

LGR4 IN BREAST CANCER STEM CELLS

A Thesis

by

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ABSTRACT

Breast cancer is the most commonly diagnosed cancer among American women. G-protein coupled receptors (GPCR) comprise a huge family protein with almost 800 members. GPCRs sense molecules or other stimuli outside the cell, and activate intracellular signals. Consequently, a large proportion of modern drugs target these receptors. Lgr4 is a GPCR implicated in the development of multiple organs; in the mammary gland, it is expressed in the basal epithelial subpopulation and controls organ development by regulating stem cell activity through the wnt/ β -catenin pathway. High breast tumor expression of Lgr4 correlated with a high risk of tumor relapse after chemokine therapy and an elevated risk of bone metastasis. We crossed mice bearing a gene trap cassette in the *Lgr4* locus with several breast cancer mouse models such as MMTV-*Wnt1* and MMTV-*PyMT* to study the consequences of Lgr4 expression ablation in breast cancer progression. We found that the absence of Lgr4 significantly delayed tumor progression in both MMTV-*Wnt1* and MMTV-*PyMT* mouse models. Meanwhile, Lgr4 ablation led to diminished lung metastases in MMTV-*PyMT* tumors and several breast cancer cell lines. Further studies revealed that the repression of tumor progression and metastasis formation was due to a decreased cancer stem cell number in tumors with Lgr4 downregulation, as well as blocking of epithelial-mesenchymal transition. Mechanistic studies suggested that Lgr4 is a master regulator which modulates multiple pathways (Wnt, EGFR, MMP) in breast cancer. Our findings clarify the role of Lgr4 in

tumor progression and metastasis formation, and provide a potential therapeutic target in breast cancer treatment.

DEDICATION

To my father.

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NOMENCLATURE

ADP	Adenosine diphosphate
APC	Adenomatosis polyposis coli
AJCC	The American Joint Committee on Cancer
ASD	Anterior segment dysgenesis
BMP	Bone Morphogenic Protein
cAMP	Cyclic adenosine monophosphate
CK1 α	Casein kinase 1 α
CREB	cAMP response element-binding protein
CSC	Cancer stem cell
DAG	Diacyl-glycerol
Dvl	Dishevelled
ECD	Extracellular domain
ECL	Extracellular loops
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal transition
ER	Endoplasmic reticulum
ER'	Estrogen receptor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor

FSHR	Follicle-stimulating hormone receptor
Fzd	Frizzled
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GI	Gastrointestinal
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
IL	Intracellular loops
IP3	Inositol 1, 4, 5-trisphosphate
ISNL	Insulin-like
LEF	Lymphoid enhancer binding factor
LGR	Leucine-rich repeats G-protein coupled receptor
LHR	Luteinizing hormone receptor
LRP	Lipoprotein receptor-related protein
LRR	Leucine-rich repeats
MMTV	Mouse mammary tumor virus
PCP	Planar cell polarity
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLAP	Placental alkaline phosphatase
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PPi	Pyrophosphate
PR	progesterone receptor
PT	Pertussis toxin
PyMT	Polyoma virus-middle T antigen Antibody
RLXN	Relaxins
RNF43	Ring finger protein 43
RSPO	R-spondin
RXFP	Relaxin and insulin-like family peptide
TCF	T cell factor
TF	Transcriptional factors
TGF	Transforming growth factor (TGF
TIC	Tumor initiating cell
TM	Transmembrane
TNBC	Triple negative breast cancer
TSHR	Thyrotropin receptor
TSP-1	Thrombospondin
Wnt	Wingless and Int
ZEB	E-box-binding homeobox
ZNRF3	Zinc and ring finger 3

TABLE OF CONTENTS

	Page
ABSTRACT	ii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
NOMENCLATURE	vi
TABLE OF CONTENTS	ix
LIST OF FIGURES.....	xi
LIST OF TABLES	xii
CHAPTER I INTRODUCTION AND LITERATURE REVIEW	1
I.1 GPCR.....	1
I.2 Leucine-Rich repeat containing G protein coupled receptors (LGRs)	8
I.3 Breast cancer	19
I.4 Stem cells and cancer stem cells (CSCs).....	23
I.5 Metastasis and Epithelial-Mesenchymal transition (EMT)	27
CHAPTER II MATERIALS AND METHODS	29
II.1 Animals and cell lines.....	29
II.2 Histology and H&E staining	31
II.3 LacZ staining	32
II.4 Immunohistochemistry	33
II.5 Dissociation of breast primary tumors.....	34
II.6 Cell Staining and Flow Cytometry	35
II.7 Limiting dilution assay	36
II.8 Tail vein injection and luciferase assay	36
II.9 Virus preparation and cell line infection	37
II.10. <i>In vitro</i> tumorsphere assay.....	37
II.11 <i>In vitro</i> 3D culture assay.....	38
II.12 RNA collection, cDNA synthesis and Quantitative PCR.....	41
II.13 Trans-well invasion assay.....	42
II.14 <i>In vivo</i> tumor size determination	43

II.15 Western blot.....	43
II.16 Drug resistance assay.....	44
II.17 Growth curve	44
II.18 Wound healing assay	44
II.19 Statistics.....	45
CHAPTER III RESULTS	46
III.1 Lgr4 is highly expressed in several breast cancer cell lines and primary MMTV- <i>Wnt1</i> tumors.....	46
III.2 Lgr4 depletion significantly delays tumor growth in MMTV- <i>Wnt1</i> and MMTV-PyMT mice.....	52
III.3 Lgr4 ablation decreases CSC numbers in MMTV- <i>Wnt1</i> tumors and several breast cancer cell lines.....	54
III.4 Lgr4 inactivation results in decreased metastasis by affecting EMT	65
III.5 Lgr4 modulates Wnt pathway, EGFR pathway, and MMPs in breast cancers	77
CHAPTER IV DISCUSION.....	81
IV.1 Overview	81
IV.2 Significance.....	83
IV.3 Limitations	83
IV.4 Future directions	84
CHAPTER V CONCLUSIONS.....	85
REFERENCES.....	86

LIST OF FIGURES

	Page
Figure 1 Lgr4 affects relapse, metastasis and survival of breast cancer patients	48
Figure 2 Tumor growth in breast cancer mouse models is delayed by loss of Lgr4.....	50
Figure 3 Deletion of Lgr4 delays tumor progression in MMTV- <i>PyMT</i> tumors	55
Figure 4 Lgr4 inactivation delays breast cancer progression in MMTV- <i>Wnt1</i> tumors ...	56
Figure 5 Lgr4 inactivation leads to decreased sphere forming cell number in MMTV- <i>Wnt1</i> tumor	60
Figure 6 Ablation of Lgr4 represses Sox2 expression in breast cancers.....	66
Figure 7 Lgr4 deletion leads to inhibition of lung metastasis formation in MMTV- <i>PyMT</i> mice	70
Figure 8 Lgr4 expression level positively correlates with <i>in vitro</i> migration ability in human breast cancer cell lines	71
Figure 9 Inactivation of LGR4 inhibits <i>in vivo</i> lung metastasis formation in MDA-231 cell line.....	73
Figure 10 Lgr4 affects EMT genes in ErbB2 and 14-3-3 ζ transformed MCF-10A 3D organoids and MMTV- <i>Wnt1</i> tumors	75
Figure 11 Lgr4 is a master regulator of breast cancer by modulating Wnt pathway, EGFR pathway and MMPs	79

LIST OF TABLES

	Page
Table 1 Quantitative PCR primers	39
Table 2 Limiting dilution assay in MMTV-Wnt1 tumors.....	59
Table 3 Limiting dilution assay using serially passaged Wnt2508 cell line spheres	61
Table 4 Limiting dilution assay using MDA-231 cell lines	62

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 GPCR

I.1.1 Basic classification and structure of GPCRs

G protein coupled receptors (GPCRs) represent the largest family of transmembrane proteins which respond to a remarkable diversity of extracellular ligands or mechanical stimuli (Iismaa & Shine, 1992). GPCRs share similar structural traits such as an N-terminal extracellular domain, three extracellular loops (ECL1-3), 7 hydrophobic transmembrane (TM) helical structures, three intracellular loops (IL1-3) and a C-terminal intracellular domain (Kobilka, 2007). GPCRs can be divided into 5 different families based on their phylogenetic sequence characteristics: Rhodopsin-like receptors, Glutamate-like receptors, Adhesion-like receptors, Frizzled/tas2 family receptors and Secretin-like receptors (Fredriksson, Lagerstrom, Lundin, & Schioth, 2003). Rhodopsin-like receptors, also called family 1 or class A, consist of the largest subgroup of receptors which mainly respond to extracellular hormones, neurotransmitters and light, smell and taste stimuli. Almost all rhodopsin-like receptors have an Asp-Arg-Tyr (DRY) motif at the cytoplasmic end of the 3rd TM (Schertler, 2005). Family 1 receptors can be further categorized into Endogenous-ligand and Olfactory or Pheromone-ligand receptors based on the nature of their ligands. Alternatively, family 1 can also be divided into 19 subgroups (A1-A19) (Joost & Methner, 2002; Katritch, Cherezov, & Stevens, 2012) based on maximum likelihood-

based phylogenetic analysis, a method to study inference of phylogeny (Day & Sankoff, 1987). Rhodopsin-like receptors have long been used as models for GPCR function and structural studies for their diversity. LGR4 (also known as GPR48) falls into this category as well. Thus, most of this introduction will focus on Rhodopsin-like receptors.

Although the universal role of the N-terminal domain in GPCRs is not fully understood, some studies show that the N-terminus has a role facilitating ligand binding and receptor activation in family 1 and family 2 receptors. For example, the N-terminus guides ligand binding and inhibits the endocytosis rate of the dopamine D2 receptor (D. I. Cho et al., 2012), and modulates agonist binding affinity and G α_q activity of the 5-HT2B Receptor (Belmer et al., 2014). The 7TMs are highly conserved based on sequence analysis, and most conformational changes also take place within these segments after agonist-induced activation of GPCRs (Katritch et al., 2012). TM1 and TM2 are relatively stable even during receptor activation, possibly because they function as location elements which regulate membrane insertion (Kobilka & Deupi, 2007). There are strong contacts between TM1 and TM7 which maintain the integrity of the TM segments. TM3, TM5, TM6, and TM7 together with ECL2 and ECL3 are the main determinants of ligand binding affinity. Unlike the TM segments, the C-terminal, N-terminal and intracellular loop have the most variable structures which contribute largely to the versatility of GPCRs. IL2 and IL3 have been shown to determine the type of G α proteins which certain GPCRs specifically couple. The C terminal domain usually contains a high frequency of serine (Ser) or threonine (Thr) residues which will recruit arrestins and shut down GPCR signaling after being phosphorylated (Strader, Fong,

Tota, Underwood, & Dixon, 1994; Wess, 1998). Despite some rare cases in which small agonists bind to the N-terminal domain, most ligands bind within the cavities between TM segments to activate GPCRs (Baldwin, 1994). Together, the complexity of both GPCR structural transformation and upstream-to-downstream signaling are determined. At present, the major obstacles in GPCR studies are to predict the activities of certain GPCRs and to determine their structure through crystallization (Lagerstrom & Schioth, 2008).

I.1.2 GPCR activation and deactivation

GPCRs interact reversibly and specifically with different G proteins at their cytoplasmic end. There are three subunits of heterotrimeric G proteins: G_{α} , G_{β} and G_{γ} . Usually the G alpha subunit stays monomeric, while G_{β} and G_{γ} form a dimeric complex known as the beta-gamma complex when GPCRs are inactive (Seifert, Wenzel-Seifert, & Kobilka, 1999). Upon ligand binding, or in response to sensory signals, GPCRs undergo conformational changes at the cytoplasmic interface involving IL1, 2, 3 and the whole C terminal domain (Kobilka, 2007). Recent studies reveal that most GPCRs have relatively conserved conformational changes upon ligand binding, in terms of the non-covalent contacts between TM and TM and/or ligand to TM (Ghanouni, Steenhuis, Farrens, & Kobilka, 2001; Unal & Karnik, 2012). These conformational changes in turn recruit Guanine nucleotide exchange factors (GEFs) to activate G proteins. GEFs catalyze transition to an active form by releasing guanosine diphosphate (GDP) from the G_{α} subunit and allowing the binding of guanosine triphosphate (GTP) instead. This

results in a short-lived dissociation of monomeric G_{α} -GTP from the $G_{\beta\gamma}$ complex (Vetter & Wittinghofer, 2001). The separation between G proteins enables both disseminated components to regulate specific biological activities by interacting with downstream signaling pathways or cellular effectors. Although most GPCRs are able to function as signal transmitters monomerically on the cell membrane, some fascinating data also show that GPCRs can form homodimers or heterodimers to activate different signaling pathways (Angers, Salahpour, & Bouvier, 2002; Damian, Martin, Mesnier, Pin, & Baneres, 2006; Terrillon & Bouvier, 2004). The fact that GPCRs form dimers further increases the diversity and complexity of GPCR function and structure.

In order to turn off activated GPCR signaling, the most well studied mechanism is the binding of arrestins to phosphorylated GPCRs in order to block the activation of G proteins. In this mechanism, G protein-coupled receptor kinases (GRKs) are recruited and phosphorylate serine and threonine residues in IL3 and the C-terminus of agonist-bound GPCRs, causing increased affinity between GPCRs and cytosolic arrestins. The binding of arrestins to GPCRs will uncouple G proteins from GPCRs, blocking further signaling (Penela, Ribas, & Mayor, 2003; Ribas et al., 2007). More studies reveal that arrestins and GRKs also mediate the recycling process of GPCRs (receptor desensitization, internalization, cytoplasmic trafficking, resensitization and re-embedding into membrane) by recruiting the vesicle coating protein Clathrin (Lohse, Benovic, Codina, Caron, & Lefkowitz, 1990; Moore, Milano, & Benovic, 2007). Along with GPCR deactivation, the inherent hydrolytic GTPase activity of G_{α} converts G_{α} -GTP back to G_{α} -GDP, which then binds to the $G_{\beta\gamma}$ complex. The reassociation of G_{α} -GDP and

the $G_{\beta\gamma}$ complex terminates all interactions with various effectors and moves the $G_{\alpha\beta\gamma}$ complex back to the GPCR IL G-protein-binding site. Thus, a full GPCR activation-deactivation cycle is completed (McCudden, Hains, Kimple, Siderovski, & Willard, 2005).

I.1.3 G proteins and signaling

Heterotrimeric guanine nucleotide binding proteins (G proteins) are membrane-receptor attached, heterotrimeric, recycling proteins involved in signal transmission. Heterotrimeric G proteins have three subunits: alpha, beta and gamma. The G alpha subunits are 40-45 kDa proteins consisting of 350-400 amino acids. The G alpha family is the key component which determines the functions of each G protein heterotrimeric complex. G_{α} proteins can be divided into 4 subtypes based on their effectors and amino acid identity: $G_{\alpha s}$, $G_{\alpha i}/G_{\alpha o}$, $G_{\alpha q}/G_{\alpha 11}$ and $G_{\alpha 12}/G_{\alpha 13}$ (Hurowitz et al., 2000). Each family contains multiple subtypes which possess different effector binding affinities, specific expression patterns and GPCR associations.

In spite of some exceptions (He et al., 2014), $G_{\alpha s}$ (stimulatory G proteins) generally bind to and activate the 12-transmembrane glycoprotein adenylyl cyclase (AC). Activated AC will in turn enzymatically hydrolyze ATP into cyclic adenosine monophosphate (cAMP) and pyrophosphate (PPi). cAMP is a secondary messenger which regulates many important cellular activities in mammalian cells by activating protein kinase A (PKA) (Nature Reviews Drug Discovery, 2004; Wettschureck & Offermanns, 2005).

$G_{\alpha i/o}$ (inhibitory G proteins) has a relatively higher expression than other G_{α} proteins, suggesting a higher signal response following activation of $G_{\alpha i/o}$ -coupled receptors. Inhibitory G proteins bind to AC and inhibit its function, causing a drop in the cytosolic cAMP level and decreased PKA activity (El-Armouche, Zolk, Rau, & Eschenhagen, 2003; E. H. Wu & Wong, 2005). Pertussis toxin (PT) is used in many experiments to prevent $G_{\alpha i}$ from binding to GPCRs on the cell membrane by catalyzing the Adenosine diphosphate (ADP)-ribosylation of the $G_{\alpha i}$ subunits. Decreased sensitivity to multiple biological stimuli is observed after PT-induced ADP-ribosylations occur on different G alpha inhibitory proteins (Mangmool & Kurose, 2011). There are several other well established negative regulatory mechanisms of the $G_{\alpha i/o}$ family, such as Gustducin ($G_{\alpha gust}$) promotion of the enzyme phosphodiesterases which degrade cAMP to 5'-AMP in response to activation of certain taste receptors, resulting in inhibition of PKA (Clapp et al., 2008; Norton, D'Amours, Grazio, Hebert, & Cote, 2000).

Four members fall into the G protein q polypeptide ($G_{\alpha q}$) family or $G_{\alpha q/11}$ because these proteins are known to be capable of inducing downstream pathways by activating phospholipase C (PLC). PLC is a class of enzymes that catalytically cleave membrane-bound phosphatidylinositol 4, 5-bisphosphate (PIP₂) into inositol 1, 4, 5-trisphosphate (IP₃) and diacyl-glycerol (DAG). IP₃, another important secondary messenger, is then dissociated from the membrane and binds to a family of IP₃-sensitive receptors (InsP₃R) on the endoplasmic reticulum (ER), causing an activation of calcium channels and release of calcium ions (Ca^{2+}) from the ER into the cytoplasm. Meanwhile, DAG stays on the membrane as a secondary messenger which, together with elevated cytosolic Ca^{2+}

levels, activates Protein kinase C (PKC) (Blaukat, Barac, Cross, Offermanns, & Dikic, 2000; Suzuki, Nakamura, Mano, & Kozasa, 2003; Wettschureck & Offermanns, 2005).

$G_{\alpha 12}$ and $G_{\alpha 13}$ belong to one family $G_{\alpha 12/13}$, they have a wide expression pattern and transduce signals from activated GPCRs to a wide range of functional effectors. In most cases, these effectors mediate neither AC nor PLC, but mainly control actin cytoskeletal rearrangements or gene expression in mammalian cells by activating Rho GTPase signaling (Bishop & Hall, 2000; Hall, 1998, 1999).

There are 5 G_{β} subunits and 12 G_{γ} subunits identified so far, which contributes to a large number of potential $G_{\beta\gamma}$ complex combinations (Gautam, Downes, Yan, & Kisselev, 1998; Wettschureck & Offermanns, 2005). G_{β} and G_{γ} form a tightly interacting heterodimer which usually serves as a negative regulator and binds to the G_{α} subunit and suppresses its activity when the $G_{\beta\gamma}$ complex is inactive (Dingus et al., 2005). After G_{α} switches to its activated GTP-binding form, freed $G_{\beta\gamma}$ activates its own signaling effectors, such as PI-3-K, ACs, PLCs, receptor kinases (RTKs) and GIRKs (Ford et al., 1998), while maintaining a relatively stable conformation.

Although there are many aspects about GPCRs that cannot be covered in this short introduction, aberrant regulation of any process discussed above is closely related with a variety of diseases and cancers (Lappano & Maggiolini, 2011; Vassart & Costagliola, 2011).

