NOTCH SIGNALING IN BREAST TUMOR INITIATING CELLS

INHIBITION OF NOTCH SIGNALING TARGETS

BREAST TUMOR INITIATING CELLS

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ABSTRACT

The cancer stem cell hypothesis claims that only a small subpopulation of cells within a tumor is responsible for tumor growth, recurrence after treatment and metastasis. These cells have been termed tumor-initiating cells or cancer stem cells and are functionally defined by their capacity to elicit the growth of tumors in immune-compromised animals that recapitulate the cellularity of the tumor from which they were isolated. Several reports demonstrate that tumor-initiating cells are resistant to most current treatments. Hence, novel therapies for breast cancer should be developed that specifically target these tumorigenic cells. The Notch signaling pathway is hyperactive in human breast cancer as well as in mouse mammary tumor-initiating cells. In this study, I have found that inhibitors of the pathway target breast tumor-initiating cells from various breast cancer subtypes and may provide a novel therapy for breast cancer. MRK-003, a gamma-secretase inhibitor that blocks Notch signaling, inhibited the self-renewal of breast tumor-initiating cells *in vitro* and reduced tumor growth in xenograft models. MRK-003 inhibited proliferation of tumor cells within xenografts and induced their apoptosis and differentiation towards the myoepithelial lineage. Expression of the Notch pathway antagonists led to similar outcome in human breast tumor cell lines. Notably, tumors in MRK-003 treated mice were devoid of tumor initiating cells, suggesting that inhibitors of Notch signaling may lead to durable cancer cures. These findings suggest that GSIs target breast tumor-initiating cells and may prove to be effective novel anti breast cancer drugs.

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PREFACE AND DECLARATION OF ACADEMIC ACHIEVEMENT

The following thesis comprises three manuscripts (sandwich thesis) as approved by the supervisory committee. The first manuscript is published as indicated below, (Oncogene, 2011) the second one is prepared for publication pending submission and the third manuscript is in preparation. The manuscript chapters are preceded by a general introduction chapter that outlines the background literature relevant for all three manuscripts. This chapter is followed by the chapter that describes experimental procedures used in this work; to avoid overlap between the chapters, these parts are taken out from the manuscripts. Each chapter is preceded by a short introduction describing the overall implications of the work in the context of the thesis. References are self-contained within each article; references for the rest of the thesis are listed in the main reference list at the end of the thesis. A discussion that describes overall conclusions and implications of this study is incorporated as a final chapter. Contribution of each of the authors to the manuscripts is explained in the beginning of each manuscript.

LIST OF ABBREVIATIONS

α-SMA – alpha Smooth Muscle Actin

BTIC – Breast Tumor Initiating Cells

CD - Cluster of Differentiation

CK - Cytokeratin

EGF- Epidermal Growth Factor

ER – Endoplasmic Reticulum

ERBB - Erythroblastic Leukemia Viral Oncogene

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

DSL – Delta/Serrate/ LAG2

FBS – Fetal Bovine Serum

FGF - Fibroblast Growth Factor

FVB – Friend Virus B Tropism

GSI – Gamma-Secretase Inhibitor

H&E – Hematoxylin and Eosin

JNK - Jun N-terminal Kinase

MAML-1 – Mastermind Like-1

MMTV – Mouse Mammary Tumor Virus

NGS - Normal Goat Serum

NICD – Notch Intracellular Domain

NOD/SCID - Non-Obese Diabetic / Severe Combined Immunodeficient

NP40 - Nonyl Phenoxypolyethoxylethanol 40

PBST- Phosphate Buffer Saline with 0.03% Tween-20

PI3K – Phosphoinositide 3-Kinase

PTEN - Phosphatase and Tensin Homolog

SCM – Stem Cell Media

T-ALL - T-cell Acute Lymphoblastic Leukemia

TIC – tumor initiating cell

CHAPTER 1

Introduction

Breast cancer and breast cancer stem cells

Despite recent advances in diagnosis and treatment, breast cancer remains the leading cause of cancer death in women worldwide (Carlson *et al.*, 2009). Current treatments include surgery, chemotherapy, radiation and hormone therapy as well as treatment with the humanized monoclonal antibody trastuzumab, which targets the ErbB2 oncogene (Valabrega *et al.*, 2007). However, little improvement has been made in the treatment of metastatic breast cancer and in a high proportion of women recurrence occurs following treatment. Novel treatments are being increasingly developed based on a growing understanding of the biology of the disease. Targeting specific signaling pathways that are deregulated in breast cancer is one promising approach to achieve durable cures (Nickoloff *et al.*, 2003; Zardawi *et al.*, 2009).

Growing evidence supports the "cancer stem cell hypothesis", suggesting that only a small subpopulation of cells within a tumor is responsible for driving tumor growth, seeding metastases, and causing relapse after remission in breast cancer patients. These "cancer stem cells" were initially identified in leukemia but have more recently been identified in a variety of malignancies including solid tumors such as those of the brain, pancreas, liver, colon, prostate, gastro-intestinal tract, skin, head and neck, ovaries and breast (Al-Hajj *et al.*, 2003; Bonnet and Dick, 1997; Collins *et al.*, 2005; Dick, 2003; Fang *et al.*, 2005; Galderisi *et al.*, 2006; Lapidot *et al.*, 1994; Ricci-Vitiani *et al.*, 2007; Singh *et al.*, 2004).

In breast cancer, a subpopulation of cells with high tumorigenic potential was sorted from the bulk tumor cells based on the expression of several cell surface markers (Al-Hajj *et al.*, 2003). Whereas only about 0.01% of bulk tumor cells were able to give rise to a tumor, the sorted cells showed a 10- to 50-fold increase in tumorigenicity when transplanted into NOD/SCID mice. Importantly, the resulting tumors contained the same diversity of cell subpopulations as the original tumors, and only a very small fraction of cells was able to reseed a new tumor. These findings suggest that anti-breast cancer therapies should be directed specifically against breast cancer stem cells since these cells are the origin of the tumor.

Targeting cancer stem cells to treat cancer recently became a popular field in research of various malignancies. However, this task might not be easy due to several intrinsic properties of cancer stem cells. First, these cells are proposed to be refractory to most current anti-cancer treatments. Since cancer stem cells are relatively quiescent (Aguirre-Ghiso, 2007; Saito *et al.*; Saito *et al.*) similar to normal stem cells (Fuchs, 2009; Yamazaki *et al.*, 2006), they remain resistant to traditional anti-cancer drugs that target rapidly proliferating cells; high expression of adenosine triphosphate – binding cassette proteins in cancer stem cells allow their more efficient efflux of drugs (Costello *et al.*, 2000; Dean *et al.*, 2005; Gottesman *et al.*, 2002). Hence, after the bulk of the tumor is eliminated during treatment, cancer stem cells persist and can reinitiate tumor growth and seed metastases. In addition, despite recent advances in isolation and propagation of cancer stem cells, not enough reliable markers exist for this cell population. In breast cancer, $CD44^{+}/CD24^{low}/ESA^{+}/lineage^{-}$ cells are significantly enriched in tumor initiating capacity (Al-Hajj *et al.*, 2003), however, still only 1 in 200 of these cells is a cancer stem

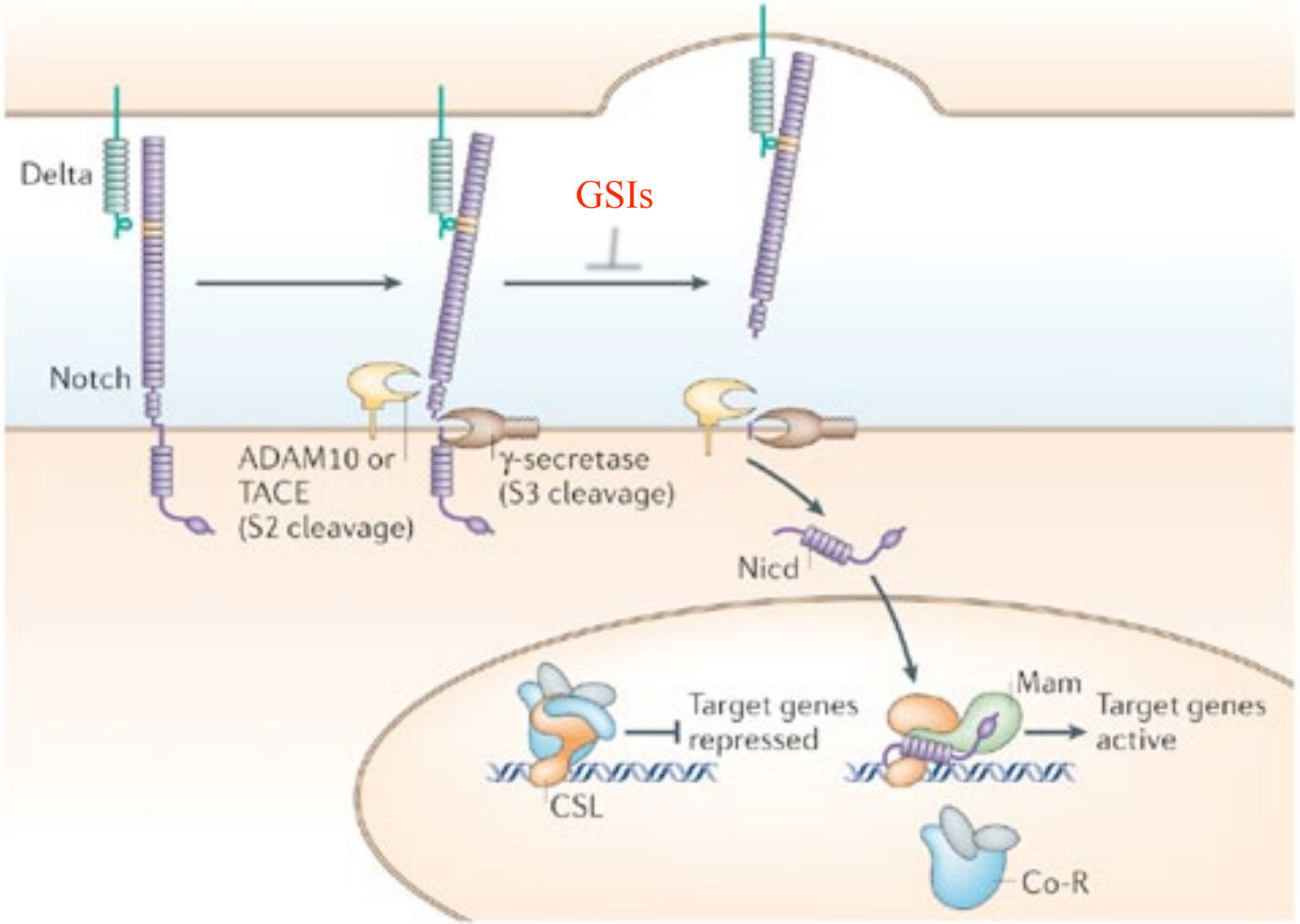
cell. Based on conditions developed for propagation of neural stem cells, cancer stem cells are commonly cultured *in vitro* in chemically defined medium and such cultures were shown to contain high fraction of tumor initiating cells (Dontu *et al.*, 2003; Dontu and Wicha, 2005; Liu *et al.*, 2007; Ponti *et al.*, 2005; Reynolds and Weiss, 1992). For human breast cancer cells 96-98% of cells in these cultures were $CD44^{+}/CD24^{-}$ while only 10-20% of the cells were able to self renew *in vitro* (Al-Hajj et al., 2003) . All these findings indicate the need to look for additional markers of cancer stem cells and to further investigate which signaling pathways are involved in their self renewal and can be targeted to specifically eliminate these cells within the tumors.

Cancer stem cells share similarities with normal stem cells such as the ability to self-renew and differentiate (Al-Hajj *et al.*, 2004; Kopper and Hajdu, 2004). Therefore, it is possible that the same signaling pathways are important in the biology of both normal and cancer stem cells. These pathways include the Wnt, Hedgehog and Notch signaling pathways, which have been found to be dysregulated in various cancers (Campbell *et al.*, 2008; Dontu *et al.*, 2004; Farnie and Clarke, 2007; Reya *et al.*, 2003; Sukhdeo *et al.*, 2007). Therapies leading to inactivation of these pathways may have an anti-tumorigenic effects by inducing the differentiation and/or causing the apoptosis of cancer stem cells. In fact, numerous inhibitors of the Wnt, Hedgehog and Notch signaling pathways are currently being developed and tested in various cancers (reviewed in (Takebe *et al.*).

The Notch signaling pathway

The Notch signaling pathway is an important developmental pathway that is conserved from Drosophila to humans (Poulson, 1937). Notch was first discovered in Drosophila as one of the "Neurogenic" genes; Notch deficient flies have hyperplasia of the nervous

Notch Pathway Overview



system and fail to develop an epidermis (Poulson, 1937). The key participants of the Notch pathway in Drosophila include the large single pass transmembrane receptor (Notch), two types of ligands – Delta and Serrate - and the transcription factor Suppressor of Hairless (Artavanis-Tsakonas, 1988). Two homologs of Notch were identified in *C. elegans* (LIN12 and GLP-1) as well as several ligands (APX-1, AGR-2, LAG-2, and F16B12.2) and the transcription factor LAG1 (Greenwald, 1998; Kimble and Simpson, 1997; Yochem *et al.*, 1988). Mammals have four Notch receptors (Notch1-4) that are structurally similar to Drosophila Notch and two families of ligands – Delta and Jagged. There are three Delta and two Jagged (Jagged1, 2) ligands in mammals (Maine *et al.*, 1995). Delta-like protein is structurally related to Delta ligands and was shown to induce expression of Notch target genes. The mammalian transcriptional factor is called CSL or RBPj kappa (κ) (Kopan, 2002; Lai, 2002a; Lewis, 1998; Maine *et al.*, 1995).

Both Notch receptors and ligands are transmembrane proteins and activation of the pathway occurs when a Notch receptor interacts with one of its ligands expressed by a neighboring cell (Artavanis-Tsakonas *et al.*, 1995). Notch is synthesized as a precursor protein that is cleaved within the ER by a furin protease and reassembles at the cell surface as a functional heterodimer (Rand *et al.*, 2000).

The Notch extracellular region contains EGF-like repeats that bind to the DSL region within the extracellular domain of the ligands. The number of these repeats varies between different Notch proteins and their posttranslational modifications regulate specificity for different ligands (Bruckner *et al.*, 2000; Moloney *et al.*, 2000). As with most transmembrane proteins, Notch undergoes N-glycosylation within the ER, which is followed by O-fucosylation of the EGF repeats by O-fucosyl transferase. The latter

modification is essential to generate functional Notch ligand binding sites. Further elongation of the polysaccharide chains of specific EGF repeats is mediated by Fringe proteins and regulates specificity of ligand binding. Three variants of Fringe exist in mammals (Lunatic, Maniac and Radical Fringe); the addition of GlcNAc residues to the EGF repeats of Notch mediated by these Fringe proteins inhibits Notch binding to Delta, but enhances its interaction with Jagged. Such regulation permits differential activation of Notch signaling by cells that express one or the other ligand. Another posttranslational modification that regulates Notch signaling is ubiquitination (Lai, 2002b). Several E3 ubiquitin ligases are involved in Notch pathway regulation. Mindbomb and Neurolized ligases ubiquitinate the cytoplasmic domain of Delta ligands and causes their internalization together with the extracellular region of Notch (Pitsouli and Delidakis, 2005). This latter event is required for Delta-mediated activation of Notch signaling in neighboring cells. Several other E3 ubiquitin ligases ubiquitinate the Notch intracellular domain causing its degradation by the proteasome and therefore negatively regulate Notch signaling. These include Deltex proteins that interact with intracellular Ankyrin repeats of Notch and target it for degradation, as well as LNX and Itch ligases that regulate the Notch pathway through interactions with Numb (McGill and McGlade, 2003). Numb is an important negative regulator of Notch signaling that will be discussed in detail below.

Upon interaction with ligands on adjacent cells, the Notch receptor undergoes a conformational change exposing an S2 cleavage site that is recognized by an ADAM10 or ADAM17 metalloprotease, resulting in release of the extracellular domain and leaving a short extracellular tail on Notch (Brou *et al.*, 2000). This partially proteolytically

processed Notch is then recognized by the gamma-secretase complex, which cleaves Notch thereby releasing the Notch intracellular domain (NICD) from the membrane (Kimberly *et al.*, 2003; Selkoe and Kopan, 2003). The NICD contains a RAM23 domain (Tamura *et al.*, 1995), CD10/Ankyrin repeats (Lubman *et al.*, 2004), a nuclear localization signal and a PEST region (Weng *et al.*, 2004). When the NICD is liberated from the membrane by the gamma-secretase it translocates to the nucleus and interacts with its main downstream effector RBPjκ through the RAM23 domain. In the absence of Notch signaling, RBPjκ is bound to promoters of Notch target genes in complexes with co-repressors that include SMRT/N-CoR, CIR, SHARP, Hairless and KyoT2 (Barolo *et al.*, 2002; Hsieh *et al.*, 1999; Kao *et al.*, 1998; Lai, 2002a; Oswald *et al.*, 2002; Taniguchi *et al.*, 1998).

Interaction between the NICD and RBPjκ causes a conformational change in both proteins and permits formation of a ternary complex with the co-activator Mastermind (Mastermind-like protein in mammals) through the CD10/Ankyrin repeats of the NICD, resulting in the release of the co-repressors (Kovall, 2007). Thereafter, the general transcriptional co-activators CBP/p300 and PCAF/GCN5 are recruited to the complex resulting in the transcription of Notch target genes (Fryer *et al.*, 2002; Kurooka and Honjo, 2000; Wallberg *et al.*, 2002). Two main families of the target genes include the Hey and Hes families of βHLH transcriptional repressors that mediate inhibition of transcription of various genes encoding proteins involved in cell differentiation (Davis and Turner, 2001; Iso *et al.*, 2003; Jennings *et al.*, 1999). In addition, other genes are directly upregulated by NICDs including ErbB2 and Cyclin D1 both of which contain RBPjκ binding elements in their promoters (Chen *et al.*, 1997; Ronchini and Capobianco,

2001). Transcription activation is terminated by ubiquitin-ligase-mediated degradation of the NICD, which is facilitated by Mastermind, and leads to disassembly of the ternary complex (Fryer *et al.*, 2002; Fryer *et al.*, 2004).

While Notch signaling is likely involved in the development of most tissues, its functions can be roughly divided into three main areas. First, Notch signaling is involved in restricting cell fate (sometimes called "lateral inhibition") (Kopan and Turner, 1996; Lewis, 1998; Muskavitch, 1994; Rooke and Xu, 1998; Sawamoto and Okano, 1996; Schlosser and Northcutt, 2000). Normally, more multipotent cells exist in tissues than are needed to provide for the loss of differentiated cells. Activation of Notch prevents the uncontrolled differentiation of these cells into specific cell types. Classic example of such a mechanism is the *Drosophila* neural-epidermal choice, where Notch inhibits differentiation of proneural cells into neurons (Parks *et al.*, 1997). Such inhibition is usually achieved by amplifying small differences in the expression of the receptor and ligand in two identical cells - cells that have higher expression of ligand will activate Notch signaling in adjacent cells and downregulate expression of their own Notch receptors.

However, in some cases Notch can promote differentiation towards specific lineages. The latter mechanism is termed "inductive signaling" and requires interaction between two non-equivalent cells (a ligand–expressing cell and a receptor- expressing cell). This interaction often leads to the creation of a new cell type at the boundary between two distinct cell populations. Well-studied examples of this signaling are the maintenance of germ line proliferation in *C. elegans* and boundary formation of the *Drosophila* wing (Greenwald, 1998; Kim *et al.*, 1996; Kimble and Simpson, 1997).

Finally, sometimes, asymmetric distribution of factors between two daughter cells during cell division can lead these cells to acquire distinct phenotypes. One example of this phenomenon is the asymmetric inheritance of the Notch inhibitor Numb during development of *Drosophila* sensory organs (Posakony, 1994). Numb plays an important role in regulating Notch signaling as recounted below. Additional examples include asymmetrical segregation of factors involved in intracellular trafficking and degradation of both receptors and ligands (Emery *et al.*, 2005; Jafar-Nejad *et al.*, 2005; Le Borgne and Schweisguth, 2003).

Inhibition of the Notch signaling pathway as anti-cancer therapy

Because Notch signaling is involved in numerous developmental processes, it is not surprising that its misregulation occurs in many human diseases. These include Alagille syndrome (Li *et al.*, 1997; Oda *et al.*, 1997a; Oda *et al.*, 1997b), spondylocostal dysostosis (Bulman *et al.*, 2000), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Joutel *et al.*, 1996a; Joutel *et al.*, 1996b) and Hajdu-Cheney Syndrome (Sympson *et al.,* 2011). Aberrant regulation of Notch signaling has also been implicated in several cancers. The first evidence for involvement of Notch in cancer came from identifying activating mutations in Notch-1 in T-ALL patients (Ellisen *et al.*, 1991; Malecki *et al.*, 2006; Weng *et al.*, 2004; Weng *et al.*, 2003). Later, activating mutations in Notch-1 were also found in CLL (Fabbri *et al.,* 2011) were they correlated with poor prognosis. Similarly, copy number increases and gain of function mutations were identified for Notch 2 in B-cell lymphoma. Aberrant regulation of the Notch signaling pathway has also been reported for many solid tumors such as those of the lung, breast, brain, pancreas, prostate and colon (Brennan *et al.*,

2009; Callahan and Egan, 2004; Dang *et al.*, 2006; Dang *et al.*, 2000; Dickson *et al.*, 2007; Fan *et al.*, 2006; Miyamoto *et al.*, 2003; Park *et al.*, 2006; Parr *et al.*, 2004; Patel *et al.*, 2005; Pece *et al.*, 2004; Proweller *et al.*, 2006; Santagata *et al.*, 2004; Stylianou *et al.*, 2006). In some cancers, such as those of the skin, pancreas and several types of the squamous cell carcinomas, Notch can act as a tumor suppressor suggesting that cellular context is important; moreover, different Notch proteins might play different roles in tumorigenesis (Fan *et al.*, 2004; Hanlon *et al.*; Harrison *et al.*; Nicolas *et al.*, 2003; Agrawal *et al.,* 2011).

Despite the variations in effects of the Notch signaling pathway under different circumstances, it remains an attractive target for developing anticancer biopharmaceutical agents. Several approaches have been taken by researchers to inhibit Notch signaling in cancer cells. Soluble receptor "decoys" and monoclonal antibodies have been developed and tested in different animal models of human disease (Dikic and Schmidt; Li *et al.*, 2008a; Phillips *et al.*, 2006; Wu *et al.*; Yin *et al.*). The main advantage of using these novel agents is that they can target specific Notch receptors thereby making the treatment more context specific and reducing possible side effects. However, the problem with soluble recombinant proteins of small molecular mass ("decoys") is their fast clearance from the organism; hence additional manipulations might be needed to stabilize these agents perhaps by their fusion to other proteins (Epstein, 1997; Mannik and Wener, 1997; Murray and Dahl, 1997). In the case of monoclonal antibodies, the main problem is their poor penetration from the blood vessels into the tissues. To solve this problem, Fab fragments or single chain antibodies are being developed (1997). Recently, one group demonstrated successful use of synthetic, cell-permeable, stabilized alpha-helical

peptides that disrupt interactions between the N1ICD and RBPjκ leading to inhibition of proliferation in both *in vitro* and *in vivo* models of Notch1-driven T-ALL (Moellering *et al.*, 2009). The problem with this approach might be the proposed existence of alternative, "non canonical" Notch signaling that does not involve RBPjκ (Raafat *et al.*, 2009; Shawber *et al.*, 1996).

As previously described, gamma secretase is a large enzymatic complex that is essential for Notch activation. It comprises a catalytic subunit (presenilin 1 or presenilin 2) as well as Aph1, Pen2 and nicastrin that stabilize the complex and participate in substrate recognition (Wolfe, 2006). Gamma secretase is not specific for the Notch receptor but has a wide variety of substrates including ErB4, CD44 as well as Notch ligands Jagged and Delta (Lammich *et al.*, 2002; Marambaud *et al.*, 2002; Ni *et al.*, 2001; Parks and Curtis, 2007; Struhl and Adachi, 2000). Since this enzyme was found to be involved in Alzheimer's disease as being responsible for cleavage of the β-amyloid precursor protein and thereby creating toxic extracellular aggregates, numerous commercially available inhibitors were developed and tested in human trials (Weihofen *et al.*, 2003; Wolfe, 2006). Later, these inhibitors were studied for their ability to inhibit tumorigenesis through the Notch pathway and some promising results were achieved. Some examples include: the tripeptide inhibitor (Z-LLNleCHO), which was shown to inhibit tumor growth in cell lines and human xenografts in mice with melanoma and Kaposi sarcoma (Curry *et al.*, 2005); N-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine *t* -butyl ester (DAPT) was found to be a potent inhibitor of all four Notch receptors and induced apoptosis and cell cycle arrest in T-ALL animal models, and inhibited sphere formation by ductal carcinoma cells indicating its ability to block self-

renewal (Farnie *et al.*, 2007; Hallahan *et al.*, 2004; O'Neil *et al.*, 2006); (S,S)-2-[2-(3,5-Difluorophenyl)acetylamino]-N-(5-methyl-6-oxo-6,7-dihydro-5-dibenzo[b,d]azepin-7-yl)propionamide (DBZ) was shown to convert proliferative crypt cells into post mitotic goblet cells, and to inhibit the growth of colon adenomas in mice (van Es *et al.*, 2005). MK-0752 is a gamma secretase inhibitor (GSI) developed by Merck, Inc. (Whitehouse Station, NJ), it also inhibits Notch signaling, and is currently in a phase I clinical trial for relapsed T-ALL patients and advanced breast cancers (Nickoloff *et al.*, 2003).

Finally, another GSI developed by Merck Inc., MRK-003, was found to potently inhibit Notch cleavage and to reduce tumorigenesis in T-ALL, a mouse model of pancreatic ductal adenocarcinoma, and human brain and lung cancers (Chen *et al.*; Cullion *et al.*, 2009; Konishi *et al.*, 2007; Lewis *et al.*, 2007; Plentz *et al.*, 2009; Rao *et al.*, 2009; Tammam *et al.*, 2009). In addition, the effectiveness of this inhibitor has been examined in several models of breast cancer (Grudzien *et al.*; Rao *et al.*, 2009; Tammam *et al.*, 2009). In my study reported herein, MRK-003 was successfully used to target Notch signaling in breast tumor cells.

