EXPRESSION OF NOTCH SIGNALING PATHWAY RECEPTORS AND LIGANDS IN HUMAN BREAST CANCER CELL LINES AND HUMAN BREAST TUMORS

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FOR THE DEGREE OF

MASTER OF SCIENCE

BY

ÖZDEN YALÇIN

SEPTEMBER, 2004

TO MY FAMILY

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Dr. Mehmet Öztürk

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ABSTRACT

Expression of Notch Signaling Pathway Receptors and Ligands in Human Breast Cancer Cell Lines and Human Breast Tumors

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M.Sc. in Molecular Biology and Genetics

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Breast cancer is the most common cancer type in women. Traditional therapies targeting proliferating cells cannot be effective in all cases and recursion is observed in 40% of breast cancers within 10 years. One possible explanation is that the origin of breast cancer is 'breast cancer stem cells', which cannot be killed by these therapies. Cancer stem cells are thought to be formed due to deregulation of normal stem cells. Breast tissue also contains normal stem cells required for its development during puberty and pregnancy; and putative breast cancer stem cells have recently been isolated. Investigation of pathways used in stem cell regulation is the first step to understand the contribution of stem cells to tumorgenesis and design new therapeutic approaches. Notch signaling is involved in stem cell maintenance and many types of human cancers. Notch activation in mouse mammary gland development and tumorigenesis lead us to its possible role in human mammary gland tumorigenesis. The expression of Notch receptors and ligands were identified by semi-quantitative RT-PCR in human breast cancer cell lines and tumor samples. It was found that Notch3 expression was strongly upregulated in cancer cells lines and tumors compared to normal cell line, while other receptors and ligands did not have significant changes in expression. Depending on the upregulation of Notch3 expression in putative breast stem cells, we may hypothesize that its activation keeps cells in a stem cell like phenotype, inhibit differentiation and increase cancer risk.

ÖZET

İnsan Meme Kanseri Hücre Hatlarında ve Meme Tümörlerinde Notch Yolağı Reseptör ve Ligandlarının Ekspresyonu

Özden Yalçın

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Bölümü

Tez Yöneticisi: Prof. Dr. Mehmet ÖZTÜRK

Eylül 2004, 69 sayfa

Meme kanseri kadınlar arasında en sık görülen kanser türüdür. Çoğalan hücreleri hedefleyen geleneksel tedavi yöntemleri her durumda etkili olamamakta ve meme kanserlerinin %40'ı 10 yıl içinde tekrarlamaktadır. Meme kanserinin temelinde bu tedavi yöntemleriyle yok edilemeyen 'meme kanseri kök hücreleri'nin olması bu duruma bir açıklama getirebilir. Kanser kök hücrelerinin, normal kök hücrelerin kontrolünün bozulması sonucunda ortaya çıktığı düşünülmektedir. Meme dokusu, ergenlik ve hamilelik sırasındaki gelişimi için gereken normal kök hücreler içermektedir, ve muhtemel meme kanseri kök hücreleri de yakın zaman önce ayrıştırılmıştır. Kök hücre kontrolünde görev yapan yolakların araştırılması, kök hücrelerin tümör oluşumuna katılımını anlamak ve yeni tedavi yöntemleri geliştirmek için gereken ilk adım olmalıdır. Notch yolağı, kök hücrelerin devamlılığında ve insanlarda bazı kanser türlerinde önemli bir role sahiptir. Farede meme dokusu gelişiminde ve tümör oluşumundaki aktivasyonu, Notch yolağının insanda da meme kanserinde bir rolü olabileceği sonucuna ulaştırmıştır. İnsan meme kanseri hücre hatları ve meme tümörlerinde Notch reseptör ve ligandlarının ekspresyonu yarı-nicel RT-PCR yöntemi ile belirlenmiştir. Diğer reseptör ve ligandların ekspresyonunda anlamlı bir değişiklik gözlenemezken, Notch3 ekspresyonunun, meme kanseri hücre hatları ve tümörlerinde normal hücre hattına göre arttığı saptanmıştır. Muhtemel meme kök höcrelerinde ekspresyonunun arttığı

göz önünde bulundurulursa, Notch3 aktivasyonunun hücreleri kök hücre fenotipinde tutup farklılaşmayı önleyerek kanser riskini artırdığı hipotezini ortaya koyabiliriz.

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ABBREVIATIONS

Bp	Base Pairs
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
DCIS	Ductal Carcinoma In Situ
ddH_2O	Double Distilled Water
dH_2O	Distilled Water
DEPC	Diethylpyrocarbonate
Dll	Delta-like
DLLC	Undifferentiated Large Light Cells
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ESA	Epithelial specific Antigen
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Caw Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HES	Hairy/Enhancer of Split
HPV	Human Papillomavirus
HSC	Haematopoietic Stem Cells
hTERT	Human Telomerase Reverse Transcriptase
ICM	Inner Cell Mass

IDC	Infiltrating (Invasive) Ductal Carcinoma
ILC	Infiltrating (Invasive) Lobular Carcinoma
LCIS	Lobular Carcinoma In Situ
LDC	Large Dark Cell
LIF	Leukemia Inhibitory Factor
LOH	Loss of Heterozygosity
LRC	Label Retaining Cells
LTR	Long Terminal Repeat
MAML	Mastermind-like protein
MMTV	Mouse Mammary Tumor Virus
NICD	Notch Intracellular Domain
NLS	Nuclear Localization Signal
NSCLC	Non-Small Cell Lung Cancer
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3-Kinase
PR	Progesterone Receptor
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCLC	Small Cell Lung Cancer
SLC	Small Light Cell
SP	Side Population
TACE	TNF-α Converting Enzyme
T-ALL	T-cell Acute Lymphoblastic Leukemia
TCRβ	T-cell receptor-β
TDLU	Terminal Duct Lobular Unit
TGF-β	Transforming Growth Factor-β
TEB	Terminal End Buds

T_m	Annealing Temperature
ULLC	Undifferentiated Large Light Cells
WAP	Whey Acidic Protein

CHAPTER 1 INTRODUCTION

The expression patterns of Notch receptors and ligands in breast cancer cell lines and human breast tumors are presented in this study. Before introducing the project, the literature will be reviewed in order to focus on some of the basic concepts about breast cancer and Notch signaling. First, development of breast and then breast cancer will be reviewed. Next, the current studies related to stem cells and cancer will be summarized. Then, specifically breast stem cells will be presented. Finally, Notch signaling and its relation to breast cancer will be introduced.

1.1 Development of Breast

The four main structures in an adult human breast are lobules, ducts, fat and connective tissue (Figure 1.1). “Nipple” surrounded by the dark tissue “areola”, is the protruding point of the breast. “Lobes” are composed of lobules or glands, which are the milk producing parts during lactation. “Ducts” are the branching tubes that are connected to lobes, and converge to the larger “collecting ducts” towards the nipple. Ducts are responsible for carrying the milk from lobes to nipple during lactation. The branching ductal system and alveoli are supported by stroma, which is consists of fat and connective tissue, blood and lymphatic vessels (www.mammary.nih.gov[a]).

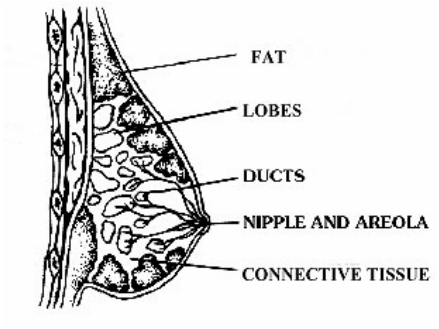

Figure 1.1 Structures of human breast. (www.mammary.nih.gov[a])

Breast tissue, in contrast to most mammalian organs, is found at a rudimentary state after birth, and completes its maturation during puberty. In human embryo, breast begins to develop at around weeks 7 to 8, and visible structures are observed at around weeks 12 to 16 (www.mammary.nih.gov[a]). The breast tissue is originated from ectoderm. First detectable structure called milk line, mammary crest or mammary band is a thickening extending across the embryo in a region, where breast bud will form. The first nipple is observed when the embryo is around 7-8mm length, it is seen as a narrow collection of ectodermal cells. Around 10mm length, the cells have a close relation with mesenchyme. Actually, the mesenchymal-epithelial interactions direct the breast development in embryo. Around day 14, following the differentiation of mesenchyme, epithelia proliferate to form a nodule that pushes mesencyme. The nodule then forms the breast bud. The bud starts to branch and form secondary buds with a clover-shape. Then secondary buds form canals and branch. The ductal morphogenesis in embryo is achieved by elongation and invasion of branches into the mesenchyme. Both female and male breast tissue shows the same development in embryo (Howard and Gusterson, 2000).

At birth, ductal system opens to the surface through a cavity on skin. The underlying mesenchyme proliferates to form nipple, and skin surrounding the nipple proliferates to form areola. The stage of breast development at birth differs from individual to individual. Some babies may have only small blunt ended tubular structures, while others may have well-developed branches. Both male and female babies may secrete some milk in response to maternal hormones. And during first

two years the lining epithelium proliferates and then involutes upon removal of the maternal hormones` effects. At the end of two years, only small ductal structures remain in a fibroblastic stroma and this state is kept until puberty (Howard and Gusterson, 2000).

Upon hormone stimulation with the beginning of the puberty, the breast structure further develops. The anatomical changes at this stage is well established, however the cellular events are not well understood. During this stage, in addition to epithelium, stroma also changes. Fibrous and fatty tissue increases that in a non-lactating breast it constitutes around 80% of the tissue. Hormones stimulate the growth of ducts, invasion into the fat pads and formation of lobular structures. The growing part of the tissue is “terminal end buds” (TEB), which consist of “body cell” mass surrounded by a layer of “cap cells”. Body cells are thought to form inner “luminal epithelial cells”, which lines the lumen. Cap cells are thought to form the outer “myoepithelial cells”, which are contractile cells found around the secretory alveoli cells and ducts; and cause squeezing the milk down to the ducts and then out of nipple upon oxytocin stimulation. Upon reaching the borders of fat pad, branching is completed and TEBs disappear (Howard and Gusterson, 2000; Smalley and Ashworth, 2003).

Human breast during pregnancy and lactation could not be studied well. In general, an increase in the number of lobules and loss of fat is observed. Lobulo-acinar structures, which have milk-secreting alveolar cells, are formed due to high proliferation followed by terminal differentiation. At weaning, removal of suckling stimulus results in involution, which means elimination of secretory epithelial cells by apoptosis and phagocytosis. At each pregnancy Terminal Duct Lobular Units (TDLUs) expand in size and then involutes. The ducts are not changed during this process. At menopause, a greater involution occurs for both lobules and ducts. The removed epithelium and interlobular connective tissue are replaced by fat (Howard and Gusterson, 2000).

It is not easy to study breast in humans. The similarities between mouse and human mammary gland development and function make mouse a good model. We have more detailed information about these processes in mice and that help us to

understand the mammary gland better. The figure below shows the dramatic changes of mouse mammary gland at different stages of development (Figure 1.2). Similar to development in human, mouse mammary gland development begins with thickening of epithelial cells and running from anterior to posterior direction in the embryo. The mammary bud forms and invades the mesenchyme, then encounters secondary mesenchyme, which is the precursor of mammary fat pad. The interactions with the mesenchyme induce a small amount of branching and form a rudimentary gland that remains unchanged till puberty. At puberty, hormones stimulate rapid elongation and branching by TEB similar to human. The ductal elongation ceases when the ducts reach to the periphery of the fat pad (Figure 1.2.c and 1.2.d), and the gland remain at this stage until pregnancy. The process from embryo to adult virgin animal can be called as "linear phase" of the mammary gland development (Lewis, 2000).

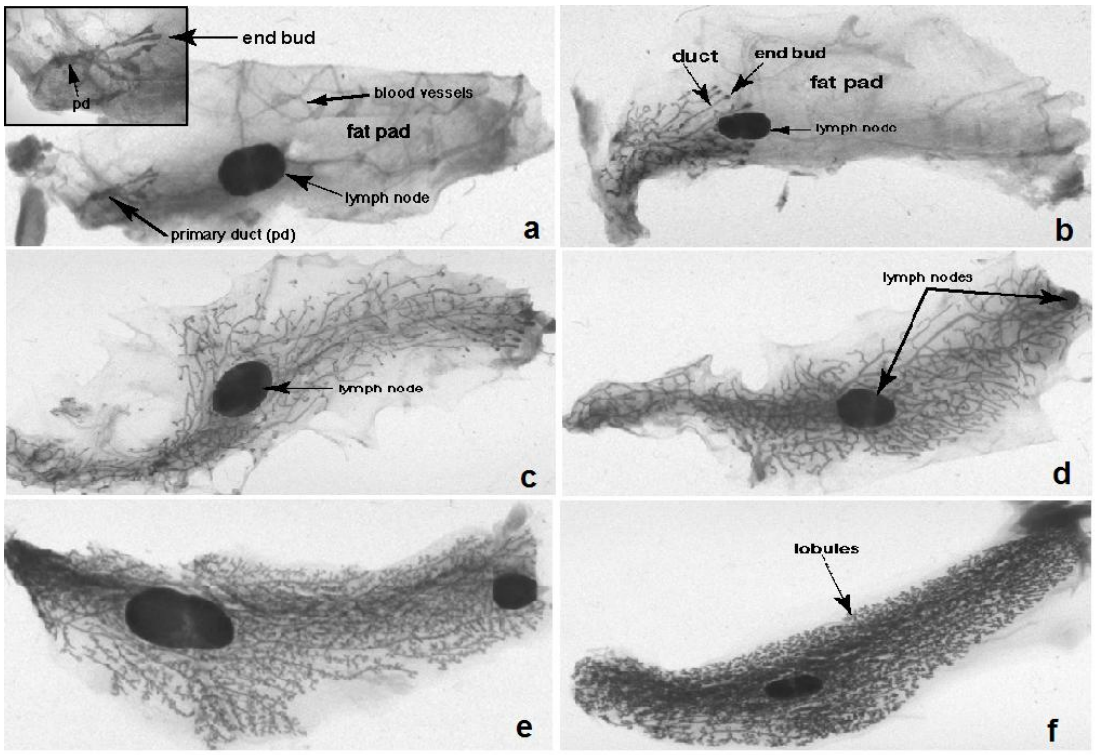

Figure 1.2 Stages of mouse mammary gland development. a. 3 weeks old mouse with slowly elongating and branching ducts. **b.** 4 weeks old virgin mouse with significantly increased growth of ducts around lymph node. **c.** 6 weeks old virgin mouse has ducts that reached to the periphery of mammary fat pad. **d.** 10 weeks old virgin mouse has ducts that completely reached to the periphery of mammary fat pad.

The ductal development decreases at that time. **e.** Day 9 pregnant mouse with an extensive and rapid proliferation of mammary ducts. **f.** Day 16 pregnant mouse has an expanded mammary epithelium (www.mammary.nih.gov[b]).

Pregnancy initiates the "cyclical phase" of the development, which is repeated in each pregnancy. The morphology of breast changes from ductal to lobuloalveolar structure (Figure 1.3). The progenitor cells proliferate and differentiate to form the alveolar buds and then alveoli. Alveolar cells gain capacity to express milk proteins at the middle of the pregnancy but secretion is inhibited until delivery. Weaning terminates the milk secretion and involution occurs by apoptosis. The gland returns to ductal morphology until next pregnancy, in which the same cycle is repeated (Lewis, 2000).