I.2 Leucine-Rich repeat containing G protein coupled receptors (LGRs)

LGRs are a class of GPCRs with a large extracellular N-terminal domain which is enriched in the hydrophobic amino acid leucine. The Leucine-rich repeats (LRRs) form multiple structurally diverse three dimensional segments called Solenoid protein domains which usually endow specific ligand-binding affinity to the LGRs (Kobe & Kajava, 2001). Roughly 15 years ago, based on phylogenetic analysis and hormone screening, three subfamilies of LGRs were identified in vertebrates: the Group A glycoprotein hormone receptors including luteinizing hormone receptor (LHR), follicle-stimulating hormone receptor (FSHR), and thyrotropin receptor (TSHR), the Group B1 R-spondins (RSPO) receptors LGR4, LGR5 and LGR6, the Group B2 Bursicon-like receptor *Drosophila* LGR2 (DLGR2) and the Group C relaxin (RLXN) and insulin-like (ISNL) peptide receptors LGR7, LGR8, GPR142 and GPR 135 (Aittomaki et al., 1995; Bathgate, Ivell, Sanborn, Sherwood, & Summers, 2005; Hsu, Liang, & Hsueh, 1998; Hsu et al., 2002; C. Liu, Chen, et al., 2003; Minegishi et al., 1990; Scott et al., 2006).

I.2.1 Group A and C LGRs

LHR and FSHR in Group A are two important receptors mainly expressed in the reproductive system, such as ovary, testis and uterus (Dufau, 1998; Simoni, Gromoll, & Nieschlag, 1997). LHR and FSHR are receptors for LH and FSH respectively, two pituitary secreted heterodimeric glycoprotein hormones which not only share a similar structure but also work synergistically in reproduction. The human LHR and rat FSHR were first cloned in 1990 (Minegishi et al., 1990; Sprengel, Braun, Nikolics, Segaloff, &

Seeburg, 1990), and further functional analysis revealed they play critical roles in both male and female germ cell maturation by inducing cAMP signaling through activation of $G_{\alpha s}$. (Aittomaki et al., 1995; Gromoll, Simoni, & Nieschlag, 1996; Heckert & Griswold, 1993; Latronico, Abell, et al., 1998; Latronico et al., 1996; Latronico, Chai, et al., 1998; Rajagopalan-Gupta, Lamm, Mukherjee, Rasenick, & Hunzicker-Dunn, 1998). The finding of several LRRs in the N-termini of LHR, FSHR as well as the TSHR (together with more recently identified LGR1, 2, 4, 5 *etc.*) led to defining LGR as a new subfamily of GPCRs (Libert et al., 1989; Nishi, Hsu, Zell, & Hsueh, 2000).

The relaxin and insulin-like family peptide (RXFP) receptors, also known as the Group C LGRs, contain 4 closely related homologues: LGR7 or RXFP1, LGR8 or RXFP2, GPR135 or RXFP3 and GPR142 or RXFP4. The Group C members are also highly conserved with the Group A LGRs in vertebrate species throughout evolution. Although LGR7 was cloned early in 2000 (Hsu et al., 2000), no ligand was found until 2002, when Sheau Yu Hsu and his colleagues reported LGR8 as a new homologue to LGR7. At the same time, relaxin was identified to be a functional ligand for LGR7 and LGR8, activating AC/cAMP/PKA signaling (Hsu et al., 2002). Later studies confirmed RLXN3 to be a high affinity agonist for LGR7 while ISNL3 is one for LGR8 (Bathgate et al., 2005; Kumagai et al., 2002). In 2003, Changlu Liu and his colleagues identified another relaxin receptor, GPR135, and its homologue GPR142. Using a ^{35}S labelled radioligand $^{35}\text{SGTP}\gamma\text{S}$, they showed that instead of activating cAMP, relaxin 3-induced GPR135 and GPR142 activation will in turn inhibit cytosolic cAMP accumulation (C. Liu, Chen, et al., 2003; C. Liu, Eriste, et al., 2003). Agonist screening showed that

INSL5 has a similar expression pattern and co-evolution with GPR142 and an even higher GPR142 affinity than relaxin3 (C. Liu et al., 2005).

I.2.2. Group B LGRs: LGR4, LGR5 and LGR6

In 1998, Qingyun Liu's group successfully cloned a 907 amino acid GPCR and named it HG38 (McDonald et al., 1998). Later in the same year, an independent group also isolated both this receptor, which they named LGR5, as well as another novel receptor, LGR4 (Hsu et al., 1998). Two years later, LGR6 and LGR7 were identified (Hsu et al., 2000). These studies showed that LGR4, LGR5 and LGR6 have a conserved primary structure, such as a relatively large number of LRRs (18 in LGR4 and LGR5, 13 in LGR6) compared to other LGRs and identical structural alignments between LRRs 1-7 in LGR6 and LRRs 1-3, 8-11 in LGR4 and LGR5. The rhodopsin-like seven TM domains in these receptors are highly conserved as well as the junction region between TM1 and the ectodomain, for example these three receptors all have PYAYQCC and GXFKPCE motifs on their C terminal cysteine-rich flanking region. The conservation between LGR4, LGR5 and LGR6 suggests a similar ligand binding preference and function across species (Hsu et al., 2000; Hsu et al., 1998; Loh, Broussard, & Kolakowski, 2001).

I.2.3 Expression pattern and functions in different organs

Complete knockout of *Lgr4* results in an increased chance of embryonic death in multiple mouse strains (Kato et al., 2006; Mazerbourg et al., 2004). To reduce this risk, several groups have used genetic engineering strategies, in which a small amount of *Lgr4* is still expressed which increases the survival rate of *Lgr4* ablated mice and therefore makes possible to study of the loss-of-function phenotypes in different organs. In a gene trap mouse model generated by Philip A. Leighton, the *Lgr4* gene is disrupted by the insertion of a gene trap vector containing two biological marker genes (LacZ and placental alkaline phosphatase (PLAP)) into the first intron of *Lgr4* (Leighton et al., 2001). This insertion results in an expression pattern of the *Lgr4* exon 1-bacterial β -galactosidase fusion protein and PLAP in heterozygous mice (one allele of wide-type *Lgr4* and one allele of disrupted *Lgr4*) that mimics the expression pattern of *Lgr4* in wide-type mice (Van Schoore, Mendive, Pochet, & Vassart, 2005). Van Schoore and colleagues showed a broad LacZ activity pattern in heterozygous mice with particularly strong activity in adrenal, skin, cartilages, kidneys, reproductive tracts and nervous system cells (Van Schoore et al., 2005). *LGR5* demonstrated a much more restricted expression pattern in mouse hair follicle (Jaks et al., 2008), inner ear (Shi, Kempfle, & Edge, 2012), uterus (Sun, Jackson, Dey, & Daikoku, 2009), mammary gland (de Visser et al., 2012), kidney (Barker et al., 2012) and gastrointestinal (GI) tract (Barker & Clevers, 2010; Barker et al., 2007; W. de Lau et al., 2011). Studies on *Lgr6* are still limited, with published papers only showing a strong expression in skin (Snippert et al., 2010) and teeth (Kawasaki et al., 2014).

Although the expression pattern of these three receptors varies, it has been shown they all have significant effects on organ development. Lgr4-null mice ($Lgr4^{Gt(pGTOTMpfs)1Wcs}$) are associated with embryonic and perinatal death accompanied by intrauterine growth retardation (Mazerbourg et al., 2004) and LGR5-null mice undergo neonatal death caused by ankyloglossia and gastrointestinal distension (Morita et al., 2004). No report so far shows embryonic or neonatal death associated with LGR6 knockout. The Lgr4 gene trap knockin mouse model mentioned above also shows defective development of the kidney, liver (Van Schoore et al., 2005), male reproductive tract (Mendive et al., 2006) and small intestine (Mustata et al., 2011), but premature ureteric bud development (Mohri, Oyama, Akamatsu, Kato, & Nishimori, 2011). Further studies using another gene trap mouse model ($Lgr4^{Gt(LST020)Byg}$) showed that deletion of Lgr4 is related to anterior segment dysgenesis (ASD) of the eye (Weng et al., 2008), abnormal eye-lid development (Jin et al., 2008), defective erythropoiesis (Song et al., 2008), impaired bone formation (J. Luo et al., 2009), increased dextran sodium sulfate(DSS)-induced Inflammatory Bowel Disease (S. Liu et al., 2013), delayed mammary gland and prostate development (W. Luo et al., 2013; Y. Wang et al., 2013), polycystic kidney lesions and renal fibrosis (Dang et al., 2014). In addition, depletion of Lgr4 in this mouse model appears to promote the white-to-brown fat transformation which regulates energy expenditure (J. Wang et al., 2013). And deletion of Lgr4 in a similar gene trap mouse model ($Lgr4^{Gt(pU-21)1Kymm}$) confirmed the developmental defects of the male reproductive tract (Hoshii et al., 2007), as well as demonstrated impaired morphogenesis of the gall bladder and cystic duct (Yamashita et al., 2009). Some recent

papers show links between these Lgr4-null defects in different organs with stem cell regulation (W. Luo et al., 2013; Y. Wang et al., 2013). One recent published paper showed that a rare nonsense mutation (c.376C>T) within the LGR4 gene in human carriers causes a series of abnormalities which partially overlap the defects observed in Lgr4-null mice (Styrkarsdottir et al., 2013). For LGR5, extensive studies have shown Lgr5 as an effective marker for Wnt-regulated adult stem/progenitor cell populations in proliferative tissue such as the small intestine, colon (Barker et al., 2007), hair-follicle (Jaks et al., 2008), uterus (Sun et al., 2009), stomach (Barker et al., 2010), mammary gland (de Visser et al., 2012), kidney (Barker et al., 2012), cochlea (Shi et al., 2012) and olfactory epithelium (Chen et al., 2014), but limited defects are reported in LGR5-null mice. LGR6 positive cells are shown to have a pattern of expression similar to LGR5 in skin, and are able to generate all cell lineages of skin epithelium (Snippert et al., 2010).

I.2.4 Tissue specific signaling pathways and their ligands

As discussed above, LGRs are extremely versatile in terms of their potential mechanisms to transmit signals. Group A and C LGRs are known to elevate the cAMP level by regulating G proteins, while Group B LGRs are much more complicated. Here I will summarize all known signaling pathways of LGR4, 5 and 6 and their ligands.

LGR4 (also known as GPR48) and LGR5 (also known as GPR49) were postulated to have a G-protein interaction domain because of the conserved Glu-Arg-Trp triplet motif in the junction between TM3 and IL 2 (Hsu et al., 1998). In 2006, screening for the effects of point mutations in the putative G-protein interaction domain, Gao and

coworkers found that expression of T755I mutant LGR4 (GPR48-T775I) into HEK293 cells increased the cAMP level, suggesting that this was a constitutively active form of Lgr4 which activates the $G_{\alpha s}$ -cAMP dependent canonical GPCR pathway (Gao, Kitagawa, Shimada, et al., 2006). Extensive following studies revealed that LGR4 activates downstream pathways through a conserved pathway, $G_{\alpha s}$ -cAMP-cAMP response element-binding protein (CREB), to mediate different transcriptional factors (TF) in different tissues. For example, it was reported that Lgr4 regulates Pitx2, a key TF in anterior segment development, through the cAMP-dependent canonical GPCR pathway mediated by $G_{\alpha s}$ /cAMP/PKA/CREB (Weng et al., 2008). In definitive erythropoiesis and bone remodeling, ATF4 is regulated by Lgr4 through the same $G_{\alpha s}$ /cAMP/PKA/CREB pathway (Song et al., 2008). Also, Lgr4 was reported to positively regulate ER_{α} expression in the male reproductive tract through this canonical pathway (X. Y. Li et al., 2010). One study shows that through the same cAMP/PKA/CREB pathway, the CD14 expression level is significantly increased in Lgr4-deficient mice which leads to elevated TLR2/4 associated innate immunity regulation (Du et al., 2013). In contrast, most recently a study showed that LGR5 regulates focal adhesion kinase, NF- κ B and c-fos through $G_{\alpha 12/13}$ -Rho GTPase (Kwon, Park, Kim, & Kim, 2013). But there is no report so far that shows G_{α} protein activation by LGR6.

The ligands which activate different canonical pathways downstream of LGR4 and LGR5 are still unknown. In a *Drosophila melanogaster* study, Bursicon, a glycoprotein-like heterodimeric cysteine knot protein, was found to be able to

functionally bind to *Drosophila melanogaster* LGR2 (DLGR2) and increase the cAMP level *in vitro* (C. W. Luo et al., 2005). Since the Bursicon gene also encodes other cysteine-knot domain proteins which resemble bone morphogenetic proteins (BMPs) in vertebrate and DLGR2 is orthologous to vertebrate LGR4, 5 and 6, it is predicted that the endogenous ligands for LGR4, 5 and 6 are members of the BMP family (Barker, Tan, & Clevers, 2013).

In 1982, in a screen of 12 viral oncogenes, Roel Nusse and Harold Varmus found a strong tumorigenic role of mouse mammary tumor virus (MMTV) insertion into a gene locus on mouse chromosome 15, and this gene was named *int1* (Nusse, van Ooyen, Cox, Fung, & Varmus, 1984; Nusse & Varmus, 1982). Several other MMTV provirus favored oncogenes were then discovered, including *int2* (Peters, Brookes, Smith, & Dickson, 1983), and *int3* (Gallahan & Callahan, 1987). But following studies revealed that *int1*, *int2* and *int3* are functionally and evolutionarily unrelated (Dickson & Peters, 1987; Gallahan & Callahan, 1997; van Ooyen & Nusse, 1984). In addition, a large family of *int1*-related genes were discovered to be expressed widely in adult mice (Christian, Gavin, McMahon, & Moon, 1991). Thus the old *int1* gene nomenclature is longer viable. In 1991, Roel Nusse adopted a new nomenclature in which *int1* and *int1* related proteins become the Wnt family of proteins due to their similarity with proteins encoded by the *Drosophila melanogaster* gene *wingless* (Rijsewijk et al., 1987), while *int2* and *int3* were renamed as FGF3 and Notch4 respectively. In the past three decades, extensive studies have revealed the great importance and versatile biological functions of wnt proteins, from *drosophila* to humans, from embryos to adults, from development to cancer

(Clevers & Nusse, 2012; Nusse & Varmus, 2012). The most well studied mechanism of wnt signaling is the Wnt/ β -catenin pathway or the canonical Wnt pathway, in which the wnt proteins carry different signals from outside of cells, with the help of wnt pathway co-receptors such as lipoprotein receptor-related protein (LRP)-5/6. Wnt binding to the N-terminal extra-cellular cysteine-rich domain of a Frizzled (Fzd) family receptor on the cell membrane allows an increase in the cytosolic β -catenin level followed by translocation of β -catenin into the nucleus to replace Groucho family co-repressors and activation of the transcription factors T cell factor (TCF)/ lymphoid enhancer binding factor (LEF) family (Brannon, Gomperts, Sumoy, Moon, & Kimelman, 1997; Cavallo et al., 1998; Riese et al., 1997; Staal & Clevers, 2000). The cytosolic β -catenin level is regulated through the activity of a “destruction complex” which is constituted of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1 α (CK1 α). Without binding of Wnt proteins to Fzd, the destruction complex is free to degrade β -catenin in the cytoplasm, thus Wnt signaling pathway is quiescent. With activation of Wnt signaling through binding of Wnt proteins to Fzd, a conformational change of the intracellular domain of Fzd will release an interacting protein, Dishevelled (Dvl), to the cytoplasm. Dvl works as a suppressor for Axin and GSK3 (Penton, Wodarz, & Nusse, 2002; Wagner et al., 1997) and disrupts the ubiquitinating ability of the destruction complex, thus stabilizing β -catenin in the cytoplasm (Kennell & Cadigan, 2009). The canonical Wnt pathway has been shown to play important roles in development and stem/progenitor cells in various organs such as the hair follicle (DasGupta & Fuchs, 1999), heart (Naito et al., 2006), kidney (Park,

Valerius, & McMahon, 2007; Stark, Vainio, Vassileva, & McMahon, 1994) and lung (Goss et al., 2009). In addition, several β -catenin independent Wnt pathways (non-canonical Wnt pathways) are of great importance in development as well, including the Wnt/PCP (Vladar, Antic, & Axelrod, 2009), Wnt/calcium (Kohn & Moon, 2005) and Wnt/RTK (Green, Nusse, & van Amerongen, 2014) pathways.

Notably, R-spondins (RSPOs) and Norrin have also shown the capability of potentiating the Wnt/ β -catenin pathway. RSPOs consists of 4 members (RSPO1-4) which share a conserved domain architecture: two cysteine-rich furin-like domains and one thrombospondin (TSP-1) motif. They are widely expressed in vertebrates, but play different roles in a tissue-specific manner (W. B. de Lau, Snel, & Clevers, 2012). Signaling studies showed that RSPOs are potentiators of Wnt/ β -catenin pathway activation (Bergmann et al., 2006; Kamata et al., 2004; Kazanskaya et al., 2004; Kazanskaya et al., 2008; Nam, Turcotte, Smith, Choi, & Yoon, 2006). Norrin, a secreted protein encoded by the Norrie disease gene, shown been shown to enhance Wnt signaling by binding to Fzd4 with a high affinity thus regulating development of the vasculature in the inner ear and retina (Q. Xu et al., 2004).

Surprisingly, RSPOs were recently found to be functional agonists of LGR4, LGR5 and LGR6, and the R-spondin binding was found to potentiate Wnt/ β -catenin signaling (Carmon, Gong, Lin, Thomas, & Liu, 2011; W. de Lau et al., 2011; Gong et al., 2012). On the other hand, norrin was reported to be the mammalian ortholog for fly *burs* and *pburs*, and a ligand for LGR4 to regulate the BMP and Wnt/ β -catenin pathways (Deng et al., 2013; Siwko, Lai, Weng, & Liu, 2013). No significant change has been

observed in either heterotrimeric G protein or β -arrestin after RSPO-induced activation of LGRs (Carmon et al., 2011; Gong et al., 2012), suggesting a non-canonical GPCR pathway is involved in this process. It is worth noting that no LGRs other than LGR4, 5 and 6 have been found to be receptors for RSPOs, while norrin is able to bind Fzd4. Furthermore, successful crystallization of the LGR4 extracellular domain (ECD) and RSPOs have provided an insight into the mechanism based on which LGR4, 5 and 6 recognize RSPOs (D. Wang et al., 2013; K. Xu, Xu, Rajashankar, Robev, & Nikolov, 2013). Another study showed that RSPO-induced activation of LGR4 and LGR5 regulate both Wnt/ β -catenin as well as Wnt/PCP pathways through Clathrin-dependent endocytosis (Glinka et al., 2011). These studies brought attention to a new area in which LGR4, 5 and 6 activate downstream signaling through Wnt/ β -catenin and the Wnt/PCP pathway in a G-protein independent manner. Later studies bring other key components, the cell-surface transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) and ring finger protein 43 (RNF43), into this “non-canonical GPCR pathway” (Hao et al., 2012). ZNRF3 and RNF43 have been previously demonstrated to be Wnt target genes (van de Wetering et al., 2002; Van der Flier et al., 2007; Yagyu et al., 2004) and putative negative regulators of the Wnt pathway which is correlated with a high frequency of mutation in various types of human cancers (W. de Lau, Peng, Gros, & Clevers, 2014; Furukawa et al., 2011; Ryland et al., 2013). The mechanism is described as follows. In the absence of RSPO, ZNRF3/RNF43 ubiquitylate Fzd and LRP5/6, leading to degradation of these Wnt signaling components. The interactions between RSPOs and the ECD of ZNRF3/RNF43 will increase the affinity between ZNRF3/RNF43 and

LGR4/LGR5; the binding of ZNRF3/RNF43 to LGR4/LGR5 results in membrane clearance of ZNRF3/RNF43; decreased membrane level of ZNRF3/RNF43 leads to stabilized LRP6 and Fzd; thus Wnt/ β -catenin signaling is potentiated (Hao et al., 2012). A more recent study showed that the IQ motif containing Ras GTPase-activating protein 1 (IQGAP1) is another key component in this complex. The Rspo-induced activation of LGR4 signaling increases the affinity between the LGR4-intracellular-domain associated protein IQGAP1 and DVL, which further potentiates Wnt signaling (Carmon, Gong, Yi, Thomas, & Liu, 2014).