The main disadvantage in using gamma secretase inhibitors is the fact that they have a wide range of effects that can lead to toxicity. First, inhibition of gamma secretase activity would likely lead to non-selective inactivation of all four Notch receptors that are known to be important in the physiology of many normal tissues. Interestingly, it was recently suggested that some degree of selectivity might exist because DAPT and DBZ blocked cleavage of Notch1 in breast tumor cell lines but did not affect the cleavage of Notch 4 (Harrison *et al.*). Nevertheless, there are known side effects of inhibiting Notch signaling with GSIs such as intestinal toxicity (Barten *et al.*, 2006; Milano *et al.*, 2004).

In the intestine, active Notch prevents differentiation of proliferative crypt cells into goblet cells. The of GSIs can cause goblet cell hyperplasia resulting in severe diarrhea. In addition, as mentioned previously, Notch can act as tumor suppressor in some cases, such as skin carcinoma, where genetic ablation of Notch1 can cause epidermal and corneal hyperplasia followed by the development of skin tumors (Nicolas *et al.*, 2003; Proweller *et al.*, 2006; Weijzen *et al.*, 2002b). Therefore, some GSIs may cause skin tumors. Besides the side effects caused by inhibition of Notch signaling, inhibition of gamma secretase may lead to attenuation of additional signaling pathways since this enzyme has a variety of substrates as previously indicated. Finally, some gamma secretase inhibitors are not specific for the gamma secretase but target other proteases such as signal peptidase (Nyborg *et al.*, 2004). All these concerns should be carefully looked at when studying the effect of gamma secretase inhibitors in preclinical models as well as in more advanced clinical trials. However, it might be possible to find a therapeutic window in which we can kill cancer cells using gamma secretase inhibitors without having any adverse effects. This suggestion is in part based on the observation that cancer cells often highly express Notch pathway components and target genes, suggestive of high Notch pathway activity, and are "addicted" to Notch signaling (Reedijk *et al.*, 2005). In this study, I tried to achieve this goal using a mouse model of breast cancer as well as human breast cancer cell lines.

Notch signaling in breast cancer

Several findings suggest that Notch signaling plays an important role in breast tumorigenesis. The viral genome DNA of the mouse mammary tumor virus randomly integrates into the host genome, causing oncogenic transformation by disrupting the

normal expression of host proto-oncogenes. When mice were infected with this virus, a high proportion of the resulting tumors were found to possess the virus integrated into the Notch 4 locus, resulting in the expression of truncated, constitutively active Notch 4 (Gallahan and Callahan, 1997; Gallahan *et al.*, 1987). Viral insertions into the Notch 1 locus resulted in the formation or maintenance of mammary tumors in MMTV/Neu transgenic mice (Dievart *et al.*, 1999; Weijzen *et al.*, 2002a). As further evidence that Notch signaling plays a causal role in tumorigenesis, transgenic mice expressing constitutively active Notch 1 (Hu *et al.*, 2006; Kiaris *et al.*, 2004) or Notch 4 alleles (Raafat *et al.*, 2004) also develop mammary tumors.

In regards to human breast cancer, over expression of Notch receptors and ligands was reported in human breast tumors as compared to normal tissues with positive correlation to tumor grade and aggressiveness (Dickson *et al.*, 2007; Reedijk *et al.*, 2005; Stylianou *et al.*, 2006). Down regulation of Numb, an inhibitor of the Notch pathway was also observed in about 50% of human mammary carcinomas (Pece *et al.*, 2004). Primary human breast tumors and human breast tumor cell lines can be divided into different molecular subtypes according to their gene expression signatures, hormone receptor expression and expression of ErbB2 (Lacroix and Leclercq, 2004; Neve *et al.*, 2006; Perou *et al.*, 2000; Sorlie *et al.*, 2001). Triple negative tumors and tumors over expressing ErbB2 are considered the most aggressive and resistant to existing treatments. Interestingly, Notch 4 was found to be over expressed in a high proportion of triple negative tumors and downregulation of Notch 3 inhibited proliferation and induced apoptosis of ErbB2 positive breast cancer cell lines (Andre *et al.*, 2009; Yamaguchi *et al.*, 2008). Moreover, downregulation of Notch 1 resensitized ErbB2 expressing trastuzumab

resistant breast cancer cells to trastuzumab suggesting possible cooperation between the two pathways in breast cancer progression (Osipo *et al.*, 2008b). *In vivo* treatment of ER^+ xenografts with gamma secretase inhibitors in combination with tamoxifen resulted in tumor regression indicating existence of a cross talk between Notch and Estrogen Receptor in breast cancer (Rizzo *et al.*, 2008).

In addition, Notch signaling seems to play a role in the resistance of breast cancer cells to conventional therapies. For example, downregulation of Notch 1 in breast cancer cell lines increased chemosensitivity to doxorubicin and docetaxel (Zang *et al.*) and irradiation of breast cancer stem cells resulted in elevated expression of Notch 1 and Jagged 1 (Phillips *et al.*, 2006).

All of these observations indicate that the Notch signaling pathway plays an important role in tumorigenesis of the breast. As mentioned above, novel anti-cancer therapies should be developed based on our understanding of the biology of breast cancer stem cells. Several groups suggested that the Notch pathway is essential for the self-renewal of breast cancer stem cells and that its inhibition may cause tumor cell differentiation and apoptosis (Chen *et al.*; Dontu *et al.*, 2004; Farnie and Clarke, 2007; Farnie *et al.*, 2007; Grudzien *et al.*; Harrison *et al.*; Phillips *et al.*, 2006; Sansone *et al.*, 2007). In the study reported here, I used various approaches to inhibit Notch signaling in breast cancer stem cells. These experiments led to accumulation of novel and exiting data that can shed a light on the role of Notch signaling in the self-renewal and differentiation of cancer stem cells in humans and mice.

Mouse models of breast cancer

High percentage of human breast tumors overexpresses ERBB2 resulting in poor

prognosis and high metastatic capacity. Therefore, several mouse models expressing Her2 in the mammary gland were generated over the years to study the effects of this oncogene on mammary tumorigenesis. Initially, many of the strains expressed activated form of ERBB2 resulting in immediate appearance of mammary tumors following expression of the transgene in the mammary epithelium (Muller *et al*., 1988; Bouchard *et* al., 1989; Bargmann *et.al.,* 1988). However, in human disease, the activating mutations in ERBB2 are very rare; instead there is an increase in copy number of the gene, which results in overexpression of the *wt* protein (Yokota *et al*., 1986, Slamon *et al*., 1987, van de Viver *et al*., 1987, Slamon *et al*., 1989). N202 transgenic mice that will be further described in the first part of my thesis overexpress the rat Her2 proto-oncogene under control of the MMTV promoter (Guy *et al.*, 1992). These mice appear with mammary tumors after prolonged latency of about a year and can serve as a more accurate model of human breast tumorigenesis than the tumors arising in mice that express activated ERBB2. Interestingly, mammary tumors in the N202 mice are particularly enriched in tumor initiating cells (Kurpios *et al.,* in preparation) - transplantation of single cell isolated from these tumors leads to formation of subcutaneous tumors in syngeneic mice in about 6 months time. All these findings suggest that N202 mice can serve as convenient and reliable model to study human breast cancer.

HYPOTHESIS

I hypothesize that the Notch signaling pathway is hyperactive in human breast tumors and inhibition of this pathway will inhibit self-renewal and induce apoptosis of breast tumor initiating cells leading to irreversible tumor regression. Use of gamma secretase inhibitors, many of which are already in clinical trials will provide a novel effective

treatment of breast cancer. In addition, development of novel Notch inhibitors that target specific components of the pathway might prove more effective and specific strategy to target breast tumor initiating cells.

CHAPTER 2

Experimental procedures

Mice

All procedures involving mice were performed with the approval of the Canadian Council on Animal Care.

Establishment and propagation of mammary epithelial cells and tumor cells in vitro

Mouse mammary glands and tumors were processed to obtain dispersed primary cells (Kurpios *et al.*, 2009; Shepherd *et al.*, 2001). Mammospheres or tumorspheres were established by plating primary cells in stem cell medium (SCM) (Reynolds and Weiss, 1992), which comprises DMEM:Ham's F-12 (3:1), 4 μg/ml of B-27, 20 ng/ml EGF, 40 ng/ml FGF-2 and 4 ng/ml Heparin (all from Invitrogen) (Kurpios *et al.*, 2009; Youn *et al.*, 2006; Youn *et al.*, 2005). Spheres arising from primary cells were passaged every 4 days by mechanical dissociation followed by plating the dispersed cells at a density of ~30 000 cells per ml in SCM. Tumorspheres and mammospheres that had been serially passaged between 2-5 times were used for the experiments reported herein.

Sphere- and colony-forming assays

The frequency of sphere-forming cells in primary mammary epithelial cell populations was determined by seeding ~100 000 cells in 1 ml of SCM in triplicate into the wells of a 24-well flat-bottom plate and then counting the number of spheres (>80 μm) that arose a week later. The frequency of sphere-forming cells in primary tumor cell populations, and established mammosphere and tumorsphere populations was determined by plating ~6 000 cells in 0.2 ml of SCM in triplicate into wells of a 96-well plate as described above. For human breast tumor cell lines, four day old sphere cultures were trypsinized

for several minutes and then mechanically dissociated and plated at 30,000 cells/mL in human SCM (low glucose DMEM: Ham's F-12 (3:1) supplemented with 1mg/ml fungizone, 1% penicillin/streptomycin, 2 μg/ml B-27, 10 ng/ml human bFGF, 20 ng/ml human EGF, 5 μg/ml Insulin, 4 ng/ml Heparin) in 96-well flat-bottom plates [Corning]. The frequency of sphere-forming cells in cell populations was calculated by dividing the number of spheres that arose by the number of cells that were seeded multiplied by 100. The frequency of the sphere-forming cells in the MRK-003- or doxycycline- exposed cell samples was normalized to that of the corresponding control cell samples exposed to the vehicle (0.1% DMSO or water, correspondingly) to establish their relative sphere-forming frequency. Colony-forming assays were performed using published protocol (Kurpios *et al.*, 2009). In short, cells were plated in colony forming medium (CFM, 1:1 ratio of DMEM (high glucose):Ham's F-12 Medium [both from Gibco], supplemented with 1 μg/mL fungizone [Gibco], 1% penicillin-streptomycin [Gibco], 5% fetal bovine serum (FBS) [Gibco], 5 μg/mL insulin [Sigma], 10 ng/mL epidermal growth factor (EGF) [Invitrogen], and 10 ng/mL cholera toxin [Sigma]) at clonogenic densities (50 cells/$cm^{2)}$ calculated using the viable cell counts on 60 mm tissue culture dishes [Corning], which were coated with 5 μg/cm^2 rat tail collagen [Roche]. Colonies, defined as 10 or more aggregated cells, were quantified using a light microscope. Alternatively, cells were fixed in 4% paraformaldehyde and immunocytochemistry was performed with indicated antibodies. Cells incubated with the vehicle (0.1% DMSO) also served as the controls in the colony-forming assays that employed MRK-003. Sphere- and colony-forming assays were performed with 3 individual preparations of primary mammary epithelial cells and primary tumor cells (from 3 independent tumors), as well as with 3

independent cultures of established mammospheres, tumorspheres or human breast tumor cell lines.

RNA isolation and analyses

Total RNA was isolated (Trizol® Reagent [Invitrogen]) from 3 populations of mammospheres and tumorspheres that originated from different mice and independent tumors. Quantitative RT-PCR was performed on each RNA sample using primer pairs specific for transcripts encoding Notch pathway components and target genes arrayed in 96-well plates as recommended by the manufacturer (RT_2 Profiler ™ PCR Array [SABiosciences]). To analyze abundance of individual transcripts, reverse transcription was performed utilizing Superscript II First-Strand Synthesis System for RT PCR [Invitrogen]. The resulting cDNA was amplified with the primers complimentary to indicated transcript using a LightCycler DNA amplification kit SYBR Green I, and a Light Cycler Instrument [Roche]. Primers for the mouse or human housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were used as a reference. Results were analysed using Light Cycler Software 4.

Histology, immunofluorescence and immunohistochemical analyses

Tumors were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 microns (Kurpios *et al.*, 2009; Shepherd *et al.*, 2001). Tumor sections were stained with H&E solution. Antigen retrieval was performed using Vector Antigen Unmasking solution [Vector lab]. Slides of tumor sections or cell colonies were blocked with 3% normal goat serum (NGS) in PBST (phosphate buffer saline with 0.03% Tween-20) for 45 minutes. Primary antibodies were diluted in 3% NGS in PBST and added to the slides for 1 hour at room temperature. Slides were washed 3 times for 5 minutes with PBST and

incubated with secondary antibody conjugated to Alex fluor 488, 595 or HRP for 1 hour at room temperature. 4', 6'-diamindino-2-phenylindole (DAPI) nuclear dye was utilized in Vectashield mounting medium [Vector Laboratories] to visualize nuclei. The staining was analysed using an inverted fluorescence microscopy [Leica] and Open Lab Improvision software to capture images.

Tumorigenesis studies

To establish subcutaneous tumors, cells were suspended in a 1:1 mixture of phosphate-buffered saline containing 5% FBS and Matrigel [BD Biosciences], and injected subcutaneously between the shoulders of 6-8 week old female FVB/N or NOD/SCID mice. The occurrence of palpable tumors was monitored weekly. Mice bearing tumors (~1 cm^3, height, width and length measured using digimatic calliper [Mitutoyo] and multiplied) were administered the vehicle (0.5% methylcellulose) or 150 mg/kg of MRK-003 freshly dissolved in the vehicle (Methyl Cellulose [Sigma]) by oral gavage for 3 consecutive days followed by a 4-day rest period (Efferson *et al.*; Lewis *et al.*, 2007; Tammam *et al.*, 2009). The dosing cycle was repeated once. Tumor volumes were measured twice a week. At the end of the treatment period, 4 days after the last dose of MRK-003, mice were sacrificed, and any residual tumor tissue was removed, weighed and processed for analyses. Mice whose tumors had completely or nearly completely regressed were monitored for tumor recurrence for one year. MRK-003 was a kind gift from Merck, Inc. For experiments with tumor cells expressing Notch pathway antagonists, 5 mg/mL of doxycycline was added to drinking water of mice bearing tumors of ~1 cm^3. Tumors of control and treated mice were measured weekly.

Western blot analysis

Whole cell lysate was prepared using NP40 lysis buffer (50mM Tris HCl pH 7.4, 250 mM NaCl, 0.5% NP40 [IGEPAL], 10% Glycerol, 4mM EDTA (pH 8.0), Roche complete protease inhibitors, Roche phosphatase inhibitors). Samples were subjected to SDS gel electrophoresis using NuPAGE Novex Bis-Tris gels (4-12%, Invitrogen, 200 V for 1 hour) and transferred onto PVDF membrane (30V for 1 hour). Membranes were blocked in blocking buffer [GE Healthcare] and incubated overnight with an indicated antibody. Anti-mouse or anti-rabbit secondary antibodies conjugated to either Alexa Fluor 680 [Invitrogen-MP] or ID-Red800 [Odyssey Lycor] were used, followed by Odyssey advanced detection system.

Flow cytometry analysis

Cells isolated from tumors were washed in PBS and resuspended in Cytofix-Cytoperm buffer [BD] and incubated at 4^0C for 30 minutes. Next, cells were washed once in PermWash buffer [BD] and incubated with indicated primary antibody diluted in PermWash buffer on ice for 45 minutes. After three washes in PermWash buffer cells were incubated with secondary antibody diluted in PermWash buffer (anti-rabbit, anti-mouse or anti-rat conjugated to either Alexa Fluor 680 or Alexa Fluor 488) for 30 minutes on ice. Then cells were washed three times in PermWash buffer, resuspended in FACS buffer [BD] and filtered through 48 micron nylon mesh [SmallParts] into 5 ml polystyrene tubes before running on CANTO flowcytometer.

Cloning and establishing of stable cell lines

Several lentiviral constructs were derived using the pSLIK platform (Shin *et al.*, 2006). HCC1954, MDA-MB-361 and MCF-7 cells were infected with these constructs using multiplicity of infection ~1 and selected with 500μg/mL of G418 [Invitrogen]. Cell

populations comprising hundreds of clones were established. To construct the dominant-negative form of RBPjκ, it was fused to the repression domain of the *Drosophila* protein Engrailed using overlap extension PCR (Higuchi *et al.*, 1988) and cloned into the pLSIK vector. Dominant negative Mastermind fused to GFP and Numb were cut out of plasmids kindly provided by Kevin Wong and Pier Paolo Di Fiore respectively and cloned into the pSLIK vector.

Data analysis

Bar diagrams in the figures show the mean plus or minus the standard deviation of a minimum of 3 biological experiments; 3 technical replicates were performed within each biological experiment. The Kruskal-Wallis test was performed followed by Dunn's multiple comparison test and the unpaired t-test with Welch's correction for unequal variances were performed to analyze the data for the tumorigenesis studies. Differences were considered statistically significant if $p < 0.05$.

CHAPTER 3

Introduction

As described above, breast cancer stem cells (also called breast tumor initiating cells (BTIC)) are the origin of tumors and they should be targeted to achieve durable cure. However, these cells are rare within human breast tumors and the conditions for their purification and *in vitro* propagation are far from being well established. Therefore, many researchers choose to use mouse models to study breast cancer. Whereas the available mouse models of breast cancer do not wholly reproduce the diversity of human breast cancer subtypes, morphological analyses (Cardiff *et al.*, 2001; Rosner *et al.*, 2002), biomarker studies (Lin *et al.*, 2003) and global transcript profiling (Herschkowitz *et al.*, 2007) suggest that they provide approximate replicas of their human counterparts. For example, mammary tumors occurring in the ERBB2 model are morphologically similar to certain human breast tumor histological subtypes (Lin *et al.*, 2003; Rosner *et al.*, 2002) and share a gene expression signature characteristic of the luminal B molecular subclass of human breast tumors (Herschkowitz *et al.*, 2007). Other mouse models similarly mirror other molecular subtypes of breast cancer (Herschkowitz *et al.*, 2007).

In the study reported in the first manuscript, I used the ERBB2 model mentioned above to study BTIC. This model is called "N202 mice" and it is based on overexpression of rat ERBB2 (Her2) oncogene under control of the MMTV promoter in FVB/N mice (Guy *et al.*, 1992) resulting in formation of mammary tumors after prolonged latency period. Our lab recently found that mammary tumors arising in several transgenic mouse models, including the described ERBB2 model constitute a very high frequency (20-50%) of TICs as determined by limiting dilution cell transplantation (Kurpios *et al*., in

preparation). Notably, 25-30% of human breast cancers over express ERBB2 (Slamon *et al.*, 1989) and the resulting tumors are particularly aggressive and resistant to therapy. Taken together, these observations suggest that the N202 mice may serve as a reliable and convenient model to study human breast cancer.

Based on a procedure developed for propagation of neuronal cancer stem cells as non adherent spheres (Reynolds and Weiss, 1996), tumorigenic breast cancer cells were also propagated *in vitro* similar to normal mammary stem cells (Ponti *et al.*, 2005). This important finding allowed studying mechanisms of self -renewal, differentiation and tumorigenesis *in vitro.* The non-adherent spheres named tumorspheres were found to result from one cell, express stem/progenitor cell markers and were able to form tumors when injected into mice subcutaneously or by orthotropic transplantation. Cells derived from sphere cultures have the ability to differentiate into different lineages and they co-fractionate with functional tumor-initiating cells (Liu *et al.*, 2007). These observations made it possible to assume that sphere formation is in correlation to self-renewal of normal or cancer stem cells (Al-Hajj *et al.*, 2003; Dontu *et al.*, 2004; Reynolds and Weiss, 1996) and sphere-forming assays are widely used to assess self-renewal *in vitro.* In this manuscript, I report that orally active gamma-secretase inhibitor MRK-003 eliminated breast tumor-initiating cells *in vitro* and *in vivo* by multiple mechanisms.

Manuscript #1

Title: Gamma-Secretase Inhibitors Target Tumor-Initiating Cells in a Mouse Model of ERBB2 Breast Cancer

Running title: MRK-003 Targets Breast Tumor-Initiating Cells

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Author contributions

Maria Kondratyev – planned, analyzed and performed most of the experiments in the paper, wrote the manuscript.

Antonija Kreso – performed some of the biological replicates for figure 1 a, c and d as well as figure 2 b and d; performed the experiment for figure 2 a, helped preparing cells and harvest the tumors in figure 3a.

Robin Hallett- helped performing the experiment and analyzing the data for figure 2 c and d, helped with *in vivo* work (treated some of the mice, helped harvesting tumors)

Adele Girgis-Gabardo – helped culturing cells, helped quantifying the colonies for figure 2 b, author of the supplementary figures 2 and 3

Maria E. Barcelon – figure 1b

Christopher Ware – figure 4c

Dora Ilieva – supplementary table 1

Pradip K. Majumder – is the supervisor of Christopher Ware, our collaborator in Merck that provided us with MRK-003 and contributed his advice and discussion to preparation of this manuscript

Abstract

Human breast tumors comprise a minor subpopulation of tumor-initiating cells (TIC), commonly termed cancer stem cells. TICs are thought to sustain tumor growth and to confer resistance to current anticancer therapies. Hence, targeting TICs may be essential to achieving durable cancer cures. To identify molecular targets in breast TICs, we employed a transgenic mouse model of ERBB2 breast cancer; tumors arising in this model comprise a very high frequency of TICs, which is maintained in tumor cell populations propagated *in vitro* as non-adherent tumorspheres. The Notch pathway is dysregulated in human breast tumors and overexpression of constitutively active Notch proteins induces mammary tumors in mice. The Notch pathway has also been implicated in stem cell processes including those of mammary epithelial stem cells. Hence we investigated the potential that the Notch pathway is required for TIC activity. We found that an antagonist of Notch signaling, a gamma (γ)-secretase inhibitor (GSI) termed MRK-003, inhibited the survival of tumorsphere-derived cells *in vitro* and eliminated TIC as assessed by cell transplantation into syngeneic mice. Whereas MRK-003 also inhibited the self-renewal and/or proliferation of mammosphere-resident cells, this effect of the inhibitor was reversible thus suggesting that it did not compromise the survival of these cells. MRK-003 administration to tumor-bearing mice eliminated tumor-resident TICs and resulted in rapid and durable tumor regression. MRK-003 inhibited the proliferation of tumor cells, and induced their apoptosis and differentiation. These findings suggest that MRK-003 targets breast TICs and illustrate that eradicating these cells in breast tumors ensures long-term, recurrence-free survival.

Results

Differential expression of Notch pathway related genes

To investigate a potential role for the Notch pathway in breast TIC, we performed quantitative RT-PCR with primer pairs homologous to transcripts encoding Notch pathway components and target genes. We compared transcript levels of Notch related genes in tumorspheres established from mammary tumors that arose in the Neu (N202) transgenic strain (Guy *et al.*, 1992) and in mammospheres derived from the mammary glands of mice (FVB/N strain). The N202 model harbors the rat Neu proto-oncogene under the transcriptional control of the Mouse Mammary Tumor Virus (MMTV) promoter in the FVB/N inbred mouse strain. During tumorigenesis deletions occur in sequences encoding the juxta-membrane region of Neu resulting in the constitutive activation of its tyrosine kinase activity (Siegel *et al.*, 1994; Siegel and Muller, 1996). Table 1 illustrates that several Notch related genes were overexpressed in tumorspheres compared to mammospheres. The latter included genes encoding Notch pathway components (*notch4*, *dtx1*, *dll1*, and *tle*); target genes (*hey1*, *runx1*, *krt1* and *il17b*); as well as *lfng* and *mmp7*, which encode proteins required for Notch glycosylation and intramembrane proteolysis (Burns *et al.*, 2005; Matsuno *et al.*, 1995; Sawey and Crawford, 2008; Sawey *et al.*, 2007; Zhang *et al.*). These findings suggested that the Notch pathway might be more active in tumorspheres compared to mammospheres.

The effect of MRK-003 on sphere- and colony-formation by mammary epithelial cell and tumor cell populations

To determine whether Notch pathway activity was required for the survival or self-renewal of tumorsphere- and mammosphere-forming cells, we used MRK-003, an orally active inhibitor of γ-secretase, which is required for the proteolytic processing of Notch receptors (Cullion *et al.*, 2009; Fan *et al.*, 2006; Konishi *et al.*, 2007; Lewis *et al.*, 2007; Rao *et al.*, 2009). We performed quantitative sphere- and colony-forming assays, which can be used to approximate the frequency of mammary epithelial stem and progenitor cells in heterogeneous cell populations (Dontu *et al.*, 2003; Reynolds and Weiss, 1992). Whereas the nature of the cells that form spheres is not known, mouse mammosphere- and tumorsphere-forming cells co-fractionate with mammary epithelial stem cells (Cicalese *et al.*, 2009; Liao *et al.*, 2007) and mammary TIC (Liu *et al.*, 2007) respectively. Moreover, agents that affect the frequency of sphere-forming cells correspondingly alter the frequency of mammary epithelial stem cells and TIC suggesting that these cells initiate sphere-formation (Cicalese *et al.*, 2009; Dontu *et al.*, 2003; Dontu *et al.*, 2004; Liao *et al.*, 2007; Mani *et al.*, 2008).

We found that cells from the mouse mammary gland and primary tumors as well as those from established populations of mammospheres and tumorspheres formed spheres directly proportional to the number of cells seeded (Supplemental Figure 1). Primary mammary epithelial cells formed mammospheres with a frequency of ~0.1% of the total cell population (Supplemental Figure 1a), whereas ~1% of primary tumor cells formed tumorspheres (Supplemental Figure 1b). The frequency of sphere-forming cells in established mammospheres and tumorspheres was ~1% when assayed at the cell densities

illustrated in Supplemental Figure 1c and d. Mammosphere- and tumorsphere-derived cells similarly formed spheres at a ~1% frequency when measured by plating single cells into the wells of 96-well plates (data not shown.)