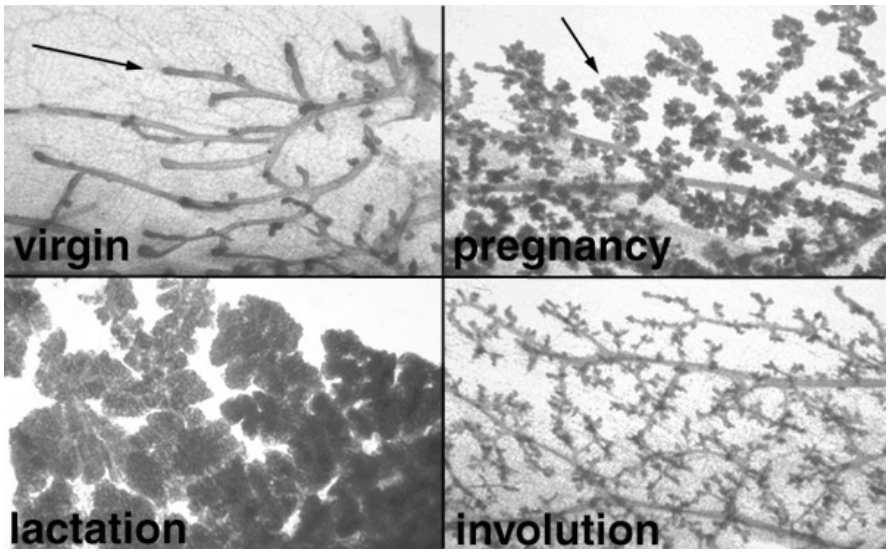

Figure 1.3 Cyclical phase of mouse mammary gland development. The predominant ductal and lobuloalveolar morphology are seen in virgin and pregnant mouse respectively. The extensively condensed milk secreting lobuloalveolar cells in lactating mouse collapsed during involution by apoptosis (www.mammary.nih.gov[b]).

1.2 Breast Cancer

1.2.1 Epidemiology of Breast Cancer

Cancer is the second leading cause of death in U.S., and constitutes for the one fourth of all deaths. In 2004, 1,363,030 new diagnoses and 563,700 cancer deaths are expected to occur in U.S. Breast cancer is one of the important cancer types that it is the most frequently diagnosed non-skin cancer in women. 215,990 new invasive breast cancer cases, which is 32% of total, are expected to be diagnosed in women, in 2004. Breast cancer is the second for cancer deaths in women after lung cancer in U.S. 40,110 women, which is 15% of total, are expected to die because of breast cancer in 2004. In addition to invasive breast cancer, 59,390 women are expected to be diagnosed with in situ breast cancer. The number of males affected from breast cancer is not very low that the expected new diagnosis of and deaths from breast cancer is 1,450 and 470 respectively in males, in 2004. According to World Health Organization, 1.2 million people will be diagnosed with breast cancer worldwide (www.imaginis.com, American Cancer Society, 2004).

In Turkey, the facts about breast cancer are similar to U.S. According to 1999 statistics of Turkish Ministry of Health, breast cancer is the most common cancer followed by stomach cancer in Turkish women with 2,390 cases that makes 24.1% of all cancers in women.

According to statistics, breast cancer risk increases by age, 94% of new diagnosed cases and 96% of deaths occur after age 40 (American Cancer Society, 2003-2004). The menstrual history is also important that early onset and long period of menstruation, and menopause at older ages increases breast cancer risk. Although non-proliferative benign breast diseases has a very little or no effect on cancer risk, proliferative lesions without atypia and atypical hyperplasia increase breast cancer risk about two or four fold, respectively. Alcohol consumption is directly proportional to breast cancer risk that 10gr of alcohol per day increases the risk by 10% (Smith-Warner *et al*., 1998). Ionizing radiation and personal or familial history of breast cancer definitely increases breast cancer risk (Key *et al*., 2001).

Breast cancer risk increases immediately after the birth; however in long term childbearing has a protective effect. In addition, the high number of pregnancies and having the first child at an earlier age decreases risk significantly. The risk for the women with five or more children is half of the risk for the women without any child (Layde *et al*., 1989; Ewertz *et al*., 1990). The younger age at first birth has a protective effect that the women, who had first child earlier than age 20 has 30% lower risk compared to women, who had after 35 (Ewertz *et al*., 1990). Breastfeeding, experiencing menopause earlier, low-fat diet, a moderate physical activity and a healthy body weight are other factors decreasing the breast cancer risk (Key *et al*., 2001; American Cancer Society, 2004).

1.2.2 Histology of Breast Cancer

Most of tumors detected in breast are benign that they do not spread and become life threatening (American Cancer Society, 2003-2004). Breast carcinomas are originated from the malignant transformation of ductal or lobular epithelial cells. Normal epithelial cells may progressively develop ductal hyperplasia, atypical ductal hyperplasia, ductal carcinoma in situ, invasive ductal carcinoma and then metastasis, or they may directly form in situ or invasive type of breast cancer (Vogelstein and Kinzler, 1998; Russo *et al*., 1998).

In situ type of breast cancer indicates the early stages of breast cancer, these tumors are cancerous but they do not spread beyond the regions they originated and almost all cases can be cured.

1.2.2.1 Ductal Carcinoma in Situ

Ductal carcinoma in situ (DCIS) is the most common noninvasive breast cancer that constitutes 85% of all in situ breast cancers. The increased rate of incidence is due to early detection by mammography screening. The epithelial cells of ducts form the tumor but the tumor does not spread beyond the duct walls through the surrounding stroma (American Cancer Society, 2003-2004; www.cancer.org[a]).

DCIS is thought to be the precursor of invasive breast carcinoma. After removal of the DCIS lesions, 30-50% of patients developed invasive breast cancer in

6-10 years at the same site of previous DCIS (Page et *al*., 1982; *Rosen et al*., 1980). Basically DCIS is subdivided into two classes called comedo and non-comedo. Comedo type includes dead cells, and necrosis inside the tumor, and it is more malignant that precedes invasion (DeVita *et al*., 2001). Comedo type DCIS lesions usually lack estrogen and progesterone receptors (Albonico *et al*., 1998), overexpress oncogene c-erbB-2 and have mutated p53 accumulation (Poller *et al*., 1993; O'Malley *et al*., 1994). Angiogenesis is observed in the stroma surrounding the comedo DCIS lesions (Engels *et al*., 1997).

1.2.2.2 Lobular Carcinoma in Situ

Lobular carcinoma in situ (LCIS) is a less common type of noninvasive in situ breast cancers. It is originated from the lobules but not spread beyond them (American Cancer Society, 2003-2004; www.cancer.org[a]).

LCIS cannot be detected by physical examination or mammography; it is usually detected in the tissues removed for other reasons. LCIS is a homogeneous mass consist of small cells containing small and rounded-oval nuclei. The most characteristic pattern of LCIS is the loss of e-cadherin protein (Vos *et al*., 1997). The women, who had LCIS have a 7-10 times more risk for developing breast carcinoma. (Vogelstein and Kinzler, 1998; DeVita *et al*., 2001).

1.2.2.3 Invasive Breast Cancer

Infiltrating (invasive) breast cancer starts in lobules or ducts and invades the surrounding tissue. There are three stages of invasive breast cancer; in local stage tumor is fixed to the breast, in regional stage tumor spread to surrounding tissue or nearby lymph nodes, and in distant stage tumor metastasized to distant organs (American Cancer Society, 2003-2004; www.cancer.org[a]).

'Infiltrating (invasive) ductal carcinoma' (IDC) is the most common type of breast cancer, covering around 80% of all invasive breast cancers. It is originated in the ducts and spread beyond the duct wall invading the fatty tissue. 'Infiltrating (invasive) lobular carcinoma' (ILC) is originated from lobules and it is a less common type. Both invasive carcinomas can metastasize to other parts of body via

blood and lymphatic vessels. The other rare types of invasive breast cancer includes 'tubular carcinoma', in which tubules are formed; 'medullary carcinoma' consists of poorly differentiated and large cells, and have immune system cells around the tumor tissue; 'mucinous (colloid) carcinoma', which is characterized by the accumulation of extracellular mucus. The prognoses for these three types are better than IDC and ILC. Another rare type of invasive breast cancer is 'inflammatory breast cancer', which results in a red and pitted appearance of breast due to the blocking of lymphatic vessels or channels in the skin (Vogelstein and Kinzler, 1998; www.cancer.org[a]).

The prognosis and treatment strategy are directly related to the stage of the invasive breast cancer. The most commonly used staging system is the TNM system stated by American Joint Committee on Cancer. The three main criteria are the size and spreading in breast or nearby organs indicated by 'T', spreading to the lymph nodes indicated by 'N', and metastasis indicated by 'M'. The evaluation of T, N and M stages gives the general stage of patient from stage I (the least advanced) to the stage IV (the worst case) (www.cancer.org[b]) .

Several therapies including surgery, radiation therapy, chemotherapy, hormone or antibody therapy are applied to breast cancer in combination. Depending on the tumor type surgery can be applied in different ways. Lumpectomy is the local removal of tumor and a part of surrounding tissue. Mastectomy is removal of entire breast, and modified radical mastectomy is the removal of lymph node under arm in addition to entire breast. Surgery is usually followed by radiation therapy or chemotherapy together with hormone therapy, in order to destroy undetectable remaining tumor cells. Tamoxifen is one of the commonly used anti-estrogen drugs. Also some drugs interfering with estrogen synthesizing enzyme are used. These therapies can also be applied before surgery to reduce the size of tumor. (American Cancer Society, 2003-2004).

1.2.3 Molecular Biology of Breast Cancer

1.2.3.1 Germ Line Mutations in Breast Cancer

BRCA-1 and BRCA-2 are breast cancer susceptibility genes, which function as tumor suppressors and involved in the repair of damaged DNA in normal cells. BRCA1 and BRCA2 mutations constitute 5% of all breast cancer cases and they are associated with hereditary breast cancer (American Cancer Society, 2004). Female carriers of BRCA1 mutation have 87% life time risk for developing breast cancer and 40-60% for ovarian cancer. Almost all mutations of this gene are germ line mutations, and depending on the rare somatic mutations we can conclude that BRCA-1 mutations do not play a major role in sporadic breast cancers in contrast to hereditary cases. However, in sporadic cancer cell lines abnormal cytoplasmic localization that it is found in nucleus in normal cells and decreased expression during progression of sporadic breast cancers are observed (Chen *et al*. 1995; Thompson *et al*., 1995). Similar to BRCA-1, BRCA-2 is not thought to have a role in sporadic breast cancer and female carriers of BRCA-2 mutations have 85% life time risk for developing breast cancer but the risk for ovarian cancer is lower (10-20%).(Vogelstein and Kinzler, 1998; DeVita *et al*., 2001).

Other germ line mutations causing breast cancer are also associated with other tumor types and diseases. Li-Fraumeni syndrome is a result of germ line mutation in TP53 gene, which in addition to breast cancer may give rise to other types of tumor like soft-tissue sarcomas, brain tumor, and leukemia. Cowden disease, increases the risk of breast cancer in addition to several different features, and carries PTEN mutation. MLH1, MSH2 are mutated in Muir-Torre syndrome, which increases the breast cancer risk and associated with skin and upper and lower gastrointestinal tumors. Ataxia-telangiectasia is another disease associated to breast cancer risk but lower than other diseases, with non-Hodgkin Lymphoma, ovarian cancer, stomach, pancreas and bladder malignancies. Mutations in ATM gene are found in Ataxia-telangiectasia (Vogelstein and Kinzler, 1998; DeVita *et al*., 2001).

1.2.3.2 Somatic Mutations in Breast Cancer

Loss of heterozygosity (LOH) in cancers is usually involved in tumor suppressor genes. In breast cancer LOH is observed in some important locus including the genes RB-1, CDKN2, CDH1, and TP53. RB-1 and CDKN2 encode retinioblastoma and p16 proteins respectively, which are involved in cell cycle regulation. CDH1 encodes E-cadherin, which regulates differentiation and tissue compartmentalization. Point mutations for TP53 and RB-1 are more common, while methylation is mostly observed in CDKN2 and CDH1. TP53 mutations are analyzed in several groups of breast cancer samples and results showed that TP53 mutations are associated with poor prognosis. Cyclin D1, another important protein involved in cell cycle regulation. It was overexpressed in some of the breast cancer cell lines and in 45% of breast tumors in a study (Buckley *et al*., 1993). Increase in cyclin D1 expression is thought to be an early event in breast cancer development. One of the most common gene amplifications in breast cancer occur in c-MYC gene, which is a protooncogene, involved in cell proliferation or apoptosis depending on the cellular context. However the detection of c-Myc at protein level is harder because of short half-life of protein (Vogelstein and Kinzler, 1998; DeVita *et al*., 2001).

Epidermal growth factor receptors (EGFR), including EGFR, erbB-2 or HER-2/neu, erbB-3, and erbB-4, behave like oncogenes in breast cancer due to gene amplifications or overexpression, and results in uncontrolled cell proliferation (Bacus *et al.,*1994; Vogelstein and Kinzler, 1998).

Fibroblast growth factors (FGF) are another group of growth factors involved in breast cancer. FGF-3 (mouse int-2), is one of the genes activated by the insertion of mouse mammary tumor virus (MMTV), and become oncogenic (Coleman-Krnacik and Rosen, 1994; DeVita *et al*., 2001).

Estrogen and progesterone are important in mammary gland development by regulating epithelial growth, differentiation and survival. In addition, these hormones are involved in the development of breast cancer. Estrogen receptor (ER) and progesterone receptor (PR), through which the hormones act, have an association with the prognosis and response to the antihormonal therapy of the patient. Estrogen

and progesterone induces the expression of protooncogenes c-MYC, c-FOS, c-JUN. In normal tissue, ER and PR are expressed in luminal epithelial cells and dividing cells are negative for both receptor. However, most of the breast tumors are steroid receptors positive (DeVita *et al*., 2001; Clarke *et al*., 2003).

1.3 Stem Cells and Cancer

1.3.1 Overview of Stem Cells

The cells, which have the ability to self-renew and to produce daughter cells that will differentiate into different cell types, are called 'stem cells'. Stem cells are classified according to their capacities. The fertilized oocyte and its first daughter cells have the capacity to generate whole embryo and the surrounding tissue of placenta, and called totipotent stem cells. Throughout the embryonic development blastocyst and inner cell mass (ICM) is formed. The ICM cells are pluripotent stem cells that can from the all cell types of three germ layers, but not supporting tissue and a complete embryo. Adult tissues are thought to have multipotential stem cells (Alison *et al*., 2002). Repair and regeneration are essential in adult tissues, and require a stock of cells, the multipotential stem cells, which have an increased lifespan, and capacity to generate necessary tissue specific cells. It is easier to detect stem cells in tissues with high cell turn over like skin, intestine and bone-marrow. In some tissues stem cells are required for regeneration in response to tissue injury like in liver, and it is harder to identify these stem cells because they are hidden and activated only after an injury or pathologic condition (Nagy, 1995; Presnell *et al*., 2002). The last group is unipotential stem cells, which can only generate one type of differentiated cell, and sometimes considered as committed progenitor cells, because of their limited proliferation and differentiation capacity (Alison *et al*., 2002).

