTC9) was initially found in our microarray analysis to be significantly up-regulated by progesterone in progesterone receptor (PR)-transfected MDA-MB-231 cells. Studies also suggested that TTC9 was a direct PR target gene. In this study, the transcript size of TTC9 gene was determined to be ~2.5 Kb in breast cancer cells. Furthermore, three transcripts of TTC9 were identified in human tissues. The protein size of TTC9 was predicted to be 222 aa, with a calculated molecular mass of 24.4 kD. Using antibodies generated against the first 50 aa and the full 222 aa of TTC9 protein that were expressed and purified in *E.coli*, Western blotting analysis detected a ~25 kD band in all the cells and tissues examined, which support the prediction on TTC9 protein.

Summary

Further studies revealed that TTC9 was a hormonally regulated gene both *in vitro* and *in vivo*. Estrogen and progesterone regulated TTC9 expression in several breast cancer cell lines. In mice, TTC9 protein was drastically up-regulated by estrogen in estrogen target tissues including the uterus, mammary glands and liver. This study also showed that growth factors regulated TTC9 expression in breast cancer cells through MAPK signaling pathways. Moreover, TTC9 mRNA was significantly over-expressed in breast cancer tissues compared with the adjacent normal breast tissue. The involvement of TTC9 in different signaling pathways indicated that TTC9 could be an important gene involved in hormone signaling and breast cancer development.

The function of TTC9 remains unknown at current stage. Sequence analysis suggested the existence of three TPR motifs at the C-terminal of TTC9 protein. By yeast-two-hybrid assay, Tm5NM-1, a member of tropomyosin gene family, was identified as one of TTC9 interacting proteins. It was also identified that the first TPR motif as well as the segment between the first two TPR motifs of TTC9 were critical for the interaction. The first 50 aa of TTC9 were also required for the interaction, possibly by maintaining the protein in proper conformation. Since the primary function of tropomyosin family proteins is to stabilize actin filament, its interaction with TTC9 may play a role in

Summary

cytoskeleton organization and affect cell shape and motility. Indeed, adherent cells expressed higher level of TTC9 compared with suspension cells, and progesterone-induced TTC9 expression was associated with increased cell motility and cell spreading. It was speculated that TTC9 might act as a chaperone protein to facilitate the function of tropomyosins in stabilizing microfilament and it could play a role in cancer cell invasion and metastasis.

The findings in this study identified and characterized a novel protein, TTC9, which is a hormonally regulated protein and could function as a chaperone protein in various cellular functions. We believe the work on TTC9 protein will be of great significance in the studying of hormone signaling mechanism in breast cancer cells, and will shed light on the understanding of breast cancer development.

CHAPTER 1

INTRODUCTION

1.1 Introduction of breast cancer

1.1.1 Incidence and prevalence of breast cancer

Breast cancer is second only to lung cancer as the leading cause of cancer death among women. It is also the most commonly diagnosed cancer in American women, except for skin cancer. It has been reported that in U.S.A., for the year 2002, more than 180,000 women were diagnosed with breast cancer, and more than 41,000 died from this disease (U.S. Cancer Statistics Working Group, 2005). In Singapore, breast cancer is the most common cancer among Singapore women. Almost 1,100 new cases are diagnosed and about 270 women die in Singapore each year from breast cancer.

Breast cancer incidence varies in different regions of the world. As reported by GLOBOCAN (a database setup by the Descriptive Epidemiology Group of International Agency for Research on Cancer, <http://www-dep.iarc.fr/>), the regions with the highest breast cancer incidence included North America, West Europe and Australia, while Africa and most regions of Asia were the areas afflicted with the least incidence.

1.1.2 Breast cancer risks

The exact cause of breast cancer has not been established yet, but there are risk

factors that may play a role. According to population studies (Kelly, 2002; Mitra et al., 2004; Nogueira and Appling, 2000; Veronesi et al., 2005; Walker, 2000), the most important breast cancer risk factors are (American Cancer Society, 2005):

- 1) Gender: Being a woman is the main risk factor for developing breast cancer. The main reason for women to develop more breast cancer is because their breast cells are constantly exposed to the growth-promoting effects of the female hormones estrogen and progesterone, thus making breast cancer much more common in women than men. Men can develop breast cancer, but this disease is about 100 times more common among women than men.
- 2) Aging: About 18% of breast cancer diagnoses are among women in their 40s, while about 77% of women with breast cancer are older than 50 at the time of diagnosis.
- 3) Personal history of breast cancer: A woman with cancer in one breast has a 3- to 4-fold increased risk of developing a new cancer in the other breast or in another part of the same breast.
- 4) Genetic risk factors: Recent studies have shown that about 5% to 10% of breast cancer cases are hereditary as a result of genetic defect. Women with an inherited BRCA1 or BRCA2 mutation have up to an 80% chance of developing breast cancer during their lifetime and at a younger age than

those women who are not born with one of these gene mutations (Coughlin et al., 1999; Shih et al., 2002; Sinilnikova et al., 2006). Other genes that might also lead to inherited breast cancers include ATM gene, which is responsible for repairing damaged DNA (Lavin, 1998; Thompson et al., 2005), and CHEK2 gene (Bogdanova et al., 2005; Mateus Pereira et al., 2004).

- 5) Family history of breast cancer: Having 1 first-degree relative (mother, sister, or daughter) with breast cancer approximately doubles a woman's risk, and having 2 first-degree relatives increases her risk by 5-fold. Although the exact risk is not known, women with a family history of breast cancer in a father or brother also have an increased risk of breast cancer. In general, about 20% to 30% of women with breast cancer have a family member with this disease.
- 6) Not having children and menstrual period effect: Women who started menstruating at an early age (before age 12) or who went through menopause at a late age (after age 55) have a slightly higher risk of breast cancer. Women who have had no children or who had their first child after age 30 have a slightly higher breast cancer risk.
- 7) Other risks include: race, dietary, obesity, breast radiation, etc. As an example, white, non-Hispanic women have the highest overall incidence

rate of breast cancer among U.S. racial groups.

1.1.3 Classification of breast cancer

Breast cancers can be classified histologically based upon the types and patterns of cells that compose them. Carcinomas can be invasive (extending into the surrounding stroma) or non-invasive or *in situ* (confined just to the ducts or lobules).

Carcinoma *in situ* can be further classified according to the tumor foci into lobular carcinoma *in situ* and ductal carcinoma *in situ*. Nearly 100% of women with cancer at this stage can be cured (Morrow and Harris, 2004).

Invasive carcinoma includes invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). In IDC, the cancer starts in a milk duct, breaks through the wall of the duct, and invades the fatty tissue of the breast. From there, it can spread to other parts of the body. IDC is the most common type of breast cancer, accounting for nearly 80% of cases. However, for ILC, the cancer starts in the mammary, or milk glands (lobules) and can spread to other parts of the body. About 10% of breast cancers belong to this type.

1.1.4 Stages of breast cancer

The most common staging of breast cancer is based on the TNM system, which uses size of tumor (T), spreading to lymph nodes (N) and metastasis (M) as parameters. According to the TNM system, breast cancer can be divided into stages 0, I, II, III, IV. The definition of each stage is listed as follows:

Stage 0: Cancer cells remain inside the breast duct, without invasion into normal adjacent breast tissue;

Stage I: Cancer is 2 centimeters or less and is confined to the breast (lymph nodes are clear);

Stage IIA: No tumor is found in the breast but it is in 1 to 3 axillary lymph nodes; or the tumor is less than 2 cm and has spread to 1 to 3 axillary lymph nodes; or cancer is found by sentinel node biopsy as microscopic disease in internal mammary nodes, but not on imaging studies or by clinical exam; or the tumor is larger than 2 cm in diameter and less than 5 cm, but hasn't spread to axillary nodes. In all cases the cancer has not spread to distant sites;

Stage IIB: The tumor is larger than 2 cm in diameter and less than 5 cm and has spread to 1 to 3 axillary lymph nodes; or cancer is found by sentinel node biopsy as microscopic disease in internal mammary nodes; or the tumor is

Introduction

larger than 5 cm and does not grow into the chest wall and has not spread to lymph nodes. In all cases, the cancer hasn't spread to distant sites;

Stage IIIA: The tumor is smaller than 5 cm in diameter and has spread to 4 to 9 axillary lymph nodes; or it is found through imaging studies or clinical exam to have spread to internal mammary nodes; or the tumor is larger than 5 cm and has spread to 1 to 9 axillary nodes, or to internal mammary nodes. In all cases, the cancer hasn't spread to distant sites;

Stage IIIB: The tumor has grown into the chest wall or skin and may have spread to no lymph nodes or to as many as 9 axillary nodes. It may or may not have spread to internal mammary nodes. The cancer hasn't spread to distant sites;

Stage IIIC: The tumor is any size, has spread to 10 or more nodes in the axilla; or to 1 or more lymph nodes under the clavicle (infraclavicular) or above the clavicle (supraclavicular); or to internal mammary lymph nodes, which are enlarged because of the cancer. All of these are on the same side as the breast cancer. The cancer hasn't spread to distant sites;

Stage IV: The cancer, regardless of its size, has spread to distant organs such as bone, liver, or lung, or to lymph nodes far from the breast.

1.1.5 Diagnosis of breast cancer

Diagnostic methods used to detect breast cancer include mammography, ultrasonography, magnetic resonance imaging (MRI) and positron emission tomography (PET).

Mammography is an x-ray technique to visualize the internal structure of the breast. It is the most important diagnostic method for screening women with no cyclic breast tenderness or other conditions that would increase breast density. It is the best method to detect small and non-palpable lesions. However, 11% of breast cancers are not detectable by mammography (Benson et al., 2004). Another technique, ultrasonography, is an effective tool to detect small tumors, especially in dense breasts (Helvie et al., 1994). MRI and PET are imaging screening tools for breast cancer tumor detection. MRI is used to locate breast tumors and provide evidence for conservative surgery. However, MRI is also subjective to high false-positive reports (Szabo et al., 2003). PET is used to measure how far breast tumors have spread. However, it is not able to identify tumors less than 5 mm in size (Wahl et al., 2004).

There are also other techniques available for the detection of breast cancer. Breast biopsy, computerized tomography (CT), blood chemical and enzyme tests, and tumor marker tests are also commonly used as clinical examination

methods for breast cancer diagnosis. They also help to determine strategies for breast cancer treatment.

1.1.6 Breast cancer therapy

In general, four types of standard treatment methods are used in breast cancer therapy, which include: surgery, radiation therapy, chemotherapy, and hormone therapy.

1.1.6.1 Surgery

Most patients with breast cancer have surgery to remove the cancer from the breast. One type of surgery is called breast-conserving surgery, which refers to an operation to remove the cancer but not the breast itself. It covers 75 – 85% of all breast cancer operations (Veronesi et al., 2005) and includes lumpectomy and partial mastectomy. Lumpectomy is a surgical procedure to remove a tumor and a small amount of normal tissue around it. Partial mastectomy is surgical procedure to remove the part of the breast that contains cancer and some normal tissue around it. This procedure is also called a segmental mastectomy. Patients who are treated with breast-conserving surgery may also have some of the lymph nodes under the arm removed for biopsy. This procedure is called lymph node dissection. It may be done at the same time as the breast-conserving

surgery or after.

Other types of surgery include total mastectomy, modified radical mastectomy and radical mastectomy, all of which remove the whole breast that contains cancer. Modified radical mastectomy also removes many of the lymph nodes under the arm, the lining over the chest muscles, and sometimes, part of the chest wall muscles. Radical mastectomy will remove chest wall muscles under the breast, and all of the lymph nodes under the arm. This procedure is sometimes called a Halsted radical mastectomy named after William S. Halsted, an eminent surgeon who developed this technique. No matter what kind of surgery performed, the patient may be given radiation therapy, chemotherapy, or hormone therapy after surgery to try to kill any cancer cells that may be left. Treatment given after surgery to increase the chances of a cure is called adjuvant therapy.

1.1.6.2 Radiation therapy

Radiation therapy is usually given after surgery to kill any possible cancer cells left. It includes external and internal radiation therapy. It has been showed that surgery followed by radiation therapy had beneficial effects on the overall survival of breast cancer patients (Overgaard et al., 1997; Overgaard et al., 1999). However, post-surgery radiation therapy remains controversial, since the

radiation-related fibrosis raises risk involved in the process of breast reconstruction (Chawla et al., 2002).

1.1.6.3 Chemotherapy

Chemotherapy is a cancer treatment that uses drugs to stop the growth of cancer cells, either by killing the cells or by stopping the cells from dividing. When chemotherapy is taken by mouth or injected into a vein or muscle, the drugs enter the bloodstream and can reach cancer cells throughout the body (systemic chemotherapy). When chemotherapy is placed directly into the spinal column, an organ, or a body cavity such as the abdomen, the drugs mainly affect cancer cells in those areas (regional chemotherapy). The way the chemotherapy is given depends on the type and stage of the cancer being treated.

1.1.6.4 Hormone therapy

Hormonal status of women is critical for breast cancer development and differentiation. Hormone therapy is a cancer treatment that removes hormones or blocks their action and stops cancer cells from growing. This strategy depends on the hormonal receptor status of the breast tumors. Current strategies of hormonal therapy are mainly focused on repression of stimulatory effect of estrogen, such as hormone therapy with tamoxifen, which is often given to

patients with early stages of breast cancer and those with metastatic breast cancer (Jolivet et al., 1978). Tamoxifen slows or stops the growth of cancer cells that are present in the body. It thus helps prevent the original breast cancer from recurring and also helps prevent the development of new cancers in the other breast. However, hormone therapy with tamoxifen or estrogens can act on cells all over the body and may increase the chance of developing endometrial cancer. Thus women taking tamoxifen should have a pelvic examination every year to look for any signs of uterine cancer.

Another strategy developed is the use of aromatase inhibitors, which suppress the activity of P450 enzyme aromatase. P450 enzyme family is essential in estradiol synthesis and metabolism. The inhibition of P450 enzyme activity thus reduces circulating estradiol levels. The newly developed third generation aromatase inhibitors have been shown to suppress plasma estradiol levels by 85-92% (Geisler et al., 2002; Geisler et al., 1998).

1.1.6.5 Other therapies being tested in clinical trials

Except for the four standard breast cancer therapies mentioned above, there are some new therapies available which is under clinical test. These include: High-dose chemotherapy with stem cell transplant and monoclonal antibodies as adjuvant therapy.

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High-dose chemotherapy with stem cell transplant is a method of giving high doses of chemotherapy and replacing blood-forming cells destroyed by the cancer treatment. Stem cells are removed from the blood or bone marrow of the patient or a donor and are frozen and stored. After the chemotherapy is completed, the stored stem cells are thawed and given back to the patient through an infusion. These reinfused stem cells will restore the body's blood cells. However, studies have shown that high-dose chemotherapy followed by stem cell transplant does not work better than standard chemotherapy in the treatment of breast cancer (Farquhar et al., 2003).

Monoclonal antibody therapy is a cancer treatment that uses monoclonal antibodies made in the laboratory. These antibodies can identify substances on cancer cells or normal substances that may help cancer cells grow. The antibodies attach to the substances and kill the cancer cells, block their growth, or keep them from spreading. Monoclonal antibodies are also used in combination with chemotherapy as adjuvant therapy. One example for monoclonal antibody therapy is Trastuzumab (Herceptin). Trastuzumab is a monoclonal antibody that blocks the effects of the epidermal growth factor receptor HER-2, which transmits growth signals to breast cancer cells. About one-fourth of patients with breast cancer have tumors that may be treated with trastuzumab combined with chemotherapy (Emens, 2005; Esteva et al., 2002).

Besides, tyrosine kinase, cyclin and proteasome inhibitors are also used in breast cancer therapy.

1.2 Nuclear hormone receptors

Small lipophilic molecules, such as steroids, thyroid hormones, retinoids, and vitamin D, play very important roles in growth, differentiation, metabolism, reproduction, etc. The receptors of these lipophilic molecules, which are located at intracellular nucleus or cytoplasm, belong to nuclear receptor superfamily.

1.2.1 Brief introduction to nuclear receptor superfamily

Nuclear receptors superfamily is composed of a series of phylogenetically related proteins (Robinson-Rechavi et al., 2003), with 21 genes in the complete genome of the fly *Drosophila melanogaster* (Adams et al., 2000), 48 in humans (Robinson-Rechavi et al., 2001), 49 in the mouse (Robinson-Rechavi and Laudet, 2003), and unexpectedly, more than 270 genes in the nematode worm *Caenorhabditis elegans* (Sluder et al., 1999). According to a phylogeny-based nomenclature, the members of this superfamily have been organized in the form of NRxyz, where x represents the sub-family, y stands for the group and z is the gene.

Nuclear receptors were divided into 7 sub-families (Table 1). Among them,

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subfamily 1 is the largest and comprises 11 groups of receptors and 27 individual genes. The 11 groups are numbered from A to K, including TR, RAR, PPAR, REV-ERB, *Drosophila* E78, RZR/ROR, the *Caenorhabditis* CNR14, ECR, VDR, the *Drosophila* DHR96 orphan receptor and the nematode NHR1 orphan receptor from *Onchocerca volvulus*. Subfamily 2 contains 6 groups (HNF4, RXR, TR2/4, DHR78, TLL, COUP-TF and EAR2) and 19 individual genes. Subfamily 3 comprises three groups and 8 genes which are the research focus in our laboratory: the estrogen receptors, the estrogen-related receptors (ERR) and the steroid receptors. ERs and ERRs were classified into separate groups because of their clear functional differences. Among the rest, subfamilies 4, 5 and 6 are generally smaller than subfamilies 1, 2 and 3, with subfamily 6 being the smallest (one group and one gene).

For all the subfamilies mentioned above, they all contain two conserved domains in nuclear receptors, i.e. DNA-binding and ligand-binding domains. However, there are 7 weird nuclear receptor genes which lack one of the conserved domains and are not represented in the phylogenetic tree (Laudet, 1997). They are divided into two groups: the receptors with only the DNA-binding domain (three *Drosophila* genes KNI, KNRL and EGON, the *Drosophila* trithorax and the *Caenorhabditis* ODR7 orphans) and the receptors with only the ligand-binding domain (DAX-1 and SHP in vertebrates) (1999).

Table 1.1 Sub-families of nuclear receptors. [modified from “A united nomenclature system for the nuclear receptor superfamily” (Cell, nuclear receptor nomenclature committee, 1999)]

Subfamily and Group	NR/Gene	Trivial Names
1A	NR1A1	TR α , c-erbA-1, THRA
	NR1A2	TR β , c-erbA-2, THRB
1B	NR1B1	RAR α
	NR1B2	RAR β , HAP
	NR1B3	RAR γ , RARD
1C	NR1C1	PPAR α
	NR1C2	PPAR β , NUC1, PPAR δ , FAAR
	NR1C3	PPAR γ
1D	NR1D1	REVERB α , EAR1, EAR1A
	NR1D2	REVERB β , EAR1 β , BD73, RVR, HZF2
	NR1D3	E75
1E	NR1E1	E78, DR-78
1F	NR1F1	ROR α , RZR α
	NR1F2	ROR β , RZR β
	NR1F3	ROR γ , TOR
	NR1F4	HR3, DHR3, MHR3, GHR3, CNR3, CHR3
1G	NR1G1	CNR14
1H	NR1H1	ECR
	NR1H2	UR, OR-1, NER1, RIP15, LXR β
	NR1H3	RLD1, LXR, LXR α
	NR1H4	FXR, RIP14, HRR1
1I	NR1I1	VDR
	NR1I2	ONR1, PXR, SXR, BXR
	NR1I3	MB67, CAR1, CAR α
	NR1I4	CAR2, CAR β
1J	NR1J1	DHR96
1K	NR1K1	NHR1
2A	NR2A1	HNF4
	NR2A2	HNF4G
	NR2A3	HNF4B
	NR2A4	DHNF4, HNF4D

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2B	NR2B1	RXRA
	NR2B2	RXRB, H-2RIIBP, RCoR-1
	NR2B3	RXRG
	NR2B4	USP, Ultraspiracle, 2C1, CF1
2C	NR2C1	TR2, TR2-11
	NR2C2	TR4, TAK1
2D	NR2D1	DHR78
2E	NR2E1	TLL, TLX, XTLL
	NR2E2	TLL, Tailless
2F	NR2F1	COUP-TFI, COUPTFA, EAR3, SVP44
	NR2F2	COUP-TFII, COUPTFB, ARP1, SVP40
	NR2F3	SVP, COUP-TF
	NR2F4	COUP-TFIII, COUPTFG
	NR2F5	SVP46
	NR2F6	EAR2
3A	NR3A1	ER α
	NR3A2	ER β
3B	NR3B1	ERR1, ERR α
	NR3B2	ERR2, ERR β
3C	NR3C1	GR
	NR3C2	MR
	NR3C3	PR
	NR3C4	AR
4A	NR4A1	NGFIB, TR3, N10, NUR77, NAK1
	NR4A2	NURR1, NOT, RNR1, HZF-3, TINOR
	NR4A3	NOR1, MINOR
	NR4A4	DHR38, NGFIB, CNR8, C48D5
5A	NR5A1	SF1, ELP, FTZ-F1, AD4BP
	NR5A2	LRH1, xFF1rA, xFF1rB, FFLR, PHR, FTF
	NR5A3	FTZ-F1
5B	NR5B1	DHR39, FTZF1B
6A	NR6A1	GCNF1, RTR
0A	NR0A1	KNI, Knirps
	NR0A2	KNRL, Knirps related
	NR0A3	EGON, Embryonic gonad, EAGLE
	NR0A4	ODR7
	NR0A5	Trithorax
0B	NR0B1	DAX1, AHCH
	NR0B2	SHP

1.2.2 Molecular organization of nuclear receptors

Nuclear receptors share a common structural organization: a highly variable N-terminal region (A/B domain), the most conserved DNA-binding domain (DBD, C domain), the largest and moderately conserved ligand-binding domain (LBD, E domain), and a less conserved D domain between the DBD and LBD, which behaves as a flexible hinge between these two domains. Some nuclear receptors may also contain an extremely variable F domain in the C-terminus of the E domain. In estrogen receptor, this F domain is suggested to be involved in ligand distinction. The domain structures of some well-known nuclear receptors are listed in Fig. 1.1.

Some activation domains are found in nuclear receptors. Activation function-1 (AF-1) is located in the A/B domain and is constitutionally active. Activation factor-2 (AF-2) lies in the E domain and is related to ligand-dependent receptor activation. In progesterone receptor isoform B (PR-B), activation factor-3 (AF-3) is located at the N-terminal region of A/B domain, contributing to some PR-B specific functions.

1.2.2.1 The A/B domain

All AF-1/N-terminal regions of NRs studied so far belong to the large category

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Fig. 1.1 Structure organization of nuclear receptors.

of intrinsically disordered activation domains (Warnmark et al., 2003). A/B domains are variable in length, from less than 50 amino acids (aa) to more than 500 aa. This domain shows promoter- and cell-specific activities, suggesting that it plays important roles in the cross-talk between different isoforms as well as cell-type specific functions. AF-1 can be activated through phosphorylation and transcription co-factor binding, so that this domain is also critical for nuclear receptor ligand-independent activation (Rochette-Egly et al., 1997; Rochette-Egly et al., 1992; Taneja et al., 1997).

1.2.2.2 DNA binding domain (DBD)

DNA binding domain is the most conserved region in nuclear receptors, which is responsible for target DNA recognition and binding. It is made up of 70-80 aa, consisting of two zinc-nucleated modules and a C-terminal extension. Residues in the first zinc module determine the specificity of the DNA recognition and residues in the second zinc module are involved in dimerisation. The C-terminal extension contains T and A boxes and is essential for DNA monomeric binding. The DNA-binding domain is furthermore involved in several other functions including nuclear localization, and interaction with transcription factors and co-activators (Claessens and Gewirth, 2004).

1.2.2.3 The hinge region

The hinge region links the ligand-binding domain and the DNA-binding domain. It contains nuclear localization signals (NLS) at the region near the DNA-binding domain. It also contains residues whose mutation abolishes the interaction with nuclear receptor co-repressors (Aranda and Pascual, 2001).