I.3 Breast cancer

I.3.1 General background

Breast cancer occurs when a group of cells from the mammary gland become more proliferative, invasive and grow out of control, eventually forming a tumor. In 2014, the estimated number of new cases of breast cancer in female patients is 232,670 and the estimated number of deaths caused by breast cancer in female patients is 40,000, which makes breast cancer the most prevalent and second deadliest cancer in women (Breast Cancer Facts & Figures, 2013-2014).

<http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-042725.pdf>). In rare cases, breast cancer also strikes male patients.

Although acquired (environmental) factors including nulliparity, age, obesity and environmental carcinogens have a significant impact on breast cancer progression (Tomatis, Melnick, Haseman, Barrett, & Huff, 2001), the hereditary factors remain

indispensable. The identification of breast cancer susceptibility genes BRCA1 and BRCA2 opened a new area for breast cancer study (Miki et al., 1994; Wooster et al., 1995). Together with *p53* and other genes identified in screening for breast cancer gene mutations (Malkin et al., 1990; Shattuck-Eidens et al., 1995), a breast cancer risk assessment system was established in which genetic testing of patients and their families became theoretical bases for breast cancer prevention and treatment (Biesecker et al., 1993; "Statement of the American Society of Clinical Oncology: genetic testing for cancer susceptibility, Adopted on February 20, 1996," 1996).

To ensure the best treatment for every individual patient, methods of classification based on different categories of features (size, histology, origin of cells, DNA profile and membrane receptors *etc.*) were introduced into clinical diagnoses (Raven, 1939a, 1939b). For this purpose, in 1977, The American Joint Committee on Cancer (AJCC) proposed a TNM staging system based on tumor size (T), node status (N), and metastasis (M) which is being reviewed and modified repeatedly, most recently in September 2014. The 7th edition of TNM classifies by the following criteria: primary tumor into 5 major categories ranging from T0 (no sign of primary tumor) to T3 (greatest dimension of primary tumor is greater than 50mm) and in addition T4 (Tumor of any size with direct extension to the chest wall and/or to the skin); regional lymph nodes status into 4 major categories ranging from N0 (No sign of regional lymph node metastases) to N3 (tumor spread to more distant or numerous regional lymph nodes); distal organ metastasis status into 2 major categories ranging from M0 (no sign of distant metastasis) to M1 (the greatest dimension of distant metastasis is greater than 0.2mm).

The combination of the values from these three categories will reflect the current staging of breast cancer ranging from 0 to 4. In addition, despite some rare cases (Paget's disease of breast), primary breast cancers can be divided into two major subgroups: ductal carcinoma and lobular carcinoma, based on their cells of origin (Haagensen, Lane, Lattes, & Bodian, 1978). IDC is the most common type of breast cancer, and it is believed to be the most dangerous type of breast cancer, making up 70 to 80 percent of breast cancer diagnosed (Borst & Ingold, 1993). Triple negative breast cancer (TNBC) is one type of IDC and does not express the receptor tyrosine-protein kinase ErbB-2 (HER2/NEU) as well as no estrogen receptor (ER) and Progesterone Receptor (PR). It spreads much more quickly than other types of breast cancers. In therapeutic treatment, TNBC lacks molecularly targeted treatments due to the absence of certain receptor expression, is highly resistant to traditional anti-estrogen hormone therapy, and often poorly responds to cytotoxic chemotherapy (Bauer, Brown, Cress, Parise, & Caggiano, 2007).

Breast cancers are versatile and heterogeneous. In order to have a deep insight into the behavior of each cancer type, gene profiles have to be assessed to define specific cancer types and their pathological nature. In 2000, Charles Perou and his colleagues investigated 8,102 genes by DNA microarrays for their expression patterns in a set of 65 breast cancer samples from 42 different individuals, based on breast cancer classification into 3 subgroups: ER positive/luminal-like, basal-like and ErbB2 positive breast cancer, and each type of cancer has its own prognosis and unique molecular portrait (Perou et al., 2000). A series of studies showed that the basal-like breast cancer patients have

shorter survival times and relapse-free times, and are more likely to form distant metastases (Minn et al., 2005; Sorlie et al., 2001; van 't Veer et al., 2002). Triple negative breast cancer (TNBC) accounts for 10–25% of all breast carcinomas (Carey et al., 2007; Haffty et al., 2006; Reis-Filho & Tutt, 2008). TNBCs make up the majority of but do not exactly equal the basal-like carcinoma group (Bertucci et al., 2008; de Ronde et al., 2010). Taken together, a breast cancer is a complicated ecosystem involves many kinds of entities (e.g. epithelial tumor cell, stromal cell, infiltrating immune cell, hematopoietic cell and endothelial cell) which have different roles during tumor progression. The cross-talk between each entity has further complicated the situation (Meacham & Morrison, 2013).

I.3.2 Use of MMTV-*Wnt1* and MMTV-*PyMT* transgenic mice as breast cancer models

The *Wnt1* gene was overexpressed in MMTV-*Wnt1* transgenic mice by mouse mammary tumor virus LTR enhancer insertion upstream of the *Wnt1* promoter. This insertion leads to constitutive activation of *Wnt1* expression in the mouse mammary gland which increases the risk for female mice to develop mammary cancer. According to previous reports, 50% of female MMTV-*Wnt1* transgenic mice develop mammary cancer by 6 months of age and metastasis to the lungs is rare (Y. Li, Hively, & Varmus, 2000). Whereas in our study, in an FVB background, *Wnt1* transgenic mice develop breast cancer at an average age of 89 days, approximately 15% of *Wnt1* transgenic mice never develop breast cancer, and 40% of transgenic mice develop lung metastasis.

In MMTV-*PyMT* transgenic mice, *PyMT* is also controlled by a mouse mammary tumor virus LRT enhancer, resulting in mammary gland-specific expression of *PyMT*. The MMTV-*PyMT* mouse model has a much more robust tumor phenotype than the MMTV-*Wnt1* model, including multifocal mammary gland adenocarcinoma formation, accelerated breast cancer initiation with 100% penetrance, shortened life-span, and increased metastasis number and size (Guy, Cardiff, & Muller, 1992).

I.4 Stem cells and cancer stem cells (CSCs)

It has been over 100 years since the term “stem cell” was used in 1908. The first experimental evidence related to this term was observed by C.E. Ford in 1956, when he reported that the lethality caused by overdose-radiation induced hematopoiesis deprivation in mouse can be rescued by bone marrow transplantation (Ford, Hamerton, Barnes, & Loutit, 1956). Therefore, together with the discovery of stem cell activity in the pig brain (Altman & Das, 1967) and of pluripotential cells in mouse embryos (Evans & Kaufman, 1981), the stem cell hypothesis was proposed: there is a rare cell population in certain organs that is capable of generating organ-specific mature cells through differentiation and maintain its immortality and potency through infinite self-renewal. This hypothesis has been best established in hematopoietic studies. Thanks to the development of cell flow cytometry technology, the isolation of a specific population using a series of cell surface markers from a large mixed pool of cells became possible (Fulwyler, 1965; Julius, Masuda, & Herzenberg, 1972). The first isolation of a stem cell enriched population was done by Gerald J. Spangrude and his colleagues in 1988, by

assessing the expression level of 3 sets of cell surface markers (Thy-1, Sca-1 and lineage markers which in this experiment include B220, Gr-1, CD4, CD8 and Mac-1). Cells with negative lineage markers, low level of Thy-1 and positive for Sca-1 were isolated from mouse bone marrow, and this population was reported to be capable of generating all blood cell types after transplantation into a hematopoiesis deprived mouse chimera (Spangrude, Heimfeld, & Weissman, 1988). Several studies further confirmed the existence of stem cells in hematopoiesis: upon different signaling stimuli, a group of high potential stem cells with expression of specific cell surface markers will undergo a series of biochemical changes and activate specific transcription factors resulting in differentiation into desired blood cell types; at the same time, stem cells perpetuate themselves through infinite self-renewal (Orkin & Zon, 2008).

The phenomena of cancer relapse after tumor incision and metastatic tumor formation lead to the suspicion that CSCs exist. The “cancer stem cell” hypothesis is derived from the idea of a “stem cell” and proposes that a rare population of tumor cells exist within certain cancers, that these cells possess an unlimited self-renewal ability and are capable of generating other lineages of cancer cells and reconstituting a new tumor. A parallel term, “tumor initiating cells” (TICs) describes a population of cancer cells which were enriched for tumorigenesis. By marking tumor cells with different sets of fluorescent-conjugated antibodies, populations with enriched tumorigenic ability were identified in different types of cancers. The first evidence of CSCs was described in human acute myeloid leukemia, in which xenotransplantation of a population of CD34 positive and CD38 negative human leukemia cells was reported to be able to cause

leukemia in recipient SCID mice (Lapidot et al., 1994); following studies using different cell surface markers showed similar results in series of other cancers such as brain (Singh et al., 2003), breast (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003), colon (O'Brien, Pollett, Gallinger, & Dick, 2007), ovary (S. Zhang et al., 2008), pancreas (C. Li et al., 2007), prostate (Collins, Berry, Hyde, Stower, & Maitland, 2005) and melanoma (Schatton et al., 2008). But in all these studies xenograft tumors in immune-deficient mice were only generated from thousands of transplanted cancer cells, thus debates were raised about the lack of evidence demonstrating that a single cancer cell is capable of generating a whole new tumor. In addition, the changed host micro-environment due to the xenograft may affect tumor growth. Further complicating interpretation of xenograft tumor initiation experiments, several reports showed that the frequency of tumor initiating cells varies depending on the strain of recipient mouse (Joyce & Pollard, 2009; Quail & Joyce, 2013). Using lineage tracing, Hans Clevers' group for the first time demonstrated that one single Lgr5 positive cell can generate other cell types in intestinal adenomas while staying at the primary cancer site (Schepers et al., 2012).

Distant metastasis are formed by a small number of disseminated cancer cells from a primary tumor, that is to say, to some extent, metastasis initiating cells must acquire stemness to generate the secondary tumor (F. Li, Tiede, Massague, & Kang, 2007). Indeed, the early disseminated cancer cell from distant metastasis are CSC-like (Balic et al., 2006), and the metastasis formation requires interactions between CSCs and their stem cell niche (Malanchi et al., 2012), and EMT endows differentiated cancer cells

with CSC-like properties (Mani et al., 2008; Morel et al., 2008). However, the studies of links between CSCs and metastasis are complicated by multiple tumor sites in one sample (Pantel, Alix-Panabieres, & Riethdorf, 2009).

Notably, CSCs also have other characteristics such as quiescence (Graham et al., 2002; Roth & Fodde, 2011), drug resistance (Dean, Fojo, & Bates, 2005), and radiation resistance (Pajonk, Vlashi, & McBride, 2010). All these features allow CSCs to avoid traditional cancer therapies and reconstitute tumors. Consequently, targeting CSCs is essential for developing effective therapies against cancers that do not respond to currently available treatments.

Characterization of tumor initiating cell-enriched populations in breast cancer has shown steady progress. After Al-Hajj and his colleagues enriched human breast cancer TICs by selecting CD24^{-low}/CD44⁺ (Al-Hajj et al., 2003), a list of cell markers were then discovered as human TIC markers in subsequent studies, including ALDH1 (Ginestier et al., 2007), ESA (Fillmore & Kuperwasser, 2008), CD133 (Wright et al., 2008), CK5 (Kabos et al., 2011), Sox2 (Leis et al., 2012) and some other features (Charafe-Jauffret et al., 2009; Lawson, Blatch, & Edkins, 2009). However, the understanding of TIC markers in mice is still relatively poor, with Sca-1 in BALB-neuT induced tumors (Grange, Lanzardo, Cavallo, Camussi, & Bussolati, 2008), CD61 in mouse mammary tumor virus (MMTV)-*Wnt1* tumors and *p53*^{+/-}-derived tumors (Vaillant et al., 2008), CD24⁻/CD90⁺ for TICs of MMTV-Polyoma virus-middle T antigen (PyMT) and MMTV-*Wnt1* mice (R. W. Cho et al., 2008; Malanchi et al., 2012) and others (Ma et al., 2012).

I.5 Metastasis and Epithelial-Mesenchymal transition (EMT)

Metastasis has been described and studied for over a century, but understanding of the deep mechanism driving metastasis is still limited. Metastasis is the process by which cancer cells from a primary tumor undergo multiple alterations resulting in cancer cell dissemination mainly into the circulatory system or lymphatic system, arrival in distant organs, and formation of a secondary cancer. The most common site for metastatic cells to reside are the brain, bone, liver and lung, but metastatic preference varies according to different cancer types (Bubendorf et al., 2000; Disibio & French, 2008; Hess et al., 2006; Nguyen, Bos, & Massague, 2009; Paget, 1889). Upon metastasis formation, the function of recipient organs is likely to be interrupted. Therefore, it is considered to be the final and the most fatal stage of cancer progression, and around 90 percent of breast cancer deaths are caused by metastasis (Bendre, Gaddy, Nicholas, & Suva, 2003).

EMT is a process through which an epithelial cell undergoes a series of gene and behavior changes accompanied by changes in cell polarity, loss of cell to cell adhesion and acquisition of increased migratory as well as invasive ability. EMT was first discovered and described during chicken embryogenesis in the 1980s, and then reported to be also important in many biological processes such as organ development and fibrosis, tissue regeneration and wound healing (Kalluri & Weinberg, 2009). More recent studies showed that EMT occurs during cancer metastasis as well (Thiery, 2002). To understand this process, the conditions that trigger EMT need to be determined. The most predominant change of cells undergoing EMT is loss of E-cadherin expression,

which is a membrane protein important for cell adhesion normally expressed in epithelial cells (Angst, Marcozzi, & Magee, 2001). Gene profiling identified a list of transcriptional factors which are able to induce EMT by regulating their downstream gene expression and eventually affect E-cadherin levels upon activation. Among them, Snail1, Snail2, zinc finger E-box-binding homeobox (ZEB) 1, ZEB2 and LEF1 are found to be able to directly repress the CDH1 promoter, resulting in a decreased level of E-cadherin (Batlle et al., 2000; Cano et al., 2000; Eger et al., 2005; Jamora, DasGupta, Kocieniewski, & Fuchs, 2003; Remacle et al., 1999). Another EMT-inducing factor family, Twist, not only is able to bind to the E-box of *CDH1* and inhibit its activity (Vesuna, van Diest, Chen, & Raman, 2008) but also directly regulates *SNAI2*, the human gene encoding SNAIL2 (Casas et al., 2011; Yang et al., 2004). In addition to these EMT-inducing transcription factors, more signaling pathways are involved in regulating EMT, such as the Wnt/ β -catenin (Kemler et al., 2004), transforming growth factor (TGF) β (J. Xu, Lamouille, & Derynck, 2009), Hedgehog (Omenetti et al., 2008), EGFR (Lo et al., 2007), and notch signaling pathways (Z. Wang, Li, Kong, & Sarkar, 2010) as well as micro RNA-dependent pathways (J. Zhang & Ma, 2012). All involved pathways form a complicated cross-talk network which controls EMT (Gonzalez & Medici, 2014).

CHAPTER II

MATERIALS AND METHODS

II.1 Animals and cell lines

Animals were maintained in the Program for Animal Resources of the Institute of Biosciences and Technology, Texas A&M Health Science Center, and were handled in accordance with the principles and procedures of the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Lgr4-null mice were generated by microinjection of an *Lgr4* gene trap embryonic stem cell clone (LST020) from Williams Skarnes (Bay Genomics) into blastocysts of C57BL/6 mice (Weng et al., 2008). *Lgr4*^{+/-} female mice were back crossed to male mice of a C57BL/6 background (Charles River, Wilmington, MA) for 5 generations or male FVB mice for 12 generations. Inbred FVB *Lgr4* null mice were used in prostate development and stem cell studies.

MMTV-*Wnt1* mice in a FVB background were kindly provided by Dr. Yi Li (Baylor College of Medicine, Department of Molecular and Cellular Biology). *Lgr4*^{+/-} mice of a FVB background were crossed with MMTV-*Wnt1* mice to generate MMTV-*Wnt1 Lgr4*^{-/-} mice for breast cancer studies. MMTV-*Wnt1 Lgr4*^{+/+} litter mates were used as controls.

MMTV-*PyMT* mice in a FVB background were obtained from The Jackson Laboratory. *Lgr4*^{+/-} mice of a FVB background were crossed with MMTV-*PyMT* mice

to generate MMTV-*PyMT* *Lgr4*^{-/-} mice for breast cancer studies. MMTV-*PyMT* *Lgr4*^{+/+} litter mates were used as controls.

All 3-week old FVB/NJ, BLAB/c and B6.Cg-Foxn1nu/J (Nude) mice for transplantation and tail vein injection assays were purchased from The Jackson laboratory.

Primers used for genotyping *Lgr4* null mice include, 5'-GGT CTT TGA GCA CCA GAG GAC ATC-3' (pGT2TMPFS R), 5'-AAA AGC CAC ATT CAA ATC TTA GTA ACC-3' (*Lgr4* WILD TYPE reverse), 5'-AAG CAC TTG ATG GTC AGA CTA CAT GC-3' (*Lgr4* WILD TYPE forward). Primers used for genotyping SV40 transgene in MMTV-*Wnt1* TG mice include 5'-GAA CTT GCT TCT CTT CTC ATA GCC-3' (*Wnt1* forward), 5'-CCA CAC AGG CAT AGA GTG TCT GC-3' (SV40 reverse). Primers used for genotyping in MMTV-*PyMT* include: 5'-GGA AGC AAG TAC TTC ACA AGG G-3' (forward), 5'-GGA AAG TCA CTA GGA GCA GGG-3' (reverse). Primers used for genotyping in Nude mice include: 5'-CTT CCG CCT TTC TCC TTC AG-3' (forward), 5'-CCT CAT GGA AGT GCC TCT TG-3' (reverse).

MDA-231 and MCF7 human breast cancer cell lines were purchased from ATCC and cultured in DMEM/high glucose medium with 10% FBS and 1% Penicillin/Streptomycin.

MDA-468 and Hs578T human breast cancer cell lines were kindly provided by Dr. Yi Li (Baylor College of Medicine, Department of Molecular and Cellular Biology), and cultured in DMEM/high glucose medium with 10% FBS and 1% Penicillin/Streptomycin.

The Wnt2508 mouse breast cancer cell line was kindly provided by Dr. Yi Li (Baylor College of Medicine, Department of Molecular and Cellular Biology), and cultured in DMEM/F12 medium with insulin, EGF 10% FBS and 1% Penicillin/Streptomycin.

MCF10A cells constitutively expressing ErbB2 and 14-3-3 ζ were kindly provided by Dr. Dihua Yu (The University of Texas MD Anderson Cancer Center).

II.2 Histology and H&E staining

II.2.1 Paraffin embedding

Mammary glands, lungs and tumors were fixed in zinc formalin for 4hrs to overnight. Tissues were then dehydrated by: (1) 70% Alcohol, overnight; (2) 2 changes of 85% alcohol, 1 hour each; (3) 2 changes of 95% alcohol, 1 hour each; (4) 2 changes of 100% alcohol, 1 hour each; (5) 50% xylene, 50% alcohol, 20 minutes; (6) 100% xylene, 1 hour; (7) 100% xylene, 30 minutes; (8) Paraffin I at 65 °C, 2 hours; (9) Paraffin II at 65 °C, overnight; (10) Paraffin III at 65 °C, 30 minutes. Tissue specimens were embedded in paraffin and sectioned at 3 μ m.

