Role of Growth factor receptor-bound protein 7 (Grb7) in mammary tumour progression

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ABSTRACT

Gene amplification and elevated expression of ErbB2 receptor tyrosine kinase has been implicated in the development and progression of human breast cancer, and correlates to poor clinical outcomes. Transgenic mouse models provide a good tool to study ErbB2 mediated mammary tumourigenesis. In both human ErbB2 positive mammary tumour and ErbB2 knock-in mouse model derived mammary tumours, Grb7 is found to be co-amplified and co-expressed with ErbB2. Here we demonstrated ectopic expression of Grb7 in MDCK cells correlates to the loss of epithelial cell polarity, and this was also found true in primary mammary epithelial cells derived from transgenic mice overexpressing Grb7. Elevated expression of Grb7 in mouse mammary gland during developing stage can lead to incomplete mammary ductal outgrowth accompanied by mammary ducts with multiple epithelial layers and myoepithelial missing. NDL transgenic overexpressing Grb7 had longer tumour latency, and it was found that NDL tumours selectively down-regulated the induced-expression of Grb7.

FRENCH ABSTRACT

L'amplification génique et l'expression élevés de récepteur tyrosine kinase ErbB2 a été impliqués dans le développement et la progression du cancer du sein humain, et corrèle avec de mauvais résultats cliniques. Les modèles de souris transgéniques constituent un bon outil pour étudier la formation de tumeurs médiées par ErbB2 dans la glande mammaire. Dans le cancer mammairehumaines positif pour ErbB2 et le modèle de cancer mammaire de souris ErbB2 knock-in, Grb7 est co-amplifié et co-exprimé avec ErbB2. Ici, nous avons démontré que l'expression ectopique de Grb7 dans les cellules MDCK corrèle avec la perte de polarité des cellules épithéliales, et qui est aussi consistent dans les cellules épithéliales mammaires primaires dérivés de souris transgéniques surexprimant Grb7. Expression élevée de Grb7 dans la glande mammaire de souris pendant la phase de développement peut causer l'incomplets excroissance canalaires mammaires accompagnés par des conduits mammaires avec de multiples couches épithéliales et myoépithéliales manquants. NDL transgéniques surexprimant Grb7 avait une plus longue délais avant formation de tumeur, et il a été constaté que les tumeurs NDL, de façon sélective, perde la capacité d`induirel`expression de Grb7.

ACKNOWLEDGEMENTS

This is the section that I am allowed to thank all those people that aided me in my quest for 'higher' learning. To those people I give my sincerest thanks.

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Finally, I want to thank my parents for their supports. After I left home 7 years ago, I only went back home twice, staying with them for no more than 5 months. Hopefully in the near future, after obtaining my degree, I can live with my parents.

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Akt: V-Akt murine thymoma viral oncogene homolog 1
cDNA: complementary DNA
CGH: Comparative genomic hybridization
DMEM: Dulbecco's modified eagle medium
DNA: Deoxyribonucleic acid
ECL: Enhanced chemiluminescence
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ErbB2: EGF receptor-like receptor 2
FAK: Focal adhesion kinase
FBS: Fetal bovine serum
Grb7: Growth factor receptor bound protein-7
H&E: Hematocylin and Eosin
HER2: Human form of ErbB2
HGF: Heregulin
MAPK: Mitogen activated protein kinase
MMTV-LTR: Mouse mammary tumor virus-long terminal repeat
Neu: Neuroblastoma (rat form of ErbB2)
NDL: Neu deletion
PBS : Phosphate buffered saline
PCR: Polymerase chain reaction
PFA: Paraformaldehyde
PVDF: Polyvinylidene difluoride
RNA: Ribonucleic acid
SDS: Sodium Dodecyl Sulfate
SH2: Src homology 2 domain
TBS: Tris buffered saline

Chapter 1

INTRODUCTION

1.1 Epidermal Growth Factor Receptor (EGFR) family of Receptor Tyrosine Kinases

Cell growth, differentiation and survival are regulated by growth factor signalling. The epidermal growth factor receptor (EGFR) tyrosine kinases are key regulatory proteins enabling the cell to respond to the system and conduct the appropriate biological functions. They are a group of proteins involved in the phosphorylation and activation of signalling proteins, and by doing so, they regulate many processes within the cell. The EGFR family consists of four receptor tyrosine kinases (RTK): ErbB1 (also known as EGFR), ErbB2, ErbB3 and ErbB4. Members of EGFR family of receptor tyrosine kinase all share the same structural scheme: they are transmembrane receptor tyrosine kinase comprising a cysteine-rich extracellular growth factor (ligand) binding domain, a lipophilic transmembrane segment and an intracellular tyrosine kinase domain with a regulatory carboxyl-terminal segment (Bargmann et al., 1986; Coussens et al., 1985; Mosesson, Y. and Y. Yarden, 2004; Schlessinger, 2000).

Members of EGFR familyshare overall 40-50% amino acid identity with the greatest homology in the tyrosine kinase domain (Earp et al., 1995). ErbB3 is an exception for it shares the least sequence homology with other members, having lost the conserved critical residues important for catalytic activity, which explains the catalytic inactivity of ErbB3 molecule (Guy et al., 1994).

Crystallographic studies have identified four subdomains (namely I, II, III, IV) within the extracellular domain. Among these subdomains, I and III contain leucine-rich repeats which are important for ligand binding; subdomain II and IV are rich in cysteine residues (Garrett et al.

2002; Ogiso et al. 2002). Without the presence of ligand, the ErbB receptors exist in an inactivated state. In this state subdomain II interacts with subdomain IV, their interaction in turn, inhibiting the interaction between subdomain I and III, and as a result, the protein's binding site is "closed" (Ferguson et al, 2003; Lemmon et al. 1997). Upon ligand binding, ErbB receptors undergo conformational changes leading to receptor dimerization. Consequently, the catalytic kinase part located in the intracellular domain is activated and "opened", they transfer phosphate groups to the tyrosine residues of nearby molecules, starting the signalling pathway (Dawson et al., 2005; Moriki et al. 2001; Ogiso et al. 2002). Two out of four ErbB receptors, ErbB1 and ErbB4 are autonomous, meaning when bound by a ligand, they undergo dimerization and generate the intracellular signals to regulate cell proliferation, differentiation, migration and survival (Feigin and Muthuswamy, 2009;Yarden. 2001). However the other two receptors, ErbB2 and ErbB3 are considered as non-autonomous. ErbB2 has no known ligand: it functions instead by forming hetero-dimerization with other members of the EGFR family of tyrosine kinase (Peles et al., 1993; Schade et al., 2007). ErbB2 is known as the preferred hetero-dimerization partner to other EGFR receptors since activated heterodimer containing ErbB2 are more stable at the cell surface. The heterodimer containing ErbB2 also has a decreased rate of ligand dissociation, resulting in stronger and more prolonged activation of EGFR signalling (Graus-Porta et al. 1997; Sako et al. 2000; Tzahar et al. 1996; Yarden and Sliwkowski 2001). ErbB3 has the capability to bind a ligand, but it cannot generate a signal by itself as the kinase function of this receptor is impaired. Interestingly, Yarden *et al.* have shown that ErbB2-ErbB3 heterodimers are the most prevalent and potent complexes (Earp et al., 1995; Holbro et al., 2003; Yarden et al. 2002).

1.2 ErbB2 and Breast Cancer

While growth factor induced EGFR signalling is important for many normal cellular processes, the aberrant activity of members of this family has been shown to play a key role in the development and progression of tumour cells (Alimandi et al., 1995; Eppenberger-Castori et al., 2001; Graus-Porta et al., 1997; Morris et al., 1999; Slamon et al., 1987; Venter et al., 1987).

Breast cancer (malignant breast neoplasm) is cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. Worldwide, breast cancer is the most common invasive cancer in women. It comprises 22.9% of invasive cancers in women and 16% of all female cancers (World Cancer Report, 2008).

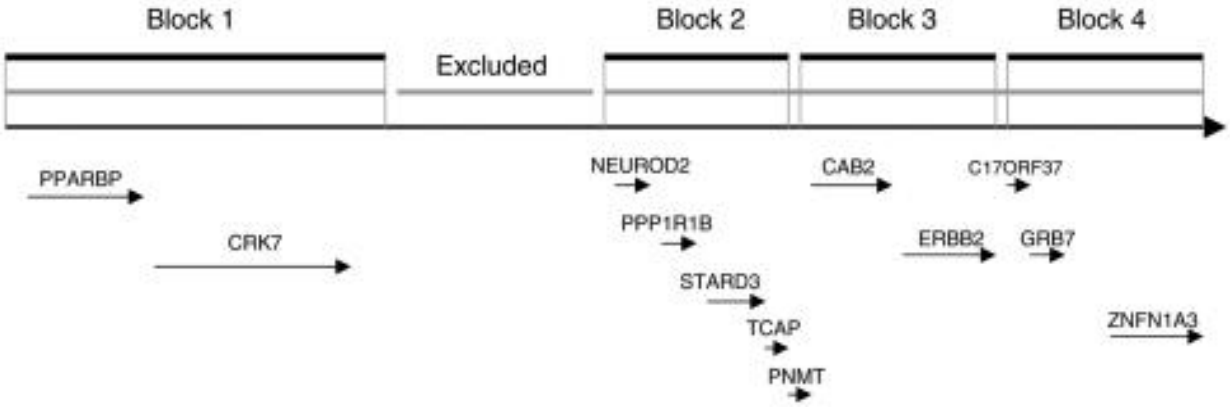
Clinical researches have revealed that among patients with breast cancer, over 20% have elevated ErbB2 expression and ErbB2 overexpression often correlates to poor clinical outcomes characterized by significantly shorter overall survival rate and relatively higher recurrence (Barnes et al., 1992; Slamon et al., 1989; Venter et al., 1987). ErbB2, also known as Human Epidermal growth factor Receptor 2 (HER2) or Neu, was originally identified as a proto-oncogene through the discovery of a point activated form (V664E point mutation in the transmembrane domain, later referred to as NeuNT) in ethylnitosourea-induced rat neuroblastomae (Bargmann et al., 1986; Schechter et al., 1985; Shih et al., 1981). ErbB2 is normally expressed in a variety of cells and tissues excluding those of hematopoietic origin. In human, the erbb2 gene is located on the long (q) arm of chromosome 17 between positions 11.2 increased HER2 protein expression. Overexpression of ErbB2 increases the number of ErbB2-

ErbB3 heterodimers, thereby increasing the ErbB2 signalling, which in turn leads to neoplastic growth (Ravdin and Chamness, 1995; Andrulis et al., 1998; Montagna and Andrechek et al., 2002).

Elevated expression of ErbB2 has been used as a valuable prognosis biomarker for predicting mammary carcinoma progression and making therapeutic plan (Slamon et al., 1987). Currently, the most effective and prevailing pharmaceutical treatment to breast cancer with elevated ErbB2 expression is with the trastuzumab (Herceptin) regime, which is a monoclonal antibody interfering with the HER2/Neu receptor (Hudis, 2007; Slamon et al., 2001).

Breast cancer, as well as almost all other cancers, has been associated with copy number aberrations, including chromosome deletion, or amplification (Hodgson, et al., 2005; Andrulis, et al., 1998). Clinical analysis of breast cancer tumours noticed that when the ErbB2 gene was amplified, a small region around ErbB2 gene was amplified, this small region now scientifically known as the ErbB2 amplicon (Montagna and Anderchek et al., 2002; Sircoulomb et al., 2010). Within the 17q12-q21 region (ErbB2 amplicon), amplification of ErbB2 Stard3, Tcap, Pnmt, Perld1, C17orf37, Grb7, Gsdml, Psmd3 and Thrap4 genes have been reported to correlate with gene expression (Figure 1-1) (Benusiglio et al., 2006; Kauraniemi et al., 2001). Among these genes, ErbB2-C17orf37-Grb7 was identified as the core region of the ErbB2 amplicon (Ramsey et al., 2010; Skotheim et al., 2002; Bai and Luoh, 2008).

Figure 1-1. ErbB2 amplicon. The 400-kb ErbB2 amplicon is split into two (regions **A** and **B**) by a 50-kb segment containing no known gene. Region **A** consists of one LD block while region **B** consists of three blocks, blocks 2, 3 and 4. More than 10 genes are co-amplified with ErbB2, among them, ErbB2-C17orf37-Grb7 was identified as the core region of the ErbB2 amplicon



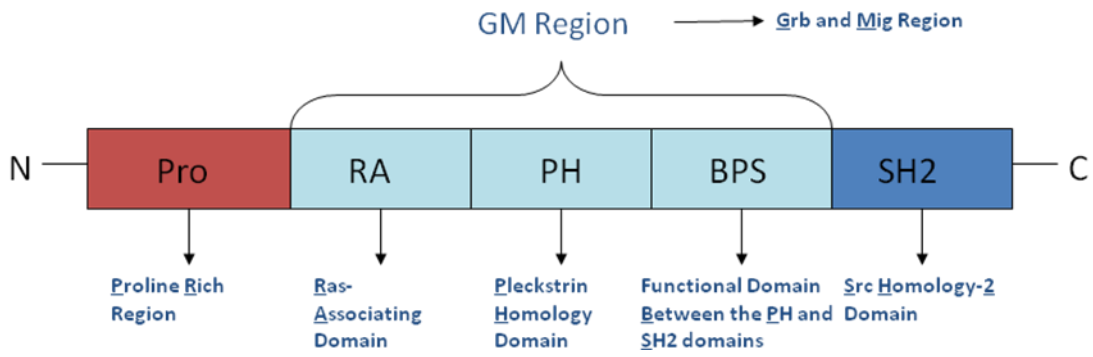
Benusiglio *et al*. 2006

1.3 Grb7 and Grb7 Family Adaptor Molecules

Growth factor receptor-bound protein 7 (Grb7) family adaptor molecules consists of Grb7, Grb10 and Grb14, and each of them has several splicing variants (Daly, 1998; Lucas-Fernandez et al., 2008). Acting as adaptors, members of Grb7 family do not have intrinsic enzymatic activity: they function instead to mediate the coupling of multiple cell surface receptors to downstream signalling pathways in the regulation of various cellular functions. All 3 members of Grb7 family share significant sequence homology with each other and a conserved molecular architecture (Daly, 1998; Li et al., 2005).

The mammalian Grb7 family members have multiple domains and show significant homology to the *Caenorhabditis elegans* protein denoted Mig10, which is involved in embryonic neuronal cell migration (Daly, 1998; Lucas-Fernandez et al., 2008). In their central section they have a region termed GM (for Grb and Mig), which includes a ras-associating (RA) domain, which allows for their interaction with members of the ras superfamily; and a Pleckstrin Homology (PH) domain whose function is to allow their binding to cell membrane phosphoinositides (Shen and Guan, 2002). At its amino terminus, Grb7 has a proline rich domain for interaction with proteins containing a Src Homology 3 (SH3) domain (Tanaka et al., 1997), distal to the GM region is located the BPS (Between PH domain and SH2 domain) domain (Ceccarelli and Sicheri, 2009; Taniguchi et al., 2006). The BPS domain of Grb10 is responsible for its interaction with the insulin and insulin-like growth factor-1 receptors (Stein et al., 2001). At the C-terminus they contain the Src Homology 2 (SH2) domain, through which Grb7 and actually all 3 members of Grb7 family can interact with phosphotyrosine residues in activated receptors and signalling proteins (Figure 1-2) (Avizienyte et al., 2004; Chu et al., 2009; Han and Guan, 2000; 2001; Janes et al., 1997). However, for Grb7 protein only, there is no direct evidence to prove that Grb7 can

Figure 1-2. Structure of Grb7 protein with multi-domains. All 3 members of Grb7 family share the same structural scheme. From N-terminus, Grb7 protein has a proline rich domain, the GM region which includes a ras-associating (RA) domain, a Pleckstrin Homology (PH) domain and BPS (Between PH domain and SH2 domain) domain; at the C-terminus they contain the Src Homology 2 (SH2) domain


GM Region
Grb and Mig Region
N
Pro
RA
PH
BPS
SH2
C
Proline Rich Region
Ras-Associating Domain
Pleckstrin Homology Domain
Functional Domain Between the PH and SH2 domains
Src Homology-2 Domain

bind ras superfamily, insulin-like growth factor receptor and SH3 containing protein.