1.2.2.4 Ligand-binding domain (LBD)

The ligand-binding domain acts in response to ligand binding, which caused a conformational change in the receptor to induce a response, thereby acting as a molecular switch to turn on transcriptional activity (Edwards, 2000). The LBD is multifunctional and much less conserved than DBD. It has a common fold that consists of 12 helices (numbered H1–H12) organized in a helical sandwich, and one beta sheet that normally consists of two short strands (Wurtz et al., 1996). The ligand-binding domain is a flexible unit, where the binding of a ligand stabilises its conformation and promotes dimer formation (Folkertsma et al., 2004). The dimerization region is involved in the homo- or hetero-dimerization of nuclear receptors and is composed of hydrophobic heptad repeats (Tanenbaum et al., 1998). Normally only dimerized hormone nuclear receptor can bind to the hormone-response element (HRE) sequence at the target gene. Hetero-dimerization of nuclear receptors includes asymmetric

dimerization of PPAR γ /RXR α (Gampe et al., 2000) and symmetric dimerization of RAR/RXR (Bourguet et al., 2000). LBD region also interacts with chaperone proteins, such as heat-shock proteins (HSPs), and mediates ligand-dependent transcriptional functions. The AF-2 domain at the C-terminal region of LBD is responsible for the ligand-dependent transcriptional activation. The binding of different ligands can alter the conformation of the LBD, which ultimately affects the DNA-binding specificity of the DBD.

1.2.3 Hormone response element (HRE)

An HRE is a binding site for the hormone-receptor complex. It is a special nuclear receptor regulatory sequence located at the upstream of the target genes. In most cases, HRE is located closely to the core promoter. However, there are also some exceptions in which HRE is found several kilobases upstream of the transcriptional initiation site. A comparison of consensus HRE sequences for the steroid receptors reveals that HRE typically contains palindromic inverted repeats of two hexameric core sequences, separated by three nucleotides (IR3) (Claessens and Gewirth, 2004). Early experiments have shown that androgen receptor (AR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and PR, which in general also called the class I receptors, all recognized a 5'-AGAACA-3' core. While other nuclear receptors (class II receptors), such as

estrogen receptor (ER), recognized a 5'-AGGTCA- 3' core (Beato, 1989; Cato et al., 1986).

With only two consensus half-site sequences and over 150 different hormone receptors, the diversity of HRE sequences is generated by varying the arrangement of the half-sites relative to one another. Inverted, everted and direct repeats all restrict the dimeric receptors that can bind (Gronemeyer and Moras, 1995; Rastinejad et al., 1995). For class II receptors, the length of spacer that separates the two hexameric half-sites also affects the diversity of HRE. The addition of 1 bp can result in a relative rotation of approximately 35° and a 3.4 Å increase in the two hexameric cores' separation (Claessens and Gewirth, 2004). The binding between HRE and nuclear receptor dimer is also determined by the "P Box" at the DBD, which is a three amino acids motif located in the first zinc module of DBD (Glass, 1994).

1.2.4 Mechanisms of nuclear receptor functioning

1.2.4.1 Pathways of nuclear receptor action

Nuclear receptors act to regulate mRNA levels by controlling gene transcription and by regulating the stability of mRNAs. To regulate gene transcription, nuclear receptors must fulfill three basic requirements. Firstly, the receptor must

be activated, which usually involves binding by a specific small molecule hormone ligand, or regulation by other cell signaling pathways. Secondly, the receptor need to binds to a specific DNA sequence, i.e. HRE. Thirdly, the receptor must undergo a conformational change as a result of ligand binding, DNA binding, and phosphorylation by cell signaling pathways. As an example of nuclear receptor action, inactivated and unliganded steroid receptors (SR) are associated with a chaperone protein complex, located in the cytoplasm. The chaperone protein complex is commonly composed of heat-shock protein 70 (Hsp70), Hsp90, p23, FKBP52, FKBP51, and probably other unknown small molecules. The complex binds to a certain region of SR LBD to maintain SR conformation and facilitate high affinity ligand binding.

Upon steroid hormone molecules passing through the cell membrane and binding to steroid receptors, the SRs dissociate from the chaperone protein complex and form homo- or hetero-dimers. Liganded receptor dimers then enter into the cell nucleus and bind to the HRE sequence of the target gene. The ligand/receptor dimer/DNA complex works together with co-regulators to activate or inhibit general transcriptional machinery (including TATA-binding protein, TATA-binding protein associated factor, RNA polymerase II and promoter-specific factor), and initiate or decrease mRNA transcription (Katzenellenbogen, 2000; Kraus et al., 1995).

When the hormone level in the cell decreases, the steroid hormone receptors are disassembled from the transcriptional machinery. It has been found that molecular chaperone p23 and maybe Hsp90 are involved in this disassembly process (Freeman and Yamamoto, 2002). The dimers are then recycled to be monomers and bind to the chaperone protein complex again. The released steroids are carried into the endoplasmic reticulum and are converted into their metabolites. The metabolic products are thereby transported to Golgi vesicles and finally released from the cell body.

Steroid hormone receptors can also be activated through a steroid-independent pathway. The activation occurs via signaling cascades from membrane regulatory molecules such as cAMP, dopamine, growth factors, cytokines and other cellular regulators acting at the membrane (Mani et al., 1994). The cascades then activate SRs in a ligand-independent manner, which is through the phosphorylation of AF-1 at receptor variable region.

Nuclear receptors are also reported to modulate gene expression in a HRE-binding-independent manner, so-called transcriptional cross-talk (Gottlicher et al., 1998). This mode of action is predominantly based on protein-protein interactions (Gottlicher et al., 1998). The nuclear receptor ER and GR are able to bind to AP-1 complex, which is composed of dimers of Jun family proteins

or Jun/Fos hetero-dimers, and play important roles in cell proliferation (Aranda and Pascual, 2001; Gaub et al., 1990; Gottlicher et al., 1998).

1.2.4.2 Nuclear receptor coactivators

Steroid receptor coactivator-1 (SRC-1) is the first nuclear receptor coactivator discovered in 1995 (Onate et al., 1995). SRC-1 is a general coactivator for all known steroid receptors and enhances transactivation of steroid hormone-dependent target genes (Shibata et al., 1997). The interaction of SRC-1 and steroid receptors is normally ligand- and AF-2-dependent. A centrally located region of SRC family proteins contains multiple LXXLL motifs (where L stands for leucine and X is any amino acid) that are responsible for ligand-dependent interaction with NRs. These motifs are also known as NR boxes (Heery et al., 1997; Onate et al., 1998; Voegel et al., 1998; Wu et al., 2005). At the C-terminus to the NR boxes is a region that was shown to contain the intrinsic transcriptional activation domain (Onate et al., 1998; Voegel et al., 1996). This domain coincides with the region responsible for the interaction of SRCs with the histone acetyltransferase activity (HAT)-containing p300/CBP cointegrators, which is critical for transcription activation mediated by SRCs (Chen et al., 1997; Li et al., 2000; Voegel et al., 1998). The coactivators also contain some proteins with molecular masses of 160 kD (p160, e.g.

SRC-1/NcoA-1) and 300 kD (p300, e.g. CBP/p300). It also has been suggested that the p160/SRC family of coactivators serve as adaptor molecules to recruit additional coactivators and basal transcription machinery to the promoter (Cavarretta et al., 2002; Torchia et al., 1997).

1.2.4.3 Nuclear receptor corepressors

The observation that the thyroid hormone and retinoic acid receptors (TR and RAR) can actively repress transcription in the absence of their cognate ligands (Banahmad et al., 1992) led to the identification of the nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT) (Chen and Evans, 1995; Horlein et al., 1995; Ordentlich et al., 1999; Park et al., 1999). Both N-CoR and SMRT contain a conserved bipartite nuclear-receptor-interaction domain (NRID) (Li et al., 1997; Seol et al., 1996; Zamir et al., 1996) and three independent repressor domains that can actively repress a heterologous DNA-binding domain (Chen and Evans, 1995; Horlein et al., 1995; Jepsen and Rosenfeld, 2002; Ordentlich et al., 1999; Park et al., 1999). Similar to the nuclear receptor coactivators, the corepressors also contain a critical L-X-X-X-I-X-X-X-I/L motif, which is predicted to form an extended α -helix one helical turn longer than the coactivator motif. In addition to nuclear receptors, N-CoR and SMRT can also be recruited to many other transcription

factors, such as serum response factor (SRF), activator protein-1 (AP-1) and nuclear factor- κ B (NF κ B) (Lee et al., 2000). In fact, both N-CoR and SMRT are components of multiple protein complexes containing histone deacetylase proteins, which provide an mechanism for N-CoR and SMRT to repress transcription of specific target genes (Jepsen and Rosenfeld, 2002). The activity of N-CoR and SMRT is regulated by cell signaling pathways, which can influence their expression levels, subcellular localization and association with other proteins (Hong and Privalsky, 2000; Lavinsky et al., 1998; Zhang et al., 1998; Zhou et al., 2001).

1.3 Progesterone receptor (PR)

PR is one of the well-studied steroid hormone receptors, belonging to the subfamily III of nuclear receptors.

1.3.1 Introduction of PR

Human PR plays a central role in the reproductive events associated with the establishment and maintenance of pregnancy. It is mapped to chromosome 11q22. PR has two different isoforms, PR-A and PR-B, both of which are encoded by the same gene sequence but using different promoters and start codons. The shorter isoform PR-A contains 768 aa. The longer isoform PR-B

contains an additional 164 aa sequence at the N-terminus of PR-A. The extra 164 aa sequence of PR-B belongs to the variable region of PR-B and is named as B-upstream segment (BUS) or activation function-3 (AF-3). The molecular organization of PR is shown in Fig. 1.2.

PR is expressed in tissues known to be progesterone responsive such as the uterus, the ovary and the breast (Duffy and Stouffer, 1995; Horwitz and McGuire, 1975; Perrot-Applanat et al., 1987; Perrot-Applanat et al., 1989; Press and Greene, 1988; Press et al., 1988). It has also been described in the brain, in the pituitary, ventromedial hypothalamus, and in the reproductive tissues like testes and vaginal tissue (Batra and Iosif, 1985; Kato et al., 1978; MacLusky and McEwen, 1980; Turner, 1977). The expression of PR is under the control of estrogen and progesterone. In most target tissues, estrogen increases, while progesterone decreases, PR expression. As an example, PR protein is increased during proestrus in the mammalian uterus (Janne et al., 1976; Leavitt et al., 1977; Leavitt et al., 1974). However, when serum progesterone levels increase during the second half of the cycle, total PR levels in the uterus decrease (Leavitt et al., 1974). On the other hand, the down-regulation effect of progesterone on total PR expression in the uterus is not observed in all cell types. For instance, PR levels do not decrease in the breast between the follicular and luteal phases (Battersby et al., 1992; Graham and Clarke, 1997;

Joyeux et al., 1990; Ricketts et al., 1991; Soderqvist et al., 1993).

Fig. 1.2 Molecular organization of PR isoform A and B.

1.3.2 Functions of progesterone receptors

Although both PR-A and PR-B bind progestins and interact with PREs, they are functionally different. Little is known regarding the unique roles of the two PR isoforms in progesterone target tissues. In general, PR-B is a much stronger activator than PR-A. However, PR-A can be a strong activator under specific cell and target gene contexts (Giangrande and McDonnell, 1999; Richer et al., 2002). The stronger activation potential of PR-B could be due to the existence of a third activation domain (AF-3) within the first N-terminal 164 aa that is unique to PR-B (Leonhardt et al., 2003; Sartorius et al., 1994). Under certain cell and target promoter contexts, PR-A is inactive as a transcription factor but can function as a ligand-independent transdominant repressor of PR-B (Leonhardt et al., 2003), which indicates that high PR-A expression may result in reduced progestin responsiveness. The repressor role of PR-A is not confined to that on PR-B, as PR-A has been shown to diminish the response of other hormone receptors to their appropriate ligands, such as androgen, glucocorticoid, mineralocorticoid, and estrogen receptors (Graham and Clarke, 1997; McDonnell and Goldman, 1994; McDonnell et al., 1994; Wen et al., 1994).

At physiological level, PR-A and PR-B are co-expressed at similar levels in

normal breast (Mote et al., 2002) and endometrial epithelial cells (Mote et al., 1999). When breast and endometrium is progressing to malignancy, the coordinated expression of PR-A and PR-B is gradually lost, and a significant proportion of carcinomas expresses a predominance of one isoform, usually PR-A (Arnett-Mansfield et al., 2001; Mote et al., 2002). This is associated with poor clinical outcomes: in endometrial cancer, the predominant expression of one PR isoform, which is usually PR-A, is significantly more common in tumors of higher grade, suggesting an association between PR isoform predominance and poor prognosis (Arnett-Mansfield et al., 2001; McGowan et al., 2004). In general, the loss of coordinated expression of PR isoforms is an early event in carcinogenesis and is evident in early pre-malignant lesions (McGowan et al., 2004; Mote et al., 2002).

1.4 Function of progesterone in breast cancer cells

1.4.1 Introduction to progesterone

The ovary is the major site of synthesis and secretion of estrogen and progesterone in the mammals and gives rise to cyclical fluctuations in the levels of these hormones in the circulation (Norman and Litwack, 1987). Progesterone is the key component in the complex regulation of normal female reproductive function. It has been known to play important physiological roles in various

tissues. In the uterus and ovary, progesterone is involved in the promotion of uterine growth and in the suppression of myometrial contractility, which in turn facilitate the release of mature oocytes, implantation, and maintenance of pregnancy (Bardin et al., 1983; Iwamasa et al., 1992; Loutradis et al., 1991; Mauvais-Jarvis, 1983; Yki-Jarvinen et al., 1985). In the mammary gland, the major physiological role of progesterone has been postulated to be the formation of lobular-alveolar structures in preparation for milk secretion and the suppression of milk protein synthesis before parturition (Topper and Freeman, 1980). In addition, progesterone is also required for the mediation of signals involved in sexually responsive behavior in the brain (DeBold and Frye, 1994a; DeBold and Frye, 1994b; Parsons et al., 1981).

1.4.2 Function of progesterone in breast cancer cells

There are still conflicting opinions regarding the function of progesterone in breast cancer cells. Progestins are found to stimulate, have no effect on, or inhibit growth in breast cancer cells (Cappelletti et al., 1995; Clarke and Sutherland, 1990; Hissom and Moore, 1987; Jeng et al., 1992; Kalkhoven et al., 1994; Schoonen et al., 1995a; Schoonen et al., 1995b; Sutherland et al., 1988). This may be due to the fact that the effects of progesterone are mediated by its receptor and that PR is induced by estrogen in most breast cancer cells (Graham

et al., 1995; Kaneko et al., 1993), which make it difficult to distinguish the effects of progesterone from those of estrogen (Otto, 1995). To delineate the functions of progestins, PR expression vectors were transfected into estrogen receptor (ER)- α and PR-negative breast cancer cells MDA-MB-231 (Lin et al., 1999b). Although no effect on growth in the vector-transfected control transfectant, progesterone markedly inhibited cell growth (Lin et al., 1999b) and induced cell changes in cell morphology and specific adhesion structures. Progesterone-treated cells became considerably more flattened and well spread than vehicle-treated control cells. This was associated with a striking increase of stress fibers and increased focal contacts (Lin et al., 2000). Furthermore, progesterone treatment also increased cell resistance to trypsin digestion and increased cell attachment to extracellular matrix proteins, such as laminin and fibronectin (Lin et al., 2001).

Further studies showed that the effects of progesterone on inhibited growth and increased focal adhesion were mediated through different molecular pathways. In PR-transfected MDA-MB-231 cells, progesterone remarkably up-regulated the protein level of p21^{CIP1/WAF1}, and dramatically decreased the protein level of cyclin A, B1 and D1, as well as the phosphorylation level of p42/p44 MAPK, all of which contributed to the progesterone-induced growth inhibition. On the other hand, the progesterone-increased cell spreading and focal adhesion is

associated with increased stress fibers and distinct up-regulation of tyrosine phosphorylation of focal adhesion protein paxillin and focal adhesion kinase. Inhibitory antibody of $\beta 1$ -integrin can reverse progesterone's effect on cell spreading and focal adhesion but had no effect on progesterone-mediated growth inhibition (Lin et al., 2000; Lin et al., 2003b). As progesterone analogs have wide clinical applications in hormone replacement therapy and breast cancer treatment, all the above findings provided a possible mechanism to design therapeutic agents targeting specific pathways of progesterone functioning to avoid possible side effects associated with progestin therapy.

1.5 Tropomyosins

1.5.1 General introduction to tropomyosins

Tropomyosins are a group of actin-binding proteins found in skeletal muscle, smooth muscle and non-muscle tissues. In skeletal muscle, tropomyosins serve to mediate the effect of Ca^{2+} on the actin-myosin interaction (Mak et al., 1979; Taylor, 1979). Instead of binding Ca^{2+} directly, they perform this function by acting as bifunctional molecules, binding to actin on the one hand, and providing specific sites for the binding of the troponin complex of regulatory proteins on the other hand (Chong and Hodges, 1982; Mak and Smillie, 1981; McLachlan and Stewart, 1976; Ohtsuki, 1979; Pearlstone and Smillie, 1982;

Pearlstone and Smillie, 1983). In non-muscle tissues, the role of tropomyosin is not well-understood. In vitro studies have shown that non-muscle tropomyosins were able to differentially protect actin from the severing action of gelsolin (Ishikawa et al., 1989) and can regulate the Mg-ATPase activity of myosins to varying degrees (Fanning et al., 1994).

All tropomyosins are dimeric proteins having a rod-shaped, α -helical coiled-coil structure. The dimers can be hetero- or homo-dimers. Usually the dimers form a head-to-tail polymer running along the major groove in the actin filament (Lin et al., 1997; Phillips et al., 1979). Actin filaments which lack tropomyosins tend to be rapidly treadmilling pools of filaments, such as those associated with neuronal growth cone filopodia and the leading edge of mammary adenocarcinoma cells (DesMarais et al., 2002; Gunning et al., 2005; Had et al., 1994; Schevzov et al., 1997). This correlates well with the role of tropomyosin in regulating many properties of actin, including stabilizing actin filaments (Cooper, 2002; Gunning et al., 2005).

1.5.2 Generation of tropomyosin diversity by alternative splicing

Mammalian and avian tropomyosins are encoded by four genes, that is, α , β , γ , δ (Pittenger et al., 1994). Historically, the tropomyosins have been divided into two classes, high-molecular-weight (HMW) and low-molecular-weight (LMW),

which are around 284 aa and 247 aa in length, respectively. This size difference is generated by the use of alternative promoters, which leads to the inclusion of exons 1a and 2a or 2b in HMW tropomyosins or exon 1b in LMW tropomyosins at the N-terminus of the protein (Fig. 1.3) (Cooley and Bergtrom, 2001; Gunning et al., 2005; Pittenger et al., 1994).

In addition to the use of alternative promoters, the genes encoding tropomyosin isoforms also utilize a mutually exclusive alternative internal splice of exons 6a versus 6b and alternative C-termini 9a and 9d in the β -tropomyosin gene, 9a, 9c and 9d in the γ -tropomyosin gene and 9a, 9b, 9c and 9d in the α -tropomyosin gene to generate over 40 isoforms (Fig. 1.3). Alternative splicing is seen with both HMW and LMW isoforms from the α -gene but has so far only been seen with LMW isoforms from the γ -gene. There are also RNA isoforms where the protein sequences are identical but the 3' UTRs are derived from different exons such as 9a9b versus 9a9d in the γ -tropomyosin gene. In this case, the splice of 9a to 9d creates a frame-shift in 9d and the whole exon now functions as a 3' UTR (Dufour et al., 1998b; Gunning et al., 2005).

The alternative exon choices within a gene display much greater sequence divergence than do the corresponding exons between genes (Pittenger et al., 1994). Thus, the N-terminal exons 1a and 1b are very dissimilar within the α -

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Fig. 1.3 Tropomyosin isoform diversity is generated by the use of four genes (a,b,g,d) and alternative splicing within at least three genes.

Alternative splicing generates multiple products through the use of alternative promoters, resulting in different N-termini, mutually exclusive internal splicing of 6a versus 6b and alternative C-termini. Colour coding is used to indicate that the 1a exon, for example, from the α Tm gene is more similar to the 1a exon from the β Tm and γ Tm genes than it is to the alternative N-terminal 1b exon from the α Tm gene. 'A' indicates polyadenylation sites, and the altered color size in the 9c exon in Tm5NM8 and Tm5NM9 reflects a frame shift in 9c translation in the 9a–9c splice compared with that in the 8–9c splice. [modified from "Tropomyosin isoforms: divining rods for actin cytoskeleton function." (Gunning et al., 2005)]

and γ -tropomyosin genes; whereas, the α -, β - and γ - gene 1a exons are very similar, as are the 1b exons from the α - and γ -tropomyosin genes. Thus, it has been comparatively easy to generate antibodies that discriminate between different exon-encoded peptides from the same gene (Gunning et al., 2005).

1.5.3 γ -tropomyosin gene

The human γ -tropomyosin gene is also referred to as TPM 3 or TMnm. It is located at human chromosome 1, proximately 42 Kb in length, with 13 exons in its primary transcripts, which are differentially processed to produce >11 mRNA isoforms (Clayton et al., 1988; Dufour et al., 1998a; Pieples et al., 2002). Most of these isoforms are expressed as LMW non-muscle tropomyosins. However, one of these isoforms is striated muscle specific with expression restricted to slow-twitch musculature, such as adult human heart (Pieples and Wieczorek, 2000; Reinach and MacLeod, 1986). This isoform is often referred to as α -TM slow because of the homology with the chicken slow-twitch muscle isoform but has also been called sk α TM.2 (Lees-Miller and Helfman, 1991; MacLeod and Gooding, 1988; Nishii et al., 1997; Pieples and Wieczorek, 2000).

By alternative mRNA splicing, the γ -tropomyosin gene produce mRNAs of two sizes, 1.3 Kb in length encoding the 284 aa sk α TM.2, and 2.5 Kb in length

encoding the about 248 aa non-muscle isoforms. In addition, the γ -tropomyosin gene is involved in the rearrangement giving rise to the *trk* oncogene, a fusion of truncated tropomyosin sequences with tyrosine kinase sequences (MacLeod et al., 1986; Martin-Zanca et al., 1986).

The functions of non-muscle tropomyosins are not well understood. The association of tropomyosins with microfilament leads people to speculate the existence of relationship between the expression level of tropomyosins and some aspects of cell phenotype, such as cell morphology and cell mobility. In the 1990s, Taniguchi et al. showed that one of the γ -tropomyosin gene products, tropomyosin 3 isoform 2 (also termed as TM5/TM30nm or Tm5NM-1), was expressed at a higher level in highly metastatic B16 mouse cell line than in mouse cell line exhibiting a lower metastasis rate, and similar result was obtained in rat cells (Miyado et al., 1996; Miyado et al., 1997). They thus suggested that TM5/TM30nm could be involved in the transformation of several types of tumor cells, probably by enhancing depolymerization of microfilaments. TM5/TM30nm was also found to increase cell spreading, promote stress fiber formation and decrease cell motility in rat cortical neuronal cells (Bryce et al., 2003). In addition, as non-muscle actin is involved in the positioning and morphology of the Golgi complex and Golgi-to-endoplasmic reticulum retrograde trafficking, one or more isoforms from the γ -tropomyosin

gene are associated with Golgi-derived vesicles because of their association with actins. One of tropomyosins identified to be associated with Golgi-derived vesicles is Tm5NM-2, which has been shown to play a role in vesicle generation (Percival et al., 2004). In summary, although the products of γ -tropomyosin gene share high homology, their exact functions could be cell- and tissue-specific.

1.6 Tetratricopeptide repeat protein 9 (TTC9)

1.6.1 The tetratricopeptide repeat (TPR)

The tetratricopeptide repeat (TPR) is a 34 aa consensus motif that is found in tandem repeats of varying number in a number of proteins (Blatch and Lassle, 1999; Lamb et al., 1995; Smith, 2004). It is found in proteins from bacteria, cyanobacteria, yeast and other fungi, insects, plants, and animals including humans (Blatch and Lassle, 1999). The functions of TPR-containing proteins also vary, which include cell cycle control, transcription repression and splicing events, stress response, protein kinase inhibition, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding (Goebel and Yanagida, 1991). In the context of steroid receptors, TPR-containing proteins, such as FKBP51, FKBP52, Hip, Hop and Cyp40, are well-known for their involvement in the process of steroid receptors maturation through the

interaction with Hsp70 and Hsp90 (Pratt and Toft, 1997; Pratt and Toft, 2003). Although not all of the detectable TPRs within one particular protein are necessarily functional, the evolutionary conservation of the TPR motif in general suggests that it is functionally and fundamentally important (Blatch and Lassle, 1999).