II.2.2 H&E staining

3 μ m sections were incubated (1) at 65 °C for 1~2 hours; (2) 100% xylene I, 5 minutes; (3) 100% xylene II, 5 minutes; (4) 100% xylene III, 5 minutes; (5) 2 changes of 100% alcohol, 2 minutes each; (6) 2 changes of 95% alcohol, 2 minutes each; (7) 70% alcohol for 2 minutes; (8) distilled water, 1 minute.

Sections were then stained in hematoxylin solution (Sigma Co, St. Louis, MO) for 3-5 minutes, rinsed with running hot water 5 times, incubated in 70% alcohol for 2 minutes, Counterstained in Eosin solution (Sigma Co, St. Louis, MO) for 20~40 seconds, placed in 2 changes of 95% alcohol, 2 minutes each followed by 2 changes of 100% alcohol, 2 minutes each and cleared in 3 changes of xylene, 5 minutes each. Slides were then mounted with xylene based mounting medium (Thermo Fisher Scientific, Waltham, MA).

II.3 LacZ staining

Tumor LacZ staining was performed in mammary glands and breast tumors at indicated ages. Mammary glands and breast tumors were fixed in freshly made fixative solution (0.2% glutaraldehyde, 5mM EGTA, 2mM MgCl₂, 2% formaldehyde in 0.1M phosphate buffer PH7.3) for 1 hour at room temperature. After 3 washes in freshly made wash buffer (0.1% deoxycholic acid, 2mM MgCl₂, 0.2% NP40 in 0.1M phosphate buffer PH7.3) for 30 minute each, samples were incubated in staining solution (1mg/ml X-gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide in wash buffer) overnight at room temperature.

Hematoxylin staining was performed on 3 μ m LacZ sections. Sections were (1) incubated at 65 °C overnight to removed paraffin roughly; (2) 3 changes in 100% xylene, 2 minutes each; (3) 2 changes of 100% alcohol, 30 seconds each; (4) 2 changes of 95% alcohol 30 seconds; (5) 70% alcohol for 1 minute; (6) stain in Hematoxylin solution for 30 seconds. (7) 2 changes of 95% alcohol, 30 seconds each; (8) 2 changes of 100%

alcohol, 30 seconds each; (9) Clear in 3 changes of xylene, 30 seconds each; (10) Mount with xylene based mounting medium.

II.4 Immunohistochemistry

II.4.1 Antibodies

Antibodies used for immuno-histochemistry include: mouse monoclonal Cytokeratin 8 antibody (Fitzgerald, Acton, MA, 1:200), rabbit monoclonal anti-Cytokeratin 5 antibody (Abcam plc, Cambridge CB4 0FL, United Kingdom, 1:250), Mouse monoclonal anti-p63 antibody (Abcam plc, Cambridge CB4 0FL, United Kingdom, 1:200), mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:100); rabbit polyclonal anti-Ki67 antibody (Novus, Littleton CO, 1:100); mouse monoclonal anti-E-cadherin antibody (Abcam plc, Cambridge CB4 0FL, United Kingdom, 1:200); mouse monoclonal anti-Cytokeratin 18 antibody (Abcam plc, Cambridge CB4 0FL, United Kingdom, 1:100).

II.4.2 IHC staining

3 μ M sections were first deparaffinized and rehydrated as described in H&E staining steps (1) through (8). Sections were then washed in PBS for 5 minutes. Antigen retrieval was achieved by boiling sections in 10 mM sodium citrate (PH 6.0) for 15~20 min. Sections were then treated with 3% H₂O₂ for 15 min at room temperature to reduce endogenous peroxidase activity. Avidin/biotin blocking kit (Vectorlabs, Burlingame, CA) was used to reduce background staining. Immunostaining was performed using the

Vectastain Elite ABC system (Vectorlabs, Burlingame, CA) and VECTOR NovaRED Peroxidase Substrate Kit (Vectorlabs, Burlingame, CA) according to protocols provided. Sections were incubated with primary antibody overnight at 4 °C. Nuclei were then counter stained with hematoxylin for 5-10 minutes. Finally, sections were dehydrated and mounted by: (1) Rinsing with running hot water for 5 times; (2) 70% alcohol for 2 minutes; (3) 2 changes of 95% alcohol, 2 minutes each; (4) 2 changes of 100% alcohol, 2 minutes each; (5) Clearing in 3 changes of xylene, 5 minutes each; (6) Mount with xylene based mounting medium.

II.5 Dissociation of breast primary tumors

Primary breast tumors were excised from euthanized MMTV-*Wnt1* tumor-bearing mice at indicated ages. Tumors were digested using the following steps: (1) Mince 0.5 cm³ tumor into small pieces no bigger than 2 mm³; (2) resuspend minced tumor with 10 mL Medium 199 containing 0.2% FBS; (3) add 50 units of DNase I (Sigma-Aldrich, St. Louis, MO), 8 units of Liberase TH (Roche Applied Science, Indianapolis), and incubate at 38 °C for 2 hours, pipetting every 30 minutes; (4) add another 50 units of DNase I, incubate 30 minutes; (5) add 40 mL RPMI medium containing 10% FBS to inactivate DNase I and Liberase TH; (6) filter all cells using 40 μM nylon cell strainer (BD Biosciences, San Jose, CA); (7) centrifuge for 5 minutes, carefully aspirate and discard the supernatant without disturbing the pellet; (8) resuspend pellet with 5 mL Red blood cell lysis buffer (eBioscience, San Diego, CA), and incubate at room temperature for 5 minutes to remove red blood cells; (9) add 10 mL HBSS

medium containing 2% FBS to dilute red blood cell lysis buffer; (10) filter all cells using 40 μ M nylon cell strainer, centrifuge for 5 minutes, remove supernatant and resuspend pellet in 2 mL HBSS medium containing 2% FBS.

II.6 Cell staining and flow cytometry

All antibodies in this procedure were from eBioscience, San Diego, CA. Single primary breast cancer cell suspension was stained with 5 biotin-conjugated cell lineage markers (2 μ L for each antibody): anti-mouse TER-119 (Ly-76), anti-mouse Ly-6G (Gr-1), anti-mouse CD11b, anti-mouse/human CD45R (B220) and anti-mouse CD3e; 0.1 μ g/200 μ l Streptavidin eFlour 450, 0.2 μ g/200 μ l Anti-Mouse CD24 (HSA) APC and 0.2 μ g/200 μ l Anti-Rat/Mouse CD90 (Thy-1) PE in 1% BSA in PBS for 0.5 hour. After staining, cells were washed with 1% BSA in PBS 3 times and re-suspended 0.5ml 1% BSA in PBS. FACS analysis was done by using the BD FACS Canto and CellQuest software. Cell sorting was conducted by BD FACS Vantage (BD Biosciences, San Jose, CA). Data from a minimum of 10,000 cells of each sample was recorded for analysis. The cell counter of the flow cytometers was used to determine cell numbers. Small samples of the triple-sorted and GFP-sorted cells were reanalyzed for purity. Final cell purity was greater than 95%. Pellets of cancer cells were resuspended in 4% PFA for fixation before flow cytometry in analysis-only experiments.

II.7 Limiting dilution assay

FVB/NJ or Nude female mice (2 months of age) were sedated by isoflurane (Abbott Laboratories, North Chicago, Illinois) through a KSC Tabletop Anesthesia System (Kent Scientific Corporation, Torrington, Connecticut). The number of cells injected in each group was calculated based on pilot experiments. Sorted cells or digested primary tumor cells or breast cancer cell lines were suspended in 50 μ L of indicated medium and then well mixed with 50 μ L growth factor reduced Matrigel (BD Biosciences, San Jose, CA). Mixed cells were injected orthotopically into the right 4th mammary gland of Nude mice using a 27_{1/2}-gauge tuberculin syringe (BD Biosciences, San Jose, CA).

II.8 Tail vein injection and luciferase assay

Nude female mice (2 months of age) were sedated by isoflurane (Abbott Laboratories, North Chicago, Illinois) through a KSC Tabletop Anesthesia System (Kent Scientific Corporation, Torrington, Connecticut). Mouse tails were soaked in warm water to dilate tail veins. GFP or luciferase labeled MDA-231 were suspended in PBS and injected into dilated tail veins. 2×10^5 MDA-231 cells stably expressing luciferase as well as either shControl or shLGR4 were injected into the tail vein of each of 5 female BALB/c nude mice per shRNA. The lung bioluminescence photon values were monitored on days 0, 10, 20, and 30 using the IVIS 200 System (Xenogen/Perkin Elmer, Boston, MA, USA). The lungs were isolated, fixed and H & E stained after mice were euthanized.

II.9 Virus preparation and cell line infection

We adopted a protocol from the Lamia laboratory (Department of Chemical Physiology, The Scripps Research Institute) to generate virus (The Lamia Lab Viral Production and Infection Protocol, http://www.scripps.edu/lamia/Lamia_website/Protocols_files/VirusProduction%26InfectionJan2012.pdf) using a calcium phosphate transfection kit (Clontech Laboratories, Mountain View, CA) and minor modifications. General steps are described as following: (1) 293T cells were grown in DMEM/high glucose medium with 10% FBS until 50% confluent; (2) transfected 6.4 μg psPAX, 3.6 μg pMD2.G and 5 μg target plasmid (5 different Lgr4 knockdown vector purchased from Thermo Fisher Scientific, Waltham, MA) per 10 cm dish into 293T; (3) changed to fresh medium with puromycin (10 $\mu\text{g}/\text{ml}$) for selection; (4) culture medium was collected and filtered with 0.4 μM strainer to remove cell debris once per day for 3 days after transfection; (5) MDA-231 or Wnt2508 breast cancer cell lines were infected with collected virus medium for 2 hours and changed to fresh medium every day for the next 3 days; (6) titration was measured at the end of the 3rd infection.

II.10. *In vitro* tumorsphere assay

10,000 cells from digested primary tumor or 1,000 cells of breast cancer cell lines were cultured in sphereculture medium for tumorsphere formation. Each generation of primary tumorspheres was cultured for 18 days, each generation of breast cancer cell line tumorspheres was cultured for one week. When passaging tumorspheres, Matrigel

was dissociated by 1mg/ml Dispase (Life Technologies, Carlsbad, CA) for 45 minutes at 37 °C. After washing with PBS, tumorspheres were dissociated with 0.25% Trypsin-EDTA 37 °C for 15 minutes, pipetting up-and-down every 5 minutes. To obtain a single cell suspension, Trypsin treated tumorspheres were passed through a 40 µm cell strainer and resuspended in sphereculture medium.

II.11 *In vitro* 3D culture assay

The general protocol for *In vitro* 3D culture assay we adopted was published on Science Direct (Jayanta Debnath 2003). Minor changes are described as follows: 6 well-plate was pretreated with a small volume of Matrigel to form a thin layer of “bed”. 50 µl of MCF10A-ErbB2-14,3,3ζ cell suspension at indicated concentration was mixed with 50 µl growth factor reduced Matrigel and seeded on the Matrigel bed. After Matrigel was solidified at 37 °C for 30 minutes, 2 mL of breast cancer 3D culture Medium was added to the culture. Medium was changed twice a week.

Table 1 Quantitative PCR primers

Sequence Name	Bases	Sequence
mCdh1 RT F	24	TCA CAG TGA AGC GGC ATC TAA AGC
mCdh1 RT R	24	ACC AGG TTC TTT GGA AAT TCG CCC
mVimentin RT F	24	ATC ATG CGG CTG CGA GAG AAA TTG
mVimentin RT R	24	TTC TTG GCA GCC ACG CTT TCA TAC
mSnail RT F	26	TTG TAA CAA GGA GTA CCT CAG CCT GG
mSnail RT R	24	TCT CTT CAC ATC CGA GTG GGT TTG
mb-catenin RT F	23	ACT GGC AGC AGC AGT CTT ACT TG
mb-catenin RT R	22	AAT TGC ACG TGT GGC AAG TTC C
mSox2 RT F	24	ATG AGA GCA AGT ACT GGC AAG ACC
mSox2 RT R	24	TCG GCA GCC TGA TTC CAA TAA CAG
m36B4 F	21	GGC ATC ACC ACG AAA ATC TCC
m36B4 R	21	AAT GCA GAT GGA TCA GCC AGG
mLgr4 F	22	AAC AGT ACC CAG TGA AGC CAT T
mLgr4 R	22	GAT GTT GTC ATC CAG CCA CAG A
mLrp5 RT F	24	ACC TCA GCA TTG ACA TCT ACA GCC
mLrp5 RT R	24	TTC GAT TCG CTT TAG GTC GGC ATC
mb-catenin RT F	23	ACT GGC AGC AGC AGT CTT ACT TG
mb-catenin RT R	22	AAT TGC ACG TGT GGC AAG TTC C
mLrp6 RT F	26	GTG ACA TCC ATG CAG TAA AGG AGC TG

Table 1 Quantitative PCR primers (Continued)

mLrp6 RT R	24	ATC TGA TTT GTC CTG GCA GTT CGC
mAxin2 RT F	24	TTC CTG ACC AAA CAG ACG ACG AAG
mAxin2 RT R	24	TAA CAT CCA CTG CCA GAC ATC CTG
mWnt2 RT F	24	ACA ACA ACA GAG CTG GAA GGA AGG
mWnt2 RT R	24	ATG TGT CAT AGC CTC TCC CAC AAC
mWnt4 RT F	24	AAA CGG AAC CTT GAG GTG ATG GAC
mWnt4 RT R	24	ACG TCC ACA AAG GAC TGT GAG AAG
mWnt5a RT F	24	TTT GGC AGG GTG ATG CAA ATA GGC
mWnt5a RT R	24	TGT AAG TTC ATG AGG ATG CGT GCG
mWnt10a F	21	GAA CAA AGT CCC CTA CGA GAG
mWnt10a R	20	CCT TCA GTT TAC CCA GAG CG
mCyclin D1 F	24	TGG AAC TGC TTC TGG TGA ACA AGC
mCyclin D1 R	24	TCT GGA AAG AAA GTG CGT TGT GCG
hMMP3-F	24	TGA GGA CAC CAG CAT GAA CCT TGT
hMMP3-R	24	ACC AAC ATC AGG AAC TCC ACA CCT
hMMP11-F	24	ACT CAC CTT TAC TGA GGT GCA CGA
hMMP11-R	24	AGG GCT GGC CAT ATA GGT GTT GAA
hLGR4-F	24	AGG CCT TGT CTG GGT TGA AAG AAC
hLGR4-R	24	TGC AGA ACT ACC AGG CTT GAA AGG

II.12 RNA collection, cDNA synthesis and Quantitative PCR

0.1 cm³ breast tumor was soaked in liquid nitrogen and milled into tissue powder, 1 mL Trizol LS reagent (Life Technology, Carlsbad, CA) was then added to lyse the tissue. 90% confluent breast cancer cell lines were lysed by adding 1 mL Trizol LS reagent per 10 cm plate. Total RNA was then extracted by the following steps: (1) Incubate at room temperature for 5 minutes; (2) Collect lysate into a 1.5 mL RNase-free Eppendorf tube and centrifuge at 12,000 rpm for 5 minutes, transfer supernatant to a new RNase-free tube; (3) Add 200 μ L chloroform, mix thoroughly with vortexing, incubate at room temperature for 15 minutes; (4) 12,000 rpm centrifuge at 4 °C for 15 minutes; (5) Carefully remove the aqueous phase of the sample and pipetting the solution into a new RNase-free tube without making any contact with the interphase or organic layer; (6) Add 500 μ L isopropanol, mix thoroughly and incubate at room temperature for 5 minutes; (7) 12,000 rpm centrifuge at 4 °C, discard supernatant; (8) Wash RNA pellet with 1 mL 70% ethanol; (9) 8,000 rpm centrifuge at 4; (10) Dissolve RNA pellet with 50 μ L RNase-free water, and proceed to determine RNA concentration and 260/280 ratio measurement using Bio-Red SmartspecTM Plus spectrophotometer (Bio-Red, Hercules, CA)

2 μ g RNA was annealed to 1 μ g Oligo (dT) 15 Primer (Promega, Madison, WI) at 70 °C for 5 minutes. Samples were put on ice immediately for 5 minutes and then subjected to cDNA synthesis by 200unit/reaction M-MLV Reverse transcriptase (Promega, Madison, WI) for 50 minutes at 42 °C. Reverse transcriptase was inactivated at 75 °C for 15 minutes.

cDNA was diluted at 1:10 and 1 μ L was subjected to each quantitative PCR reaction. Real time quantitative PCR was done with GoTaq® qPCR Master Mix (Promega, Madison, WI). Quantitative PCR was performed on Mx3000P QPCR System (Agilent Technologies, Santa Clara CA) and with MxPro QPCR Software (Agilent Technologies, Santa Clara CA). The ratio between expression levels in the two samples was calculated by relative quantification, by using housekeeping genes 36B4 in mouse QPCR and β -actin in human QPCR as reference for normalization.

II.13 Trans-well invasion assay

Growth factor reduced Matrigel (BD Biosciences, San Jose, CA) was mixed with serum free MEM Eagle medium at 1:4. 100 μ l of the mixture was added to the bottom of cell culture insert with 8 μ m pore (BD Biosciences, San Jose, CA). Cell culture inserts were incubated at 37 °C for 1 hour.

Cells were serum starved in DMEM/high glucose medium containing 0.2% FBS for 12 hours. Starved cells were pre-treated with mytomycin C (Sigma Co, St. Louis, MO) at 20 μ g/ml for 4 hours. Starved cells were harvested and resuspended at 1 \times 10⁵ cells/ml in DMEM/high glucose medium containing 0.2% FBS. 100 μ l of cell suspension was seeded into cell culture insert with Matrigel at bottom. 500 μ l complete DMEM/high glucose medium containing 10% FBS was added to lower chamber. 10% FBS was served as chemo-attractant.

Cell invasion was checked every 12 hours until a significant amount of cells invaded through 8 μ m pore on cell culture insert. Remove cells from the top of the filter

with cotton swab. Cells at the bottom of the insert were fixed by immersing cell culture inserts into 4% paraformaldehyde for 15 minutes at room temperature. Cell culture inserts were then washed in PBS. Invaded cells were stained with 0.05% Crystal Violet (Sigma Co, St. Louis, MO) in distilled water for 30 minutes. After 2 washes in PBS, invaded cells were visualized under microscope.

II.14 *In vivo* tumor size determination

Primary MMTV-*Wnt1* tumors or xenograft tumors were measured by calibration ruler. We defined the day palpable tumors were detected as day 0, the width (W) and length (L) of tumors were measured and recorded once a week. To calculate the Volume of tumors (V), the function was adopted: $V=L*W^2$.

II.15 Western blot

Cells were starved overnight in DMEM/high glucose medium containing 0.2% FBS and washed twice by 4 °C PBS before cell lysis. Cell lysate was collected by RIPA buffer (10mM Tris, PH 7.2, 0.1% SDS, 0.1% v/w Triton X-100, 0.1% Deoxycholate, 5mM EDTA) with protease inhibitor cocktail (EMD Millipore Corporation, Billerica, MA). After protein concentration was determined by Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), the Western blot procedure was carried out as described in the protocol obtained online (Western blotting - a detailed guide, Abcam).

II.16 Drug resistance assay

MDA-231 breast cancer cell line was infected with ShLgr4 or ShControl virus. After knockdown efficiency was measured, cells were trypsinized and seeded into 96 well-plates with the cell concentration of 10,000 per well. Cells were cultured in DMEM/high glucose medium containing 10% FBS for 6 hours and then starved in DMEM/high glucose medium with 0.2% FBS for 4 hours. Starved cells were then treated with indicated concentrations of Doxorubicin or Docetaxel in 10% FBS containing fresh medium for 12 hours. Remove the medium and proceed to growth curve determination.