Although all 3 members of Grb7 family share the same multidomain structural scheme, the binding potential and binding partners of each member are quite different. For instance, the SH2 domain of Grb7 shares about 70% amino acid identity with the SH2 domains of Grb10 and Grb14. However, the different family members seem to have different preferences for binding partners: Grb7 binds to ErbB2 strongly through SH2 domain, but Grb14, which also has SH2 domain, cannot associate with ErbB2. In addition, Grb7 has been shown to be able to associate with Focal Adhesion Kinase (FAK) via its SH2 domain, but Grb10 does not bind to FAK. These observations suggest that the different Grb7 family members have evolved to interact with specific functional partners (Daly, 1998; Li et al., 2005).

The high homology of Grb7 family proteins with the *C. elegans* protein Mig-10, which is involved in the regulation of neuronal cell migration during embryonic development, suggests that Grb7 may play a role in mammalian cell migration. This is supported by the observation that the SH2 domain of Grb7 can directly interact with autophosphorylated Tyr^{397} of FAK, and increases the phosphorylation of FAK. In addition, several research groups also demonstrated that Grb7 can interact with phosphatidylinositol phosphates through its PH domain both *in vitro* and in intact cells, and this interaction is thought to play a role in migration. A study by Kao and Pollack demonstrated that knockdown of GRB7 resulted in decreased cell proliferation and cell-cycle progression (Han and Guan, 2000, 2001; Kao and Pollack, 2006)**.**

Recently clinical studies have shown that increased expression of Grb7 correlates with a lower survival rate in breast cancer patients with tumour with either high or low expression of ErbB2.

In addition, clinically, Grb7 has been used as a prognostic marker and therapeutic target in breast cancer (Nadler et al., 2010; Ramsey et al., 2010; Skotheim et al., 2002).

It was suggested that Grb7 protein by itself may contribute to the tumour phenotypes independent of ErbB2 protein overexpression. A research group studying a subset of testicular germ cell tumours shown that in these tumours, Grb7 mRNA, rather than ErbB2 mRNA was overexpressed. This observation strengthened the hypothesis that Grb7 gene, rather than ErbB2 gene, is the target of gene amplification and selective retention. In other words, Grb7 might be the driving force of ErbB2 amplicon amplification (Skotheim et al., 2002).

1.4 14-3-3 sigma in ErbB2 mediated breast cancer

As previously mentioned, in addition to ErbB2 amplification, tumourigenesis in the ErbB2 knock-in model is frequently associated with recurrent deletions of chromosome 4. Refined mapping of chromosome 4 deletion revealed that 14-3-3σ the tumour suppressor was frequently lost (Genuardi et al., 1989; Montagna and Anderchek et al., 2002). Interestingly, loss of 14-3-3 σ expression has been noted in a large proportion of primary human breast cancers (Vercoutter-Edouart et al., 2001). 14-3-3 σ is involved in the cytosolic sequestration of the EGR2 transcription factor, a critical factor in the up-regulation of ErbB2 expression. Thus, ErbB2 induced tumours may select against 14-3-3 σ expression to prevent its inhibitory effects on ErbB2 expression (Dillon et al., 2007). Ectopic expression of 14-3-3 σ tumour suppressor was able to restore epithelial polarity in ErbB2 tumour cells (Fergin et al., 2009). Conversely, the targeted ablation of 14-3-3 σ in established epithelial cells also leads to disruption of epithelial cell polarity. It was also demonstrated that disruption of 14-3-3 σ tumour suppressor in

mammary epithelium is associated with loss of epithelial polarity that was further correlated with an increase in proliferative capacity of these cells (Ling et al., 2010).

1.6 Mouse Models studying Neu-mediated mammary tumourigenesis

Elevated expression of ErbB2 was first found to be associated with a subtype of breast cancer in 1987 (Slamon et al.), since then, over the past 20 years, researchers have done extensive work studying the role of ErbB2 as well as its related signalling pathway.

The first major transgenic mouse model studying ErbB2 in breast cancer was to express point mutation activated Neu (NeuNT) using a strong viral promoter called Mouse Mammary Tumour Virus Long Terminal Repeat (MMTV-LTR) (Muller et al., 1988). The use of MMTV-LTR promoter ensured high levels of activated Neu expression primarily in the mammary epithelium. This transgenic mouse model developed mammary tumours with a very short tumour latency (3 months). It was the first transgenic mouse model shown that enhanced expression of activated Neu was sufficient to promote mammary tumourigenesis. However, this mouse model had its limitation by requiring the use of a point activated form of Neu (NeuNT), which had not been observed to be expressed in human breast cancers.

Another mouse model used a similar approach, but to express wild type Neu under the MMTV-LTR promoter (Guy et al., 1992). The resulting transgenic mice developed focal mammary tumours with a latency of approximately 190 days. It was demonstrated that the activation of Neu in these transgenic mice occurred through somatic mutations located within the transgene. Sequence analysis of these mutations revealed that they contain in-frame deletions of 7 to 12 amino acids in the extracellular region proximal to the transmembrane domain. It is believed that

the in-frame deletions resulted in an imbalance of cysteine residues, this imbalance in turn lead to constitutive Neu activation through the formation of disulphide-bridged homodimers, resulting in the induction of metastatic mammary tumours (Siegel et al., 1994; Siegel et al., 1999).

In order to determine the role of these somatic mutations in inducing mammary tumourigenesis, transgenic mice, named NDL, for Neu Deletion mutants, were generated to express Neu transcript with a 16 amino acid in-frame deletion in the extracellular domain under the control of MMTV-LTR promoter (Siegel et al., 1999). As expected, multi-focal mammary tumours developed within approximately 6 months, which was shorter than transgenic mice expressing wild type Neu. It was believed that the shortened tumour latency was due to the fact that NDL transgenic mice express a somatic mutant form of Neu, bypassing the time required to induce the somatic mutations. Biochemical analysis has shown that when in-frame deletion occurs, the mutant Neu was constitutively activated, and displayed 10- to 15- fold increase in tyrosine phosphorylated ErbB3 receptor in mammary tumours, resulting increased ErbB3. In the parallel study with human primary breast tumour samples, the co-expression of ErbB2 with ErbB3 was found as well (Quinn, et al., 1994).

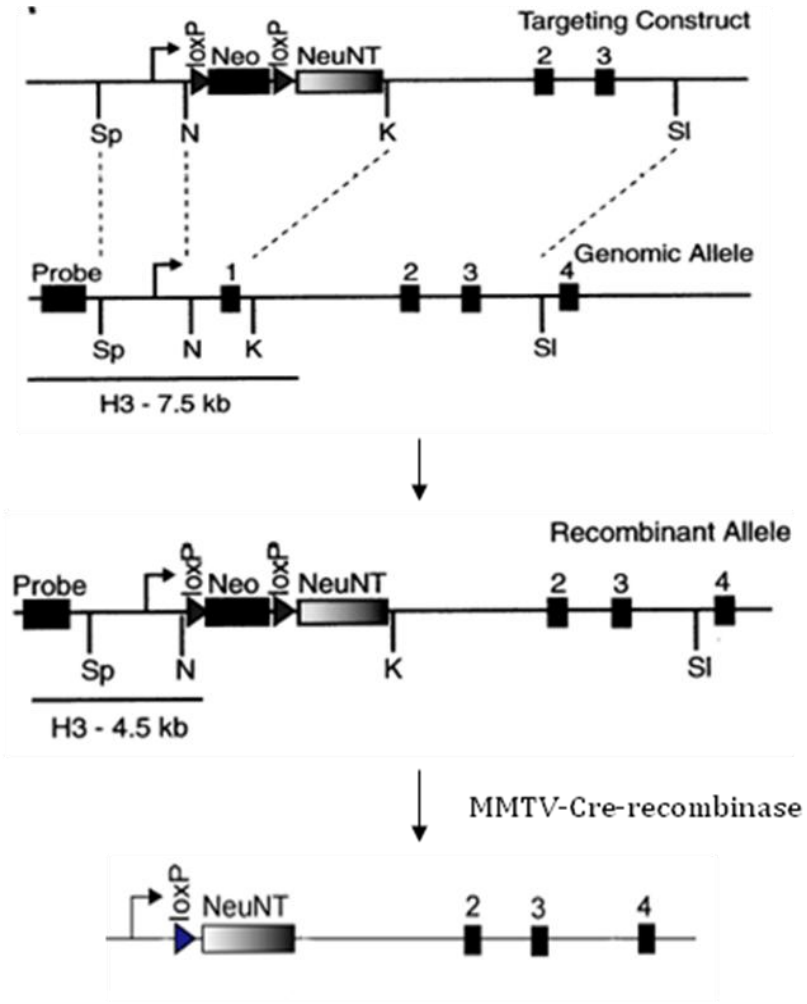
Interestingly, in an attempt to find similar somatic mutations in human breast cancer, although researchers failed to detect comparable somatic mutations in ErbB2 in human breast cancer, they successfully identified the existence of an alternatively spliced ErbB2 isoform encoding a 16 amino acid in-frame deletion in the juxtamembrane domain, which closely resembled the NDL mutation (Kwong and Hung, 1998; Siegel et al., 1999; Zito et al., 2008). All of these mouse models provided useful information about ErbB2 and its role in mammary tumourigenesis; however, the use of the MMTV-LTR as promoter to drive transgene expression limited these mouse models in featuring human breast cancers. This is because firstly, MMTV promoter is a

strong viral promoter which is not likely to be relevant to human breast cancers. Secondly, as when the MMTV driven-expressing-ErbB2 construct was integrated into the mouse genome, depending on the integration sites the construct was located, it can exhibit different expression patterns and have quite different phenotypes (in term of tumour latency). Thirdly, it has been reported that MMTV promoter is hormonally regulated, therefore may be indirectly impacted by the hormonal status of the mice.

Then in 2000, another mouse model called ErbB2 knock-in model was generated. Compared with former mouse models, this knock-in model achieved mammary-specific expression of activated NeuNT under the control of the endogenous promoter, but is transcriptionally silenced by an upstream loxP flanked neomycin cassette (Anderchek et al., 2000). The mammary specific expression of NeuNT is achieved by crossing those animals with MMTV-CRE transgenic mice, the cre recombinase excising the neomycin cassette, specifically in mammary gland, resulting in single copy expression of the NeuNT transgene under endogenous promoter (figure 1-3).

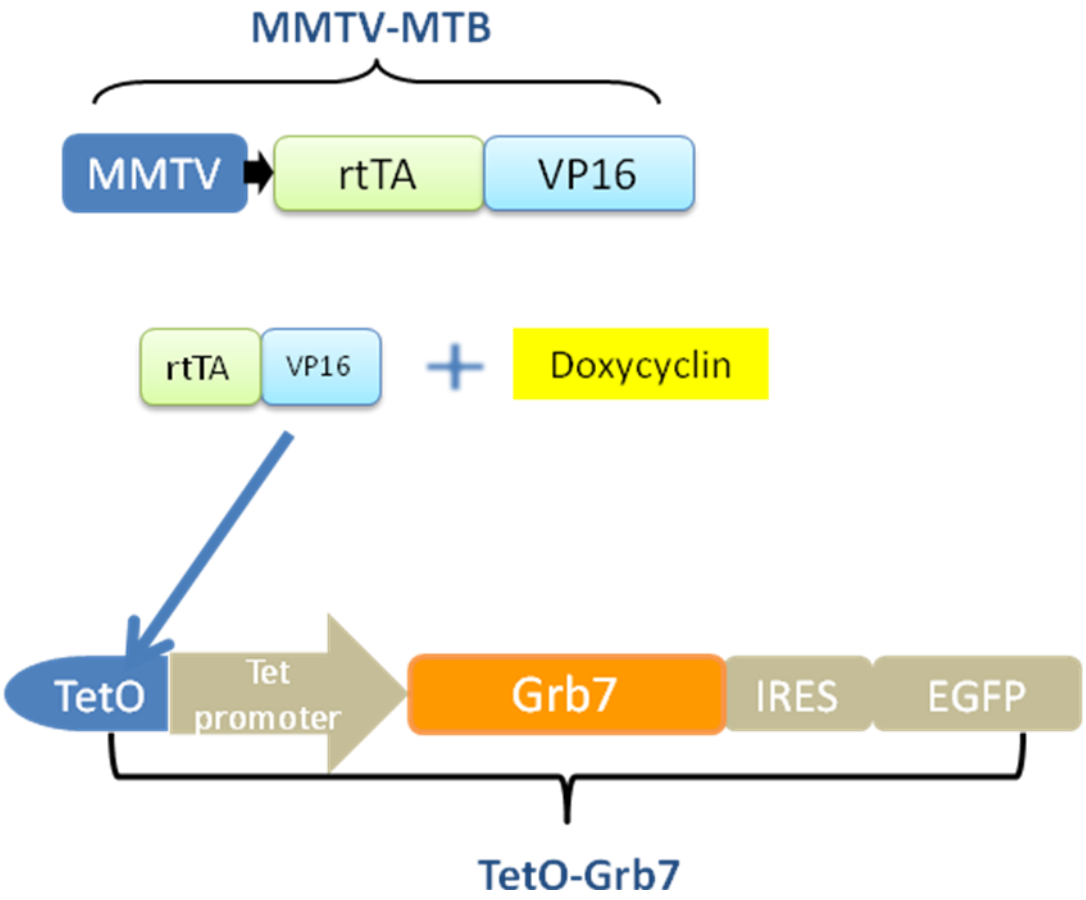
The induction of mammary tumours in this model is associated with increased expression of the activated Neu allele. In 85% of the mammary tumours, increased transgene expression is achieved through the selective amplification of NeuNT (2-22 copies). The remaining 15% of breast tumours show increased NeuNT protein expression without genomic amplification. This model however has long tumour latency, about 16 months. The ErbB2 knock-in model shows several key similar features that are seen in human breast cancers including frequent deletion of part of chromosome 4 and ErbB2 amplicon amplification on chromosome 11 (Anderchek et al 2000).

Figure 1-3. ErbB2 knock-in mouse model. This model achieves mammary-specific expression of activated NeuNT under the control of the endogenous promoter, but is transcriptionally silenced by an upstream loxP flanked neomycin cassette. The mammary specific expression of NeuNT is achieved by crossing those animals with MMTV-CRE transgenic mice, the cre recombinase excising the neomycin cassette, specifically in mammary gland, resulting in single copy expression of the NeuNT transgene under endogenous promoter


Targeting Construct
loxP
Neo
loxP
NeuNT
2
3
Sp
N
K
Sl
Probe
1
2
3
4
Genomic Allele
Sp
N
K
Sl
H3 - 7.5 kb
Recombinant Allele
Probe
loxP
Neo
loxP
NeuNT
2
3
4
Sp
N
K
Sl
H3 - 4.5 kb
MMTV-Cre-recombinase
loxP
NeuNT
2
3
4

To address the issue of whether the sustained expression of Neu was required for the maintenance of tumour phenotype, researchers also generated a mouse model that can express activated Neu in an inducible fashion. This is achieved by using the bi-transgenic (TetO-NeuNT / MMTV-MTB) mouse that conditionally expresses NeuNT in the mammary epithelium under the dual control of the MMTV promoter and a tetracycline regulatory element (Figure 1-4): the tetracycline regulatory element functions as in the presence of doxycycline, and the reverse tetracycline dependent trans-activator (rTA) can bind DNA at a TetO operator. Once bound, the TetO operator will activate a promoter coupled to itself, activating the transcription of the nearby gene. By adding doxycycline, the expression of NeuNT could be controlled (Gunther et al., 2002). Although this bi-transgenic system failed to prove that sustained expression of Neu was required for mammary tumour progression as many of the mice developed Neu-independent mammary tumours, this unique mouse model provides a valuable tool to study protein functions and their role in mammary tumourigenesis. In this thesis we have characterized similar transgenic mice that express Grb7 in an inducible fashion: we have successfully generated transgenic mice named TetO-Grb7, which replaces the NeuNT cDNA with mouse Grb7 cDNA. It has been tested that the bi-transgenic mice (TetO-Grb7 mice / MMTV-MTB) can express elevated level of Grb7 in the presence of doxycycline in the drinking water, and cannot express elevated Grb7 when doxycycline is not available (Figure 1-4). This mouse model provides us a useful tool to directly assess the importance of Grb7 in ErbB2 mammary tumour progression.

