REGULATION OF EPITHELIAL-MESENCHYMAL TRANSITION AND DNA DAMAGE RESPONSES BY SINGLEMINDED-2s

A Dissertation

by

BRIAN LAFFIN

Submitted to the Office of Graduate Studies of Texas A&M University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Toxicology
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Approved by:

Chair of Committee, Weston W. Porter
Committee Members, Robert C. Burghardt
Stephen H. Safe
Timothy D. Phillips
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ABSTRACT

Regulation of Epithelial-Mesenchymal Transition and DNA Damage Responses by Singleminded-2s.

(August 2008)

Brian Laffin, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Weston W. Porter

Virtually all signaling pathways that play key roles in development such as the transforming growth factor (TGF)-beta, notch, and wnt pathways also influence tumor formation, implying that cancer is in a sense development gone awry. Therefore, identification and elucidation of developmental pathways has great potential for generating new diagnostic tools and molecular therapy targets. Singleminded-2s (SIM2s), a splice variant of the basic helix-loop-helix / PER-ARNT-SIM (bHLH/PAS) transcriptional repressor Singleminded-2, is lost or repressed in approximately 70% of human breast tumors and has a profound influence on normal mammary development. In order to gain a better understanding of the mechanisms by which SIM2s restricts malignant transformation and progression in breast cancer, we depleted SIM2 RNA in MCF-7 cells using a retroviral shRNA system and examined gene expression and functional abilities of the SIM2-depleted MCF-7 cells (SIM2i) relative to a control MCF line expressing a non-specific “scrambled” shRNA (SCR). Depletion of SIM2 resulted in an epithelial-mesenchymal transition (EMT)-like effect characterized by increased
migration and invasion, altered morphology, and loss of epithelial markers concomitant with gain of mesenchymal markers. The root of this effect may be loss of SIM2-mediated repression of the E-cadherin repressor slug, as SIM2 is able to bind and repress transcription from the slug promoter, and slug expression is dramatically elevated in SIM2i MCF-7 cells. Consistent with the previously established role of slug in resistance to various cancer therapies, SIM2i cells are resistant to the radiomimetic doxorubicin and appear to have elevated self-renewal capacity under certain conditions. Intriguingly, SIM2 protein levels are elevated by treatment with DNA damaging agents, and SIM2 interacts with the p53 complex via co-regulation of specific p53-target gene such as p21/WAF1/CIP1. These results provide a plausible mechanism for the tumor suppressor activity of SIM2, and provide insight into a novel tumor suppressive transcriptional circuit that may have utility as a therapeutic target.
I dedicate this work to my son, Nathaniel W. Laffin, who brings me happiness in any situation
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Weston Porter, for his support, guidance and willingness to allow me to test my ideas. I would like to thank my committee members, Dr. Robert Burghardt, Dr. Stephen Safe and Dr. Timothy Phillips for doing whatever they could to assist me and giving generously of their time. I also thank Dr. Rick Metz and Elizabeth Wellberg for being the absolute best co-workers I can conceive of having. I appreciate the advice and hard work of our collaborators, Dr. Pepper Schedin, Dr. Mike Lewis, and Dr. Jeffrey Rosen. Finally, I would like to thank my wife, Teresa, and all my extended family, without whose support I could not have achieved the completion of this work.
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<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>CCAAT/enhancer binding protein, beta</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CME</td>
<td>Central midline element</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DS</td>
<td>DS</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta/Serrate/lag-2</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ETS2</td>
<td>V-ets erythroblastosis virus E26 oncogene homolog 2</td>
</tr>
<tr>
<td>HIF1-α</td>
<td>Hypoxia-inducible factor 1 alpha</td>
</tr>
<tr>
<td>HIF2-α</td>
<td>Hypoxia-inducible factor 2 alpha</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of kappaB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of kappaB kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>PAS</td>
<td>Per-Arnt-Sim</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3'-kinase</td>
</tr>
<tr>
<td>SIM1</td>
<td>Single-minded 1</td>
</tr>
<tr>
<td>SIM2</td>
<td>Single-minded 2</td>
</tr>
<tr>
<td>SIM2s</td>
<td>Single-minded 2, short isoform</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic response element</td>
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CHAPTER I
INTRODUCTION