MRK-003 reduced the frequency of sphere-forming cells in a dose-dependent fashion in both primary mammary epithelial cell and tumor cell populations without any evidence of selectivity (Figure 1a). MRK-003 did not affect the size of any spheres that formed suggesting that it did not block the proliferation of cells during sphere formation. Similar results were obtained when cells from established mammospheres and tumorspheres were used in sphere-forming assays (Figure 1b).

To determine whether the effect of MRK-003 was reversible, we scaled up the sphere-forming assays to include many more freshly isolated primary cells in an attempt to recover spheres that formed in the presence of the compound. Any spheres formed in the presence of the vehicle or various concentrations of MRK-003 were dissociated, and the same number of viable dispersed cells from these samples was seeded into fresh medium lacking MRK-003 to generate secondary spheres.

Cells from both vehicle- and MRK-003-treated primary mammospheres gave rise to secondary spheres with similar frequencies approximating 1% (Figure1c). By contrast, cells dissociated from MRK-003-treated primary tumorspheres displayed a reduced sphere-forming efficiency directly proportional to the concentration of the compound that was used in the primary sphere-forming assays (Figure 1d). Hence MRK-003 irreversibly affected the activity of the tumorsphere-forming cells, but reversibly affected that of mammosphere-forming cells.

To determine whether MRK-003 altered the capacity of progenitor cells to proliferate and differentiate we performed colony-forming assays with primary mammary epithelial cell and tumor cell populations (Kurpios *et al.*, 2009; Smalley *et al.*, 1998; Stingl *et al.*, 1998). Colonies arise from a subset of single cells when they are plated at clonal densities. We found that primary mammary epithelial cells from 6-8 week-old virgin mice reproducibly yielded colonies at a frequency of between 5-10% (Supplemental Figure 2a). By contrast, the frequency of colonies seeded by primary tumor cells varied from one tumor to another from a low of 5% to a high of 20% (Figure 2b). The overall frequency and size of colonies formed by the primary mammary epithelial cells or primary tumor cells was unaffected by the presence MRK-003 (Figure 2a). These findings suggested that MRK-003 did not alter the viability or proliferation of the colony-forming cells present in either primary mammary epithelial cell or tumor cell populations under the conditions used for these assays.

We also assessed whether MRK-003 altered the distribution of the various progenitor cells that have been identified in the mouse mammary epithelium (Smalley *et al.*, 1998). Primary mouse mammary epithelial cells form 4 types of morphologically distinct colonies representing different progenitor cell populations. Type A and D colonies comprise only luminal or myoepithelial cells respectively, and hence likely arise from unipotent progenitor cells; whereas, type B and C colonies are composed of both luminal and myoepithelial cells, and thus are the progeny of bipotent progenitor cells. We have independently confirmed the cellular composition of the various colony types arising from primary mammary epithelial cells by co-staining the cells with antibodies to

α-Sma and Ck8, markers of myoepithelial and luminal lineage cells respectively (Kurpios *et al.*, 2009).

We compared the frequency of the various colony types arising from primary mammary epithelial cells in the presence of the vehicle or of MRK-003 (2 μmol/L). MRK-003 increased the frequency of D-type colonies and reduced that of C-type colonies (Figure 2b). The morphology of the colonies seeded by primary tumor cells were very similar to each other and was unaffected by MRK-003. To determine whether MRK-003 altered the cellular composition of the tumor cell colonies we co-stained them with antibodies to myoepithelial and luminal lineage markers. The majority (~85%) of the colonies seeded by the vehicle-treated primary tumor cells comprised cells that only expressed the luminal marker (Figure 2c, top-most panel labeled vehicle-treated), whereas the remaining colonies were composed of cells that expressed either the luminal or the myoepithelial cell marker (Figure 2c, bottom-most panel labeled vehicle-treated). The latter results are consistent with the observation that mammary tumors arising in this model primarily comprise cells that express luminal lineage markers (Cardiff, 2003). When MRK-003 was present during colony formation, the frequency of tumor cell colonies comprising only luminal-lineage cells declined from ~85% to ~40%, whereas that of the colonies comprising both luminal and myoepithelial cells increased from ~15% to ~60% (Figure 2c, MRK-003 treated panels, and Figure 2d). Hence, MRK-003 skewed the differentiation of bipotent mammary epithelial progenitor cells as well as that of progenitor-like tumor cells toward the myoepithelial lineage.

MRK-003 targets TIC in vitro

To determine whether MRK-003 targets TIC, we exposed freshly-prepared primary tumor cells to the vehicle or to various concentrations of MRK-003 for 4 days, dissociated any spheres that arose, injected ~10 000 viable dispersed cells from the spheres subcutaneously into 8 syngeneic immune-competent mice, and thereafter monitored tumor formation. When a tumor estimated to constitute 10% of the weight of any individual mouse arose, all the mice in the experiment were sacrificed and the volume of the tumors was determined. We chose to inject the mammary tumor cells subcutaneously because this method can be accomplished much more rapidly and is much less invasive than performing orthotopic cell transplantation. Importantly we have found that TIC frequency in tumors of the N202 model determined by orthotopic or subcutaneous tumor cell transplantation is the same (Kurpios et al., in preparation). Moreover, the morphology (Supplementary Figure 3), cellular composition (described in Figure 3 below) and transcript profiles of the primary mammary tumors and those resulting from orthotopic or subcutaneous cell transplantation were essentially identical (Supplementary Table 1). Indeed tumors that formed after subcutaneous tumor cell injection were invariably found in the fat pad of the #2 mammary gland, a stromal environment similar to that of mammary tumors in the transgenic strain or those resulting from orthotopic tumor cell transplantation (data not shown).

All 8 mice injected with the vehicle-treated tumor cells formed tumors, and whereas these varied in volume, they averaged 240 mm^3 (Figure 3a). By contrast, mice injected with MRK-003-treated tumor cells formed tumors at lower frequencies and smaller volumes directly proportional to the concentration of MRK-003 to which they

were exposed. No tumors arose from transplant of tumor cells treated with the highest doses of MRK-003 (5.0 μmol/L) used in these experiments.

We were able to recover viable cells from the tumors of mice injected with tumor cells exposed to the lowest concentration (0.125 μmol/L) of MRK-003; subcutaneous injection of ~10 000 cells from each of 4 such tumors did not yield any tumors in syngeneic mice 6 months post-transplantation. By contrast, transplant of the same number of cells isolated from tumors of the vehicle-treated tumor cells led to 1-2 gram tumors in 3 of 4 recipient mice after 4 months. Hence exposure of primary tumor cells to MRK-003 *in vitro* eliminated TIC from these cell populations as assessed by tumor cell transplantation.

H&E staining of tumor sections revealed that the morphology of the tumors formed by the MRK-003-treated tumor cells was dramatically different from that seeded by vehicle-treated tumor cells (Figure 3b). There were many localized cell-free areas or lumens that often contained material in the tumor sections arising from the MRK-003-treated tumor cells. Notably these cell-free areas appeared to be encircled by a ring of tumor cells. Hence transient incubation of tumor cells with MRK-003 *in vitro* for 4 days was manifest many months later in an altered histopathology of tumors arising from transplant of these cells.

We also examined the cellular composition of the subcutaneous tumors. Like mammary tumors occurring in N202 transgenic mice (Cardiff, 2003), those formed subcutaneously by the vehicle-treated tumor cells comprised predominantly Ck8-positive luminal-like cells (Figure 3c, sections of 2 independent tumors are shown). There were very few Ck14- or α-Sma-positive myoepithelial lineage cells in these tumors. By

contrast, the tumors formed after transplant of the MRK-003-treated tumor cells comprised many myoepithelial lineage-restricted cells, which were frequently organized into duct-like structures that stained brightly with antibodies to Ck14 or α-Sma (Figure 3d, examples of 3 independent tumors are shown). It is not clear whether these duct-like structures visualized by staining of the myoepithelial-lineage markers are the same as those identified by H&E staining of the tumor sections. Most of the cells comprising these tumors expressed both Ck8 and Ck14, or Ck8 and α-Sma.

These data suggest that exposure of tumor cells to MRK-003 for a short period *in vitro* initiated a differentiation program, whose manifestation was evident months later in tumors seeded by these cells, and which may account in part for the loss of TIC in these tumor cell populations.

MRK-003 shrinks and eliminates tumors in mice

To learn whether MRK-003 targets TIC in tumors, syngeneic mice bearing subcutaneous tumors (~1 cm^3) were administered the vehicle or MRK-003 for 3 consecutive days followed by a 4-day recovery; this dosing regimen was repeated once (Efferson *et al.*; Lewis *et al.*, 2007; Tammam *et al.*, 2009). The tumors in all the vehicle-treated mice (n=13) increased in volume (average of 225%), whereas those in the MRK-003-treated cohort either shrank (n=6) or completely regressed (n=10) during the 2-week dosing period (Figure 4a). Three mice in the MRK-003 treatment group possessed very small, but palpable tumors, and were included with the 10 mice whose tumors had completely regressed to monitor tumor recurrence. The tumors completely disappeared in the 3 mice with small palpable tumors during the 2-week period after the last dose of MRK-003 had been administered. Tumors did not recur in any of these 13 mice for up to a year after

MRK-003 administration had been discontinued suggesting that MRK-003 effectively eliminated TIC in the tumors of the vast majority of the mice.

To determine the mechanism whereby tumor regression occurred, histological analyses were performed on sections of tumors from vehicle-treated and MRK-003-treated mice, whose tumors partially regressed during the treatment period. As observed previously the morphology of the subcutaneous tumors in the vehicle-treated mice was very similar to that of mammary tumors occurring in the transgenic strain (Figure 4b, top panels) (Cardiff, 2003). By contrast, tumor sections from mice administered MRK-003 comprised many localized cell-free areas (Figure 4b), reminiscent of the morphology of tumors seeded by tumor cells that had been exposed to MRK-003 *in vitro* (Figure 3b). No histological differences were observed in sections of the mammary glands of the tumor-bearing mice treated with either the vehicle or MRK-003, indicating that the compound did not affect their gross morphology (Figure 4b, bottom panels).

The occurrence of many cell-free areas in the tumors of MRK-003-treated mice suggested that the compound induced cell death. To determine whether cell death occurred by apoptosis we stained sections of these tumors with an antibody specific to active caspase-3 (Figure 4c). We observed a dramatically increased frequency of active caspase-3 positive cells in the tumors of MRK-003-treated mice (~25%) compared to those administered the vehicle (~1%) (Figure 4c, bar diagram). TUNEL assays confirmed the latter findings (Figure 4c).

Exposure of tumor cells to MRK-003 *in vitro* promoted their differentiation toward the myoepithelial lineage. To determine whether MRK-003 had a similar effect *in vivo,* tumor sections were co-stained with antibodies to luminal (Ck-8) and myoepithelial

(α-Sma) markers. The tumors from vehicle-treated mice comprised predominantly Ck8-positive cells (Figure 4d, top panels), whereas those from MRK-003-treated mice contained areas of cells that expressed only α-Sma, or more generally both markers (Figure 4d, bottom panels; Supplementary Figure 4). We observed duct-like rings of brightly stained α-Sma-expressing cells in tumors of mice administered MRK-003 similar to those identified in tumors that arose from transplant of tumor cells that had been treated with MRK-003 *in vitro* (Figure 4d). These data suggest that MRK-003 altered the fate of the luminal-like, tumor-resident cells resulting in their differentiation towards the myoepithelial lineage.

We also stained tumor sections from vehicle- and MRK-003-treated mice with antibodies to Ki67, a marker of cell proliferation. However, we did not observe any difference in the percentage of Ki-67 positive cells between these samples. Because the tumor sections were prepared from mice that had been sacrificed 4 days after the last dose of MRK-003 had been administered, any short-term effect of the GSI on cell proliferation may have been missed. Hence we treated tumor-bearing mice with the vehicle or a single dose of MRK-003, and prepared tumor sections from these mice 2-days thereafter. The frequency of Ki-67-positive cells was dramatically reduced in the tumor sections of the MRK-003 treated mice compared to those of the vehicle-treated mice (Figure 5a and b).

To learn whether administration of MRK-003 to tumor-bearing mice affected Notch signaling, we measured the transcript levels of *Hey1*, a Notch target gene, in their tumors. Mice bearing subcutaneous tumors were administered the vehicle or MRK-003, and 2 days later tumor RNA was isolated and Hey1 RNA abundance determined by

quantitative RT-PCR normalized to that of glyceraldehyde 3-phosphate dehydrogenase. Hey1 transcripts were reduced by about 6-fold in two independent tumors of mice that were administered MRK-003 compared to tumors from the vehicle-treated mice suggesting that MRK-003 attenuated Notch signaling in tumor-resident cells (Figure 5c).

Discussion

MRK-003 reduced the frequency of tumorsphere-initiating cells in primary tumor cell populations and in established tumorspheres *in vitro* by an irreversible mechanism. MRK-003 did not affect the frequency of colony-forming cells present in primary tumor cell populations but skewed their differentiation toward the myoepithelial lineage. Exposure of primary tumor cells to MRK-003 *in vitro* dramatically reduced the frequency of TIC as assessed by cell transplantation into syngeneic mice. Any tumors that arose in mice transplanted with tumor cells exposed to relatively low concentrations of MRK-003 were devoid of TIC as assessed by tumor cell transplantation. Interestingly, unlike tumors seeded by transplant of the vehicle-treated cells, those seeded by MRK-003-treated tumor cells comprised a high fraction of cells positive for myoepithelial lineage markers (Ck14 or α-Sma) consistent with our observations of a differentiation-altering effect of MRK-003 *in vitro* during colony formation. Administration of MRK-003 to tumor bearing mice resulted in rapid tumor shrinkage and generally to complete and durable tumor remissions. MRK-003 inhibited tumor cell proliferation, and induced their apoptosis and differentiation towards the myoepithelial lineage. Taken together these observations are consistent with the hypothesis that MRK-003 targets TIC by multiple mechanisms that culminate in their death and loss of tumorigenicity. Whether any or all of the effects of MRK-003 on mouse mammary TIC are a consequence of Notch signaling inhibition

remains unresolved; effort are underway to determine whether tumor cells expressing the Notch 1 or 4 intracellular domain are refractory to the effects of MRK-003.

MRK-003 reduced the capacity of primary mammary epithelial cells or established mammosphere-resident cells to form mammospheres, but the effect of the compound was reversible in these cells. Antagonists of Notch signaling also reduce the frequency of human mammosphere-forming cells, but the reversibility of these inhibitors was not addressed in a previous study (Dontu *et al.*, 2004).

MRK-003 altered the fate of progenitor cells in mammary epithelial cell populations by skewing their differentiation towards the myoepithelial lineage. MRK-003 seemed to specifically affect bipotent progenitor cells that form type C colonies; the frequency of type C colonies was reduced and that of type D colonies, which comprise only myoepithelial cells, was increased by exposing these cells to MRK-003 during colony formation. MRK-003 similarly altered the differentiation of tumor cells, which normally express only luminal-lineage markers. Recent findings reveal that luminal progenitor cells may be the cells of origin of the Neu-induced tumors in the Neu (N202) transgenic strain (Jeselsohn *et al.*, 2010). The effect of MRK-003 on mammary epithelial progenitor cell and tumor cell differentiation is consistent with an effect on Notch signaling; numerous studies have demonstrated that this pathway commits normal human and mouse mammary epithelial progenitor cells to the luminal fate and suppresses their differentiation toward the myoepithelial lineage (Bouras *et al.*, 2008; Buono *et al.*, 2006; Raouf *et al.*, 2008; Yalcin-Ozuysal *et al.*, 2010).

MRK-003 caused rapid tumor regression in the vast majority of mice treated with the compound. Using the dosing regimen developed previously (Efferson *et al.*; Lewis *et*

al., 2007; Tammam *et al.*, 2009), we observed no weight loss or other obvious manifestations of the compound during the 2-week treatment period. The effect of MRK-003 on tumor regression was durable; none of the mice whose tumors completely regressed experienced recurrences during a 1-year follow-up period. We also did not observe any overt morphological changes in the mammary glands of mice that had been administered MRK-003. Mice whose tumor completely regressed remained healthy for a year following MRK-003 administration before the experiment was terminated, suggesting that the activity of any essential adult stem cells was not compromised during the treatment period.

Conventional breast cancer chemotherapies initially inhibit tumor growth but patients frequently relapse years after treatment. One explanation for these findings is that breast tumors comprise a population of chemo-resistant TIC. Indeed recent studies of patient tumors before and after therapy support the contention that breast TIC are chemo-resistant and underscore the need to develop therapies that target these cells (Chang *et al.*, 2005; Li *et al.*, 2008b) (Creighton *et al.*, 2009). Our findings illustrate that MRK-003 targets breast TIC as well as non-tumorigenic tumor cells without any deleterious consequences for adult stem cells and thus provides proof-of-principle that eliminating these cells can afford long-lasting cancer cures.

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Table and Figure Legends

Table 1 Notch pathway-related transcripts are overexpressed in tumorspheres compared to mammospheres. The values shown are the average fold changes between transcript levels in 3 independent RNA preparations of mammospheres and tumorspheres normalized to that of glyceraldehyde 3-phosphate dehydrogenase (p-value < 0.05).

Figure 1 MRK-003 irreversibly reduces sphere formation. P-values were calculated using one-way ANOVA and are indicated with asterisks where applicable. (**a**) MRK-003 reduced the frequency of sphere-forming cells to the same extent in primary mammary epithelial cell ($p = 1.03x10^{-5}$) or tumor cell populations ($p = 9.235x10^{-9}$). (**b**) MRK-003 reduced the frequency of sphere-forming cells in established mammospheres ($p=1.2x10^{-9}$) and tumorspheres ($p=2.4x10^{-8}$). No spheres arose from cells treated with 10 μmol/L MRK-003 regardless of their source. (**c**) MRK-003 reversibly affected sphere-formation by mammosphere-derived cells. The numbers above the bars identify the concentration of MRK-003 (μmol/L) used in the primary sphere-forming assay. (**d**) MRK-003 irreversibly affected sphere-formation by tumorsphere-derived cells ($p=1.13x10^{-7}$).

Figure 2 MRK-003 induces the differentiation of progenitor cells along the myoepithelial lineage. **(a**) MRK-003 did not affect the frequency of colony-forming cells in primary mammary epithelial cell or primary tumor cell populations. (**b**) MRK-003 altered the differentiation of distinct progenitor cells in primary mammary epithelial cell populations. The effect of MRK-003 treatment was dependent on colony type (two-way ANOVA Interaction: treatment x colony type p = 0.02985) (as indicated by the asterisk). (**c**) MRK-003 affected the cellular composition of colonies formed by primary tumor cells. The colonies were co-stained with antibodies to Ck8 and α-Sma. The top-most

panels illustrate the cellular composition of colonies from the vehicle-treated tumor cells, whereas the panels below illustrate the appearance of colonies arising from the MRK-003-treated tumor cells. The top panels in each set of panels illustrate the major colony type from each cell source. (**d**) Quantification of the nature of colonies from the vehicle- and MRK-003-treated tumor cells. Colonies comprising both luminal and myoepithelial cells are designated mixed. The effect of MRK-003 treatment was dependent on colony type (two-way ANOVA; interaction: treatment x colony type $p = 4.41x10^{-6}$) (as indicated by the asterisk).

Figure 3 MRK-003 targets tumor-initiating cells *in vitro*. **(a)** The volume of tumors resulting from transplant of tumor cells that had been incubated *in vitro* with the solvent (0.1% DMSO) or various concentrations of MRK-003. Each dot represents the volume of a tumor borne by an individual mouse; the horizontal bar designates the average tumor volume for each treatment group. The numbers on top of each treatment group indicate the number of mice that developed tumors (numerator) among 8 cohorts (denominator) transplanted with tumor cells. (**b**) H&E stained tumor sections. The top panels illustrate the appearance of sections from 2 independent tumors seeded by tumor cells exposed to the vehicle, whereas the bottom panels illustrate the histology of 2 independent tumors resulting from transplant of MRK-003-treated tumor cells. Scale bar (inset) represents 40 μm in all panels unless otherwise indicated. (**c**) Cellular composition of two tumors resulting from transplant of vehicle-exposed tumor cells. Sections of 2 independent tumors were co-stained with antibodies to Ck8 and Ck14, or Ck8 and α-Sma. (**d**) Cellular composition of tumors resulting from transplant of MRK-003-exposed tumor cells. Sections were co-stained with antibodies to Ck8 and Ck14, or Ck8 and α-Sma. The red

arrow illustrates the ring-like structures found in tumors resulting from transplant of the MRK-003-exposed tumor cells.

Figure 4 MRK-003 effects tumor regression by inducing tumor cell apoptosis and differentiation. (**a**) Final tumor volumes as a percentage of their initial volume in vehicle- and MRK-003-treated mice. (**b**) H&E staining of tumor and mammary gland sections from the vehicle- and MRK-003-treated mice. (**c**) Apoptotic cells in tumor sections from vehicle- and MRK-003-treated mice. P-value was calculated using the Student's t-test and is indicated by asterisk; P=0.0205. (**d**) Tumor sections from vehicle- and MRK-003-treated mice co-stained with antibodies against Ck8 and α-Sma.

Figure 5 MRK-003 inhibits proliferation of tumor-resident cells. (**a**) Sections of tumors from vehicle- and MRK-003 treated mice were stained with an anti-Ki67 antibody. (**b**) Bars represent the fraction of Ki-67 positive cells in tumors from the vehicle- and MRK-003-treated mice. The effect of MRK-003 on the fraction of Ki67-positive tumor cells was determined in tumor sections prepared from the tumors of a vehicle-treated mouse and two MRK-003-treated mice. The tumor-bearing mice were administered a single dose of MRK-003 and sacrificed 48 hours thereafter. (**c**) MRK-003 inhibits expression of the Notch target gene Hey-1 in tumor-resident cells.

Supplementary Table and Figure Legends

Supplementary Table 1 Gene expression profiles were obtained from primary tumors (N202) arising in the transgenic mice, and from tumors resulting from transplant of tumor cells from N202 tumors to orthotopic (Ortho) sites (#4 mammary fat pad) or a subcutaneous (SubQ) sites. Pair-wise Pearson correlation coefficients were calculated between each pair of samples. Primary N202 tumor samples that were used to seed

tumors at a different site are highlighted by the same color. N202 tumors transplanted to sites in 2 different recipient mice are designated with a number and letter (a or b). Gene expression profiles were obtained using Affymetrix GeneChips MOE430 2.0.

Supplementary Figure 1 The frequency of spheres formed after seeding cells from several sources into wells is directly proportional to the number of dispersed cells that were plated. (**a**) Varying numbers of primary mammary epithelial cells (500; 1 000; 2 500; 5 000; 50 000; and 100 000) were seeded into wells and the number of spheres formed a week thereafter determined. (**b**) Varying numbers (100; 500; 1 000; 5 000; and 10 000) of primary tumor cells were seeded into wells and the number of spheres formed a week later determined. (**c**) Varying numbers (100; 250; 500; 1 000; 2 000; and 6 000) of cells dissociated from mammospheres were plated into wells, and the number of spheres formed a week thereafter determined. (**d**) Varying numbers (500; 1 000; 2 500; 5 000; and 10 000) of cells dissociated from tumorspheres were seeded into wells and the number of spheres formed a week thereafter determined. The sphere-forming efficiency (SFE) of each cell population is shown as an inset in each panel. The error bars represent the standard deviation of the mean of triplicate samples.

Supplementary Figure 2 Colony formation by dispersed primary mammary epithelial cells or primary tumor cells is directly proportional the number of cells plated. (**a**) Varying numbers (100; 500; 1 000; and 5 000) of primary mammary epithelial cells were seeded into wells and the number of colonies that arose thereafter determined. (**b**) Varying numbers (100; 500; 1 000; and 2 500) of primary tumor cells were seeded into wells and the number of colonies that arose thereafter determined. The error bars

represent the standard deviation of the mean of triplicate samples. The colony forming efficiency (CFE) of the cell populations is shown as an inset in each panel.

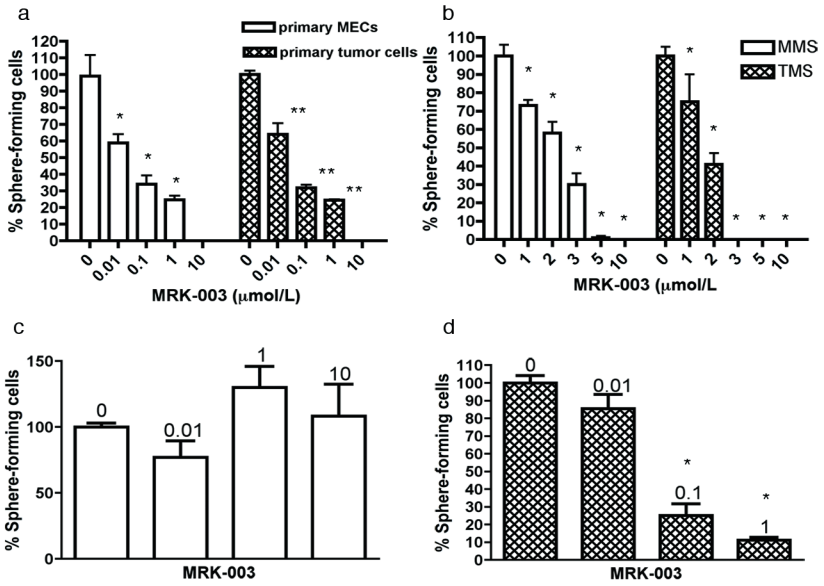
Supplementary Figure 3 H&E-stained section of a primary mouse mammary tumor and that of a tumor formed after subcutaneous injection of mouse mammary tumor cells. (**a**) Section of a primary tumor that arose in the #4 fat pad of a transgenic mouse. (**b**) Section of a tumor resulting from subcutaneous transplant of mammary tumor cells. Scale bar (inset) represents 40 μm in both panels.