Figure 1-4. The tet-on mouse system. The controlled expression of Grb7 in mammary gland is achieved by crossing MMTV-MTB transgenic mice with TetO-Grb7 transgenic mice. The tet-on system functions in the presence of doxycycline, and the reverse tetracycline dependent trans-activator (rTA) can bind DNA at a TetO operator. Once bound, the TetO operator will activate a promoter downstream, activating the transcription of the nearby gene. Enhanced-GFP (EGFP) serves as a reported gene.



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EXPERIMENTAL RATIONALE

The fact that elevated expression of ErbB2 has been implicated in 20 to 30% of primary breast cancers and correlates with a poor prognosis and clinical outcome have urged many researchers to study the signalling mechanisms that are involved in the ErbB2 mediated mammary tumourigenesis. ErbB2 overexpression in human breast cancer has been shown to occur through genomic amplification. In addition, the human ErbB2 positive breast cancers is highlighted by the existence of ErbB2 amplicons comprising approximately 10 genes that are closely linked to the ErbB2 locus.

Among these genes co-amplified with ErbB2, Grb7 is thought to play an important role in ErbB2 signal transduction. Through its unique SH2 domain, Grb7 has been demonstrated to physically associate with tyrosine phosphorylated ErbB2. Grb7 family members participate in important cellular functions such as signal transduction, cell motility and tumour progression, but the precise role of the Grb7 adapter molecule is still not clear. Several research groups studying oesophageal carcinoma previously pointed out the importance of co-overexpression of Grb7 with ErbB2 in oesophageal carcinogenesis. In addition, our laboratory has recently demonstrated that ectopic expression of Grb7 in polarized MDCK epithelial cells resulted in a loss of epithelial polarity that was further accelerated by growth factor stimulation. Conversely, using RNA interference against Grb7, the polarity of these Grb7 expressing MDCK cells was restored. Given that loss of epithelial polarity is a critical hallmark in tumourigenesis (Lee et al., 2008; Huber et al., 2005; Herzig et al., 2007), it is possible that the selection for co-expression of Grb7 and ErbB2 in ErbB2 tumour induction reflects this important requirement for loss of cell polarity. Recently, the expression of 14-3-3σ, a tumour repressor, was found to be down-regulated in

Grb7 expressing cells. Taken together, these results and observations from previous research urgently state the necessity to investigate the role of overexpression in Grb7 in ErbB2 positive breast cancer.

The importance of ErbB2 in mammary tumour development has been demonstrated through the use of transgenic mouse models expressing various constitutively active forms of the receptor in the mammary epithelium by use of the mouse mammary tumour virus (MMTV) promoter-enhancer. In order to mimic more closely the events involved in ErbB2 induced mammary tumour progression, transgenic mice that carry Cre inducible activated ErbB2 under the transcriptional control of the endogenous ErbB2 promoter (ErbB2 knock-in model) were created. The similarity of this mouse model to ErbB2 initiated human breast cancer is highlighted by the fact that both human and mouse ErbB2 amplicons comprise the same core 10 genes that are closely linked to the ErbB2 locus. Furthermore, expression analysis of mouse tumours have revealed that of these 10 genes, some genes (Stard3, Grb7, Perld1, and ErbB2) are expressed at elevated levels in the ErbB2 knock-in model, which is also observed in human breast cancers. Therefore, the mouse models available allow us to investigate the role of Grb7 in mammary tumourigenesis in addition to *in vitro* approaches and clinical studies.

Chapter 2

MATERIALS & METHODS

Cell Cultures.

MDCK cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Wisent), TM15 cells were maintained in DMEM with 10% fetal bovine serum, and 100ug/mL of streptomycin, 100 IU/mL penicillin, 10ug/mL insulin (Sigma-Aldrich, St.Louis, MO, USA). Primary mammary epithelial cells were obtained from mouse mammary glands. Mammary glands were harvested and processed with McILWAN Tissue Chopper, and later disassociated in DMEM with 2.4mg/ml Collagenase B (Roche) and 2.4mg/ml Dispase II (Roche) at 37℃ for 3 hours. Disassociated cells were washed with PBS/EDTA (1mM). They were maintained in DMEM with 2% fetal bovine serum and MEGS supplement (Cascade Biologics).

Fugene Transfection

Cells were previously plated in a 6 centimetre plate one day prior to transfection in order to achieve 60-80% confluency. The next day, 9 uL of Fugene agent was added to 291 uL of DMEM without contact with plastic, and allowed to incubate for 5 minutes at room temperature. Later 3 ug of DNA was added to the mixture, and allowed to incubate at room temperature for 30 minutes. Finally the mixture was pipetted dropwise onto the cells. Selection was performed after 2 days.

Monolayer Immunofluorescence staining

First cover slips were placed into each well of a 24 well plate (NUNC), then 1 x 10^5 cells of interest was plated into each well. Cells were allowed to grow for 2 days, once cells had reached optimal density, medium was aspirated and followed by 2 washes with 1X PBS (pH 7.4) and cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature. After 3 washes with PBS, the sample was permeabilized with PBS containing 0.2% TritonX-100 for 10 minutes. The sample was rinsed 3 times with PBS/Glyciine (100 mM glycine in PBS) then incubated with IF buffer (PBS + 0.2% Triton X-100+ 0.05% Tween-20) plus 2% BSA for 30 minutes at room temperature. The sample was incubated with primary antibody in the blocking solution for 1 hour at room temperature and followed by 3 washes with IF buffer. It was then incubated with fluorescent conjugated secondary antibody in blocking buffer for 45 minutes. After 3 times of IF buffer wash, the sample was incubated with PBS containing 0.5ng/ml 4'6-diamidino-2-phenylindole (DAPI) (Jackson Laboratories) for 5 minutes at room temperature. Finally the cover slip was mounted with immu-mount (Thermo Scientific) and allow to dry overnight.

3-Dimensional culture and Immunofluorescence staining

For 3D collagen cultures, 24-well plates (NUNC) were utilized. For each well, 350µl 80% collagen I (Inamed) in PBS (PH7.4) was plated and solidified at 37^0C without CO_2 for 45 minutes. Subsequently, 3000 mono-dispersed cells mixed with 500µl collagen were seeded on top, and incubated at 37^0C without CO_2 for 30 minutes. 500µl Leibovitz's L-15 medium (Invitrogen) was applied into each well, and changed every 4 days. For HGF stimulation, after 12 days' culture, HGF (provided by Dr. Morag Park) was added in the medium to a final

concentration of 10 units/ml, and cells were cultured with HGF for an additional 2 days before subsequent staining. For 3D Matrigel cultures, 8-well Chamber slides (NUNC) were utilized. For each well, 100µl Matrigel (BD Biosciences) was plated and solidified at 37℃ for 30 minutes. Later, 1500 mono-dispersed cells mixed in 300µl DMEM with 2% fetal bovine serum (BSA), 2% Matrigel, and MEGS supplement were seeded on top. Medium was changed every 4 days. Cells were stained after 10 days of culture.

Samples (matrigel or collagen) were fixed with 2% paraformaldehyde in PBS, permeabilized in 0.5% Triton X-100 in PBS, followed by washes of 100 mM glycine in PBS. Blocking was performed with IF buffer (PBS, 0.1% BSA, 0.2% Triton X-100 0.05% Tween-20) + 2% BSA followed by 60 minute incubation at RT with the following primary antibodies diluted in IF buffer:14-3-3 sigma (1:100; Millipore); E-Cadherin (1:100; BD Transduction Laboratories); ZO-1 (1:100; Zymed). Cells were washed in IF buffer and incubated in a humidified chamber with the appropriate Alexa-fluor conjugated secondary antibodies (1:1000; Molecular Probes) diluted in IF buffer for 45 minutes at room temperature (RT). The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Jackson Laboratories).

Migration/ Invasion assay using Boyden Chamber

In a sterile 24- well plate (Falcon), a sterile Boyden chamber (Falcon) was added to each well. For invasion assay, 50 uL of 5% v/v matrigel in DMEM was added to each chamber filter disk, and allowed to incubate at 37 ^{0}C for 1 hour to polymerize.

800 uL of media containing 10% FBS (or desired chemoattractant) was added to the bottom chamber, and 500 uL of serum-free medium containing a specific amount of cells was added to

the top chamber. After 1-day incubation in 37 ^{0}C, the media from both top and bottom chambers were aspirated and washed 3 times with PBS, cells were immediately fixed in 10% formalin (Surgipath) for 20 minutes. After 3 washes with PBS, chambers were stained with crystal violet stain for 20 minutes. The chamber were washed with distilled water and cells left on top chamber were scraped away, leaving only those that migrated (or invaded, if matrigel was added) through the membrane filter. Quantification was done using microscope.

Cell lysis for Protein Extraction

Once cells have reached optimal density, cell plate was placed on ice, and the medium was aspirated and washed once with cold 1X PBS solution. All subsequent steps were performed on ice. One millilitre of cold NP-40 lysis buffer (0.5% Nonident P 40, 50mM Tris-HCl with pH 7.5, 2mM EDTA, amM NaF, 150mM NaCl, 1mM Na3VO4, 10mg/ml of aprotinin, 10ug/ml of leupeptin) was added. Cell plate with lysis buffer was allowed to incubate for 10 minutes, and stirred every 5 minutes. Cells were scrapped with a sterile plastic scraper into a microcentrifuge, and centrifuged at 4 ^{0}C and 13,200 RPM for 10 minutes. Supernatant was collected and quantified by the Bradford protein assay system (BioRad, Hercules, CA, USA). Protein lysates were stored at -80 ^{0}C.

Protein Extraction from frozen animal tissues

Liquid nitrogen frozen samples of mammary gland or tumours was placed in a pre-chilled pestle and crushed to powder with a pre-chilled mortar while still submerged in a small portion of liquid nitrogen. The resulting mixture of liquid nitrogen and sample in powder form were

transferred to a 50 mL Falcon tube sitting on ice to allow the liquid nitrogen to dry. Then the cold NP-40 lysis buffer was added (1 mL for tumour piece, 300uL for mammary gland). The sample was allowed to sit on ice for 15 minutes to lyse, with being vortexed every 5 minutes. After lysis the lysate was transferred to a microcentrifuge tube and centrifuge at 4^0C and 13,200 RPM for 10 minutes. The supernatant was collected and quantified using the Bradford protein quantification assay (BioRad, Hercules, CA, USA). Protein lysates were stored at -80 ^{0}C.

Western Blotting

Previously extracted protein lysate was resuspended in 6X SDS-PAGE loading buffer in appropriated ratio, and was denatured by incubating sample in 95^0C dry bath incubator (Fisher Scientific) for 10 minutes. Sample was then loaded and separated by 10% polyacrylamide gel. Separated protein on the PAGE gel was then transferredonto a polyvinylidene difluoride (PVDF) membrane (Immobilon FL) using a wet transfer (Bio-Rad) with transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) for 90 minutes at 1.25 A. The membrane was later blocked with 5% skim milk in TBS-T (or 5% BSA in TBS-T) (TBS-T is TBS solution with 0.1% Tween-20) for 1 hour. Then the membrane was incubated in primary antibody overnight at 4 ^{0}C. After washed 3 times with TBS-T, membrane was incubated with secondary antibody for 45-60 minutes, followed by 3 times of wash with TBS-T. The membrane was then incubated in the enhanced chemiluminescence (ECL, Amersham) reagent and exposed onto a film (Kodak Biomax Light).

Immunohistochemistry (IHC).

Paraffin embedded sections were deparaffinized in 3 changes of xylenes. Sections were heated in 10 mM sodium citrate (pH 6), followed by incubation in 3% H_2O_2 for 20 minutes. Samples were incubated in primary antibodies of CK8, CK14 (1:100; Covance) and Ki67 (1:100; Cedarlane) diluted in PBS/2% BSA for 1 hour at RT. For IHF, samples were incubated in a humidified chamber with the appropriate Alexa-fluor conjugated secondary antibodies (1:1000; Molecular Probes) diluted in IF buffer for 45 minutes at RT, followed by nuclei counterstaining with DAPI. For IHC, samples were subject to incubation at RT with HRP-conjugated secondary antibody (1:1000 in PBS/2% BSA; Jackson Laboratories). Immunoreactivity was visualized using the DAB+ substrate chromagen system (DAKO) and the tissues were counterstained with hematoxylin. For TUNEL IHC staining, ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Serological) was used.

Confocal Imaging

Confocal imaging was performed using an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) with 63X/1.4 plan-APOCHROMAT objectives equipped with a confocal microscope system (LSM 510 Meta confocal microscope; Carl Zeiss MicroImaging, Inc.). Image analysis was carried out using the LSM 5 image browser (Empix Imaging).

Tail DNA extraction

A tail size of 0.5 cm was excised from transgenic mouse and placed in an Eppendorf tube. 500ul tail buffer (10mM Tris-HCl [pH 8.0], 10mM EDTA, 100mM NaCl, 0.5% SDS) and 10 ul of 20mg/mL proteinase K (Invitrogen) were added and left to digest in 55 ^{0}C waterbath for 1 day. After digestion, 200 uL of 5M NaCl solution was added and vortexed to mix, sample was centrifuged at 13,200 RPM at 4 ^{0}C for 10 minutes. After centrifugation, supernatant was collected and 800uL of 100% ethanol was added. Sample was mixed by inverting a couple of times and then was centrifuged at 13,200 RPM at 4 ^{0}C for 10 minutes. The ethanol was removed and the DNA pellet was resuspended in TE buffer.

Mouse breeding & Mouse Colony Maintenance

For mouse colony maintenance, either male or female transgenic mice were interbreed with FVB (except for MMTV-Neu-NDL2-5 mice, for only males are good breeders) to generate more offspring. 5 males and 5 females were kept as stock.

For generating MMTV-MTB/TetO-Grb7 mice, female MMTV-MTB bi-transgenic mice were crossed with male TetO-Grb7 mice, or vice versa.

For generating MMTV-MTB/TetO-Grb7/MMTV-Neu-NDL2-5 tri-transgenic mice, the male MMTV-Neu-NDL2-5 mice were crossed with female MMTV-MTB/TetO-Grb7.

For generating MMTV-MTB/TetO-Grb7/MMTV-CRE/loxp-NeuNT tetra-transgenic mice, the male MMTV-CRE/loxp-NeuNT mice were crossed with MMTV-MTB/TetO-Grb7 female mice.

Mammary gland wholemounts

Mice were euthanized before necropsy under the guidelines of the Canadian Council for Aniamal Care (CCAC). The number 4 mammary gland of the virgin female mice was excised, spread upon a glass plate and dried for 20 minutes. Then the glass plate with mammary gland was allowed to fully submerged in acetone for at least one day to remove the fat connective tissue. After this, the glass plate was stained with hematoxylin (Harris' Modified, Fisher Scientific) overnight. The stained mammary gland was placed in 70% ethanol containing 1% v/v HCl to destain until the background was clear and the ductal outgrowth of the mammary gland was well-defined. The mammary gland was hydrated in 100% ethanol for 1 hour and then placed in xylene solution overnight. The mammary gland was mounted under a coverslip using permount (Fisher Scientific) and left to dry.