Breast cancer is the second most common malignancy in women and the second leading cause of cancer death in women, with an estimated 178,480 new cases of invasive disease and 62,030 cases of in situ disease along with approximately 40,460 deaths from breast cancer in 2007 American Cancer Society. Breast cancer retains its position as the second most common malignancy despite introduction of improved estimation methods, which have resulted in new case figures dramatically lower than the 2005 estimate. Early detection and new treatments have significantly improved the mortality rate of breast cancer patients, however, it is becoming increasingly clear that many current treatments simply diminish the bulk of the tumor without destroying the so-called tumor initiating cells that are primarily responsible for tumor growth, invasion, angiogenesis, metastasis, and recurrence (1-5). This makes understanding the developmental pathways that are hijacked or dismantled by tumor-initiating cells to acquire de-differentiated stem cell-like properties critical to development of therapies that can truly destroy tumors. Understanding how these pathways function in normal development and the adult organism is also of great importance for creating targeted therapies that will cause the least of amount of suffering possible for the patient while delivering the maximum therapeutic effect.

This dissertation follows the style of Cancer Research.
1.1 Normal mammary gland development

The first morphological descriptions of mammary gland development were undertaken in the 1900s, proceeding through investigations of tissue interaction in the mammary placodes and young virgins glands in the 1960s up to the molecular studies of the present day (6, 7). The mammary gland has evolved in egg-laying synapsids during the Pennsylvanian epoch as a means of hydrating and nourishing thin-shelled eggs that would otherwise dry out (8, 9). In mammals, the ectoderm-derived mammary gland functions primarily to provide nutrients to offspring, but also confers immune protection through transfer of maternal antibodies to the young. The mammary gland develops in three distinct stages (Fig. 1), embryonic, pubertal, and adult, the latter two of which are under the control of circulating hormones and growth factors.

The embryonic stage of mouse mammary development begins with the formation of mammary placodes along the so-called “milk line”, which is defined by expression of Wnt10b (10). In female mice, the epithelium then condenses into bulb-like mammary buds at distinct locations on the milk line, while in male mice androgen acting on the mammary mesenchyme causes bud regression (11-13). The bulb elongates under control of interaction between parathyroid hormone related protein (PTHrP) and bone morphogenetic protein (BMP) signaling, invades into the fat pad beneath the skin, and forms a nipple with a lumen that opens to the skin (14). This PTHrP – BMP interaction also functions to suppress hair follicle development in the vicinity of the nascent gland, via induction of MSX2 (14). Invasion into the fat pad is led by dense epithelial structures called terminal end buds, or TEBs. TEBs, which appear at the tips of elongating mammary ducts in young virgin animals, consist of multiple layers of body cells.
surrounded by a cap cell layer. Proliferation occurs exclusively in the cap cell layer, which expresses enzymes that facilitate fat pad invasion, leaving the elongating duct behind (15-17). Interaction between the TEB and the surrounding stroma guides invasion into the fat pad, and may also instruct clefting events that result in bifurcation and trifurcations that in concert with side branching events give rise to the ductal tree.

Figure 1 Post-natal mammary ductal tree morphology. TEB outgrowth in early virgin glands along with side branching events results in a ductal tree that reaches to the end of the fat pad in the adult virgin. Hormonal changes during pregnancy initiate the formation of lobuloalveolar units for milk production, which by the time of lactation have almost completely filled the fat pad. After pups are weaned, the gland returns to the pre-pregnant state via the process of involution.

This process, termed branching morphogenesis, occurs in many organs such as the lungs and kidneys (18, 19), suggesting that a conserved branching transcriptional module exists and is heavily involved in the execution of all developmental branching
processes. This branching module orchestrates expression of the tissue remodeling factors such as matrix metalloproteases (MMPs) that drive ductal extension and branching (15-17). Broad inhibition of MMPs does not block initial formation of lateral branches, but ductal extension appears to require MMP activity (15). MMP2 null mice display impaired ductal extension as expected (15), and extensive lateral branching in MMP2-/- glands suggests that MMP2 supresses lateral branching. Surprisingly, these effects appear to be mediated through effects on cell survival and proliferation within the TEB, despite localization of the MMP2 activator MMP14 at the tips of TEBs (15).

MMP3 acts antagonistically to MMP2 and promotes lateral branching, as MMP3 null mice had greatly reduced numbers of branch points (15). However, the number of TEBs and duct length in MMP3 null mice was unchanged, implying that MMP3 is not involved in TEB invasion or bifurcation (15). Other factors can compensate for MMP3, as MMP3 null mice eventually catch up to wild type controls in ductal development (ref).