II.17 Growth curve

10,000 cells were seeded and cultured overnight in DMEM/high glucose medium with 10% FBS. Cells were serum starved for 4 hours before experiments. After RSPO2 or wnt3a or drug treatments, the cell number was determined by MTT based *in vitro* toxicology assay kit (Sigma Co, St. Louis, MO).

II.18 Wound healing assay

Cells were cultured in 6 well plate until 100% confluent, followed by overnight starvation in medium containing 0.5% FBS. Starved cells were pre-treated with mytomyacin C (Sigma Co, St. Louis, MO) at 20ug/ml for 4 hours. A wound was made by scratching cells with a 10 μ L tip. Cells were then washed with PBS twice and migration

was induced by complete medium containing 10% FBS. Cell migration was checked every 12 hours.

II.19 Statistics

Statistical analysis on single gene expression was performed using Student's T-test between the two groups. p value equal or less than 0.05 was considered significant. Comparison of multiple gene expressions in qPCR experiment was performed using Holm's test. The tumor free curve was carried out by Kaplan-Meier estimate (Goel, Khanna, & Kishore, 2010).

CHAPTER III

RESULTS

III.1 Lgr4 is highly expressed in several breast cancer cell lines and primary MMTV-*Wnt1* tumors

To investigate the function of Lgr4 in breast cancer, we first assessed Lgr4 expression both *in vivo* and *in vitro*. First, in an analysis of a published human breast cancer microarray database (Y. Wang et al., 2005), LGR4 expression is elevated in human breast cancer patient samples. High expression of LGR4 in human breast cancer patients is correlated with high risk of cancer recurrence (Figure 1 A), increased distant metastasis formation (Figure 1 B) and poor overall survival rate (Figure 1 C).

Lgr4 has previously been implicated in cell invasion in colorectal and several cancer cell lines (Gao, Kitagawa, Hiramatsu, et al., 2006; J. Wu et al., 2013). We therefore examined whether Lgr4 expression in a panel of breast cancer cell lines would correlate with the relative invasive capability of each cell line. By using QPCR, six different human breast cancer cell lines were tested for LGR4 mRNA level. The invasive and TNBC cell lines tended to have a relatively higher expression of Lgr4 than other breast cancer subtypes (Figure 1 D), suggesting that the correlation between invasiveness and Lgr4 expression extends to breast cancer.

In the mouse study, we used an Lgr4 gene-trap mouse model. In this mouse model, the CD4 transmembrane domain is fused with the β -galactodase gene in a gene trap vector that also contains the placental alkaline phosphatase gene behind an IRES,

and the gene trap was inserted between exon1 and exon2 of the *Lgr4* gene (Weng et al., 2008). The insertion results in disruption of *Lgr4* transcription and expression of the biochemical markers Lac-Z and PLAP which allows examination of the *Lgr4* expression pattern by Lac-Z staining in *Lgr4*^{+/-} mice. The insertion was confirmed by Southern blot, and genotyping of the experimental mice was done as described in materials and methods. We have previously published the expression pattern of *Lgr4* in the normal mammary gland in adult mice (Y. Wang et al., 2013), where *Lgr4* is expressed in most basal epithelial cells. Three MMTV-*Wnt1* *Lgr4*^{+/-} mice with *Wnt1* tumors were sacrificed at the age of 5 months, and Lac-Z staining was performed on these tumors; one *Wnt1* tumor from *Lgr4*^{+/+} mouse at the age 5 months was also stained for Lac-Z activity as a negative control. The Lac-Z staining in MMTV-*Wnt1* hyperplastic mammary gland showed that a rare (around 7%) population were *Lgr4* positive cells, and all p63-positive cells were also *Lgr4* positive (Figure 1 E) which supported our previous report (Y. Wang et al., 2013). Furthermore, the *Lgr4* level is elevated in MMTV-*PyMT* induced breast cancer when compared with normal mammary gland (Figure 1 F). Taken together, these results suggest that *Lgr4* may play an important role in breast cancer progression.

Figure 1 Lgr4 affects relapse, metastasis and survival of breast cancer patients

A, C & D) 286 human breast cancer patients reported in the database described by Wang et al. (Y. Wang et al., 2005) were segregated into two groups based on relative tumor LGR4 mRNA expression and subsequent relapse (A), distant metastasis (C) and overall survival (D) were analyzed. Patients with tumors expressing high levels of LGR4 had significantly worse outcomes in all three metrics. B) mRNA level of LGR4 in 6 different human breast cancer cell lines. Cell lines with weak invasion ability are marked blue; cell lines with strong invasion ability are marked red. Bar graph shows that the relative expression level of LGR4 mRNA in three highly invasive cell lines are significantly higher than 3 poorly invasive cell lines. Error bar shows the standard deviation between PCR reaction triplicates. E) In hyperplastic mammary gland, Lgr4 expression (shown by LacZ staining) is partially co-localized with p63 (shown by Immunohistochemistry). Arrows indicate Lgr4 and p63 co-localization. F) Bar graph shows relative mRNA level \pm S. E. of Lgr4 in normal breast tissues and tumors from 7-week old MMTV-*PyMT* mice. A-D courtesy of Ying Wang.

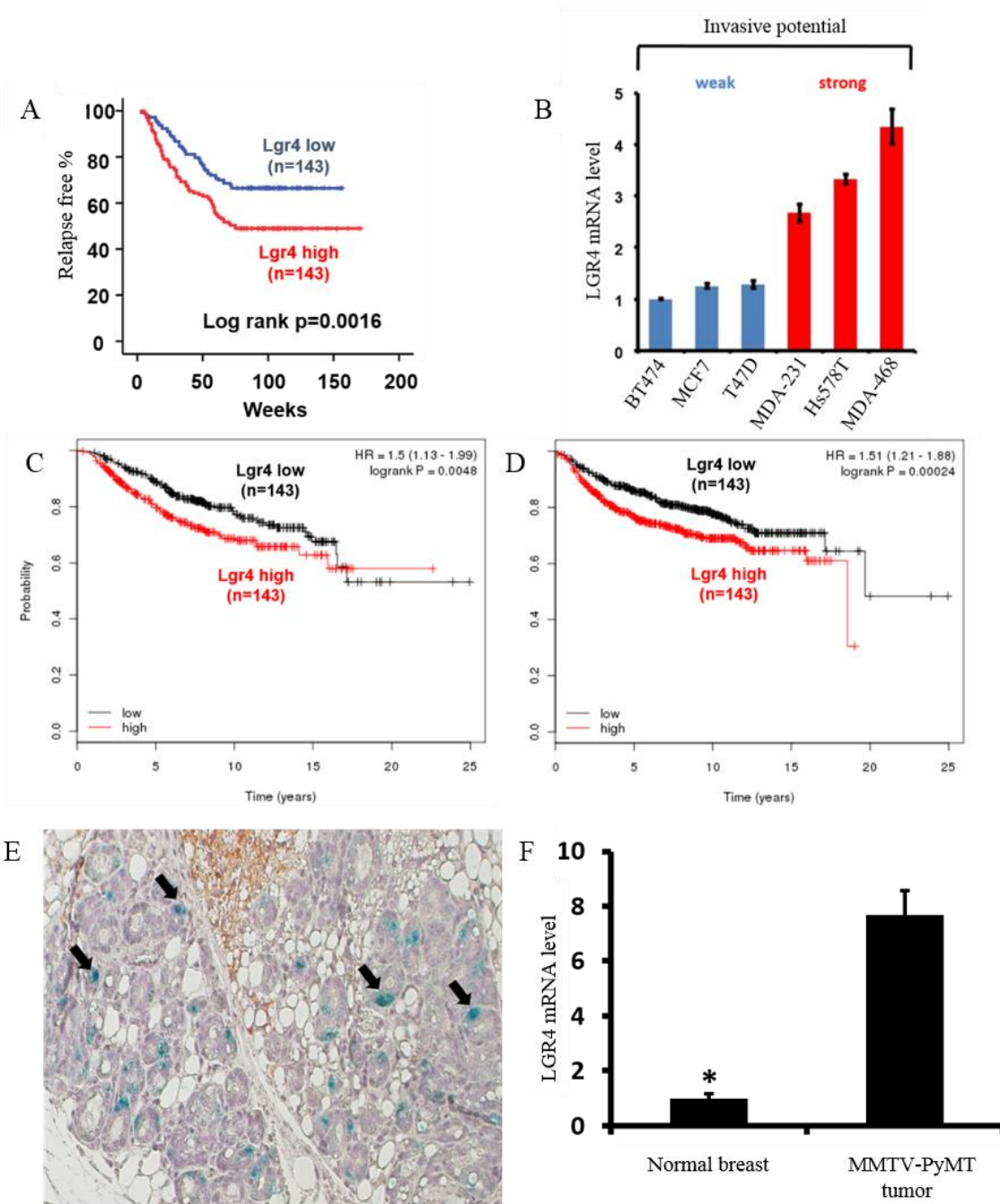
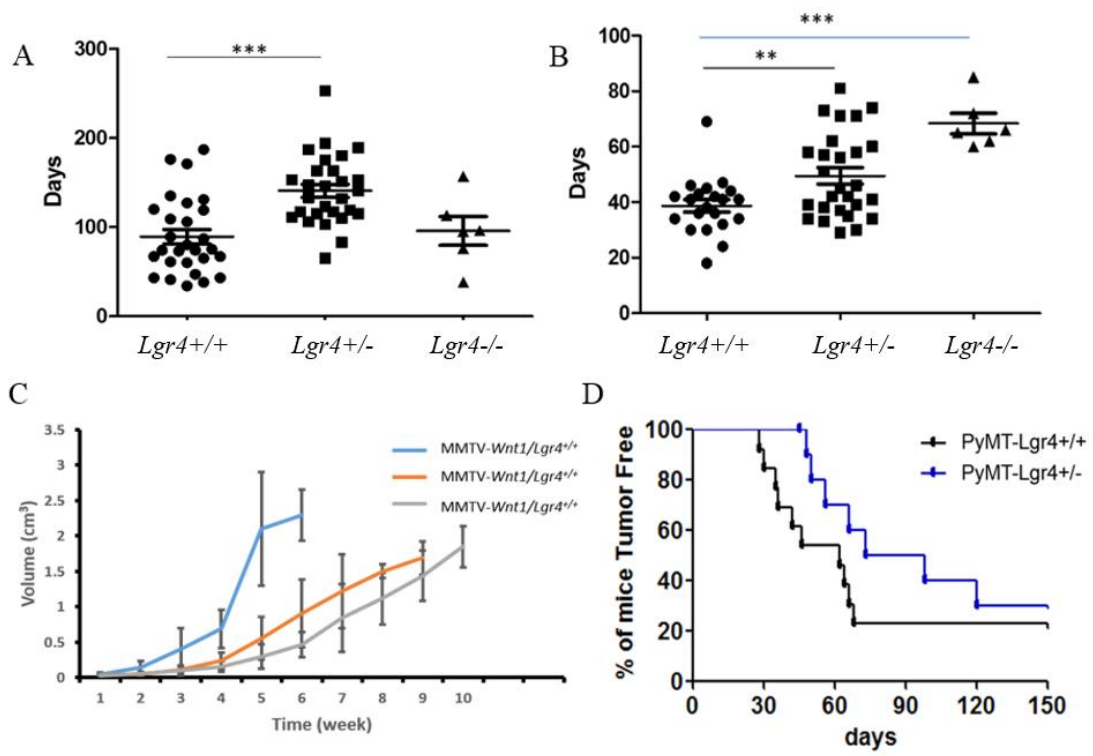


Figure 2 Tumor growth in breast cancer mouse models is delayed by loss of *Lgr4*

A) The age at which MMTV-*Wnt1* mice develop palpable breast cancers. Mice were palpated twice every week after weaning; the age of the mice at detection of palpable tumors (usually 0.3 cm at greatest dimension) was recorded (T1), *** $p \leq 0.001$ B) The length of time for MMTV-*Wnt1* tumors to grow from initial detection to 1.5 cm length at greatest dimension. The tumors were measured once every week. Once the tumor size reached 2 cm at greatest dimension, the mice were sacrificed and the ages of mice were recorded (T2), ** $p \leq 0.01$, *** $p \leq 0.001$ C) Breast tumor volumes were compared between *Lgr4*^{+/+}, *Lgr4*^{+/-} and *Lgr4*^{-/-} MMTV-*Wnt1* tumors. The breast tumor width (W) and length (L) were measured in *Lgr4*^{+/+}, *Lgr4*^{+/-} and *Lgr4*^{-/-} MMTV-*Wnt1* tumors once a week, and tumor size (V) was calculated using the function: $V=L*W^2$. Using the equation: $V=L*W^2$. D) The Kaplan–Meier estimate showing MMTV-*PyMT* tumor free rate in *Lgr4*^{+/+}, *Lgr4*^{+/-} and *Lgr4*^{-/-} mice, courtesy of Ying Wang.



III.2 *Lgr4* depletion significantly delays tumor growth in MMTV-*Wnt1* and MMTV-*PyMT* mice

In our preliminary observations, MMTV-*Wnt1* *Lgr4*^{-/-} and MMTV-*PyMT* *Lgr4*^{-/-} mice with genotype have a much slower growth of mammary cancer than the corresponding *Lgr4*^{+/+} mice. To investigate the effect of *Lgr4* ablation on *Wnt1*-driven tumor growth, we observed 86 MMTV-*Wnt1* mice with different *Lgr4* genotypes (33 *Lgr4*^{+/+} mice, 37 *Lgr4*^{+/-} mice and 6 *Lgr4*^{-/-} mice) from date of birth (DOB), through time of tumor initiation (T1) to length of time for tumor growth from initiation to 2 cm at greatest dimension (T2). Based on published data, half of MMTV-*Wnt1* wild-type mice with an SJL strain background start to develop tumors at an age of 6 months and the rest develop tumors in the following half a year (Y. Li et al., 2000). In our study all MMTV-*Wnt1* TG mice are in a FVB background, *Lgr4*^{+/+} mice have an average of 89 ± 43 days to T1 and 15.6% (N=5) of mice do not develop palpable tumors by the age of 1 year; *Lgr4*^{+/-} showed an delayed development over *Lgr4*^{+/+} with an average of 140 ± 39 days to T1 and 24.3% (N=9) of mice do not develop palpable tumors by the age of 1 year. MMTV-*Wnt1* *Lgr4*^{-/-} mice have an average of 67 ± 39 days to T1 and all 6 *Lgr4*^{-/-} mice developed tumors before 160 days (Figure 2 A). Therefore, loss of *Lgr4* appeared to accelerate the initial appearance of mammary tumors in MMTV-*Wnt1* mice, although our numbers of MMTV-*Wnt1* *Lgr4*^{-/-} mice are too small to draw a firm conclusion on this point. When comparing T2, MMTV-*Wnt1* *Lgr4*^{+/+} mouse tumors have an average time of 39 ± 10.2 days to grow from initial detection to 2cm at the largest dimension; MMTV-*Wnt1* *Lgr4*^{+/-} mice have an average of 50 ± 15.3 days; while MMTV-*Wnt1*

Lgr4^{-/-} mice have an average of 68 ± 9.1 days (Figure 2 B). Furthermore, the tumor size in MMTV-*Wnt1* *Lgr4*^{+/+} tumors grew significantly faster than MMTV-*Wnt1* *Lgr4*^{-/-} tumors (Figure 2 C). It suggests that the *Lgr4* expression level correlated with the speed of tumor growth in MMTV-*Wnt1* mice. Meanwhile, compared to MMTV-*PyMT* *Lgr4*^{+/+} mice, haploinsufficiency of *Lgr4* in MMTV-*PyMT* mice is enough to increase the T1 by 33 days (Figure 2 D).

We next sought to determine whether cancer progression is affected by *Lgr4*. The tumor histology, assessed by H&E staining, was compared between MMTV-*PyMT* *Lgr4*^{+/+} and MMTV-*PyMT* *Lgr4*^{-/-} in different stages of breast cancer for tumor progression. Pathologic studies showed MMTV-*PyMT* *Lgr4*^{+/+} mice started to develop hyperplasia by the age of 6 weeks, MMTV-*PyMT* *Lgr4*^{+/-} started by the age of 9 weeks, while *Lgr4* showing no sign of hyperplasia development (Figure 3). Meanwhile, MMTV-*Wnt1* *Lgr4*^{-/-} mammary glands showed a decreased hyperplasia formation compared to MMTV-*Wnt1* *Lgr4*^{+/+} (Figure 4 A). Therefore, tumor progression in MMTV-*Wnt1* and MMTV-*PyMT* correlated with *Lgr4* expression level.

In order to study whether *Lgr4* ablation leads to decreased tumor size and delayed cancer progression, proliferation of epithelial cells was determined by immunohistochemical staining of the proliferation marker Ki67 in MMTV-*Wnt1* tumors (Figure 4 B). The frequency of Ki67 positive cells in MMTV-*Wnt1* *Lgr4*^{+/+} tumors was 33 ± 7.4 per hundred cells, and the number in MMTV-*Wnt1* *Lgr4*^{-/-} tumors was decreased to 17.3 ± 5.6 per hundred cells (Figure 4 C). Therefore, mammary tumor proliferation is reduced in the absence of *Lgr4*.

III.3 Lgr4 ablation decreases CSC numbers in MMTV-*Wnt1* tumors and several breast cancer cell lines

Lgr4 knockout leads to a decreased number of stem cells in the mammary gland and impaired prostate stem cell differentiation (W. Luo et al., 2013; Y. Wang et al., 2013). In order to investigate whether the attenuated tumor size and delayed cancer progression in MMTV-*Wnt1* *Lgr4*^{-/-} and MMTV-*PyMT* *Lgr4*^{-/-} mice are caused by effects on cancer stem cells, we first evaluated CSC numbers in MMTV-*Wnt1* tumors.

The limiting dilution transplantation assay has been used for a long time as a standard method for stem cell frequency calculation (Fazekas de St, 1982; Finney, 1951; Ploemacher, van der Sluijs, Voerman, & Brons, 1989). Here we used limiting dilution transplantation into nude mouse recipients to assess the CSC frequency in MMTV-*Wnt1* tumors, MDA-231 cell lines and wnt2508 cell lines which were generated and immortalized from a MMTV-*Wnt1* mouse tumor.

5 pairs of littermate MMTV-*Wnt1* *Lgr4*^{+/+} and MMTV-*Wnt1* *Lgr4*^{-/-} mice with tumors were sacrificed when tumors reached 1.5cm at the greatest dimension. Tumors were excised and digested into single cell suspensions as described in Materials and Methods. The cell suspensions were injected orthotopically into 3-month old Nude mouse mammary gland with different cell numbers as indicated in Table 2. All mice were sacrificed when xenograft tumors reached 1.5 cm at greatest dimension or 3 months after injection. The tumor outgrowths were recorded and the putative CSC numbers were calculated by a webtool extreme limiting dilution assay (<http://bioinf.wehi.edu.au/software/elda/>).