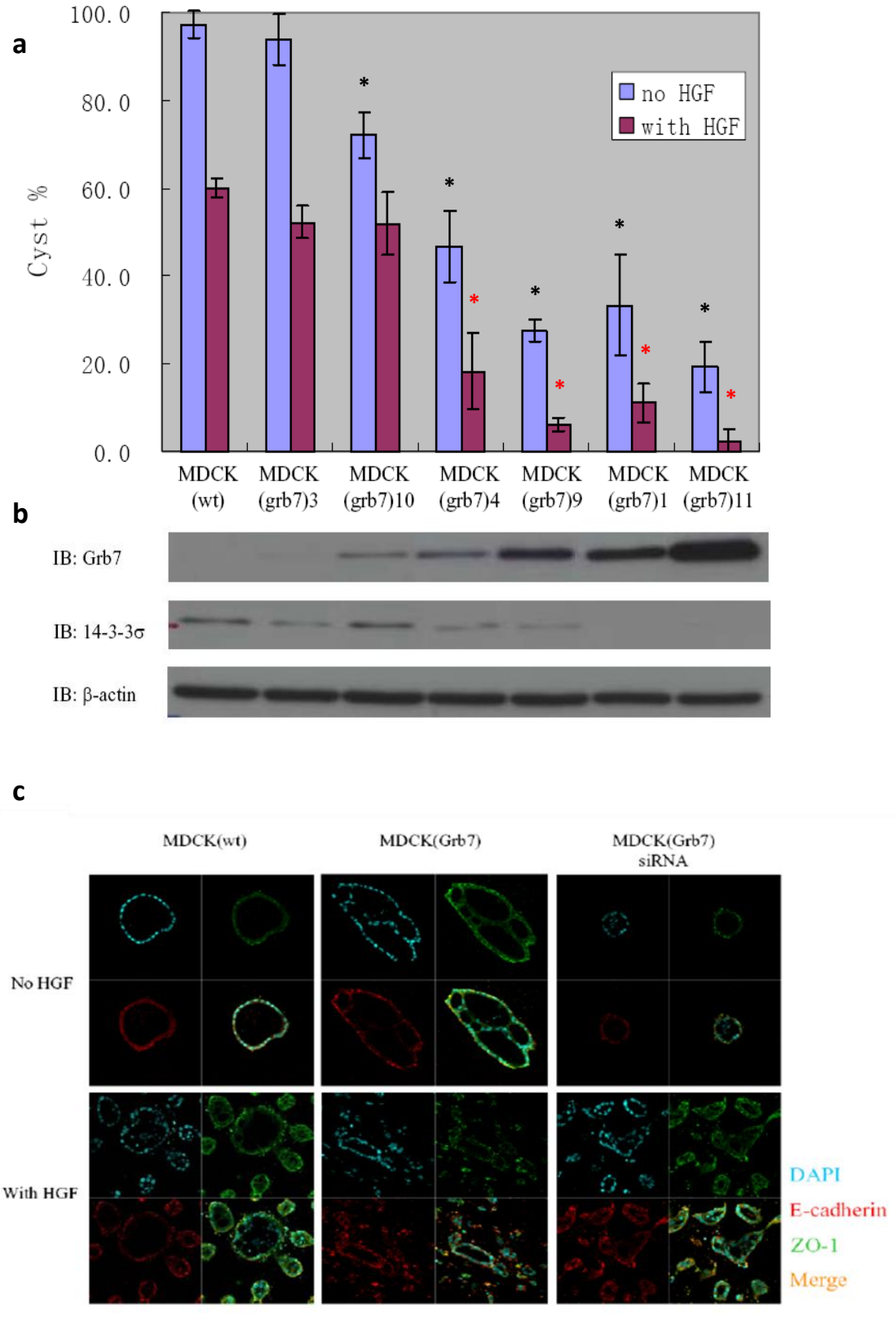
Chapter 3

RESULTS

3.1 Ectopic expression of Grb7 in MDCK cell lines disrupts cellular polarity and down-regulates 14-3-3 σ expression.

After Chen Ling found that 14-3-3 σ can act as a tumour suppressor, regulating epithelial polarity, and expression of 14-3-3 σ was down-regulated by Grb7 expression, MDCK cell lines expressing different level of Grb7 were generated by transfecting MDCK cell lines with pcDNA 3. plasmid (Invitrogen) containing mouse Grb7 cDNA. It was confirmed that as protein level of Grb7 increased, the expression of 14-3-3 σ decreased (Figure 3-1b). As demonstrated by Chen Ling, down-regulating 14-3-3 σ can result in loss of cell polarity, the logical experiments following this would be to examine whether elevated expression of Grb7 correlates with cell polarity. Through 3-Dimensional collagen assay, it was observed that the extent of disruption in cellular polarity is proportional to Grb7 expression. When cellular polarity status is normal, in collagen, cells can form nicely outlined hollow cyst, with regular pattern of expression of adherent junction marker E-cadherin and tight-junction marker ZO1. The disruption of cell polarity is characterized by multi-hollowed structure or solid structures with irregular expression or weak expression of E-cadherin and ZO1 restored (Figure 3-1 b). In an effort to confirm the observed phenotypes were caused by Grb7 expression, we used siRNA targeting Grb7. As Grb7 was knocked down, the cellular polarity was restored (Figure 3-1 c). HGF here was used as a scattering factor: it was noticed that when HGF was added, the disruption effect could be

Figure 3-1. Morphogenic impact of Grb7 over-expression and knock-down on MDCK cells in 3-demensional collagen culture (a) Grb7-expressing MDCK stable cell lines were established by transfection. After 7 days collagen culture, most MDCK clones showed fewer polarized cyst structures before and/or after 10u/ml HGF treatment for 24h. All cultures were performed three times. (b) The Grb7 and 14-3-3σ protein levels in all MDCK (Grb7) clones are showed by immuno-blotting with Grb7 and 14-3-3 σ antibodies. β-actin was used as loading control; (C) The Grb7 and 14-3-3 σ protein levels in different clones was shown by immuno-blotting; (c) Representing immuno-flourescence images of those three MDCK stable cell lines in collagen 3-D cultures before and after HGF treatment and after Grb7 was knocked-down stably expressing specific siRNAs. DAPI (blue) was stained for nuclei, ZO1 (green) for tight junction, E-cadherin (red) for adhesion junction.


a
100. 0
80. 0
60. 0
40. 0
20. 0
0. 0
Cyst %
no HGF
with HGF
MDCK (wt)
MDCK (grb7)3
MDCK (grb7)10
MDCK (grb7)4
MDCK (grb7)9
MDCK (grb7)1
MDCK (grb7)11
b
IB: Grb7
IB: 14-3-3σ
IB: β-actin
c
MDCK(wt)
MDCK(Grb7)
MDCK(Grb7) siRNA
No HGF
With HGF
DAPI
E-cadherin
ZO-1
Merge

exaggerated, making it easier for us to quantify. This part of the experiments was mostly performed by Chen Ling

Taken together, these observations suggest that up-regulation of Grb7 leads to down-regulation of 14-3-3 and loss of epithelial polarity.

3.2 Ectopic expression of Grb7 in MDCK decreases migration rate.

Grb7 has been implicated in a role of regulating cell migration. It shows homology to the *Caenorhabditis elegans* protein denoted Mig10, which is involved in embryonic neuronal cell migration. After previous observations that Grb7 can disrupt cellular polarity and knowing that cellular polarity plays an important role in migration, the next step was to examine the effect of Grb7 on migration rate.

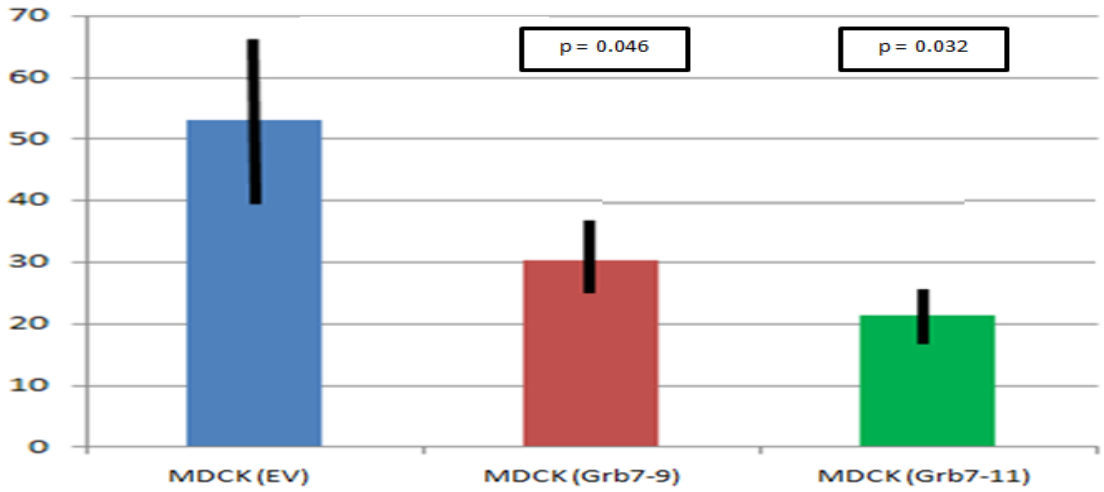
Firstly, using Boyden Chamber, it was shown that as Grb7 was up-regulated, the migration rate of MDCK cells was decreased. This was further confirmed with another migration assay using xCELLigence (Figure 3-2). These results suggest that up-regulation of Grb7 protein alone does not increase cells' migration capability, it may require other proteins to co-operate to achieve increased migration. After *in vitro* studies, we decided to use animal model for *in vivo* studies are clinically more meaningful compared with *in vitro* approaches.

3.3. MMTV-MTB/ TetO-Grb7 bi-transgenic mice mammary gland developmental studies.

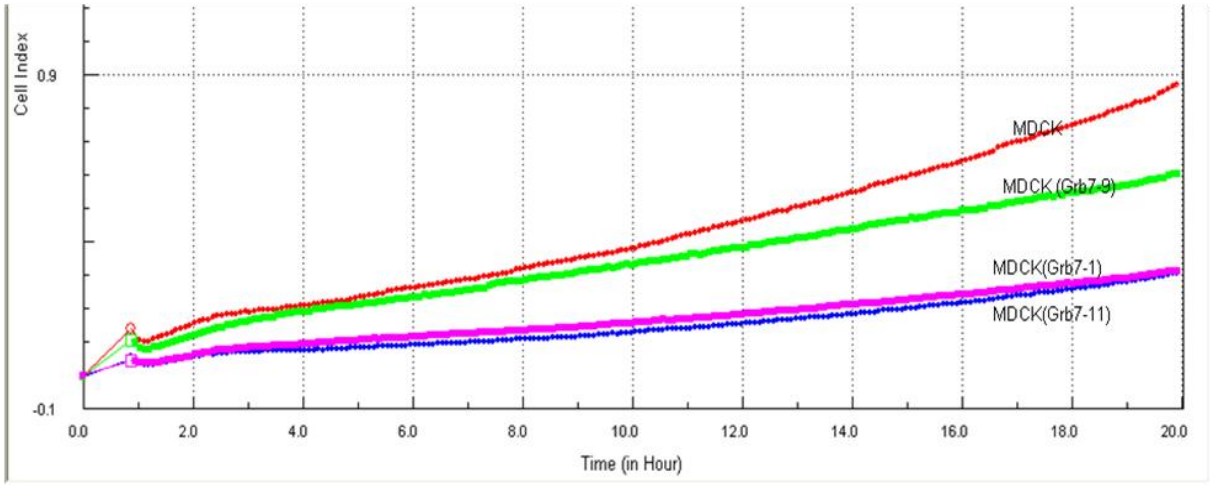
The TetO-Grb7 transgenic mice was generated and characterized by former graduate student, Allison Bergen. There was no publication reporting the effect of Grb7 expression on mammary

Figure 3-2. Migration assay studying the effect of elevated expression of Grb7 protein. (a) Using Boyden chamber, 150,000 of MDCK cells expressing Grb7 at different level were plated, and after 24 hours incubation, analysis was conducted. It showed that as Grb7 expression increased, the migration capability decreased. The same results were suggested by migration assay with xCELLigence (b).

a


70
60
50
40
30
20
10
0
p = 0.046
p = 0.032
MDCK (EV)
MDCK (Grb7-9)
MDCK (Grb7-11)

b


Cell Index
0.9
-0.1
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
Time (in Hour)
MDCK
MDCK (Grb7-9)
MDCK(Grb7-1)
MDCK(Grb7-11)

gland development, therefore it was important to know this not only because this would provide new information that we had not known but also because it was required for us in order to design further experiments.

After cohorts of female bi-transgenic mice was generated, Grb7 expression was induced by adding doxycycline in the drinking water starting at 3 week of age, and equal number of their littermates left uninduced, serving as control. The mice were sacrificed at 4, 6, 8, 10 weeks of age and the number 4 mammary gland was used to make wholemount, and protein lysates were also extracted from the remaining mammary glands.

Western blot confirmed that MMTV-MTB/TetO-Grb7 bi-transgenic mice with doxycyline had elevated Grb7 expression in the mammary gland, while their littermate control did not have elevated Grb7 expression (only endogenous Grb7 expression observed) (Figure 3-3a).

It was found that overexpression of Grb7 resulted in impaired mammary gland ductal outgrowth (Figure 3-3 b & c). To confirm, bi-transgenic mice were induced with doxycycline for 1 year starting at 3 week of age, at which point none of them had completed mammary gland outgrowth. However, this did not impair the lactation function as 2 MMTV-MTB/TetO-Grb7 bi-transgenic were induced with doxycycline starting at 3 weeks of age, and mated with FVB male after 10 weeks of age. 2 out of 2 had viable and healthy litters (data not shown).

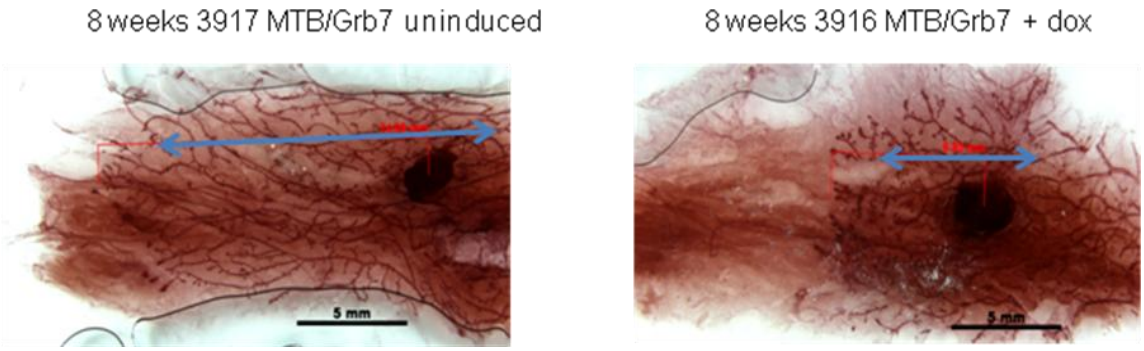
It was also found that in later stages (10 week of age), Grb7 expressing mice had more branching mammary glands compared with their uninduced littermate controls (Figure 3-4). This quantification was done by counting the number of branching within 2 centimeters long ductal outgrowth taken randomly from the mammary gland wholemount image. In total, 5 were taken from each mammary gland and average was calculated.