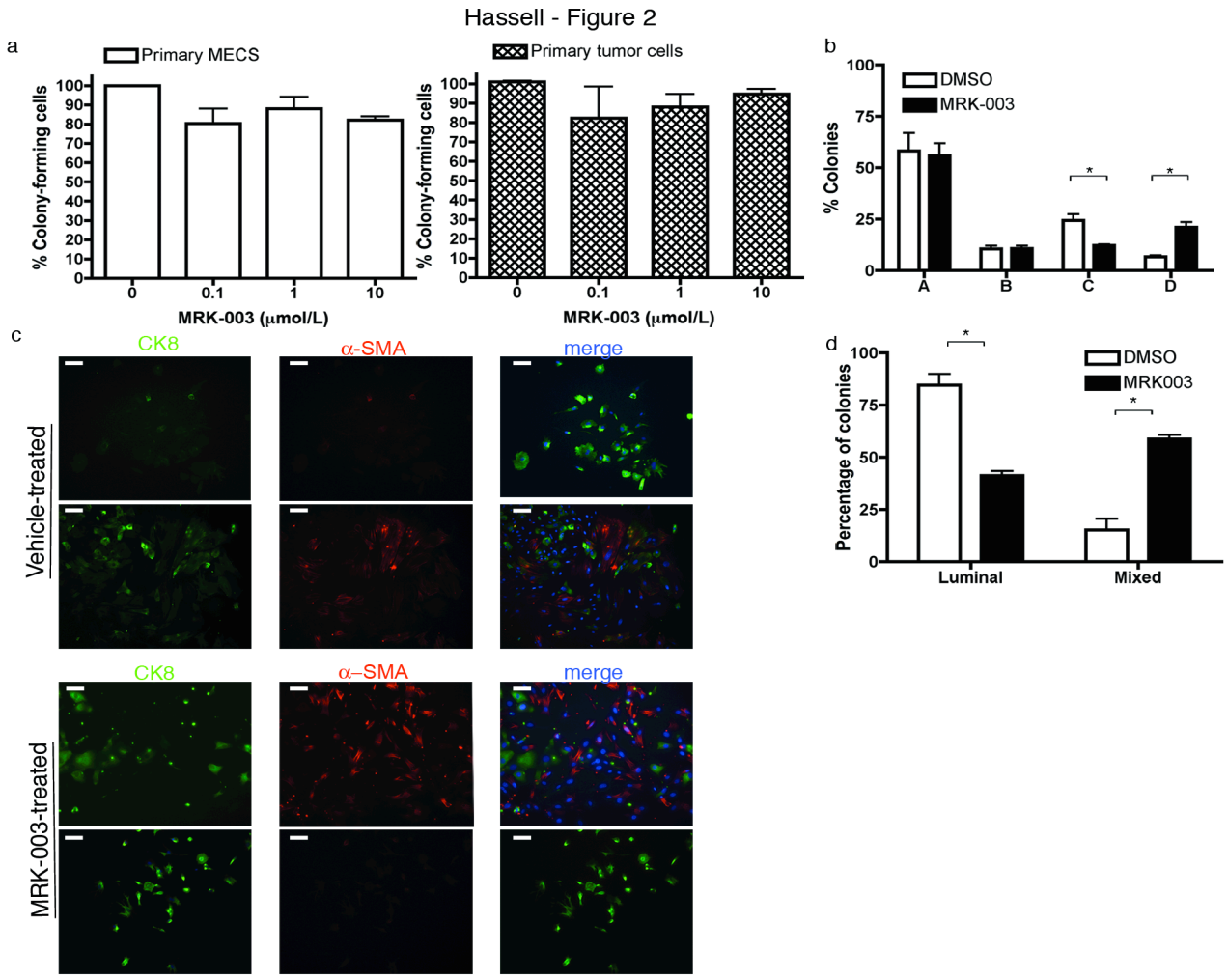
Supplementary Figure 4 Tumor sections from MRK-003-treated tumor bearing mice co-stained with antibodies against Ck8 and α-Sma. Note that most of the cells co-express both mammary epithelial lineage markers suggesting that MRK-003 skewed the differentiation of the tumor luminal progenitor-like cells towards the myoepithelial lineage. Scale bar (inset) represents 40 μm in all panels.

Table 1. Notch pathway-related transcripts are more highly expressed in tumorspheres compared to mammospheres.

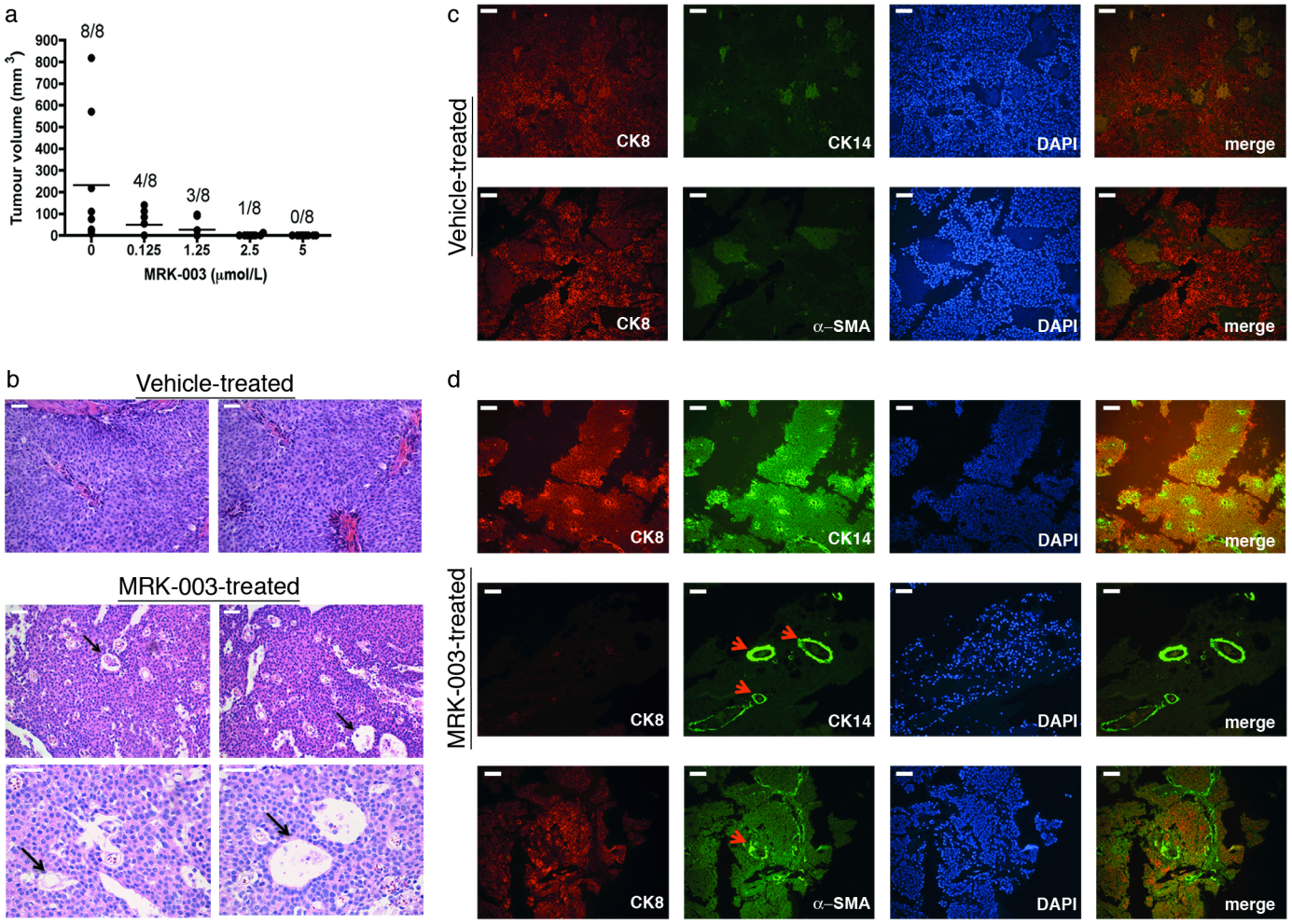
Gene	***Fold Change***
Ill7b	82.4
Dtx1	40.2
Hey1	14.1
Lfng	11.1
Krt1	6.8
Dll1	6.7
Mmp7	5.1
Notch 4	2.9
Runx1	2.5
Tle	2.1

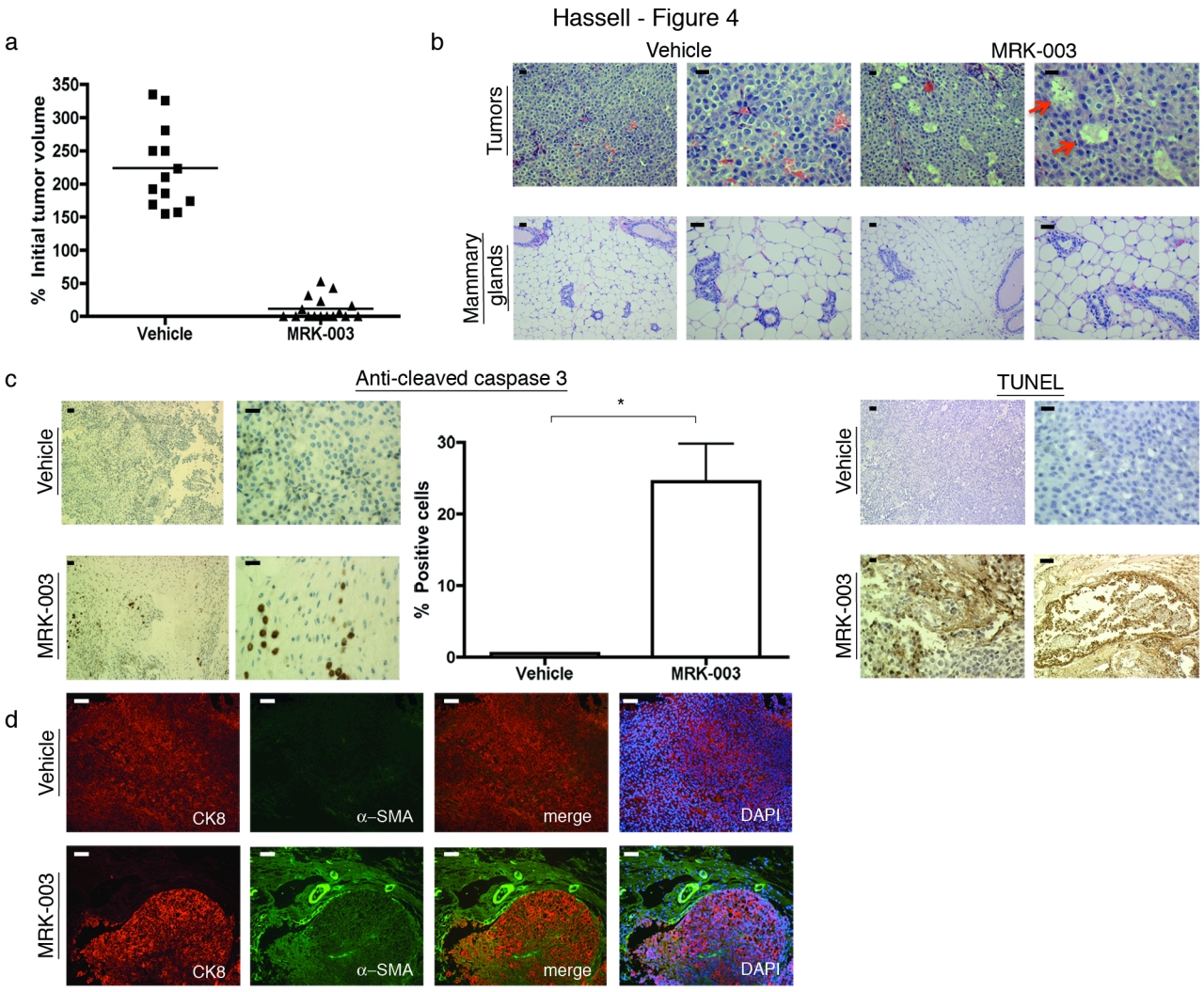
Hassell - Figure 1



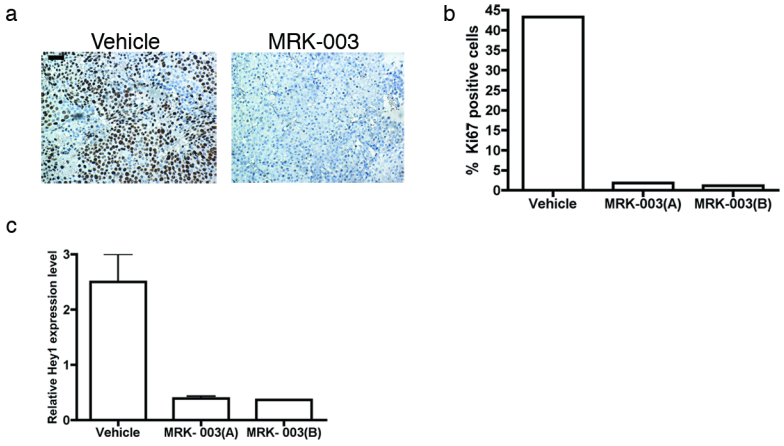

Hassell - Figure 2
a
Primary MECS
% Colony-forming cells
MRK-003 (μmol/L)
Primary tumor cells
% Colony-forming cells
MRK-003 (μmol/L)
b
DMSO
MRK-003
% Colonies
A
B
C
D
c
CK8
α-SMA
merge
Vehicle-treated
CK8
α–SMA
merge
MRK-003-treated
d
DMSO
MRK003
Percentage of colonies
Luminal
Mixed

Hassell - Figure 3




Hassell - Figure 4
a
% Initial tumor volume
350
300
250
200
150
100
50
0
Vehicle
MRK-003
b
Vehicle
MRK-003
Tumors
Mammary glands
c
Anti-cleaved caspase 3
Vehicle
MRK-003
% Positive cells
30
20
10
0
*
Vehicle
MRK-003
TUNEL
Vehicle
MRK-003
d
Vehicle
MRK-003
CK8
α–SMA
merge
DAPI
CK8
α–SMA
merge
DAPI

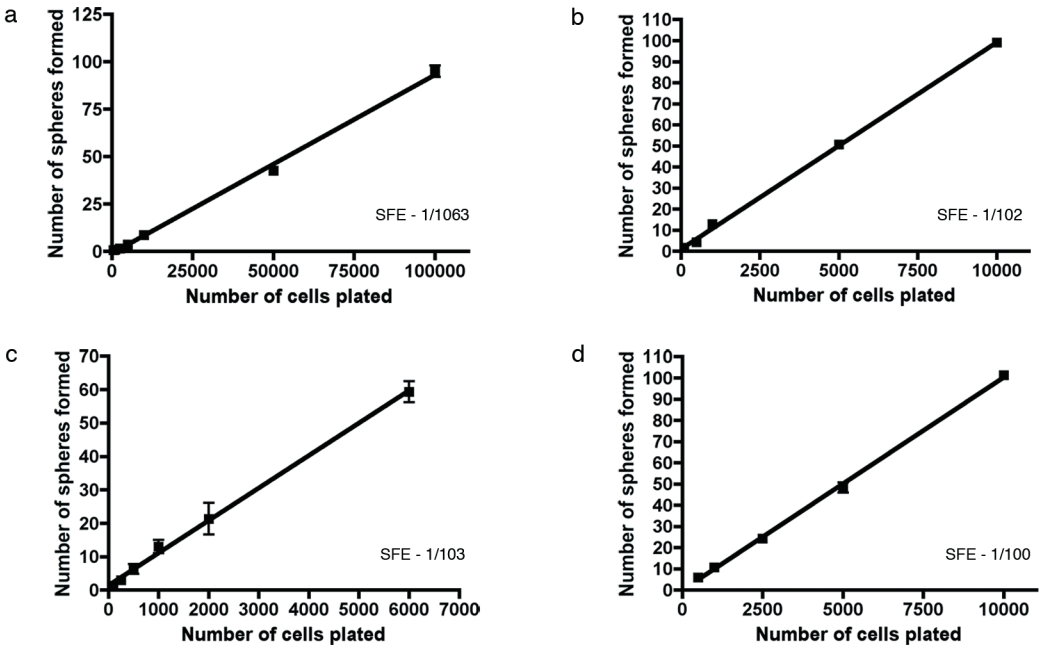
Hassell - Figure 5



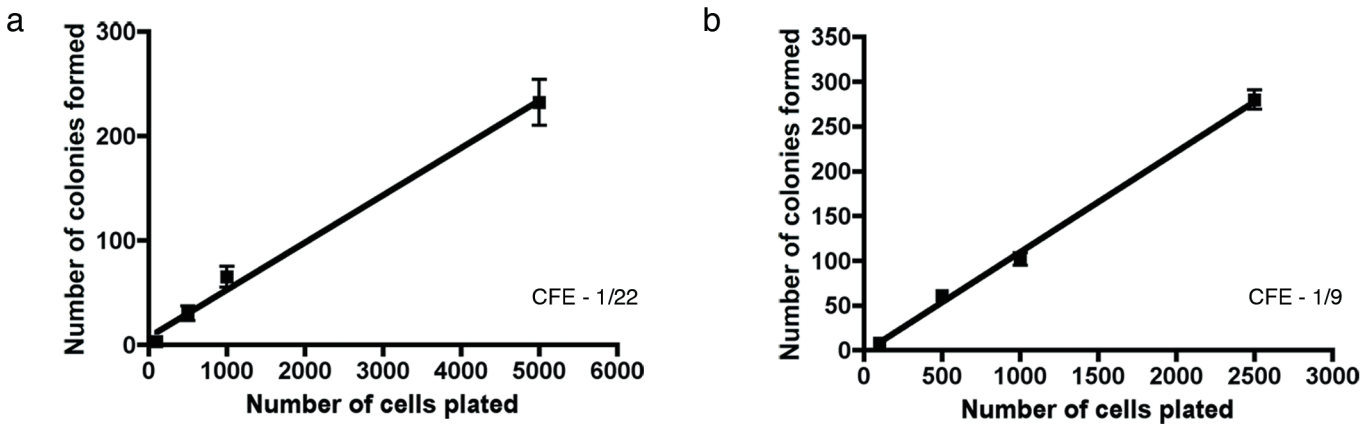
Supplementary Table 1. Pearson correlation coefficients for primary tumors and corresponding orthotopic or subcutaneous tumors.

Array Name	N202-1	Ortho-1a	Ortho-1b	N202-2	Ortho-2a	Ortho-2b	N202-3	Ortho-3	N202-4	SubQ-4a	SubQ-4b	N202-5	SubQ-5a	SubQ-5b	N202-6	SubQ-6a	SubQ-6b
N202-1	1																
Ortho-1a	0.99	1															
Ortho-1b	0.94	0.93	1														
N202-2	0.98	0.98	0.94	1													
Ortho-2a	0.97	0.97	0.95	0.98	1												
Ortho-2b	0.99	0.99	0.94	0.98	0.97	1											
N202-3	0.98	0.98	0.95	0.98	0.97	0.98	1										
Ortho-3	0.97	0.97	0.94	0.97	0.98	0.97	0.96	1									
N202-4	0.99	0.99	0.95	0.99	0.98	0.99	0.99	0.97	1								
SubQ-4a	0.98	0.98	0.96	0.98	0.98	0.98	0.98	0.98	0.99	1							
SubQ-4b	0.98	0.98	0.96	0.98	0.99	0.99	0.98	0.98	0.98	0.99	1						
N202-5	0.98	0.99	0.94	0.99	0.98	0.98	0.98	0.98	0.99	0.97	0.99	1					
SubQ-5a	0.98	0.98	0.97	0.98	0.98	0.99	0.99	0.97	0.99	0.98	0.99	0.97	1				
SubQ-5b	0.98	0.98	0.96	0.98	0.98	0.98	0.98	0.98	0.99	0.97	0.99	0.98	0.98	1			
N202-6	0.97	0.98	0.96	0.98	0.98	0.98	0.97	0.98	0.98	0.98	0.99	0.98	0.98	0.98	1		
SubQ-6a	0.98	0.99	0.95	0.98	0.98	0.99	0.98	0.98	0.99	0.98	0.99	0.99	0.99	0.99	0.99	1	
SubQ-6b	0.99	0.99	0.96	0.99	0.99	0.99	0.99	0.98	1.00	0.99	1.00	0.99	0.99	0.99	0.99	0.99	1

Hassell - Supplementary Figure 1

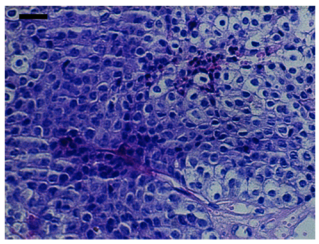


Hassell - Supplementary Figure 2

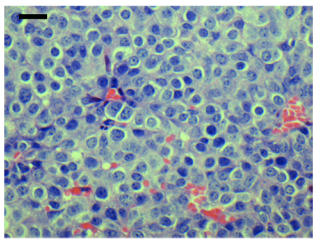


Hassell - Supplementary Figure 3

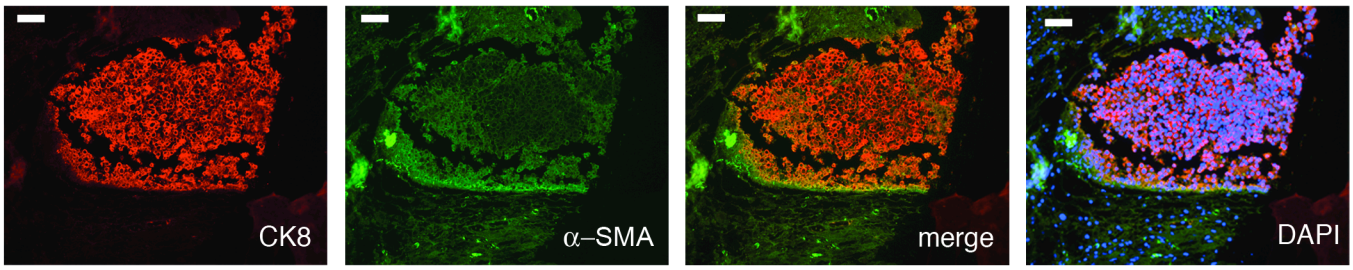
a



b



Hassell - Supplementary Figure 4



CHAPTER 4

Introduction

Human breast cancer is a heterogeneous disease; several molecular subtypes of tumors have been identified and characterized according to their histological profiles, marker expression and gene expression signature (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Identification of molecular subtypes of breast cancer has clinical relevance because the various subtypes differ significantly in their prognosis and responsiveness to treatments. To overcome low accessibility and small sample sizes of primary tumor tissue, human breast tumor cell lines are commonly used to study molecular mechanisms of the disease. Numerous lines were developed from different sources such as primary tumors, recurrences and pleural effusions (Lacroix and Leclercq, 2004; Neve *et al.*, 2006). Interestingly, human breast cancer cell lines have been shown to retain molecular characteristics of primary tumors and, just as with primary human tumor samples, can be classified into molecular subtypes according to their gene expression profiles (Lacroix and Leclercq, 2004; Neve *et al.*, 2006). Moreover, breast cancer cell lines are often organized as cellular hierarchies, comprising rare cells with stem cell properties as well as more differentiated progeny (Charafe-Jauffret *et al.*, 2009; Fillmore and Kuperwasser, 2008) and form tumors in immuno-compromised NOD/SCID mice that recapitulate histology and progression of human breast tumors (Kuperwasser *et al.*, 2005; Sheridan *et al.*, 2006). Culturing human breast cancer cell lines in chemically defined media previously developed for *in vitro* propagation of mammary epithelial stem and progenitor cells enriches for cells with tumor-initiating capacity (Dontu *et al.*, 2003). All these

observations make these cell lines a convenient and reliable model to study human breast cancer.

I report here that the gamma-secretase inhibitor, MRK-003, reduced the tumorigenic potential of human tumor cell lines representative of various molecular subtypes. Notch signaling is hyper activated by multiple mechanisms in human breast tumors, therefore, use of Notch signaling inhibitors may provide a novel therapy for breast cancer.

Manuscript #2

Title: Gamma-Secretase Inhibitors Target Tumor-Initiating Cells in Xenograft Models of Human Breast Cancer

Running title: MRK-003 Targets Breast Tumor-Initiating Cells

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Author contributions

Maria Kondratyev – planned, analyzed and performed most of the experiments in the manuscript, wrote the manuscript.

Adele Girgis-Gabardo – helped culturing cells and preparing them for injections, performed sphere-forming assays with human mammospheres, BT549 and MDA-MB-231 (figure 1A), author of figure 3C, helped with immunostainings in all figures.

Robin Hallett - helped with *in vivo* work (treated some of the mice, helped harvesting tumors)

Bonnie Bojovic – prepared the N1ICD construct and verified its expression in the cell lines (figure 2A)

Pradip K. Majumder – is our collaborator in Merck that provided us with MRK-003 and contributed his advice and discussion to preparation of this manuscript

Abstract

Notch signaling is deregulated in a high fraction of breast tumors and consequently agents targeting this pathway such as gamma-secretase inhibitors (GSIs) are being explored as anticancer drugs. The efficacy of anticancer therapies may hinge on their capacity to eradicate cancer stem cells also termed tumor-initiating cells (TICs). To learn whether Notch pathway antagonists affected breast TIC (BTIC) survival we investigated the capacity of a GSI, termed MRK-003, to affect their activity. *In vitro* studies revealed that MRK-003 inhibited sphere formation, a property of stem/progenitor cells, by multiple human breast tumor cell lines. MRK-003 also reduced the frequency of sphere-forming cells resident in human mammospheres and in an immortalized non-tumorigenic human mammary epithelial cell line. The effect of MRK-003 on sphere formation was irreversible in the breast tumor cell lines but reversible in the non-tumorigenic cell line suggesting that MRK-003 was cytotoxic for the former cell lines but cytostatic for the latter. The expression of the Notch-1 intracellular domain in the breast tumor cell lines ameliorated the inhibitory activity of MRK-003, demonstrating that MRK-003 reduced Notch signaling in these cell lines. MRK-003 inhibited the growth of xenografts seeded by multiple human breast tumor cell lines. Using a sensitive transplantation protocol we found that tumor cells isolated from xenografts of mice administered MRK-003 did not yield secondary xenografts even when a million cells were transplanted. By contrast, as few as 10 000 tumor cells from xenografts of mice administered the vehicle yielded secondary xenografts in all recipient mice. MRK-003 inhibited xenograft-resident tumor cell proliferation, increased their apoptosis and altered their differentiation suggesting that these processes eliminated BTICs. Notch signaling is deregulated in many cancers

including both hematological and solid tumors. Hence Notch pathway inhibitors may provide a novel therapy for breast cancer as well as other malignancies.