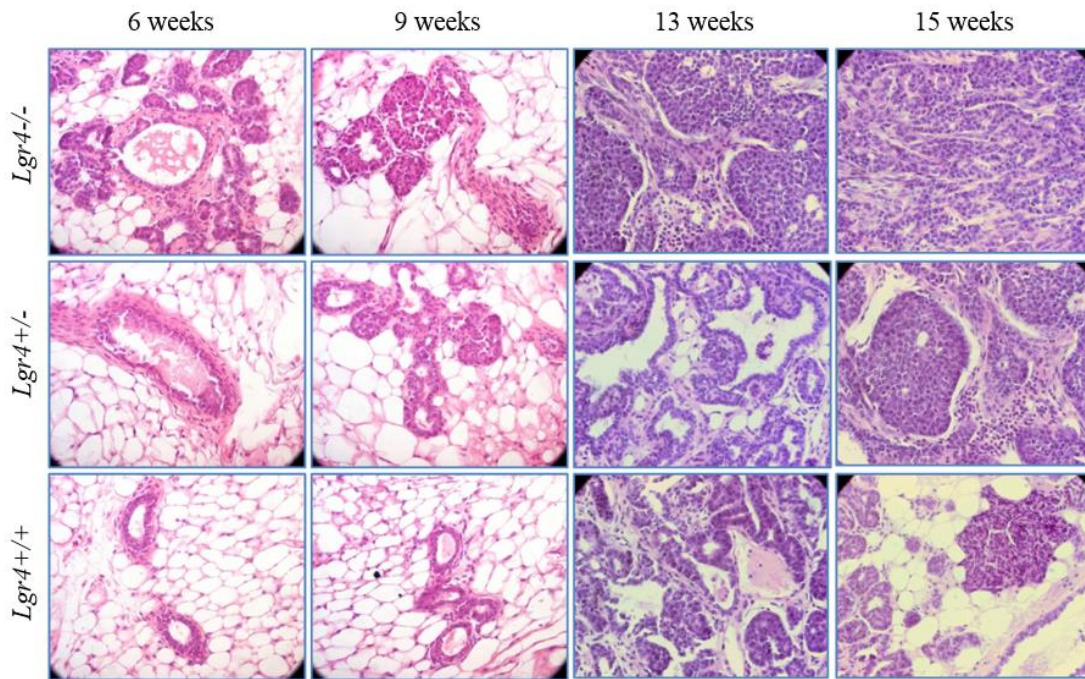


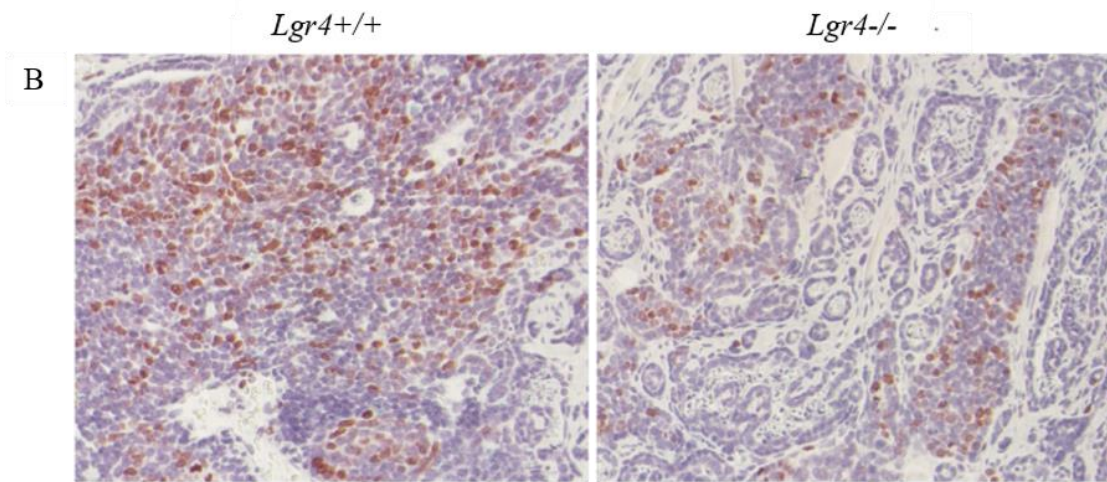
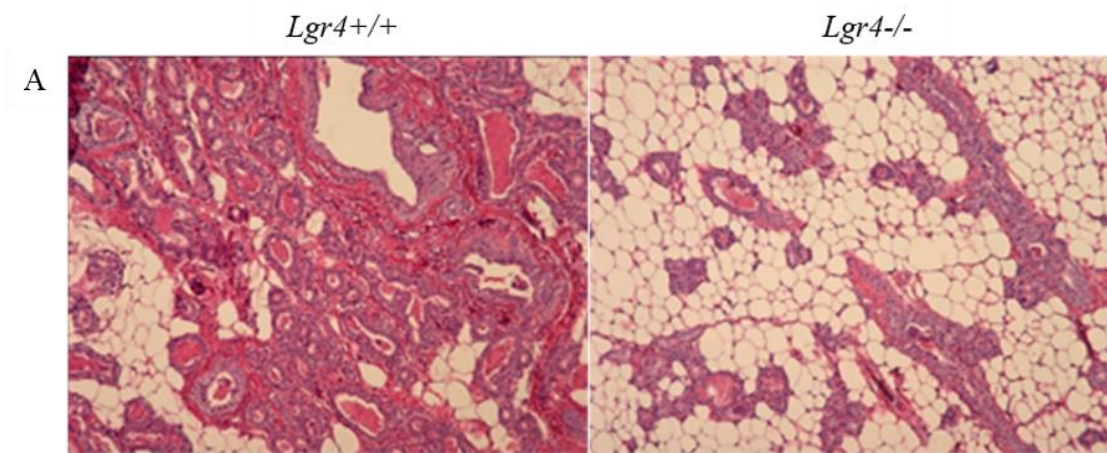
Figure 3 Deletion of *Lgr4* delays tumor progression in MMTV-*PyMT* tumors

Breast tissues or breast tumors from indicated ages and genotypes were collected for H&E staining, and histopathological analysis was used to evaluate tumor progression. At each time point analyzed, the extent of tumor progression was delayed in *Lgr4*^{-/-} mice.

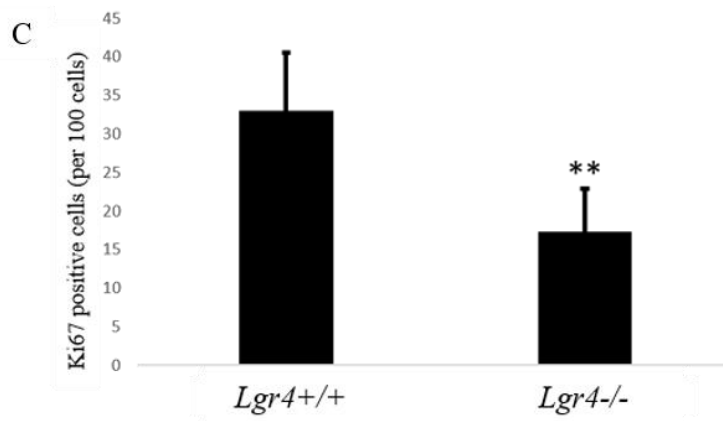
Courtesy of Zengjin Yuan.

Figure 4 *Lgr4* inactivation delays breast cancer progression in MMTV-*Wnt1* tumors

A) Breast tissues or breast tumors from two-month old MMTV-*Wnt1* mice were collected and subjected to H&E staining. The area of hyperplasia was compared between *Lgr4*^{+/+} and *Lgr4*^{-/-}. B) Proliferating cells were detected by Ki67 immunohistochemistry in late stage (1.5 cm at greatest dimension) MMTV-*Wnt1* tumors. C) Bar graph showing quantitation of Ki67 positive cells \pm S. E. per one hundred cells in MMTV-*Wnt1* tumors, ** $p \leq 0.01$. 3 fields were counted for Ki67 positive cells in each mouse, and 3 mice were counted in both *Lgr4*^{+/+} and *Lgr4*^{-/-} genotypes.



Ki67



The data showed that the CSC frequency in MMTV-*Wnt1* *Lgr4*^{+/+} tumors (1 CSC per 2,758 cells) was 8 times higher than in MMTV-*Wnt1* *Lgr4*^{-/-} tumors (1 CSC per 21,963 cells) (Table 2).

The sphereculture assay first developed in neurogenesis research (Reynolds & Weiss, 1992) and later adopted for mammary gland study in which stem or progenitor cell populations can be enriched by sphereculture (Dontu, Al-Hajj, Abdallah, Clarke, & Wicha, 2003). Extensive studies showed that sphereculture can also enrich CSCs from several human breast cancer cell lines (Charafe-Jauffret et al., 2009; Fillmore & Kuperwasser, 2008). In order to assess the impact of *Lgr4* ablation on the number of CSCs in MMTV-*Wnt1* tumors, primary tumors from MMTV-*Wnt1* *Lgr4*^{+/+} mice and MMTV-*Wnt1* *Lgr4*^{-/-} mice were digested, single tumor cell suspensions were made, and the *in vitro* tumorsphere assay was performed. As shown in Figure 5 A and B, the sphere formation was greatly reduced from 12 ± 3.6 spheres/10,000 cells in MMTV-*Wnt1* *Lgr4*^{+/+} tumors to 1.3 ± 1.15 spheres/10,000 cells in MMTV-*Wnt1* *Lgr4*^{-/-} tumors, suggesting a depletion of CSCs in MMTV-*Wnt1* tumors by ablation of *Lgr4* (Figure 5 A B).

Table 2 Limiting dilution assay in MMTV-Wnt1 tumors

<i>Lgr4</i> ^{+/+}					Estimated CSC frequency
cell number	10 ²	10 ³	10 ⁴	10 ⁵	1 in 2758
Outgrowth	0/7	3/11	21/22	10/10	(1546-4919)
<i>Lgr4</i> ^{-/-}					
cell number	10 ²	10 ³	10 ⁴	10 ⁵	1 in 21,963
Outgrowth	0/8	1/12	11/22	9/10	(12,059-40,001)

p=3.42e-07

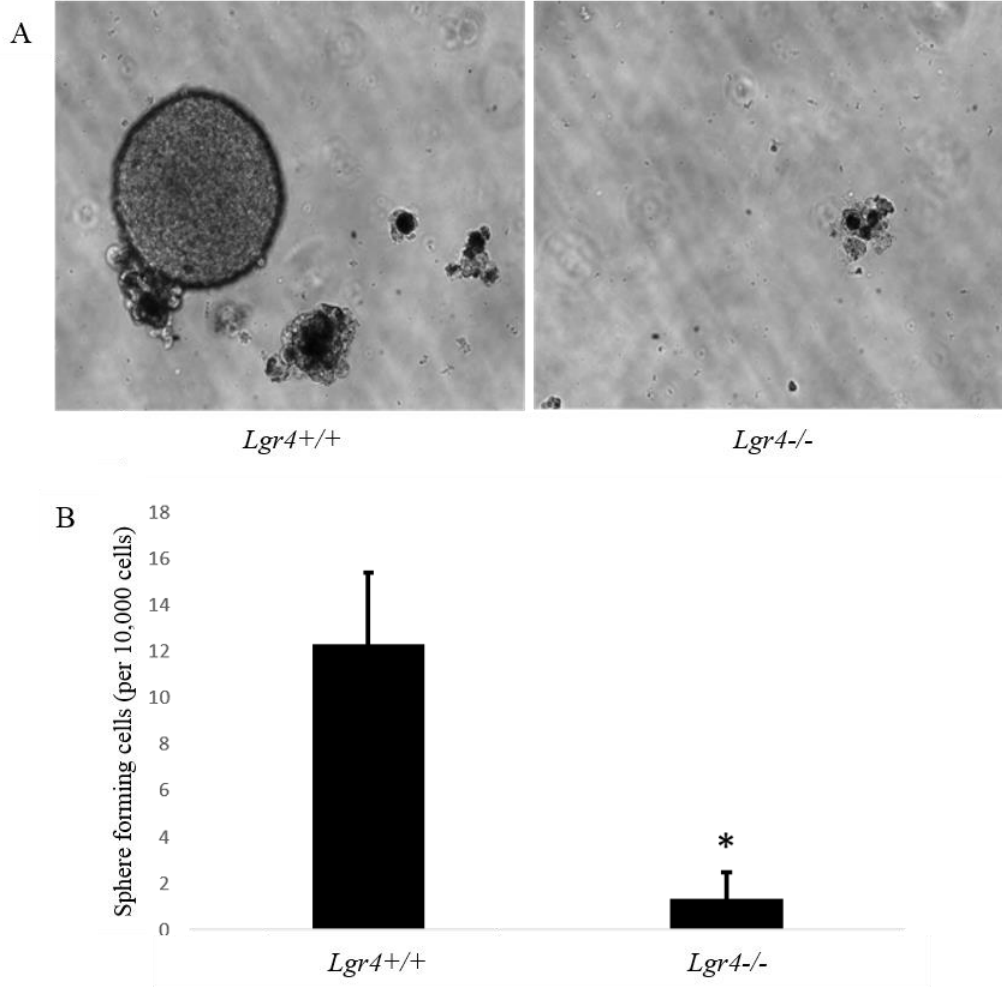


Figure 5 *Lgr4* inactivation leads to decreased sphere forming cell number in MMTV-*Wnt1* tumor

A) MMTV-*Wnt1* tumors under indicated genotypes were digested and cultured in tumorsphere medium as described. Pictures were taken at day 18 after seeding.

Magnification: 200X. B) Bar graph showing number of sphere forming cells \pm S. E. per 10,000 cells in MMTV-*Wnt1* tumors under indicated *Lgr4* genotypes, N=3, * $p \leq 0.05$.

Table 3 Limiting dilution assay using serially passaged Wnt2508 cell line spheres

2D culture					Estimated CSC frequency
Cell number	50	500	5,000	50,000	
ShControl	1/3	2/3	3/3	3/3	1 in 334.8 (93.3 – 1,021)
ShLgr4	0/3	0/3	3/3	3/3	1 in 11,786 (2,892 – 48,027)
1 st passage					
Cell number	50	500	5,000	50,000	
ShControl	0/3	3/3	3/3	3/3	1 in 208.6 (5.2 – 746.5)
ShLgr4	0/3	1/3	3/3	3/3	1 in 1,170 (285 – 4,802)
4 th passage					
Cell number	50	100	500	1,000	
ShControl	1/3	0/3	3/3	3/3	1 in 219.9 (83.3 – 576.9)
ShLgr4	0/3	1/3	1/3	2/3	1 in 859 (307 – 2402)
7 th passage					
Cell number	50	100	500	1,000	
ShControl	1/5	3/5	4/5	3/3	1 in 208.9 (97 – 454)
ShLgr4	2/5	1/5	1/5	2/3	1 in 778 (327 – 1851)

2D culture p=0.0105

1st passage p=0.0225

4st passage p=0.0552

7st passage p=0.0166

Table 4 Limiting dilution assay using MDA-231 cell lines

ShControl					Estimated CSC frequency
cell number	10^3	10^4	10^5	10^6	1 in 45,903
Outgrowths / # injected mammary glands	0/6	2/6	5/6	6/6	(18,996 – 110,925)
ShLgr4					
cell number	10^3	10^4	10^5	10^6	1 in 569,691
Outgrowths / # injected mammary glands	0/6	0/6	1/6	5/6	(235,901 – 1,375,781)

p=9.8e-05

To confirm the result that Lgr4 inactivation led to decreased CSC frequency in MMTV-Wnt1 tumor, we also examined the effect of Lgr4 knockdown on CSCs in the mouse breast cancer cell line Wnt2508. We infected Wnt2508 cells with ShLgr4 or ShControl lentivirus. Serial sphereculture assay was adopted to enrich CSCs and then transplantation assays were performed using infected Wnt2508 cells grown in 2D culture, 1st passage sphere culture, 4th passage sphere culture, and 7th passage sphere culture to assess how Lgr4 knockdown affects CSC frequency and enrichment following serial sphereculture. An initial passage of sphere culture resulted in an increased CSC frequency from 1 in 335 (standard deviation was 93 to 1021) to 1 in 209 (standard deviation was 5.2 to 747) in ShControl, and from 1 in 11,786 (standard deviation was 2892 to 48,027) to 1 in 1,170 (standard deviation was 285 to 4802). However, over several sphereculture passages, we noted a general increase in CSC frequency in both ShLgr4 and ShControl-infected cells. CSC frequency was significantly decreased after Lgr4 knockdown in every transplantation assay (Table 3). Therefore, our results from the limiting dilution tumorigenesis assay using MMTV-*Wnt1* tumor or Wnt1 induced mouse mammary cancer cell line suggested Lgr4 affects CSC number in MMTV-*Wnt1* induced mammary cancer.

We then tested if Lgr4 knockdown has a similar effect in human breast cancer cell lines. With a lentivirus containing an LGR4 targeting ShRNA (shLGR4) or a Nonsense control sequence produced as described in Materials and Methods, MDA-231 cells were infected transiently with ShRNA lentivirus or ShControl lentivirus (efficiency shown in Figure 6). Varying dilutions of cells were injected into the mammary glands of

2-month old Nude mice. The numbers of cells injected and recipients are indicated in Table 4. The numbers of tumor outgrowths were recorded and putative CSC numbers were calculated by ELDA. *Lgr4* knockdown led to a 92% decrease in CSC frequency in MDA-231 cells (Table 4).

Sox genes encode a family of transcription factors which play important roles in development by affecting stem cells (Sarkar & Hochedlinger, 2013). Nanog, Oct4 and Sox2 were reported to be key genetic regulators in human embryonic stem cells (Rodda et al., 2005). Further studies showed that Sox2 fosters various cancers by promoting cellular proliferation, evading apoptosis, and enhancing invasion and migration (Weina & Utikal, 2014). And two papers reported Sox2 to be a CSC marker in breast cancer (Leis et al., 2012) and squamous cell carcinoma (Boumahdi et al., 2014). We have previously reported a decrease in Sox2 expression of mammospheres cultured from primary mammary cells of *Lgr4*^{-/-} mice as compared to those from *Lgr4*^{+/+} mice (Y. Wang et al., 2013). We therefore examined whether Sox2 levels are affected by loss of *Lgr4*. Indeed, Sox2 mRNA expression levels were significantly decreased in MMTV-*Wnt1 Lgr4*^{-/-} tumors when compared with MMTV-*Wnt1 Lgr4*^{+/+} tumors (Figure 6 A), and Sox2 positive cells indicated by immunohistochemistry also demonstrated a dramatic decrease from 10.2% ± 1.43% in MMTV-*Wnt1 Lgr4*^{+/+} tumors to 5.7% ± 1.41% in MMTV-*Wnt1 Lgr4*^{-/-} tumors (Figure 6 B&C). Similar results were observed as a result of LGR4 knockdown in MDA-231 cells and Wnt2508 cells (Figure 6 D&E), suggesting that Sox2 is regulated by *Lgr4* in MMTV-*Wnt1* induced mammary cancer and MDA-231 cells. We next examined whether MMTV-*Wnt1* tumor cells that express

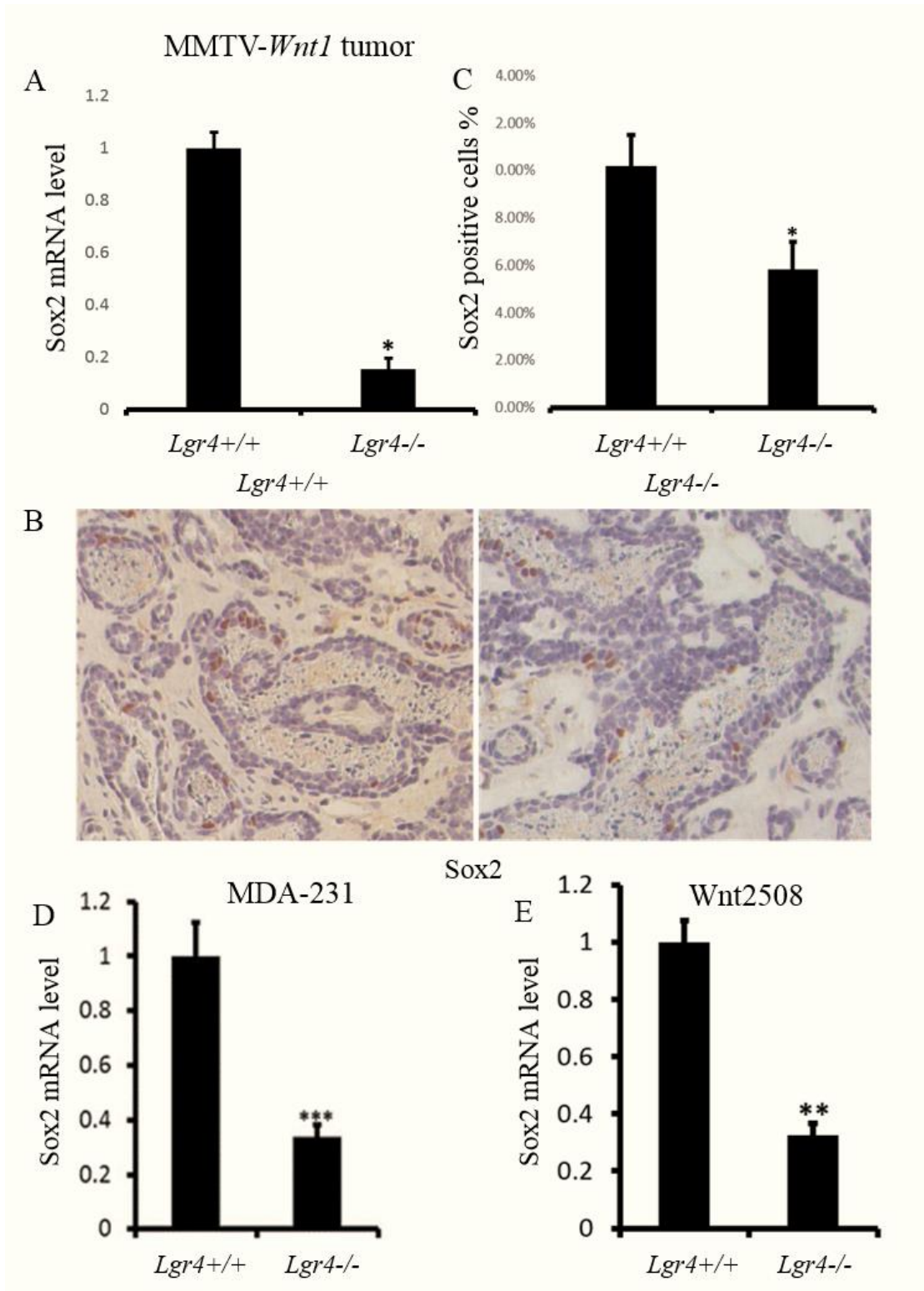
Lgr4 are also Sox2⁺. We performed immunohistochemistry for Sox2 in β -gal stained MMTV-*Wnt1* Lgr4^{+/-} tumors. Notably Sox2 does not co-localize with Lgr4 in MMTV-*Wnt1* tumors (data not shown), suggesting that a paracrine signaling pathway regulated by Lgr4 is involved in control of Sox2 expression in MMTV-*Wnt1* tumors.