Additionally, there is evidence that as the tree penetrates into the fat pad, morphogen gradients created by the geometry of the extending tree have a strong influence on branch point selection and the frequency of side branching (20). Growth of the mammary ductal tree slows or ceases altogether with the reaching of the end of the fat pad and puberty-associated rises in estrogen. Although elevated estrogen levels contribute to stopping ductal tree outgrowth, estrogen signaling is critical to virgin mammary development, as estrogen receptor-α (ERα) null mice lack TEBs and are deficient in ductal invasion into the fat pad (21, 22). This phenotype appears to be due in part to a lack of ERα -driven expression of the estrogen-responsive EGFR ligand amphiregulin, as the ductal phenotype of ERα, amphiregulin, and EGFR null mice are highly similar (23-26).
Amphiregulin function requires proteolytic release from the cell membrane by ADAM17/TACE, consistent with the phenotypic similarity of ADAM17-/- mice to ERα, amphiregulin, and EGFR null mice (27). Consistent with all of these observations, lack of mammary gland development in ovariectomized mice can be rescued by EGF or TGFα pellets, but not by insulin or albumin containing pellets (28). This demonstrates that circulating hormones exert their effects by driving expression of paracrine-acting growth factors. Apoptosis within the body cells left behind by invading TEBs has been proposed as the mechanism by which lumen formation occurs, and indeed, deletion of the pro-apoptotic protein Bim delays lumen formation significantly (29, 30). The ducts in Bim null mice eventually form lumens, demonstrating that functionally redundant apoptotic processes ensure eventual lumen formation. After puberty, estrogen cycling gives rise to waves of side-branch formation and apoptotic dieback, mimicking on a small scale the dramatic changes that occur during pregnancy, lactation, and involution. The amount of this estrous-related side branching that occurs varies widely between mouse strains, but can be quite extensive.

During pregnancy, the hormones progesterone and prolactin drive the gene expression and morphologic changes necessary to effect lactation, namely the formation and differentiation of alveolar subunits, where milk production occurs. Progesterone, through the action of its receptor, is a prime mover in the formation of side branches and alveolar units during pregnancy (31). Progesterone also acts in concert with prolactin (PRL) signaling through the prolactin receptor to promote differentiation of the alveolar cells, which produce the large quantities milk needed during lactation (31, 32). During
lactation, PRL-induced phosphorylation of STAT-5 and subsequent milk protein mRNA transcription interact with translational control mechanisms to generate elevated amounts of caseins and other milk proteins, resulting in production of up to 5 mL of milk per day (33-39). As the offspring grow and are weaned, milk accumulation triggers involution, a cell death and fatty tissue regeneration process that returns the gland to its pre-pregnant state. Involution occurs in two distinct stages which are defined by the point at which suckling is no longer able to reverse the involution process (40). The first, reversible stage lasts roughly 48 hrs and characterized by decreased expression of pro-survival proteins and increased expression of pro-apoptotic proteins (41). The second, irreversible stage starts with clearance of apoptotic cells and excess milk by the epithelium followed by macrophage and MMP-mediated tissue remodeling that reverses the pregnancy-associated epithelial expansion as the animal resumes estrous cycling (40, 42). In addition to the radical changes in the epithelium of the mammary gland during pregnancy and lactation, the extracellular matrix (ECM) simultaneously undergoes dynamic changes that contribute to mammary gland function and affect the phenotype of pregnancy-associated breast cancer (43, 44).