Figure 3-3 Effect of Grb7 overexpression on mammary gland development. (a) Western blot confirmed the inducible expression of Grb7 in MMTV-MTB/TetO-Grb7 bi-transgenic mice. (b & c) Overexpression of Grb7 can slow mammary gland development, resulting in incomplete mammary gland outgrowth. E-cadherin was used as loading control.

a


TM15 (+control)
MTB (– control)
3887 (dox+)
3886 (dox-)
3888 (dox+)
3889 (dox-)
3901 (dox+)
3903 (dox-)
E-Cadherin
Grb7

b


8 weeks 3917 MTB/Grb7 uninduced
8 weeks 3916 MTB/Grb7 + dox
5 mm
5 mm

c

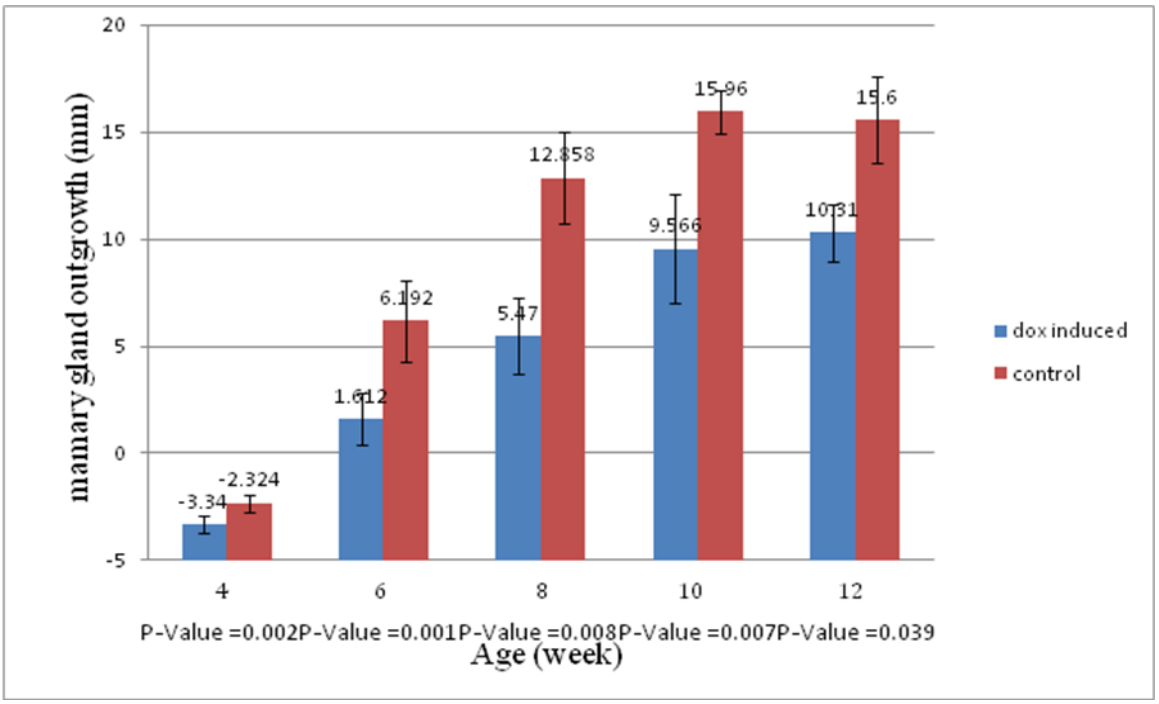
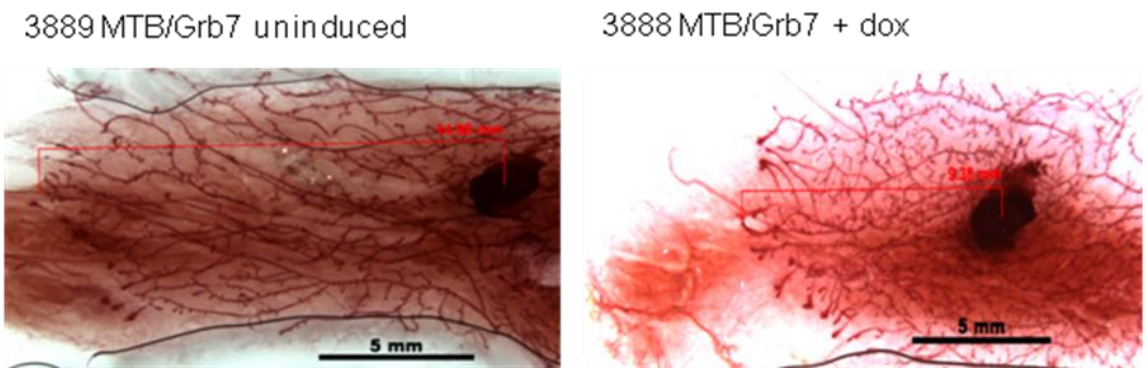
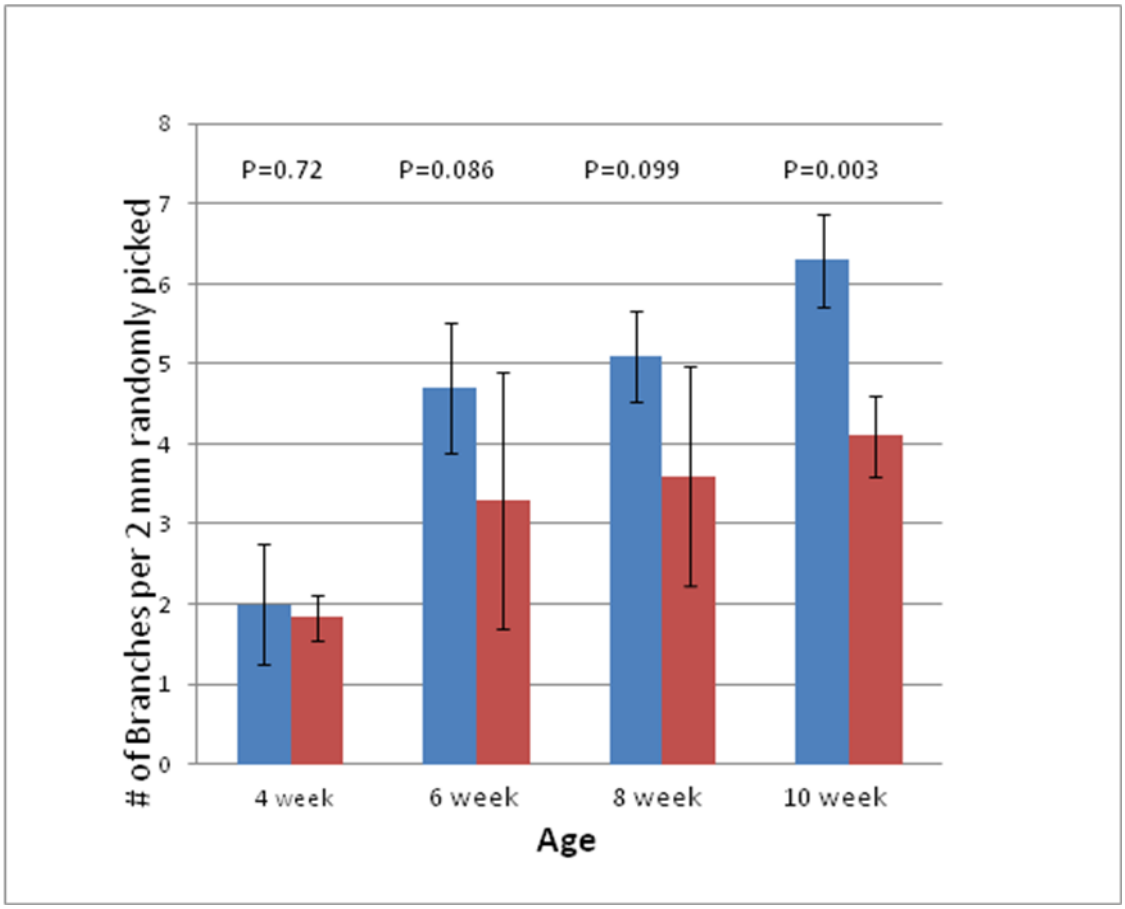

mamary gland outgrowth (mm)
20
15
10
5
0
-5
-3.34
-2.324
1.612
6.192
5.47
12.858
9.566
15.96
10.31
15.6
4
6
8
10
12
P-Value =0.002P-Value =0.001P-Value =0.008P-Value =0.007P-Value =0.039
Age (week)
dox induced
control

Figure3-4. Effect of Grb7 overexpression on mammary gland branching. (a) The mammary gland wholemount of one MMTV-MTB/TetO-Grb7 bi-transgenic mouse overexpressing Grb7 and its littermate control. Compared with its control, Grb7 overexpressing mouse was observed to have more branching in the mammary ductal outgrowth. (b) Quantification results of mammary gland branching showing that at later stage of mammary gland development, mice with Grb7 overexpressed had significantly more branching.

a


3889 MTB/Grb7 uninduced
3888 MTB/Grb7 + dox
5 mm
5 mm

b


P=0.72
P=0.086
P=0.099
P=0.003
# of Branches per 2 mm randomly picked
0
1
2
3
4
5
6
7
8
4 week
6 week
8 week
10 week
Age

Furthermore, H&E staining of the paraffin embedded mammary gland revealed that compared with the normal mammary gland in control mice, which is characterized by luminal structure and single layer of luminal epithelial cells, Grb7 expressing mice have been observed to have ducts with multiple epithelial layers, while this was rarely seen in their control of normal mammary glands (Figure 3-5).

Immunohisto-fluorescence assay of these paraffin embedded mammary glands revealed that compared with the normal ducts in control mice, in which the luminal epithelial cells stained by CK8, is enveloped by myoepithelial cells, stained by CK14, Grb7 expressing mice have ducts with disrupted myoepithelial cell layers (Figure 3-6).

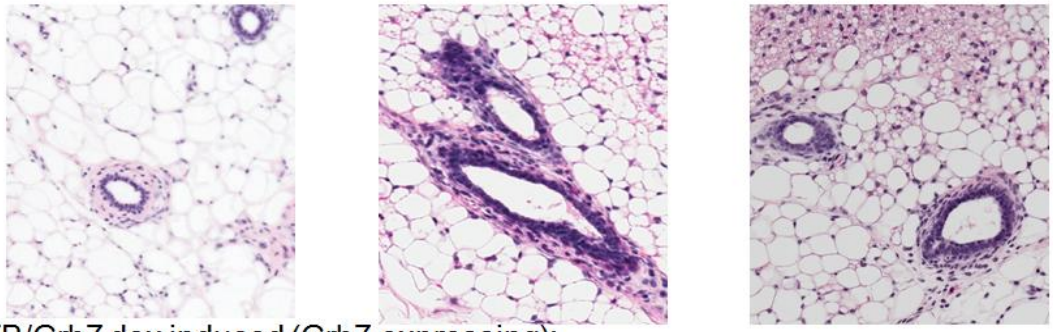
The proliferation and apoptotic cell death were also examined by doing Ki67 and TUNEL assays, however, I failed to observe any significant difference between Grb7 overexpressing mice and their control (Figure 3-7).

We have successfully shown using MDCK cells that overexpression of Grb7 can disrupt cell polarity, and decrease migration rate. However, these data were derived from MDCK cells, which are Madin-Darby Canine Kidney (MDCK) epithelial cell lines. Because MDCK cell lines are well characterized and easy to experimentally manipulated, they are the preferred cell lines when studying epithelial cellular polarity. It is important to examine if these observations are true when it applies to primary mammary epithelial cells. Therefore, I next dissociated primary mammary epithelial cells, and then for polarity study, I cultured them in matrigel for 7-10 days before immunofluorescence (Figure 3-8a); for migration assay, I cultured them in 24-well plate (NUNC) for 24 hours before plating them in Boyden chamber, after another 24 hours incubation, the data was collected (Figure 3-8b). these data agreed with our previous finding on MDCK

Figure 3-5. H&E staining of mammary gland. During the developing stage (4, 6, 8 weeks of age), mice overexpressing Grb7 had mammary ducts with multiple epithelial layers, while the same phenotype was not observed in their littermate control.

MTB/Grb7 uninduced:

4 weeks 6 weeks 8 weeks



MTB/Grb7 dox induced (Grb7 expressing):

4 weeks 6 weeks 8 weeks

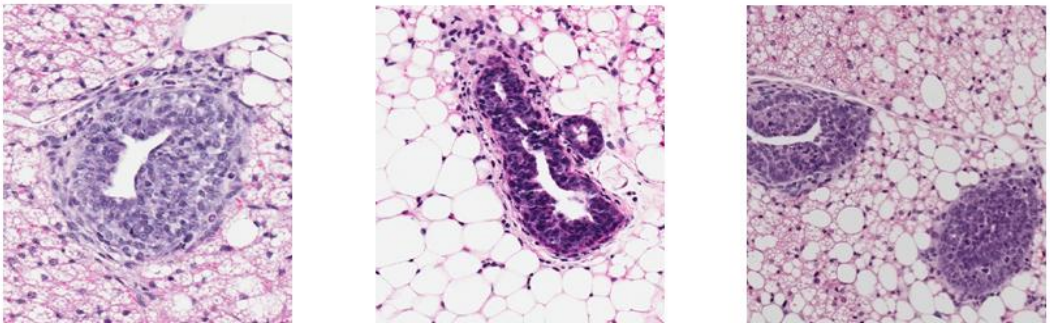
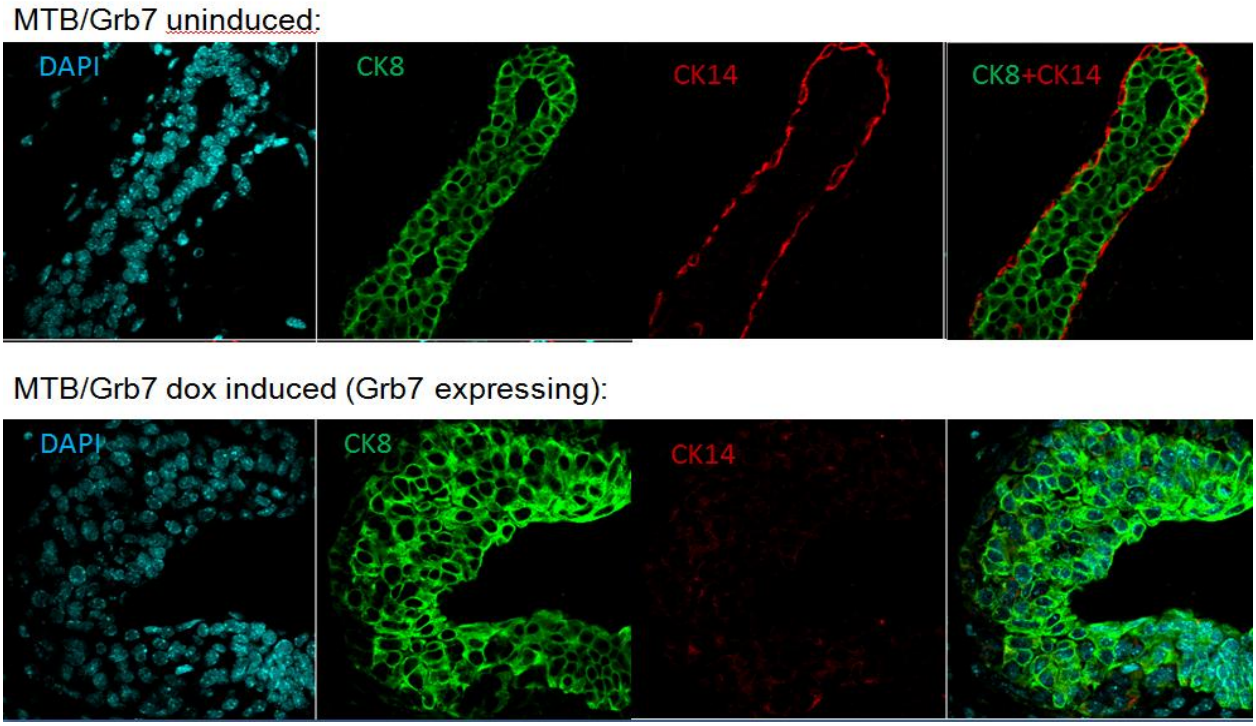
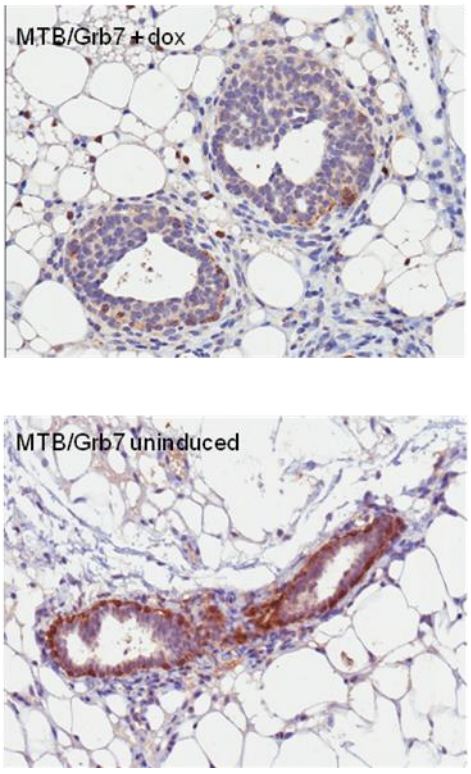


Figure 3-6. Effect of Grb7 overexpression on myoepithelial cells development. (a) Immunofluorescence assay using CK8 antibody (luminal epithelial cell marker, green stain) and CK14 antibody (myoepithelial cell marker, red stain) showed that transgenic mice with elevated Grb7 expression had irregular epithelial structure: normal mammary glands, as seen in MMTV-MTB/TetO-Grb7 uninduced, have a single layer of luminal epithelial cells enclosed by another layer of myoepithelial cells, but with Grb7 overexpressing mice have ducts with myoepithelial cells missing, resulting in multiple luminal epithelial layers. (b & c) Quantification analysis was performed using samples subjected to IHC. Statistic analysis showed there was significantly higher proportion in Grb7 overexpressing mice missing myoepithelial cell expression.