The intermediate cells between the stem cells and the terminally differentiated cells are called "progenitor cells", "transit cells" or "transit amplifying cells". A stem cell can generate one stem cell (self-renewal property) and one progenitor cell (differentiating daughter cell producing property) by asymmetric division, or can generate two identical types of cells by symmetric division. The asymmetric division conserves the stem cell compartment, while a symmetric

division generating two stem cells cause expansion or symmetric division generating two progenitor cells cause depletion of the stem cell compartment (Smalley and Ashworth, 2003).

Stem cells are undifferentiated and usually do not function like their progeny. They are thought to be found in very small numbers in the tissues, like 1 in 10,000 in haematopoietic tissue, and have a slow rate of proliferation compared to progenitor cells (Alison *et al*., 2002).

The stem cells are thought to be found in a specific part of the tissue, 'stem cell niche', which include extracellular matrix (ECM) and supporting cells with specific signaling functions. The base of the small intestine is an example for the stem cell niche. It was shown that in the case of stem cell depletion, the stem cell niche have the capacity to induce the formation of stem cells or can induce the progenitor cells to behave like stem cells (Smalley and Ashworth, 2003).

Stem cells should keep a balance between self-renewal and differentiation. Uncontrolled self-renewal will cause problems like tumor formation. It is crucial to understand pathways behind the decision of two fates in order to understand the stem cell derived tumor formation. The most detailed studies in stem cells are done in haematopoietic stem cells (HSC), which are isolated both from mice and human. The knowledge about stem cells mostly comes from HSC studies. Activation of notch signaling pathway by Jagged-1 ligand promoted HSC self-renewal or maintenance of multipotentiality (Varnum-Finney *et al*., 2000; Karanu F.N., *et al*., 2000). Sonic hedgehog signaling is another pathway that can increase self-renewal capacity of human HSC *in vitro* (Bhardwaj *et al*., 2001). Overexpression of activated β-catenin, which functions at the downstream of Wnt signaling, increased the cell population that can reconstitute haematopoietic system *in vivo* and have stem cell markers in isolated HSC. In addition, inhibition of Wnt signaling due to Axin overexpression resulted in inhibition of HSC proliferation and reduction in *in vivo* reconstitution. Activation of Wnt signaling results in increased self-renewal and decreased differentiation in epidermal (Zhu and Watt, 1999) and intestinal stem cells (Sancho *et*

al., 2003). We may conclude that Wnt signaling plays a role in self-renewal of stem cells.

1.3.2 Stem Cells and Cancer

Tumors are also tissues that consist of heterogeneous cell populations, they can form the required structures like blood vessels to survive, they can metastasize to new tissues and form similar tumor tissues, and they can regenerate after surgical removal. All these features require a group of cells that have capacity to survive for a longer time and differentiate into several distinct cell types. As explained in section 1.3.1, stem cells have a high capacity to replicate, and live for a longer time compared to differentiated cells. These properties give them a greater potential to accumulate mutations, which is the main requirement for cancer formation. Trott expanded the hypothesis that only a small part, around 1%, of tumor cells behave like tumor stem cells, which can proliferate to form both tumor stem cells and progenitor cells to differentiate and form heterogeneous cell types of a tumor (Trott, 1994; Presnell *et al*., 2002; Smalley and Ashworth, 2003).

The dedifferentiation of differentiated adult cells was first thought to be the origin of cancer, they are thought to gain the ability to proliferate rapidly and give rise to different types of cells due to dedifferentiation. However cancer formation occurs within several months or years, and requires more than one 'hit' that changes the cellular behavior. Even when mature cells get the first 'hit' they will probably die before secondary hits occur. However; stem cells can live long enough to accumulate mutations (Sell and Pierce, 1994). Skin is one of the best examples for the stem cell origin of cancer. Skin cancer is mostly observed in the middle or late life time; however the initial promoting events occur early in life. The mature skin cells have a high turn over that, a fully differentiated keratinocyte derived from daughters of stem cells, removed from the skin in a day. Since the differentiated skin cells do not have enough time to accumulate mutations, there should be long-lived skin stem cells that begin to accumulate mutations early at life and give rise to cancer several years later (Presnell *et al*., 2002).

Another advantage of stem cells is that they already have self-renewal capacity, in contrast to differentiated cells. And uncontrolled activation of self-renewal will cause tumor formation due to proliferating stem cells. Stem cells require fewer mutations to maintain self-renewal capacity compared to differentiated cells, which should gain this capacity *de novo*. So, stem cells are more susceptible for being the origin of cancer (Reya *et al*., 2001).

Normal stem cells and cancer cells have many similar properties. First of all, both have ability to self-renew that stem cells use tightly controlled proliferation capacity for organogenesis, adult tissue maintenance, repair, and regeneration; while uncontrolled self-renewal of cancer cells cause tumor formation. Second, both can differentiate to form different types of tissue specific cells, or organogenesis in the case of stem cells and tumor heterogeneity in the case of cancer cells. Third, both have active antiapoptotic pathways and telomerase activity, which results in long life and high risk of mutation accumulation. Fourth, they have resistance to damaging agents. Stem cells have an increased transporter activity to exclude toxic agents, and cancer cells can remove chemotherapeutics and become resistant. Last, both can survive independent of anchorage and migrate, which gives cancer cells ability to metastasize (Dontu *et al*., 2003a).

Tu and colleagues hypothesize that heterogeneity of the tumors depends on the origin stem cell. If the origin is an early stem cell the tumor will be more heterogeneous than the tumors derived from a late stem cell with a narrower capacity. The early stem cells will generate tumors containing all cell types of germ layers, like in germ cell tumors; intermediate stem cells will generate tumors with restricted cell phenotypes, like in respiratory and gastrointestinal cancers; and a late stem cell will generate tumors containing only one type of cell, like in basal cell skin carcinoma (Tu *et al*., 2002).

The signaling of stem cell niche may contribute to tumor formation too. Because an abnormality in the signaling can cause accumulation of stem cells and that will increase the risk of tumor formation. The repopulation of stem cells by progenitor cells in response to stem cell niche signals will also increase the tumor formation risk according to the Cairns hypothesis. Cairns hypothesis states that after

asymmetric divisions, always stem cells take the original DNA strand, which will serve as template for the following divisions. In that case daughter progenitor cells always carry the newly synthesized DNA strand, which have more error due to the nature of the replication and accumulate mutations (Smalley and Ashworth, 2003).

Current therapeutic approaches target proliferating cells. However; stem cells have a relatively low proliferation rate and will not be affected by traditional therapies. Reducing the tumor size will not cure the cancer because it will recur as long as the stem cells exist in the tissue. However; a novel therapy targeting the stem cells will be effective against the origin of tumorigeneis and prevent recursion. A therapy targeting the stem cells may use the advantage of common phenotypic properties of stem cells. Instead of designing different therapies for different genotypic aberration in each cancer case, removing the stem cells directly may be easier. Some cases, like in breast cancer, a preventive treatment can be applied to the women in risk group. Since breast is not a vital organ, eliminating cancer prone stem cells at early ages will prevent tumor formation. However, these kinds of applications should be discussed deeply because depleting whole stem cells may generate serious side effects. For example repopulation of stem cells, will result in the accumulation of more mutations according to Cairns hypothesis, and this will increase the risk of tumorigenesis (Smalley and Ashworth, 2003).

1.4 Breast Stem Cells

1.4.1 Breast Stem Cells in General

Stem cells are mostly studied in detail in blood tissue, because studying with blood cells is easier than studying with the solid tissues. As a result, proliferation and differentiation process of haematopoietic stem cells are explained in detail, but the stem cells of solid tissues are not well identified and studied (Smalley and Ashworth, 2003).

The idea of breast stem cells first comes from a study done in 1959, by DeOme. He did the first transplantation experiment in breast, and showed that

epithelium isolated from different regions of postnatal mammary gland, can generate functional mammary epithelial structures. (Welm *et al*., 2003)

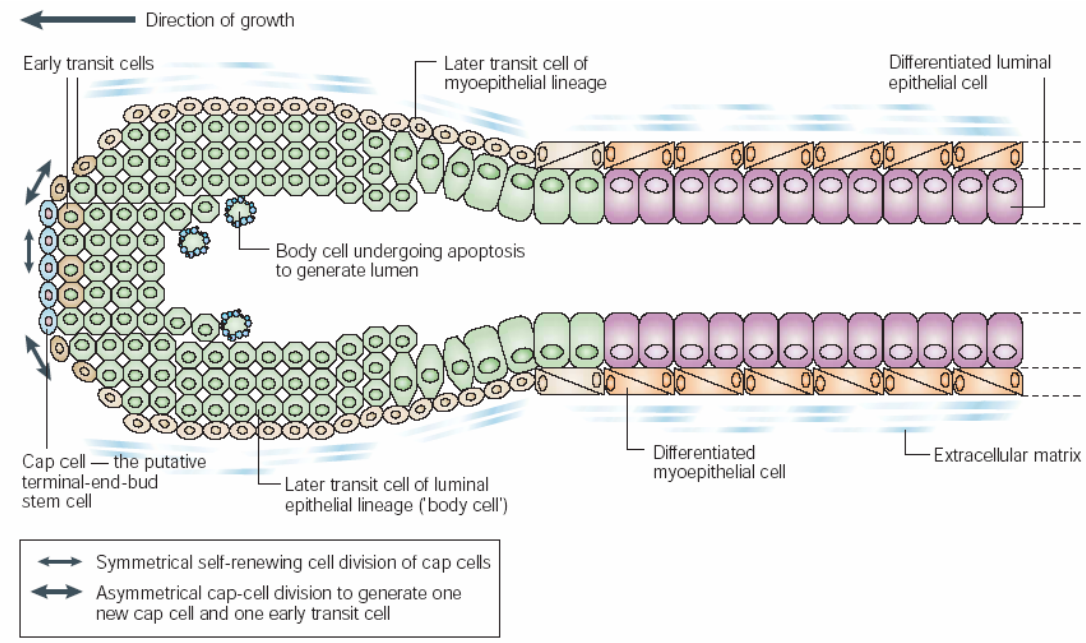

Figure 1.4 Formation of a duct in developing breast. The inner luminal epithelial cell layer and outer myoepithelial cell layer are formed from TEB. Cap cells at the tip of the TEB generate two different types of transit cells; first type on the outer site differentiate into myoepithelial cells, and second type at the center, called "body cells" differentiate into luminal epithelial cells. Central body cells undergo apoptosis while outer layer differentiate into the luminal epithelial cells in order to form the ductal lumen (Smalley and Ashworth, 2003).

A stem cell population cannot be identified in breast tissue yet; however it is thought that there are cell populations that behave like stem cells in specific microenvironments. In developing mammary gland, depending on the structure and function of TEB, we can say that the TEB are most probably the structures that contain the stem cell activity in breast tissue (Figure 1.4). In terminal end buds, the cap cells are the candidates for stem cells because it was shown that they migrated into the body cell mass (Smalley and Ashworth, 2003).

In addition to developing mammary gland, breast stem cells should be found in adult mammary gland for at least three processes. During pregnancy, side

branches are developed and secretory alveoli are generated to produce milk. Upon weaning involution occurs and these structures are removed by apoptosis. In each pregnancy this mammary cycle is repeated and dramatic changes occur in breast tissue (Figure 1.3). Only stem cells have the capacity to proliferate and differentiate into different structures for many times during the life of a female animal. In addition, non-pregnant animals have a similar alveolar bud expansion and regression process during each menstrual cycle. The other process is the replacement of epithelial cells, which are shed into the lumen during cell turnover. The cells may be lost during lactation, since they may be found in the milk, or they may be lost in resting gland (Smalley and Ashworth, 2003).

1.4.2 Evidences for Breast Stem Cells

The experimental techniques like cleared fat pad transplantation, retroviral tagging and x-chromosome inactivation patterning gave some evidences about breast stem cells. Cleared fat pad transplantation depends on the growth of the epithelia after birth, and enables removal of all epithelial cells of host breast and transplantation of new epithelial cells. The primary epithelial cells transplanted into the cleared fat pads resulted in mostly complete ductal system generation that can respond to hormones during pregnancy and generate alveolar cells, less commonly only alveoli without ducts, and rarely only ducts without alveoli generation. These results show that in the transplanted population there are some cells, which are able to form all or a few parts of an adult breast. Retroviral tagging system, in which retroviral sequence is incorporated into the host genome and enables marking of the cells, and monitoring the progeny of cells transplanted to the cleared fat pads. This system showed that entire mammary epithelial growth could be clonal in origin (Kordon and Smith, 1998). X chromosome inactivation patterns also showed that terminal duct lobular units (TDLU) are clonal, so there may be adult stem cells distributed to the mammary gland (Diallo *et al*., 2001; Welm *et al*., 2003; Smalley and Ashworth, 2003).

The histological and microscopic studies in mouse and rat identified a cell population, which morphologically is a candidate for a stem cell population. The small light cells (SLC), are undifferentiated cells in luminal cell layer, near

myoepithelial cells, and small in size. SLCs have a pale cytoplasm and do not have specialized organelles. They are found together as homogeneous or heterogeneous pairs, probably representing symmetric and asymmetric divisions respectively. SLC can differentiate into undifferentiated large light cells (ULLC), differentiated large light cells (DLLC) and large dark cells (LDC). It is thought that ULLC and DLLC are the progenitor/transit cells. Cells similar to SLC are identified in cattle and human mammary glands too (Smith and Chepko, 2001).

Another candidate cell population is identified by bromodeoxyuridine (BrdU) labeling experiment, in which the dye is incorporated into DNA and diluted by each cell proliferation. Some cells were shown to retain the label for a longer time, and that indicates a slow rate of proliferation, which is a property of stem cells. Some of these label-retaining cells (LRC) did not express neither luminal nor myoepithelial markers, while some express both. So it is thought that undifferentiated LRC stem cells can form differentiated transit cells (Welm *et al*., 2002; Smalley and Ashworth, 2003).

As mentioned before, luminal cells are thought to be the candidate for stem cell population. Freshly isolated human luminal and myoepithelial cells can be grown in specific mediums. When luminal cells are switched to the myoepithelial medium, after a certain time some cells with myoepithelial markers, and also some other cells with both myoepithelial and luminal markers are observed, indicating the intermediate transit cells. However, myoepithelial cells do not give this result (Pechoux *et al*., 1999). In addition, some cells positive for ESA (epithelial specific antigen, a marker for luminal cells), negative for MUC1 (luminal epithelial marker) and positive for cytokeratin 19, can generate $ESA^+/MUC1^+$ cells and myoepithelial cells. And when these $ESA^+/MUC1^-$/Cytokeratin 19^+ cells are grown on three-dimensional cultures and xenografts, they can form TDLU like structures, and are thought to be TDLU progenitors (Gudjonsson *et al*., 2002; Smalley and Ashworth, 2003).

A common property of stem cells is their ability to efflux chemicals like chemotherapeutics or Hoechst dye out of their cytoplasm. Side population (SP), is accepted as a universal stem cell marker, because it is found in putative stem cells of

many tissues. SP cell population also pumps chemicals out of cell. This phenomenon is thought to be due to ABC transporter protein ABCG. SP cells are also found in breast epithelium of human and mouse, in which they are undifferentiated and express none of the cytoskeletal markers of myoepithelial and luminal epithelial cells. Depending on the efflux of Hoechst dye SP cells can be isolated from mouse mammary gland by FACS. The isolated SP cells were shown to reconstitute mammary gland and form ductal and alveolar cell populations. Breast SP cells are rich for expression of Sca-1, a stem cell marker. The Sca-1 enriched cells were able to reconstitute mammary gland. However since 20-30% of breast cells are positive for Sca-1, we can say that this is mixed population containing stem cells. (Welm *et al*., 2003; Smalley and Ashworth, 2003).