Comparison of TPRs from a variety of proteins reveals eight loosely conserved consensus residues at position 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (A/S/L), and 32 (P/K/E) (Sikorski et al., 1990). These residues are conserved in terms of their size, hydrophobicity and spacing (Lamb et al., 1995). Above the basic eight TPR consensus residues, individual TPR has a specific overall structure, which might associate with functional implications. Adjacent TPRs within the same protein are typically related only by the consensus residues, whereas individual TPRs are extensively conserved in evolution in a way that is specific to that individual TPR (Lamb et al., 1995). As an explanation, TPRs 5 and 7 in CDC27 only show homology in these eight consensus residues mentioned above. However, when TPRs 5 or 7 of CDC27 and its orthologs from *Saccharomyces cerevisiae* (Lamb et al., 1994), *Schizosaccharomyces pombe* (Hirano et al., 1990), *Aspergillus nidulans* (O'Donnell et al., 1991), *Drosophila melanogaster* and humans (Tugendreich et al., 1993) are compared, there is a high degree of sequence conservation that is

not limited to the consensus residues, but is also present throughout all 34 aa of the individual TPR (Lamb et al., 1995).

Circular dichroism (CD) studies indicate that TPR motifs are approximately 50% α -helical structures with little or no β -sheet formation (Hirano et al., 1990). The TPR motif was thus proposed to form two α -helical domains. This has been confirmed by the decipherment of crystallographic structures for numerous TRP-containing proteins, which revealed that TPR motif generally form an antiparallel α -helical hairpin (Das et al., 1998; Scheufler et al., 2000). Clustering of these hairpins in tandem generates a domain with a grooved surface and dimension that can conveniently grasp another polypeptide. Generally, by generating a flexible, mutable domain that can facilitate specific protein-protein interactions, the TPR motif presents an elegant evolutionary solution contributing to the fundamental biological importance of coordinating interactions among gene products (Smith, 2004).

1.6.2 TPR-containing protein in steroid receptor complexes

TPR-containing proteins are well known for their involvement in the steroid receptor maturation process. Around 1985, Hsp90, which is a TPR-binding protein, was identified to be associated with the PR and GR. This is the first indication for the involvement of chaperones in unactivated steroid receptor

complexes. In 1990's, both Toft and Smith elaborated the assembly process of GR and PR with the assistance of different chaperone proteins (Pratt and Toft, 1997; Smith, 2000). In a cell-free system, an ordered assembly pathway was identified for PR, which could be divided into three stages. In an early stage, Hsp70 binds to PR and starts the assembly of hormone receptor complex. The binding of Hip to Hsp70 promotes the Hop-Hsp90 complex recruitment to the early stage Hsp70-PR complex (Prapapanich et al., 1998). Here Hop functions as an adaptor that targets Hsp90 to Hsp70 (Chen et al., 1996; Chen and Smith, 1998). The presence of Hsp70, Hsp90, Hip and Hop in the PR complex forms the intermediate stage of PR assembly. Once Hsp90 is bound to PR, p23 binds to Hsp90 and permitting, or maybe promoting, the loss of Hsp70, Hip and Hop from the PR complex and the concomitant incorporation of an immunophilin, such as FKBP51, FKBP52 and Cyp40, or immunophilin-like PP5, to form a mature PR-Hsp90-p23-immunophilin complex (Smith, 2000). In this process, Hip, Hop and each of the immunophilin are TPR-containing proteins. The TPR region in Hip is involved in Hsp70 binding (Irmer and Hohfeld, 1997; Prapapanich et al., 1996). Hop has two TPR regions, one of which binds Hsp70 and the other Hsp90 (Chen et al., 1996; Lasse et al., 1997). FKBP51, FKBP52, Cyp40 and PP5 all have a single TPR region required for Hsp90 binding (Nair et al., 1997; Owens-Grillo et al., 1995; Ratajczak and Carrello, 1996).

1.6.3 TPR-containing proteins participate in diverse cellular functions

Except for their regulatory roles in hormone receptor assembly, TPR-containing proteins also participate in many other aspects of cell functioning.

1.6.3.1 TPR-containing proteins in cell cycle control

The involvement of TPR-containing protein in cell cycle control is well studied in the anaphase promoting complex, which includes the TPR proteins CDC16, CDC23, and CDC27. All of these three proteins interact with one another in yeast and contain multiple, tandem TPRs in the C-terminus, with one or two additional TPRs in the N-terminus (Lamb et al., 1994). Studies have shown that mutations in the TPR domains of these proteins disrupted the interactions among these proteins and/or the function of these proteins, indicating the importance of TPRs for the proper function of the complex. A mutation at the non-consensus position 6 and an insertion between position 6 and 7 in TPR7 of CDC27 disrupts its interaction with CDC23, supporting the protein-protein interaction mediated by TPRs (Lamb et al., 1994). Mutations at position 8 of TPR 5 and 7 of CDC23 (Sikorski et al., 1993), and position 20 of TPR9 of CDC16 (Samejima and Yanagida, 1994) result in mitotic arrest at the metaphase to anaphase transition. This could be due to the disruption of protein function resulted from the mutation-induced incorrect packing of neighbouring α -helices

within the TPR motif (Das et al., 1998).

1.6.3.2 Transcription repression complex

The yeast proteins Tup1 and TPR protein SSN6 form a transcription repressor complex involved in the regulation of a variety of genes (Edmondson et al., 1996; Keleher et al., 1992). They gain specificity of action by interacting with sequence-specific DNA-binding proteins. Once targeted to DNA, Tup1 acts as a global transcriptional repressor (Edmondson et al., 1996; Friesen et al., 1997; Keleher et al., 1992; Komachi et al., 1994; Redd et al., 1996), while SSN6 functions as an adaptor between Tup1 and DNA-binding proteins and has no transcriptional repression activity of its own (Keleher et al., 1992; Tzamarias and Struhl, 1995). The N-terminus of SSN6 contains 10 TPR motifs that are essential for its function (Schultz and Carlson, 1987; Schultz et al., 1990). Distinct combinations of TPR motifs are required for direct interaction with Tup1 and for repression of distinct classes of genes (Tzamarias and Struhl, 1995). For example, the first three TPR motifs are sufficient for binding to Tup1 (Tzamarias and Struhl, 1995) and to Mat α 2 (Smith et al., 1995) and for repression of mating-type regulated genes (Tzamarias and Struhl, 1995). Repeats 1-7 are necessary for the repression of oxygen-regulated genes, whereas all the TPR motifs are required for repression of DNA

damage-regulated genes (Jabet et al., 2000; Tzamarias and Struhl, 1995).

1.6.3.3 TPRs in protein transportation

The mitochondria import receptor complex involving the TPR protein Tom20 and Tom70 binds to proteins and transports them across mitochondria membranes (Bauer et al., 2000; Wiedemann et al., 2004). Both of these two proteins are located at the outer membrane of mitochondria. Recent studies suggested that Tom70 family proteins are characterized by 11 TPR motifs. These 11 motifs are organized with three TPRs in an N-terminal “clamp” domain, five in a “core” domain and three in a C-terminal domain (Chan et al., 2006). The TPR motifs are functionally important for binding to substrate as well as for proper functioning of Tom70. Truncation of the C-terminal TPR domain of Tom70 results in a polypeptide that is transported correctly to yeast mitochondria but is nonfunctional (Riezman et al., 1983).

For proteins to be transported into the peroxisomal matrix, they possess a peroxisomal targeting signal (PTS) that directs them to peroxisomes. Two different PTSs have been identified, PTS1 and PTS2, which are recognized and bound in the cytosol by specific receptor proteins, Pex5p (Dodt and Gould, 1996; McCollum et al., 1993; Terlecky et al., 1995) and Pex7p (Elgersma et al., 1998; Marzioch et al., 1994; Purdue et al., 1997), respectively. Pex5p contains

an array of TPR in the C-terminal part of the protein, which is responsible for binding to PTS1 and targeting them to the peroxisomal membrane (Gatto et al., 2000; Klein et al., 2001; Klein et al., 2002).

1.6.4 Basic knowledge of TTC9

Tetratricopeptide repeat protein 9 (TTC9) was first reported as a hypothetical gene KIAA0227 by Nagase et al (Nagase et al., 1996) based on the sequence analysis of a cDNA clone isolated from a brain cDNA library. It was also reported later by the National Institutes of MGC Program (Strausberg et al., 2002). It is now referred to as TTC9 because of its sequence homology to a family of TTC proteins which contain TPR domains. Human TTC9 gene is located on chromosome 14q24.2, with three exons and spanning a region of about 33 Kb. Its transcript sizes are predicted to be 5217 bp and 2428 bp (<http://www.pubmed.com>), which encode predicted proteins of 336 aa and 277 aa respectively. The recent updated NCBI entry predicted a 5510 bp transcript that encodes a 434 aa protein (GenBank accession no. XM_027236.6). Using Vector NTI software, we deduced the ORF of TTC9 mRNA (GenBank accession no. D86980) to be from nucleotide 344 to 1012, encoding a protein of 222 aa. ScanProsite analysis of TTC9 protein sequence predicted the existence of three tandem TPR domains between aa 57-197 (aa 57-90, aa

128-161 and aa 164-197). Blast searches using the amino acid sequence 1–51, which is outside of the TPR domains of TTC9, yielded no sequence similarity to any protein domains in the database, suggesting that TTC9 is a unique novel protein.

Previous results from our lab have shown that progesterone could significantly inhibit cell proliferation and induce remarkable cell spreading and focal adhesion in PR-transfected MDA-MB-231 cells which were originally ER- and PR-negative (Lin et al., 2000; Lin et al., 1999b). Microarray analysis suggested that these effects were coupled with a drastic up-regulation of the mRNA of TTC9 in the cells, as confirmed by the results from real-time RT-PCR analysis. It was then hypothesized that TTC9 mRNA was actively translated and the protein might play some roles in mediating the function of PR on cell growth and focal adhesion. Further studies revealed that not only by progesterone, the expression level of TTC9 was also regulated by different hormones, growth factors and serum factors in different breast cancer cell lines. In addition, TTC9 was ubiquitously expressed in human tissues. In breast cancer cells, it was predominantly concentrated to the endoplasmic reticulum (Cao et al., 2006). All these results suggested that TTC9 could be an important functional protein in different tissues and cell types.

1.7 Scope of study

In this study, it is believed that TTC9 is an important gene involved in progesterone, and probably other hormones, functioning. The objectives of this study are:

1. Characterization of TTC9 gene, including determination of its transcript size and protein size;
2. Preliminary results already shown that TTC9 is a progesterone-regulated gene in PR-transfected MDA-MB-231 cells. It will be interesting to investigate whether TTC9 is only involved in PR signaling pathway, or it is broadly involved in different signaling pathways in various breast cancer cell lines. The effects of different hormones, factors on the expression of TTC9 were thus examined both *in vitro* and *in vivo*;
3. Generation of TTC9 polyclonal antibodies;
4. Cellular localization of a protein normally will give some clues on the protein function. As a newly identified protein, it is important to know the cellular localization of TTC9 in breast cancer cells, which will definitely assist in future studies on TTC9 function. Immunostaining and cell fractionation was performed to localize TTC9 in the cells;

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5. Hunting for interacting proteins is a common way to get some hints on the function of an unknown protein. Yeast-two-hybrid assay was used to identify TTC9 interacting proteins. GST-pull down assay and co-immunoprecipitation were performed to further confirm the interactions;
6. Investigation of TTC9 functions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Bovine serum albumin (BSA), dextran, charcoal, RNase A, pepstatin A, leupeptin, aprotinin, Na_3VO_4 , NaCl, phenylmethanesulphonylfluoride (PMSF), β -mercapthoethanol, ethidium bromide, formaldehyde, diethylpyrocarbonate (DEPC), ethylenediaminetetraacetic acid (EDTA), NaF, sorbitol, zymolyase, Tris base, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Tween 20, 17β -estradiol (E_2), 17β -estradiol benzoate, progesterone, dexamethasone, aldosterone, RU486, ZK98299, basic fibroblast growth factor-b (bFGF), SB203580, PD98059, FITC-conjugated anti-mouse IgG and other common chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) were bought from Fermentas International Inc. (Hanover, MD). Trichloroacetic acid (TCA) was purchased from Fisher Scientific (Hampton, NH). Epidermal growth factor (EGF) was obtained from ProSpec-Tany TechnoGene Ltd., Israel. ICI182, 780 was bought from Tocris Biosciences (Ellisville, MO). Triton X-100 was bought from Merck KGaA. (Darmstadt, Germany). RedivueTM deoxycytidine 5'-[α - ^{32}P]-triphosphate (~3000 Ci/mmol) was obtained from Amersham BioSciences Inc. (Buckinghamshire, UK). The hexyl ester of rhodamine B and Alexa Fluo 647 donkey anti-goat IgG were obtained from Molecular Probes, Inc. (Eugene, OR).

2.2 Cell culture

MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) in 1995 at passage 147 and 28, respectively. BT-20 cells were also bought from ATCC. Breast cancer cell lines MDA-MB-435, ZR-75.1, T47D were generous gift from Dr. Suet Feung Chin at Department of Oncology, University of Cambridge, Addenbrooke's Hospital, Cambridge. HL-60 and HT-29 were obtained from Dr. Shali Shen at Department of Physiology, National University of Singapore, Singapore. Other cell lines were kindly given by professors from School of Biological Sciences, Nanyang Technological University, Singapore, which include Hep G2 from Dr. William Chen Wei Ning, COS-7 cells from Dr. Koh Cheng Gee, PC12 cell line from Dr. Klaus Heese, MOLT-4 from Dr. Tan Siet Mien and Jurkat cells from Dr. Kristen Sadler.

MDA-MB-231-C2 (clone 2) cells were subcloned from MDA-MB-231 cells using single cell dilution method. This clone was transfected with PR expression vectors hPR1 and hPR2 that contain human PR cDNA coding for PR isoform B and isoform A, respectively, in pSG5 plasmid (Kastner et al., 1990). PR-transfected cell clone ABC28 was used in the present study. ABC28 cells expressed approximately 660 fmol PR per milligram protein as determined by

enzyme immunoassay (Abbott Laboratories, Abbott Park, IL). CTC15 cells are MDA-MB-231 clone 2 cells stably transfected with empty vectors, and they were used as transfection controls (Lin et al., 1999a).

Most of the cell lines were routinely maintained in phenol red and high D-glucose-containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7.5% fetal calf serum (FCS), 2 mM glutamine and 40 mg/L gentamicin. PC12 cells were routinely maintained in phenol red-containing DMEM supplemented with 5% FCS, 5% horse serum, 2 mM glutamine and 40 mg/L gentamicin. HL-60 and HT-29 cells were grown in RPMI 1640 medium containing 10% FCS, 2 mM glutamine and 40 mg/L gentamicin.

As phenol red has weak estrogenic effects, and estrogen is known to crosstalk with growth factor pathways in breast cancer cells, for all the experiments on the effects of steroid compounds and growth factors, cells were grown in phenol red-free DMEM containing 2 mM glutamine, 40 mg/L gentamicin, and 5% dextran charcoal-treated FCS (DCC-FCS). FCS was treated with dextran-coated charcoal to remove the endogenous steroid hormones. Cells were treated with various steroid compounds from 1000-fold stock in ethanol. This gave a final concentration of ethanol at 0.1%. Treatment controls received 0.1% ethanol only. SB203580 and PD98059 were prepared as 1000-fold stock in DMSO.

Treatment controls received 0.1% DMSO only. Growth factors were added to the cell culture from 1000 stock in ddH₂O.

All cell culture reagents were from Gibco BRL, Life Technologies Inc (Gaithersburg, MD). Fetal calf sera were from Hyclone (Logan, UT) or PromoCell GmbH (Heidelberg, Germany). All cell culture plastic wares were purchased from Falcon (Becton Dickinson, San Jose, CA), NUNC (Nalge Nunc International, Rochester, NY), or Corning (Corning, NY).

2.3 siRNA transfection

The day before transfection, ABC28, MDA-MB-231 or MCF-7 cells were plated into phenol red-free DMEM supplemented with 5% DCC-FCS and incubated at 37°C. Cells reached 40-50% confluence at the time of transfection. The transfection was carried out using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each well of 6-well plate, 6 µl Lipofectamine 2000 reagent and 125 nmol siRNA were added into a final 2.5 ml transfection medium. Cells were harvested at 72 hr post-transfection.

Non-targeting siRNA was bought from Ambion, Inc (Austin, TX) and was used as a negative control for siRNA transfection. TTC9 siRNA was designed and

synthesized from Ambion, Inc. The sequence for the siRNA is as follows:

Name	Sequence
TTC9 siRNA	Target sequence: ACTATGAACGAGTCAAGGA sense: 5'-ACTATGAACGAGTCAAGGAtt-3' antisense: 5'-UCCUUGACUCGUUCAUAGUtt-3'

2.4 Transfection of COS-7 cells

COS-7 cells were seeded in phenol red containing DMEM supplemented with 7.5% FCS the day before transfection. The confluence of cells at transfection reached 40%-50%. The transfection was carried out by FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. For each well of six-well plate, 3 µl FuGENE 6 and 1 µg DNA was used. Cells were harvested at 48 hr post-transfection. The expression of protein was examined by Western blotting.

2.5 Immunoblotting

2.5.1 Cell lysates preparation and protein concentration quantification

Cells were lysed on ice with cold lysis buffer containing 100 mM NaF, 50 mM HEPES (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF and the cocktail of proteinase inhibitors (5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2

$\mu\text{g/ml}$ aprotinin and 1 mM Na_3VO_4). The cell debris was discarded by centrifuging at 14,000 rpm for 12 min at 4°C. The supernatants were immediately frozen down in liquid nitrogen and were stored at -80°C for future use. Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL).

2.5.2 Western blotting analysis

Lysates containing equal amount of total proteins were separated on SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham Biosciences Inc.). Membranes were blocked in 5% non-fat milk. The antibodies used are listed as follows:

Primary antibodies:

- a) anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): from Ambion Inc. (Austin, TX).
- b) anti-His antibody: from Amersham Biosciences Inc.
- c) anti-human calnexin: AF18 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).
- d) anti-cytochrome oxidase subunit IV: 20E8 from Molecular Probes, Inc.
- e) anti-flag M2: from Sigma-Aldrich (St. Louis, MO)

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f) anti-Tm5NM-1/2: bought from Chemicon (Temecula, CA)

Secondary antibody:

HRP (horseradish peroxidase)-conjugated anti-mouse IgG: from Amersham Biosciences Inc.

HRP-conjugated anti-goat IgG: bought from Santa Cruz Biotechnology Inc.

Signal detection was carried out by using either enhanced chemiluminescence (ECL) or ECL plus system (Amersham Biosciences Inc.) followed by exposing to X-ray films (Eastman Kodak Co., New Haven, CT). Band intensities were determined by Bio-Rad Molecular Image Analyzer (Hercules, CA).

2.6 Immunofluorescence microscopy

Cells were seeded onto 6-well plates equipped with sterile glass cover-slips. After rinsing with PBS, the cells were fixed in 4% paraformaldehyde for 10 min and were permeabilized with 0.2% Triton X-100 for 10 min. This was followed by incubation with 2% normal FCS in PBS for 1 hr to block nonspecific binding. Cells were then incubated with antibody to TTC9 or to calnexin (Santa Cruz Biotechnology Inc.) in blocking buffer for 1 hr at 37°C, followed by incubation with FITC-conjugated antimouse IgG (Sigma) or Alexa Fluo 647

donkey anti-goat IgG (Molecular Probes, Inc.) in blocking buffer for 1 hr at 37°C. After washing in PBS, the cells were stained in the hexyl ester of rhodamine B (Molecular Probes, Inc.) at 1 µg/ml for endoplasmic reticulum. Stained cells were viewed and photographed using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Gottingen, Germany).

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

All the incubation should be done in a humid box and the plate should not get dry during any procedure. Antigen protein was prepared in PBS (containing 0.05% w/v NaN₃) at 4 µg/ml. The day before the assay, 50 µl antigen solution was added into each well of the 96-well polysorb ELISA flat bottom plate (Nalge Nunc International, Rochester, NY) and the plate was incubated overnight at 4°C. On the day of assay, the coating antigen solution was decanted, followed by blocking the plate with 200 µl/well blocking buffer (1% w/v BSA, 0.3% v/v Tween 20 and 0.05% w/v NaN₃ in PBS) at 37°C for 1 hr. During blocking, antisera from mice were diluted sequentially in blocking buffer. After wash the plate three times in ELISA washing buffer (0.025% v/v Tween 20 in PBS), 50 µl diluted primary antibody was added into each well and the plate was incubated for 2 hr at 37°C. As a negative control, pre-immune serum was included at 1:50 dilution. The plate was then washed eight times in ELISA

washing buffer and 50 μ l alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) was added into each well at 1:2000 dilution in PBS. After 1 hr incubation at 37°C, the plate was washed in ELISA washing buffer for five times. The substrate, PNPP (Sigma), was freshly prepared as 1 mg/ml solution in diethanolamine buffer (4.85% v/v diethanolamine, 0.01% w/v NaN_3 and 0.04% w/v $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) and was added to each well (50 μ l/well). The plate was allowed to develop in the dark and was read at OD_{405} .

2.8 Real-time RT-PCR

2.8.1 RNA extraction from cell and tissue

Total RNA was extracted using TRIzol reagent (Life Technologies Inc.) according to the manufacturer's instructions. Pelleted RNA was dissolved with 0.2% DEPC treated sterile double distilled water. Quantification was performed on a spectrophotometer (Beckman Coulter Inc., Fullerton, CA) at the wavelength of 260 nm. The ratio of 260 nm/280 nm was calculated with the value greater than 1.70 considered acceptable.

2.8.2 Real-time RT-PCR

cDNAs were synthesized from 5 μ g of total RNA using Superscript II reverse transcriptase and random primers (Invitrogen Inc.) according to the

manufacturer's instruction. Real-time PCR was performed with SYBR Green master mix on an ABI Prism 7000 sequence detection system (PE Applied Biosystems, Foster City, CA). cDNAs were amplified by incubation at 95°C for 10 min to activate the Hot Start AmpliTaq Gold DNA polymerase, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 1 min. PCR for each gene fragment were performed in triplicate, and each primer set was repeated three times. To ensure the specificity of PCR products, melting curves were generated after amplification followed by agarose gel electrophoresis. Both melting curve and gel electrophoresis showed a single, specific product for each gene. An example of melting curves for both TTC9 and 36B4 genes was shown in the appendices (Fig. A.1). The changes in fluorescence of the SYBR Green I dye in each cycle were monitored by ABI 7000 system, and the threshold cycle, which is defined as the cycle number at which the amount of amplified target reaches a fixed threshold, was obtained for each gene. Primer sets for the 36B4 gene, which codes for human acidic ribosomal phosphoprotein P0, were included in each experiment as a control for normalizing the quantity of cDNA used. The relative amount of PCR products generated from each primer set was determined on the basis of the threshold cycle value (C_t). The $2^{-\Delta\Delta C_t}$ method was adopted to analyze the data. ΔC_t is equal to the difference in threshold cycles for target and

control genes (C_t of target gene – C_t of 36B4). Subsequently, the $\Delta\Delta C_t$ values were calculated by subtracting the ΔC_t values of the controls from the ΔC_t values of the samples. Changes in gene expression were reported as fold increases ($2^{-\Delta\Delta C_t}$) relative to controls.

The primers used for the quantitative PCR were designed as follows:

Name of Gene	Primer Sequences	Annealing temperature (°C)	Length of product
36B4	Forward: 5'-GATTGGCTACCCAACTGTTGCA-3' Reverse: 5'-CAGGGGCAGCAGCCACAAAGGC-3'	60	158 bp
TTC9	Forward: 5'-CACATGTCTATAACGATTTCC-3' Reverse: 5'-TGCAGGAAACAGGGGACTCTC-3'	60	154 bp

2.9 Cell fractionation of MCF-7 cells

All steps of this procedure were carried out at 4°C. All buffers and equipments were pre-cooled to 4°C. 6 x 10⁷ MCF-7 cells were suspended in 5 ml lysis buffer containing 1 M sorbitol, 10 mM HEPES (pH7.4) and 1 mM EDTA and incubated on ice for 30 min. Cells were then passed through 21 G needle for 40 times and centrifuged at 1000×g for 20 min to pellet nuclei and unbroken cells.