III.4 Lgr4 inactivation results in decreased metastasis by affecting EMT

As the final and the most fatal step of tumor progression, metastasis is associated with pain, distant metastasized organ malfunction and lethality. In order to form a metastasis at distant organ, the metastatic cells from the primary tumor must undergo several steps: (1) intravasation from primary tumor site; (2) survival and dissemination in the circulation; (3) extravasation; (4) colonization in distant organs and generation of metastases (Klein, 2008). Loss of Lgr4 has been reported to cause diminished invasiveness in several cancer cell lines as well as in colon cancer (Gao, Kitagawa, Hiramatsu, et al., 2006; J. Wu et al., 2013).

Figure 6 Ablation of Lgr4 represses Sox2 expression in breast cancers

A) Bar graph showing relative mRNA level in MMTV-*Wnt1* tumors under indicated genotypes. B) Sox2 expression (brown) in MMTV-*Wnt1* tumors under indicated genotypes are shown by immunohistochemistry. C) Bar graph showing % of cells expressing Sox2 (Sox2 positive cell percentage) in MMTV-*Wnt1* tumors under indicated Lgr4 genotypes. Bar graph showing the mean Sox2⁺ cell percentage ± S. E., * p≤0.05. D & E) Bar graph showing Sox2 relative mRNA level ± S. E in MDA-231 (D) and Wnt2508 cells (E), **p≤0.01, ***p≤0.001.



III.4.1 Breast cancer metastasis to lungs is repressed by Lgr4 depletion in MMTV-*Wnt1* and MMTV-*PyMT* mice

A previous report showed that Lgr4 knockdown blocks invasion in several cancer cell lines (Gao, Kitagawa, Hiramatsu, et al., 2006). To explore whether Lgr4 has the same function in breast cancer, we first evaluated the lung metastasis in MMTV-*Wnt1* and MMTV-*PyMT* mice bearing mammary tumors measuring 1.5 cm at the greatest dimension. By H&E staining, the pathological analysis showed that MMTV-*PyMT* *Lgr4*^{+/-} mice had a dramatic reduction in metastasis formation when compared with MMTV-*PyMT* *Lgr4*^{+/+} (Figure 7 A). The number of metastases per field dropped from an average of 19 in *Lgr4*^{+/+} to 4 in *Lgr4*^{+/-} (Figure 7 B). Although no significant difference was detected in the MMTV-*Wnt1* mice due to low sample size, only MMTV-*Wnt1* *Lgr4*^{+/+} developed metastasis in the lung (3 out of 8) and no metastases were detected in MMTV-*Wnt1* *Lgr4*^{-/-} mice (0 out of 6) (data not shown). By using QPCR, Lgr4 mRNA levels in 6 different breast cancer cell lines were measured. As shown in Figure 1 B, the three most invasive breast cancer cell lines have the highest Lgr4 expression, which suggests a strong correlation between Lgr4 mRNA level and breast cancer invasiveness. Therefore, these data strongly suggested that Lgr4 expression is positively related with breast cancer metastasis.

III.4.2 Lgr4 knockdown in MDA-231 cells results in decreased cell migration and attenuated lung metastasis formation

To better demonstrate Lgr4 function in metastasis, we then examined the effect of Lgr4 knockdown on migration *in vitro*. In the the trans-well migration assay, Lgr4 knockdown significantly inhibited cell migration of MDA-231, MDA-468 and Hs578T cells (Figure 8 A&B). The inhibition of migration by inactivation of LGR4 was then confirmed in MDA-231 wound healing assay (Figure 8 C&D).

We next sought to determine whether Lgr4 knockdown MDA-231 cells were impaired in metastasis formation. We injected luciferase-expressing MDA-231 cells also stably expressing either shLGR4 or shNC (a non-specific control shRNA) into the tail vein of nude mice. LGR4 knockdown resulted in significantly decreased luciferase activity detected in the lungs 30 days after injection when compared with ShControl (Figure 9), suggesting that the metastasis formation abilities of MDA-231 cells are heavily impaired by knocking down Lgr4.

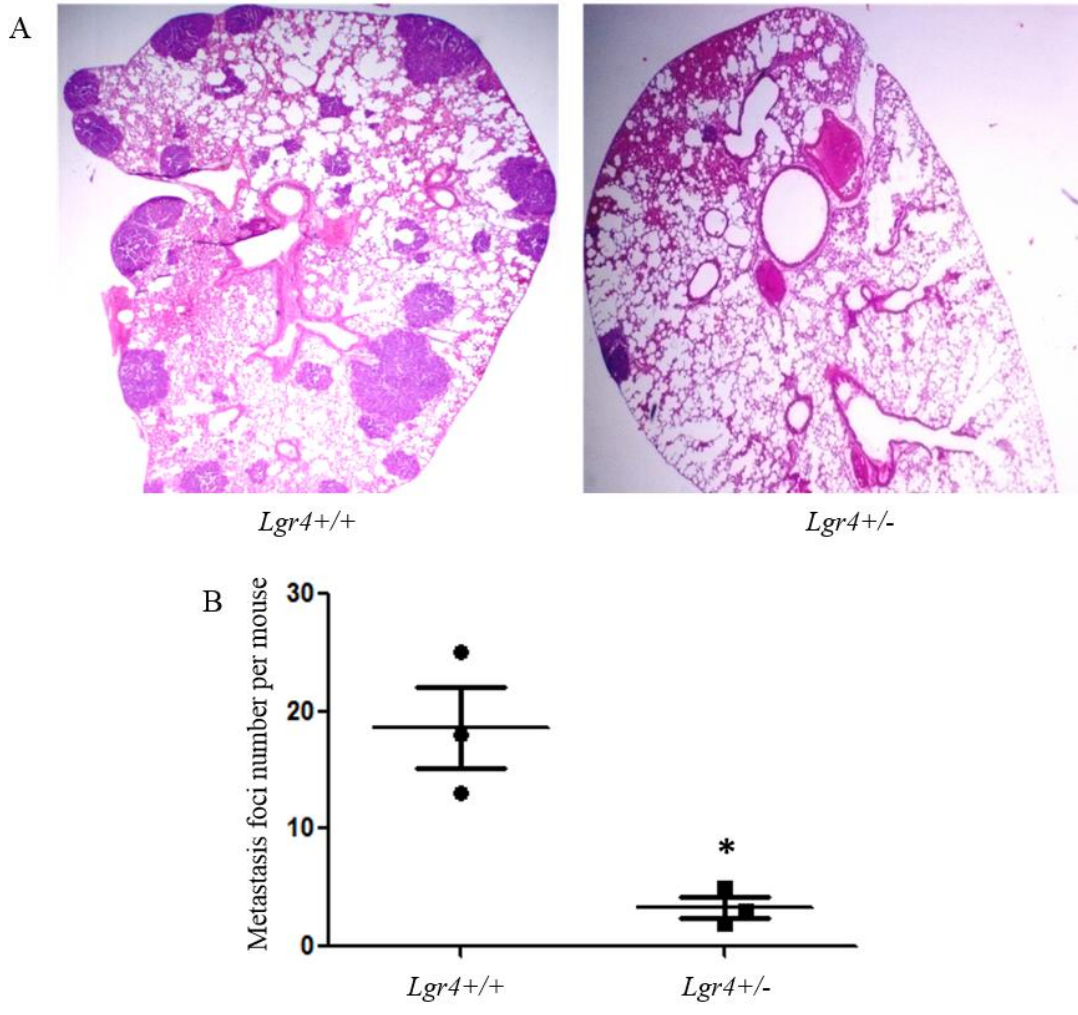


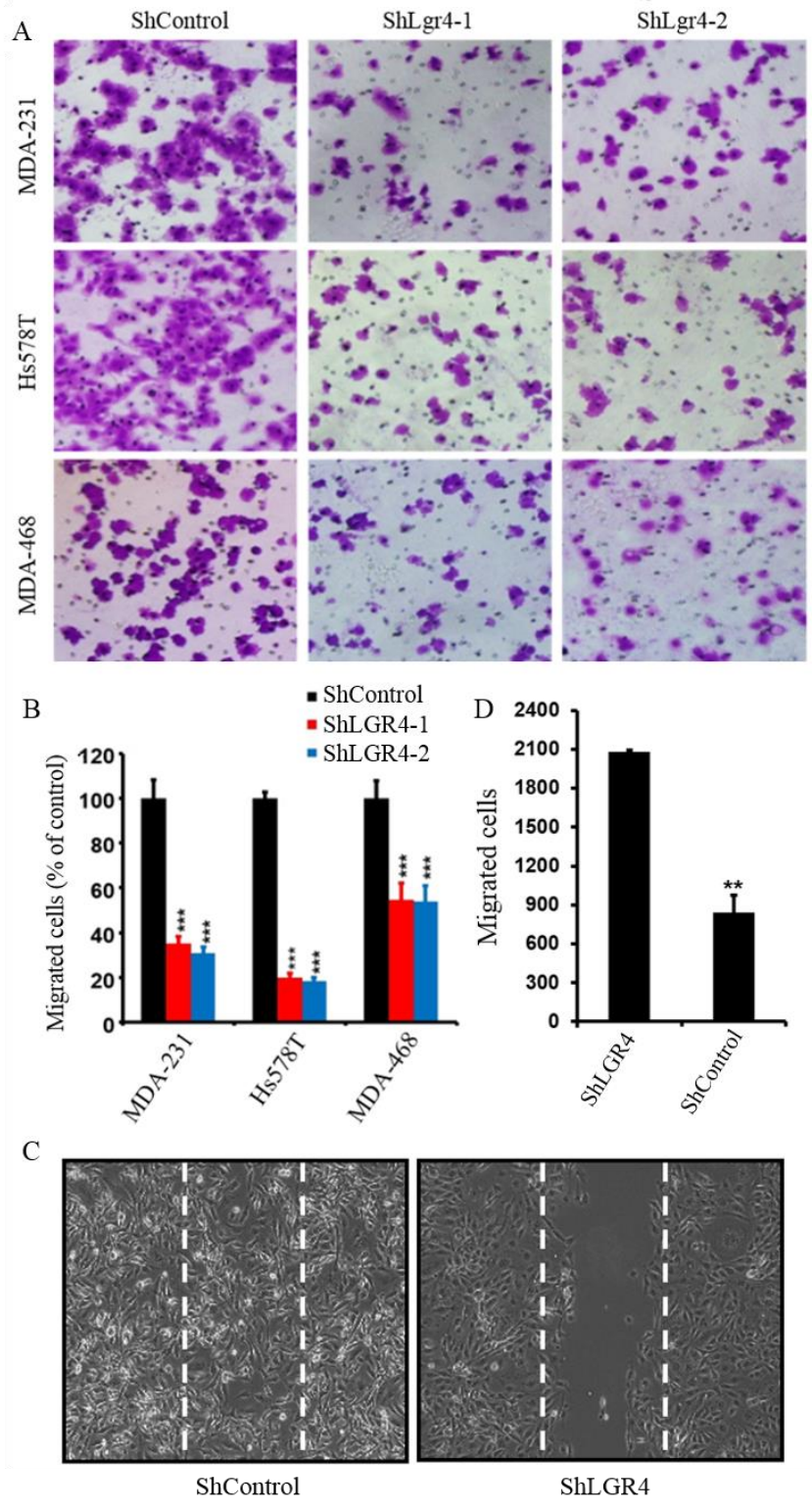
Figure 7 *Lgr4* deletion leads to inhibition of lung metastasis formation in MMTV-*PyMT* mice

A) Hematoxylin & Eosin staining showing lung metastasis under indicated genotypes.

B) Graph showing number of metastatic foci ± S.E. per mouse under indicated genotypes, * $p \leq 0.05$.

Figure 8 Lgr4 expression level positively correlates with *in vitro* migration ability in human breast cancer cell lines

A) Trans-well assay using MDA-231, MDA-468 and Hs578T cells infected with indicated ShRNA containing lentivirus; migrating cells are indicated with Crystal Violet staining. B) Bar graph showing relative percentage \pm S.E. of control migrated cells in transwell assay. *** $p < 0.001$. C) Wound healing assay using MDA-231 cells infected with indicated ShRNA containing lentivirus. Wound edges are indicated with white dashed lines. D) Bar graph showing number of migrated cells \pm S.E. ** $p < 0.01$. A-B Courtesy of Yuanzhang Fang.



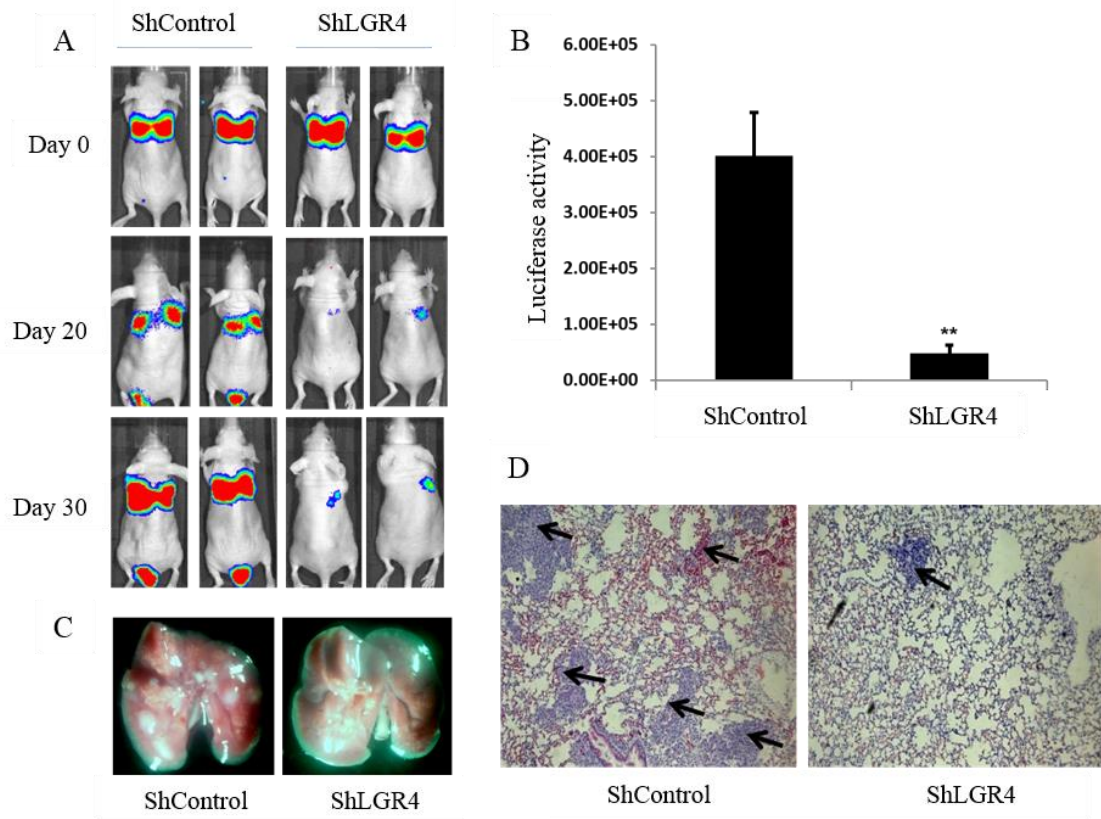


Figure 9 Inactivation of LGR4 inhibits *in vivo* lung metastasis formation in MDA-231 cell line

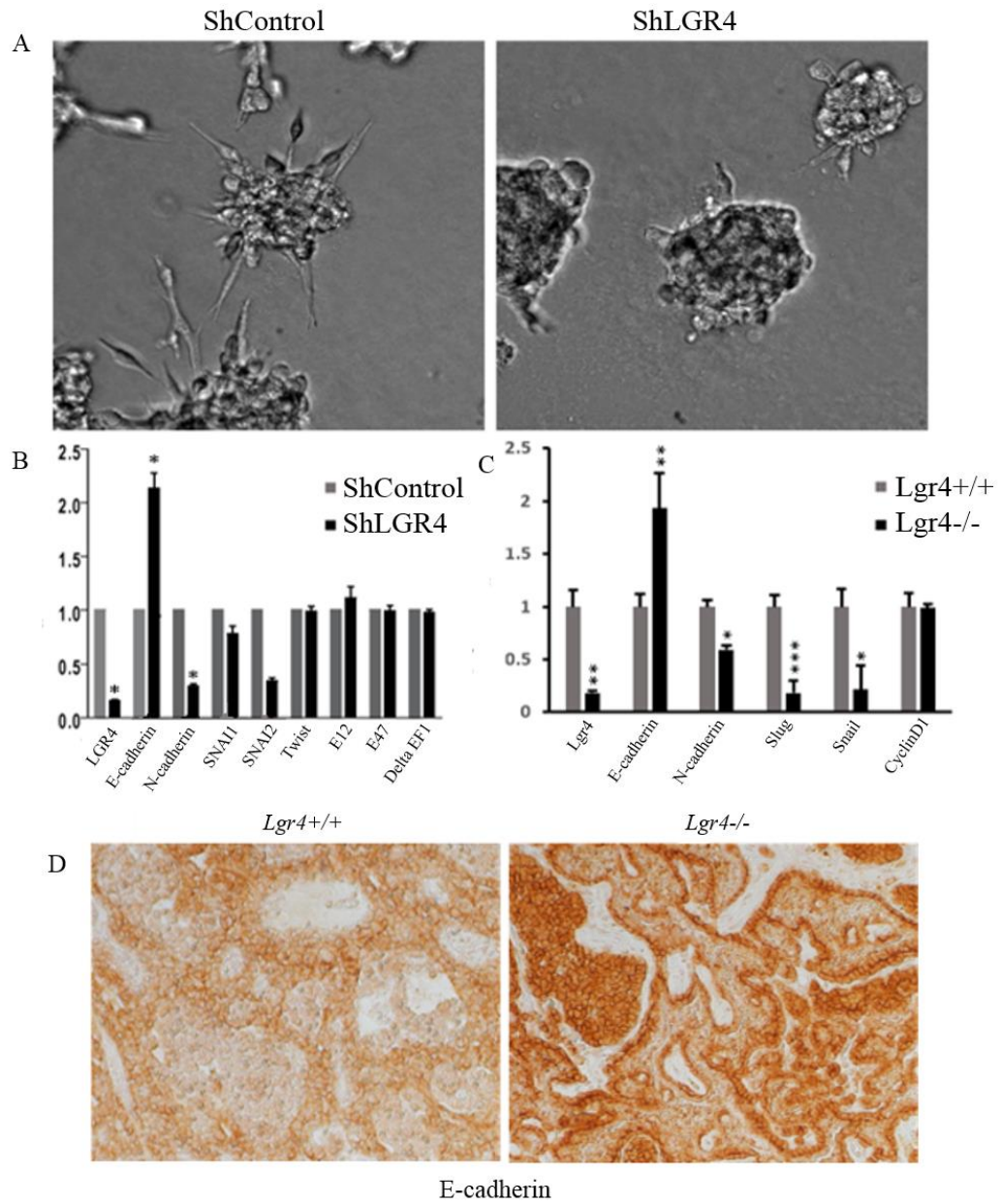
A) The lung bioluminescence photon values on days 0, 20, and 30 in ShControl and ShLGR4 MDA-231 tailvein injection assay. B) Bar graph showing luciferase activity \pm S.E. in lung on day 30. ** $p < 0.01$. C) Lung metastasis comparison between ShControl and ShLgr4 MDA-231 tailvein injection on day 30. White spots indicate macroscopic lung metastases. D) Hematoxylin & Eosin staining of lung tissues from indicated tailvein injection group; black arrows indicate metastases. Courtesy of Zhiying Yue.