Results

MRK-003 irreversibly inhibits BTIC survival and self-renewal in vitro *by blocking Notch signaling*

To determine whether the Notch pathway is required for the survival and self-renewal of BTIC we assessed the capacity of MRK-003 to affect sphere-formation, an *in vitro* surrogate assay for stem cell self-renewal and/or progenitor cell proliferation (Pastrana *et al.*, 2011).To this end we first established spheres, termed tumorspheres (Youn *et al.*, 2006), from a number of human breast tumor cell lines representative of the various molecular subtypes of breast cancer. Such established tumorspheres comprise a stable frequency of sphere-initiating cells and a higher frequency of functional BTICs compared to the same cells propagated in serum-containing medium (Charafe-Jauffret *et al.*, 2009; Fillmore and Kuperwasser, 2008). We also established human mammospheres from the epithelial cells of breast reduction surgeries (Dontu *et al.*, 2003) and from a non-tumorigenic immortalized human mammary epithelial cell line, HMLE (Elenbaas *et al.*, 2001). Whereas the nature of the cells that form spheres is not known, mammosphere- and tumorsphere-forming cells co-fractionate with mammary epithelial stem and progenitor cells (Cicalese *et al.*, 2009; Liao *et al.*, 2007) and BTICs (Liu *et al.*, 2007) respectively. Moreover, agents affecting the frequency of sphere-forming cells correspondingly alter the frequency of mammary epithelial stem cells and BTICs as defined by cell transplantation experiments suggesting that these cells posses the capacity to initiate sphere formation (Cicalese *et al.*, 2009; Dontu *et al.*, 2003; Dontu *et al.*, 2004; Liao *et al.*, 2007; Mani *et al.*, 2008) (Cicalese *et al.*, 2009; Dontu *et al.*, 2004; Liao *et al.*, 2007; Liu *et al.*, 2007; Mani *et al.*, 2008).

MRK-003 inhibited sphere formation by cells from all the breast tumorsphere cell lines that were tested independent of their molecular subtype (Figure 1A). MRK-003 also inhibited sphere-formation by mammospheres that were freshly prepared from primary human mammary epithelial tissue as well as by the HMLE cell line. The lack of any selectivity of MRK-003 for the tumorspheres or mammospheres and HMLE spheres was reminiscent of our previous observation that mouse mammospheres are as sensitive to MRK-003 inhibition of sphere formation as are tumorspheres established from the tumors of a transgenic mouse model of ERBB2-positive of breast cancer (Kondratyev *et al.*, 2011). Our previous experiments also revealed that the effect of MRK-003 was to reversibly inhibit mammosphere formation by either primary mammary epithelial cells or established mammosphere-derived cells, but to irreversibly inhibit tumorsphere formation from primary tumor cells or from established tumorsphere-derived cells. To determine whether the latter was also true of the human breast cell populations, serial sphere-forming assays were performed with 3 different human breast tumorsphere cultures (HCC1954 - basal and HER2-positive; MDA-MB-361 - luminal and HER2 positive; and MCF-7 cells - luminal and HER2 negative). Because our human mammosphere preparations established from individual patients did not comprise a sufficiently abundant cell population to perform these experiments, we used the non-tumorigenic immortalized HMLE cell line to this end.

Each cell line was cultured under sphere-forming conditions in the presence of the vehicle or various concentrations of MRK-003, and 4 days later any spheres that formed were dissociated and the same number of dispersed viable cells from each culture was placed in fresh medium lacking MRK-003 to generate secondary spheres. Cells from

each of the 3 the tumorsphere populations displayed a decreased sphere-forming capacity directly proportional to the concentration of MRK-003 used in the primary sphere-forming assays (Figure 1B). By contrast, MRK-003 treated HMLE cells formed secondary spheres with similar efficiency (1-2%) as that of the vehicle-treated cells. These data demonstrate that MRK-003 irreversibly affected sphere-formation by the human breast tumorsphere lines whereas its effect on sphere-formation by their quasi-normal counterparts (HMLE) was reversible.

Whereas MRK-003 has been shown to inhibit Notch signaling in multiple studies, the gamma-secretase complex proteolytically processes multiple protein substrates; hence it was important to learn whether the effect of MRK-003 on sphere formation in the breast tumor cell lines we studied was due to inhibition of Notch pathway activity (Lammich *et al.*, 2002; Marambaud *et al.*, 2002; Ni *et al.*, 2001; Parks and Curtis, 2007; Struhl and Adachi, 2000; Wolfe, 2006). To address the latter, the HCC1954, MDA-MB-361 and MCF-7 breast tumor cell lines were genetically manipulated using a lentivirus vector to inducibly express a constitutively active form of Notch1 (Notch1 intracellular domain –N1ICD) (Shin *et al.*, 2006). The inducible expression of the N1ICD protein in each of the 3 cell lines was confirmed by Western blot analyses (Figure 2A).

Each of the cell lines was sensitive to MRK-003 inhibition of sphere formation in the absence of doxycycline, but they were all insensitive to the effect of MRK-003 when doxycycline was added to the medium, conditions that led to the induction of N1ICD expression (Figure 2 B). These data suggest that MRK-003 reduced the sphere-forming capacity of human breast tumor cell lines by inhibiting signaling through one or more Notch receptors.

MRK-003 inhibits the growth and enhances the shrinkage of breast tumor xenografts

To learn whether MRK-003 affected tumor growth, we used 3 of the breast tumor cell lines (HCC1954, MDA-MB-361 and MCF-7) to generate tumor xenografts in immune-compromised NOD/SCID mice. When the tumor xenografts were approximately 1 cm^3 the mice were orally administered the vehicle or MRK-003 once a day for 3 successive days followed by a 4-day recovery period; this dosing regimen was repeated once. The tumor xenografts of the vehicle-treated mice increased in volume whereas those in the MRK-003 treated cohort either stopped growing or shrank during the 2-week dosing regimen (Figure 3A). To determine the mechanism by which tumor xenograft growth arrest and shrinkage occurred, the mice were sacrificed four days after the last dose of MRK-003 had been administered. The remaining xenografts were harvested and portions of these fixed in formaldehyde, embedded in paraffin and sectioned; other fragments of the tumor tissue were viably frozen for further analyses.

Histological analyses were performed on tumor xenograft sections from vehicle-treated and MRK-003-treated mice. Inspection of the latter revealed areas bereft of tumor cells and seemingly containing extracellular matrix (Figure 3B). Immune cells, likely macrophages, could be seen in these areas of tissue remodeling.

Tumor sections were also stained with an antibody to Ki67, a marker of cell proliferation. A significant decrease in the fraction of Ki67-positive tumor cells was observed in tumor xenograft sections from mice that were administered MRK-003 compared to those that were administered the vehicle (Figure 3C; Supplemental Figure 1). Whereas the fraction of Ki67 positive cells in tumor xenografts of the vehicle-treated mice varied contingent on the nature of the cell lines that initiated their growth, the

companion cell line seeded xenografts of mice that had been administered MRK-003 comprised a minimum of a 3-5 fold reduction in the fraction of Ki67-positive cells.

Because there were fewer tumor cells per unit area in the xenografts of the MRK-003-treated mice, we enquired whether MRK-003 affected tumor cell apoptosis. To this end TUNEL assays were performed. The sections prepared from the tumor-bearing mice that had been administered MRK-003 comprised a much higher fraction of apoptotic cells than those of tumors from the mice administered the vehicle (Figure 3C). Taken together these findings demonstrate that MRK-003 inhibited tumor cell proliferation and increased tumor cell apoptosis, which likely resulted in the cessation of tumor growth and tumor regression.

MRK-003 affects the distribution of luminal and myoepithelial lineage tumor cells resident in xenografts

Our analyses of the effect of MRK-003 on mouse mammary tumors arising in the ERBB2 model, which comprise a homogeneous luminal-lineage restricted cell population (Jeselsohn *et al.*, 2010), revealed that this GSI skewed the differentiation of these tumor progenitor cells toward the myoepithelial lineage (Kondratyev *et al.*, 2011). These findings are consistent with an inhibitory effect of MRK-003 on Notch signaling, which is required to commit progenitor cells to a luminal fate and to suppress their differentiation towards the myoepithelial cell fate (Buono *et al.*, 2006) (Bouras *et al.*, 2008) (Raouf *et al.*, 2008) (Yalcin-Ozuysal *et al.*, 2010). Hence we enquired whether MRK-003 similarly affected the differentiation of xenograft-resident tumor cells seeded by human breast tumor cell lines. We grossly estimated the cellular composition of xenografts from vehicle treated and MRK-003 treated mice by staining tumor xenograft

sections simultaneously with combinations of antibodies to markers of the luminal (CK8) and myoepithelial (CK14, CK5 or α-SMA) lineages. To quantify the effect of MRK-003 on the cellular composition of tumor xenografts flow cytometry was performed using antibodies to luminal and myoepithelial lineage markers.

MCF-7

The MCF-7 cell line (ER-positive and ERBB2-negative) has been molecularly classified as being luminal-like (Hollestelle *et al.*, 2010; Neve *et al.*, 2006). Consistent with this classification, MCF-7 tumor xenograft sections from the vehicle-treated mice comprised primarily luminal lineage cells as revealed by expression of CK8 in the majority of the tumor cell population (Figure 4A). The myoepithelial lineage markers CK14 and CK5 were not expressed, whereas a-SMA was expressed in some of the cells comprising the xenograft sections. Few, if any of the a-SMA positive cells seemed to co-express CK8.

By contrast, the majority of the cells in the tumor xenograft sections of MRK-003- treated mice comprised cells that expressed the CK14 and a-SMA myoepithelial lineage markers (Figure 4B). Interestingly CK8 and CK14 appeared to be co-expressed in the cells comprising these sections (Figure 4B). No CK5 positive cells were discernable in tumor xenograft section of either the vehicle- or MRK-003-treated mice (Figure 4 A and B).

To quantify the nature of the cells comprising the MCF-7 xenografts from the vehicle- and MRK-003-treated mice we performed flow cytometry with cells isolated from the xenografts and antibodies to CK14 and CK8. Figure 4C illustrates that ~70% of the cells in MCF-7 tumor xenografts from the vehicle-treated mice only expressed the CK8 luminal lineage marker, whereas the remaining ~30% of cells expressed neither the

CK14 nor CK8 markers. (Very few cells expressed only CK14 or both CK14 and CK8.)

Similar analyses of the xenograft-resident cells from MRK-003-treated mice showed a marked increase in "double-positive" CK8/CK14 co-expressing cells, which approximated 70% of the total cell population. The other 30% cell population in these xenografts did not express either marker: very few of the cells independently expressed CK8 or CK14. These findings illustrate that MRK-003 induced the expression of the CK14 myoepithelial lineage marker in the primarily luminal lineage MCF-7 tumor cells.

HCC1954

The HCC1954 cell line (ER-negative and ERBB2-positive) has been molecularly classified as being of the basal-like subtype (Neve *et al.*, 2006). Sections of HCC1954 tumor xenografts from vehicle-treated mice comprised both luminal- and myoepithelial-lineage derived cells as revealed by their staining with antibodies to luminal and myoepithelial cell markers (Figure 5A). Inspection of the xenograft sections revealed expression of the CK14 myoepithelial cell marker in a subset of the cells and that of CK5 and a-SMA in a much higher fraction of the cell population. The difference in the frequency of tumor cells expressing each of the myoepithelial lineage markers may be a consequence of their relative position in the differentiation hierarchy in these tumor xenografts. CK14 is thought to be a marker of more primitive proliferative myoepithelial progenitor cells, whereas a-SMA marks more differentiated quiescent myoepithelial cells (Kendrick *et al.*, 2008). The CK8 luminal lineage marker was also expressed in many of the cells comprising the xenografts of vehicle-treated mice, but few cells seemingly co-expressed the luminal lineage marker and any of the myoepithelial markers. The

occurrence of cells in these xenografts that express luminal- and myoepithelial-lineage markers suggests that these cells originate from bipotent progenitor cells.

We similarly examined HCC1954 tumor xenografts of mice that had been administered MRK-003. The most striking difference between the xenograft sections from the vehicle- and MRK-003 treated mice was a reduction in the fraction of tumor cells expressing the CK8 luminal-lineage marker and an increase in the frequency of tumor cells expressing the CK14 myoepithelial marker (Figure 5B). The fraction of cells expressing CK5 appeared unaltered in the xenografts from the MRK-003-treated mice. Visual inspection of the sections did not reveal any change effected by MRK-003 on the "double-positive" tumor cell population.

To quantify the effect of MRK-003 on the cellular composition of HCC1954 tumor xenografts we used flow cytometry to estimate the frequency of cells expressing each, both or neither luminal epithelial and myoepithelial lineage markers. These analyses demonstrated that approximately 40% or 50% of the xenograft-derived tumor cells from the vehicle-treated mice expressed the luminal CK18 or CK8 markers respectively, whereas ~1% expressed the CK14 lineage marker and ~16% expressed the CK5 myoepithelial lineage markers (Figure 5C). Six percent of the cells in the xenografts from vehicle-treated mice were "double positive" for both the CK14 and CK18 lineage markers, whereas 1% expressed both the CK5 and CK8 lineage markers. The remaining ~ 40% of cells did not express either marker.

By contrast, HCC1954 tumor xenografts from the MRK-003-treated mice comprised fewer "luminal-like" cells that expressed the luminal CK18 (~ 19%) and CK8 (~ 29)% but were negative for the myoepithelial markers and significantly higher fraction

of cells expressing the CK14 myoepithelial-lineage marker (20%) compared to 1% in the vehicle-treated mouse xenografts (Figure 5D). Whereas the tumor xenografts from vehicle-treated mice comprised fewer than 6% double-positive CK14/CK18 cells, those from mice administered MRK-003 were composed of roughly 30% such double-positive cells. Hence administration of MRK-003 to mice bearing HCC1954 tumor xenografts also promoted differentiation of tumor resident cells along the myoepithelial lineage.

MDA-MB-361

An effect of MRK-003 on the expression of lineage markers was also observed in xenografts resulting from transplant of MDA-MB-361 breast tumor cells (ER-positive and ERB2-positive), which have been molecularly classified as luminal-like (Hollestelle *et al.*, 2010; Neve *et al.*, 2006). In accordance with their classification, the tumor xenografts from the vehicle-treated mice comprised very few cells expressing the CK14, CK5 or a-SMA myoepithelial markers; instead the majority of cells expressed the CK8 luminal lineage marker (Figure 6A). Analyses of sections of xenografts from mice that had been administered MRK-003 revealed an apparent increase in the CK14-positive myoepithelial lineage cell population and a dramatic decrease in the CK8-positive luminal lineage cell population (Figure 6B). The fraction of CK5 and a-SMA expressing cells was not dramatically different between the xenografts of vehicle and MRK-003-treated mice.

Flow cytometric analyses of cells isolated from tumor xenografts from the vehicle- and MRK-003-treated mice confirmed the latter impressions. The fraction of CK8- or CK18-expressing cells declined from ~40% and ~60% respectively in xenografts of vehicle-treated mice (Figure 6C) to roughly 3% in those of mice that had been

administered MRK-003 (Figure 6D). Correspondingly the population of CK14-positive cells in xenografts increased from ~1% in xenografts from vehicle-treated mice to ~15% in xenografts from MRK-003-treated mice.

Taken together the data from the analyses of xenografts initiated by transplant of three different breast tumor cell lines suggested that MRK-003 alters the expression of both luminal and myoepithelial lineage markers in xenograft resident cells. Generally, the effect of MRK-003 was to decrease the frequency of cells expressing the luminal lineage marker and to increase that of cells expressing myoepithelial lineage markers.

Staining with an antibody against human mitochondria confirmed that most of the cells within xenografts are of human origin; double staining of sections with this antibody together with anti-α-SMA antibody showed that at least some α-SMA positive cells within xenografts are tumor cells that differentiated towards myoepithelial lineage rather then mouse stromal cells that invaded the tumors (Supplemental figure 2).

MRK-003 Reduces BTIC Frequency in Tumor Xenografts

To learn whether MRK-003 targeted BTICs resident in xenografts, we initially assessed the sensitivity of our transplantation assay. To this end limiting dilutions of MCF-7 tumor cells were transplanted into immune-compromised mice. Because breast tumor cells propagated in chemically defined medium comprise a higher BTICs frequency than companion cultures propagated in serum-containing medium, we compared the BTICs fraction in these two cell sources. As few as 10 MCF-7 cells cultured as spheres in chemically defined medium were sufficient to seed a tumor in 25% (1/4) of the mice injected with these cells (Table 1). All the mice injected with 100 or more MCF-7 cells from the sphere cultures initiated tumor growth in recipient mice. Assuming a single-hit

model we estimate that 1/615 MCF-7 cells from spheres is a BTIC. By contrast, 10 000 MCF-7 from the adherent cultures were required to form a tumor xenograft in 1 of 4 injected mice, suggesting that 1/30000 cells is a BTIC.

To determine whether MRK-003 had any effect on the fraction of BTICs within tumor xenografts equal numbers of viable cells isolated from the MCF-7 xenografts of mice administered either the vehicle or MRK-003 were injected into NOD/SCID mice at doses of 10^6 or 10^4 tumor cells in quadruplicate and the mice monitored weekly for the appearance of palpable tumors. All the mice (4/4) injected with 10^6 MCF-7 cells isolated from two independent xenografts of mice that had been administered the vehicle formed palpable tumors after about 7 weeks. Similarly 4/4 mice injected with 10^4 cells from tumor xenografts of these same mice formed palpable tumors, albeit after 10 weeks. Tumor xenografts did not form during a period of 20 weeks in any of the mice that had been injected with cells isolated from the xenografts of mice that had been administered MRK-003. These data illustrate the sensitivity of the transplantation assay described above and demonstrate that MRK-003 substantially reduces the frequency of BTICs resident in MCF-7 tumor xenografts.

Discussion

Taken together, the data presented here suggest that MRK-003 targets tumor-initiating cells within human breast tumor cell lines irrespective of their molecular subtype. As tested *in vitro,* this effect is irreversible for cancer cells but reversible for normal cells and is mediated specifically by inhibition of Notch 1 signaling.

The treatment of mice with MRK-003 resulted in inhibition of tumor growth and in some cases rapid and significant tumor regression; the latter effect seems to be more profound in tumors seeded by cells that belong to the luminal subtype. Remarkably, the remaining mass of tumors seeded by MCF7 cells is depleted of tumor initiating cells as assessed by secondary transplantation assay. This finding suggests that MRK-003 eradicated the subpopulation of tumor-initiating cells within the tumors. The likely mechanism for this effect of MRK-003 is inhibition of cell proliferation and induction of apoptosis as suggested by a decrease in Ki-67-staining and an increase in TUNEL positive cells in the tumors of treated mice. Interestingly, MRK-003 also induced differentiation of tumor cells towards the myoepithelial lineage as revealed by staining with antibodies against several myoepithelial and luminal markers using both immunofluoresence and flow-cytometry assays. A similar phenomenon was observed in mouse mammary tumors from N202 transgenic mice as reported previously (Kondratyev *et al.*, 2011). This effect is consistent with the observations of several other groups regarding the role of Notch signaling in differentiation of mammary epithelial cells. First, in both mouse and human mammary tissue, Notch1 is predominantly expressed in luminal cells (Bouras *et al.*, 2008; Buono *et al.*, 2006; Harrison *et al.*). Knockdown of RBPj in mouse mammary epithelial cells resulted in expansion of basal progenitors while

expression of activated form of Notch 1 in luminal progenitors resulted in hyperplasia and tumorigenesis (Bouras *et al.*, 2008). In addition, loss of RBPj and the Notch post-translational regulator Pofut1 led to abnormal expression of myoepithelial markers in the mammary tissue that acquired a basal phenotype during pregnancy (Buono *et al.*, 2006). All these data suggest that Notch 1 activity blocks differentiation of bi-potent progenitors towards myoepithelial lineage and hyper-activation of Notch may lead to extensive expansion of immature luminal cells resulting in tumorigenesis. Evidence exist that luminal cells have the capacity to adopt a basal fate under certain circumstances, such as not getting appropriate signals from the surrounding myoepithelial cells (Pechoux 1999). Therefore, alternative explanation to our findings might be that inactivation of Notch signaling in luminal cells results in their transdifferentiation towards the myoepithelial lineage. Notably, MRK-003 had its strongest effect on breast tumors belonging to luminal molecular subtype, which include N202 mammary tumors that share a gene expression signature characteristic of luminal B molecular subtype of breast cancer (Herschkowitz *et al.*, 2007). However, it also caused growth arrest of HCC1954-seeded tumors that belong to basal A molecular subtype and caused a significant decrease in the fraction of luminal cells within these tumors. Moreover, *in vitro* experiments revealed that MRK-003 inhibited sphere formation by breast tumor cell lines regardless of their molecular subtype (Figure 1). Therefore, it is plausible that inhibition of Notch signaling has anti-tumorigenic effect in both luminal and basal tumors, while this effect is less profound in basal tumors and the mechanism of this effect remains unclear. Since HCC1954-seeded tumors comprised both luminal and myoepithelial cells it is possible that the luminal subpopulation contributed to tumor growth and its elimination led to growth arrest upon

treatment with MRK-003. In addition, MRK-003 induced apoptosis in tumor cells, which might represent separate mechanism of action of this inhibitor, either related or unrelated to its ability to induce differentiation.

Taken together, our data suggest that pharmacological inhibition of Notch signaling results in loss of tumorigenic potential of breast cancer stem cells. This phenomenon was demonstrated in human breast cancer cells from various molecular subtypes and it provides a rationale for further development of anti-breast cancer therapies based on inhibition of Notch.

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Table Legend

Table 1. MRK-003 targets tumor-initiating cells in MCF-7-seeded xenografts. To evaluate the fraction of tumor initiating cells within populations of MCF-7 cells cultured adherently and as spheres, limiting dilution analysis was performed. Indicated number of cells from adherent or sphere cultures was injected subcutaneously into NOD/SCID mice in quadruplicates. Cells were isolated from MCF-7 tumor xenografts of mice that had been administered either the vehicle or MRK-003. NOD/SCID mice were injected with either 10^6 or 10^4 tumor cells in quadruplicate and the mice monitored weekly for the appearance of palpable tumors. Time till appearance of palpable tumors and number of takes for both experiments are summarized in the table. TIC frequency was calculated by ELDA (Hu and Smyth, 2009).

Figure Legends

Figure 1. MRK-003 irreversibly inhibits BTIC survival *in vitro*. P-values were calculated using one-way ANOVA and are indicated with asterisks where applicable (<0.001). (A) MRK-003 inhibits sphere formation by human breast tumor cell lines from various molecular subtypes. (B) MRK-003 reversibly affected sphere-formation by HMLE cells but irreversibly affected sphere-formation by human breast tumor cell lines.

Figure 2. MRK-003 inhibits sphere formation by human breast tumor cell lines specifically through inhibition of Notch 1 signaling. (A) Western blot analysis confirmed inducible expression of Notch1 intracellular domain in indicated cell lines. (B) The expression of the N1ICD protected the cells from the effect of the inhibitor.

Figure 3. MRK-003 inhibits the growth and enhances the shrinkage of breast tumor xenografts. (A) Tumor volumes as percentage of their initial volumes in MRK-003- and

vehicle-treated mice. (B) Sections of xenografts from MRK-003 and vehicle-treated mice were stained with antibody against Ki67 (C) TUNEL staining of sections of xenografts from MRK-003 and vehicle-treated mice.

Figure 4. MRK-003 induces differentiation of tumor cells in MCF-7-seeded xenogratfs towards the myoepithelial lineage. Sections of MCF-7-seeded xenogratfs from vehicle- (A) or MRK-003- (B) treated mice were stained with antibodies against luminal (CK8) and myoepithelial (CK14, CK5, α-SMA) markers. Flow Cytomerry analysis was performed on cells isolated from xenogratfs from vehicle- (C) and MRK-003-(D) treated mice using antibodies against luminal (CK8) and myoepithelial (CK14) markers.

Figure 5. MRK-003 induces differentiation of tumor cells in HCC1954-seeded xenogratfs towards the myoepithelial lineage. Sections of HCC-1954-seeded xenogratfs from vehicle- (A) or MRK-003- (B) treated mice were stained with antibodies against luminal (CK8) and myoepithelial (CK14, CK5, α-SMA) markers. Flow Cytometry analysis was performed on cells isolated from xenogratfs from vehicle- (C) and MRK-003- (D) treated mice using antibodies against luminal (CK8, CK18) and myoepithelial (CK14, CK5) markers.

Figure 6. MRK-003 induces differentiation of tumor cells in MDA-MB-361-seeded xenogratfs towards the myoepithelial lineage**.** Sections of MDA-MB-361-seeded xenogratfs from vehicle- (A) or MRK-003- (B) treated mice were stained with antibodies against luminal (CK8) and myoepithelial (CK14, CK5, α-SMA) markers. Flow Cytometry analysis was performed on cells isolated from xenogratfs from vehicle- (C) and MRK-003-(D) treated mice using antibodies against luminal (CK8,CK18) and myoepithelial (CK14, CK5) markers.