1.2 The history of breast cancer and chemotherapy

Breast cancer was one of the first tumors to be described in ancient medical writings. The Edwin Smith Papyrus, which is the earliest known medical writing of any kind, was written around 1600 BC and describes 8 cases of breast ulcers or tumors in humans, one of whom was a man. While Egyptian doctors were sometimes able to remove the tumors by cauterizing them with an instrument called a “fire drill”, the
disease was essentially incurable at that time. This state of knowledge and care for breast cancer patients remained largely unchanged for centuries, until the work of Jean Louis Petit and Benjamin Bell in the early 18th century. Bell and Petit were the first to advocate removal of surrounding tissue, lymph nodes, and muscle in addition to the tumor mass itself to combat recurrence and metastasis. Their work was continued and expanded upon by Dr. William Halsted, who performed the first complete mastectomy in 1882. The procedure that bears his name, the Halsted radical mastectomy, is still performed to this day. Breast cancer treatment took its next major step forward in 1946. The port-mortem observation of lymphoid and myeloid depletion in persons exposed to nitrogen mustard gas led to seminal work by Goodman, Gilman, and Linskog which demonstrated that lymphomas could be shrunk by injection of nitrogen mustard compounds and established the feasibility of cancer chemotherapy (45, 46). In 1952 the first major contribution to improving the mental well being of breast cancer patients and survivors was made by Terese Lasser when she founded the Reach to Recovery program (47). Reach to Recovery employs trained volunteers, many of whom are mastectomy patients and cancer survivors, to provide emotional support and guidance to breast cancer patients and help them to maintain their feeling of personal dignity. This program and others like it have raised awareness of the devastating impact cancer has on a patient’s entire life and had a profound positive impact on the quality of life had by breast cancer patients and survivors.

Additional steps forward in cancer therapy such as the discovery of the anticancer activity of antifolates, 6-mercaptopurine, and vinca alkaloids led congress to establish the
National Cancer Chemotherapy Service Center (NCCSC) at the National Cancer Institute (NCI) in 1955. The NCCSC was the first federal program promoting cancer drug discovery, and generated numerous animal models and cell lines in its pioneering drug development efforts, leading to the relatively rapid development of additional chemotherapeutic drugs such as the taxanes in 1964 and camptothecins in 1966. Cisplatin and Tamoxifen were also discovered during this time, but were not approved for use in human cancers until the 1970s. 1965 saw a major paradigm shift in cancer chemotherapy: contemplating the problem of mutation-induced drug resistance in tumors, James Holland, Emil Freireich, and Emil Frei extrapolated the multi-drug treatment regimens used to treat tuberculosis to treatment of leukemias by simultaneously administering methotrexate, vincristine, 6-mercaptopurine (6-MP) and prednisone— together referred to as the POMP regimen — and induced long-term remissions in children with acute lymphoblastic leukaemia (ALL). Bernard Fisher and Gianni Bonadonna independently followed this and additional work by Dr. Frei in osteosarcomas by showing that adjuvant chemotherapy after complete surgical resection of breast tumours significantly extended survival — particularly in more advanced cancer (48, 49).

From the 1970s to the 1990s, several other classes of drugs were developed such as the anthracyclines, nitrosoureas, and epipodophyllotoxins, as well as supporting therapies such as anti-nausea drugs. With the advent of genomics, proteomics, and other high-throughput techniques, knowledge of tumor signaling networks has exploded to the point that therapies that target specific weak points of tumors with greatly reduced damage to normal tissue are now possible. Examples of this are tyrosine kinase inhibitors
such as imatinib, which specifically targets the oncogenic fusion protein BCR-ABL, and humanized antibodies such trastuzumab, which inactivates the product of the HER2/neu oncogene in breast tumors (50, 51). The promise of these targeted therapies makes understanding tumor signaling networks of the utmost importance, so as to elucidate new targets and create the most possibilities for combination therapies in the infinitely heterogeneous tumor signaling networks encountered in patients.

1.3 Types and subtypes of breast cancer

Breast cancer is a remarkably heterogeneous disease, with well over 50 types and subtypes of invasive disease currently recognized by the World Health Organization (52), a summary of which appears in Table 1(53-102) (103). Invasive ductal carcinomas represent the vast majority of breast tumors (52). While these tumors fall into the same category histologically, they are quite variable in clinical behavior, underscoring that these tumors cannot be broadly treated in the same way as might be warranted in rarer subtypes that have a more uniform set of genetic abnormalities. Groundbreaking work by Perou et al (104) and Sorlie et al (105) has provided a new approach to the problem posed by the morphologic similarity and behavioral heterogeneity of invasive ductal carcinomas. These studies demonstrated that gene expression profiling could sort invasive ductal carcinomas into 5-6 molecular subtypes - luminal A, luminal B, luminal C, normal breast-like, HER2 overexpressing, and basal-like – and that these groupings had significant predictive value for patient outcome. Luminal subtype may be viewed as a subset of luminal B tumors. Abbreviated molecular definitions of these subtypes and their relative prognosis is presented in table 2.
### Table 1. Types and subtypes of invasive breast carcinomas.