Mammospheres, which is based on the principle that normal epithelial cells cannot survive in the absence of a substratum to attach; but in contrast stem cells are anchorage independent that they can survive and proliferate in this condition, was used to enrich the candidate breast stem cells. Cultured mammospheres lost the normal epithelial cells and enriched in the undifferentiated cells. These cells were able to differentiate and form myoepithelial, ductal epithelial and alveolar epithelial cells. In addition, they had self-renewal capacity that they could form new mammospheres containing cells with the same differentiation capacity. These studies should be expanded for *in vivo* experimental conditions as well. The expression profiles of mammospheres gave some clues about the pathways important in breast stem cells that TGF-β, and growth hormone signaling were activated, and Wnt-1 and Notch3 were among the signaling proteins upregulated (Dontu *et al*., 2003b).

Depending on these experimental results, we can conclude that there are breast stem cells, which are most probably luminal origin, can differentiate into both luminal epithelial and myoepithelial cells, and can generate TDLU like structures. The probable stem cells are located at the base of luminal epithelial layer, next to myoepithelial layer, and do not have a contact with lumen or basement membrane. These cells do not express all markers of differentiated luminal epithelial or myoepithelial cells, but may express some of them together. They generate transit cells, which express markers of both lineage and then differentiate into luminal

epithelial or myoepithelial cells. And these cells also express some common markers for stem cells (Smalley and Ashworth, 2003).

In spite of these experimental evidences, we still cannot say that there were breast stem cells in adult breast. The self-renewal capacity of the candidate stem cells is not shown yet. In order to prove the presence of breast stem cells exactly, the breast cells with multipotent differentiation and self-renewal capacity should be isolated.

1.4.3 Breast Cancer Stem Cells

Breast stem cells are strong candidates for the origin of breast cancer. In addition to the general ideas explained in section 1.3.2., some observations also supports this idea. For example, women exposed to ionizing irradiation as teenagers are more susceptible to breast cancer compared to women exposed as adults. In 40% of breast cancer cases, recursion is observed in 10 years after the diagnosis and removal of the tumor. So, the origin should have an extremely long life and should be found in young women more, with a higher proliferative capacity (Welm, 2003). These observations direct us to the breast stem cells as origin of breast caner.

Al-Hajj et al identified a group of candidate "breast cancer stem cells" in human breast tissue. In solid tumors only a small proportion of cells are able to from colonies *in vitro*, and large number of cells are required in transplantation to form tumor in animals. So, they think that in a tumor there is small number of cells, which have capacity to proliferate and from new tumors. They isolated different group of cells depending on the heterogeneous expression of cell surface markers from human breast tumors. A specific group of cell, which is positive for cell adhesion molecule CD44, negative or has low expression for CD24, and negative for lineage markers (CD2, CD3, CD10, CD16, CD18, CD31, CD64, CD140b, which are associated to normal cell type and not expressed in cancer cells) was isolated. It is interesting that the transplantation of 10^3 $CD44^+/CD24^{-/low}/Lineage^-$ cells generated tumor in all cases, however transplantation of 10^4 mixed tumor cells generated tumors in minority of cases. In ESA^+ subpopulation, even 200 cells give rise to tumor. The complexity of the new tumor with respect to ESA, CD24, and CD44 expression

patterns is similar to the original tumor, from which $CD44^{+}/CD24^{-/low}/Lineage^{-}$ cells were isolated. The newly formed tumor contained tumorigenic $CD44^{+}/CD24^{-/low}/Lineage^{-}$ cells, representing the self-renewal property. And also the tumor contained non-tumorigenic cells with different cell surface marker expression patterns, representing the differentiation property. The experiments strongly states that $CD44^{+}/CD24^{-/low}/Lineage^{-}$ group of cells have stem cell properties, however in order to prove definitely a single cell transplantation should be able to generate tumor (Al-Hajj *et al*., 2003). A possibility is that this group of cells is enriched in stem cells, but may also contain non-tumorigenic cells. So, further analysis is required for the isolation of exact stem cells within $CD44^{+}/CD24^{-/low}/Lineage^{-}$ group.

Do the tumor stem cells originated from normal adult stem cells? The multipotent cells of breast are also known to express ESA and CD44, which are positive in putative tumor stem cells as well (Al-Hajj *et al*., 2003). In addition, depending on the experimental results we can say that they are probably originated from normal stem cells. When the epithelial cells of a Wap-TGFβ1 transgenic mouse are transplanted to a cleared fat pad, reduced repopulation is observed, and this is thought to be due the premature stem cell senescence. And these transplanted animals are resistant to MMTV induced tumorigenesis. Another evidence for the normal adult stem cell origin of breast cancer stem cells is the protective role of early pregnancy against breast cancer. Pregnancy causes terminal differentiation, and decreases the proliferative capacity. Early pregnancy results in the increase in number of terminally differentiated cells depleting the stem cell compartments before they accumulate mutations and begin tumorigenesis (Smalley and Ashworth, 2003).

1.4.4 Regulation of Breast Stem Cells

The regulation of stem cell self-renewal and differentiation is based on the balance between signaling pathways and many factors like environment. Understanding the pathways involved in regulation of self-renewal and differentiation will be important to establish the relation between breast stem cells and tumorigenesis, and will direct us to novel therapeutic approaches.

Wnt signaling is involved in the self-renewal of haematopoietic, epidermal and intestinal stem cells as explained in section 1.3.1. Mammosphere experiments showed that Wnt pathway components are differentially expressed in putative breast stem cells. In addition overexpression of Wnt in the mouse mammary gland by MMTV promoter increased tumor formation (Schroeder *et al*., 2002). So, abnormal Wnt signaling may be involved in tumor formation due to stem cell deregulation in mammary gland (Dontu *et al*., 2003a).

Leukemia Inhibitory Factor (LIF), which stimulates self-renewal of neural stem cells and maintenance of embryonic stem cells in undifferentiated state, was shown to be involved in mammary gland tumorigenesis. Overexpression of LIF in transgenic mouse caused mammary tumor development and its overexpression is observed in human breast cancers (Dhingra *et al*., 1998). PTEN, is another protein shown to be involved in self-renewal of neural stem cell self-renewal, and formation of neurospeheres, have mutation in breast cancer (Dontu *et al*., 2003a).

Notch signaling pathway is an emerging topic in breast stem cells and tumorigenesis and will be discussed in the next section.

1.5 Notch Pathway

1.5.1 Overview of Notch Pathway

1.5.1.1 The Structure of Notch Receptors and Ligands

A *Drosophila* strain with notches at the end of its wings was first described in 1917. Later it was identified that this phenotype is related to a partial loss of function of Notch gene. Notch gene is evolutionary conserved from flies to mammals (Nam *et al*., 2002; Radtke and Raj, 2003).

Notch receptor is single-pass transmembrane protein, which is synthesized as a single precursor protein but cleaved during transportation to the membrane by a furin-like protease in trans-Golgi. This first cleavage of Notch occurs at around 70 amino acids external to the transmembrane domain and generates two subunits, extracellular and transmembrane. The subunits are not dissociated but transported to the membrane as a heterodimeric receptor, which is held together by non-covalent

interactions and calcium. Notch consists of highly conserved structural motifs (Figure 1.5). Extracellular subunit of Notch receptor contains 29-36 N-terminal epidermal growth factor (EGF)-like repeats, which are involved in ligand binding. EGF-like repeats are followed by three tandemly repeated LIN-12 motifs, which are required to inhibit activation in the absence of ligand binding. First cleavage site is around 100 residues after LIN-12 repeats. The extracellular part of transmembrane subunit is very short and consists of conserved cysteine residues. The intracellular part of transmembrane subunit contains RAM domain followed by ankyrin/CDC10 repeats, both of which are involved in binding to the transcription factors in the nucleus. Ankyrin/CDC10 repeats are flanked by two nuclear localization signals (NLS) and followed by transactivation domain and PEST (PEST for proline, glutamate, serine and threonine) sequence (Kadesch, 2000; Nam *et al*., 2002).

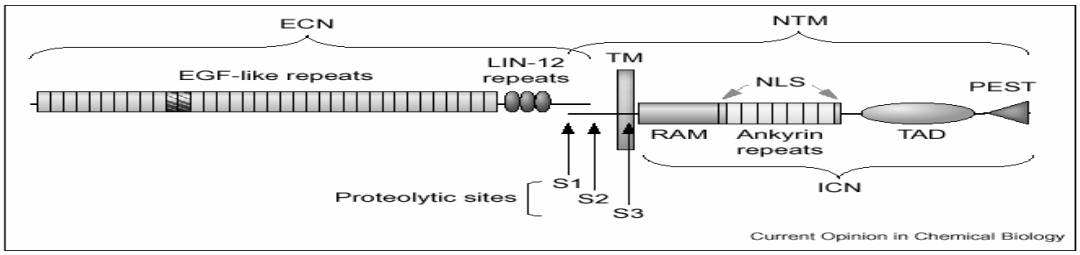

Figure 1.5 Structure of Notch Receptor. ECN and NTM show the extracellular and transmembrane subunits of Notch receptor, respectively. S1, S2 and S3 represent the sites of three cleavages in order (Nam *et al*., 2002).

Drosophila has only one Notch gene, while *C.elegans* have two (glp-1 and lin-12) and mammals have four (Notch1-4), which are thought to be evolved by gene duplication. Mammalian Notch receptors are highly homologues, but have some differences in extracellular and cytoplasmic domains (Figure 1.6). Notch1 and Notch2 have 36 EGF-like repeats while Notch3 has 34 and the shortest one Notch4 has 29 repeats. Notch1 has the strongest transactivation domain, Notch2 has a weaker one, but Notch3 and Notch4 do not have. Ankyrin/CDC10 repeats are the least conserved part of the receptors and affects transactivating activity (Nam *et al*., 2002; Radtke and Raj, 2003).

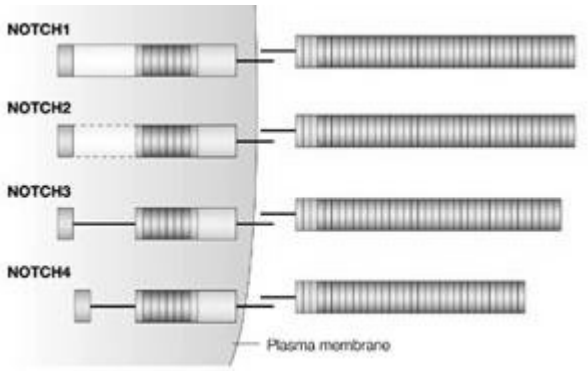

Figure 1.6 Human Notch Receptors. Human Notch receptors are highly conserved but have differences in the number of extracellular EGF repeats and cytoplasmic Ankyrin repeats and transactivating domain (Radtke and Raj, 2003).

Notch ligands, DSL, are also single-pass transmembrane proteins. *Drosophila* has two ligands Delta and Serrate, while in human, two Serrate homologues Jagged1 and Jagged2, and three Delta homologues Delta-like1, Delta-like3, and Delta-like4, are found. Each of the ligands contain N-terminal DSL motif, which is required for receptor interaction. DSL is followed by EGF-like repeats, and then transmembrane domain. The cytoplasmic tail of ligands is shorter and consists of 70-215 amino acids. The number and spacing of EGF-like repeats are different in ligands. Jagged1 and Jagged2 have additional cysteine rich domain below EGF-like repeats (Nam *et al*., 2002; Radtke and Raj, 2003).

1.5.1.2 The Notch Signaling Pathway

In contrast to usual signaling pathways, Notch signaling does not contain an enzymatic amplification step, and Notch receptor itself directly involved in the transduction of the signal to the nucleus (Figure 1.7).

Notch signaling is activated upon interaction with ligand represented by adjacent cells. Receptor-ligand interaction disrupts the heterodimeric structure of Notch and induces the second proteolytic cleavage. A member of ADAM family of proteases, TNF-α converting enzyme (TACE) in human and Kuzbanian in *Drosophila*, cleaves the transmembrane subunit of receptor from a site adjacent to membrane. This cleavage induces the third cleavage within transmembrane region,

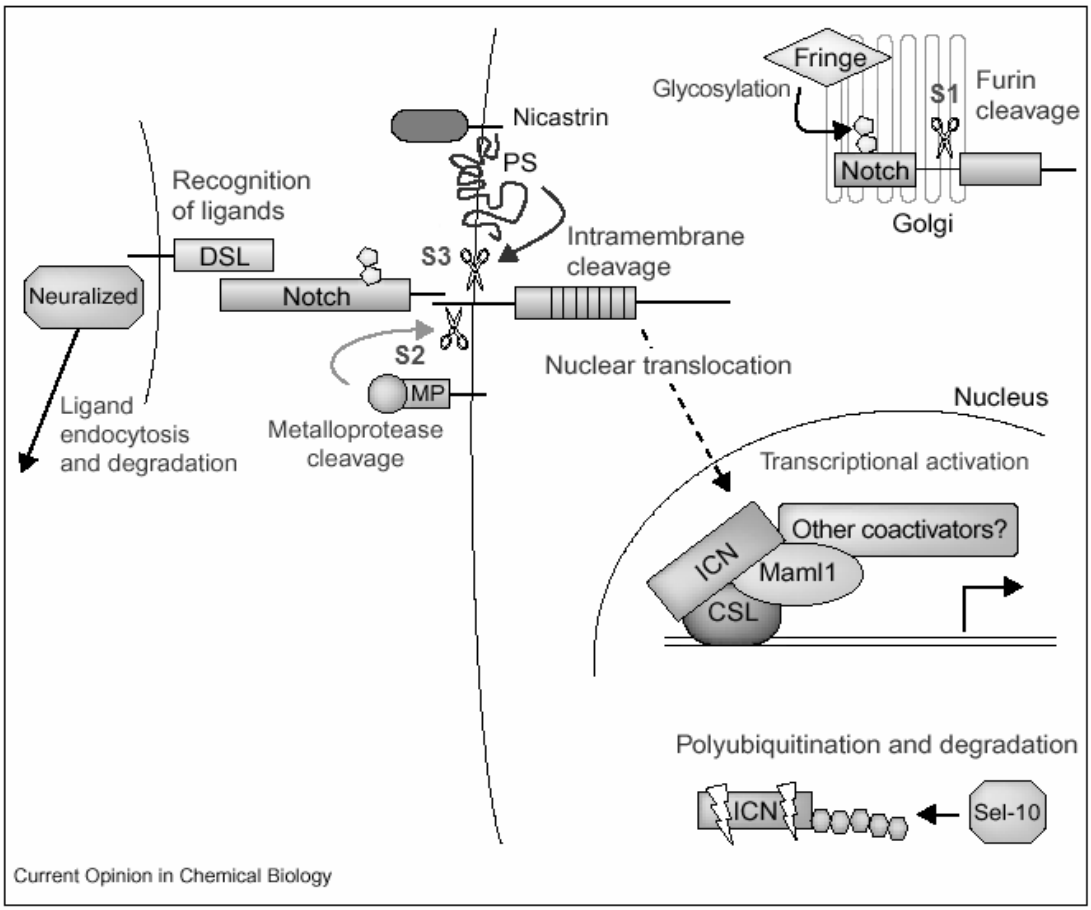

Figure 1.7 Notch Signaling Pathway. S1, S2, and S3 represents the three cleavages in order. ICN represents the released intracellular domain of Notch (NICD) (Nam *et al*., 2002).

which is catalyzed by a complex of Presenilin, nicastrin, Aph1, and Pen2 protein complex and releases functional cytoplasmic part, Notch intracellular domain (NICD). NICD translocates to the nucleus and interacts with CSL (CSL stands for CBF1 in mammals, Su(H) in *Drosophila*, and Lag-1 in *C.Elegans*) transcription factors. In the absence of Notch signaling CSL binds to the target genes' promoters in a complex with several corepressors and histone deacetylases to inhibit the transcription. NICD competes with the inhibitory proteins, like CIR, KyoT2, and SMRT. Then displace them and recruit coactivators and histone acetylases like CBP/p300, pCAF, GCN5 and Mastemind like protein (MAML) converting CSL from repressor to activator, and initiate transcription. The signaling is terminated by the rapid degradation of NICD in the nucleus due to ubiquitination by human Sel-10,

an E3 ubiquitin ligase (Kadesch, 2000; Nam *et al*., 2002; Radtke and Raj, 2003; Wu and Griffin, 2004).