Supplementary Figure Legends

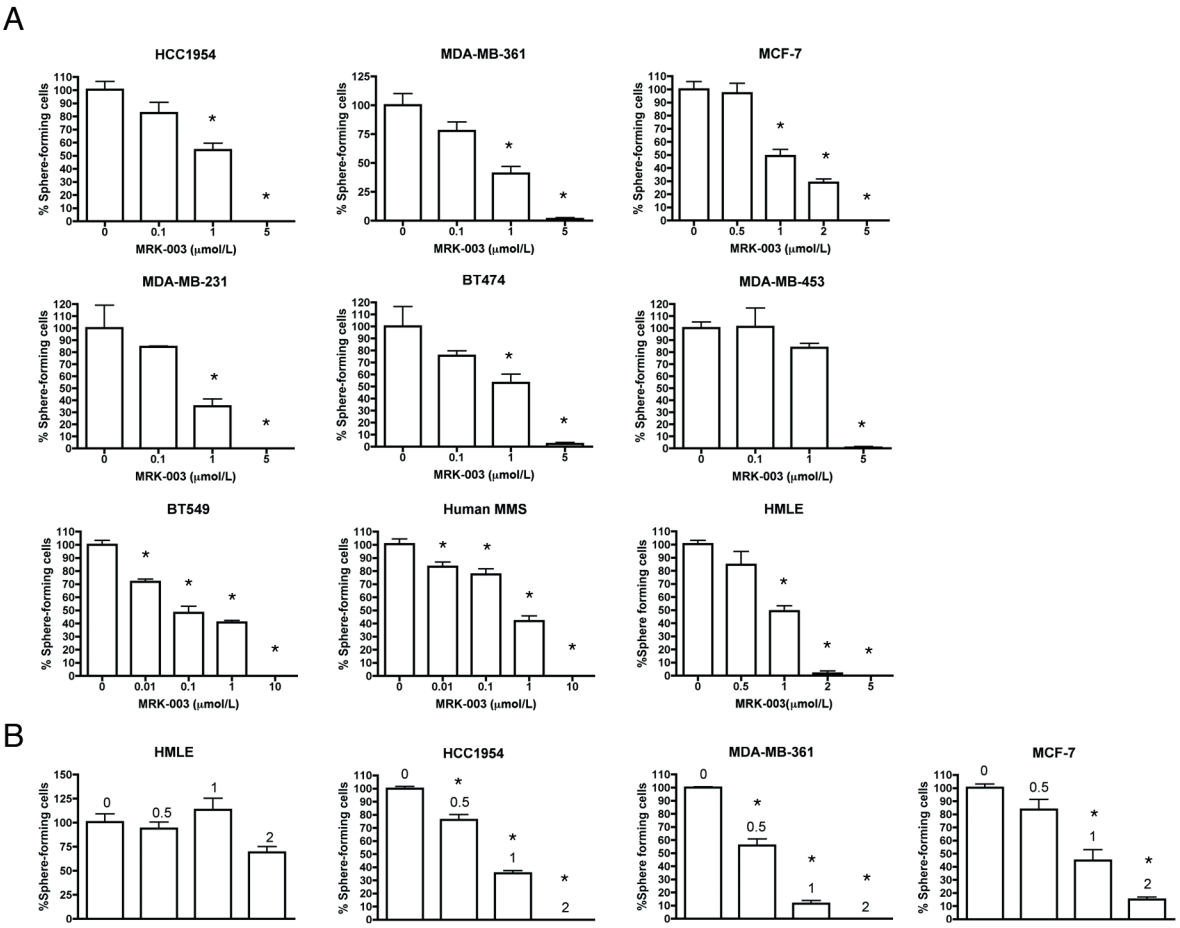
Supplementary figure 1. The fraction of Ki-67 cells in tumors from MRK-003 and vehicle-treated mice. Percentage of Ki67 positive cells within sections of xenografts from MRK-003 and vehicle-treated mice. P-values are calculated using two-way ANOVA and indicated by asterisks (<0.001).

Supplementary figure 2. The fraction of cells in tumors from MRK-0003 and vehicle-treated mice that are positive for human mitochondrial markers. Sections of (A) HCC1954-, (B) MDA-MB-361-, and (C) MCF-7-seeded xenografts from vehicle and MRK-003-treated mice were stained with antibodies against human mitochondria (green) and α-SMA (red). Flow Cytometry analysis was performed on cells isolated from HCC1954- and (E) MDA-MB-361-seeded xenogratfs from vehicle- and MRK-003-treated mice using antibodies against human mitochondria and luminal marker CK8.

Hassell - Table 1

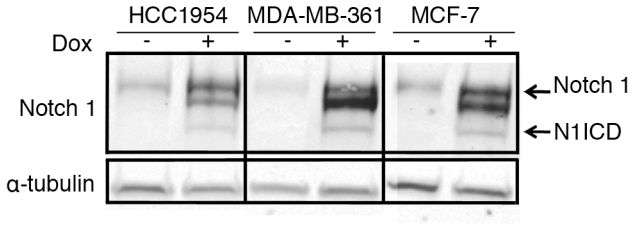
Cell type	Number of cells transplanted	Fraction of cells engrafted	Weeks until appearance of palpable tumors	Frequency (Confidence interval)
MCF-7 adherent	$1X10^5$	4/4	10	1/27,830 (1/89,552 - 1/8,649)
	$1X10^4$	1/4	12	
	$1X10^3$	0/4		
	$1X10^2$	0/4		
	10	0/4		
	1	0/4		
MCF-7 spheres	$1X10^5$	4/4	6	1/615 (1/1,822 - 1/208)
	$1X10^4$	4/4	8	
	$1X10^3$	2/4	10	
	$1X10^2$	2/4	14	
	10	1/4	31	
	1	0/4		
Cells from vehicle-treated MCF-7 xenografts:				
Tumor A	10^6	4/4	7	1 (1/15,617 - 1)
	10^4	4/4	10	
Tumor B	10^6	4/4	7	1 (1/15,617 - 1)
	10^4	4/4	10	
Cells from MRK-003 treated MCF-7 xenografts:				
Tumor A	10^6	0/4		Impossible to calculate (everything is 0)
	10^4	0/4		
Tumor B	10^6	0/4		Impossible to calculate (everything is 0)
	10^4	0/4		

Hassell - Figure 1



Hassell - Figure 2

A



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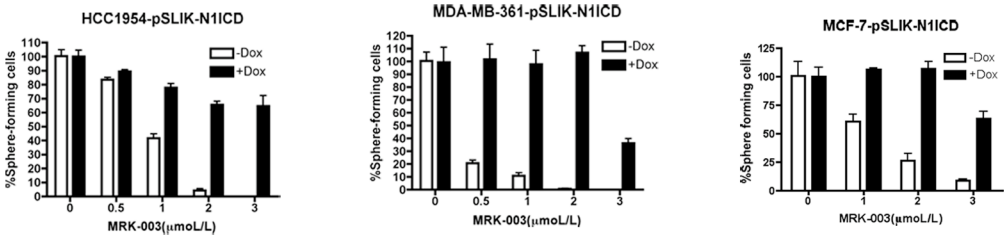

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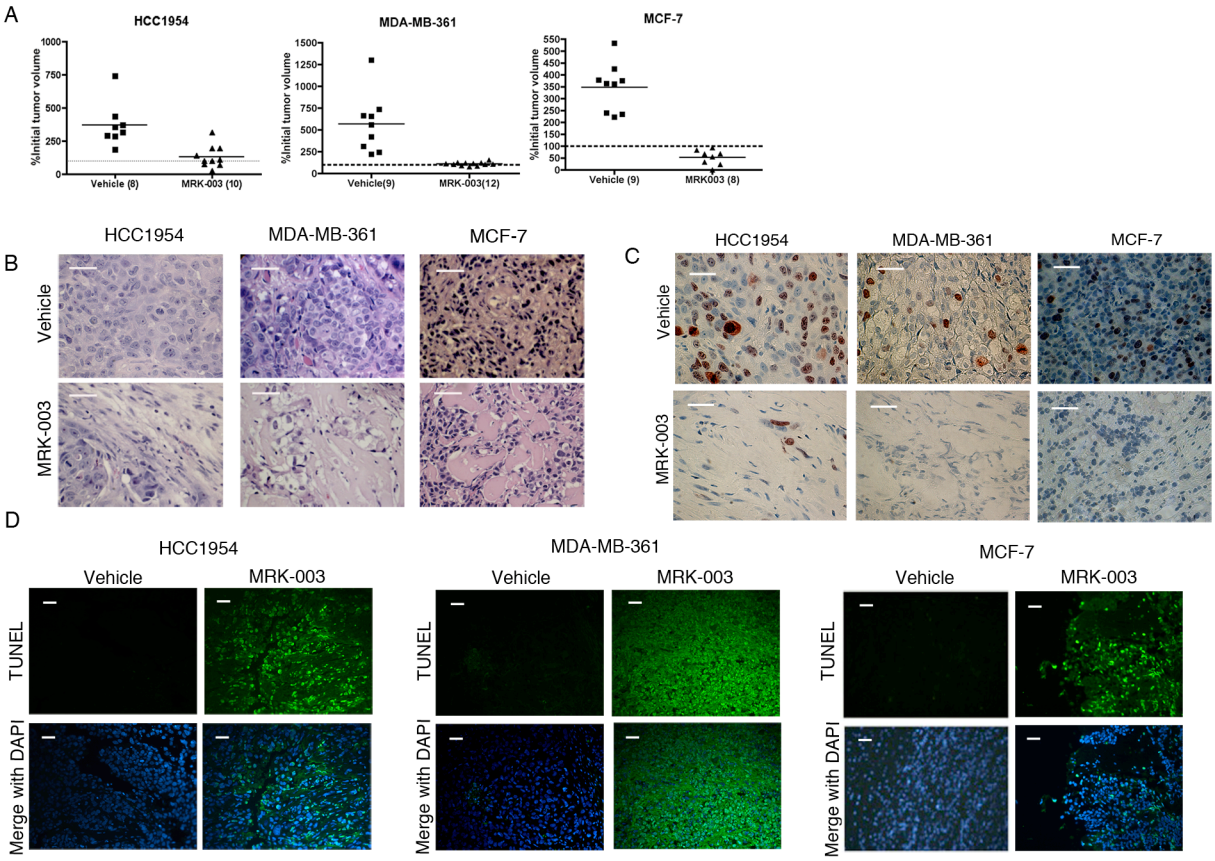

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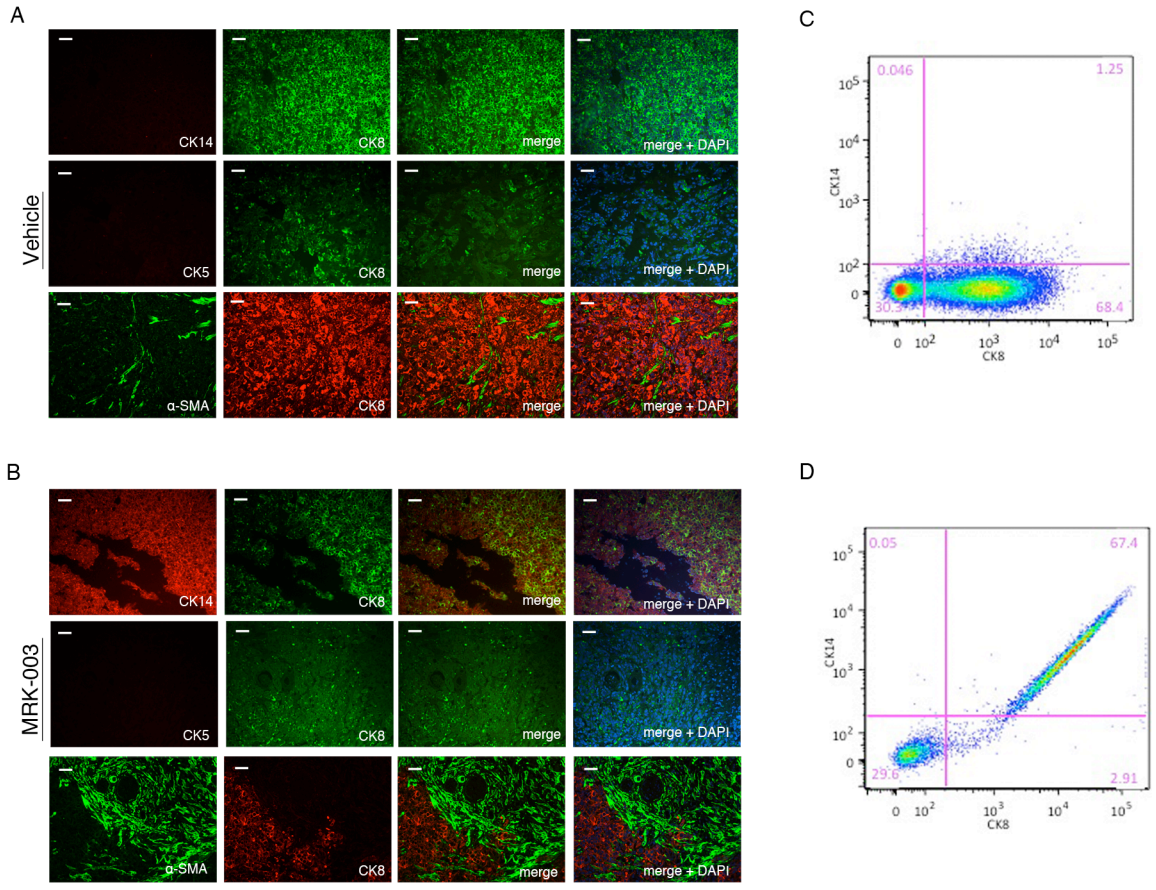
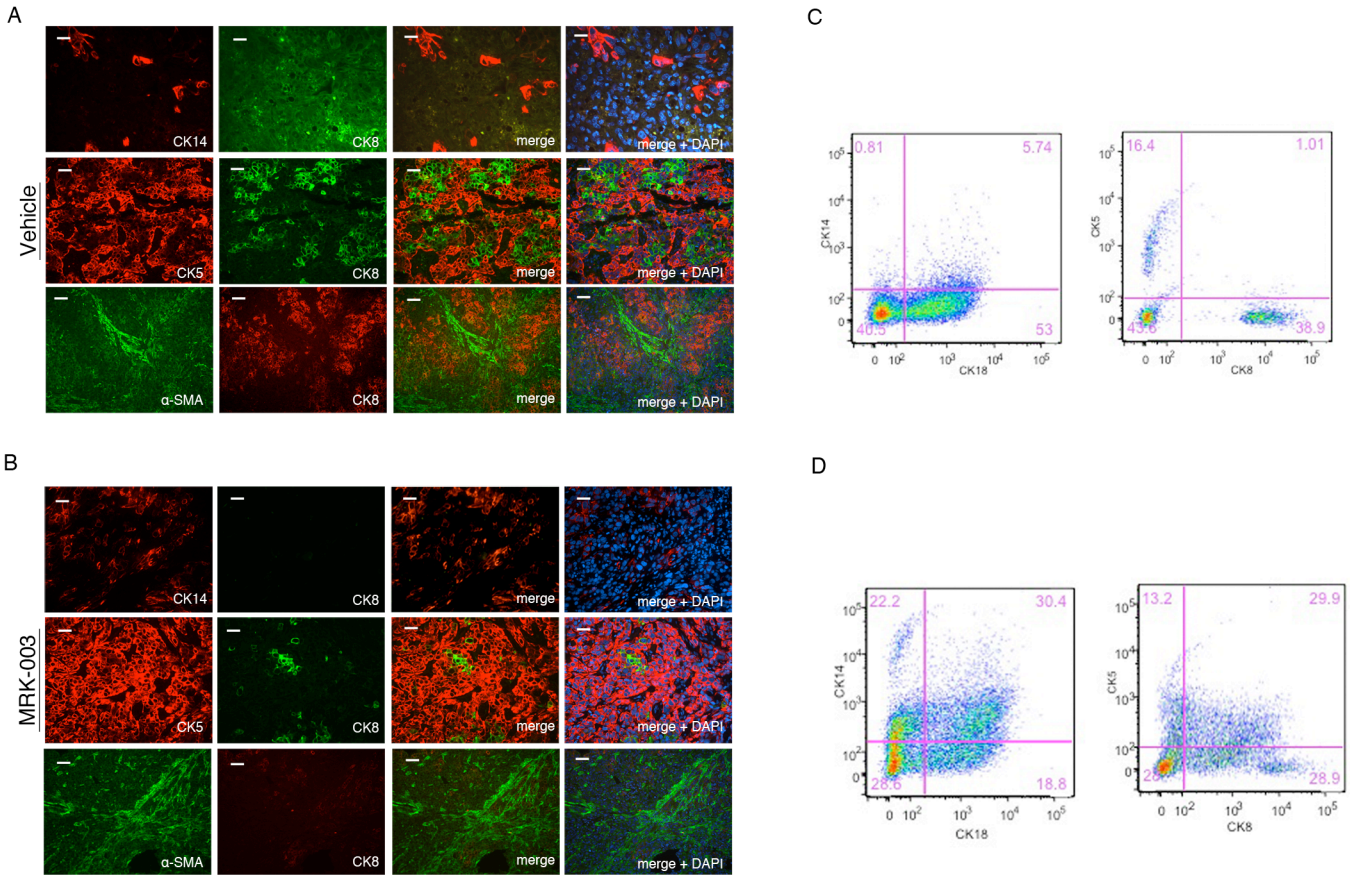


Hassell - Figure 5



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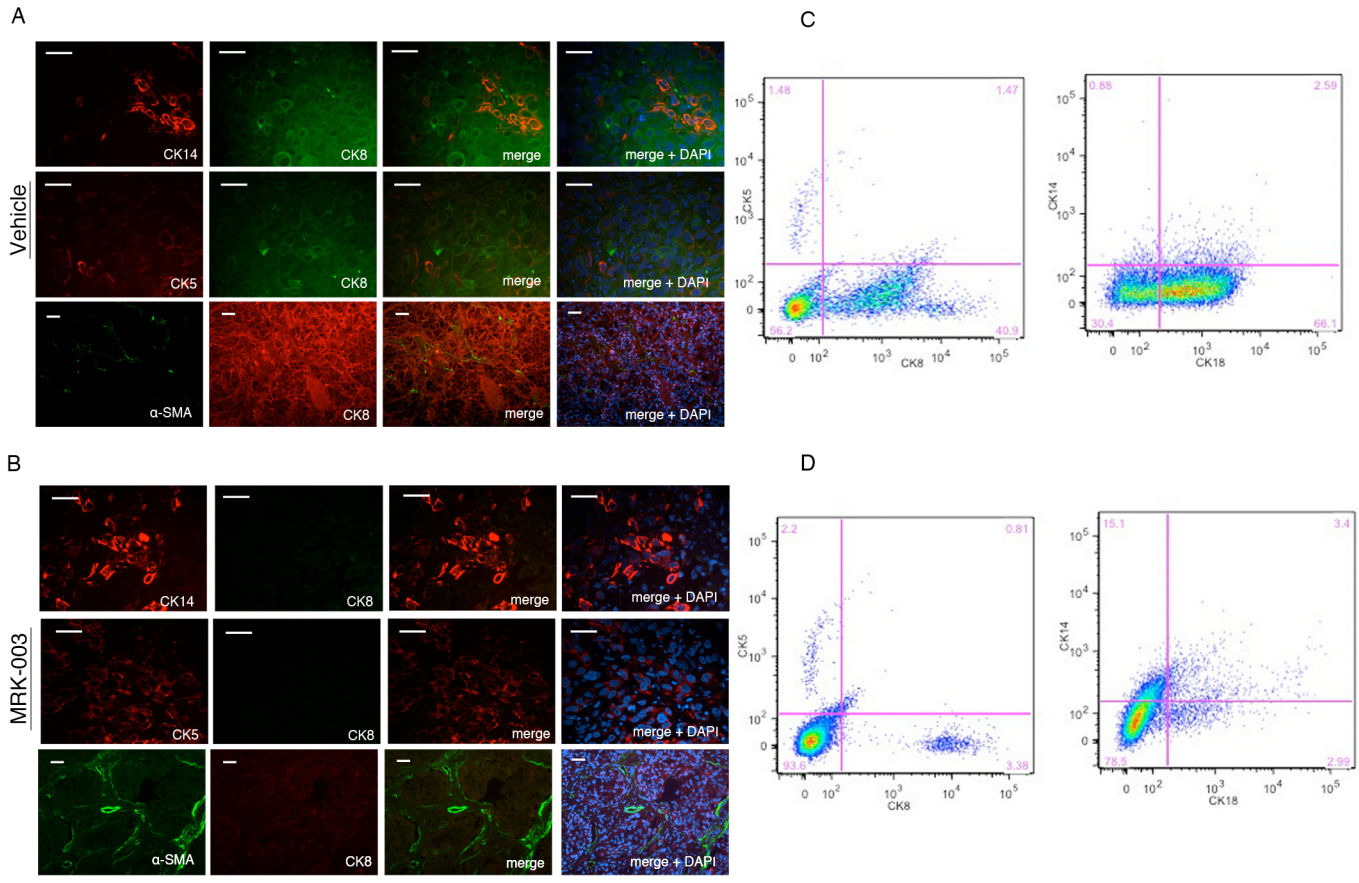
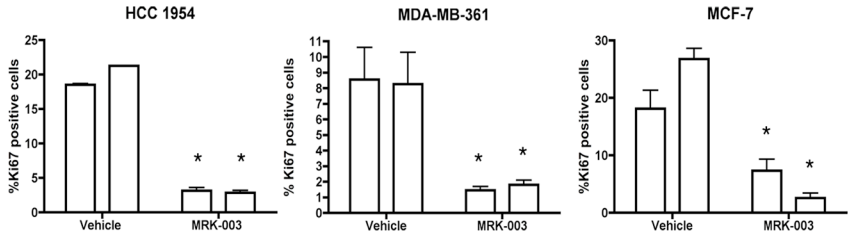
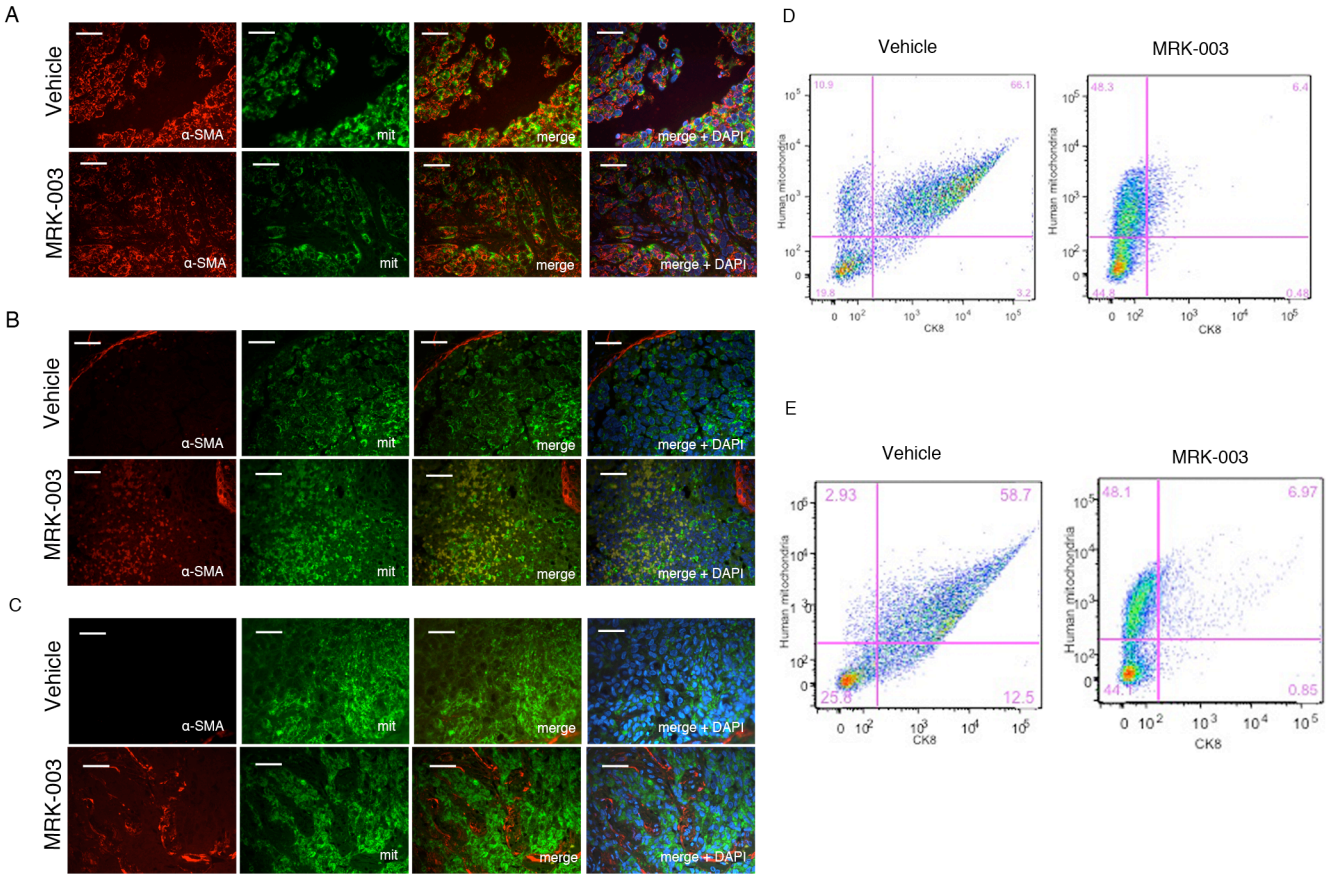


Hassell - Supplemental Figure 1



Hassell - Supplemental Figure 2



CHAPTER 5

Introduction

Aberrant Notch signaling occurs in many cancers including breast cancer. However, its role can be either oncogenic or anti-tumorigenic, depending on both cellular and tissue context. In most cases, activation of the pathway has oncogenic consequences and consequently its inhibition represents a potential strategy to treat cancer. Previous chapters discussed small molecular inhibitors of Notch signaling, namely inhibitors of the gamma secretase enzyme complex (GSIs), and their effects on breast tumor-initiating cells. Genetic experiments may provide a more specific means of interfering with Notch signaling. Hence we adopted the latter strategy to reduce Notch signaling in human breast cancer cell lines.

RBPjκ is the principal nuclear transcriptional mediator of Notch signaling (Lai, 2002a). In the absence of Notch signaling, this protein is bound to DNA in complex with co-repressors thus inhibiting Notch target gene transcription. However, Notch pathway activation results in the interaction of the NICD with RBPjκ, which then undergoes a conformational change resulting in the release of co-repressors, recruitment of co-activators and subsequent transcription of Notch target genes. A common approach for the conversion of transcription factors into their dominant negative forms is based on the use of the repressor domain of the *Drosophila* Engrailed protein (Han and Manley, 1993; John *et al.*, 1995; Markel *et al.*, 2002; Wang *et al.*, 2004). Translational fusions of both mammalian and plant transcriptional factors to this sequence repress their respective target genes. In this work, the effect of an RBPjκ-Engrailed fusion protein was assessed in human breast tumor cell lines.