a


MTB/Grb7 uninduced:
DAPI
CK8
CK14
CK8+CK14
MTB/Grb7 dox induced (Grb7 expressing):
DAPI
CK8
CK14

b


MTB/Grb7 + dox
MTB/Grb7 uninduced

c

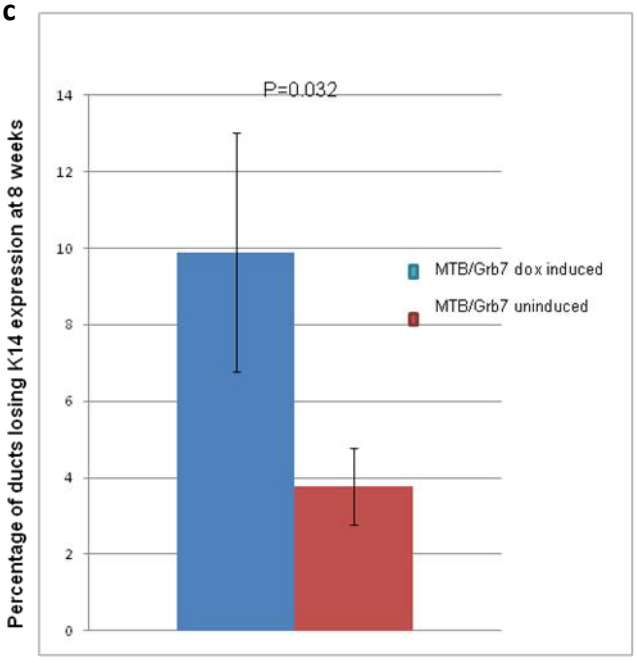
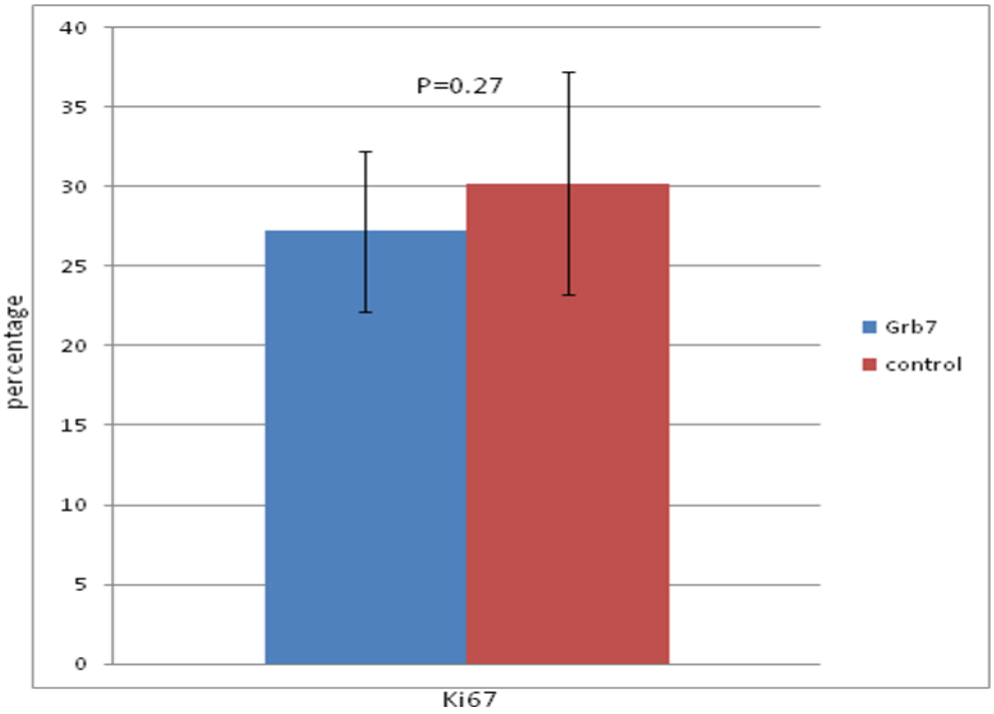

P=0.032
Percentage of ducts losing K14 expression at 8 weeks
0
2
4
6
8
10
12
14
MTB/Grb7 dox induced
MTB/Grb7 uninduced

Figure 3-7. Effect of Grb7 overexpression on proliferation and apoptotic cell death in primary mammary epithelial cells (Ki67 and TUNEL assay). (a) Ki67 assay of Grb7 overexpressing primary epithelial cells and their controls showed no significant difference. (b) TUNEL assay of Grb7 overexpressing primary epithelial cells and their controls showed no significant difference.

a


40
35
30
25
20
15
10
5
0
P=0.27
percentage
Grb7
control
Ki67

b

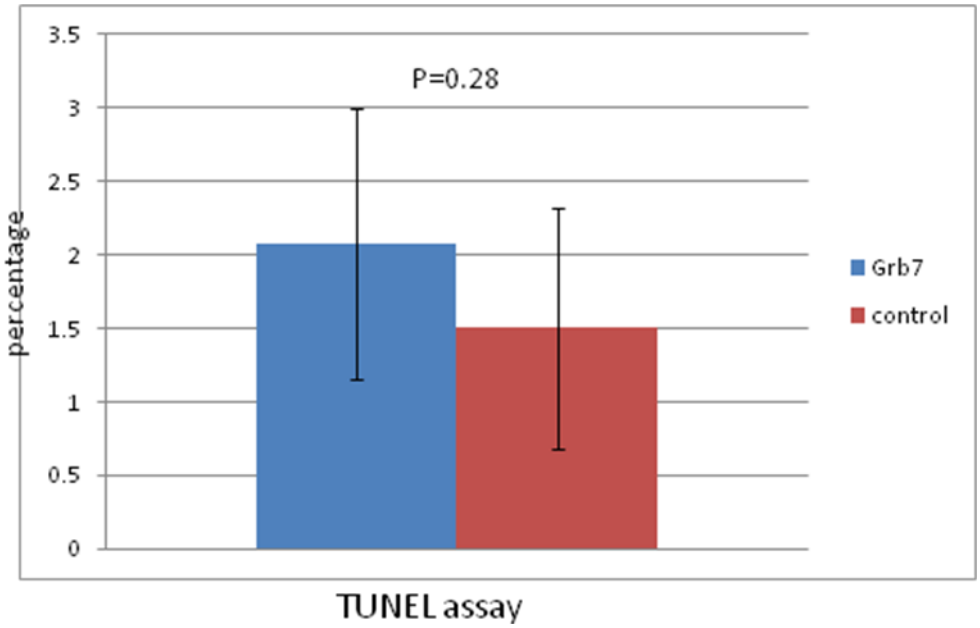
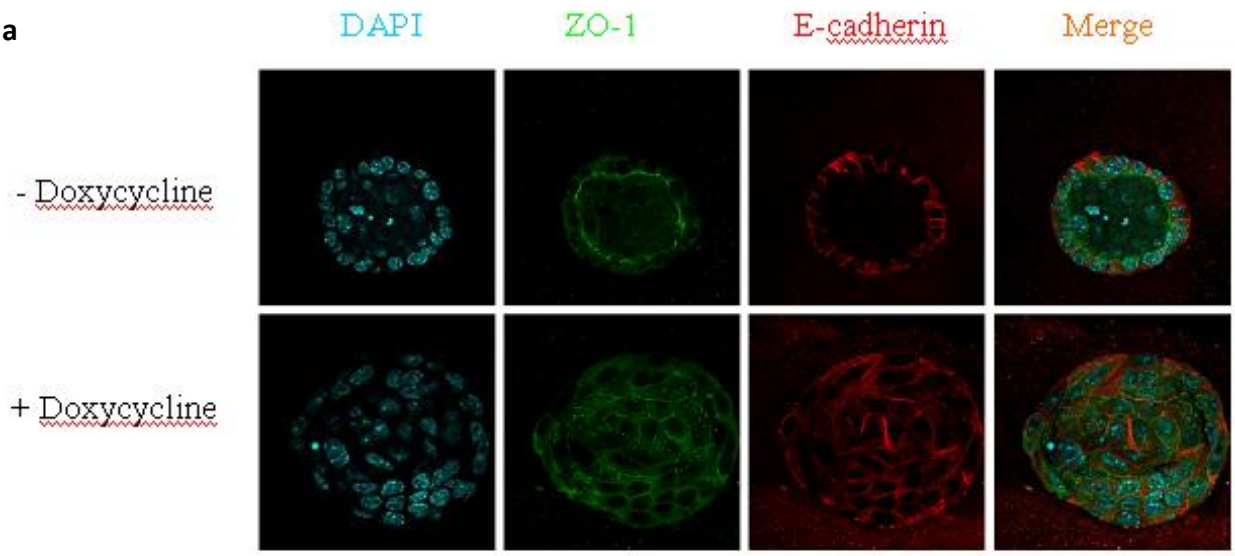
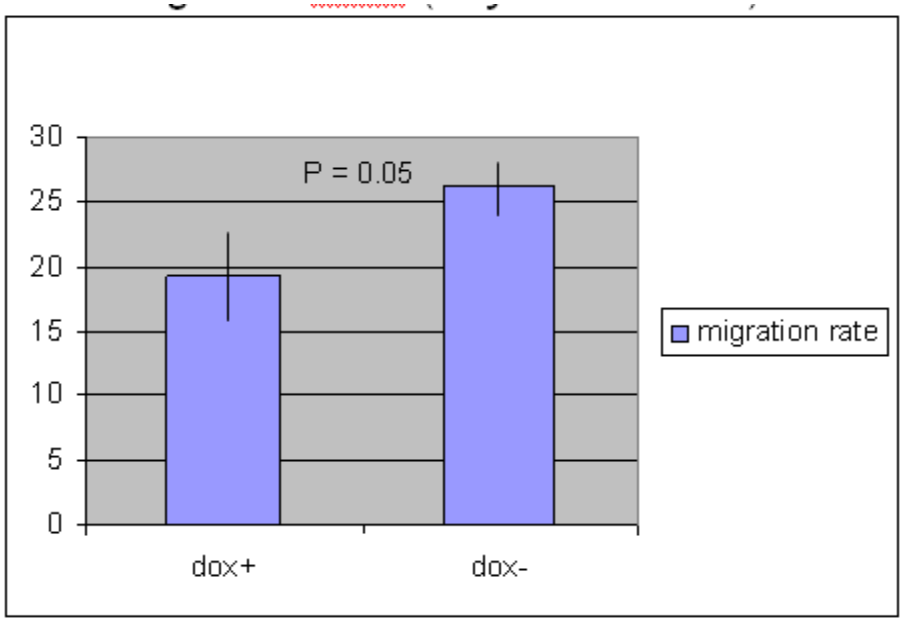

3.5
3
2.5
2
1.5
1
0.5
0
P=0.28
percentage
Grb7
control
TUNEL assay

Figure 3-8. Effect of Grb7 overexpression of cellular polarity and migration in primary mammary epithelial cells. (a) 3-Dimensional matrigel assay showed with elevated Grb7 expression, primary mammary epithelial cells had their polarity disrupted, which was characterized by cysts with solid lumen. (b) Boyden chamber migration assay showed primary mammary epithelial cells with elevated Grb7 expression had significantly reduced migration capability.

a


DAPI
ZO-1
E-cadherin
Merge
- Doxycycline
+ Doxycycline

b


30
25
20
15
10
5
0
P = 0.05
migration rate
dox+
dox-

cells that elevated expression of Grb7 can disrupt cell polarity in vivo, and overexpressing Grb7 alone does not lead to increased migration, instead, it caused decreased migration, which might explain the slow ductal outgrowth we observed in doxycycline induced MMTV-MTB/TetO-Grb7 bi-transgenic mice.

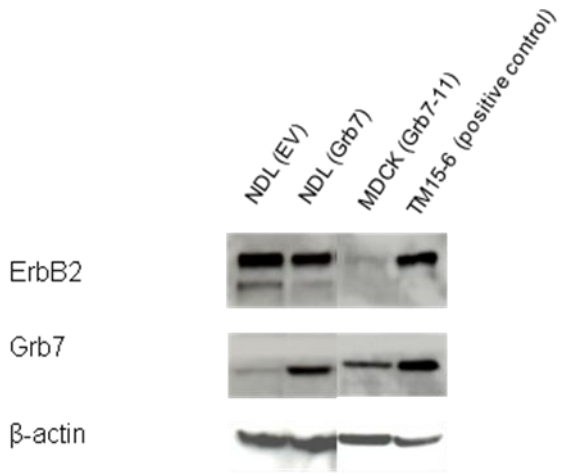
3. 4 Overexpression of Grb7 in NDL tumour cell lines

While the previous experiments examined the effect of overexpressing Grb7 alone, given the fact that Grb7 was often found co-overexpressed with ErbB2, it was important to study the effect of Grb7 overexpression accompanying ErbB2 overexpression. In our laboratory, we have established NDL tumour cell lines which are derived from MMTV-Neu-NDL 2-5 transgenic mice. Western blot of NDL tumour cell lines has revealed that NDL tumour cells have slightly elevated Grb7 expression, but to a much lesser extent when compared to TM15 cell lines. TM15 cell lines are derived from ErbB2 knock-in mice. Therefore, NDL tumour cell lines provided a good candidate to study the effect of ectopic expression of Grb7 on ErbB2 positive tumour cells.

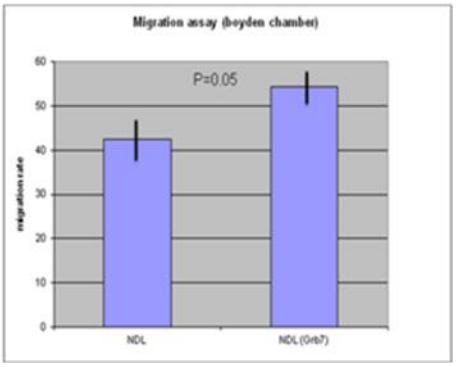
Through transfection, stable NDL tumour cell lines overexpressing Grb7 to similar extent as in TM15 were established. Proliferation, migration and invasion assays were conducted using different experimental approaches, and consistent results were obtained. It was observed that NDL cells with Grb7 highly overexpressed had a higher proliferation rate (72 hour-assay), lower migration rate but higher invasion capability (Figure 3-9). This result agreed with other researchers' finding that Grb7 overexpression coupled ErbB2 overexpression can increase proliferation and invasion rates. The proliferation assay showed that within the first 24 hours, there was no significant difference between 2 cell lines. This can further validate our finding in

Figure 3-9. Effect of Grb7 overexpression on proliferation, migration and invasion in NDL tumour cells. (a) Western blot showing transfected NDL tumour cells (with pcDNA 3.-Grb7) has elevated Grb7 expression compared with NDL tumour cells (with empty vector). Immunoblotting against β-actin was used as a loading control. (b) Grb7 overexpressing NDL tumours cells had reduced migration rate. (c) Elevated expression of Grb7 in NDL tumour cells increased the proliferation rate. (d) Grb7 overexpression in NDL tumour cells showed enhanced invasion capability.