The supernatant was collected and centrifuged sequentially at 12,000×g and 100,000×g for 1 hr, respectively, to obtain the fractions 12P (pellet after centrifugation at 12,000×g, containing mitochondria), 100P (pellet after centrifugation at 100,000×g, containing microsome and endoplasmic reticulum) and 100S (supernatant after centrifugation at 100,000×g, cytoplasm). 20 µg total protein from each fraction was analyzed by Western blotting.

2.10 Northern blotting analysis

Cells at 60-70% confluence were treated with hormones or control vehicle for various time periods. Total RNA was isolated from the cells as mentioned before. 12 µg of total RNA from each sample were separated on a 1.5% Northern gel in duplicates and transferred to a nylon Hybond-N membrane (Amersham Biosciences). ³²P-labeled TTC9 and ³²P-labeled 36B4 probes were generated by random priming reaction (Amersham Biosciences) using PCR-amplified cDNA fragments of the individual genes.

The TTC9 probe was the 669 bp complete coding sequence for TTC9 protein. The TTC9 cDNA clone was a generous gift from Dr. Takahiro Nagase, Kazusa DNA Research Institute Foundation, Japan. The probe for 36B4 was a 158 bp cDNA fragment located between nucleotides 802 and 959 of the 36B4 mRNA (NCBI accession number: BC019014.2). The probes were sequentially

hybridized to the membrane using Ultrahyb solution of Ambion, Inc (Austin, TX) and the results were analyzed using Bio-Rad Molecular Image Analyzer (Hercules, CA).

2.11 TTC9 expression in breast cancer tissues

Human tissue samples were obtained from the Tissue Repository at the National Cancer Centre (NCC), Singapore. Tissue samples were harvested at the time of mastectomy or breast conserving surgery with prior signed informed consent from the patients. Matched pairs of malignant tissue and the adjacent normal breast tissue were harvested and confirmed histologically by a pathologist and were snap frozen in liquid nitrogen. The cases utilized in this study were collected between January 2002 and December 2003. Clinicopathological data such as tumor size, nuclear grade and hormone receptor status were obtained from a prospective database. This study was approved by the ethics committee at NCC.

25 matched pairs of breast tissues were mashed using a mortar and pestle. Total RNA was extracted using TRIzol reagent (Life Technologies Inc.) according to the manufacturer's instructions. Five micrograms of total RNA from each sample were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) with a 30 μ l total reaction volume. 1 μ l cDNA

produced from each RT reaction were amplified by PCR. The primers used here for TTC9 were the same as those used in real-time RT-PCR. 10 µl PCR products corresponding to individual breast tissue sample were separated on 1% agarose gel and transferred to nylon Hybond-N membrane (Amersham Biosciences). ³²P-labeled TTC9 were generated by random priming reaction (Amersham Biosciences) using the same PCR product of TTC9. The band intensities were analyzed using Bio-Rad Molecular Image Analyzer. As internal controls, 36B4 and GAPDH genes were also included for normalization.

2.12 Cloning of expression vectors

To obtain different expression vectors for TTC9 or Tm5NM-1, the inserts were firstly amplified from respective template with specific primers by polymerase chain reaction (PCR). The amplified inserts were loaded onto 1% agarose gel and the band containing the products were extracted by Gel Extraction Kit (Qiagen GmbH, Germany). Purified inserts were then digested with restriction endonucleases identical to those used to digest target vectors. Digested inserts were purified with PCR Purification Kit (Qiagen GmbH). Digested vectors were separated by electrophoresis and the band containing the linearized vector was extracted by Gel Extraction Kit (Qiagen GmbH). To reduce the

transformation background, linearized vectors were dephosphorylated by alkaline phosphatase (CIP, New England Biolabs). Inserts were thus cloned into the targeting vector by Quick Ligation Kit (New England Biolabs) according to the manufacturer's instructions. The positive clones were verified by restriction endonuclease digestion and sequencing.

The primers used for cloning were designed as follows:

Name of insert	Primer sequences	Enzyme digestion site	Target vector	Purpose
TTC9	Forward: 5'-ggaattccatatggagagaaagggtcggcgg-3' Reverse: 5'-ccgctcgagcatggcttcttttctctctggga-3'	Nde I Xho I	pET24b(+)	Expression of TTC9-(His) ₆ for antibody preparation
TTC9	Forward: 5'-ttaggatccctatggagagaaagggtcgg-3' Reverse: 5'-gcgcggaattcttacatggcttcttttctctc-3'	BamH I EcoR I	pGEX-5X-3	GST-TTC9 expression for GST pull-down
TTC9	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-ccgctcgagttacatggcttcttttctctctggga-3'	BamH I Xho I	pXJ-FLAG	flag-TTC9 expression for co-immunoprecipitation

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TTC9	Forward: 5'-ttaggatccctatggagagaaagggtcg-3' Reverse: 5'-ccgctcgagttacatggcttcttttctctctggga-3'	BamH I Xho I	pLexA	Yeast two hybrid
TTC9 (1-50)	Forward: 5'-aattgccatggagagaaagggtcggcg-3' Reverse: 5'-gcgcgaaattcttacatggcttcttttctctc-3'	Nco I EcoR I	pET32a(+)	Express amino acid 1-50 of TTC9 protein for antibody preparation
TTC9 (1-50)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-aatctcgagttacggctcggccgccgccc-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co-immunoprecipitation
TTC9 (1-115)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-aatctcgagttagaggcgccgggttcag-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co-immunoprecipitation
TTC9 (51-222)	Forward: 5'-taggatccatggcggagctcatccgacga-3' Reverse: 5'-ccgctcgagttacatggcttcttttctctctggga-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co-immunoprecipitation
TTC9 (116-222)	Forward: 5'-taggatccatgtcggaggagcagagcaag-3' Reverse: 5'-ccgctcgagttacatggcttcttttctctctggga-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co-immunoprecipitation

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TTC9 (51- 115)	Forward: 5'-taggatccatggccgagctcatccgacga-3' Reverse: 5'-aatctcgagttagaggcgccgggcttcag-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-70)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttagtcctttagcactgcgc-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-85)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttacagcaacgcccgggtgta-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-90)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttacagcccttcagctccag-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-95)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttacccggggggcggcagcag-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-100)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttagtccttcctccgttcccc-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation

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TTC9 (1-105)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttaggaggccgcgcgagtc-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-110)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttacagggccccagcgggga-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-128)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttagatctcgatggcttcac-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-161)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttacccttcttctcaagac-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
TTC9 (1-197)	Forward: 5'-taggatcccgaatggagagaaagggtcgg-3' Reverse: 5'-atactcgagttagtctgttggtgttggt-3'	BamH I Xho I	pXL-Flag	Truncated TTC9-flag expression for co- immunopre- cipitation
Tm5 NM-1	Forward: 5'-ttactcgagatggctgggatcaccacat-3' Reverse: 5'-agaaagcttggcatctcattcaggtcaagc-3'	Xho I Hind III	pcDNA3.1/ myc-His(-) B	Tm5NM-1 -(His) ₆ expression for co- immunopre- cipitation

2.13 Protein purification

2.13.1 Purification of TTC9-(His)₆

The 669 bp TTC9 cDNA sequence was amplified and cloned into pET-24b(+) (EMD Biosciences, Inc., Germany) for the expression of TTC9-(His)₆ proteins. Selected clones were verified by sequencing for correct sequences as well as reading frame. The TTC9-(His)₆ expression vector was transformed into BL21 (DE3) *E. coli* competent cells for the expression. A seed culture was prepared by inoculating one colony into 25 ml LB with Kanamycin (50 µg/ml), incubating overnight at 37°C with vigorous shaking (220 rpm). The second day, 10 ml of overnight seed culture was transferred to 1 liter LB with Kanamycin (50 µg/ml) and was incubated at 37°C with shaking (220 rpm) until OD₆₀₀ reached 0.6-0.8. Expression was induced by adding IPTG at a final concentration of 1 mM. After 4 hr incubation at 30°C, cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C and pellets were resuspended in 40 ml PBS, followed by sonication (SONICS) for 20 min. Cell lysates were cleared by centrifuging at 18,000 rpm for 20 min. The supernatant was loaded over a Ni-NTA agarose column (Qiagen GmbH) pre-equilibrated with resuspension buffer containing 20 mM TrisCl, pH 7.4, 0.5 M NaCl. The column was then washed with 20 mM TrisCl (pH 7.4), 1 M NaCl, 20 mM Imidazole (pH 6.0) and 3 mM β-mercaptoethanol.

TTC9-(His)₆ protein was eluted using a buffer containing 20 mM Tris·Cl (pH 7.4), 0.5 M NaCl, 0.5 M Imidazole (pH 6.0) and 3 mM β-mercaptoethanol, followed by dialyzing in buffer A [20 mM Tris·Cl (pH 7.0), 3 mM β-mercaptoethanol]. The protein was then loaded onto a cation exchange chromatography column (HiTrap SP HP, Amersham Biosciences) pre-equilibrated with buffer A. Elution was carried out using a NaCl gradient of buffer B [20 mM Tris·Cl (pH 7.0), 3 mM β-mercaptoethanol, 1 M NaCl]. Fractions containing the protein were pooled and concentrated by ultrafiltration using an Amicon Ultra centrifugal filter device (Millipore, Billerica, MA) with a molecular weight cutoff of 10 kD. The final purity of TTC9-(His)₆ is over 90%. The purified protein was then dialyzed in PBS for antibody production.

2.13.2 Purification of TTC9 peptide

For the purification of the peptide consisting of the first 50 aa of TTC9 protein (TTC9 (1-50)), the first 150 bp TTC9 cDNA sequence was amplified and cloned into pET32b(+) (EMD Biosciences, Inc.), which has a gene encoding the thioredoxin (Trx) protein and a linker consisting six histidine residues and an enterokinase cleavage site at the N-terminal of the peptide. After sequencing and verification, the TTC9 (1-50) expression vector was transformed into BL21 (DE3) *E.coli* competent cells for expression. A seed culture was prepared by

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inoculating one colony into 50 ml LB with Ampicillin (100 µg/ml), shaking overnight at 37°C. The second day, 30 ml of overnight seed culture was transferred to 3 liter LB with Ampicillin (100 µg/ml) and was incubated at 37°C with shaking (220 rpm) until OD₆₀₀ reached 0.6-0.8. Expression was induced by adding IPTG at a final concentration of 1 mM. After 4 hr incubation at 30°C, cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C and pellets were resuspended in 120 ml PBS, followed by sonication (SONICS) for 60 min. Cell lysates were cleared by centrifuging at 18,000 rpm for 20 min. The supernatant was loaded over a Ni-NTA agarose column (Qiagen GmbH) pre-equilibrated with resuspension buffer containing 20 mM Tris·Cl, pH 7.4, 0.5 M NaCl. The column was then washed with 20 mM Tris·Cl (pH 7.4), 1 M NaCl, 20 mM Imidazole (pH 6.0) and 3 mM β-mercaptoethanol. TTC9 (1-50) protein was eluted using a buffer containing 20 mM Tris·Cl (pH 7.4), 0.5 M NaCl, 0.5 M Imidazole (pH 6.0) and 3 mM β-mercaptoethanol. Eluted protein was dialyzed in 20 mM Tris·Cl (pH 8.0), 50 mM NaCl and 2 mM CaCl₂ and the tag was removed by enterokinase (New England Biolabs). Digestion was performed at 4°C for overnight. HiTrap Benzamidine FF column (Amersham Biosciences) pre-equilibrated with 50 mM Tris·Cl (pH 7.4), 0.6 M NaCl and 3 mM β-mercaptoethanol was used to remove the enterokinase. Enterokinase bound to the column was eluted using 6 M guanidine hydrochloride. Proteins in

the flow through were then dialyzed in buffer A [20 mM Tris·Cl (pH 7.0), 3 mM β -mercaptoethanol] and were loaded onto the cation exchange chromatography column (HiTrap SP HP, Amersham Biosciences) to separate the TTC9 (1-50) fragment and Trx protein. Elution was carried out using a NaCl gradient of buffer B [20 mM Tris·Cl (pH 7.0), 3 mM β -mercaptoethanol, 1 M NaCl]. Fractions containing the peptide were pooled and concentrated by ultrafiltration using a Centriplus centrifugal filter device (Millipore, Billerica, MA) with a molecular weight cutoff at 3 kD. The final purity of TTC9 (1-50) is over 90%. The protein was dialyzed in PBS for later conjugation to carrier protein.

2.13.3 Purification of GST-TTC9

The 669 bp TTC9 coding sequence was cloned into pGEX-5X-3 (Amersham Biosciences) for the expression of GST-TTC9 protein. Protein expression was induced and crude supernatant was collected as mentioned in 2.13.1 and 2.13.2. Glutathione S-transferase (GST) or GST-TTC9 protein was purified with Glutathione Sepharose 4B (Amersham Biosciences). Prior to the addition of crude supernatant, 50% Glutathione Sepharose 4B slurry was prepared according to the manufacturer's instructions. For the binding of GST or GST-TTC9 protein, 1 ml of the 50% slurry prepared was added to 50 ml bacteria sonicate. The mixture was incubated at room temperature for 30 min with gentle

suppression was set to 100 μ s for the water suppression. Other parameters were set according to the program from Bruker. NMR data were processed and analysed using Bruker Topspin software.

2.15 GST pull-down assay

COS-7 cells were transfected with Tm5NM-1-(His)₆ expression vector or control vector (pcDNA3.1/myc-His(-) B) respectively. Total cell lysates were collected at 48 h post-transfection as mentioned before. 60 μ g GST-TTC9 protein was immobilized onto 12 μ l Glutathione Sepharose 4B gel by gentle rotation at 4°C for 2 hr. 300 μ g total cell lysates collected were then added and the total reaction volume was brought up to 1 ml by PBS. The reactions were incubated overnight at 4°C with gentle rotation. Nonspecific binding proteins were removed by washing in cold washing buffer (100 mM NaF, 50 mM HEPES (pH 7.5), and 150 mM NaCl) for four times, followed by one more wash in PBS. Proteins bound to the beads were eluted with 12 μ l 2 $\times$ SDS sample buffer, boiled for 5 min and were separated on an SDS-PAGE gel. GST protein expressed by empty pGEX-5X-3 vector was included as a negative control.

2.16 Co-immunoprecipitation with anti-flag M2 Affinity Gel

Anti-flag M2 Affinity Gel was bought from Sigma-Aldrich. Co-immunoprecipitation was carried out according to the manufacturer's instructions. Briefly, COS-7 cells were transfected with flag-TTC9 or flag-TTC9 fragments and Tm5NM-1-(His)₆ expression vectors using FuGENE 6 Transfection Reagent. The amount of Tm5NM-1-(His)₆ vector is two times more than those of flag-TTC9 or flag-TTC9 fragments vectors. Cell lysates were collected at 48 hr post-transfection. 400 µg total protein lysates were mixed with 15 µl anti-flag M2 Affinity Gel and the total reaction volume was brought up to 1 ml by PBS. After overnight incubation with the cell lysates at 4°C, the affinity gel was washed three times with 0.5 ml TBS. Proteins bound were eluted with 15 µl 2×SDS-PAGE sample buffer, boiled for 5 min, and were loaded onto an SDS-PAGE gel. COS-7 cells transfected with empty pXL-Flag vector and Tm5NM-1-(His)₆ expression vector were used as a negative control.

2.17 Co-immunoprecipitation of endogenous TTC9 and Tm5NM-1-(His)₆

COS-7 cells were transfected with flag-TTC9 expression vector using FuGENE 6 Transfection Reagent according to the manufacturer's instructions. Cell lysates were collected at 48 h post-transfection as mentioned before. 500 µg of total protein lysates collected were mixed with 5 µl anti-Tm5NM-1/2 antibody

agitation. The gel with the protein bound was sediment by centrifuging at 500×g for 5 min and was washed 3 times with 10 bed volumes of PBS. To elute the protein, 1 ml of glutathione elution buffer (0.154 g reduced glutathione dissolved in 50 ml 50 mM Tris-HCl, pH 8.0) was added to 1 ml bed volume of Glutathione Sepharose 4B, followed by mixing gently at room temperature for 10 min. Eluted protein was collected by centrifuging at 500×g for 5 min and supernatant was reserved. For complete elution, the elution step was performed for a total of three times. Eluted protein was pooled and dialyzed in PBS for future experiment.

2.14 Nuclear magnetic resonance (NMR) analysis of TTC9-(His)₆ protein

Purified TTC9-(His)₆ protein was concentrated to 0.2 mM. The buffer for the sample was changed to 20 mM Na-PO₄, pH 6.0, 20 mM NaCl, 1 mM DTT, 0.01% NaN₃ and 10% D₂O. The 1D ¹H NMR experiment was recorded at 298 K on a Bruker Avance AV600 spectrometer. The pulse program was “zgpgw5” using water suppression with watrgate W5 pulse sequences with gradients. The experiment was run with parameters: NS = 32, DS = 4 TD (size of fid) = 32 K, and SW = 16 ppm. The o1p was 4.7 ppm which was decided with the gs mode. The receiver gain was obtained by the command rga. The number of scan was decided according to the concentration of sample. The D19 delay for water

or goat pre-immune serum for 4 hr at 4°C with gentle rotation. 40 µl protein A/G plus-agarose beads were then added to the cell lysates and antibody mixture and were incubated overnight with gentle rotation at 4°C. The agarose beads were washed four times with washing buffer containing 100 mM NaF, 50 mM HEPES (pH7.5), 150 mM NaCl, 0.01% Triton X-100. Proteins bound were eluted with 18 µl 2×SDS-PAGE sample buffer, boiled for 5 min, and were loaded onto an SDS-PAGE gel.

2.18 Generation of polyclonal antibody against TTC9 protein

2.18.1 Conjugation of TTC9 peptide to carrier protein

Peptides normally cannot stimulate an immune response alone. They can be made fully immunogenic by coupling them to a suitable carrier molecule such as keyhole limpet hemocyanin (KLH). Inject[®] Maleimide Activated mcKLH (Pierce, Rockford, IL) is for conjugating a sulfhydryl-containing peptide to elicit an immune response and antibody production against the peptide. Compare with KLH, mcKLH is a more environmentally friendly product.

Conjugation was carried out according to the manufacturer's instructions. The Maleimide Activated mcKLH was firstly reconstituted in water to a final concentration of 10 mg/ml. Equal amount of TTC9 (1-50) in PBS and mcKLH

was then mixed. The volume of TTC9 (1-50) should be 1.0-2.5 times the volume of reconstituted mcKLH. The conjugation reaction mix was incubated at room temperature for 2 hr followed by dialyzing in PBS.

2.18.2 Polyclonal antibody production

TTC9-(His)₆ or TTC9 (1-50)-mcKLH was used as immunogen and injected into male BALB/C inbred mice or male New Zealand White rabbit. Both mice and rabbits were about 8 weeks old at the time of first injection. For each injection, 50 µg immunogen was used for one mouse, while 100 µg immunogen was used for each rabbit. Immunogens were prepared with Freund's Complete Adjuvant (Pierce) at 1:1 ratio for the initial injection, and were prepared with Freund's Incomplete Adjuvant (Pierce) at 1:1 ratio for the subsequent injections. Intraperitoneal injection was adopted for mice, while subcutaneous injection was used for rabbit, with 5 injection sites on the animal's back. Blood was withdrawn from the tail vein of mice (~200 µl) or from the ear artery of rabbits (~20 ml).

Non-immune serum was collected at day 0, and initial injection was made on day 3. At day 17, animals were boosted with immunogens again and sera were collected at 10 days post-injection. Together with the initial injection, each animal was boosted 4 times at 14-days interval. Sera collected were tested by

either ELISA or Western blotting analysis.

2.19 Yeast-two-hybrid assay screen

Yeast two hybrid assay screen was carried out using MATCHMAKER LexA Two-Hybrid System from Clontech Laboratories, Inc. (Mountain View, CA) according to the manufacturer's instructions. Briefly, Yeast *Saccharomyces cerevisiae* MAT α strain EGY48 was transformed with bait plasmid containing TTC9 coding sequence (TTC9-pLexA) and cDNA libraries of human breast cancer cell line MCF-7 (OriGene Technologies, Inc., Rockville, MD) using the lithium acetate method (Gietz et al., 1995). Transformed EGY48 was plated onto SD/Gal/Raf/-His/-Trp/-Ura/-Leu+X-Gal plates. An interaction was considered positive when two reporter genes, LEU2 and *lacZ*, were activated. The interactions were further verified by co-immunoprecipitation or GST pull-down assay.

2.20 Protein extraction from yeast cells

Yeast cells in their exponential phase were lysed by incubating with 240 μ l cold lysis buffer (1.85 M NaOH and 1.06 M β -mercaptoethanol) for 10 min. Equal volume of 20% TCA was then added and the mixture was incubated on ice for another 10 min. After centrifugation, the pellet was washed in acetone and

protein was extracted using 2×SDS-PAGE sample buffer.

2.21 Plasmid isolation from yeast cells

Yeast was grown on suitable plate to form colonies at least 2 mm in diameter. To lyse the cell, one colony was scraped from the plate and was resuspended in 50 µl S-buffer (10 mM KHPO₄, pH 7.2, 10 mM EDTA, 50 mM β-mercaptoethanol and 1.5 mg/ml zymolyase). After incubating at 37°C for 30 min, cells were lysed by 50 µl lysis solution (0.25 M TrisCl, pH 7.5, 25 mM EDTA, 2.5% SDS), and the reaction was incubated at 65°C for 30 min. Cell debris was collected by the addition of 88 µl NaAc, followed by incubating on ice for 10min and spinning at 14,000 rpm for 10 min. DNA in the supernatant was precipitated by 100% ethanol and was dissolved in TE buffer containing 20 ng/ml RNase A.

2.22 Hormone injection into BALB/C mice

Female mice of the BALB/C strain were obtained from the Center for Animal Resources of National University of Singapore. Proestrus mice at 14 days old were used, which weigh about 10 g. 17β-estradiol benzoate was prepared at 2 mg/ml in benzyl alcohol and diluted to 2 µg/ml in sesame oil before injection. Sesame oil with 0.1% benzyl alcohol was injected as control. Mice were

weighed before injection. The injection volume was adjusted, so that the final amount of 17 β -estradiol benzoate used for each mouse was at 20 μ g/kg of mice weight. Injection was repeated 24 hr later. Mice were sacrificed by cervical dislocation at 24 hr post the second injection. The uteri, mammary glands and livers were taken, followed by homogenization in cold lysis buffer containing 100 mM NaF, 50 mM HEPES (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF and the cocktail of proteinase inhibitors (5 μ g/ml pepstatin A, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin and 1 mM Na₃VO₄). Protein lysates were cleared by centrifuging at 14,000 rpm for 20 min at 4°C. Protein concentrations were determined by BCA protein assay kit (Pierce). 20 μ g of protein lysates from each sample was separated on an SDS-PAGE gel.

Mice were used in accordance with the National Resource Council guidelines for use of laboratory animals (Institute of Laboratory Animal Resources (U.S.). Committee on Care and Use of Laboratory Animals.)

2.23 Statistical analysis

The experiment for TTC9 expression in human breast cancer tissues and adjacent normal tissues were analyzed by the Mann-Whitney nonparametric test using the SPSS program for Windows, version 11.5. Difference between the expression of TTC9 in normal and cancer tissues was considered as significant

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when the P value is less than 0.05.

Results

CHAPTER 3

RESULTS

3.1 General introduction to TTC9

3.1.1 Identification of TTC9

Human TTC9 gene was first identified by Nagase et al from cell line KG-1 (a human immature myeloid cell line) and human brain, and was designated as KIAA0227 at that time (Nagase et al., 1996). It was later referred to as tetratricopeptide repeat protein 9 (TTC9) because of the presence of three TPR domains in the protein sequence. The gene was found to be interesting in breast cancer cells as the microarray analysis result from our lab revealed that TTC9 mRNA level was up-regulated by 17 folds in progesterone-treated ABC28 cells.