As mentioned, upon interaction with the NICD, RBPjκ is converted from transcriptional repressor into transcriptional activator. This change is achieved through formation of tertiary complex between RBPjκ, the NICD and Mastermind-like (MAML). MAML is a co-activator of transcription for both Notch target genes and those of other transcription factors such as p53, the MADS box transcription enhancer factor (MEF) 2C, and ß-catenin (Alves-Guerra *et al.*, 2007; Shen *et al.*, 2006; Wu *et al.*, 2000; Wu *et al.*, 2002; Zhao *et al.*, 2007). There are three MAML proteins in humans (MAML1-3), all of which are involved in Notch signaling and were found to play a role in a variety of cancers (Conkright *et al.*, 2003; Metzler *et al.*, 2008; Nemoto *et al.*, 2007; Sivasankaran *et al.*, 2009; Tonon *et al.*, 2003). Two alpha helixes in the N-terminus of MAML1 interact with the ankyrin repeats of NICD (Kovall, 2007) resulting in recruitment of p300 histone acetyl transferase and other general transcriptional activators (Fryer *et al.*, 2002; Wallberg *et al.*, 2002). Previous analyses demonstrated, that a truncated form of the Mastermind-like protein containing only the N-terminal region of the protein functions as a dominant-negative inhibitor of Notch signaling likely because it is incapable of recruiting co-activators (Fryer *et al.*, 2002; Weng *et al.*, 2003). Importantly, expression of the dominant-negative form of Mastermind-like 1 inhibits all MAML proteins by competing for binding to RBPjκ and other coactivators (McElhinny *et al.*, 2008). The effect of the dominant-negative Mastermind-like1 on human breast tumor cells was tested and will be discussed in this chapter.

An alternative approach to inhibit Notch signaling is based on overexpression of Numb, a well-known antagonist of the pathway (McGill and McGlade, 2003; O'Connor-Giles and Skeath, 2003). This evolutionary conserved protein was first identified in

Drosophila were it was found to be asymmetrically segregated during cell division leading to activation of Notch in one cell and its down regulation in the other (Posakony, 1994). In both *Drosophila* and mammals, Numb recruits the E3 ubiquitin ligase Itch to membrane-bound Notch, causing polyubiquitination and degradation of the NICD (McGill and McGlade, 2003; Nie *et al.*, 2002). In addition, Numb may be involved in endocytosis of Notch receptors and ligands thereby suppressing their function, possibly by linking Notch to alpha-adaptin, a component of the endocytic complex that is responsible for cargo sorting in the vesicular transport system (Berdnik *et al.*, 2002; O'Connor-Giles and Skeath, 2003). Loss of Numb expression was found in about 50% of human mammary carcinomas, suggesting its importance in human breast cancer (Pece *et al.*, 2004). The effect of Numb overexpression in breast tumor cells will be demonstrated in this chapter.

Manuscript #3

Title: Genetic inhibition of Notch signaling targets breast tumor-initiating cells

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Author contributions

Maria Kondratyev – planned, analyzed and performed most of the experiments in the manuscript, wrote the manuscript.

Bonnie Bojovic – prepared some of the constructs (pSLIK-Mastermind-GFP and pSLIK-Numb) and helped testing expression of the transgenes (figure 1A), author of figure 3B

Robin Hallett - helped with *in vivo* work (treated some of the mice, helped harvesting tumors)

Adele Girgis-Gabardo – helped culturing cells and preparing them for injections, performed the staining for figure 4 B and C

Results

Expression of Notch pathway antagonists in breast tumor cell lines causes reduction in the levels of Notch target genes.

To complete genetic experiments aimed at assessing the effects of Notch signaling pathway knock-down on breast tumor-initiating cells, we generated HCC1954, MDA-MB-361 and MCF-7 cell populations that inducibly expressed negative regulators of the Notch signaling pathway. The pSLIK-RBPjκ-Engrailed, pSLIK-Mastermind-GFP and pSLIK-Numb were generated as described in Materials and Methods. Inducible expression of each transgene was validated using Western blot analysis (figure 1A), qRT-PCR (figure 1B) and fluorescent imaging (figure 1C). Western blot analysis was not performed for RBPjk-Engrailed fusion protein because none of the commercially available antibodies were proved capable of detecting the fusion protein. However very significant induction in the level of the fusion transcript was observed when qRT-PCR was performed using primers for *Drosophila* Engrailed transcript (figure 1B). qRT-PCR was not performed for Mastermind-GFP transcripts because expression of the fusion protein could be verified using both Western blot analysis (figure 1A) and fluorescent imaging (figure 1C).

To test whether the expression of the Notch antagonists affected the levels of Notch signaling in breast tumor cell lines, qRT-PCR was performed on both induced and non-induced cell populations using primers for *Hey1* and *Hes1* transcripts. To do so, indicated cell populations were cultured for four days in serum-containing media in the presence or absence of doxycycline. Total RNA was isolated from the cells using RNeasy extraction kit [Qiagen], cDNA was prepared using Invitrogen Reverse Transcription kit

and quantitative RT PCR was performed using primers for indicated transcript. Figure 2 illustrates expression levels of *Hey1* (A) and *Hes1* (B) transcripts normalized to the levels of GAPDH. As shown, expression of both genes was reduced in the cells upon addition of doxycycline, indicating that Notch signaling is inhibited by the expression of transgenes.

Expression of the Notch pathway antagonists inhibits sphere formation by human breast tumor cells.

As discussed above, human breast tumor cell lines can be cultured in chemically defined medium as non-adherent spheres (Dontu *et al.*, 2003). Such cultures are enriched in tumor-initiating cells and sphere-forming assays can be used to assess the survival and self-renewal of these cells (Cicalese *et al.*, 2009; Dontu *et al.*, 2004; Liao *et al.*, 2007; Liu *et al.*, 2007; Mani *et al.*, 2008), (Kondratyev *et al.*, in preparation). To test whether genetic inhibition of Notch signaling reduced the *in vitro* tumorigenic potential of human breast tumor cells, we performed sphere-forming assays using the described cell populations in the presence and absence of doxycycline. To do so, the cells were propagated as spheres in human SCM for 3 passages and then plated in 96 well plates in the presence or absence of doxycycline as described in Materials and Methods. Number of spheres formed by the cells was established a week later and is shown in figure 3A. As evident from this figure, addition of doxycycline inhibited sphere formation by all cell lines that were tested. This result indicates that active Notch signaling is essential for sphere formation, and suggests that downregulation of the pathway may reduce TIC survival and/or self-renewal in vitro.

Expression of the Notch pathway antagonists does not affect colony-forming capacity of human breast tumor cells.

As discussed in previous chapters, inhibition of Notch signaling using the GSI MRK-003 did not affect the frequency of colony formation by cells derived from mouse mammary tumors, indicating that the likely cellular target of its action was the sphere-initiating cells (TIC) rather than progenitor cells. To test whether the antagonists of Notch signaling mimicked the effect of MRK-003, colony-forming assays were performed with the described cell populations in the presence and absence of doxycycline. Cells from the indicated cell population were plated in clonogenic densities following published protocol (Kurpios *et al.*, 2009), colonies that were formed by cells in the presence and absence of doxycycline were counted a week later. As illustrated in figure 3B, in most cases expression of the transgene did not result in a reduction in the number of colonies. This finding indicates that dominant-negative protein antagonists of the Notch pathway do not affect viability or proliferation of colony forming cells in tumor cell populations.

Expression of the dominant negative Mastermind-like protein in human xenografts inhibits tumor growth and induces differentiation of tumor cells towards the myoepithelial lineage.

As discussed above, expression of the dominant negative Mastermind-like protein in human breast tumor cells resulted in downregulation of Notch target genes and reduced sphere formation by these cells. We previously reported that inhibition of Notch signaling using GSI blocks tumor growth and causes tumor regression in mouse and human models of breast cancer (Kondratyev *et al.*, 2011), (Kondratyev *et al*., in preparation). To test whether the expression of dominant-negative MAML-1 affected tumorigenesis HCC1954

cells carrying the transgene were injected subcutaneously into NOD/SCID mice. When the tumors were formed and reached ~1cm^3, the mice were given 5 mg/mL of Doxycycline in their drinking water for the period of 1 month; the volumes of the tumors were measured weekly. Figure 4A shows the final volume of each tumor plotted as percentage of initial tumor volume for control mice and mice treated with doxycycline. As evident from this graph, induction of transgene expression in the tumor cells significantly inhibited tumor growth.

Our previous study using GSI MRK-003 revealed that inhibition of Notch signaling in tumor cells skews their differentiation towards the myoepithelial lineage (Kondratyev *et al.*, 2011), (Kondratyev *et al*., in preparation). To test whether the expression of dominant negative MAML-1 in HCC1954-seeded xenografts had a similar effect, sections of tumors harvested from control and doxycycline-treated mice were stained with antibodies against markers of luminal (CK8) and myoepithelial (CK5 and CK14) lineages. HCC1954 cell line belongs to basal molecular subtype and control tumors seeded by these cells contain cells positive for luminal and cells that are positive for the myoepithelial markers (figure 4B). However, tumors from doxycycline-treated mice comprised cells that express myoepithelial markers but did not contain any cells positive for the luminal marker CK8 (figure 4C). These data suggest that inhibition of the Notch signaling in these tumors through expression of the dominant-negative MAML-1 induced differentiation of tumor cells towards the myoepithelial lineage similar to the effect of GSI.

Discussion

Our data demonstrate that expression of the dominant-negative effectors and over-expression of an antagonist of the Notch signaling pathway led to decreased sphere formation in three different breast tumor cell lines (figure 3A). Interestingly, the colony-forming capacity of the cells was generally not affected by expression of the transgene (figure 3B). These data are in accordance with observations that were made testing the effect of MRK-003 on colony-forming capacity of tumor cells from the N202 mouse mammary tumors (Kondratyev *et al.*, 2011). These findings also indicate that inhibition of Notch signaling did not affect the viability or proliferative potential of colony forming cells within breast tumor cell line populations. Evidence exists that breast tumor cell lines retain a cellular hierarchy characteristic of the primary tumors comprising cells with stem cell properties as well as differentiated progeny (Charafe-Jauffret *et al.*, 2009; Fillmore and Kuperwasser, 2008). While a large proportion of cells within the cell lines were capable of forming colonies (about 50%, data not shown), the frequency of sphere formation is only about 1-2%, and an even lower proportion of cells are capable of seeding a tumor in NOD/SCID mice. Since it was shown that culturing human breast tumor cell lines under sphere-forming conditions enriches for cells with tumor-initiating capacity, sphere formation may roughly correlate with tumorigenic potential (Charafe-Jauffret *et al.*, 2009; Dontu *et al.*, 2003; Fillmore and Kuperwasser, 2008; Reynolds and Weiss, 1992). Therefore it is plausible that Notch signaling is important for the self-renewal of tumor-initiating cells but is not required for the proliferation of differentiating non-tumorigenic cells. However, expression of RBP-Engrailed fusion protein led to significant reduction in number of colonies formed by MCF-7 cells and to a lesser extent

those that were formed by the MDA-MB-361 cell line. Interestingly, RBP-Engrailed had no effect on colony formation by basal cell line HCC1954. These data may suggest that the dominant negative RBPjκ inhibited the proliferation of progenitor cells in two luminal breast tumor cell lines, suggesting that it may affect tumor cells by different or additional mechanisms as compared to other antagonists of the pathway and GSIs.

Expression of the dominant-negative MAML-1 in HCC1954-seeded xenografts inhibited tumor growth likely by inhibiting Notch signaling (figure 4A). Interestingly, the tumors induced to express dominant-negative MAML-1 differed in their cellular composition and did not contain any cells expressing luminal markers unlike control tumors, which comprised both luminal and myoepithelial-like cells. These observations are in accordance with several reports suggesting that Notch signaling promotes a luminal differentiation program in both mammary epithelial stem/progenitor cells and tumor cells (Bouras *et al.*, 2008), (Kondratyev *et al.*, in preparation). Notch1 is preferentially expressed in the luminal compartment of mouse mammary glands, and overexpression of the N1ICD in the mouse mammary epithelium resulted in the expansion of luminal progenitors and led to hyperplasia and tumorigenesis (Bouras *et al.*, 2008).

Knockdown of RBPjκ and Pofut1 (a fucosyltrasnferase essential for Notch activity) skewed differentiation of mammary epithelial progenitor cells towards the myoepithelial lineage during pregnancy (Buono *et al.*, 2006). Finally, we previously reported that MRK-003 induced the differentiation of mouse and human breast tumor cells towards the myoepithelial lineage, and also inhibited their proliferation and induced their apoptosis (Kondratyev *et al.*, 2011), (Kondratyev *et al.*, in preparation). Our data suggest that expression of the dominant negative MAML-1 also induced the

differentiation of breast tumor cells towards the myoepithelial lineage. Hence the effect of both pharmacological inhibitors and dominant-negative antagonists suggest that attenuation of Notch signaling accounts for the effect of these agents. The latter notion is supported by the fact that Notch target genes are downregulated in cells after induction of the transgenes encoding proteins that interfere with Notch signaling (figure 2).

Numerous observations suggest that the Notch signaling pathway is an attractive target for anti-cancer therapies (reviewed in (Nickoloff *et al.*, 2003; Shih Ie and Wang, 2007)). Current approaches to mediate Notch signaling for therapeutic purposes include the use of small molecules (gamma- secretase inhibitors (Farnie *et al.*, 2007; Hallahan *et al.*, 2004; O'Neil *et al.*, 2006) and inhibitors of ADAM metalloproteases (Moss *et al.*, 2008)), antibodies against specific pathway components or small peptides that interrupt NICD/RBPjk interactions (Dikic and Schmidt; Li *et al.*, 2008a; Phillips *et al.*, 2006; Wu *et al.*; Yin *et al.*). Our data confirms the involvement of Notch signaling in breast tumorigenesis and suggest that its inhibition reduces the frequency of tumor-initiating cells both *in vitro* and *in vivo.* Manipulations of three different Notch pathway components were completed in this study to inhibit the Notch signaling pathway leading to reduction in the self-renewal of tumor initiating cells *in vitro.* Experiments performed with xenografts generated by cells expressing dominant-negative MAML-1 confirmed that Notch pathway inhibition correlated with the reduction in tumorigenic potential *in vivo*. Therefore, we suggest that agents interrupting the activity of Mastermind and RBPjκ as well as those upregulating Numb can be used as novel breast cancer treatments.

Recently, extensive studies were performed to reveal the molecular structure of the tertiary complex between NICD, MAML-1 and RBPjκ (Kovall, 2007; Saint Just

Ribeiro *et al.*, 2007). This knowledge can contribute to developing peptides that will interrupt formation of the complex; such peptides can be delivered into tumor cells as a potential anti-breast cancer therapy. Several reports suggested that Notch might have activity independent of RBPjκ that might contribute to its role in tumorigenesis (Aster *et al.*, 1997; Blanco *et al.*, 2002; Jeffries and Capobianco, 2000; Kurooka *et al.*, 1998; Ordentlich *et al.*, 1998; Ross and Kadesch, 2001; Yamamoto *et al.*, 2001). While these data are contradictory at this point, alternative approaches to inhibit Notch signaling that do not involve RBPjκ might prove effective. In this regard upregulation of Numb activity can be explored. In addition to the data shown here, it was reported that overexpression of Numb in human breast tumor cells completely blocked the ability of these cells to form xenografts upon transplantation into NOD/SCID mice (Harrison *et al.*), confirming its ability to target tumorigenic cells. As mentioned above, the loss of Numb is observed in a high proportion of human breast carcinomas (Pece *et al.*, 2004). Interestingly, the downregulation of Numb activity in cancer is usually the result of its increased ubiquitin-mediated degradation (Colaluca *et al.*, 2008; Westhoff *et al.*, 2009). Therefore, drugs targeting enzymes of the ubiquitination machinery might be efficient in upregulating Numb and thus inhibiting Notch-mediated tumorigenesis.

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Figure legends

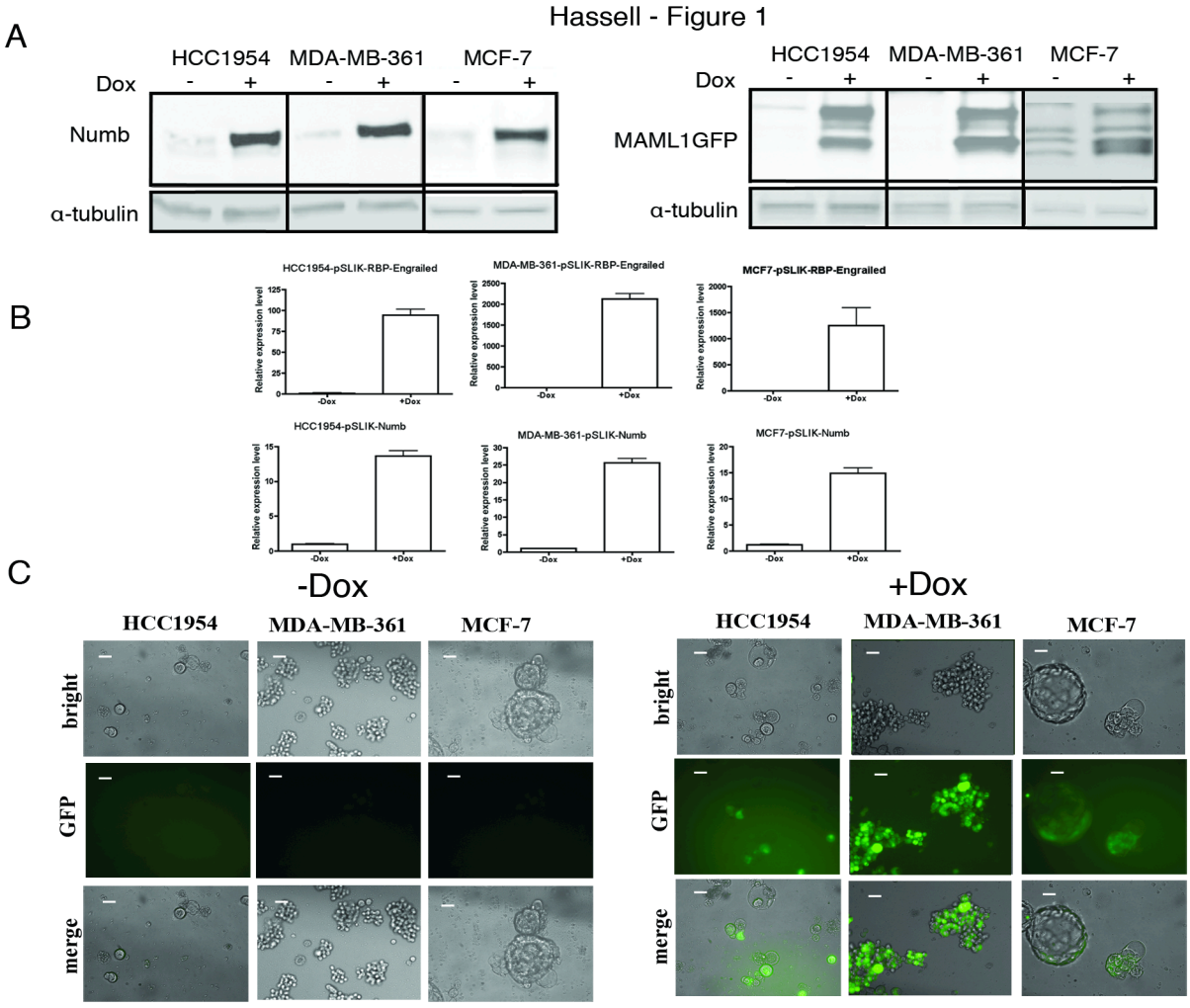
Figure 1. Validation of expression of Notch pathway components introduced into human breast tumor cell lines via lentiviral infection. (A) Western blot analysis and (B) quantitative RT PCR confirmed inducible expression of indicated transgene in indicated cell line. (C) Cell populations infected with lentiviral construct encoding the dominant negative MAML-1-GFP (pSLIK-Mastermind-GFP) were cultured in SCM in the presence and absence of Doxycycline for 72 hours. Inducible expression of GFP was validated by photographing spheres under Leica Fluorescent Microscope.

Figure 2. Expression of dominant-negative forms of the Notch pathway components and over expression of the pathway antagonist Numb downregulated expression of Notch target genes in human breast tumor cells. Levels of Hey1 (A) and Hes1 (B) transcripts were measured in indicated cell lines using quantitative RT PCR after cells were cultured for 72 hours in the presence and absence of doxycycline.

Figure 3. Expression of the Notch pathway antagonists inhibits sphere formation by human breast tumor cells but do not affect their colony forming capacity. (A) Sphere forming assays were performed with indicated cell populations in the presence and absence of doxycycline. In all cases, expression of the transgene reduced number of spheres formed. (B) Colony forming assays were performed with the indicated cell populations in the presence and absence of doxycycline. In most cases, colony-forming frequency was not affected by the expression of transgene.

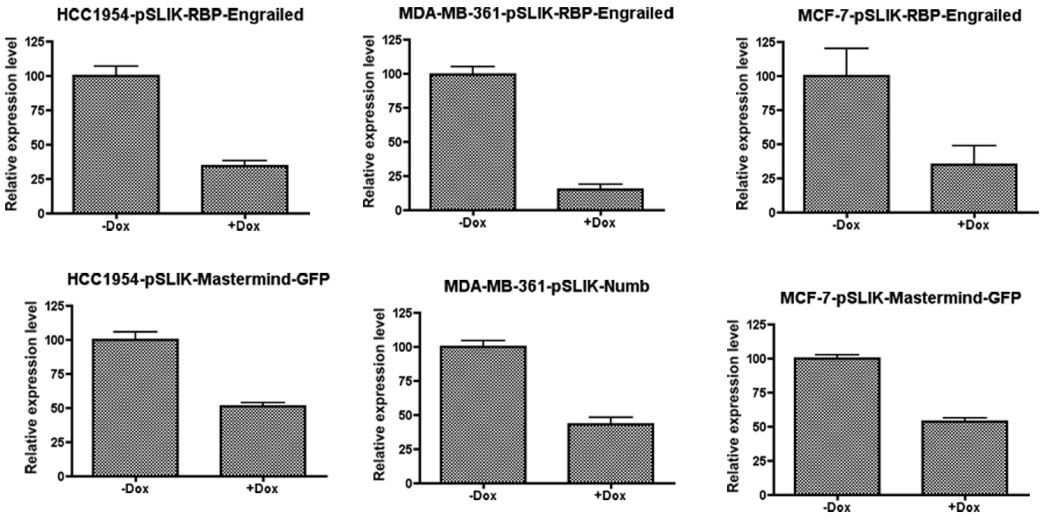
Figure 4. Expression of dominant negative Mastermind-like protein in human xenografts inhibits tumor growth and induces differentiation of tumor cells towards the myoepithelial lineage. NOD/SCID mice bearing subcutaneous tumors seeded by

HCC1954 cells carrying dominant negative Mastermind-GFP transgene were given 5 mg/mL of doxycycline in their drinking water for 1 month. (A) Final volumes of tumors as percentage of initial tumor volumes are plotted for control and doxycycline-treated mice. Doxycycline caused significant inhibition of tumor growth, p-value = 0.0128, unpaired t test with Welch's correction. The mice were sacrificed after one-month treatment; the tumors were harvested, sectioned and stained with the antibodies against indicated markers (CK8, CK5 and CK14). (B) – sections of tumors from control mice, (C) – sections of tumors from mice that had Doxycycline in their drinking water.