The first identified target gene of Notch signaling is HES (Hairy/enhancer of split) basic helix-loop-helix transcription factors, which inhibits expression or function of Neurogenin, Mash, and MyoD proteins. Notch signaling is thought to activate different targets at different tissues. For example in keratinocytes Notch1 was shown to induce $p21^{Waf1}$, which has CSL binding site, inhibiting proliferation and inducing terminal differentiation (Rangarajan *et al*., 2001a). CSL binding site is also described in cyclin D1 gene, which is upregulated by Notch signaling in rat kidney epithelial cells (Ronchini and Capobianco, 2001). Notch1 was shown to activate phosphatidylinositol 3-kinase (PI3K), which confers resistance to anoikis and p53 mediated apoptosis. ERBB2 and NF-κB2 promoters have CSL binding sites, and induced by Notch1 (Chen *et al*., 1997; Oswald *et al*., 1998). ERBB2 stimulates proliferation, while NF-κB2 is mainly involved in development of lymphoid organs and induces expression of many genes, some of which have anti-apoptotic roles (Radtke and Raj, 2003; Hansson *et al*., 2004).

There are also evidences for CSL-independent signaling of Notch. NICD lacking CSL-interacting domain can still function in blocking myogenesis. Deltex protein is involved in CSL-independent signaling (Radtke and Raj, 2003; Hansson *et al*., 2004).

1.5.1.3 Regulation and Crosstalks of Notch Signaling

Notch receptors are glycosylated by addition of fucose to serine and threonine residues of EGF-like repeats by O-fucosyl transferase. The glycsoylation is essential for the regulation of Notch activity that loss of function or overexpression of the particular enzyme may inhibit Notch activity. Fringe is another enzyme involved in modification of O-linked fucose on EGF-like repeats, which are important in ligand binding. Modification by Fringe increases the affinity of Notch for Delta while decreasing the affinity for Serrate in *Drosophila* (Schweisguth, 2004). The orthologues of Fringe in human are Radical, Manic and Lunatic Fringe, which perform gycosylation in Golgi. Similar to *Drosophila*, modification of Notch inhibits

activation by Jagged1 and Jagged2 but not by Delta-like (Dll) ligands in mammals (Mumm and Kopan, 2000; Nam *et al*., 2002).

The internalization of receptors by endocytosis, and degradation by ubiqitination is another mechanism of Notch regulation. Numb is one of the proteins that are thought to be involved in endocytosis. The exact mechanism of Numb function is not clear but it segregates differentially to daughter cells and affect the cell fate by inhibiting Notch (Hansson *et al.*, 2004; Schweisguth, 2004).

The crosstalk with several pathways may alter the result of Notch signaling depending on the cellular context. For example, in *C.elegans* Ras activation downregulates Notch. However, in cultured human cell lines Ras activates Notch signaling and Notch is required to maintain Ras induced neoplastic phenotype (Weijzen *et al*., 2002). Wnt signaling has an inhibitory effect on Notch signaling, and NICD has a week interaction with LEF-1, and GSK-3β, which is thought to phosphorylate and stabilize NICD (Hansson *et al.*, 2004).

1.5.1.4 Function of Notch Signaling

Notch is one of the pathways involved in development in coordination with the pathways Wnt, Hedgehog, Transforming Growth Factor-β (TGF-β), and Receptor Tyrosine Kinase/Phosphatase. Notch signaling is important in several cellular processes like maintenance of stem cells, specification of cell fate, differentiation, and proliferation depending on the cell type (Mumm and Kopan, 2000; Radtke and Raj, 2003).

Maintenance of an undifferentiated state is one of the major roles of the signaling. Activated Notch signaling prevents differentiation neuronal progenitor cells, while inhibition results in excessive neurogenesis and depletion of progenitor cells. In a hematopoietic cell population, induction of Notch signaling by Jagged1, increases the stem cell ratio. So, Notch signaling keeps progenitor cells in a stem cell like character (Radtke and Raj, 2003).

Table 1.1 Knockout Mice of Notch Components

Gene	Knockout Phenotype	Reference
Notch1	Embryonic lethality	Swiatek *et al*., 1994; Conlon *et al*., 1995
Notch2	Embryonic lethality	Hamada *et al*., 1999
Notch3	No apparent phenotype	Krebs *et al*., 2003
Notch4	No apparent phenotype	Krebs *et al*., 2000
Notch1 and Notch4	More severe phenotype than Notch1 null mice	Krebs *et al*., 2000
Notch1 and Notch3	Embryonic lethality similar to Notch1 null mice	Krebs *et al*., 2003
Jagged1	Embryonic lethality	Xue *et al*., 1999
Delta like1	Embryonic lethality	Hrabe *et al*., 1997
Delta like3	Skeletal defects	Kusumi *et al*., 1998; Dunwoodie *et al*., 2002
CBF-1	Embryonic lethality	Oka *et al*., 1995

Notch signaling is also involved in binary cell fate decision, which means the determination of a cell fate between two possible options. During development in *Drosophila*, the fate of precursor cells, which can differentiate into neuronal-precursor or epidermal cells, are determined by Notch signaling. In lateral inhibition, Notch receptor and ligands are expressed equally in all cells at the beginning, but the concentrations changes over time and cells begin to express either receptors or ligands dominantly. The cells expressing ligand differentiate into neuronal cells,

while the differentiation in cells with activated Notch signaling is inhibited and they adopt epidermal cell fate (Kimble and Simpson, 1997). In inductive cell fate determination, two distinct cell types expressing either Notch receptor or ligand adopt different cell fates. In bipotential neurol crest stem cells, the Notch expressing cells differentiate into glial cells instead of neurons. Notch signaling induces terminal differentiation in skin (Radtke and Raj, 2003).

The knockout mouse models of Notch signaling components established its critical role in development that most of the knockouts died (Table 1.1). Depending on the double knockout mice, Notch1$^{-/-}$/Notch4$^{-/-}$, we can conclude that Notch1 can only partially compensate Notch4 functions. However, Notch3 function during development can be completely compensated by other Notch receptors that Notch3 knockout mice have no apparent phenotype, and Notch1$^{-/-}$/Notch3$^{-/-}$ mice do not have more severe phenotype than Notch1$^{-/-}$ mice.

1.5.1.5 Possible Therapeutic Applications Related to Notch Signaling

In the cases that Notch is functioning as oncogene, or inducing tumorigeneis due to induction of stem-cell self-renewal, inhibition of the pathway may be an effective therapeutic strategy. The inhibition may be at different steps of signaling like ligand binding, receptor cleavage, or NICD function. Competitive inhibitors consist of EGF-like repeats may compete with full-length Notch and interfere with ligand binding (Garces *et al*., 1997). The enzymes involved in second or third cleavage of Notch receptor may also inhibit signaling. Truncated Notch, which is already activated NICD, is observed in many cancer cases. So, inhibition of NICD interaction with CSL may be effective in inhibition. Notch can also function as tumor suppressor depending on the tissue. In these cases, activation of the signaling may be required by inhibition of NICD degradation in nucleus, activation of CSL independently of Notch, or induction of signaling upon increase in ligand may be applied (Nam *et al*., 2002). The multiple function of Notch signaling and enzymes involved in Notch cleavage should be considered when designing a therapeutic strategy in order to prevent unexpected results.

1.5.2 Notch Pathway in Diseases

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is a hereditary disease, which has features like subcortical ischemic strokes and progressive dementia. Most of the CADASIL patients have point mutations at the EGF-like repeats of Notch3 extracellular domain, and that causes addition or deletion of a cysteine residue (Joutel *et al*., 1997). Since disulfide bonds formed between cysteine residues are important in protein folding, mutation is thought to affect the folding and cause loss of function for Notch3. Extracellular Notch3 accumulation, deficiency in first proteolysis and intracellular accumulation of Notch3 is observed in several CADASIL brains and cell lines (Nam *et al*., 2002; Hansson *et al.*, 2004).

Alagille's Syndrome is a rare autosomal dominant disease that is caused by frameshift mutations in ligand Jagged-1, mainly affecting cysteine residues in EGF-like repeats. Alagille's Syndrome caused developmental defects in many organs like liver, heart, eye, skeleton, and kidney (Nam *et al*., 2002).

Spondylocostal Dysostosis is a malsegmentation syndrome related a Notch ligand, Delta-like-3 (Dll-3) mutation. Homozygous mutations in Dll-3 result in developmental defects of axial skeleton (Nam *et al*., 2002).

1.5.3 Notch Pathway in Cancer

Notch signaling may induce expression of different proteins, interact with other signaling pathways and as a result involved in distinct cellular mechanisms including differentiation or proliferation as explained in section 1.5.1. The diversity of cellular functions affects Notch activity in cancer that it may act both as a tumorsuppressor or oncogene.

Notch signaling has a potential to function as an oncogene that some of the components of siganling were shown to be overexpressed in several cancers like renal cell carcinoma, head and neck squamous cell carcinoma, endometrial cancer. Another evidence for oncogenic character of Notch signaling is the similarity between Epstein-Barr virus, which immortalizes the infected cells. Epstein-Barr virus

nuclear antigen 2 functions similar to NICD that it binds to CSL and converts it from transcriptional repressor to activator (Brennan and Brown, 2003). In many type of cancers Notch play complex roles related to its function in development of the particular tissue.

1.5.3.1 T-cell Leukemia

Notch was first described as an oncogene in T-cell acute lymphoblastic leukemia (T-ALL). It was found that t(7;9)(q34;q34.3) translocation results in the breaking of Notch gene, and fusion to the promoter/enhancer region of T-cell receptor-β (TCRβ). The breakpoint results in high-level expression of constitutively active Notch1, NICD domain (Reynolds *et al*., 1987; Ellisen *et al*., 1991). Since all T-ALL patients with t(7;9) translocation have the same feature, overexpression of active Notch1 is thought to be responsible for T-ALL (Radtke and Raj, 2003).

Similar to translocation, viral integrations can also generate truncated Notch genes. Integration of Moloney murine leukemia virus and feline leukemia virus into the Notch1 and Notch2 genes, respectively, cause expression of truncated Notch proteins under viral promoters. The abnormal activation of the Notch signaling causes T-cell leukemia (Girard *et al*., 1996; Rohn *et al*., 1996).

Mice expressing truncated Notch1 in haematopoietic progenitor cells developed T-cell leukemia. B-cell development was inhibited in these mice, and T-cells were arrested at an immature stage (Radtke, 1999). Notch1 is required for T-cell lineage commitment but should be downregulated for complete differentiation of T-cells. The arrested T-cells with active Notch1 were polyclonal origin and did not form tumors for a while, but in time developed monoclonal aggressive tumors. Most probably Notch1 activation arrests cells before terminal differentiation and allow their survival and additional mutations achieve transformation in T-cell leukemia (Radtke and Raj, 2003; Zweidler-McKay and Pear, 2004).

Hes1 was shown to be important in T-cell development as a target of Notch signaling. However, overexpression of Hes1 did not induce T-cell tumors indictaing that other target genes of Notch should be involved in T-cell leukemia. Deltex1 is one of the candidates that inhibit T-cell differentiation when overexpressed, but loss

of function studies are required to determine its exact role. NF-κB may play a role in Notch induced T-cell leukemia. Notch can inhibit NF-κB activity by acting like IκB. In addition, active Notch3 may cause constitutive activation of NF-κB (Zweidler-McKay and Pear, 2004).

1.5.3.2 Neuroblastoma

Neural stem cells give rise to neuron and glial cells and Notch is important in cell fate decision during this process. Notch signaling mainly blocks differentiation, keeps cell at undifferentiated state, and also may induce glial cell fate. Notch signaling was shown to be active in neuroblastoma cell lines and Notch3 is expressed almost 50% of neuroblastoma cell lines. The experimental results support the idea that Notch signaling function to keeping cells in a more stem cell like phenotype, but further evidences like neuroblastoma formation upon Notch activation is needed (Pahlman *et al*., 2004).

1.5.3.3 Skin Cancer

Notch signaling induces differentiation and keratinocyte stem cells commitment to transient amplifying cell populations. Activation of Notch signaling in mouse keratinocytes cause cell cycle arrest by p21 expression, and induce terminal differentiation (Lefort and Dotto, 2004).

Mice with deficient Notch1 in epithelia develop basal-cell carcinoma like tumors, become more susceptible to chemical-induced carcinogenesis in skin (Nicolas *et al*., 2003). As a result we can conclude that Notch function as a tumor suppressor skin, that its absence may not be the direct cause of cancer but mediates the accumulation of mutations due to increased proliferation of cells.

1.5.3.4 Lung Cancer

During lung development Notch1 is involved in airway epithelial development by inducing expression of HES1. Aberrant lung morphology in mice with Hes-1 mutation and expression of Notch receptors and ligands, Dll1 and Jagged1, suggests that Notch signaling plays a role in normal lung development.

Notch signaling is thought to down-regulate epithelial differentiation in lung (Collins *et al*., 2004).

Notch may function both as an oncogene or tumorsuppressor depending on the type of lung cancer. Non-small cell lung cancer (NSCLC) cell lines express significant levels of Hes-1 while small cell lung cancer (SCLC) cell lines express at low or undetectable levels. Notch1, Notch2 and Notch3 are frequently expressed in NSCLC tumors but not in SCLC. Notch signaling seems to play a growth promoting role in NSCLC; however overexpression of activated Notch1 and Notch2 inhibits growth of SCLC cells representing a tumorsuppressor function (Collins *et al*., 2004).

1.5.3.5 Cervical Cancer

Oncoproteins E6 and E7 of HPV, which is responsible for almost 99% of cervical cancers, require active Notch to be able to fully transform human keratinocyte cell line HaCaT (Rangarajan *et al*., 2001b). In early stage cervical cancer tumors, cytoplasmic and nuclear staining of Notch1 and Notch2 was detected (Zagouras *et al*., 1995). These results indicate that Notch contributes to initiation of cervical tumors together with HPV. However, invasive cervical tumors and malignant HPV-positive cervical cancer cell lines had a lower Notch1 expression, indicating a reduction in Notch1 level by the progression of cancer. Notch1 expression reduced E6 and E7 expression (Talora *et al*., 2002). Notch1 activity may be required for the initiation of cervical cancer but disappear for progression.