3.1.2 Sequence characteristics of TTC9 cDNA

The transcript sizes of TTC9 gene are predicted to be 5217 bp and 2428 bp (<http://www.pubmed.com>), which encode predicted proteins of 336 aa and 277 aa respectively. The predictions are based on the mRNA information of GeneBank entry D86980 and BC047950, which were suspected to have incomplete 5' mRNA sequence. Using gene prediction method, GNOMON, the updated NCBI entry (GenBank accession no. XM_027236.6) predicted a 5510 bp transcript based on automated computational analysis of an annotated genomic sequence (GenBank accession no. NT_026437), supported by mRNA

and EST evidence. This transcript is predicted to encode a 434 aa protein.

Using Vector NTI software, we deduced the ORF of TTC9 mRNA (GenBank accession no. D86980) to be from nucleotide 344 to 1012, encoding a protein of 222 aa (Fig. 3.1 and 3.2). A polyadenylation signal (AATAAA), which indicates the addition of poly-A tail, was identified in the mRNA sequence. In addition, analysis of the promoter sequence of TTC9 identified a putative PRE sequence (5'-AGTAACAGGTGTTCT-3'), which is located between 889 bp and 875 bp upstream of the start codon, suggesting TTC9 could be one of the direct target genes of PR.

3.1.3 Characteristics of TTC9 protein

The hydropathy plot analysis (Kyte and Doolittle, 1982) of the human TTC9 protein sequence revealed no presence of transmembrane domain, which suggested TTC9 is a soluble protein (Fig. 3.3). The hydropathy plot shown in Fig. 3.3 was drawn with the window size of 19. At this window size, peaks with scores greater than 1.8 indicate possible transmembrane regions.

Using the SMART algorithm, three TPR domains were identified in the 222 aa TTC9 protein (residues 57-90; 128-163; 164-197). Motif searches by the PROSITE program identified a cAMP- and cGMP-dependent protein kinase