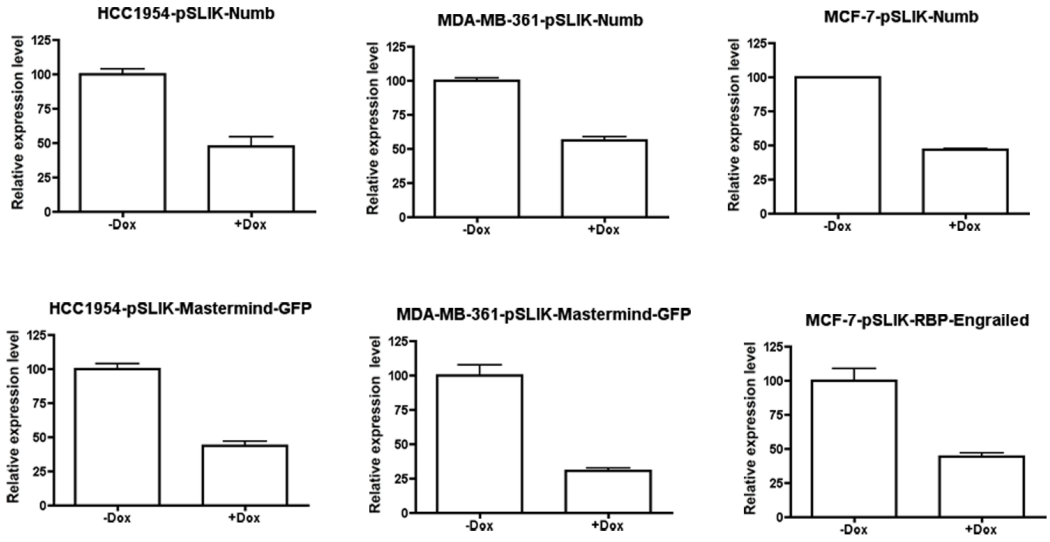

Hassell - Figure 1
A
HCC1954
MDA-MB-361
MCF-7
Dox
- + - + - +
Numb
α-tubulin
HCC1954
MDA-MB-361
MCF-7
Dox
- + - + - +
MAML1GFP
α-tubulin
B
HCC1954-pSLIK-RBP-Engrailed
MDA-MB-361-pSLIK-RBP-Engrailed
MCF7-pSLIK-RBP-Engrailed
HCC1954-pSLIK-Numb
MDA-MB-361-pSLIK-Numb
MCF7-pSLIK-Numb
Relative expression level
-Dox
+Dox
C
-Dox
+Dox
HCC1954
MDA-MB-361
MCF-7
bright
GFP
merge

Hassell - Figure 2

A

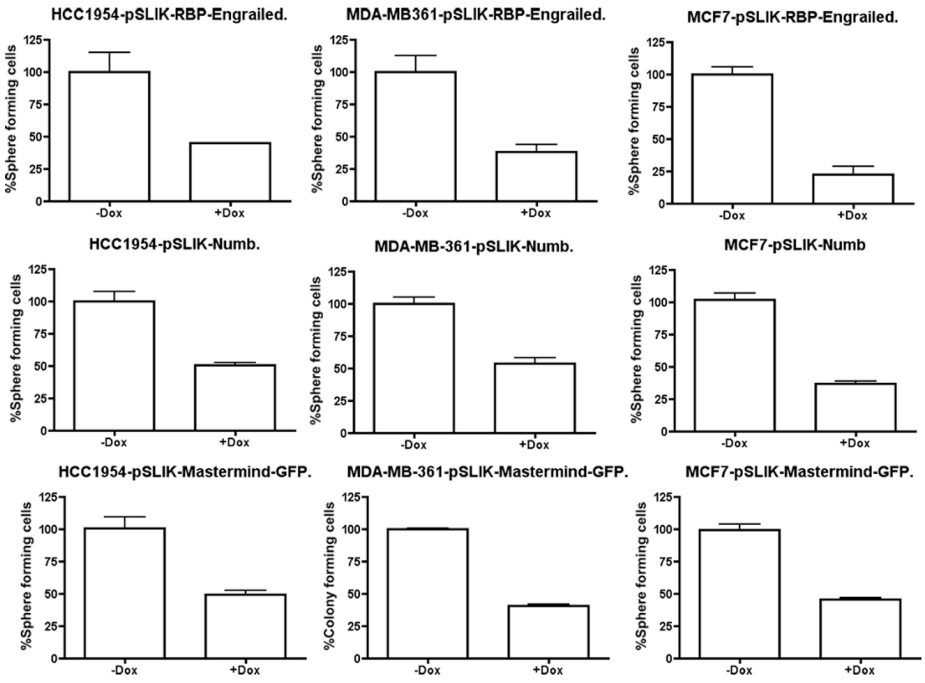


B



Hassell - Figure 3

A



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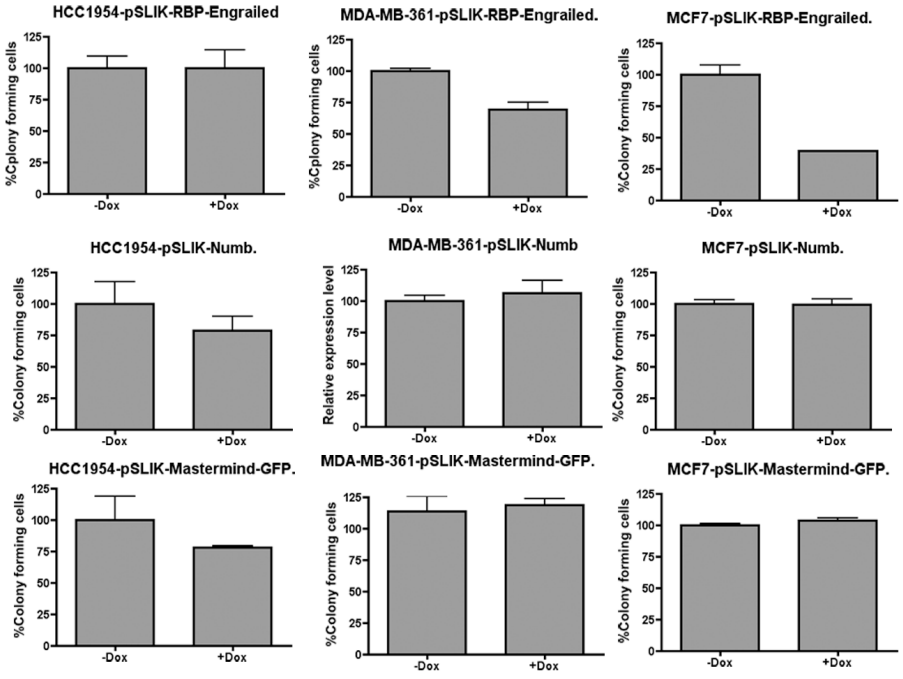
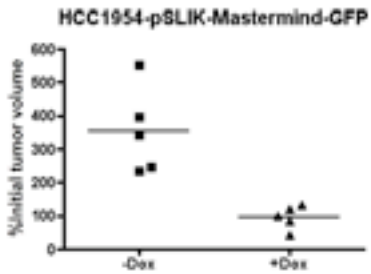
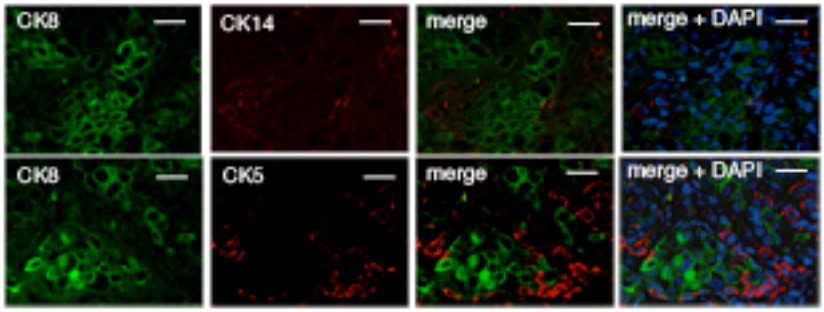


Hassell - Figure 4

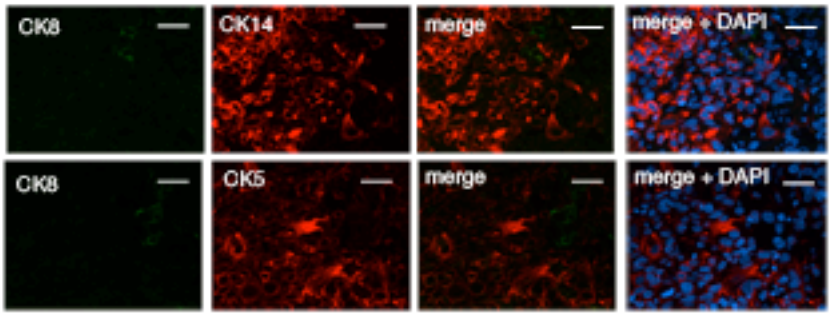
A



B



C



CHAPTER 6

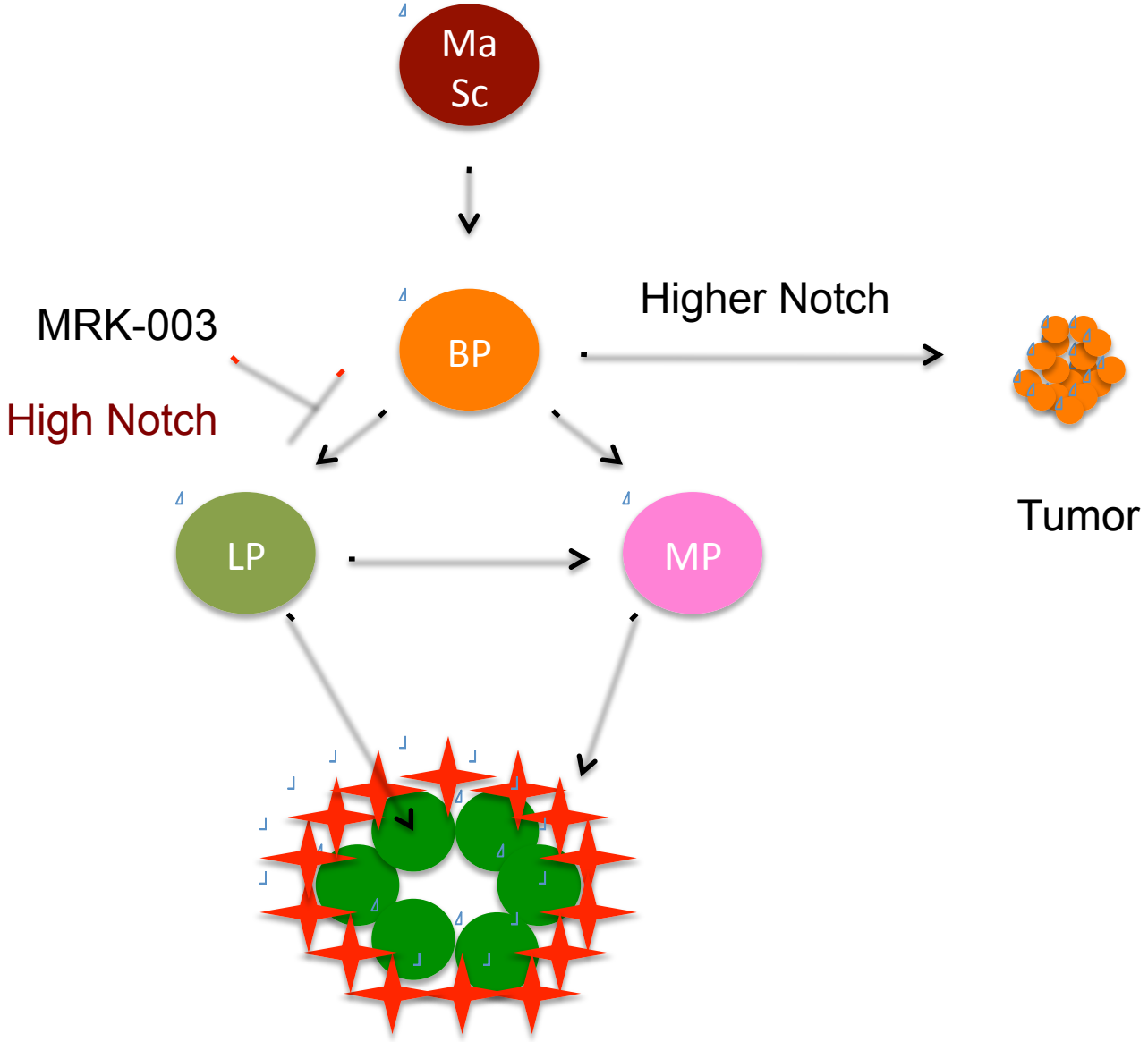
Discussion

In this work, I performed careful and detailed analysis on the effects of inhibiting Notch signaling in breast tumor cells. First, a transgenic mouse model was used to test the effect of the GSI, MRK-003 on breast tumor-initiating cells (Guy *et al.*, 1992). The main advantages of this model include an easy access to high numbers of independent primary tumors that comprise a high frequency of tumor-initiating cells (Kuprios et al., in preparation). The components and target genes of the Notch signaling pathway were found to be overexpressed in tumorsphere cultures as compared to their normal counterparts (mammospheres) (table 1, manuscript #1). Since N202 tumorspheres are enriched in tumor-initiating cells similar to the primary tumor they are derived from (Kuprios et al., in preparation), this finding indicates that Notch signaling is important for self renewal of the mammary tumor initiating cells and plays a role in mammary tumorigenesis. Taken together with the data published by several groups that demonstrated the involvement of Notch signaling in breast tumorigenesis, these results provided a rationale to develop novel anti-breast cancer therapies based on inhibition of Notch.

Two different approaches were taken to achieve this goal. First, the effect of GSI, MRK-003 was explored in mouse and human breast tumor cells. MRK-003 inhibited sphere formation by both mouse tumorspheres and spheres derived from human breast tumor cell lines, indicating its effect on self- renewal of these cells *in vitro* (manuscript #1 figure 1, manuscript #2 figure 1). Similarly, MRK-003 reduced sphere formation by control cell populations, such as mouse and human mammospheres derived from either

the primary mammary epithelial cells or from the HMLE cell line. However, tumor cells exposed to MRK-003 for four days *in vitro* failed to form secondary spheres in inhibitor-free media, while their normal counterparts completely recovered from incubation with MRK-003. Moreover, when MRK-003 treated N202 tumor cells were injected subcutaneously into syngeneic FVB mice, they failed to form tumors or formed smaller tumors than the vehicle-treated cells, depending on the dose of MRK-003 used for pre-treatment (manuscript #1, figure3). These data suggest that exposure to GSI has a long-term consequences for tumor cells, irreversibly inhibiting their self-renewal and tumorigenic potential.

The mechanism by which MRK-003 has differential effects on tumor cells and their normal mammary epithelial cell counterparts is still not completely clear. I demonstrated here, that MRK-003 inhibited proliferation and induced apoptosis and differentiation of tumor cells in both mouse and human models of breast cancer. The THe model of Notch signalingEach one or the combination of these processes can contribute to the selectivity and irreversibility of the effect of MRK-003 on tumor cells. The resistance of tumor cells to physiological or therapeutically induced apoptosis is a well-known feature of many malignancies. Evidence exists, that Notch signaling is involved in activation of anti-apoptotic pathways in tumor cells (Efferson *et al.*; Guy *et al.*, 1992; Kim *et al.*, 2005; Meurette *et al.*, 2009; Osipo *et al.*, 2008a; Palomero *et al.*, 2008; Ramdass *et al.*, 2007; Song *et al.*, 2008). The most studied mechanism by which Notch signaling confers resistance of tumor cells to apoptosis is its cross talk with p53. In T-ALL cells, treatment with GSIs reduced expression of Hes1 leading to the increased expression of PTEN, a phosphatase that negatively regulates the PI3K/Akt signaling



The model of the effects of Notch inhibitors in BTICs. In normal mammary gland high levels of Notch signaling are observed in the luminal progenitors. Upon inhibition of Notch, bipotent progenitors fail to differentiate towards luminal progenitors and either become myoepithelial progenitors or commit apoptosis. Alternatively, luminal cells can transdifferentiate towards myoepithelial cells upon inhibition of Notch.

pathway that is known to promote survival by multiple mechanisms including inactivation of p53 (Palomero *et al.*, 2008). In breast epithelial cells, activation of AKT was shown to be involved in Notch-induced resistance to apoptosis (Meurette *et al.*, 2009) and in breast cancer, GSIs caused a decrease in levels of phospho-AKT and phospho-S6RP in mouse mammary tumors (Efferson *et al.*). Other pathways involved in Notch-mediated inhibition of apoptosis in tumor cells include JNK (Kim *et al.*, 2005) and NF**κ**B (Osipo *et al.*, 2008a; Ramdass *et al.*, 2007; Song *et al.*, 2008). Since Notch signaling is hyper-activated in both mouse mammary tumors (manuscript#1) and human breast cancer (Dickson *et al.*, 2007; Reedijk *et al.*, 2005), it is plausible that GSIs relieve Notch-mediated protection of these cells from programmed cell death causing them to exit the cell cycle and undergo apoptosis. Normal breast cells express lower levels of Notch pathway components and target genes and thus are less dependent on the pathway for their survival.

MRK-003 skewed differentiation of tumor cells towards the myoepithelial lineage. This effect was observed in various cells and under different circumstances. *In vitro*, more myoepithelial colonies (type D) are formed by mouse primary mammary epithelial cells in the presence of MRK-003 at the expense of colonies that comprise both luminal and myoepithelial cells (type C) (manuscript #1, figure 2B). Primary N202 tumor cells formed colonies that comprised both luminal and myoepithelial cells in the presence of MRK-003 as opposed to colonies that contained luminal cells only that formed in the presence of the vehicle (manuscript #1, figure 2C,D). Interestingly, when primary mouse tumor cells exposed to MRK-003 for short term in culture (4 days) were injected subcutaneously into syngeneic mice, they formed tumors that were drastically different

from those formed by vehicle-treated cells, containing multiple ring like structures and cells expressing markers of the myoepithelial lineage (manuscript #1, figure 3). This result indicates, that *in vitro* inhibition of Notch signaling in these cells changed their fate, inducing a differentiation program that manifested several months later when the cells formed small tumors in mice. Importantly, these tumors were devoid of tumor initiating cells as established by secondary transplantation assay. This finding confirms the *in vitro* effect of MRK-003 on self-renewal of tumor-initiating cells and suggests that this inhibitor eliminates the fraction of tumor-initiating cells in the tumor cell population. The most dramatic effect of Notch inhibition in tumors was observed when mice bearing subcutaneous tumors seeded by N202 cells were treated orally with MRK-003. Such treatment resulted in rapid regression and in most cases complete elimination of the tumors (manuscript #1, figure 4). Importantly, the tumors did not grow back after the treatment was stopped, indicating that MRK-003 eliminated the fraction of tumor initiating cells in these tumors. When NOD/SCID mice bearing subcutaneous tumors seeded by human breast tumor cell lines were treated with MRK-003, it caused growth inhibition and in some cases regression of the tumors (manuscript #2, figure 3). Generally, the effect was more profound when cells derived from a cell line belonging to luminal molecular subtype seeded the tumor, albeit only a small number of cell lines were tested. This is an interesting observation since the N202 cells share their gene expression signature with human tumors belonging to luminal molecular subtypes (Herschkowitz *et al.*, 2007) and these cells were most significantly affected by MRK-003.

The mechanism by which MRK-003 affects cells at different stages of

differentiation remains to be investigated, however the analysis of expression of lineage markers in tumors seeded by cells from different subtypes in treated versus untreated mice can shed some light on this phenomenon. Tumors seeded by MCF-7 cells in NOD/SCID mice are most similar to N202 cells since they solely comprise cells that express luminal markers (CK8 and CK18). Upon treatment with MRK-003, the tumors shrunk and most of the cells became double positive for CK8 and CK14, while some of the cells only expressed α-SMA, which is considered to be a marker of more mature myoepithelial cells (Kendrick *et al.*, 2008). Importantly, cells isolated from these tumors were devoid of tumor initiating cells, as established by secondary transplantation assay (manuscript 2, table 1). MDA-MB-361 tumors contained a few cells that are positive for myoepithelial markers whereas most of the cells expressed luminal markers in accordance with the luminal nature of the cell line. Administration of MRK-003 to the mice caused complete growth arrest of the tumors. Tumors in the treated mice did not contain any cells that expressed luminal markers and the fraction of cells expressing myoepithelial markers was increased compared to control tumors. Similarly, HCC-1954 tumors that comprise both luminal and myoepithelial-like cancer cells and belong to basal molecular subtype, were devoid of luminal-like cells upon treatment with MRK-003 and contained higher fraction of either double positive cells or cells that only express the myoepithelial markers.

It was previously reported that active Notch signaling inhibits differentiation of mammary epithelial stem cells towards the myoepithelial lineage and it maintains luminal progenitors in an undifferentiated state (Bouras *et al.*, 2008). Therefore, it is plausible that inhibition of the pathway in cancer cells that originated from or at least share

similarities with bipotent progenitors, will also result in their differentiation towards the myoepithelial lineage. While it is not yet possible to pinpoint the cell of origin for the diversity of molecular subtypes that exist in breast cancer, further analysis of the effect of GSIs on expression of lineage markers in breast tumor cells might contribute greatly to such investigation.

As discussed above, MRK-003 had more pronounced effect on tumors that belong to the luminal molecular subtype. Generally, human breast tumors that belong to this subtype are less aggressive and generally have a better prognosis (Perou *et al.*, 2000; Sorlie *et al.*, 2001), which might diminish the importance of our observations. However, it is important to notice that MDA-MB-361 cells and N202 tumors are strongly affected by MRK-003. While these tumors belong to the luminal subtype according to their gene expression signature, they also overexpress ERBB2 (Guy *et al.*, 1992; Neve *et al.*, 2006). Tumors overexpressing this oncogene are very common in human breast cancer (~30%), and are also known to be particularly aggressive and resistant to many available treatments (Lacroix and Leclercq, 2004; Neve *et al.*, 2006; Perou *et al.*, 2000; Sorlie *et al.*, 2001). Several groups reported a possible connection between Notch and ERBB2 signaling, suggesting that inhibition of Notch might be particularly effective in killing tumor cells overexpressing ERBB2 (Chen *et al.*, 1997; Florena *et al.*, 2007; Yamaguchi *et al.*, 2008; Zardawi *et al.*). In addition, it was reported, that a subset of luminal tumors (B subtype) is in fact correlated with a poor disease outcome, especially in relation to relapse, and luminal C subtype share similarities with ERBB2 overexpressing and basal tumors according to expression of set of genes (Sorlie *et al.*, 2001). Moreover, as demonstrated in this work, both treatment with GSI and alteration of the Notch signaling

through genetic manipulations led to inhibition of self-renewal in breast cancer cell lines representing the various molecular subtypes *in vitro* and inhibition of Notch signaling in mice bearing HCC1954 (basal) seeded tumors resulted in growth inhibition and apoptosis of tumor cells. All these data suggest that Notch signaling is involved in tumorigenicity of various subtypes of breast cancer and its inhibition has a valuable therapeutic potential.

Finally, it is important to address a question whether the effect of MRK-003 on breast tumor-initiating cells was through inhibition of the Notch signaling. As mentioned above, GSIs might have multiple effects on cells including inhibition of enzymes other then the gamma secretase complex (Nyborg *et al.*, 2004) as well as attenuation of additional signaling pathways activated by the gamma secretase (Lammich *et al.*, 2002; Marambaud *et al.*, 2002; Ni *et al.*, 2001; Parks and Curtis, 2007; Struhl and Adachi, 2000). However, expression of constitutively active form of Notch1 (the N1ICD) in human breast tumor cells protected them from the effect of MRK-003 *in vitro*, suggesting that this effect is mediated through Notch1 signaling (manuscript #2, figure 2). Several observations were made regarding the differential effect of different Notch receptors in cancer. For example, Notch 1 and Notch 2 have opposite effects in embryonic brain tumors and in colorectal cancer (Chu *et al.*; Fan *et al.*, 2004). In regards to breast cancer, all four Notch receptors seem to play a role in initiation and progression of the disease (Florena *et al.*, 2007; Fu *et al.*; Harrison *et al.*; Hu *et al.*, 2006; Imatani and Callahan, 2000; O'Neill *et al.*, 2007; Raafat *et al.*, 2004; Reedijk *et al.*, 2005; Weijzen *et al.*, 2002a; Yamaguchi *et al.*, 2008). However, contradictive evidence exists regarding the importance of each particular Notch under different circumstances. One group reported that Notch4 is preferentially activated in the tumor initiating fraction of human breast

tumor cell lines and primary tumor samples, while Notch1 was mainly activated in more differentiated cells (Harrison *et al.*). In addition, they showed that inhibition of Notch4 had more robust effect on initiation of tumor formation and tumor growth as compared to Notch1. Interestingly, gamma secretase inhibitors did not affect cleavage of Notch4, but inhibited Notch1 cleavage, suggesting that they can only block signaling through Notch1 receptor but not through Notch4. However, in contrast to their findings, I demonstrated here that treatment with GSI MRK-003 led to elimination of the fraction of tumor initiating cells in both mouse mammary tumors and xenografts seeded by human breast tumor cell lines. It is important to notice that while in my study the effect of GSI was measured by treating mice bearing tumors and then following tumor shrinkage and recurrence, in the described paper the mice were treated with the inhibitors from the day of injection. Therefore, it is possible that the effect of Notch1 inhibition in breast tumor initiating cells is dependent on their environment and/or on a stage of progression. Another group suggested that Notch3 but not Notch1 is important for proliferation of ERBB2 negative human breast tumor cell lines (Yamaguchi *et al.*, 2008). According to this study, the downregulation of Notch1 had no significant effect on either ERBB2 positive or ERBB2-negative cells while the downregulation of Notch3 led to significant inhibition of proliferation of ERBB2 negative cells. However, this conclusion was based on looking at *in vitro* proliferation of cell lines rather then on the *in vitro* or *in vivo* tumorigenic potential of tumor-initiating cells. Since only very small fraction of cells within a cell line has tumor initiating capacity (Charafe-Jauffret *et al.*, 2009; Fillmore and Kuperwasser, 2008), it is plausible that the observed effect was on proliferation of more differentiated, non-tumorigenic cells. As discussed above, inhibition of Notch signaling

with MRK-003 did not affect proliferation of tumor cells in colony forming assay (manuscript #1, figure 2), but only inhibited their self-renewal when grown under conditions enriching for tumor-initiating cells. Thus, it is possible that while the downregulation of Notch3 inhibits proliferation of progenitor cells, Notch1, which is inhibited by MRK-003 and other GSIs, has a specific role in self -renewal of cancer stem cells.

While the inhibitory effect of GSIs on breast tumor initiating cells was analyzed in manuscripts #1 and #2, manuscript #3 accessed whether inhibition of Notch signaling by expression of dominant-negative pathway components or by overexpression of the pathway antagonist Numb would lead to similar outcomes. Interestingly, the various methods used to inhibit Notch signaling led to reduction in sphere formation by human breast tumor cells. Colony forming capacity of tumor cells was generally not affected by either GSI or induction of expression of pathway antagonists. Since sphere forming assay measures *in vitro* self renewal of tumor initiating cells (Al-Hajj *et al.*, 2003; Dontu *et al.*, 2004; Reynolds and Weiss, 1996) while the colony forming capacity correlates with the proliferation potential of progenitors (Hamburger and Salmon, 1977; Smalley *et al.*, 1998; Smalley *et al.*, 1999), it is plausible that Notch signaling is more important for less differentiated tumorigenic cells within tumor cell population. This hypothesis is further supported by the fact that the N202 mouse mammary tumors, that were shown to be enriched in tumor initiating cells, rapidly regressed and did not grow back after treatment with MRK-003, while human breast tumor xenografts, that contain only a small fraction of tumor initiating cells, were more moderately affected by the inhibitor. Importantly, induction of expression of dominant negative Mastermind-like protein in HCC1954-

seeded xenografts led to growth inhibition and induced differentiation of cells within the tumors towards the myoepithelial lineage, similar to the effect of MRK-003 (manuscript #3, figure 4). This finding not only further supports the *in vitro* data showing that MRK-003 acts through inhibition of the Notch signaling, but it provides additional rationale for the use of different Notch inhibitors to target breast tumor initiating cells.

Taken together, the data presented in this thesis suggest that Notch signaling is essential for self-renewal and tumorigenic potential of breast tumor initiating cells. Inhibition of the Notch signaling pathway using either small molecules or genetic manipulations targeted these cells *in vitro* and *in vivo*, blocking tumor growth and inducing apoptosis and differentiation. This phenomenon was demonstrated using a transgenic mouse model as well as human breast tumor cells belonging to various molecular subtypes, suggesting that novel anti-breast cancer treatments could be developed based on inhibition of Notch.

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