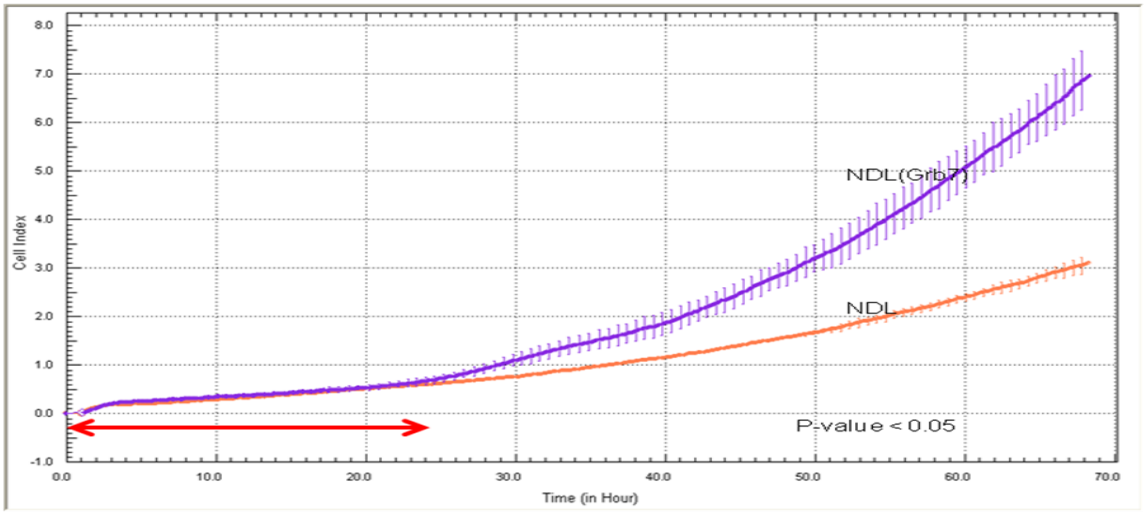
a


NDL (EV)
NDL (Grb7)
MDCK (Grb7-11)
TM15-6 (positive control)
ErbB2
Grb7
β-actin

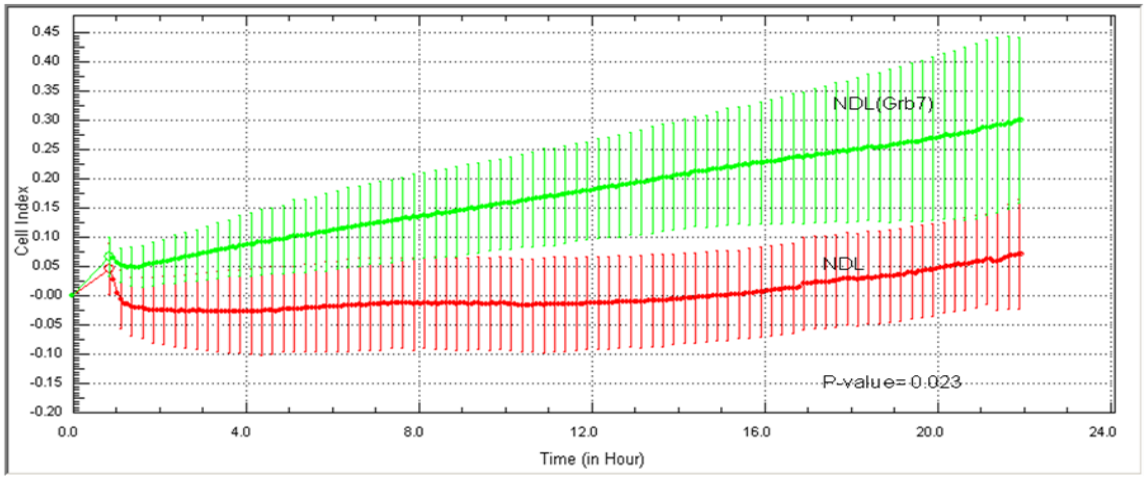
b


Migration assay (boyden chamber)
P=0.05
migration rate
NDL
NDL (Grb7)

c


Cell Index
Time (in Hour)
NDL(Grb7)
NDL
P-value < 0.05

d


Cell Index
Time (in Hour)
NDL(Grb7)
NDL
P-value= 0.023

migration and invasion assays (24 hours long), ruling out the possibility that any observed difference in migration and invasion is due to a difference in cell proliferation.

3.5 MMTV-MTB/TetO-Grb7/ MMTV-Neu-NDL 2-5 tri-transgenic mice

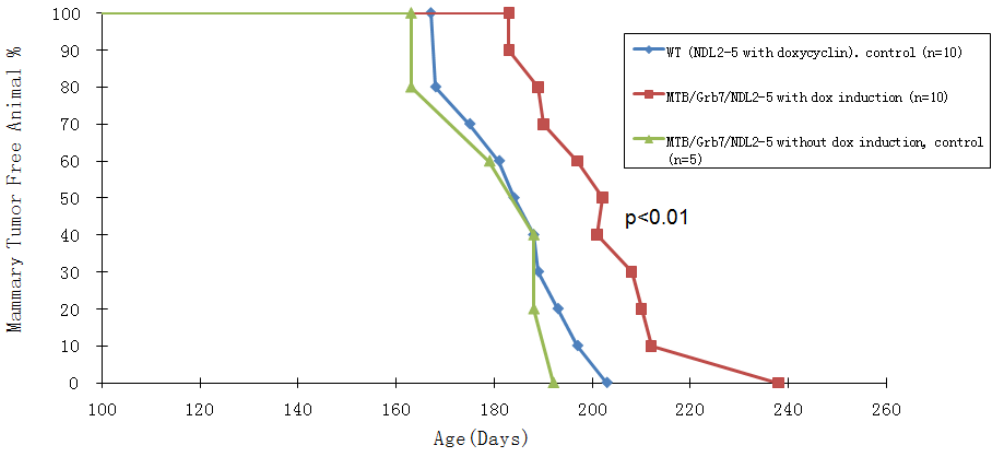
As just described, ectopic expression of Grb7 in established NDL tumour cell lines showed that elevated expression of Grb7 in ErbB2 positive tumours can increase proliferation and invasion. Similar results were obtained by other research groups studying Grb7 as well, using various breast cancer cell lines, for instance, MCF-10A and BT-474. These previous data state the necessity to investigate whether overexpressing Grb7 in advance of tumourigenesis could affect the development and progression of ErbB2 positive breast tumours, especially for tumour onset and metastasis. Transgenic techniques made this possible. By adding doxycycline into the drinking water of MMTV-MTB/TetO-Grb7/ MMTV-Neu-NDL2-5 tri-transgenic mice, we can easily control the expression of Grb7. Because it was noticed that starting elevated expression of Grb7 at 3 weeks of age would cause mammary gland defect, the induction starting point was decided to be at 9 weeks of age, by which point the mammary gland development should be finished. Tumour palpation was routinely performed twice a week. The tumour onset time was recorded. After tumours reached end point (6 weeks postpalpation or tumour volume reaches limit, whichever comes first), the mouse was sacrificed and tumour sample was taken.

It was shown that overexpressing Grb7 in NDL mice did not accelerate mammary tumourigenesis (figure 3-10a). The average tumour onset of those induced tri-transgenic mice was 204 ± 15 (n=10) days, while tumour onset of the control cohorts was 182 ± 12 days (n=15). In addition, lung metastasis analysis failed to show any difference between these induced mice

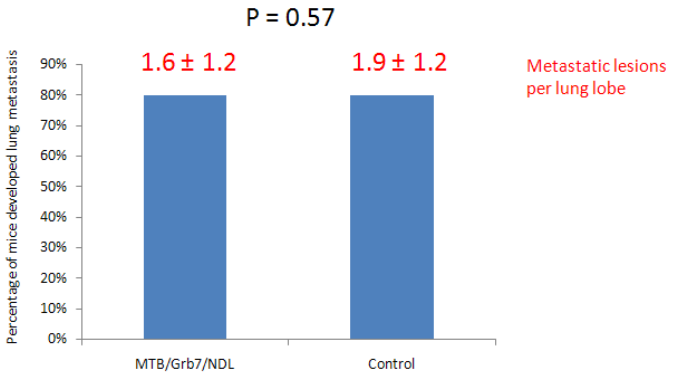
and their control: both induced cohort and control cohort, 80% of mice showed lung metastasis, and the average counting of lung metastasis pads are statistically comparable (Figure 3-10b). Interestingly, biochemistry analysis of tumours samples revealed that the Grb7 protein in those induced tri-transgenic mice was expressed at the same level as their control (Figure 3-10c). In other words, the inducible system was not functioning as we expected. However, MMTV-MTB/TetO-Grb7 bi-transgenic mice induced for up to 12 months reported strong Grb7 expression (data not shown), as well as in adjacent normal (ErbB2 was not overexpressed) mammary gland in tri-transgenic mouse (ID#2018), Grb7 overexpression was observed (Figure 3-10c). Moreover, 6 out of 10 tumours derived from induced NDL mice had strong GFP expression, a reporter gene of inducible system (refer to figure 1-4). Here the Western blot only shows 5 induced and 5 uninduced. These observations suggested that some NDL tumours selectively down-regulated the inducible expression of Grb7 protein.

Figure 3-10. Tumour onset, metastasis, and biochemistry analysis of mammary tumours of MMTV-MTB/TetO-Grb7/MMTV-Neu-NDL2-5 tri-transgenic mice and tumour curve. (a) The average tumour onset of those induced tri-transgenic mice was 204 ± 15 (n=10) days, while tumour onset of the control was 182 ± 12 days (n=15). (b) Lung metastasis counts revealed that in both Grb7 overexpressing NDL 2-5 mice and their controls, 80% of mice developed lung metastasis. Digital numbers (in red ink) represent average metastatic lesion per lung lobe, and p-value equalling to 0.57 suggests there was no significant difference in metastatic lesion counts. (c) Western blot showed Grb7 overexpression due to Tet –on inducible was down-regulated. The level of ErbB2, ErbB3, 14-3-3 σ and total and phospho- AKT, MAPK in these mammary tumours showed no difference. Immunoblotting against β-actin was used as a loading control.

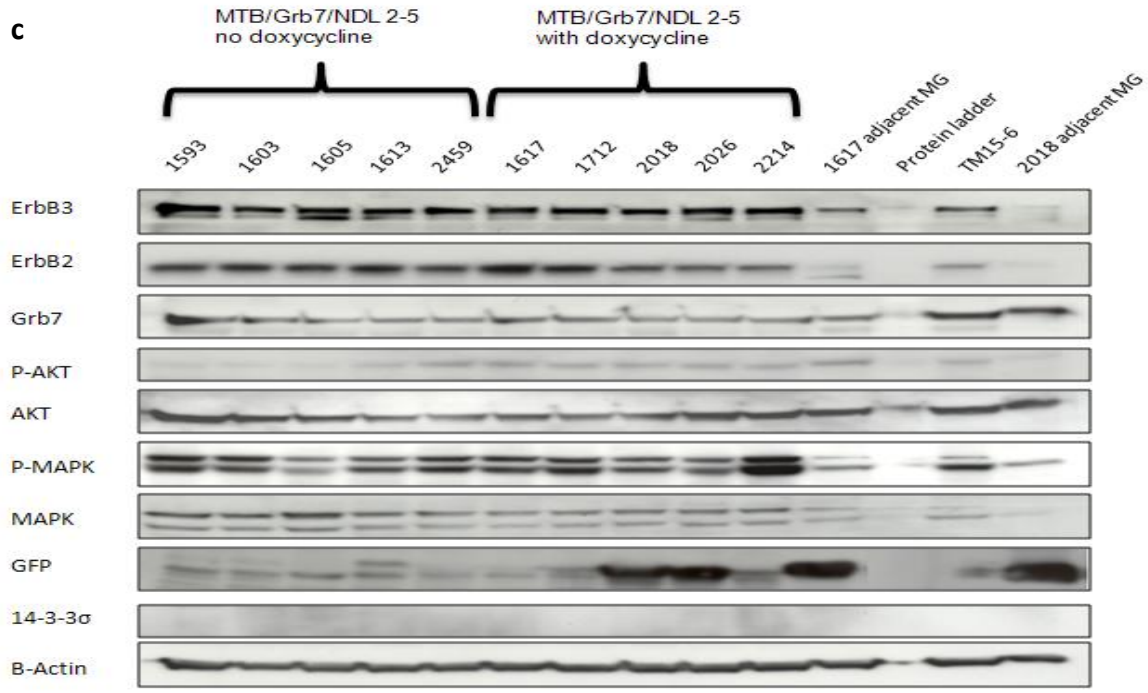
a


Mammary Tumor Free Animal %
100
90
80
70
60
50
40
30
20
10
0
100
120
140
160
180
200
220
240
260
Age (Days)
WT (NDL2-5 with doxycyclin). control (n=10)
MTB/Grb7/NDL2-5 with dox induction (n=10)
MTB/Grb7/NDL2-5 without dox induction, control (n=5)
p<0.01

b


P = 0.57
1.6 ± 1.2
1.9 ± 1.2
Metastatic lesions per lung lobe
Percentage of mice developed lung metastasis
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
MTB/Grb7/NDL
Control

c


MTB/Grb7/NDL 2-5 no doxycycline
MTB/Grb7/NDL 2-5 with doxycycline
1593
1603
1605
1613
2459
1617
1712
2018
2026
2214
1617 adjacent MG
Protein ladder
TM15-6
2018 adjacent MG
ErbB3
ErbB2
Grb7
P-AKT
AKT
P-MAPK
MAPK
GFP
14-3-3σ
B-Actin

Chapter 4

DISCUSSION

4.1 Overview

Amplification and overexpression of ErbB2 in a subtype of breast cancer was detected in the last century, and since then the role and mechanism of ErbB2 signalling has been studied extensively. Researchers have gained a lot of useful information about ErbB2 and cancer therapies targeting ErbB2 are commonly used clinically. Grb7 was found to be co-amplified and co-overexpressed with ErbB2 in many mammary tumours as well as in oesophageal, ovarian, colon cancers. Grb7 overexpression was thought to contribute to tumour phenotypes, due to the clinical findings that Grb7 protein overexpression is associated with larger tumour size, more lymph node involvement, higher tumour grade, and inferior breast cancer-free interval (Ramsey et al., 2010). This was experimentally confirmed by the results that ectopic expression of Grb7 in mammary epithelial cells increased proliferation and invasion. Conversely, a study by Kao and Pollack (2006) demonstrated that knockdown of Grb7 resulted in decreased cell proliferation and cell-cycle progression**.** In addition, the human and mouse chromosomal loci where Grb7 reside are evolutionarily conserved regarding the order and orientation, so from an evolutionary view, Grb7 gene locating within ErbB2 amplicon was kept this way through evolution, being favoured by selection, maybe because the retained amplification of Grb7 gene might contribute to tumour phenotypes, providing selective growth advantage to tumour cells.

Mouse models can act as pre-clinical models for the development of new anti-cancer treatments. The goal of this project was to gain more understanding about the role of Grb7, specifically in

ErbB2 mediated mammary tumourigenesis, and to confirm the findings previously derived from *in vitro* studies by using *in vivo* mouse models.

4.2 Cellular polarity and tumour development

In both MDCK cells and primary mammary epithelial cells, we successfully showed that overexpression of Grb7 protein could lead to loss of cell polarity. As our knowledge about cancer expands, we now know that cancer development is a process involving multiple steps. Loss of cell polarity has been commonly observed in advanced tumours and correlates with their invasion into adjacent tissues and the formation of metastasis (Aranda et al., 2006; Benton et al., 2003; McCaffrey et al., 2009; Wodarz and Nathke, 2007). More and more evidence suggests that loss of cell polarity may be important in early stages of cancer progression (Lee and Vasioukhin, 2008; Zhan et al., 2008). Therefore Grb7's capability to disrupt cell polarity is thought to contribute to tumour phenotypes.

Epithelial tissues are characterized by a specific architecture: epithelial cells align to each other with junctions between them. Each epithelial cell has apical and basolateral membrane domains that vary in protein and lipid content (Hurd et al., 2003). This causes the cell to have polarity: a front and a back. Cell polarity is crucial to normal cell function. Cell polarity enables cells to proliferate as programmed, to migrate towards a specific direction with an ordered manner. Without it, cells would move in all directions, or spread, which may result in uncontrolled proliferation. In fact, important hallmarks of advanced cancerous tumours are the loss of epithelial character and the appearance of more mesenchymal like cells, a process known as Epithelial-Mesenchymal transition (EMT). Typical EMT includes loss of cell-cell adhesion and

apical-basal cell polarity. This makes the tumour cells more mobile and easier to penetrate basal membrane and enter blood streams (Wodarz et al., 2007). Several research groups had demonstrated that when Grb7 expression was elevated, cells became more proliferative and/or more invasive, and our results may partially explain the reason and provide a feasible mechanism: Grb7 overexpression causes disruption of cell polarity, which renders epithelial cells with increased proliferation and invasion potential (Aranda et al., 2006; Feigin and Muthuswamy, 2009).