1.5.4 Notch Pathway in Breast Development and Breast Cancer

The first evidence about the contribution of Notch signaling to mammary gland tumorigenesis is the identification of MMTV integration sites in Czech II mice (Gallahan *et al*., 1987). In 20% of tumors developed in those mice, MMTV was shown to be integrated into the int-3 locus, Notch4 gene. Notch1 was later characterized as an integration site for MMTV (Dievart *et al*., 1999). In both cases, MMTV integrates within the Notch genes and results in the expression of truncated Notch protein, which is the active form of receptor. These first studies suggested that aberrant activation of Notch signaling may contribute to mammary gland tumorigenesis (Callahan and Raafat, 2001; Politi *et al*., 2004).

MMTV integration disrupts several genes like Wnt-1, Wnt-3, Wnt-10B, FGF3, and FGF4; and generates mammary tumors. The long terminal repeat (LTR) of MMTV fuses to the affected gene and act as a promoter/enhancer to initiate transcription of altered gene. The expressed truncated Notch proteins have a gain function mutation and are constitutively active (Politi *et al*., 2004).

Several studies used MMTV integration in order to characterize the role of Notch4 in mammary gland development and tumorigenesis. Overexpression of truncated Notch4 in transgenic mice resulted in incomplete differentiation of mammary epithelium and induced proliferation of immature ductal cells (Smith *et al*., 1995). Another study used mice with MMTV integrated Notch4 gene, and showed that in TEB of virgin animals cannot be detected and lower level of branching is observed. During pregnancy, ductal epithelium expanded through fat pad due to hormonel stimulation, but no alveoli development and expression of milk proteins occurred. Within 4-6 months all of the mice developed poorly differentiated mammary adenocarcionomas (Jhappan *et al*., 1992). Truncated Notch4 expression is activated by WAP (whey acidic protein) promoter in order to activate Notch4 signaling only in secretory mammary epithelia of pregnant mice. The ductal growth was normal in virgin mice, but secretory lobule growth and differentiation was inhibited. Mammary tumors similar to the tumors formed in MMTV activated Notch4 mice, occurred within 6 months (Gallahan *et al*., 1996). The experimental results of transgenic mice studies state that Notch4 signaling has an inhibitory role in differentiation of ductal and alveolar epithelium in mammary gland.

TAC2 is cultured mammary epithelial system, which can form branching morphology *in vitro* in response to FGF. The TAC2 cells with activated Notch4 supported the transgenic experiments that differentiation and branching was inhibited (Uyttendaele *et al*., 1998; Soriano *et al*., 2000).

Truncated Notch4 expression is detected by Northern blott analysis in some of the breast cancer cell lines (BT474, MDA MB 231, MDA MB 468, SK BR3, T47D, ZR-75-1). This truncated form was able to transform normal human mammary epithelial cell line MCF-10A, which gain the capacity to grow on soft agar (Imatani and Callahan, 2000).

The experimental evidences states that Notch4 activity is involved in mammary epithelial differentiation in ductal branching, lobuloalveolar differentiation and lactation. Notch4 staining detected expression at the proliferating TEBs in mammary gland, which are the structures containing putative stem cells (Smith *et al*., 1995). Notch4 activation also induced tumor formation in mice models. As a result we can conclude that the activated Notch4 signaling keeps mammary epithelial cells in an undifferentiated, proliferative state, in a stem-cell like phenotype and that makes cells more susceptible to accumulation of additional mutations increasing cancer risk.

Introducing Notch1 NICD encoding cDNA to HC11 mouse mammary epithelial cells transform the cells and they became able to form colonies on soft agar. This effect requires CSL binding region and NLS of Notch1, and was specific to mammary epithelial cell line that rat fibroblasts did not gain the same property by Notch1 NICD expression. However the transplantation of HC11 cells expressing activated Notch1, did not generate tumors in mice (Dievart *et al*., 1999). These results support the idea that Notch activation can generate the necessary conditions like undifferentiated and proliferative phenotype, and additional mutations are required for tumor formation (Politi *et al*., 2004).

Notch1 signaling was shown to be positively affected by Ras signaling that Ras positive breast tumors had an increased Notch1 expression. In cell culture, inhibition of Ras signaling blocked upregulated Notch1 NICD, and overexpression of Ras increased Dll1 ad Presenilin1 expression, which may activate Notch signaling (Weijzen *et al*., 2002). The Ras-Notch interaction may play a role in mammary gland tumorigensis.

Notch1 and Notch4 expression was detected in human breast tumors. Both receptors were negative in normal breast tissue while Notch1 was positive in 67% and Notch4 was positive for 44% of DCIS samples (Siziopikou *et al*., 2002).

Musashi-1 is an RNA binding protein, and identified as a stem cell marker in neuronal stem cells. Msi-1, the human homologue of *Drosophila* Musashi-1 protein is involved in Delta/Notch pathway during asymmetric cell division. Msi-1 interacts

with Numb RNA and induces its degradation. Msi-1 was shown to be enriched in human breast LRC, which are putative breast stem cells. Inhibition of Msi-1 translation releases the repression on Delta/Notch signaling in asymmetric cell division. When Msi-1 is found in the cells Notch1 was found to be released from membrane and activated. It is suggested that in a breast stem cell division, the daughter cell expressing the Msi-1 will have the active Notch-1 and replace the stem cell, while the Msi-1 negative daughter cell will be committed to differentiation. Aberrant Notch signaling may cause symmetric division instead of asymmetric division (Clarke *et al*., 2003).

Mainly Notch1 and Notch4 are studied in breast cancer. Notch3 was found to be upregulated in mammospheres, which is enriched in breast stem cells (Dontu *et al*., 2003b). So, Notch3 may play a critical role in breast tumorigenesis originated from breast stem cells. Other components of the Notch pathway should be investigated further for their possible roles in breast cancer.

CHAPTER 2 AIM OF THE STUDY

2.1 Aim

Recent evidences hypothesize that there are cancer stem cells in the origin of cancer. In contrast to classical hypothesis, it is being clarified that not all of the cells in a tumor have capacity to proliferate, but cancer stem cells with deregulated proliferation and differentiation properties are forming tumors. The adult stem cells are trying to be isolated in breast tissue. A candidate population of breast cancer stem cells, which are thought to be originated from adult breast stem cell, have recently been isolated. Investigation of pathways behind the tumor formation of cancer stem cells is the initial step in the generation of novel therapeutic approaches against cancer. Notch signaling is one of the pathways involved in stem cell maintenance, and its activity in some of the human tumors was established. Activation of Notch signaling in mouse mammary tumors due to MMTV integration, and its role in mammary gland development lead us to the possible role of Notch signaling in human breast cancer. There is no complete data about any expression alteration of Notch receptors and ligands in human breast cancer.

This study tries to answer the question if Notch signaling has a role in human breast cancer, and if so, how it contributes to tumorigenesis? The specific aim is to investigate the expression status of all Notch receptors and ligands in human breast cancer cell lines and breast tumors in order to identify any abnormality that may direct us to the significance of Notch signaling in breast cancer.

2.2 Strategy

Six breast cancer cell lines (T47D, BT 474, MCF-7, BT 20, MDA MB 468, MDA MB 453), and eight breast tumor samples were used to study expression of four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five ligands (Jagged1, Jagged2, Delta-like protein1, Delta-like protein3, and Delta-like protein4). A normal mammary epithelial cell line, hTERT-HME1, immortalized with hTERT expression was used as normal control for both cell lines and tumor samples. The expression was detected by semi-quantitative RT-PCR.

CHAPTER 3 MATERIALS AND METHODS

3.1 Tissue Culture

Six breast cancer cell lines, listed in Table 3.1, were grown as monolayer in 150 mm culture dishes. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.1 mM non-essential amino acids, is used as medium.

Table 3.1 Human Breast Cancer Cell Lines Used in This Study

Cell Lines	Cancer Type
T-47D	Metastasis to pleural effusion ductal carcinoma
BT 474	Breast ductal carcinoma
MCF 7	Metastasis to pleural effusion adenocarcinoma
BT 20	Breast carcinoma
MDA MB 468	Breast adenocarcinoma
MDA MB 453	Metastasis to pericardial effusion metastatic carcinoma

hTERT-HME1 (Clontech), a normal mammary epithelial cell line immortalized with hTERT expression, was used as normal control for both cancer cell lines and breast tumors. hTERT-HME1 was grown in a specific medium prepared with equal amounts of HAM'S F-12 Medium and DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM HEPES Buffer, 3.5 ng/ml insuline, 0.1 mg/ml EGF, and 0.5 μg/ml hydrocortisone.

All cell lines were kept in an incubator at 37^{o}C and 5% CO_2, and all applications were performed under sterile hoods in cell culture facility. All mediums and solutions were warmed at 37^{o}C, and wiped with 70% alcohol before placing under the hood. For all washing steps PBS, prepared as below, was used.

Phosphate Buffered Saline (PBS):

10 X Stock solution (1 lt): 80g NaCl

2 g KCl

7.64 g $Na_2HPO_4.2H_2O$

2 g KH_2PO_4

1 X working solution: 10 X stock solution is diluted to 1 X with ddH_2O and pH is adjusted to 7.4. Solution is autoclaved and kept at 4^{o}C.

3.1.1 Thawing of Cells

The vial of the frozen cell line was taken from liquid nitrogen tank and immediately immersed in 37^{o}C water bath and frozen cell suspension was thawed. Then cells were transferred to 15 ml falcon tubes containing 10 ml fresh DMEM or specific medium of hTERT-HME1, and centrifuged at 1500 rpm for 5 minutes at 4^{o}C. The supernatant was discarded and cell pellet was resuspended in 10 ml fresh medium. Cells were plated into 75 mm culture flasks including 10 ml medium. The cells were left in the incubator overnight and medium was refreshed on the following day.

3.1.2 Subculturing of Cells

The medium of cells were refreshed every two or three days. Cells were splitted when they reached 80-90% confluency. The medium was aspirated and cells were washed two or three times with PBS (pH 7.4). Then enough trypsin/EDTA solution to cover plate surface is added. Cells were incubated in the incubator till they detach from the plate (1 to 5 minutes depending on the cell line). Trypsin was immediately inactivated by addition of some fresh medium. Then cells are plated in new 150 mm culture dishes at the desired dilution.

3.1.3 Freezing of Cells

The cells were grown in 150 mm culture dishes in order to obtain 70-80% confluency at the end of two or three days. The medium was aspirated and cells were washed with PBS two or three times. Enough amount of trypsin/EDTA solution was added and cells were incubated in the incubator till detachment. 10 ml fresh medium was added onto the detached cells immediately and cells were centrifuged at 1500 rpm for 5 minutes at 4^{o}C. Supernatant were aspirated and cells were resuspended in 1 ml ice-cold freezing medium consists of 10% DMSO, 20% FCS, and 70% medium (DMEM or specific medium of hTERT-HME1). Cells were transferred to cryotubes, first kept at -20^{o}C till the solution freeze (not more than two or three hours), then put into -80^{o}C overnight, and placed into the liquid nitrogen tank on the following day.

3.1.4 Preparation of Cell Pellets for RNA Isolation

The cells were grown in 150 mm culture dishes in order to obtain 70-80% confluency at the end of two or three days. The medium was aspirated and cells were washed with PBS four or five times. Enough amount of trypsin/EDTA solution was added and cells were incubated in the incubator till detachment. Cells were collected into a 15 ml tube with 10 ml ice-cold PBS, and centrifuged at 1500 rpm for 5 minutes at 4^{o}C. Supernatant was aspirated. Cell pellet was resuspended in 1 ml ice-cold PBS, transferred to a 1.5 ml eppendorf tube, and centrifuged at 13000rpm for 3 minutes at 4^{o}C. Supernatant was aspirated. Cell pellet was directly immersed in liquid nitrogen and then kept at -80^{o}C till RNA isolation.

3.1.5 Tumor Samples

Eight human breast tumor samples were obtained from Dr. Betül Bozkurt from Ankara Numune Eğitim ve Araştırma Hospital. The available data about the patients are listed in Table 3.2.

Table 3.2 Human Breast Tumor Samples Used in This Study

Tumor	Age	Type	ERBB status
T-29	58	IDC	Positive
T-43	43	IDC	Positive
T-44	50	IDC	Positive
T-47	37	IDC	Positive
T-57	51	mucinous (colloid) carcinoma	Negative
T-58	62	IDC	Not Known
T-59	34	Mixed IDC and ILC	Positive
T-94	37	IDC	Negative

3.2 RNA Isolation and Quantification

RNA isolation from cell pellets and frozen tissues was performed with NucleoSpin RNA II Kit (Macherey-Nagel) according to the instructor's manual. At the last step, resuspension of RNA was done with 40 or 50 µl of Rnase free water supplied by the kit. Isolated RNA was immediately immersed in liquid nitrogen and the stored at -80^{o}C. For the spectrophotometric measurement of RNA concentration 4 µl sample was diluted in 400 µl 0.1% DEPC treated ddH_2O, and measured with BECKMAN DU 640.

3.3 cDNA Synthesis

4 µg RNA was used for cDNA synthesis in 30 µl reaction volume. cDNA synthesis was done with RevertAid First Strand cDNA Sybthesis Kit (Fermentas) according to the instructor's manual. cDNAs were stored at -20^{o}C.

3.4 Semi-Quantitative RT-PCR

3.4.1 Reaction Conditions and Primers

Semi-quantitative RT-PCR is used to compare gene expression levels of samples with each other at RNA level. GAPDH, which is a housekeeping gene, was used as equal loading control. GAPDH expression levels should be equal in cDNAs synthesized from equal amount of RNA. The cycle number of RT-PCR reactions should be optimized to detect expression levels before saturation of amplification. The optimized cycle number is 24 for GAPDH and 35 for other genes. PCR reactions were performed in the conditions below:

94^{o}C 5 minutes

94^{o}C	30 seconds	} Optimized number of cycles
T_m	30 seconds	
72^{o}C	30 seconds	

72^{o}C 10 minutes

Forward and Reverse primers were designed from different exons for each gene by using primer3 program. The sequence of primers, annealing temperatures (T_m) and product sizes are listed in Table 3.3.

Negative control in each RT-PCR reaction contains all components but not cDNA. Instead of cDNA, equal amount of sterile PCR water was used.

Table 3.3 Sequences of Primers, Annealing Temperatures and Product Sizes

Gene		Sequence (5' to 3')	T_m	Product Size (bp)
Notch1	Forward	cgc ctt tgt gct tct gtt ctt	61°C	209
	Reverse	cat tct ggt tgt cgt cca tga		
Notch2	Forward	ttg aca acc gcc agt gtg tt	63 °C	183
	Reverse	tga caa cag caa cag caa gga		
Notch3	Forward	att gcc gtc agt gga ctc aa	61 °C	239
	Reverse	gat cag gtc gga gat gat gct a		
Notch4	Forward	ggc tga aga aaa gct agg agg a	63 °C	133
	Reverse	gga caa atc cac acc cat ga		
Jagged1	Forward	gcc gtt gca gaa gta aga gtt c	60 °C	101
	Reverse	caa cag atc caa gcc aca gtt a		
Jagged2	Forward	gtc aag gtg gag acg gtt gt	60 °C	146
	Reverse	ctc ctc tcc cgc tct ttc ct		
Dll1	Forward	ggg tgg aga agc atc tga aa	59 °C	116
	Reverse	agt ctt gcc atc tca ctt cca		
Dll3	Forward	aac aac cta agg acg cag ga	59 °C	119
	Reverse	gcg tag atg gaa gga gca gat a		
Dll4	Forward	aca gca aaa cca cac att gg	59 °C	144
	Reverse	atc cga cac tct ggc ttt tc		
GAPDH	Forward	ggc tga gaa cgg gaa gct tgt cat	62 °C	143
	Reverse	cag cct tct cca tgg tgg tga aga		

3.4.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze RT-PCR results. 2% (w/v) agarose gel was prepared with 1X TAE Buffer, and 30 ng/ml ethidium bromide solution was added for visualization. Samples were prepared by addition of 5 µl 6 X loading buffer to each 25 µl product, and 20 µl was loaded to each well. 5 µl of GeneRuler 100 bp DNA Ladder (Fermentas) was used as DNA size marker. Agarose gel was run at 80V for 40 minutes and visualized under BioRad Transilluminator. MultiAnalyst software was used to take photographs of the gels.