III.4.3 Breast cancer EMT is blocked by inactivation of Lgr4

Recently published reports revealed that EMT is closely associated with CSCs, and plays important roles in cancer invasion and metastasis (Mani et al., 2008; Scheel & Weinberg, 2012). Since ablation of Lgr4 decreased CSC number as well as metastasis, we suspected that Lgr4 positively regulates EMT, resulting in promotion of invasion and migration. In order to test this hypothesis, we first performed the *in vitro* 3D colony formation assay (Debnath, Muthuswamy, & Brugge, 2003) using MCF10A cells stably expressing both ErbB2 and 14-3-3 ξ (MCF10A-ErbB2-14-3-3 ξ), which is a viable model for EMT studies (Lu et al., 2009). By measuring the length and number of branches invading into the surrounding Matrigel from the acinar structured colonies of MCF10A-ErbB2-14-3-3 ξ cells, we can evaluate the invasiveness. Strikingly, the invasive branches in LGR4 wild-type MCF10A colonies were greatly decreased by LGR4 knockdown (Figure 10 A). We examined the effect of LGR4 knockdown on colony expression of EMT markers. mRNA analysis of selected EMT markers showed decreased expression of N-cadherin, SNAL1, SNAL2 and increased E-cadherin after LGR4 knockdown, which supported a reversal of EMT in cells expressing shLGR4 (Figure 10 B). We then examined EMT marker expression in MMTV-*Wnt1* mammary tumors. mRNA levels of both Snail and Slug were decreased in tumors from Lgr4^{-/-} mice, whereas E-cadherin mRNA was elevated in tumors lacking Lgr4, supporting an essential role for Lgr4 in tumor EMT. We also observed a dramatically increased E-cadherin protein expression in MMTV-*Wnt1* Lgr4^{-/-} tumors compared to MMTV-*Wnt1* Lgr4^{+/+} tumors (Figure 10 C&D). Taken together our data suggests that Lgr4 promotes metastasis through EMT.

Figure 10 Lgr4 affects EMT genes in ErbB2 and 14-3-3 ζ transformed MCF-10A 3D organoids and MMTV-*Wnt1* tumors

A) Representative 3D culture organoids from plating 1000 cells/well of ErbB2 and 14-3-3 ζ transformed MCF-10A cells infected with indicated ShRNA containing virus. Length and number of invading branches in surrounding Matrigel from the acinar structure were measured for invasive potential evaluation. B) Bar graph showing relative mRNA level \pm S.E of indicated genes in ErbB2 and 14-3-3 ζ transformed MCF-10A 3D organoids, * $p \leq 0.05$. C) Bar graph showing relative mRNA level \pm S.E of indicated genes in MMTV-*Wnt1* tumors under indicated Lgr4 genotypes, * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$. D) E-cadherin expression (brown) in MMTV-*Wnt1* tumors under indicated genotypes is shown by immunohistochemistry. A-B courtesy of Ying Wang.



III.5 Lgr4 modulates Wnt pathway, EGFR pathway, and MMPs in breast cancers

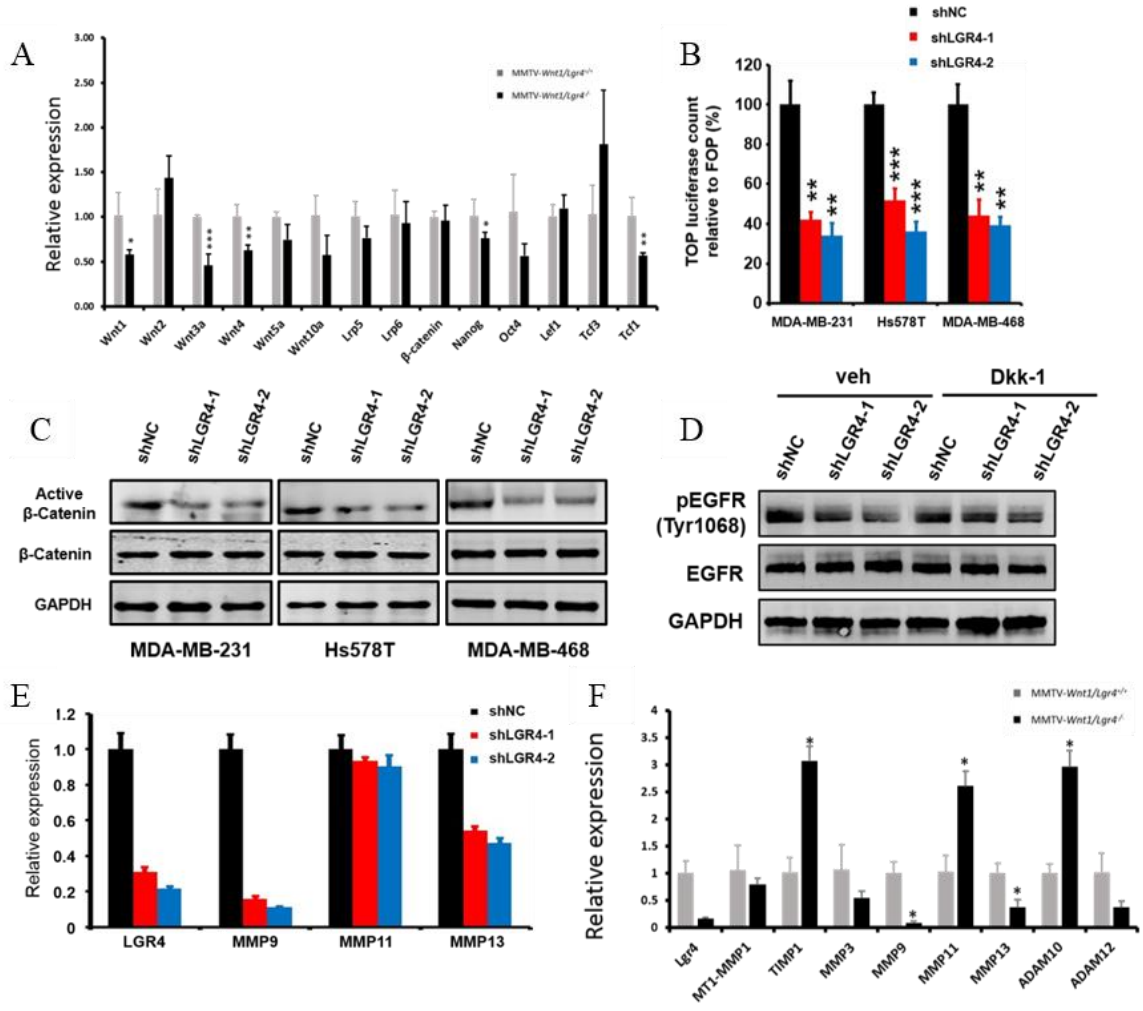
The Wnt signaling pathway is a key regulator in cancer progression (Anastas & Moon, 2013), cancer stem cells (Holland, Klaus, Garratt, & Birchmeier, 2013) and EMT (Y. Wu et al., 2012), and our previous study showed that Lgr4 regulates Sox2 expression level in mammary gland development through wnt signaling (Y. Wang et al., 2013). In order to investigate whether the Wnt pathway mediates Lgr4 regulated breast cancer progression, wnt target gene mRNA levels were measured in MMTV-*Wnt1* tumors under *Lgr4*^{+/+} and *Lgr4*^{-/-} genotypes. Quantitative PCR showed that the mRNA level of wnt1, wnt3a, wnt4, Nanog and TCF1 are significantly decreased in MMTV-*Wnt1* *Lgr4*^{-/-} tumors compared with MMTV-*Wnt1* *Lgr4*^{+/+} tumors (Figure 11 A). Furthermore, the TOP-Flash assay in MDA-231 cells also showed decreased the Wnt signaling pathway reporter activity in MDA-231, MDA-468 and Hs578T human breast cancer cell lines after knockdown of LGR4 (Figure 11 B). Furthermore, Western blot analysis showed a decreased level of the active form of β -catenin in these three cell lines after Lgr4 inactivation (Figure 11 C).

Epidermal growth factor receptor (EGFR) pathway is a downstream target of the canonical Wnt pathway (Schlange, Matsuda, Lienhard, Huber, & Hynes, 2007), and also actively participates in cancer progression (Masuda et al., 2012) and as well as in EMT (Lo et al., 2007). In order to explore whether the EGFR pathway is affected by Lgr4 in breast cancer, we checked the level of phosphoY1068-EGFR, a marker of EGFR activity, in MDA-231 cells after LGR4 knockdown. The Western blot showed a decreased level of phosphorylatedY1068 EGFR after LGR4 knockdown, and DKK

treatment showed no significant difference in terms of pY1068-EGFR level, suggesting that the EGFR pathway in MDA-231 cells is regulated at least in part through Wnt-independent mechanisms (Figure 11 D). Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases which are known to play important roles in tumor cell invasion and survival. Among them, the active form of MMP9 has been shown be able to activate EGFR and disrupt E-cadherin expression in ovarian cancer (Cowden Dahl et al., 2008). Here, our quantitative PCR results also demonstrated MMP9 and MMP13 mRNA level are consistently decreased in MDA-231 and MMTV-Wnt1 tumors after inactivation of Lgr4 (Figure 11 E&F). Taken together, our results suggested Lgr4 is a master regulator of breast cancer.

Figure 11 Lgr4 is a master regulator of breast cancer by modulating Wnt pathway, EGFR pathway and MMPs

A) Bar graph showing relative mRNA level \pm S.E of indicated wnt target genes * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. B) Bar graph showing Top luciferase activity \pm S.E in indicated cell lines with indicated ShRNA. C) Western blot showing expression level of active β -catenin level (clone 8E7) in indicated cell lines. D) Western blot showing pEGFR level with or without treatment of DKK-1. E) Bar graph showing relative mRNA level \pm S.E of indicated MMPs in MDA-231. F) Bar graph showing relative mRNA level \pm S.E of indicated MMPs in MMTV-*Wnt1* tumors, * $p \leq 0.05$. B-E courtesy of Yuanzhang Fang.



CHAPTER IV

DISCUSSION

IV.1 Overview

Our lab has been focused on the function of *Lgr4* in development of various organs for a long time. This study, as well as another prostate cancer study conducted by Weijia Luo, was the first time we investigated the link between *Lgr4* and cancer. The first clue leading us to this study is a paper published on 2006 that *Lgr4* promotes invasiveness and metastasis in several carcinoma cell lines (Gao, Kitagawa, Hiramatsu, et al., 2006). And our first evidence of *Lgr4* cancer-related phenomenon was finding that the tumor growth in *Lgr4*-null MMTV-*PyMT* mice is significantly delayed when compared with tumor growth in *Lgr4* wild-type MMTV-*PyMT* mice. Combined with an earlier publication and our data-base-analysis that *Lgr4* expression level is correlated with migration ability in several breast cancer cell lines and breast cancer recurrence in human patients (Gao, Kitagawa, Hiramatsu, et al., 2006), we suspected that *Lgr4* may affect breast cancer progression and metastasis as well. Subsequent studies gave data that supported our hypothesis, and even more striking findings: loss of *Lgr4* heavily impairs metastasis to lungs in MMTV-*PyMT* mice and MDA-231 tail vein injection assay. At that time, several labs reported that LGR4 potentiates the Wnt signaling upon binding of RSPOs (W. B. de Lau et al., 2012), and Wnt signaling pathway had been shown to regulate stem cell and CSC activities (Holland et al., 2013). Then our lab published two papers which reported *Lgr4* to be a stem cell regulator through Wnt

pathway in both the prostate and mammary gland; and notably Sox2 is directly regulated by Lgr4 in mammary gland stem cells (W. Luo et al., 2013; Y. Wang et al., 2013). Sox2 was then reported as a CSC marker for breast cancer and squamous (Boumahdi et al., 2014; Leis et al., 2012). All these studies supported a link between Lgr4 and breast CSCs. Since Lgr4 is mainly expressed in mammary gland basal cells, we crossed our Lgr4 knockout mice with MMTV-*Wnt1* mice which is a popular basal-originated breast cancer model (Y. Li et al., 2000). In MMTV-*Wnt1* mice, tumor progression is repressed as well. We aimed to evaluate the CSC numbers in MMTV-*Wnt1* tumors with different Lgr4 genotypes. Therefore we performed transplantation assay and *in vitro* sphereculture assay which both showed decreased CSC frequency in Lgr4 low groups. Furthermore, Lgr4 knockdown in MDA-231 showed a similar outcome in Xenograft assay and tumorsphere assay. All these results revealed a strong correlation between reduction in Lgr4 level and decreased CSC frequency. On the other hand, we aimed to explore whether Lgr4 affected metastasis through EMT which is one hallmark of CSCs (Mani et al., 2008). To assess the role which Lgr4 plays in migration and invasion, we adopted trans-well assay and *in vitro* 3D culture assay. Lgr4 knockdown in all breast cancer cell lines we tested showed decreased motilities which supported our earlier observation of metastasis abrogation in MMTV-*PyMT* *Lgr4*^{-/-} mice.

IV.2 Significance

Our findings generate new insight into LGR4 in breast cancer and the overall understanding of GPCRs. More specifically, our results for the first time showed that LGR4 is a key regulator of breast cancer stem cells. Furthermore, our results also showed that LGR4 regulates breast cancer progression in different ways: 1. LGR4 affects breast cancer frequency 2. LGR4 affects metastasis 3. LGR4 affects cancer cell EMT. Finally, our signaling experiments in breast cancer showed that LGR4 affects the Wnt pathway, EGFR pathway and several MMP proteins level as well. Thus we provided a potential therapeutic target for breast cancer treatment. By targeting LGR4, we may be able to inhibit several cancer promoting pathways, reduce tumor promotion and block distant metastasis.

IV.3 Limitations

Lack of human breast cancer data. All our experiments were performed using cell lines and breast cancer mouse models. Although these experiments covered both *in vivo* and *in vitro* conditions, a human patient based experiment which confirms our results in mouse and cell lines would be essential to support the clinical validity of our findings.

Lack of effective methods to isolate genuine cancer stem cells. Although our results suggested that we enriched cancer stem cells up to 1 in 220 cancer cells in serial sphereculture and transplantation assay, we still cannot exclude the possibility that those 220 cancer cells work as a group to generate a xenograft tumor. In addition we could not sort out viable CD24⁺/CD90⁺ cells from MMTV-*Wnt1* tumors. Possibly due to technical

difficulties, we cannot reproduce the data in two previous papers (R. W. Cho et al., 2008; Malanchi et al., 2012). The development of more definitive CSC separation protocols would allow an evaluation of Lgr4 function in breast CSCs in greater depth.

IV.4 Future directions

One of our future directions will be focused on how Lgr4 functions in EMT regulation *in vivo*. Our results have shown EMT gene expression are affected by Lgr4 inactivation, but we have not yet demonstrated direct evidence that Lgr4 affects EMT *in vivo*. To achieve this, we will isolate circulating tumor cells from MDA-231 xenograft recipient blood by using GFP, and then analyze the EMT markers altered in those cells expressing either shNC or shLGR4. This experiment will give us direct evidence of LGR4 regulating EMT *in vivo*. Furthermore, we have shown that the EGFR and Wnt pathways are affected by Lgr4 in breast cancer, but we have not yet answered whether the GPCR canonical cAMP/CREB pathway also participates in Lgr4-mediated cancer regulation. To answer these questions, Lgr4-T775I, a mutated Lgr4 construct which constitutively activates the cAMP/CREB pathway, will be used in this study. With wnt antagonist or EGFR antagonist controls, we will be able to study the impact of each pathway on EMT and cancer progression.

Another future direction will be to study the role of LGR4 in drug resistance. Our previous data has shown that loss of LGR4 impairs resistance of MDA-231 and MCF7 against the breast cancer drugs doxorubicin and docetaxel.

CHAPTER V

CONCLUSIONS

Group B LGRs have been shown as stem cell markers in proliferative tissues such as the intestines and hair follicles. *Lgr4* participates in the development of multiple organs, and recently *Lgr4* has been reported as a stem cell regulator in the mammary gland and prostate. Two papers reported that *Lgr4* promotes invasion or metastasis in several cancer cell lines and colorectal cancer, one paper reported that LGR4 non-sense mutation in human patients is related with several diseases and cancers, but the overall mechanism how *Lgr4* affects cancer is still largely unknown. This is the first investigation that focused on LGR function in breast cancer stem cells. In this study, we report that *Lgr4* inactivation in the MMTV-*Wnt1* mouse model decreased the number and tumorigenic ability of CSCs. Metastasis, the final and the most fatal step of cancer, is always a priority in breast cancer treatment. Especially in those patients who developed TNBC, finding a method to delay metastasis formation is very challenging. Here, our results from several TNBC cell lines studies revealed that LGR4 affects multiple signaling pathways. By knocking down LGR4 in these TNBC cell lines, metastasis was attenuated. Last but not least, we found that *Lgr4* positively regulated the Wnt signaling pathway, EGFR pathway and MMPs in breast cancer. Taken together, this study is the first investigation on LGR4 function in breast cancer stem cells which may help future clinical strategies against breast cancer progression and metastasis.

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