<table>
<thead>
<tr>
<th>Type / Subtype</th>
<th>Frequency</th>
<th>Prognosis</th>
<th>Description / characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive ductal carcinoma (not otherwise specified)</td>
<td>35-50%</td>
<td>—</td>
<td>Generally ER-positive, Epithelioid</td>
</tr>
<tr>
<td>Basal-like carcinoma</td>
<td>7-20%</td>
<td>Poor</td>
<td>Expression of high-molecular weight cytokeratins 5, 14 and 17, Triple negative (ER, PR, p53)</td>
</tr>
<tr>
<td>Mixed type carcinoma</td>
<td>&lt;1%</td>
<td>Varies</td>
<td>Varies widely</td>
</tr>
<tr>
<td>Pleomorphic carcinoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Frequent E-cadherin gene inactivation / LOH</td>
</tr>
<tr>
<td>Carcinoma with osteoclastic giant cells</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Osteoclastic giant cells present in stroma surrounding tumor</td>
</tr>
<tr>
<td>Carcinoma with choriocarcinomatous features</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Elevated human chorionic gonadotropin-β expression, presence of multinucleated syncytiotrophoblast-like giant cells</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>10%</td>
<td>Similar</td>
<td>Small, “lens-like” nuclei, mucin-filled acini. Rows of cells sandwiched between collagen fascicles, poorly defined margins</td>
</tr>
<tr>
<td>Tubular carcinoma</td>
<td>5%</td>
<td>Good</td>
<td>Highly differentiated, Low recurrence rate, low potential for metastasis,Generally ER positive</td>
</tr>
<tr>
<td>Invasive cribriform carcinoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Mucin positive, laminin negative cystic appearance. Luminal epithelial ultrastructural appearance.</td>
</tr>
<tr>
<td>Medullary carcinoma / Atypical medullary carcinoma</td>
<td>6%</td>
<td>Good</td>
<td>Predominant syncytial growth pattern, featuring broad anastamosing sheets, microscopically complete circumscription, marked mononuclear stromal infiltrate, nuclear grade II or III.</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>&lt;5%</td>
<td>Good</td>
<td>Monomorphic ductal epithelial tumor cells “floating” in abundant background mucin pool, comprising more than 90% of tumor. Low rate of metastatis, generally hormone receptor positive.</td>
</tr>
<tr>
<td>Cystadenocarcinoma and columnar cell mucinous carcinoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Predominantly tall, columnar mucinous epithelium. Presence of both intracellular and extracellular mucin</td>
</tr>
<tr>
<td>Signet ring cell carcinoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Abundant intracellular mucin. Metastasis to the peritoneum, gastrointestinal tract, lung, and gynecologic organs.</td>
</tr>
<tr>
<td>Neuroendocrine tumors</td>
<td>&lt;1%</td>
<td>Varies</td>
<td>Positive IHC for neuroendocrine markers. Small cell / oat cell carcinoma is the most agressive type.</td>
</tr>
<tr>
<td>Invasive papillary carcinoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Fibrovascular stromal core lined by epithelial and myoepithelial cells, attached to the wall of the duct and extending into the duct lumen</td>
</tr>
<tr>
<td>Invasive micropapillary carcinoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Micropapillary epithelial architecture surrounded by lymphatic duct–like empty space. Aggressive behavior with frequent lymph node metastasis</td>
</tr>
<tr>
<td>Apocrine carcinoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>ER, PR negative. Elevated expression of Androgen Receptor, psoriasin, S100A9, and p53. Low proliferative index.</td>
</tr>
<tr>
<td>Metaplastic carcinomas</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Tumor cells display differentiation along multiple lineages. Generally aggressive clinical course, haematogenous metastasis pattern (lung and bone).</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Presence of cells with squamous differentiation forming a keratinizing tissue, frequent lymph node metastasis, rapid growth</td>
</tr>
</tbody>
</table>