The exact mechanism how Grb7 disrupt cell polarity is still unclear, whether Grb7 achieves this by down-regulation of 14-3-3 σ, a potent tumour suppressor suggested to participate in establishing cell polarity by Chen Ling and other researchers (Benton et al., 2003; Hurd et al., 2003; Ling et al., 2010), or through another unknown mechanism. For example, among the new uncovered functional roles of the Grb7 protein it has been recently described that through SH3 domain, it can act as a translational repressor upon binding to the 5'-UTR of a targeted mRNA (k-opioid receptor), thereby blocking the recruitment of the translation eukaryotic initiation factor 4E and silencing the protein expression, the end result is mRNA translation suppressed (Tsai et al., 2007). It was also suggested that the SH3 domain of Grb7 has other binding targets. It is possible that elevated expression of Grb7 can down-regulate proteins involved in cell polarity establishment by suppressing mRNA translation. Further studies are required to answer this question.

4.3 Mammary gland developmental study

In mammary gland developmental study, it was shown that Grb7 overexpression can cause mammary gland defect, histological analysis of Grb7 overexpressing epithelium revealed an increase in the number of luminal epithelial cells, a phenomenon which may be correlated with our previous finding that elevated expression of Grb7 causes loss of cell polarity. Indeed, 3-dimensional matrigel assay demonstrated that Grb7 overexpression can disrupt cell polarity in primary mammary epithelial cells (figure 3-8a). During mammary gland development, without polarity, Grb7 overexpressing epithelium partially grows inwards into the lumen instead of growing into the fat pad. Considering that there was no significant change in proliferation and cell death observed from TUNEL and Ki67 assays, this could slow down the speed of mammary gland outgrowth. Try to imagine that the newly generated cells are used to fill the ductal lumen instead of building up the end bud, elongating the mammary ducts. As a result, at each stage, mammary gland ductal outgrowth was shorter in induced mice compared to the control. Migration assay on primary mammary epithelial cells showed that elevated Grb7 expression decreased migration rate, which provided direct explanation for the delayed mammary outgrowth.

Chu et al. (2009) have reported that through the SH2 domain, Grb7 interacted with Focal Adhesion Kinase and was phosphorylated by FAK. The Grb7-FAK complex plays an essential role for promoting cell migration and proliferation in epidermal carcinoma cells. Our finding seemed controversial with their finding. However, keep in mind that our observations were derived from an *in vivo* source, a complex and dynamic system. The elevated level of a single protein can have complex outcomes, especially Grb7 has multi-domains, enabling it to interact with various proteins. Another possible reason is in our experiment, Grb7 was overexpressed in primary mammary epithelial cells, while Chu et al. used A431 cells, which express abnormally

high level of EGFR. Taken together, our data suggests that in normal conditions Grb7 does not promote migration by itself, instead requiring interaction with other proteins.

In addition, immuno-fluorescent and IHC assays revealed that a higher portion of mammary ducts lost myoepithelial cells. Typical mammary gland duct has two layers: an inner luminal epithelial layer; and an outer myoepithelial layer, which can act like a boundary, to restrict the progression of epithelial cells. By losing it, cells have the potential to penetrate the basal membrane, spreading into the surrounding tissue (including blood streams), a progression very similar to invasive tumour cell progression (Berman, 2009; Feigin and Muthuswamy, 2009). Again, this correlates with our previous finding that Grb7 overexpressing epithelium had an increase in the number of luminal epithelial cells.

As I noticed the effect of expressing Grb7 on mammary gland development, the initiation of *in vivo* expression of Grb7 in NDL was delayed till 9 weeks of age, so that both the induced and the control mice could have completed mammary ductal outgrowth, and any change we observed between these two groups should be due to Grb7 expression.

4.4 Ectopic expression of Grb7 in NDL tumour cell lines

As we just discussed, overexpressing Grb7 in normal mammary epithelium and in EGFR receptor overexpressed epithelium can have very different outcomes. It is known that through SH2 domain, Grb7 can interact with ErbB2, mediating the ErbB2 related signalling, although the exact pathway and mechanism are still unclear. After obtaining results from MMTV-MTB.TetO-Grb7 bi-transgenic mice, it was necessary to examine the effect of Grb7 overexpression in ErbB2 positive breast cancer cells using an *in vivo* system. However, before putting it into mouse model,

we needed to overexpress Grb7 ectopically in ErbB2 positive breast tumour cells. *In vitro* study provided the results to be expected from *in vivo* study. While most research groups studying Grb7 using various human mammary cell lines (transformed or non-transformed, ErbB2 positive or ErbB2 negative) (Bai et al., 2008; Chu et al., 2009; Kao and Pollack, 2006; Nencioni et al., 2010; Porter et al., 2007), in this thesis, NDL tumour cells were used. It was found that NDL tumour cells with elevated Grb7 level had higher proliferation and invasion capability, which agreed with other researchers' data (Bai et al., 2008; Porter et al., 2007). Based on these observations, I expected NDL mice overexpressing Grb7 to have shorter tumour latency and higher metastatic penetrance. Also, by obtaining similar results, I was more confident to use a mouse model as they are comparable to human mammary epithelium for showing similar phenotypes.

4.5 Overexpressing Grb7 in MMTV-Neu-NDL2-5 transgenic mice

It was attempted to accelerate mammary tumourigenesis and promote metastasis by overexpressing Grb7 concurrently with expressing activated ErbB2 in NDL transgenic mice. However, compared with regular MMTV-Neu-NDL2-5 mice and tri-transgenic mice without induction, induced tri-transgenic mice had significant longer tumour latency and non-differentiable lung metastatic conditions.

In addition, from Western blot, it was noticed Grb7 was not "overexpressed" as it was supposed, and it seemed that the inducible system did not work as designed. However, I had MMTV-MTB/TetO-Grb7 bi-transgenic mice induced with doxycycline for up to 1 year, and they did have Grb7 overexpressed to a much higher level. In addition, preliminary cell lines set up by

dissociating tumours derived from MMTV-MTB/TetO-Grb7/MMTV-Neu-NDL tri-transgenic mice had Grb7 overexpressed with doxycycline was added. Moreover, the western blot of adjacent mammary gland of induced mouse (ID# 2018) (Figure 3-9b) failed to have elevated ErbB2 expression but had Grb7 overexpressed; in contrast, another adjacent mammary gland of induced mouse (ID#1617) had elevated ErbB2 expression but failed to have Grb7 overexpressed. Western blot suggests these two adjacent mammary glands were at different stage in term of mammary tumourigenesis, and it is possible that the mammary gland of 1617 used in Western blot was in the process of developing tumours, while the one of 2018 was still considered normal. Finally, 7 out of 10 tumours derived from NDL mice overexpressing Grb7 had strong GFP expression, indicating the inducible system functioned properly. All these findings suggest that inducible Grb7 expression was selectively down regulated in some NDL tumours for reasons we do not know so far. It is possible that *in vivo* overexpression of Grb7 can trigger the cell stress response. Cell stress response describes how cells can respond to stress in a variety of ways ranging from the activation of survival pathways to the initiation of cell death that eventually eliminates damaged cells. One of the molecules playing critical role in cell stress response is Ca^{2+}: Ca^{2+} is an important second messenger participating in many cellular activities; when physicochemical insults deregulate its delicate homeostasis, it acts as an intrinsic stressor, producing/increasing cell damage. Damage elicits both repair and death responses. Li et al. (2005) found that Grb7 is a Ca^{2+}-dependent calmodulin-binding protein. Overexpression of Grb7 may complicate with Ca^{2+} to trigger cell death, which may account for the observation that inducible system worked but Grb7 level was not elevated, because NDL tumour cells with elevated Grb7 were dead.

In addition, there may be a mechanism for Grb7 self-regulation as reported in yeast 2-hybrid assays showing that upon interaction with Hax-1, Grb7 can exist in a head-to-tail conformational state (Siamakpour-Reihani et al., 2011). Examining the mRNA level of Grb7 in these mice may provide us more information and help us to explain this observation.

About the longer tumour latency, it could be caused by the cell stress response which was just mentioned: a proportion of tumour cells were dead, causing a overall slow progression of breast cancer. It is also possible by titrating effect: most protein-protein interactions are known to be an equilibrium system, and presence of excess Grb7 protein could interfere (most likely by competing for binding partners) with interaction of proteins essential for tumour development. Or it could simply be the result that cells used too much resource to overexpress Grb7, exhausting the cells. In addition, a new function of Grb7 has been identified recently: it was found that through the proline rich domain, Grb7 can bind RNA (mRNA), and modulate translation of certain proteins which may by advantageous to tumour progression (Tsai, et al., 2007; 2010). Further studies need to be done to determine the exact reason for the longer tumour latency in Grb7 induced NDL mice.

4.6 Overexpressing Grb7 in ErbB2 knock-in transgenic mice

As explained in the introduction, ErbB2 knock-in mouse model expresses activated ErB2 under the control of endogenous promoter (not driven by MMTV), and shows several features with human ErbB2 positive breast cancer, including frequent deletion of chromosome 4 and extra gene copies of a small section in human chromosome 11, known as the ErbB2 amplicon. Therefore, ErbB2 knock-in mice provide a very good and valuable model to study the human

ErbB2 positive breast cancer. Despite our results from MMTV-MTB/TetO-Grb7/MMTV-Neu-NDL tri-transgenic model, it is worthwhile to overexpress Grb7 in ErbB2 knock-in transgenic mice. Grb7 is within the smallest region being amplified with ErbB2 gene. Clinical studies have shown that increased expression of Grb7 correlates with a lower survival rate in breast cancer patients with tumour with either high or low expression of ErbB2. Grb7 protein is thought to play an auxiliary role in ErbB2 positive mammary cancer, contributing tumour phenotype (more invasive or proliferative). This question could also be answered by the NDL model overexpressing Grb7. However, amplification of Grb7 gene and the resulting elevated expression of Grb7 protein may be a mechanism necessary to drive ErbB2 amplication. This was suggested by a research group studying a subset of testicular germ cell tumours. They showed that in these tumours, Grb7 mRNA, rather than ErbB2 mRNA was overexpressed. This observation strengthened the hypothesis that Grb7 gene, rather than ErbB2 gene, is the target of gene amplification and selective retention (Ramsey et al., 2010; Skotheim et al., 2002). In other words, Grb7 might be the driving force of ErbB2 amplicon amplification. This question, however, can only be investigated in a MMTV-MTB/TetO-Grb7/ErbB2 knock-in model.

By crossing ErbB2 knock-in model with a MTB/Grb7 bi-transgenic model, we can generate a tetra-transgenic model that has a separated machinery to overexpress Grb7 when induced with doxycycline, and possibly bypasses the amplification of endogenous Grb7 gene located within ErbB2 amplicon. If the resulting tetra-transgenic mice indeed have no or delayed overexpression of ErbB2, then it provides evidence suggesting that Grb7 gene amplification promotes the amplification of the ErbB2 amplicon, and therefore the amplification of the ErbB2 gene.

4.7 Clinical significance of Grb7 protein

Due to more and more evidence supporting that Grb7 is strongly related to aggressive phenotype of ErbB2 positive breast cancer (as well as other forms of ErbB2 positive cancer, for instance, oesophageal cancer and ovarian cancer), now clinically Grb7 has been used as a prognostic marker in breast cancer (Tanaka et al., 1997, 1998, 2000; Wang et al., 2010). In addition, combination treatment with Grb7 peptide and Doxorubicin or Trastuzumab (Herceptin) has shown to result in cooperative cell growth inhibition in breast cancer cells (Pero *et al*., 2007).

Trastuzumab (Herceptin) is a monoclonal antibody targeting ErbB2, and efficiently increases the survival rate of ErbB2 mediated breast cancer patients. However, Trastuzumab not only targets ErbB2 on tumour cells, but also normal cells expressing ErbB2. This leads to severe side effects (common for most, if not all cancer therapy). One of the significant complications of Trastuzumab is its effect on the heart. Trastuzumab is associated with cardiac dysfunction in 2-7% of cases (Seidman, A, *et al.* 2002). Compared with the extensive expression sites of ErbB2, Grb7 protein is normally expressed in fewer tissues, including liver, kidney and gonads. This makes the side effect of cancer therapy targeting Grb7 less severe as it will affect fewer tissues besides target cancer cells.

4.8 Other genes in ErbB2 amplicon

Besides Grb7, there are other genes co-amplified and co-expressed with ErbB2. Within the 17q12-q21 region (ErbB2 amplicon), amplification of ErbB2 Stard3, Tcap, Pnmt, Perld1, C17orf37, Grb7, Gsdml, Psmd3 and Thrap4 genes have been reported to correlate with gene

expression (Figure 1-1). Some of them have also been suggested to play important role in ErbB2 mediated mammary tumourigenesis.

C17orf37 open reading frame encodes a 12-kDa protein (known as C35), C35 overexpression is linked with ErbB2 amplification in most cases, and it has also been observed in breast carcinomas that do not overexpress ErbB2, and particularly in early stage and infiltrating lobular carcinomas that typically do not overexpress ErbB2 (Evans et al., 2006). This suggests that like Grb7, C17orf37 could provide advantages to tumour phenotypes, and in particular, amplification of C17orf37 could be the driving force of the ErbB2 amplicon.

Stard3, also known as MLN64, is another protein that is thought to be important in ErbB2 positive breast cancer. Like Grb7, RNA interference studies have suggested that expression of Stard3 may contribute to the tumour phenotype (Kao and Pollark, 2006) and has been found elevated in lymph node breast cancer metastasis (Moog-Lutz et al., 1997).

In addition to Grb7, C117orf37 and Stard3 also provide good candidate for breast cancer research. And I believe my project will also be beneficial to researchers studying these two proteins as well as other proteins in the ErbB2 amplicon.

4.9 Future directions

The first thing to do is to continue generating the MMTV-MTB/TetO-Grb7/ErbB2 knock-in tetra-transgenic mice. As previously noticed, ErbB2 knock-in model has tumours quite different from one case to another in term of tumour latency, protein expression and so on. Therefore in order to obtain meaningful information, a large cohort size is mandatory.

We have successfully demonstrated that Grb7 overexpression can disrupt cell polarity. However, the exact mechanism on how Grb7 disrupts cell polarity is still unclear. It is believed that microtubules and filamentous actin are important for establishing and maintaining a cell's polarity. Whether Grb7 disrupts cell polarity by directly interfering with microtubules and filamentous actin, or indirectly by down-regulating 14-3-3σ, further studies are required.

Similar to ErbB2, as well as many natural proteins, Grb7 exists in isoforms due to splicing differences during translation. The only known isoform of Grb7, namely Grb7V, has a truncated SH2 domain. Overexpression of Grb7 and Grb7V have both been detected in carcinomas, and correlated with tumourigenesis. Particularly, study of oesophageal carcinoma by Tanaka et al. (1998) showed data suggesting that Grb7V was strongly correlated with invasive tumour phenotype. Similar results were obtained in ovarian cancers (Wang et al., 2010). So the next step following this project is to investigate the role of Grb7V in mammary tumourigenesis, possibly by generating mouse model overexpressing Grb7V cDNA.

It is interesting to see that NDL mice selectively down-regulated the induced Grb7 expression, especially this happened when ErbB2 was up-regulated. Even though I do not know the exact reason, but it is definitely worthy to investigate.

4.10 Conclusion

Through ectopic expression of Grb7 in MDCK cells, we demonstrated that elevated expression of Grb7 protein correlates to the loss of epithelial cell polarity, and this was also found true in primary mammary epithelial cells derived from transgenic mice overexpressing Grb7. Grb7 overexpression in mammary gland was found to cause mammary ductal outgrowth defect, with

lactation still functional. In addition, H&E and immunoblotting identified a higher proportion of mammary ducts of transgenic mice overexpressing Grb7 had multiple epithelial layers and myoepithelium cells were missing. We further demonstrated that overexpression of Grb7 in NDL tumour cells increases their proliferative and invasive capacities, which is consistent with the results of other research groups studying Grb7 using human mammary epithelial cells.

NDL mice inducible-expressing Grb7 were generated by crossing MMTV-Neu-NDL2-5 model with MMTV-MTB/TetO-Grb7 bi-transgenic mouse. Doxycycline was added into drinking water to induce the overexpression of Grb7. It was found that Grb7 inducible system was selectively down-regulated in NDL tumours. Instead of having shorter tumour latency, Grb7 overexpressing NDL mice had delayed tumour onset (204 days) compared with control cohort (182 days). Further experiments are required to explain this observation. In addition, no significant difference was reported regarding lung metastasis.

Chapter 5

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