Tris Acetic Acid-EDTA (TAE) Buffer:

50 X Stock solution (1 lt): 242 g Tris Base

57.1 ml Glacial Acetic Acid

37.2 g $Na_2EDTA.2H_2O$

1 X working solution: 50 X stock solution is diluted to 1 X with dH_2O.

6 X Gel Loading Buffer (10 ml): 0.020 g Bromophenol Blue

0.020 g Xylene cyanol

6 ml Glycerol

1 mM EDTA

CHAPTER 4 RESULTS

4.1 Expression of Notch Receptors and Ligands in Human Breast Cancer Cell Lines Detected by Semi-Quantitative RT-PCR

Expression of Notch receptors and ligands were detected in six breast cancer cell lines (T47D, BT 474, MCF-7, BT 20, MDA MB 468, MDA MB 453) and one normal mammary epithelial cell line, hTERT-HME1. hTERT-HME1, which is immortalized with hTERT expression, was used as a normal control. GAPDH was used as an equal loading control.

The expressions of three Notch receptors were detected by semi-quantitative RT-PCR (Figure 4.1). Notch4 RT-PCR did not give positive results in any of the cell lines. Notch1 is weekly expressed in hTERT-HME1, while Notch2 has a relatively strong signal, and Notch3 is not expressed. Notch1 is expressed strongly by six breast cancer cell lines, so we can say that Notch1 expression is increased in cancer cell lines compared to hTERT-HME1. Notch2 is expressed almost equally in cancer cell lines and no change is observed compared to hTERT-HME1. The faint band at around 371 bp in Notch2 occurs due to genomic DNA contamination of RNA. Primers also amplified the genomic DNA including 188 bp intronic sequence. Notch3 has the strongest signal among receptors. All six breast cancer cell lines express Notch3 extensively. So, we can say that Notch3 is upregulated in breast cancer cell lines.

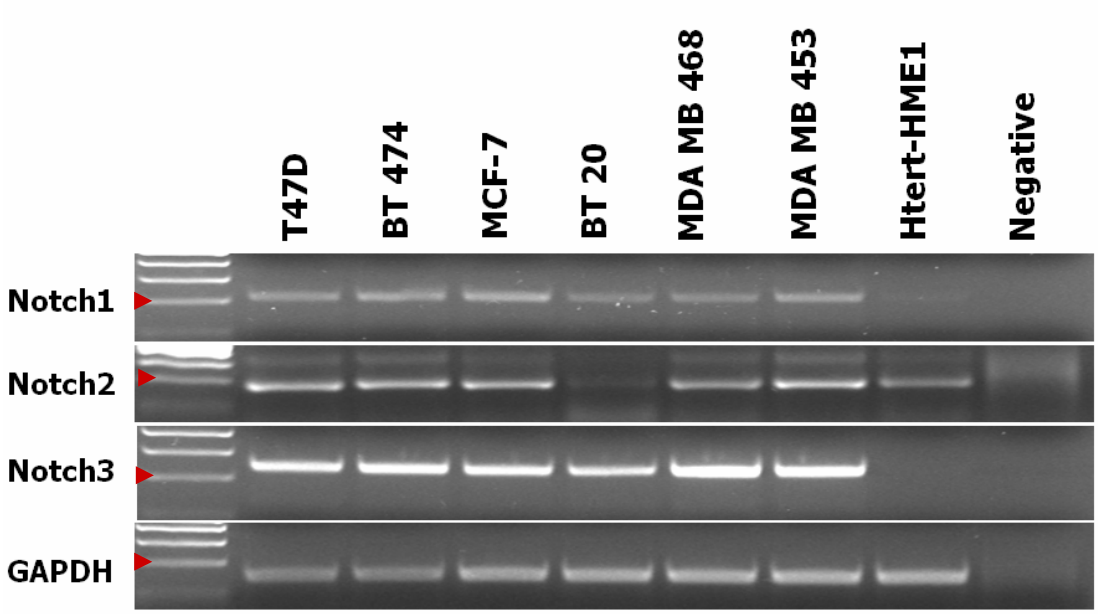

Figure 4.1 Expression of Notch receptors in breast cancer cell lines detected by semi-quantitative RT-PCR. First lane shows the marker and last lane shows the negative control. The sizes of amplified fragment for Notch1, Notch2 and Notch3 are 209, 183, and 239 respectively. The upper band around 371, in Notch2 figure is the genomic DNA amplification product. GAPDH is used as equal loading control. The red arrow head shows the 200 bp size marker.

Figure 4.2 represents the expression of four Notch ligands detected by semi-quantitative RT-PCR. Similar to Notch4, Dll4 RT-PCR gave negative results for all cell lines too. In contrast to receptors, hTERT-HME1 expresses all of the ligands at high levels. Jagged1 is expressed in all of the cell lines, so there is no change compared to hTERT-HME1. Jagged2 is negative only in one cancer cell line, MCF7, and no change is observed for others. Dll1 is also negative only in one cancer cell line, MDA MB 468, and not changed in others. Dll1 has a genomic DNA contamination at around 241, which is the size of expected fragment including 125 bp intronic sequence. Dll3 is expressed weaker in two of the cell lines, MCF-7 and BT 474, and it is negative in two of the cancer cell lines, T47D and MDA MB 453.

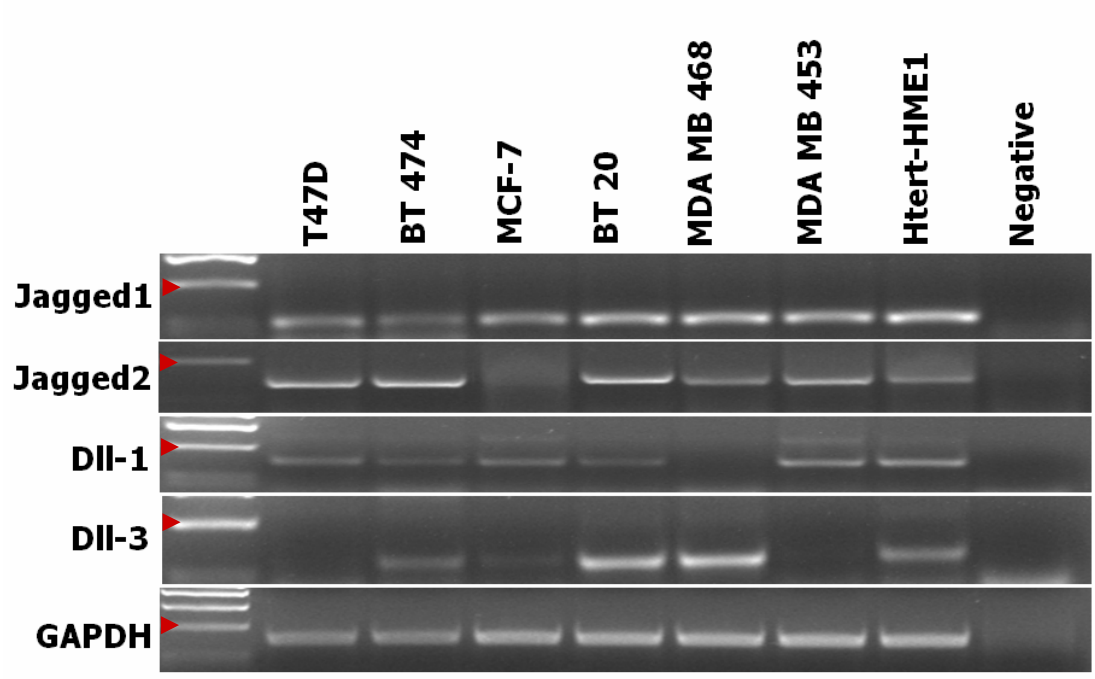

Figure 4.2 Expression of Notch ligands in breast cancer cell lines detected by semi-quantitative RT-PCR. First lane shows the marker and last lane shows the negative control. The sizes of amplified fragment for Jagged1, Jagged2, Dll1 and Dll3 are 101, 146, 116, and 119, respectively. The upper band around 241 in Dll1 figure is the genomic DNA amplification product. GAPDH is used as equal loading control. The red arrow head shows the 200 bp size marker.

4.2 Expression of Notch Receptors and Ligands in Human Breast Cancer Tumor Samples Detected by Semi-Quantitative RT-PCR

Eight different human breast tumor samples are used in semi-quantitative RT-PCR experiments. The results are compared with hTERT-HME1 as a normal control. GAPDH is used for equal loading control.

Figure 4.3 represents the expression of three Notch receptor expression in breast tumor samples. Notch1 has a weak expression generally. Notch1 levels in six of the tumors are almost equal to hTERT-HME1, while two of them, T-57 and T-59, have a lower expression. Notch2 is negative only in one of the tumors, T-43, and has

significant increase in four of them, T-29, T-47, T-57 and T-58, compared to hTERT-HME1. A genomic DNA amplification is seen at around 371, similar to the Notch2 results in cell lines. Notch3 has the most interesting result that six of eight tumors (75%) have significantly upregulated compared to hTERT-HME1.

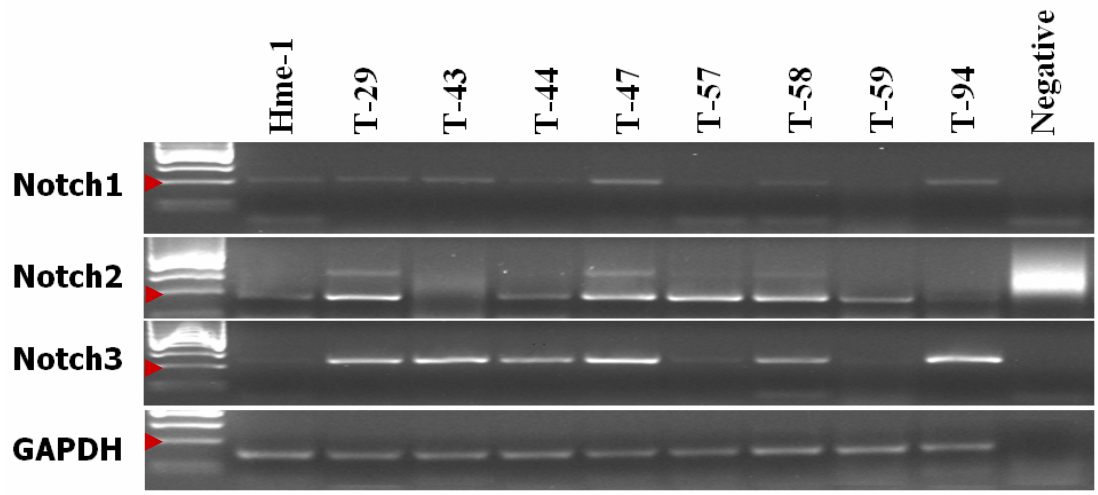

Figure 4.3 Expression of Notch receptors in human breast tumor samples detected by semi-quantitative RT-PCR. First lane shows the marker and last lane shows the negative control. 'T' indicates the 'Tumor'. The sizes of amplified fragment for Notch1, Notch2, and Notch3 are 209, 183, and 239, respectively. The upper band around 371, in Notch2 figure is the genomic DNA amplification product. GAPDH is used as equal loading control. The red arrow head shows the 200 bp size marker.

Figure 4.4 represents the expression of four Notch ligands in eight breast tumor samples. Jagged1 is expressed in all of the tumor samples almost at equal levels in four of the tumors and a slight decrease is observed in other four samples, T-29, T57, T-58 and T-94 with hTERT-HME1. Jagged2 is negative only in T-58, has a lower expression in T-43, T-44 and T-47, and equally expressed in four other tumor samples. Only two of the eight tumors, T29 and T-43, express Dll1, and expression is quite weak. Dll3 is expressed by all tumor samples except T-44, the expression level is slightly lower than hTERT-HME1 in five of the tumors, T-29, T-43, T-47, T-58, T-94.

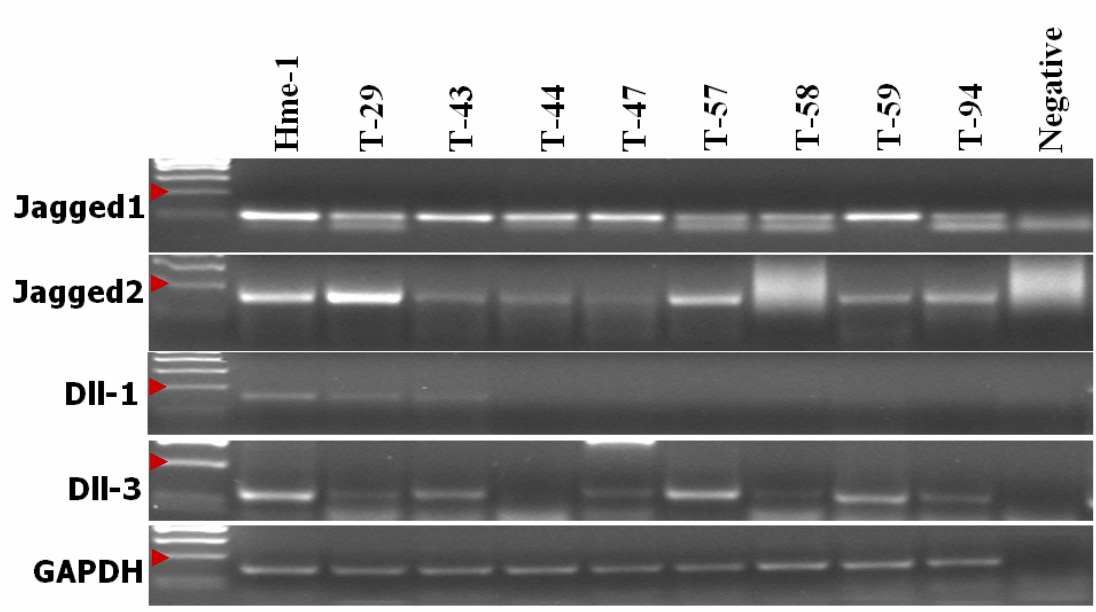

Figure 4.4 Expression of Notch ligands in human breast tumor samples detected by semi-quantitative RT-PCR. First lane shows the marker and last lane shows the negative control. 'T' indicates the 'Tumor'. The sizes of amplified fragment for Jagged1, Jagged2, Dll1 and Dll3 are 101, 146, 116, and 119, respectively. GAPDH is used as equal loading control. The red arrow head shows the 200 bp size marker.