All WHO-recognized histological types and subtypes for which there is sufficient published data to make statements about prognosis are shown. Prognosis shown is relative to not otherwise listed invasive ductal carcinomas.
<table>
<thead>
<tr>
<th>Type / Subtype</th>
<th>Frequency</th>
<th>Prognosis</th>
<th>Description / characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosquamous carcinoma</td>
<td>&lt;1%</td>
<td>Similar</td>
<td>Combination of glandular and squamous differentiation</td>
</tr>
<tr>
<td>Mucoepidermoid carcinoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Nesting pattern with multiple well-circumscribed squamous nests containing numerous clear cells. Prominent mucin-secreting component frequently present. Myoepithelial differentiation common.</td>
</tr>
<tr>
<td>Mixed epithelial/mesenchymal metaplastic carcinomas</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Tumor cells display epithelial (carcinoma) and mesenchymal (sarcoma) differentiation. Generally aggressive clinical course.</td>
</tr>
<tr>
<td>Secretary carcinoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Frequent presence of oncogenic ETV6-NTRK3 fusion kinase. Most common in children.</td>
</tr>
<tr>
<td>Glycogen-rich clear cell carcinoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Finely granular eosinophilic cytoplasm or foamy to clear cytoplasm with well-defined cytoplasmic membranes, moderate to marked nuclear pleomorphism with prominent nucleoli.</td>
</tr>
<tr>
<td>Inflammatory carcinoma</td>
<td>&lt;10%</td>
<td>Poor</td>
<td>Presence of skin erythema and oedema. Rapidly growing tumors that are highly angiogenic, angio-invasive, numerous tumour emboli filling the dermal lymphatics causing inflammatory signs.</td>
</tr>
<tr>
<td>Myofibroblastoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Densely packed large cells with a solid or trabecular growth pattern, focally arranged in nests, with abundant eosinophilic glassy cytoplasm and sharp cellular borders. Large and round nuclei, prominent nucleoli. P63 and vimentin positive.</td>
</tr>
<tr>
<td>Inflammatory myofibroblastic tumor</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Large cohesive sheets of histiocytes intermingled with clusters of spindle cells and mixed inflammatory cells. Lack of granulomas and ductal epithelial cells.</td>
</tr>
<tr>
<td>Granular cell tumor</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Granular cytoplasm. Fat necrosis, duct ectasia.</td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>Schwann cell origin. Composed of spindle cells with thin, often wavy nuclei. S100 positive, generally well-circumscribed.</td>
</tr>
<tr>
<td>Schwannoma</td>
<td>&lt;1%</td>
<td>Good</td>
<td>S100 positive. Compact and spongy biphasic pattern, nuclear palisading, interlacing bundles of numerous elongated spindle shaped cells.</td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Rapid growth. Malignant endothelial cells lining vascular channels are flat, and most nuclei are pale and small. Contains anastomosing vascular channels that surround and invade lobules.</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>&lt;1%</td>
<td>Variable</td>
<td>Mature adipocytic proliferation, variable cell size, focal nuclear atypia of adipocytes and stromal cells.</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>&lt;1%</td>
<td>Poor</td>
<td>Osteoid production present. Broad eosinophilic seams were that may display mature bone formation. Frequent mitoses, early recurrence, haematogenous metastasis pattern (lung and bone).</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>&lt;1%</td>
<td>Similar</td>
<td>Pleomorphic spindle cells, hyperchromatism and elongated nuclei, eosinophilic cytoplasm, large nucleoli and numerous mitoses.</td>
</tr>
</tbody>
</table>
In addition to their prognostic value, these classifications have implications for targeted therapies that will grow more important as understanding of tumor signaling pathways increases. Indeed, larger, more refined gene expression profiling studies centered around analysis of kinase gene expression can not only differentiate the subtypes previously described, but predict prognosis within the luminal A subtype (106). Many of these kinases, which were selected in an unbiased fashion for their ability to discriminate between the subtypes, are already therapeutic targets under investigation while others are potential targets.

Table 2. Molecular subtypes of invasive ductal carcinoma.

<table>
<thead>
<tr>
<th>IDC subtype:</th>
<th>Highly expressed genes:</th>
<th>Weakly expressed genes:</th>
<th>Average relative prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal breast-like</td>
<td>Adipose tissue genes Basal epithelial genes</td>
<td>Luminal epithelial genes</td>
<td>Good</td>
</tr>
<tr>
<td>Luminal A</td>
<td>ERα Gata-3 TFF3 Keratin 8/18 Luminal epithelial genes</td>
<td>Basal epithelial genes Non-epithelial genes</td>
<td>Best</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Luminal epithelial genes</td>
<td>ER cluster (moderate expression)</td>
<td>Average</td>
</tr>
<tr>
<td>Luminal C</td>
<td>Luminal epithelial genes Subset of basal epithelial genes of unknown function</td>
<td>ER cluster (moderate expression)</td>
<td>Poor</td>
</tr>
<tr>
<td>Basal-like</td>
<td>Keratin 5 Keratin 17 FABP7 Laminin</td>
<td>ER cluster PR ERBB2 cluster</td>
<td>Worst</td>
</tr>
<tr>
<td>ERBB2+</td>
<td>ERBB2 Others in ERBB2 amplicon</td>
<td>ER cluster</td>
<td>Poor</td>
</tr>
</tbody>
</table>
1.4 Basal-like carcinomas