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1  WWW
  MERKGSAAAGAKGNFSPPAAGEGQRPPPLCVPGGGGAPARGQVGAAAEPAELIRRAHEFKSQGA 65
66  QCYKDKKFREAIGKYHRALLELKGLLPPPGERERDSRPASPAGALKPGR LSEEQSKTVEAIEIDC 130
131 YNSLAACLLQAE LVNYERVKEYCLKVLKKEGENFKALYRSGVAFYHLGDYDKALYYLKEARTQQP 195
      ***WWW
196 TDTNVIRYIQLTEMKLSRCSQREKEAM 222
      .....

```

Fig. 3.2 Predicted amino acid sequence of human TTC9 protein.

Human TTC9 gene encodes a putative protein of 222 aa. The fragments in bold are the three TPR domains. The amino acids underlined with solid lines are the cAMP- and cGMP-dependent protein kinase phosphorylation site, while the ones underlined with dashed lines are the casein kinase II phosphorylation site. The residues marked under the “*” represent the protein kinase C phosphorylation site. The regions in red are the N-myristoylation sites. Two endoplasmic reticulum membrane retention signals (marked under the arrow) are identified at the N-terminal and the C-terminal.

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Fig. 3.3 Hydrophobicity profile of human TTC9 protein (Kyte and Doolittle, 1982).

The hydrophobicity profile of human TTC9 protein was analyzed using online software with a window size at 19. X-axis is numbering of the amino acids with the translation starting site numbered as 1. Peaks with scores greater than 1.8 (the dotted line) indicate possible transmembrane regions.

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phosphorylation site (residues 3-6), a protein kinase C phosphorylation site (residues 215-217), a casein kinase II phosphorylation site (residues 215-218), and four N-myristoylation sites (residues 5-10; 33-38; 36-41; 42-47). Furthermore, two endoplasmic reticulum membrane retention signals (residues 2-5; 218-221) were identified using PSORT II program.

The Fourier Transform Infrared (FT-IR) analysis was applied to analyze the secondary structure of TTC9 protein. As shown in Fig. 3.4, the FT-IR spectra of TTC9 protein in D₂O solution displayed a major peak at 1656 cm⁻¹, suggesting the majority of the protein was α -helix. The minor peaks at 1634 cm⁻¹ and 1608 cm⁻¹ represented for β -sheet and aggregated β -sheet respectively. This result was in accordance to the facts that TTC9 contains three TPR domains and that TPR motif generally forms an antiparallel α -helical hairpin (Das et al., 1998; Scheufler et al., 2000).

3.1.4 Sequence analysis of TTC9 genomic DNA

Searches in Ensembl database revealed that human TTC9 gene was located on chromosome 14q24, spanning a region of 33.57 Kb (70,178,257-70,211,830). It has three exons ranging in sizes from 749 to 4285 bp. The start codon was located in exon 1 and the stop codon was positioned in exon 3. The gene also has three introns varying greatly from sizes 3329 to 25,028 bp. The GT-AG

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splicing signal rules applied to every exon/intron boundaries.

Fig. 3.4 FT-IR analysis suggested the secondary structure of TTC9 protein was mainly α -helix.

The secondary structure of purified TTC9-(His)₆ protein was predicted by FT-IR analysis. The peak appeared at ~ 1650 cm⁻¹ corresponds to α -helix, which is the major peak. The small peaks at ~ 1630 and 1610 cm⁻¹ represent β -sheet and aggregated β -sheet respectively. Blue line is the curve fitting.

3.1.5 Conservation of TTC9 protein during evolution

Searches in protein databases revealed that the predicted 222 aa TTC9 protein sequence was highly conserved during evolution. It shares a high degree of homology with its homologues in *Gallus gallus* (GenBank accession no. XP_001231669), *Mus musculus* (GenBank accession no. NP_001028321), *Rattus norvegicus* (GenBank accession no. XP_576070), *Pan troglodytes* (GenBank accession no. XP_001139297) and *Macaca mulatta* (GenBank accession no. XP_001082201). As shown in Fig. 3.5, human TTC9 protein shares 88.3% identity with the corresponding homologue of *Mus musculus* and 69.4% identity with that of *Gallus gallus*. Compared with human TTC9 protein, there are some extra N-terminal fragments in the amino acid sequences of TTC9 homologues in *Rattus norvegicus*, *Pan troglodytes* and *Macaca mulatto*, all of which are predicted on NCBI website (www.pubmed.com). However, the antibody produced against human TTC9 could also recognize the corresponding protein in rat, with the same size as that of human TTC9 (Fig. 3.21). We thus concluded that the extra sequence predicted by computer might not present in the mature protein in *Rattus norvegicus*, and probably does not present in TTC9 homologs of *Pan troglodytes* and *Macaca mulatta*. The identities of human TTC9 protein and its homologues in *Rattus norvegicus*, *Pan troglodytes* and *Macaca mulatta* are as high as 91%, 89.8% and 98.6% respectively, without

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considering the N-terminal fragment.

Human	(1)	-----
Gallus gallus	(1)	-----
Mus musculus	(1)	-----
Rattus norvegicus	(1)	MDTGDKAEAGEQIVCMPSPPFRVSDNHHHSBRAGCPLEHQQGQARTG----SGSS
Pan troglodytes	(1)	-----LTHSVRCSPSLMSVIEFAQLR-----AH8
Macaca mulatta	(1)	-----MEQGGKEECPKKEGWQLIQINDIMAPRRASIDTGDEAVREWIGCCRYT
Human	(1)	-----
Gallus gallus	(1)	-----
Mus musculus	(1)	-----
Rattus norvegicus	(48)	RGQVAGAEKNGDEGGRRGSGGSESEFRDRRRRQFPRAAGDEDGSAARHGA
Pan troglodytes	(25)	TRIHRLPETYGAPHATRRASQSGIEWERAGFSLSLILF-SFGQAKLLPGQRF
Macaca mulatta	(47)	AHAHTGSQNTAPHTLYSTRASGRENGHTHRDTFQRFQFGSTKRTKLLPGQRF
Human	(1)	-----
Gallus gallus	(1)	-----
Mus musculus	(1)	-----
Rattus norvegicus	(99)	QGHGSGVCGARQRALLKFB-----
Pan troglodytes	(75)	SPPSSTRSPRASSGPASTGP-----
Macaca mulatta	(98)	SPPSSTRSPRASSGPASTGPREFQVAEGGGRERAAWSGEAGEGRASEA
Human	(1)	-----
Gallus gallus	(1)	-----
Mus musculus	(1)	-----
Rattus norvegicus	(123)	-----R-----
Pan troglodytes	(99)	-----Q-----
Macaca mulatta	(149)	AAAAAAAAAGAAAGSGSGENGSAQRQTAAGCHGRHEACEARGRRFRLLNPGAR
Human	(1)	-----MERKSSAGSKNPSPTAAGGQSPPELCVE
Gallus gallus	(1)	-----MERASLARGKNAAGGAGDGHGI
Mus musculus	(1)	-----MERKLLARRSCNPSPTALGEGFVPPPOVE
Rattus norvegicus	(124)	-----ARG--AGGGRMERKLLARRSCNPSPTALGEGFVPPPOVE
Pan troglodytes	(100)	-----TPELCVE
Macaca mulatta	(200)	GGGGGGQIAARTRRGGGAERKSSAGSKNPSPTAAGGQSPPELCVE
Human	(33)	GGGSGAPARGQVGAADPEELIIRAEFFKSQGAQCYKDKKFRDAIGKYHRA
Gallus gallus	(25)	AGGQPRRAAAAGGAEEGLIGRALDFKSQGAQCYKDKKFRDAIGKYHRA
Mus musculus	(33)	SGG--AEPGQAGTAADPEELIIRAEFFKSQGAQCYKDKKFRDAIGKYHRA
Rattus norvegicus	(164)	SGG--AEPGQAGTAADPEELIIRAEFFKSQGAQCYKDKKFRDAIGKYHRA
Pan troglodytes	(107)	GGGSGAPARGQVGAADPEELIIRAEFFKSQGAQCYKDKKFRDAIGKYHRA
Macaca mulatta	(251)	GGGSGAPARGQVGAADPEELIIRAEFFKSQGAQCYKDKKFRDAIGKYHRA
Human	(84)	LLELNGLLEPPGERERDSRAASAGALKFGSLSEEQSKTVEAIEIDCYN5L
Gallus gallus	(78)	LLELNGLLIS---Q-EGGQRANAAAG--LSEEQRCQAVEAIEIDCYN5L
Mus musculus	(83)	LLELNGLL--SQEERDANFASAGVFNSSLSSEEQSKTVEAIEIDCYN5L
Rattus norvegicus	(214)	LLELNGLLPPGERERDAFASAGIFASLSSEEQSKTVEAIEIDCYN5L
Pan troglodytes	(158)	LLELNGLLPPGERERDSRAASAGALKFGSLSEEQSKTVEAIEIDCYN5L
Macaca mulatta	(302)	LLELNGLLPPGEREQDSRAASAGALKFGSLSEEQSKTVEAIEIDCYN5L
Human	(135)	AACLLQASLVNYERVKEYCLKVLKEGENFKALYRSGVAFYHLGDYKALY
Gallus gallus	(121)	AACLLQASLVNYERVKEYCLKVLKEGENFKALYRSGVAFYHLGDYKALY
Mus musculus	(132)	AACLLQASLVNYERVKEYCLKVLKEGENFKALYRSGVAFYHLGDYKALY
Rattus norvegicus	(265)	AACLLQASLVNYERVKEYCLKVLKEGENFKALYRSGVAFYHLGDYKALY
Pan troglodytes	(209)	AACLLQASLVNYERVKEYCLKVLKEGENFKALYRSGVAFYHLGDYKALY
Macaca mulatta	(363)	AACLLQASLVNYERVKEYCLKVLKEGENFKALYRSGVAFYHLGDYKALY
Human	(186)	YLKEAR--QPD TNVIRYIQLTEMKLSRCSQREKEAL
Gallus gallus	(172)	YLKEAR--QPD TNVIRYIQLTEMKLSRCSQREKEAL
Mus musculus	(183)	YLKEAR--QPD TNVIRYIQLTEMKLSRCSQREKEAL
Rattus norvegicus	(318)	YLKEAR--QPD TNVIRYIQLTEMKLSRCSQREKEAL
Pan troglodytes	(260)	YLKEAR--QPD TNVIRYIQLTEMKLSRCSQREKEAL
Macaca mulatta	(404)	YLKEAR--QPD TNVIRYIQLTEMKLSRCSQREKEAL

Fig. 3.5 Comparison of TTC9 orthologues.

TTC9 homologues in human, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes* and *Macaca mulatta* are compared. Residues highlighted in yellow are identical amino acids, while those highlighted in blue are conservative residues among species. Amino acids highlighted in green represent the similar residues. Amino acids in black letters are non-similar residues and those in green are weakly similar residues.

3.2 Purification of TTC9 proteins

3.2.1 Purification of TTC9-(His)₆ protein

As described in “materials and methods”, the coding sequence for the 222 aa TTC9 protein was cloned into the pET-24b(+) vector (EMD Biosciences, Inc., Germany), which added a histidine tag to the C-terminus of the protein. Colonies were verified by restriction enzyme digestion as well as sequencing. The correct TTC9-(His)₆ expression vector was transformed into *E.coli* strain BL21 for the expression of TTC9-(His)₆ protein. Fig. 3.6A verified the induction of TTC9-(His)₆ protein expression by 1 mM IPTG. After the addition of IPTG, a band around 26 kD was induced, which is in agreement of the calculated molecular weight of TTC9-(His)₆. In addition, the protein induced is mainly in the supernatant fraction, especially when the induction was carried out at 30°C, suggesting it is soluble under these conditions. In later experiments, protein was induced at 30°C.

TTC9-(His)₆ expressed from the pET-24b(+) vector was first purified through Ni-NTA agarose column (Qiagen GmbH) for the binding of His-tagged protein. Fig. 3.6B showed the elution profile and purity of TTC9-(His)₆ after this step of purification. The identity of the protein eluted was verified by Western blotting analysis using anti-His antibody (Amersham) (Fig. 3.6B, lower panel).

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Fig. 3.6 Induction and purification of TTC9-(His)₆ using Ni-NTA agarose column.

(A) The expression of TTC9-(His)₆ was induced by 1 mM IPTG at 30°C and 37°C for 3 hrs. Cells were harvested and lysed by sonication. The expression was induced at both temperature, but protein was easier to get precipitated at 37°C. (B) Elution profile of TTC9-(His)₆ from Ni-NTA agarose column. The protein purified is confirmed to be TTC9-(His)₆ by Western blotting analysis with anti-His antibody (lower panel).

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The elutes from Ni-NTA agarose column were pulled and subjected to cation exchange chromatography with HiTrap SP HP column (Amersham) for further purification of the target protein. Recombinant protein was only eluted after the gradient reached 100%. The elution profile and purity of TTC9-(His)₆ were shown in Fig. 3.7. The final purity of the protein reached over 90%. NMR analysis was then performed to test whether the protein purified was in correct conformation. Shown in Fig. 3.8, The 1D ¹H spectrum of TTC9-(His)₆ showed very good dispersion of the peaks, indicating that the recombinant protein was correctly folded.

3.2.2 Purification of TTC9 (1-50)

The coding sequence for TTC9 (1-50) peptide was cloned into pET-32b(+) for the expression of TTC9 (1-50) with a thioredoxin (Trx) tag and a six-histidine linker on its N-terminal. The tag and the linker can later be removed by enterokinase digestion. The purification of TTC9 (1-50) peptide included 3 steps. The crude cell lysates were first pass through Ni-NTA agarose column for the separation of His-tagged protein from other proteins. This was followed by enterokinase digestion to remove the Trx tag and linker. Finally, the 50 aa peptide was separated from the cutoff by cation exchange chromatography.

Results

Fig. 3.7 Cation exchange chromatography of TTC9-(His)₆.

Upper: Cation exchange elution profile of TTC9-(His)₆. Lower: The final purity of the protein reached over 90%.

Fig. 3.8 1D ^1H spectrum of TTC9-(His) $_6$.

Purified TTC9-(His) $_6$ protein was concentrated to 0.2 mM. The buffer for the sample was changed to 20 mM Na-PO $_4$, pH 6.0, 20 mM NaCl, 1 mM DTT, 0.01% NaN $_3$ and 10% D $_2$ O. The 1D ^1H NMR experiment was recorded at 298 K on a Bruker Avance AV600 spectrometer. The pulse program was “zgpgw5” using water suppression with watergate W5 pulse sequences with gradients. The experiment was run with parameters: NS = 32, DS = 4 TD (size of fid) = 32 K, and SW = 16 ppm. The o1p was 4.7 ppm which was decided with the gs mode. The receiver gain was obtained by the command rga. The number of scan was decided according to the concentration of sample. The D19 delay for water suppression was set to 100 μs for the water suppression. Other parameters were set according to the program from Bruker. NMR data were processed and analysed using Bruker Topspin software.

Results

Fig. 3.9A is the elution profile of Trx-TTC9 (1-50) from Ni-NTA agarose column. The calculated molecular weight and the histidine linker is ~18 kD, making the molecular weight of the total protein about 23 kD. Protein eluted was then dialyzed in the buffer suitable for enterokinase digestion as described in “materials and methods”. In order to maximize the yield of TTC9 (1-50) peptide and avoid the undesirable secondary cleavage in the peptide, the reaction time of the enterokinase digestion was optimized. As shown in Fig. 3.9B, there were still some protein remained uncut after 6 hr digestion at room temperature. The reaction was then carried out overnight at 4°C and was stopped by removing the enterokinase through HiTrap Benzamidine FF column (Amersham Biosciences) which is specifically designed for the removal of serine proteases. Fig. 3.9C shows the profile of the purification. The first few fractions containing the cut-off TTC9 (1-50) peptide were combined for future purification.

The peptide was further purified by cation exchange chromatography using HiTrap SP HP column (Amersham Biosciences). Fig. 3.9D is the elution profile of the purification. Portions corresponding to TTC9 (1-50) was pooled and dialyzed in PBS for antibody production.

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Fig. 3.9 Purification of TTC9 (1-50).

(A) Purification of TTC9 (1-50) with Trx tag using Ni-NTA column. FT: flowthrough fraction; W: washing fraction. (B) Removal of Trx tag from TTC9 (1-50) using enterokinase. The reaction was carried out at room temperature for 1 hr and 6 hr. (C) Removal of enterokinase. Enterokinase was removed by HiTrap Benzamidine FF column. The first lane of the SDS-PAGE gel was the protein digested overnight. Little TTC9 (1-50)-Trx remains uncut. (D) Purification of TTC9 (1-50) peptide using cation exchange chromatography.

3.2.3 Purification of GST and GST-TTC9 protein

TTC9 full length coding sequence was cloned into pGEX-5X-3 (Amersham Biosciences) for the expression of GST-TTC9 protein, while the empty vector was used for the expression of GST protein. Both the empty vector and the GST-TTC9 expression vector were transformed into BL21 (DE3) *E.coli* competent cells for protein expression. The expression was induced by 1 mM IPTG at 30°C. As shown in Fig. 3.10A, the expression of GST-TTC9 was induced under this condition, with a band appearing at ~50 kD, while the induced GST protein appear to be ~26 kD. The proteins induced were purified by Glutathione Sepharose 4B (Amersham Biosciences). Fig. 3.10B and 3.10C are the purification profiles of GST (Fig. 3.10C) and GST-TTC9 (Fig. 3.10B), which revealed that the proteins reached over 90% purity. The purified proteins were dialyzed in PBS and stored at -80°C for GST-pull down experiments.

3.3 Generation of TTC9 polyclonal antibodies

3.3.1 Generation of mouse TTC9 polyclonal antibody using TTC9-(His)₆

Purified TTC9-(His)₆ in PBS was used as antigen and was injected into mice for the production of TTC9 polyclonal antibody. As illustrated in “materials and methods”, the antigen was prepared in Freund’s complete adjuvant for the first

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Fig. 3.10 Purification of GST-TTC9 and GST protein using Glutathione Sepharose 4B.

(A) Induction of protein expression at 30°C with 1 mM IPTG. (B) Purification of GST-TTC9. The bands between 25-35 kD are the GST-tag and TTC9 protein respectively, which may result from the degradation of GST-TTC9 protein. (C) Purification of GST-tag.

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injection and in Freund's incomplete adjuvant for the subsequent boosts. Five mice were used for this purpose. Mouse sera were taken after the second boost and the quality of the polyclonal antibody was analyzed by ELISA. The ability of the antibody produced from each mouse to recognize the antigen was measured by the OD reading at 405 nm. As shown in Fig. 3.11A, antibodies from all the five mice were capable of recognizing the antigen at various dilutions, indicating the antibodies produced were functional. The specificity of the antibodies was suggested by the ability of the sera to react with the antigen in a concentration-dependent manner. Unless specified, this batch of antibody was used for further experiments.

3.3.2 Antibody production in rabbits

To produce large amount of TTC9 antibody, purified TTC9-(His)₆ was injected into New Zealand White rabbit as described in "materials and methods". The quality of antibody produced was examined by Western blotting analysis. As shown in Fig. 3.11B, the antibodies produced from both rabbits detected a band appearing at ~25 kD in ABC28 cells, which is up-regulated by progesterone treatment. As TTC9 is known to be up-regulated by progesterone in ABC28 cells (Fig. 3.12), the band detected should correspond to the endogenous TTC9 in this cell line.

3.3.3 Generation of TTC9 antibody using TTC9 (1-50) epitope

As the C-terminal of TTC9 protein contains three TPR motifs and shows homology with many other proteins, we speculated whether it was possible to get a more specific antibody with less nonspecific bands using TTC9 peptides. The first 50 aa of TTC9 protein was also used as antigen to induce antibody production in mice. As most peptides with a molecular weight lower than 5 kD are not large enough to stimulate an immune response on their own (Harlow and Lane, 1988; Sell, 1987), the purified TTC9 (1-50) peptide was first conjugated to carrier molecule KLH before injected into mice as illustrated in “materials and methods”. Similarly, the quality of antibodies produced was examined by Western blotting analysis. Shown in Fig. 3.11C, sera collected detected a band at ~25 kD. In addition, the ~25 kD band also showed down-regulation in TTC9 siRNA transfected MCF-7 cells, which indicated the specificity of the antibody.

Results

Fig. 3.11 Test of polyclonal antibodies produced from mice and rabbits.

(A) Titration test of mice polyclonal antibodies against TTC9-(His)₆. Five mice were injected with TTC9-(His)₆ as antigen and sera were collected 10 days after the second boost. The quality of the antibodies to their antigen was tested by Elisa at series dilutions. (B) Western blot analysis using rabbit polyclonal antibodies produced against TTC9-(His)₆. Cell lysates from progesterone-treated ABC28 were analyzed, in which TTC9 should show an up-regulation after progesterone treatment. E: control vehicle; P: progesterone. (C) Western blot analysis using mouse polyclonal antibodies against TTC9 (1-50). MCF-7 cells were transfected with TTC9 siRNA and harvested at 72 hr post transfection. TTC9 showed a down-regulation in TTC9 siRNA-transfected cells. T: TTC9 siRNA-transfected MCF-7; C: MCF-7 cells transfected with control siRNA.

3.4 TTC9 is hormonally regulated in ABC28 cells

3.4.1 Progesterone dramatically up-regulated the expression of TTC9 in ABC28 cells

TTC9 first attracted our attention as a gene whose expression showed dramatic up-regulation by progesterone in ABC28 cells in microarray analysis (unpublished data). Real-time RT-PCR was then performed to confirm the microarray analysis result. Fig. 3.12A presents the result of real-time RT-PCR, which showed that in ABC28 cells, progesterone up-regulated the mRNA level of TTC9 by 22-fold after 3 hr treatment. At 24 hr after treatment, the relative TTC9 mRNA level in progesterone-treated cells was 56-fold higher than that in controls. Using specific ^{32}P -labelled TTC9 probe, Northern blot analysis further confirmed the real-time RT-PCR results. Fig. 3.12B shows that progesterone induced a drastic increase in TTC9 mRNA level in ABC28 cells following 8 hr and 24 hr of treatment. The band subjected to progesterone regulation appeared at ~2.5 Kb, which probably corresponds to the shorter transcript of 2567 bp predicted by Ensembl software. The ~2.5 Kb transcript was also the only one detected in other breast cancer cell lines such as MCF-7, T47D and ZR-75.1 (Fig. 3.15A). However, the TTC9 probe did not detect any band corresponding to the longer transcript of either 5217 bp or 5510 bp in these breast cancer cell

lines, as is published on the NCBI website.

In order to verify that the ~2.5 Kb TTC9 transcript is actually translated into TTC9 protein in ABC28 cells, we used Western blot analysis to study the protein level of TTC9 with self-generated mouse polyclonal antibody. In ABC28 cells, the increase of TTC9 protein level became evident as early as 8 hr after progesterone treatment. The magnitude of induction after 24 hr and 48 hr of progesterone treatment was about 8-10 folds in this experiment (Fig. 3.12C). In addition, when ABC28 cells were treated with progesterone at 1 nM, 10 nM and 100 nM, the expression of TTC9 was induced at all the doses tested (Fig. 3.12D). However, progesterone has no effect on TTC9 expression in control cell line CTC15 (Fig. 3.13A), which suggested that the up-regulation effect of progesterone on TTC9 was mediated through PR.

3.4.2 The regulation of TTC9 protein level by other steroid hormones

It has been reported that MDA-MB-231 cells and its derivatives ABC28 and CTC15 express glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), and that glucocorticoid and mineralocorticoid could cross-talk with PR to induce progesterone-like effects in ABC28 cells (Leo et al., 2004). The effect of aldosterone and dexamethasone, which bind to MR and GR respectively, on

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Fig. 3.12 Progesterone up-regulates the expression of TTC9 in ABC28 cells in a time-dependent and concentration independent manner.

(A) Cells were treated with control vehicle and 0.1 μ M progesterone for 3, 12 and 24 hours respectively. The transcription level of TTC9 was analyzed by real-time RT-PCR. The expression level is expressed relative to vehicle-treated controls which are given the value of 1. The results are the means of 3 replicates. (B) Cells were treated with 0.1 μ M progesterone (+) or control vehicle (-) for various time periods. 12 μ g of total RNA from each sample were separated on a Northern gel and transferred to nylon membrane. 32 P-labelled TTC9 probe was hybridized to the membrane. Molecular size ladders of RNA were indicated on the right. (C) Cell lysates were collected from ABC28 cells treated with 0.1 μ M progesterone (+) or control vehicle (-) for various time periods and were analyzed by Western blot analysis. GAPDH was used as a loading control. The numbers associated with the blot are densitometry values of TTC9 relative to that of the vehicle-treated cells, which is given the value of 1. (D) The TTC9 protein level is up-regulated by different concentrations of progesterone in ABC28 cells. Cells were treated with 1 nM, 10 nM or 100 nM progesterone for 24 hours and TTC9 expression levels were analyzed by Western blot analysis with GAPDH as a loading control.

TTC9 expression, was examined in ABC28 cells. Fig. 3.13A shows that dexamethasone and aldosterone had no obvious effect on TTC9 protein level in CTC15 cells. On the contrary, aldosterone and dexamethasone treatment significantly up-regulated TTC9 protein level in the PR-positive ABC28 cells (Fig. 3.13B), indicating the effect of aldosterone and dexamethasone is mediated by PR. Progesterone antagonist RU486, on the other hand, had no effect alone but completely abolished the effect of progesterone on TTC9 expression. These data again support the earlier notion that TTC9 is a PR target gene.

3.4 The expression of TTC9 in breast cancer cell lines

As TTC9 is a hormonally-regulated gene in ABC28 cells, the expression of TTC9 protein was then examined in other breast cancer cell lines with different ER and PR status. As shown in Fig. 3.14, TTC9 expression level in three ER- and PR-positive cell lines (T47D, MCF-7 and ZR-75.1) and two ER- and PR-negative cell lines (MDA-MB-231 and MDA-MB-435) was studied. All the cells were grown in DCC-FCS containing medium for two days before lysates collection. The polyclonal antibody also detected a protein band of ~ 25 kD in various breast cancer cell lines. It appears that ER- and PR-positive breast cancer cells express more TTC9 than the hormone receptors negative cells

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(MDA-MB-435 and MDA-MB-231), but more cell lines need to be tested in order to confirm the notion.

Fig. 3.13 Effects of different hormones on TTC9 expression in ABC28.

Cells were treated with different hormones for 48 hours and the expression levels of TTC9 were analyzed by Western blotting. GAPDH was used as loading control. The numbers associated with each blot are densitometry values of TTC9 relative to that of the vehicle-treated cells, which is given the value of 1. C, control vehicle; A, 0.1 μ M aldosterone; D, 0.1 μ M dexamethasone; E2, 1 nM 17 β -estradiol; P, 0.1 μ M progesterone; RU, 0.1 μ M RU486; ZK, 0.1 μ M ZK98,299; P+RU, 0.1 μ M progesterone plus 0.1 μ M RU486. (A). The expression of TTC9 was not regulated by hormone treatment in CTC15 cells. (B). Progesterone and aldosterone dramatically up-regulated TTC9 expression

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in ABC28 cells.

Fig. 3.14 The expression of TTC9 in breast cancer cell lines.

Cells were plated in DCC-FCS containing medium and cell lysates were collected two days after plating. 20 μ g lysate from each cell line was loaded onto SDS-PAGE gel and analyzed by Western blotting.

3.5 TTC9 is hormonally regulated in MCF-7 cells

In order to find out whether TTC9 is regulated by steroid hormones in other breast cancer cell lines, the expression pattern of TTC9 in different breast cancer cell lines with different hormone receptor status were then studied. Fig. 3.15A shows the effects of different hormones on the mRNA level of TTC9 in ABC28, MCF-7, MDA-MB-435, ZR-75.1 and T47D cells. In accordance with what was shown in Fig. 3.14, TTC9 mRNA was expressed at a relatively higher level in MCF-7, T47D and ZR-75.1 cells compared with that in the ER-negative cell lines. Very little TTC9 mRNA was detected in MDA-MB-435 cells with different hormone treatment. In MCF-7 cells, estradiol-17 β (E₂) and progesterone (P) alone decreased TTC9 mRNA level to some extent, while the treatment with estradiol-17 β together with progesterone (E₂+P) further lowered the gene transcription level. No evident difference of TTC9 expression was observed in ZR-75.1 cells upon hormone treatment. Similar to that in MCF-7 cells, E₂+P decreased TTC9 mRNA level in T47D cells, although either hormone alone has no effect. Thus the hormonal effects on the expression of TTC9 gene vary in cell lines with different hormone receptor status.

Western blot analyses were then performed to examine TTC9 protein level in MCF-7 cells. Similar to the results from Northern blot analysis, in MCF-7 cells,

estradiol-17 β (E2) and progesterone (P) alone or in combination (E2+P) all decreased the TTC9 proteins level by approximately 50-70% after 48 hr treatments (Fig. 3.15B).

Focus on MCF-7, we then studied the hormonal regulation on TTC9 expression in some more detail. Time course experiment showed that the regulation appeared as early as 24 hr after hormone treatment and lasted throughout the 96 hr period tested with 1 nM E2 or 1 nM E2 plus 100 nM P (Fig. 3.16A). Furthermore, various doses of E2 (1 nM, 10 nM and 100 nM) are all effective in down-regulating the TTC9 protein level (Fig. 3.16B).

3.6 TTC9 is over-expressed in breast cancer tissues compared with the adjacent normal tissues

Breast cancer was recognized to be a hormone-dependent malignancy as early as 1896, when Beatson reported that the removal of the ovaries caused the regression of disseminated breast cancer (Beatson, 1896). Thus hormone regulation is an important factor involved in the occurrence of breast cancer. Previous results have shown that TTC9 is a hormonally regulated gene *in vitro*. We asked the question if TTC9 is over-expressed in breast cancer tissues and if its expression is correlated with hormone receptor status. 25 matched pairs of

Results

Fig. 3.15 The expression of TTC9 was hormonally regulated in different breast cancer cell lines.

Different breast cancer cell lines were treated with control vehicle (E), 100 nM progesterone (P), 100 nM 17 β -estradiol (E2) or 100 nM progesterone together with 100 nM 17 β -estradiol (E2+P) for 48 hr before RNA or protein extraction. (A) 12 μ g total RNA from each sample was analyzed by Northern blotting using 32 P-TTC9 probe. 36B4 was used as a loading control. (B) 20 μ g cell lysate from each sample was analyzed by Western blotting. GAPDH was used as a loading control. The numbers associated with each blot are densitometry values of TTC9 relative to that of the vehicle-treated cells, which is given the value of 1.

Results

Fig. 3.16 TTC9 protein expression was hormonally-regulated in MCF-7 cells as analyzed by Western blotting.

The numbers associated with each blot are densitometry values of TTC9 relative to that of the vehicle-treated cells, which is given the value of 1. (A) MCF-7 cells were treated with control vehicle, 1 nM E2 and 1 nM E2+100 nM P for 24, 48 and 96 hours. The treatment medium was changed after 48 hr in culture. (B) Various concentrations of E2 decreased the TTC9 level in MCF-7 cells. MCF-7 cells were treated with 1 nM, 10 nM or 100 nM E2 for 48 hours.

Results

human breast cancer tissue and the adjacent normal tissue were analyzed for TTC9 mRNA expression. The results presented in Fig. 3.17 were obtained by RT-PCR as illustrated in the “materials and methods” and the PCR products were quantitated by Southern blotting analysis. 36B4, which codes for human acidic ribosomal phosphoprotein P0, was used as a control for cDNA input (Fig. 3.17A). Similar results were obtained when TTC9 expression is normalized to GAPDH expression (Fig. 3.17B). It is to be noted that the expression of 36B4 and GAPDH vary among samples. In general, the expression levels of these housekeeping genes are higher in tumor tissues compared to the corresponding normal tissues. This variation has been reported before. For example, GAPDH expression is 3.3-fold higher in seminoma compared to normal testis (Vila et al., 2000). Similarly, GAPDH transcription was significantly greater in both colonic adenomas and cancers than in normal mucosa (Neuvians et al., 2005). Nonetheless, the results revealed that the relative TTC9 mRNA, when normalized to either 36B4 or GAPDH, was significantly higher in breast cancer tissue compared to its adjacent normal tissue ($P < 0.00001$). It is also notable that nearly all the tumor tissues express higher levels of TTC9 mRNA compared to its adjacent controls. However, we found no correlation of TTC9 expression with other clinic pathological data such as tumor size, nuclear grade, axillary's lymph node status or hormone receptor expression.

Results

Fig. 3.17 The expression level of TTC9 mRNA was significantly higher in breast cancer tissues than that in the adjacent normal breast tissues.

Total RNA was extracted from human breast cancer tissues and the matched adjacent normal breast tissues. Equal amount of RNA from each sample was subjected to reverse transcription and cDNA produced was amplified by PCR using TTC9, 36B4 or GAPDH primers. 10 μ l PCR products were separated on an agarose gel and analyzed by Southern blotting. Band intensity was analyzed by Bio-Rad Molecular Image Analyzer. The figure shows the expression levels of TTC9 in 25 pairs of normal and tumor tissue samples after normalized to those of 36B4 (A) or GAPDH (B). Each pair of bars represents samples from one patient.

3.7 Regulation of TTC9 expression in MCF-7 cells

3.7.1 TTC9 is serum and growth factors inducible