4.3 Summary of Results

Table 4.1 summarizes the expression results of Notch receptors and ligands in breast cancer cell lines and human breast tumors. Notch1 is increased in cell lines, but not in tumors, while Notch2 is increased in half of the tumors but no change is observed in cell lines. However Notch3 is significantly upregulated in both cancer cell lines and tumors. Ligands are positive in hTERT-HME1, but no increase is observed in cell lines or tumors. The expression of ligands generally do not change or negative in cell lines, and decreased or negative in tumor samples.

Table 4.1 Summary of Expression Results.

Gene Family	Genes	HTERT-HME1	Breast Cancer Cell Lines	Human Breast Tumors
Receptors	Notch1	Weakly Positive	Increased (6/6)	Decreased (2/8) No change (6/8)
	Notch2	Positive	No change (6/6)	Increase (4/8) No change (4/8)
	Notch3	Negative	Increased (6/6)	Increased (6/8)
	Notch4	Not Detected	Not Detected	Not Tested
Ligands	Jagged1	Positive	No change (6/6)	Decrease (4/8) No change (4/8)
	Jagged2	Positive	No change (5/6) Negative (1/6)	Decreased (3/8) Negative (1/8)
	Dll1	Positive	No change (5/6) Negative (1/6)	No change (2/8) Negative (6/8)
	Dll3	Positive	Decrease (2/6) Negative (2/6)	Decrease (5/8) Negative (1/8)
	Dll4	Not Detected	Not Detected	Not Tested

CHAPTER 5 DISCUSSION AND FUTURE PERSPECTIVES

Expression of Notch receptors and ligands in human breast cancer cell lines and breast tumor samples was detected by semi-quantitative RT-PCR to investigate any alterations compared to normal cell line, hTERT-HME1.

hTERT-HME1 was immortalized by stable expression of human telomerase reverse transcriptase (hTERT) in primary human epithelial cells. It has the indefinitely division capacity of transformed cell lines but normal in function and phenotype. Transformation requires coexpression of hTERT and other oncogenes. Five pathways, including pRb, p53, Ras, hTERT and PP2A should be disrupted in order to obtain a transformed cell line. hTERT-HME1 has only hTERT expression and has normal p53 expression. hTERT-HME1 has an extended life span but in contrast to transformed cell lines it is anchorage dependent, genetically stable, has a normal diploid karyotype, does not grow in soft agar and form tumor in nude mice (ClONTECHniques, 2000; http://web.mit.edu). Using hTERT-HME1 has many advantages over using normal tissues as control. For example, it is easy to grow cell line and obtain enough material for RNA or protein isolation. It is usually harder to obtain enough normal tissue and isolate qualified RNA. Normal breast tissue also contains fat, and epithelial part may not be separated effectively. However; immortalized cell line has some limitations too. hTERT-HME1 results cannot be as informative as normal tissue because comparison of tumors with their normal pairs

directly shows the altered expression patterns specific to tumor cells. In addition, ectopic hTERT expression may cause some alterations in the cell. For example, in a study, decrease in p16 expression was observed in late passages of immortalized human mammary epithelial cell line (Kim *et al*., 2002). Indeed p16 expression is negative in hTERT-HME1. In this study, we planned to use normal breast tissues of each tumor sample for normal-tumor comparison. However, RNA and cDNA quality of normal samples were not good enough, GAPDH RT-PCR results was not equal to tumors and there were not enough cDNA. So, hTERT-HME1 was used as normal control for both cell lines and tumor samples.

Expression of Notch receptors and ligands in hTERT-HME1 can represent expression patterns of normal mammary epithelial cells generally as explained above. Notch1 is weekly expressed, while Notch2 expression is significantly positive and Notch3 expression is negative. Expression of all ligands are positive, especially Jagged1 has strong expression in hTERT-HME1.

Notch1 expression increased in cancer cell lines compared to hTERT-HME1, but no change is observed in tumor samples that they express Notch1 as weak as hTERT-HME1. The increase in cancer cell lines was expected because of the previous data. Notch1 was one of the MMTV disrupted gene, and overexpression causes mammary gland tumorigenesis in mice (Dievart *et al*., 1999). In another study, Notch1 overexpression is detected in DCIS samples (Siziopikou *et al*., 2002). In this study, *in vivo* results are not consistent with the increase in cell lines. However, Notch1 is still positive in all tumor samples, and comparison of tumors with immortalized cell line may be the reason of the inconsistency with the overexpression in the previous studies. The expression status of Notch1 in normal breast tissue should be identified to further analyze this data.

Notch2 expression does not change in cell lines, but an increase is observed in 50% (4/8) of the tumor samples. Notch2 was not extensively studied in breast, but it is known to be involved in other cancer types. Activation due to viral integration causes T-cell leukemia in cats, and expression in NSCLC and early cervical tumors was detected (Rohn *et al*., 1996, Collins *et al*., 2004; Zagouras *et al*., 1995). However, we cannot be sure if the increase detected in this study is significant

because it is not supported by cell lines. The number of tumor samples should be increased and expression of Notch2 should be identified in normal breast tissue too.

Exposure of receptors to ligands is another way to activate Notch signaling. So, the expression of Notch ligands, Jagged1, Jagged2, Dll1, and Dll3 are also checked by RT-PCR. In contrast to receptors, all ligands are strongly expressed in hTERT-HME1.

Jagged1 expression has no change in all cell lines, and only a slight decrease is observed in 50% (4/8) of tumor samples. Jagged1 mutations were identified in Alagille's syndrome, so it is important in development (Nam *et al*., 2002). However, an abnormality in Jagged1 expression was not reported in cancer. As a result, we can conclude that Jagged1 is expressed by all of the cell lines and tumor samples, so it may have a role in the activation of Notch signaling.

Jagged2 is almost equally expressed in all cell lines, and negative only in one of them. One of the tumor samples does not express Jagged2, and 37.5% (3/8) has decreased expression. We cannot conclude that it is increased or decreased. However, we can say that Jagged2 is present in 5 of 6 cell lines, and 7 of 8 tumor samples. Similar to Jagged1, it may activate Notch receptors.

Dll1 is expressed by 5 of 6 cell lines, but negative in 75% (6/8) of tumor samples. In a study, it was shown that Dll1 was upregulated upon Ras overexpression, which also increases Notch1 expression. So, Dll1 may be involved in tumorigenesis upon Notch1 activation (Weijzen *et al*., 2002). However, our results are not confirmed by this study that it was expected to be upregulated or at least expressed in tumors. It is possible that Dll1 upregulation in the other study is related to overexpression of Ras, and does not occur under physiological conditions, like human tumor samples.

Dll3 is not expressed by two of the cell lines and one of the tumors. There is a decrease in 62.5% (5/8) of tumors, but *in vitro* results do not support this observation. Dll3 mutations are found in Spondylocostal Dysostosis, which is characterized by developmental defects, but not reported in cancers. So, we can say

that Dll3 has potential to activate Notch signaling since its expression is positive in breast cancer cell lines and tumor samples.

Notch3 expression is not detectable in hTERT-HME1, but it is expressed strongly in all of the six cell lines and 75% (6/8) of tumor samples. Notch1 and Notch4 are mostly studied in breast cancer, however Notch3 expression was not identified before. In this study, it was shown that Notch3 expression is upregulated in breast cancer cell lines and tumors. The next question should be whether Notch3 is active. Jagged1-Notch3 interactions were shown to be important in vascular smooth muscle cells that some of the Notch3 mutations decrease Jagged1 interaction in CADASIL (Joutel *et al*., 2004). In addition Dll1 was shown to activate Notch3 in T-cell development (Maekawa *et al*., 2003). So, Jagged1 and Dll1 can bind and activate Notch3. Dll1 is expressed by 5 of 6 breast cancer cell lines, but negative in 75% of tumor samples. So, Dll1 may activate Notch3 in most of the cell lines but not in tumors. However Jagged1 is strongly expressed by all of the cell lines and tumor samples. Hence, Notch3 may be activated by Jagged1 in the breast cancer cell lines and tumors.

Knockout mice studies shows that Notch3 does not have a critical role in development that Notch3 null mice have no apparent phenotype (Krebs *et al*., 2003). Most probably its function is compensated by other Notch receptors during development. Mutations found in CADASIL patients represent the possible role of Notch3 in vascular smooth muscle cells in brain (Joutel *et al*., 1997). Notch3 activation was also shown to upregulate c-FLIP, which inhibits Fas ligand induced apoptosis in vascular smooth muscle cells (Wang *et al*., 2002). The data about Notch3 activity in cancer is not as detailed as other Notch receptors. Notch3 expression is detected in neuroblastoma cell lines and overexpression is frequently observed in NSCLC (Pahlman *et al*., 2004; Collins *et al*., 2004). Transgenic mice expressing Notch3 NICD was shown to induce T-cell leukemia (Bellavia *et al*., 2003). Notch3 functions as an oncogene in these cancer types. Similarly, its activation may contribute to human breast cancer as well. The upregulation of Notch3 in mammospheres represents its activity in putative breast stem cells (Dontu *et al*., 2003b). Together with the overexpression of Notch3 in breast cancer cell lines

and 75% of tumor samples presented in this study, we can conclude that Notch3 activation is involved in human breast tumorigenesis. Upregulation of Notch3 in putative breast stem cells fits to the cancer stem cell hypothesis, which is abnormal Notch activity in breast stem cells induces the maintenance of stem cell properties and inhibits differentiation, which will promote tumor formation.

Notch4 and Dll4 RT-PCR reactions did not give any positive results in cell lines. The reason may be that all cell lines I used are negative for the expression of both genes. Since Notch4 and Dll4 were shown to be important in vascular endothelial cells, it may not be expressed by mammary epithelial cell lines. However, I could not use a positive control that certainly expresses Notch4 or Dll4. So, the other possibility is that RT-PCR reaction did not work due to a problem, most probably related to the primers.

The RT-PCR results show that both cell lines and tumor samples express at least one of the three Notch receptors. They also express at least one of the Dll ligands, except T-44, and one of the Jagged ligands. All of the samples have receptor and ligand, so theoretically have capacity to activate Notch signaling. However RT-PCR represents the expression at RNA level and the presence of receptors and ligands should be confirmed at protein level. Even if proteins exist, the receptors should be transported to the membrane and processed properly to be active.

As a next step it was decided to test Notch receptors' and ligands' expression at protein level by western blotting and localization by immunofluoresence. However, the antibodies ordered from Santa-Cruz Biotechnolgy, did not work well. Polyclonal antibodies gave too many non-specific bands in western blotting. Notch2 and Notch3 staining in immunofluoresence gave positive results at a high primary antibody dilution (1:10) in MCF-7 cell line, but the specificity of the signal was not satisfying. Western blotting and immunofluoresence experiments should be repeated with different, preferably monoclonal antibodies. Antibodies raised against active Notch receptors, NICD, may be used to directly determine the presence of active Notch. In addition, higher number tumor tissues with their normal counterparts should be used to confirm these results, and see Notch3 expression status in normal breast tissue.

The activity of Notch3 in cancer cell lines and tumor samples should be confirmed. Reporter assays detecting the CSL transcriptional activation could be designed to examine Notch3. However, Notch signaling does not have an enzymatic amplification step. Activation is achieved by a few molecules, which makes the detection of reporter signal harder. Localization of the intracellular domain of Notch3 may reflect the activity of the receptor. Localization in membrane reflects the inactivity, while localization in nucleus indicates the active Notch3. Antibodies specific to intracellular domain of Notch3 should be used for immunofluoresence experiments.

Functional studies should be designed in order to identify the role of Notch3 in breast cancer. Knockdown of Notch3 by RNAi technique in a cell line strongly expressing Notch3, may be used to observe the phenotypic and expression profile changes in the absence of Notch3. hTERT-HME1, which does not express Notch3 can be transfected with activated Notch3. Expression profile of main cell cycle regulatory genes; Notch target genes including cyclinD1, ERBB2, and p21; and phenotype should be compared in wild-type and Notch3 transfected hTERT-HME1. Notch3 transfected hTERT-HME1 is expected to show transformed cell line phenotype like growth on soft agar and tumor formation in mice.

Other components of the signaling may also affect the activity of Notch3. For example, glycosylation and three proteolytic cleavages are critical in Notch activity. Any deficiency in the enzymes performing these steps, like Fringe, Furin protease, TACE or presenilins, can inhibit Notch activation. So, further expression and functional analysis of these enzymes may help us to comment on Notch activation in breast cancer. MAML coactivators are also important in initiation of transcription upon Notch activation. It was shown that dominant negative MAML1 could inhibit Notch signaling in leukemia cells transformed with activated Notch1, and increased MAML2 activity due to a translocation induces mucoepidermoid carcinoma (Wu and Griffin, 2004). MAML transcriptional coactivators may be important in Notch signaling activation in breast cancer as well. So, the expression status of MAML may be investigated in breast cancer cell lines and tumor samples.

It was shown that Notch signaling can activate apoptosis by a p53 dependent pathway in neuronal progenitor cells (Yang *et al*., 2004). All of the breast cancer cell lines used in this study have mutated p53, except MCF 7, which has wild type form. There is no correlation between p53 status of cell lines and the expression of Notch receptors and ligands. The induction of p53 dependent apoptosis by Notch signaling may be specific to neuronal tissue. Induction of apoptosis by Notch signaling is not expected in these cell lines because Notch most probably behaves like an oncogene in breast tissue. However, the p53 expression status should be checked in human breast tumor samples to observe if any correlation exists.

An important target gene of Notch signaling is ERBB2, which has a CSL binding site at the promoter and shown to be upregulated by Notch signaling (Chen *et al*., 1997). Induction of ERBB2 may also contribute to Notch induced tumorigenesis in breast. Five of the tumor samples, T-29, T-43, T-44, T-47 and T-59 are ERBB positive, and two tumors, T-57 and T-94, are ERBB negative. However, there is no correlation between ERBB status and expression of Notch receptors and ligands. The number of tumor samples should be increased to investigate any correlation between ERBB2 expression and Notch activity. Cell lines may also be examined for ERBB2 induction. In addition, all Notch3 expressing tumors are invasive ductal carcinoma samples, while two negative tumors are mucinous carcinoma and mixed invasive ductal carcinoma and invasive lobular carcinoma. So, Notch3 expression may be specific for invasive ductal carcinoma type of breast cancer, however; sample size should be increased in order to investigate correlation between Notch3 expression and specific types of breast cancer.

Most of the studies focused on Notch1 and Notch4 in breast cancer. Their significant importance comes from the MMTV integration, and resulted activation in mouse mammary tumorigenesis. Upregulation in expression of two receptors in a group of DCIS samples and Notch4 expression in some of the breast cancer cell lines were detected before (Imatani and Callahan, 2000; Siziopikou *et al*., 2002). However there is no complete data about the expression status of all Notch receptors and ligands in human breast cancer cell lines and tumors. I investigated expression status

of all Notch receptors and ligands in six human breast cancer cell lines and eight breast tumor samples.

In this study, or the first time, Notch3 expression was found to be significantly upregulated in breast cancer cell lines and tumors. Since, Notch3 may have a function in breast stem cell maintenance, its activation may cause breast cells to be arrested at a stem cell like phenotype, gain advantage to accumulate mutations and form tumors. Targeting the proliferating cells only and reducing the size of tumor by classical therapeutic applications cannot cure the cancer and prevent recursion of the disease as long as the cancer stem cells exist. It would be worthy to further analyze Notch3 activity in breast cancer in order to establish a relation between breast stem cells and tumorigenesis, which may mediate generation of novel therapeutic approaches.

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