The higher expression level of TTC9 in breast cancer tissues led us to hypothesize that this may be in some way associated with enhanced mitotic activities in the tumor tissue. Since serum contains growth-stimulation factors, we tested if TTC9 expression is serum-inducible. We chose MCF-7 cells as study model as TTC9 is expressed at a medium level (Fig. 3.14) and is subject to hormonal regulations (Fig. 3.16) in this cell line. In the first experiment, MCF-7 cells were treated with DCC-FCS at different concentrations. The reason for using DCC-FCS instead of FCS is that compared with FCS, DCC-FCS has already had the steroid hormones in the serum removed, so that the effects of serum growth factors and steroid hormones can be distinguished. Fig. 3.18A shows that DCC-FCS at 5%, 10% and 20% enhanced the levels of TTC9 protein in MCF-7 cells in a concentration-dependent manner, which indicated serum growth factors could play some role in the regulation of TTC9 expression. To further verify this point, the effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were tested in MCF-7 cells. As shown in Fig. 3.18B, these two growth factors also enhanced the protein level of TTC9 by 3- to 5- fold after 48hr treatment.

3.7.2 Growth factor-induced TTC9 expression is via the activation of ERK1/2 signaling pathway

The treatment of EGF and bFGF can activate several downstream signaling pathways. To investigate through which pathway the expression of TTC9 was regulated, different inhibitors were added to the cell medium together with the growth factors. Inhibition of ERK phosphorylation by PD98059 alone did not have notable effect on TTC9 protein level. However, the effects of EGF and bFGF were abolished by the concurrent treatment with PD98059 (Fig. 3.18C). This suggests that growth factor-induced TTC9 expression is via the activation of ERK1/2 signaling pathway. On the other hand, p38 kinase inhibitor SB203580 enhanced TTC9 level alone and the effects of SB203580 and growth factors are synergistic in increasing the TTC9 level (Fig. 3.18D), indicating that the expression of TTC9 is differentially regulated through different signaling pathways.

3.7.3 The regulations by growth factors and estrogen on TTC9 protein level are mediated through separate pathways

As shown in Fig. 3.18A, FCS without DCC treatment had little effect on TTC9 protein expression at all the concentrations tested. We postulated this may due to the antagonistic effect of serum estrogens which have been shown to

Results

Fig. 3.18 TTC9 protein was serum and growth factor inducible in MCF-7 cells.

Total cell lysates were collected following various treatments and subjected to Western blotting analysis. The numbers associated with each blot are densitometry values of TTC9 relative to that of the vehicle-treated cells, which is given the value of 1. (A) The effect of serum on TTC9 protein level. MCF-7 cells were grown in serum-free medium and in media containing various concentrations of DCC-FCS or FCS as indicated for 48h. (B) EGF and bFGF increased the level of TTC9 protein in MCF-7 cells. MCF-7 cells were treated with 100 ng/ml EGF and 50 ng/ml bFGF, respectively, for 48 h. (C) Growth factor-induced TTC9 expression was abolished by ERK inhibitor PD98059. MCF-7 cells were treated with 100 ng/ml EGF, 50 ng/ml FGF in the presence or absence of PD98059 (50 μ M) as indicated for 48 h. PD98059 was added to the cell medium 30 min before the addition of growth factors. (D) p38 kinase inhibitor SB203580 could not inhibit the growth factor-induced TTC9 expression. MCF-7 cells were treated with 100 ng/ml EGF, 50 ng/ml FGF in the presence or absence of SB203580 (10 μ M) as indicated for 48 h. SB203580 was added to the cell medium 30 min before the addition of growth factors.

decrease TTC9 level in MCF-7 cells. This speculation is supported by the results in Fig. 3.19A showing that E2 was able to abolish the effect of DCC-FCS on the up-regulation of TTC9 protein. Intriguingly, E2 also reversed the effects of EGF and bFGF to the control level (Fig. 3.19B). This effect of E2 is perhaps through a similar mechanism as its effect on DCC-FCS-induced increase of TTC9.

In order to understand whether E2 contradicts the growth factors up-regulation on TTC9 through the same pathway or different pathways, the effects of specific inhibitors were tested. Antiestrogen ICI182,780 inhibits cell proliferation of MCF-7 cells (Varshochi et al., 2005). It up-regulated TTC9 protein level when used alone and also abolished the effect of E2 in decreasing the TTC9 level. Furthermore, ICI182,780 exerted additional effect on EGF- and bFGF-induced TTC9 protein expression when it was added together with these growth factors (Fig. 3.19B). All these results indicate that the regulations by growth factors and estrogen on TTC9 protein level are mediated through separate pathways.

3.8 Subcellular localization of TTC9

Cell fractionation study was performed using MCF-7 cells (Fig. 3.20A). Mitochondria are present mainly in 12,000 g pellet as evidenced by the presence of mitochondria marker cytochrome c oxidase subunit IV (COX IV).

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Fig. 3.19 Growth factors and E2 regulate TTC9 expression through different pathways.

MCF-7 cell lysates were collected at 48 hr after various treatments and subjected to Western blotting analysis. The numbers associated with each blot are densitometry values of TTC9 relative to that of the vehicle-treated cells, which is given the value of 1.(A) The up-regulation of TTC9 protein level by 10% DCC-FCS was abolished by 1 nM E2. (B) The up-regulation of TTC9 protein level by growth factors was counteracted by E2 but not by ICI182,780. MCF-7 cells were treated with 100 ng/ml EGF, 50 ng/ml FGF, together with either 1 nM E2, 0.1 μ M ICI182,780 (ICI) or both as indicated for 48 hr.

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On the other hand, endoplasmic reticulum is present in both the 12,000 g pellet and 100,000 g pellet as shown by the presence of calnexin, a well-known endoplasmic reticulum marker (Katiyar et al., 2004). TTC9 is present in both the 12,000 g pellet and the 100,000 g pellet fractions. Since both of the 12,000g and 100,000 g pellet fractions contain endoplasmic reticulum, we can conclude that TTC9 is concentrated mainly to the endoplasmic reticulum. Nonetheless, a lighter band of TTC9 was also observed in the 100,000 g supernatant fraction, which is the cytoplasmic fraction.

The subcellular localization of TTC9 in MCF-7 cells was also examined by immunostaining. Hexyl ester of Rhodamine B is known to stain endoplasmic reticulum at high concentrations (Terasaki and Reese, 1992). Since Rhodamine B (Fig. 3.20B, green) perfectly overlaps with the staining pattern of endoplasmic reticulum marker protein calnexin (red), we used Rhodamine B as an endoplasmic reticulum marker to avoid the non-specific staining associated with the use of antibodies to calnexin. The immunostaining of TTC9 (green) largely overlaps with the staining of Rhodamine B (red, Fig. 3.20B), which is in accordance with the results of cell fractionation studies which localize TTC9 mainly to the endoplasmic reticulum.

3.9 Identification of TTC9 transcript and protein in human tissues

Since the ~2.5 Kb mRNA transcript seems to be the only transcript of TTC9 in breast cancer cells, the transcription of TTC9 gene was further examined in

Results

Results

Fig. 3.20 Subcellular localization of TTC9.

(A) Cells were subfractionated as described in “materials and methods”. 12P is the pellet fraction after centrifugation at $12,000\times g$; 100P is the pellet fraction after centrifugation at $100,000\times g$; 100S is the supernatant after centrifugation at $100,000\times g$. Calnexin and COX IV were used as markers for endoplasmic reticulum and mitochondria, respectively. (B) MCF-7 cells were grown on glass coverslips and stained for calnexin together with the hexyl ester of Rhodamine B (top panel), or stained for TTC9 together with the hexyl ester of Rhodamine B (lower panel). Calnexin was probed with anti-calnexin antibody and detected by Alexa Fluo 647 donkey anti-goat IgG (red). TTC9 was probed with TTC9 antibody and detected by FITC-conjugated anti-mouse IgG (green). Rhodamine B staining completely overlaps with that of calnexin, supporting its use as an endoplasmic reticulum stain at the concentration of $1\text{ }\mu\text{g/ml}$. The images were obtained using Zeiss confocal laser scanning microscope model LSM510 (*bar*, $10\mu\text{m}$).

Results

human tissues to see whether it is the same case *in vivo*. Similar to the results in cells, Northern blotting analysis using specific ^{32}P -labelled TTC9 probe also identified a band of ~2.5 Kb in some of the 12 tissues of the commercial mRNA blot (Fig. 3.21A). Different from the results from cell lines, an estimated 5.1 Kb transcript was detected at the same time. In addition, a 5.9 Kb band was also seen in cardiac and skeletal muscle tissues. TTC9 gene expression is the highest in brain tissue. However, the RNA in some of the tissues such as the colon looks degraded. It is difficult to assess the relative expression of TTC9 among various tissues using this blot.

At the protein level, the TTC9 antibody also identified a ~25kD protein in human tissues of a commercial Western blot (Fig. 3.21B). However, it also detected a prominent double band of 36-37 kD proteins in human brain but their identity remained to be investigated. In accordance with the mRNA expression, TTC9 protein is also most abundant in the brain tissue. Though liver tissue expresses little TTC9, the protein seems to be ubiquitously expressed in all the tissues studied.

3.10 Detection of TTC9 protein in mice and rats tissues

As shown before, there is about 90% homology among human TTC9 protein and its homologues in mice and rats. It is thus natural to speculate whether the TTC9 antibody, which is generated against human TTC9 protein, can recognize its homologues in mouse and rat tissues. Mice and rats at 8 weeks old were

Results

Fig. 3.21 Identification of TTC9 transcripts and protein in the commercial human tissue blots.

(A) TTC9 mRNA levels were determined using 32 P-labelled TTC9 probe. Human Northern RNA blot was obtained from OriGene Technologies, Inc. (Rockville, MD). (B) TTC9 protein expression pattern in eight human normal tissues. Human tissue blot was bought from ProSci Inc. (Poway, CA). Molecular weight markers (left) indicated the TTC9 band appeared at ~25 kD.

Results

dissected and different tissues were homogenized in protein lysis buffer [100mM NaF, 50 mM HEPES (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF] with protease inhibitors (5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM Na₃VO₄). 20 µg total protein from respective tissue was analyzed by Western blotting. In accordance with our speculation, the antibody also recognized a band appearing at ~25 kD. As shown in Fig. 3.22, the expression pattern of TTC9 protein was similar in mouse (Fig. 3.22A) and rat (Fig. 3.22B) tissues. The patterns were also similar to that in human tissues, i.e. TTC9 was ubiquitously expressed in every tissue tested and it was expressed at a high level in brain tissue and was low in liver. Furthermore, although sequence alignment showed that the computationally-predicted TTC9 homologue in rats has an extra fragment compared to the TTC9 protein in human (Fig. 3.5), the size of the protein detected here by the antibody revealed that the homologue was of the same length to human TTC9, indicating that the extra sequence might not exist.

3.11 TTC9 is also hormonally regulated in mouse uterus

The hormonally regulation of TTC9 expression in breast cancer cells, together with the finding that TTC9 is expressed at a higher level in human breast cancer tissues compared with adjacent normal tissues, suggest steroid hormones do effect the expression of TTC9 *in vivo* and *in vitro*. To further confirm this point, the steroid hormone effect on TTC9 expression was studied in mice uterus, liver and mammary gland, which are target organs of estrogen. Female BALB/C

Results

Fig. 3.22 TTC9 expression in mice and rats tissues.

Tissues from 8 weeks old mice or rats were homogenized in protein lysis buffer with protease inhibitors. 40 μ g lysate from each tissue were analyzed by Western blotting with the mouse-generated polyclonal antibodies against human TTC9 protein. Molecular marker revealed that the bands detected in mice and rats tissues also appeared at ~25 kD. (A) TTC9 in mice tissues. (B) TTC9 in rat tissues.

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mice, which are fourteen days old and still in their proestrus stage, were injected with E₂-benzoate in sesame oil at 20 µg/kg. Mice were sacrificed at 72 hr post injection and the weights of uterus were recorded. An increase in the weight of uterus indicates E₂-benzoate is taking effect. The expression of TTC9 in the uterus, liver and mammary gland was analyzed by Western blotting. As shown in Fig. 3.23, the weight of the uteri from E₂-benzoate injected mice were two times more than those from control vehicle injected mice. For all the estrogen target tissues, TTC9 expression level was dramatically higher in E₂-benzoate treated mice, comparing with those from control mice. It can thus be concluded that TTC9 is a hormonally regulated gene both *in vitro* and *in vivo*.

3.12 TTC9 interacting proteins

Although previous results suggest TTC9 could be an important protein functioning in different tissues and cell types, the exact role of TTC9 remains unclear. Hunting for interacting proteins is a common way to get some hints on the function of an unknown protein. Here we made use of yeast-two-hybrid system to look for TTC9 interacting proteins in human breast cancer cell line MCF-7 cDNA libraries (OriGene).

In yeast-two-hybrid screening, the 669 bp coding sequence of TTC9 protein was cloned into pLexA vector, which contains the DNA-binding domain. TTC9 functions as a bait protein by the fusion to the DNA-binding domain. On the

Results

Fig. 3.23 TTC9 is up-regulated by E₂-benzoate in mice uterus.

14 days old BALB/C mice were injected with E₂-benzoate in sesame oil at 20 µg/kg as described in “materials and methods”. Mice were sacrificed 72 hr after injection. The uteri, mammary glands and livers were taken and homogenized for Western blot analysis. The increase of uterus weight after hormone injection indicated the E₂-benzoate was taking effect.

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other hand, MCF-7 cDNA libraries were fused to the activation domain. The bait vector and the cDNA libraries were transformed into yeast strain EGY48 together with the lacZ reporter gene (Fig. A.2 in Appendices). Transformants grown on SD-HUW plates were harvested and re-spread onto X-Gal containing SD-HUWL plates to test protein-protein interactions. 400 blue colonies were picked from the X-Gal plates and were re-dotted onto X-Gal containing SD-HUWL plates to further confirm the interaction. The time at which each colony turned blue was noted. Plasmids were then isolated from the first 100 colonies turned blue, which also showed the strongest blue color. As each yeast cell could contain more than one expression vectors, the plasmids isolated from each yeast colony were then transformed into KC8 *E.coli* cells, which carry *trpC* mutation and thus can survive on tryptophan dropout selective plates only when yeast TRP1 gene is present. The library plasmids, which bear TRP1 gene, were then separated from the plasmid mixture isolated from the yeast co-transformants that contain bait, reporter and library plasmids. 5-6 KC8 colonies from each transformation plate, which contains plasmids from one yeast colony, were amplified for plasmid extraction, followed by digestion with EcoRI and XhoI. It was considered to only have one library plasmid in a yeast colony if the digestion pattern for the plasmids isolated from one transformation plate was identical. Such library plasmids were sent for sequencing and the cDNA sequences were subject to blast searches and in-frame analysis. Table 2 shows the library plasmids that interacted with TTC9 and were also in frame with the activation domain.

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The library plasmids listed in Table 2 were then re-transformed into EGY48 cells together with TTC9 bait vector (TTC9-pLexA) or empty bait vector (pLexA) for another round of confirmation. Colonies were dotted onto an X-Gal containing SD-HUWL plate in duplicate and were allowed to grow at 30°C for 96 hr (Fig. 3.24). The time for each colony to turn blue was recorded. Colonies which contain TTC9 bait vector and turned blue at earlier time points than corresponding control colonies (with empty bait vector) were considered to be TTC9 interacting proteins. Among them, 4 library proteins showed very strong interactions with TTC9, i.e. Tm5NM-1, PKC binding protein 1 (PRKCBP1), thymosin β 4 and thioredoxin-like 2 (high-lighted in Table 2).

3.13 TTC9 can interact with cellular Tm5NM-1

Among all the four proteins, Tm5NM-1 is of particular interest. Studies have shown that Tm5NM-1 is associated with stress fibers (Percival et al., 2004) and progesterone have been shown to dramatically up-regulate TTC9 expression level as well as to increase cell stress fibers (Cao et al., 2006; Lin et al., 2000). We hypothesized that the interaction between TTC9 and Tm5NM-1 might be involved in the mechanism of progesterone induced cell spreading. The binding of TTC9 to Tm5NM-1 was further examined by GST-pull down assay. Here GST-TTC9 was used as a “bait” to pull down the cellular expressed Tm5NM-1

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Table 3.1. Possible TTC9 interacting proteins as indicated by yeast-two-hybrid assay. Listed are the plasmids turned out to be positive in the first-round selection of yeast-two-hybrid assay. High-lighted plasmids are positive library proteins in the second-round verification.

Colony No.	Corresponding gene
1	Homo sapiens DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B)
2	Homo sapiens tropomyosin 3, mRNA
3	Human thymosin beta-4 mRNA
4	Homo sapiens ATP synthase, H ⁺ transporting, mitochondrial F0, complex, subunit b, isoform 1
5	Homo sapiens solute carrier family 7 (cationic amino acid transporter, γ^+ system), member 5
6	Homo sapiens calcium and integrin binding 1 (calmyrin)
7	Homo sapiens gene for 14-3-3 protein
8	Homo sapiens mitochondrial DNA, complete genome,NADH dehydrogenase subunit 1
9	Homo sapiens hAZ-brain mRNA for ornithine decarboxylase antizyme
10	Homo sapiens tropomyosin 3
11	Homo sapiens X-box binding protein 1 (XBP1)
12	Homo sapiens signal-transducing adaptor protein-2 (STAP2), transcript variant 2
13	Homo sapiens heterogeneous nuclear ribonucleoprotein C (C1/C2)(HNRPC), transcript variant 2
14	Homo sapiens patched related protein TRC8 (TRC8) gene
15	Homo sapiens PKC ζ -interacting protein PICOT (PICOT)
16	Homo sapiens thioredoxin-like 2
17	Homo sapiens isolate 14_L0f(Tor65) mitochondrion, COX II
18	Homo sapiens signal transducer and activator of transcription 5B (STAT5B)
19	Homo sapiens isolate 9_N21(Tor57) mitochondrion, complete genome.16S ribosomal RNA
20	Homo sapiens eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)
21	Homo sapiens BRMS2 mRNA
22	Homo sapiens aldolase A, fructose-bisphosphate (ALDOA), transcript variant 2
23	Homo sapiens tropomyosin 3
24	Homo sapiens Janus kinase 1 (a protein tyrosine kinase) (JAK1)
25	Homo sapiens tropomyosin 3
26	Homo sapiens dCMP deaminase (DCTD), transcript variant 2
27	Homo sapiens dolichyl-diphosphooligosaccharide-protein glycosyltransferase (DDOST)
28	Homo sapiens DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B), transcript variant 2
29	Homo sapiens transcriptional repressor BSR/RACK7/PRKCBP1 isoform o
30	Homo sapiens tropomyosin 3

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No.	-	-	+	+	No.	-	-	+	+	No.	-	-	+	+
1	24	24	39	39	2	72	72	63	63	3	-	-	72	63
4	96	96	-	-	5	48	-	-	-	6	72	72	96	-
7	63	72	72	-	8	96	96	-	96	9	96	96	-	-
10	72	72	39	50	11	63	63	72	96	12	63	72	72	72
13	96	63	72	96	14	63	63	96	96	15	63	96	63	96
16	63	96	39	39	17	-	63	72	72	18	-	48	-	-
19	63	96	72	-	20	63	63	96	-	21	72	-	72	63
22	72	72	63	63	23	-	72	39	39	24	-	96	-	96
25	63	-	39	39	26	63	63	72	96	27	63	-	-	96
28	15	15	39	39	29	63	72	24	24	30	63	63	-	63

Fig. 3.24 Second-round verification of library plasmids listed in Table 2.

Library plasmids listed in Table 2 were re-transformed into EGY48 together with bait vector TTC9-pLexA (testing colonies) or empty pLexA vector (negative control). Transformants from each transformation reaction were dotted onto X-Gal plate in duplicates (upper figure). The time point for each dotted colony to turn blue after inoculation was recorded (lower chart). The numbers showing on the figure and the chart respond to the library plasmid numbers in Table 2. Testing colonies were labeled as red numbers in the figure and “+” in the chart. Negative controls were represented by blue numbers in the figure and “-” in the chart.

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protein. As we did not have specific antibody for Tm5NM-1 initially, the possible interacting candidate was cloned into pcDNA3.1/ myc-His(-) vector for the expression of His-tagged protein, which can then be detected by anti-His antibody. As shown in Fig. 3.25A, Tm5NM-1 was pulled down by GST-TTC9, but not by the GST-tag, suggesting that GST-TTC9 does interact with cellular expressed Tm5NM-1. The specificity of the interaction was further confirmed by GST pull-down assay with increasing amount of bait protein. Fig. 3.25B showed that the protein amount of Tm5NM-1 pulled down by GST-TTC9 increased proportionally to the amount of bait protein used in the assay.

3.14 TTC9 interacts with Tm5NM-1 in mammalian cells

In the case of yeast-two-hybrid assay and GST pull-down, the TTC9 protein was expressed in yeast and *E. coli* respectively, which lacks post-translational modification. To confirm that cellular expressed TTC9 can indeed interact with Tm5NM-1, co-immunoprecipitation was performed between cellular expressed TTC9 and Tm5NM-1 proteins. Here TTC9 was cloned into pXL-Flag, which added an 8 aa flag-tag to its N-terminal. This expression vector for TTC9 was co-transfected into COS-7 cells with Tm5NM-1-(His)₆ expression vector. Co-immunoprecipitation was carried out using anti-flag agarose beads (Sigma). As shown in Fig. 3.26A, cellular expressed TTC9 showed prominent interaction

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Fig. 3.25 GST-TTC9 interacts with cellular expressed Tm5NM-1.

(A) COS-7 cells were transfected with Tm5NM-1-(His)₆ expression vector and GST-TTC9 was used to pull down cellular expressed Tm5NM-1 as described in “materials and methods”. The pull-down reaction with GST-tag was used as a control. (B) GST-TTC9 specifically interact with Tm5NM-1. GST-pull down assay was carried out with 2 µg and 20 µg GST-TTC9 as bait protein. The amount of Tm5NM-1-(His)₆ pulled down was proportional to the amount of bait protein used.

with cellular Tm5NM-1.

In another experiment, flag-TTC9 expression vector was transfected into COS-7 cells. The interaction between endogenous Tm5NM-1 and cellular expressed TTC9 was examined by co-immunoprecipitation with anti-Tm5NM-1/2 antibody. Fig. 3.26B revealed that cellular expressed TTC9 could also bind to endogenous Tm5NM-1.

3.15 The linker fragment between the first two TPR domains of TTC9 is important for the interaction with Tm5NM-1

To specify the TTC9 domains interacting with Tm5NM-1 in the cell, four different constructs of TTC9 were transfected into COS-7 cells together with Tm5NM-1 expression vector. TTC9 (1-50) stands for the first 50 aa of TTC9 protein, which is outside of the TPR domains. TTC9 (1-115) expresses the first 115 aa of TTC9, with one TPR domain included. TTC9 (51-222) contains all three TPR domains in the C-terminus, while TTC9 (116-222) includes two consensus TPR domains. As shown in Fig. 3.28A, TTC9 (1-50) did not interact with Tm5NM-1 protein. TTC9 (1-115) showed the stronger interaction with target protein than the full-length TTC9. TTC9 (51-222) also interacted with Tm5NM-1, but the interaction was weaker than that of TTC9. TTC9 (116-222) only weakly bound to Tm5NM-1 under the reaction conditions.

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Fig. 3.26 Cellular expressed TTC9 can bind to Tm5NM-1.

(A) Expression vectors for flag-TTC9 and Tm5NM-1-(His)₆ were co-transfected into COS-7 cells. Co-immunoprecipitation was carried out with anti-flag agarose beads (Sigma) and Tm5NM-1 was detected by anti-His antibody. Upper panel: Tm5NM-1-(His)₆ was expressed at similar level in control vector and flag-TTC9 transfected COS-7 cells; lower panel: Tm5NM-1-(His)₆ was pulled down by flag-TTC9. (B) Expression vector for flag-TTC9 was transfected into COS-7 cells. Co-immunoprecipitation was carried out with anti-Tm5NM-1/2 antibody (Chemicon) and flag-TTC9 was detected by anti-flag antibody (Sigma). Co-immunoprecipitation with goat pre-immune serum was included as a negative control.

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As TTC9 (1-115) appeared to be the strongest fragment interacting with Tm5NM-1 and TTC9 (1-50) showed no interaction, TTC9 (51-115) was thus constructed to test whether this fragment alone was necessary and sufficient for the binding between these two proteins. As shown in Fig. 3.28A, TTC9 (51-115) did not interact with Tm5NM-1, which indicated the first 50 aa of TTC9 was required for the proper folding of the protein. We thus speculated that the first 50 aa of TTC9 could be involved in determining either the functional specificity of TTC9, or the stereological conformation of this protein.

We then went on to examine which region was involved in the interaction of TTC9 (1-115) with target protein. Fig. 3.28B revealed that TTC9 (1-70) and TTC9 (1-85) did not interact with Tm5NM-1, while TTC9 (1-100) interacted with Tm5NM-1 to some extent, weaker than that of full-length TTC9.

It could be concluded from previous results that either the fragment between aa 85 and 100 was important for the interaction, or the first TPR domain of TTC9 was required for the binding to Tm5NM-1. Furthermore, it seems that the second and third TPR domains had an inhibitory effect on the binding of TTC9 to Tm5NM-1 (Fig. 3.28A). To confirm these points, more truncations of TTC9 protein were generated. Fig. 3.28C revealed that TTC9 (1-90) showed prominent interaction with Tm5NM-1, indicating it is the complete first TPR domain which is required for the interaction. In addition, the linker peptide, which is between the first and the second TPR, facilitated the binding between these two proteins, as TTC9 (1-128) showed stronger pull-down compared with

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TTC9

Fig. 3.27 Truncation constructs of TTC9 protein.

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Fig. 3.28 Domain analysis of TTC9 binding to Tm5NM-1.

TTC9 truncates in pXL-Flag vector were transfected into COS-7 cells together with Tm5NM-1-(His)₆ expression vector. The interaction between flag-TTC9 truncates and Tm5NM-1-(His)₆ was analyzed by co-immunoprecipitation with anti-flag agarose beads. The upper panels are Western blot analysis of

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Tm5NM-1 in the cell lysates. The lower panels are the co-immunoprecipitation result.

(1-90). The inclusion of the second and the third TPR domain of TTC9 weakened the interaction, since TTC9 (1-161) and TTC9 (1-197) pulled down less Tm5NM-1 than TTC9 (1-128) did.

3.16 TTC9 was expressed at a higher level in adherent cells compared with that in suspension cells

The interaction of TTC9 with Tm5NM-1, together with the fact that in ABC28 cells, TTC9 is up-regulated by progesterone, accompanied with an increase in stress fibers, both in quantity and diameter (Lin et al., 2000), led us to hypothesize that TTC9 may be involved in cell cytoskeleton organization and cell adhesion. To verify the hypothesis, we examined the expression level of TTC9 in different cell lines, including both adherent and suspension cells. All these cells were growing in their normal growth medium before harvest. Indeed, TTC9 is expressed at a higher level in adherent cells, including BT20, COS-7, MCF-7, MDA-MB-231, PC12 and T47D, compared with that in suspension cells, such as HL-60, MOLT-4, myeloma and Jurkat cells (Fig. 3.29). In addition, in adherent cells, the expression level of TTC9 seems to correlate with the “strength” of attachment, that is, TTC9 is expressed at a higher level in cells which need more time to detach from the dish when treated with trypsin.

Results

However, this point needs to be examined by further experiments.

Fig. 3.29 TTC9 is expressed at a higher level in adherent cells compared with that in suspension cells.

Cells were grown in their normal growth medium before harvest. 20 μ g total cell lysate from each cell line was separated on SDS-PAGE gel and TTC9 expression was analyzed by Western blotting.

CHAPTER 4

DISCUSSION

4.1 Sequence characteristics of TTC9

4.1.1 mRNA size of TTC9 transcript

TTC9 was a hypothetical protein first predicted from a brain cDNA clone some 10 years ago (Nagase et al., 1996). Microarray analysis revealed that in PR-transfected breast cancer cell line MDA-MB-231, TTC9 mRNA was dramatically up-regulated by progesterone (unpublished data), suggesting this gene could be importantly involved in PR signaling pathways. Although three transcript sizes for this gene are reported online, i.e. 5510 bp, 5217 bp and 2428 bp, Northern blot analysis using specific TTC9 probe only detected transcripts of one size, ~2.5 Kb, in breast cancer cell lines. The ~2.5 Kb band is also subjected to hormonal regulation in different breast cells. Similar to that in cells, a ~2.5 Kb transcript was also detected in human tissues. However, two other transcripts, which appeared to be ~5.1 Kb and ~5.9 Kb, were detected in human tissues as well. The identity of the ~5.9 Kb transcript remains to be investigated. The result of our Northern blot analysis in terms of size was similar to the online Northern blot data (<http://www.kazusa.or.jp/huge/gfpage/KIAA0227/>), which also detected transcripts of ~2.5 Kb as well as of 4.5-5 Kb. We speculated that the ~2.5 Kb transcript could be corresponding to the 2428 bp transcript, while the ~5 Kb one might be the 5217 bp transcript. The ~5 Kb

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band present in some tissues could be a tissue-specific isoform generated by the use of alternate promoters of TTC9 gene. Further analysis need to be done to confirm this notion.

The abundance of different transcripts in our study differs from the online Northern data. The ~2.5 Kb transcript in our Northern blot was detected in all tissues, whereas the ~2.5 Kb band was only prominent in heart and skeletal muscles in online Northern blot. Instead, the ~5 Kb transcripts was detected in all the tissues and seemed to be the only band in most tissues in the online data. This difference may be due to different RNA preparation: the commercial blot we bought was mRNA blot whereas the online data could have used total RNA blot. There may be a preferential enrichment of the shorter transcript during the mRNA preparation due to the length of poly A tail. It is not known if the longer transcripts are expressed in the normal breast tissue as the mRNA sample is not included in the commercial Northern blot used in this study.

4.1.2 Characteristics of TTC9 protein

4.1.2.1 Protein length of TTC9

Three TTC9 proteins of different sizes are predicted and published on NCBI website (www.pubmed.com). These include TTC9 of 336 aa and 277 aa, both

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of which are suggested to lack complete sequence at the N-terminus, and TTC9 at 434 aa. Using Vector NTI software, we predicted the size of TTC9 protein to be 222 aa, with a calculated molecular weight at 24.4 kD. This prediction was confirmed by Western blot analysis with mouse polyclonal antibodies generated against the predicted 222 aa TTC9, which detected a ~25 kD band regulated by progesterone in ABC28 cells. With the antibody produced we confirmed that almost all human tissues also express an approximately 25 kD protein, corresponding to the 222 aa TTC9, with the highest expression in brain and the lowest expression in liver. In the human tissues, a band ~39 kD was also detected in human brain. The identity of this band remains to be determined. It could be a nonspecific band, or a longer protein variant of TTC9 protein which is specifically expressed in the brain, although none of the online predicted TTC9 protein has a molecular weight of 39 kD. It at least can be concluded at current stage that the major protein product of TTC9 gene is a protein with 222 aa.

4.1.2.2 TTC9 is mainly localized in endoplasmic reticulum

Both cell fractionation and immunostaining results reveal that in spite of its presence in the cytosolic fraction, TTC9 is most concentrated to endoplasmic reticulum. TTC9 may not reside in the lumen of endoplasmic reticulum since the protein lacks the typical C-terminal KDEL sequence, which is an

endoplasmic reticulum lumen retention signal (Munro and Pelham, 1987). However, TTC9 could be concentrated to the peripheral of endoplasmic reticulum by anchoring to the membrane as two putative endoplasmic reticulum membrane retention signals (XXRR-like in the N-terminal and KKXX-like motif in the C-terminal) are identified at the N-terminal and C-terminal of the protein. On the other hand, analysis by PROSITE program indicates several potential sites for N-myristoylation, which refers to the attachment of myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine of the protein (Farazi et al., 2001; Shrivastav et al., 2003). It is also possible that TTC9 is anchored to the endoplasmic reticulum membrane through N-myristoylation.

4.1.2.3 Secondary structure of TTC9 protein

Three TPR motifs are identified in TTC9 by SMART algorithm. TPR motif has been suggested to compose of two α -helical domains with little or no β -sheet formation (Das et al., 1998; Hirano et al., 1990; Scheufler et al., 2000). It can be predicted that the majority of TTC9 secondary structure should be α -helices. This hypothesis is confirmed by the FT-IR study, which revealed that the secondary structure of TTC9 is mainly α -helices. We have tried to crystallize TTC9-(His)₆ protein for further structural analysis. However, the protein was difficult to crystallize and it was degraded easily. Further effort will be made to

analyze the protein structure by either NMR or crystallization.

4.1.3 TTC9 is located on human chromosome 14

TTC9 gene is located on human chromosome 14q24. Abnormalities of chromosome 14q including chromosome breakage, rearrangement, amplification or overrepresentation, and loss of 14q DNA have been reported in a number of tumors, including breast cancers (Knuutila et al., 2000; Pollack et al., 2002; Tanner et al., 1998; Xie et al., 2002), chondrosarcomas (Larramendy et al., 1997), prostate cancer (Saramaki et al., 2001), fibrosarcomas (Schmidt et al., 2002), gastrointestinal stromal tumors (El-Rifai et al., 2000), and neuroblastomas (Thompson et al., 2001). These findings suggest that both oncogenes and tumor suppressor genes may exist at 14q. Examples for these genes include AKT1 gene, which is a proto-oncogene of the viral oncogene *v*-AKT, and is located at 14q32 (Staal et al., 1988). In addition, two tumor suppressor genes on chromosome 14q12-13 and 14q32 were indicated to be involved in ovarian carcinomas (Bandera et al., 1997).

4.2 The involvement of TTC9 in hormone signaling

4.2.1 In ABC28 cells, TTC9 is up-regulated by progesterone through PR

Several lines of evidence suggested TTC9 is a gene mediated by PR. Firstly, the up-regulation of progesterone on TTC9 mRNA expression appears as early as 3 hrs post-treatment, and the obvious increase in protein level is observed after 8-hour treatment. Furthermore, the expression of TTC9 is subject to progesterone treatment even at low concentrations. These suggest that in addition to be a gene involved in progesterone signaling, TTC9 could also be a PR direct target gene. This suggestion is supported by the identification of a putative PRE in the upstream sequence of TTC9 ORF and preliminary results indicate the removal of the putative PRE result in dramatic decrease of the promoter activity (data not shown). Secondly, in ABC28 cells, RU486, which is a well-known PR antagonist, can inhibit the up-regulation effect of progesterone on TTC9 expression, although it does not have any effect on TTC9 expression alone. In addition, another PR antagonist, ZK98059, is also capable of antagonizing progesterone's effect (data not shown). It can be concluded that progesterone increases TTC9 expression level through PR. Thirdly, although aldosterone and dexamethasone can increase TTC9 expression in ABC28 cells, they have no effect on TTC9 expression in control

cell line CTC15. As both CTC15 and ABC28 are GR- and MR-positive, this result verifies the up-regulation effect of aldosterone and dexamethasone is not through GR or MR. Since it has already been shown that GR and MR can crosstalk with PR in breast cancer cells (Leo et al., 2004), it is of high possibility that aldosterone and dexamethasone increase TTC9 expression through PR signaling pathway.

4.2.2 TTC9 is down-regulated by E₂ in MCF-7 cells

Although TTC9 is up-regulated by progesterone in ABC28 cells, it is down-regulated by progesterone alone or E₂ plus progesterone in MCF-7 cells. The exact mechanism involved is unclear. It is possible that TTC9 is also a gene involved in ER signaling pathways, and estrogen down-regulates its expression. The down-regulation by estrogen could dominate over the progesterone effect on TTC9 expression. The involvement of TTC9 in ER signaling pathway is supported by the result which reveals that ICI 182780, a pure antiestrogen, is capable of inhibiting the down-regulating effect of E₂ on TTC9 expression. Alternatively, the presence of ER may inhibit the up-regulation effect of progesterone by interacting with PR. Thirdly, in 1980s, Meyer et al reported that steroid hormone receptors could compete for transcription factors when two or more receptors are present in the cell (Meyer et al., 1989). In MCF-7

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cells, because of the dominant ER amount over that of PR, the competition for transcription factors between ER and PR might result in the inhibition of TTC9 expression by E2 plus P.

One point to be noted the up-regulation of TTC9 by ICI 182780 in MCF-7 cells. When treated together with E₂, ICI 182780 not only inhibited the down-regulating effect of E₂ on TTC9 expression, but also further increased TTC9 expression level. This regulatory pattern of gene transcription by ER agonist and antagonist is consistent with other reports on estrogen action (Jakacka et al., 2001; Paech et al., 1997). The regulation of E2 and ICI 182780 on TTC9 expression may be through a nonclassical pathway, in which ER interacts with other transcription factors bound to their response elements, such as SP1, AP1 and NF- κ B, in the target genes. In 2001, Jakacka et al. proposed that ligands traditionally considered ER agonists or antagonists have an opposite effect on the nonclassical AP1 pathway, where agonists repress and antagonists activate transcription (Jakacka et al., 2001). Analysis of TTC9 promoter using online software have identified quite a few SP1 sites in TTC9 promoter, which provides a possibility that E₂ and ICI 182780 may regulate TTC9 expression through the SP1 nonclassical pathway.

4.2.3 TTC9 is involved in MAPK signaling pathways

4.2.3.1 Growth factors increased TTC9 expression

Both DCC-FCS and growth factors including EGF and FGF increase TTC9 expression. It is possible that the effect of DCC-FCS on the increase of TTC9 level may be mediated through serum growth factors. There are two possible mechanisms for the effect of growth factors on TTC9 expression. Growth factors could crosstalk with ER signaling pathway to affect TTC9 expression. Various studies have shown that the actions of EGF-EGFR complex occasionally mimics the effects of estrogen and these effects are most likely ER-dependent but estrogen-independent phenomena (Apostolakis et al., 2000; Ignar-Trowbridge et al., 1993; Levin, 2003; Vignon et al., 1987). However, the up-regulation of TTC9 by EGF and FGF is abolished by PD98059, which is a specific inhibitor for MEK1/2, suggesting that effects of growth factors on TTC9 are unlikely mediated through the crosstalk with ER signaling pathway.

Another mechanism is that TTC9 is also involved in the classic MAPK signaling pathway. This mechanism is supported by the fact that TTC9 is up-regulated by various growth factors, the ability of PD98059 to abolish the up-regulation effects, and the inability of ER antagonist, ICI182780, to inhibit the regulation.

4.2.3.2 TTC9 is differentially regulated by different MAPK signaling pathways

MAPK signaling pathways including several cascade: the MAPK/Erk signaling cascade involved in growth and differentiation; the G-protein-coupled receptors signaling to MAPK/Erk; Stress-activated protein kinases (SAPK)/Jun N-terminal kinases (JNK) signaling cascade and signaling pathways activating p38 MAPK. The inhibition of growth factors-increased TTC9 expression by PD98059 indicates TTC9 is one of the downstream genes of MEK1/2, which is involved in the MAPK/Erk or the G-protein-coupled cascades. On the other hand, the increase of TTC9 expression by SB203580, a specific inhibitor of p38 MAPK, and the additive effect of growth factors and SB203580 on TTC9 expression, reveals that TTC9 is also regulated by the p38 MAPK pathway.

4.2.4 TTC9 is hormonally regulated *in vivo*

The expression of TTC9 is also regulated *in vivo*. This is firstly supported by the fact that TTC9 mRNA is significantly over-expressed in human breast cancer tissues compared with their adjacent normal controls. The mechanism involved is not clear yet. The increased TTC9 expression level induced by serum and growth factor may be one of the reasons. Breast cancer tissues are commonly associated with increased MAPK activity compared with their

benign adjacent controls (Foekens et al., 1989; Resnik et al., 1998), thus the increased TTC9 expression in breast cancer tissues could be the result of enhanced growth factor activity. Another possibility is that in breast cancer tissues, TTC9 mRNA might have enhanced stability. The transcription rate of TTC9 gene could be the same in breast cancer tissues and adjacent normal tissues, while TTC9 mRNA is preferentially protected from degradation in breast cancer tissues. It is also possible that gene amplification is a mechanism of TTC9 overexpression in breast cancer tissues. Gene amplification is highly correlated with mRNA overexpression and on the whole, as little as a 2-fold change in DNA copy number is associated with a corresponding 1.5-fold change in mRNA levels (Pollack et al., 2002; Xie et al., 2002).

It is unexpected that the up-regulation of TTC9 in breast cancer tissues shows no correlation with ER and PR status, although TTC9 is the target gene of both estrogen and progesterone. The up-regulation also shows no correlation with tumor size, nuclear grade or other clinicopathological parameters. One possibility we can think of is that TTC9 might involved in the oncogenic cell transformation process, which is the initial step in tumor generation. The up-regulated TTC9 expression could assist in certain changes in the cell characteristics, such as cell skeleton organization.

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In mice uterus, TTC9 expression is also increased by estrogen, which provides another example for hormone regulation *in vivo*. In this study, mice at proestrus stages are chosen, so that the effect of E₂ can be studied without the interference from endogenous steroid hormones. The up-regulation of TTC9 by estradiol treatment might be associated with uterine cell growth and differentiation, as estradiol has been shown to increase the stromal and epithelial cell proliferation in immature mouse uterus (Quarmby and Korach, 1984).

It is not surprising to find that the effects of E₂ on TTC9 expression were opposite in breast cancer cell line MCF-7 and mice uterus. Other researchers have also observed the different effects of E₂ on protein expression in breast cancer cells and uterus. For example, Hyder et al and others have reported that E₂ was unable to induce vascular endothelial growth factor (VEGF) expression in MCF-7 or T47D cells (Bermont et al., 2001; Bogin and Degani, 2002; Hyder et al., 1998), while it was capable of up-regulating VEGF expression in rat uterus (Hyder et al., 1999). It could be that the effect of E₂ on TTC9 expression was cell- and tissue-type specific. Factors other than ER may also be involved in the estrogen response of TTC9 gene.

In summary, the regulation of TTC9 expression by estrogen and progesterone via their specific receptors in breast cancer cell lines, the over-expression of

TTC9 mRNA in human breast cancer tissues, the E₂-regulated TTC9 expression in mouse uterus and the involvement of TTC9 in growth factor signaling pathways, suggest that TTC9 is commonly regulated by steroid hormone and growth factors. The findings suggest that TTC9 may be involved in the mechanism of hormone signaling and breast cancer development.

4.2.5 Estrogen and growth factors regulate TTC9 expression through different pathways

Although both estrogen and growth factors regulate the level of TTC9 proteins and E₂ diminished the effect of serum factors, EGF and bFGF on the induction of TTC9 protein, the findings do not suggest a cross-talk between estrogen receptor and growth factor pathways. Firstly, it has been shown by various studies that E₂ can transiently activate MAPK signaling pathways (Migliaccio et al., 1996). However, in MCF-7 cells, E₂ and growth factors show contradictory effects on TTC9 expression and E₂ abolished the inductive effect of growth factors on TTC9 expression. Secondly, if the effect of growth factors is through cross-talk with estrogen receptor, antiestrogen should be able to block the signaling pathway and hence we expect to see an inhibition of growth factors' effect by ICI182780. On the contrary, we observed an additive effect when the cells were treated with both ICI 182780 and growth factors together

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compared with cells treated with growth factors alone. It is likely that the regulations by growth factors and estrogen on TTC9 protein level are mediated through separate pathways.

4.3 Identification of TTC9 domains involved in the interaction with Tm5NM-1

4.3.1 The first 50 amino acids of TTC9 is important for the interaction

The first 50 amino acids of TTC9 do not interact with Tm5NM-1, as shown by the result of co-immunoprecipitation. However, several lines of evidence suggest that this fragment is required for the interaction. Firstly, the truncated peptide TTC9 (51-222), shows much weaker interaction with Tm5NM-1, compared with full-length TTC9. In addition, although TTC9 (1-115) shows strong interaction with the partner protein, the removal of the first 50 amino acids almost abolish the interaction. As region containing the first 50 amino acids of TTC9 is located outside of the TPR domains, it is understandable that this region is not involved in the direct interaction with Tm5NM-1, or most probably, with other TTC9-interacting proteins. On the other hand, the requirement of this region for the binding of TTC9 to Tm5NM-1 indicates that although does not directly bind to Tm5NM-1, the first 50 amino acids could be necessary for the maintenance of TTC9 protein in a proper conformation, which facilitates the binding of TTC9 to other proteins.

4.3.2 The second and third TPR domains of TTC9 shows inhibitory effect on the interaction between TTC9 and Tm5NM-1

Results from co-immunoprecipitation studies revealed that the region containing amino acids 116 to 222, which includes the second and third TPR domains of TTC9, only had weak interaction with Tm5NM-1. This indicates that the second and third TPR domain may not required for the interaction. Moreover, results from this study also indicate this region might exert some negative effect on the binding of TTC9 protein to Tm5NM-1. This speculation is supported by two facts: the interaction between TTC9 (1-115) and Tm5NM-1 is stronger than that of full-length TTC9; and secondly, truncates TTC9 (1-167), which includes the first two TPR domains, and truncates (1-197), containing all the three TPR domains, shows weaker binding to Tm5NM-1 than TTC9 (1-128), which only contains the first TPR domain. However, the binding of TTC9 (51-222) to the partner is better than that of TTC9 (51-115). This may be due to the loss of the first 50 amino acids, which has been indicated to be involved in the maintenance of proper conformation of the protein.

It is not surprising that instead of assisting in binding, the second and third TPR domains actually exert inhibitory effect on the binding of TTC9 to Tm5NM-1, and probably to other TTC9-interacting proteins. As it is quite common that not

all the TPR domains in a protein are functional, the presence of these two TPR domains might form a stereological hindrance to the TTC9 interacting partners. Alternatively, instead of binding to Tm5NM-1 directly, the last two TPR motifs of TTC9 may provide a binding site for regulatory factors to mediate the interaction between these two proteins.

4.3.3 The first TPR domain and the linker region between the first two TPR domains are important for the interaction

Contrary to the second and third TPR domains, the complete first TPR domain is sufficient and necessary for the binding of TTC9 to Tm5NM-1. The first TPR domain is sufficient for the interaction as TTC9 (1-90), which only includes the first TPR domain, shows prominent interaction with Tm5NM-1, although the interaction is weaker than that of full-length TTC9. However, the truncate TTC9 (1-85) containing the incomplete first TPR domain, is incapable of binding to Tm5NM-1. Other truncates, which contain incomplete first TPR domain (TTC9 (1-50), TTC9 (1-70) and TTC9 (1-85)), also fail to interact with the partner protein. These findings suggest a complete first TPR domain is necessary for TTC9/Tm5NM-1 interaction.

Except for the first TPR domain, the linker segment between the first and second TPR domains also appears to facilitate the interaction. This is supported

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by the result that the binding between TTC9 (1-128) and Tm5NM-1 is stronger than that between TTC9 (1-90) and Tm5NM-1.

In summary, the results from the co-immunoprecipitation study revealed that the segment containing the N-terminal 128 amino acids of TTC9 is both necessary and sufficient for binding of TTC9 to Tm5NM-1, or probably to other TTC9 interacting partners. Although not involved in the direct binding of TTC to Tm5NM-1, the second and third TPR domains may participate in the determination of the specificity of TTC9 interaction proteins.

4.4 TTC9 and cytoskeleton organization

The higher expression of TTC9 in adherent cells compared with that in suspension cells gives the possibility for the involvement of TTC9 in the cell attachment process or the cytoskeleton system. Several factors, including the regulation of TTC9 expression by estrogen and progesterone in different breast cancer cell lines, the involvement of TTC9 in growth factor signaling pathways and the interaction between TTC9 and Tm5NM-1, may contribute individually or together to affect cell cytoskeleton.

From previous studies, Lin et al have reported in ABC28 cells, progesterone was capable of inhibiting cell growth, inducing remarkable changes in cell morphology and increasing cell attachment to extracellular proteins as well as cell migration through matrix protein-coated membranes (Lin et al., 2001; Lin et al., 2000; Lin et al., 1999b). These finding are accompanied by a dramatic increase of TTC9 expression. It is not clear whether the up-regulated TTC9 expression is specifically associated with progesterone-induced cell morphological changes in ABC28 cells. Furthermore, the decrease of TTC9 expression by specific siRNA does not show any obvious reversal effects on progesterone-induced changes in ABC28 cells (data not shown). We thus suspect the involvement of TTC9 in progesterone signaling pathway may be

indirect, which requires the cooperative functioning of TTC9 and other proteins.

Tm5NM-1 and other tropomyosin family members are well-known for their association with the cytoskeleton system. The multiple isoforms of tropomyosin present in all nonmuscle cells may be necessary for the cells to carry out fine regulation of cell shape and cell motility. Cells may perform the regulation by controlling the amounts of different tropomyosin isoforms expressed. For example, an elevated level of Tm5NM-1 has been found in high-metastatic mouse melanoma cells and transformed rat fibroblastic cells, which suggests a function of Tm5NM-1 in inhibiting the polymerization and/or the formation of the bundles of actin microfilaments (Miyado et al., 1996; Miyado et al., 1997). Also, reduced expression of HMW tropomyosins is very common in highly malignant cells (Franzen et al., 1996; Hughes et al., 2003; Leavitt et al., 1987; Wang et al., 1996). Alternatively, cells may distribute specific isoforms in a localized area to affect the performance of particular microfilament functions. Studies have shown LMW tropomyosin isoforms bind actin filaments more weakly than the HMW isoforms and are localized more abundantly in highly motile regions (peripheral ruffles) (Lin et al., 1988). Isoform-specific sorting is best characterized in neuron cells, with specific isoforms sorting to particular regions at different stages of neuronal maturation (Gunning et al., 1998; Had et

al., 1994; Hannan et al., 1998; Hannan et al., 1995; Schevzov et al., 1997; Weinberger et al., 1996). Another possibility is that the multiple isoforms of nonmuscle tropomyosin may play a role in modulating the organization of microfilaments in cells by regulating the interaction between actin and other actin-binding proteins, such as filamin, spectrin, caldesmon, gelsolin, DNase I (Hitchcock et al., 1976; Koteliansky et al., 1983; Maruyama and Ohashi, 1978; Nomura et al., 1987; Puskin et al., 1978). The interaction of TTC9 to Tm5NM-1 suggests TTC9 could also participate in the complex cytoskeleton regulation mechanism.

Except for ABC28 cells, studies have shown that in cells overexpressing the PR-A isoform, cells acquired rounded morphology and there was decreased adherence of cells to tissue culture flasks (McGowan and Clarke, 1999). Altogether, these findings suggest that the cell cytoskeleton is a target of progesterone action. As the actin-tropomyosin microfilament system is the most critical cytoskeletal system in transducing information from the cellular environment (Hall, 1998), the involvement of tropomyosins in the PR signaling pathways is highly expected. Evidence is provided by the hormone regulation on the relative levels of Tm5a and Tm5b in PR-A overexpressed T47D cells (McGowan et al., 2003). However, our preliminary results do not indicate any change in the expression level of Tm5NM-1. It is possible that in ABC28 cells,

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progesterone induces the expression of TTC9, which in turn binds to Tm5NM-1, regulates its activity and affect microfilament organization. It is also possible that TTC9 may be capable of binding to other members of tropomyosin family and regulates cytoskeleton system. Alternatively, instead of binding to tropomyosins directly, TTC9 could be involved in other signaling pathways, which also result in changes in the cytoskeleton organization. Future investigations, like disrupting the interaction between TTC9 and Tm5NM-1, are needed to explore the functional significance of the interaction.

As part of the metastatic process, tumor cells must escape the primary lesion, escape immune surveillance in the circulation, and penetrate and proliferate in tissues at distant sites (Fidler, 1978; Liotta, 1984). This process involves cell detachment, cell migration and cell attachment, all of which are closely related to the complex cytoskeleton. The obvious overexpression of TTC9 in breast cancer tissues compared with that in adjacent normal tissues suggest the possible function of TTC9 in cell transformation, migration and attachment.

In addition, TTC9 could also participate other aspects of cell functioning. In ABC28 cells, progesterone also induces cell differentiation with increased number of rough endoplasmic reticulum (Lin et al., 2003a). The localization of TTC9 to endoplasmic reticulum indicates some more possible functions of

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TTC9. TTC9 could be involved in the expression regulation of proteins downstream of PR signaling pathways, as endoplasmic reticulum is an important organelle for protein synthesis. TTC9 can also function in the differentiation process through the interaction with tropomyosins. It may also play a role in Golgi-to-endoplasmic reticulum retrograde trafficking, as one or more isoforms from Tm5NM gene are associated with Golgi-derived vesicles (Heimann et al., 1999). In general, although the exact function of TTC9 remains to be determined, findings in this study suggest TTC9 could be a chaperone protein participating in different cellular functions through its interaction with cell cytoskeleton system.

4.5 Conclusions

In this study, we identified and characterized TTC9 as a novel hormonally regulated protein in breast cancer cells.

Human TTC9 is a gene located at chromosome 14, and it shows high level of conservation during evolution. It is ubiquitously expressed in human, mouse and rat tissues. Northern blot analysis revealed that although human tissues appear to express three mRNA variants, breast cancer cells expressed only one mRNA transcript (2.5 Kb). Computer analysis predicted the protein size of TTC9 to be 222 aa, and this was confirmed by Western blotting with the mouse and rabbit polyclonal antibodies that were generated with purified TTC9 protein.

The TTC9 protein is predominantly concentrated to the endoplasmic reticulum, and its expression was regulated by a number of factors including steroid hormones, serum and growth factors. ERK pathway appears to be involved in growth factor-mediated regulation of TTC9 expression. In human, TTC9 is expressed at a higher level in breast cancer tissues compared with adjacent normal tissues. In mouse uterus, the expression of TTC9 is up-regulated by E₂ treatment. We propose that TTC9 may play an important role in steroid hormone and growth factor signaling in breast cancer. Since breast cancer

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growth is critically regulated by steroid hormones and growth factors (Fabian and Kimler, 2005; Nicholson et al., 1999), elucidating the function of TTC9 may lead to the discovery of novel mechanisms that govern the regulation of breast cancer development and treatment.

In the effort to look for TTC9 interacting proteins, yeast-two-hybrid assay identified Tm5NM-1 as one of the candidate. The interaction was further confirmed by GST-pull down and co-immunoprecipitation. Domain analysis revealed that it is the first TPR domain of TTC9 and the linker peptide between the first two TPR domains that is important for the interaction. As Tm5NM-1 is known to be associated with stress fibers (Percival et al., 2004) and progesterone have been shown to dramatically up-regulate TTC9 expression level as well as to increase cell stress fibers (Cao et al., 2006; Lin et al., 2000), the interaction between TTC9 and Tm5NM-1 could be involved in the process of progesterone induced cell spreading and cell adhesion. The higher expression of TTC9 in adherent cells compared with that in suspension cells supports this hypothesis to some extent. In summary, although the exact function of TTC9 remains unknown at current stage, the existing findings support the idea that TTC9 might act as a chaperone protein to facilitate the function of tropomyosins in stabilizing microfilament and could play a role in cancer cell invasion and metastasis.

4.6 Future studies

In the present study, we identified and characterized TTC9 protein and indicated its possible function. More studies can be done for further characterization of the protein function.

In ABC28 cells, the up-regulation effect of progesterone on TTC9 expression appears as early as 8 hr after treatment, which gave rise to the possibility that TTC9 could be a direct gene target of progesterone signaling. This possibility could be confirmed by identifying PRE in the gene's promoter. The promoter analysis of TTC9 gene is currently being conducted. Being a direct target gene of PR further supports the idea that TTC9 plays an important role in progesterone signaling pathways.

Furthermore, it is not clear whether TTC9 is regulated by PR-A or PR-B or both in ABC28 cells. Exploring the individual effects of PR-A and PR-B on TTC9 gene expression will provide information on how these two receptors function differently.

Although TTC9 is regulated by different steroid hormones *in vitro* and *in vivo*, the significance of this involvement is not well understood. Elucidating the role TTC9 plays in the hormone signaling pathways will help us better understand

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the mechanism of hormone function and the relationship between different signaling pathways.

Although Tm5NM-1 has been identified as one of TTC9 interacting proteins, the significance of the interaction remains unknown. Understanding what TTC9 does when binding to Tm5NM-1 could reveal how TTC9 is involved in cytoskeleton organization, cell attachment, cell morphology and other aspects of cells.

The functions of TTC9 *in vivo* could also be revealed by TTC9 knock-out mice. TTC9 is expressed ubiquitously among human, mice and rats tissues. Other results from our lab also showed that TTC9 was already expressed at a high level during embryonic stages. These findings support the idea that TTC9 could be an important protein functioning at different stages of development.

TTC9 is a newly discovered protein and till now we only has predicted secondary structure of the protein. Analyzing its crystal structure will provide valuable information on the prediction of TTC9 function and designing of its specific inhibitors.

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APPENDICES

RECIPE OF SOLUTIONS

1×PBS (pH 7.4)

NaCl	9.00 g/L
Na ₂ HPO ₄ ·7H ₂ O	0.795 g/L
KH ₂ PO ₄	0.144 g/L
ddH ₂ O	

1×DPBS (pH 7.4)

KCl	0.20 g/L
NaCl	8.00 g/L
KH ₂ PO ₄	0.20 g/L
Na ₂ HPO ₄	1.15 g/L
ddH ₂ O	

Cell lysis buffer

NaF	100 mM
HEPES (pH 7.5)	50 mM
NaCl	150 mM
PMSF	1 mM
Na ₃ VO ₄	1 mM
pepstatin A	5 µg/ml
leupeptin	5 µg/ml

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aprotinin 2 µg/ml

Triton X-100 1%

ddH₂O

2×SDS-PAGE sample buffer

SDS 4% (w/v)

Glycerol 20% (v/v)

Tris·Cl (pH 6.8) 0.125 M

β-mercaptoethanol 10% (v/v)

bromophenol blue 0.04% (w/v)

ddH₂O

5×SDS-PAGE sample buffer

SDS 10% (w/v)

Glycerol 50% (v/v)

Tris·Cl (pH 6.8) 0.25 M

β-mercaptoethanol 25% (v/v)

bromophenol blue 0.1% (w/v)

ddH₂O

1×running buffer for immunoblotting

Tris base 6 g/L

Glycine 28.8 g/L

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SDS 1 g/L

ddH₂O

1×transferring buffer for immunoblotting

Glycine 14.41 g/L

Tris base 3.03 g/L

Methanol 10% (v/v)

ddH₂O

TBST

Tris·Cl 10 mM

NaCl 100 mM

TWEEN 20 0.1%

ddH₂O

Stripping buffer for immunoblotting

Tris·Cl (pH 6.8) 62.5 mM

SDS 2 %

β-mercaptoethanol 0.8%

1×TBE buffer (pH 8.35)

Tris base 90 mM

Boric acid 90 mM

EDTA 2 mM

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ddH₂O

ELISA blocking buffer

BSA	1% (w/v)
TWEEN 20	0.3% (v/v)
NaN ₃	0.05% (w/v)
PBS	

ELISA washing buffer

TWEEN 20	0.025% (v/v)
PBS	

Diethanolamine buffer

Diethanolamine	4.85% (v/v)
NaN ₃	0.01% (w/v)
MgCl ₂ ·6H ₂ O	0.04% (w/v)
ddH ₂ O	

Lysis buffer for cell fractionation

sorbitol	1 M
HEPES (pH7.4)	10 mM
EDTA	1 mM
ddH ₂ O	

Resuspension buffer (Ni-NTA agarose column)

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Tris·Cl (pH 7.4)	20 mM
NaCl	0.5 M
ddH ₂ O	

Wash buffer (Ni-NTA agarose column)

Tris·Cl (pH 7.4)	20 mM
NaCl	1 M
Imidazole (pH 6.0)	20 mM
β-mercaptoethanol	3 mM
ddH ₂ O	

Elution buffer (Ni-NTA agarose column)

Tris·Cl (pH 7.4)	20 mM
NaCl	0.5 M
Imidazole (pH 6.0)	0.5 M
β-mercaptoethanol	3 mM
ddH ₂ O	

Buffer A

Tris·Cl (pH 7.0)	20 mM
β-mercaptoethanol	3 mM
ddH ₂ O	

Buffer B

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Tris·Cl (pH 7.0)	20 mM
NaCl	1 M
β-mercaptoethanol	3 mM
ddH ₂ O	

Glutathione elution buffer

reduced glutathione	0.308% (w/v) 0.154 g
Tris·Cl (pH 8.0)	50 mM
ddH ₂ O	

TBS

Tris·Cl	10 mM
NaCl	100 mM
ddH ₂ O	

S-buffer

KHPO ₄ (pH 7.2)	10 mM
EDTA	10 mM
β-mercaptoethanol	50 mM
zymolyase	1.5 mg/ml
ddH ₂ O	

Lysis solution for yeast

Tris·Cl (pH 7.5)	0.25 M
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EDTA	25 mM
SDS	2.5% (w/v)
ddH ₂ O

Fig. A.1 Melting curves for TTC9 and 36B4 genes.

Melting curves (dissociation curves) for both TTC9 and 36B4 genes were generated after real-time PCR amplification. One peak represents for one single, specific product for each gene. The left peak is the melting curve for TTC9 gene, while the right peak is the one for 36B4 gene.

Fig. A.2 Maps of plasmids used in yeast-two-hybrid assay.