Basal-like carcinomas are a subset of invasive ductal carcinomas that consistently have the poorest prognosis of all breast carcinomas whether grouped by histological appearance or molecular signature (104, 106-108). Basal-like carcinomas are also more aggressively metastatic than other invasive ductal carcinomas (109, 110). While recent studies have provided a fairly robust molecular definition for basal-like carcinomas, tumors displaying basal / myoepithelial differentiation were first described in the 1970s (111, 112). The basal phenotype is defined histologically by pushing margins, frequent central necrosis and lymphatic infiltrate, expression of high molecular weight keratins such as 5, 14 and 17, overexpression of EGFR / HER1, and frequently triple negative status for ER, PR, and ERBB2 / HER2 (113, 114). On the molecular level, basal-like breast carcinomas frequently feature inactivation of the Rb tumor suppressor pathway, up-regulation of slug and other EMT-like changes (113-117). These abnormalities are frequently present in the in situ lesion (118-120), and may explain why basal-like carcinomas are highly sensitive to some treatments despite their poor prognosis (121, 122). It has been proposed that basal-like carcinoma may have a more stem-cell-like phenotype, due to the significant overlap in their respective gene expression profiles (114, 123, 124). However, there is no published evidence that basal-like carcinomas have functionally greater stem cell capacity than luminal tumors, and the basal phenotype has an inverse correlation with some stem cell markers (108). Basal-like tumors have a more consistent phenotype in terms of metaplastic EMT-like elements. While the relevance of strictly-defined EMT to human breast carcinogenesis remains controversial (125), evidence is mounting that transient EMTs or EMT-like events are real phenomena that
are associated with metastasis, invasion, disease progression (114, 117, 126-132). These findings challenge assumptions made about the biology of basal-like carcinomas, and demonstrate the need for additional study into their molecular origins to develop targeted therapies and animal models for basal-like tumors, which are thus far confined to BRCA1 models (133, 134).

1.5 Epithelial-mesenchymal transition

The importance of epithelial-mesenchymal transitions in embryonic development has been appreciated since work done in the laboratory of Elizabeth Hay in 1967 (135), when the role of EMT in gastrulation was established. Since that time, multiple studies have revealed a crucial role for EMT in the patterning of multiple mature and embryonic tissues including the heart, palate, and neural crest (136-139). During gastrulation, epiblast cells ingressing through the primitive groove undergo EMT and migrate into the blastocoel to form the mesoderm (140), until Noggin-mediated suppression of BMP signaling halts gastrulation (138). Blockage of EMT via disruption of BMP signaling leads to failure in either mesoderm or neural crest formation (141-143), demonstrating the centrality of EMT to these processes. During heart development, endocardial cushion mesenchyme formation occurs via EMT in cells residing in the atrioventricular (AV) canal, which invade into the underlying cardiac jelly and form the septal and valvular primordia via process dependent upon cross talk between the TGF-beta and Notch pathways (144-149). TGF-beta3 induced EMT is necessary for palate fusion, and involves induction of a LEF1-Smad2-Smad4 transcriptional complex that represses E-cadherin and up-regulates vimentin and fibronectin in a beta-catenin independent fashion.
A central feature of EMT during gastrulation and other developmental events, as well as in pathological EMTs, is loss or suppression of E-cadherin and other epithelial adhesion molecules (127-130, 137, 139, 154-158) and acquisition of mesenchymal markers such as vimentin and N-cadherin (131, 159-162). These gene expression changes have functional consequences as in the case of cadherins, which have different adhesion strengths that can affect invasion and metastasis (163).

EMT is thought to be associated with the most aggressive cancers (117), and is likely to be more heterogenous in manifestation than EMTs in developmental settings, leading to the proposal that EMT-like events in tumor cells generate “metastable” cells that are an abnormal blending of epithelial and mesenchymal phenotypes and transcriptomes (164). This hypothesis is supported by the involvement of EMT-related factors in the metastasis of epithelioid cancers (126, 127, 162, 165-170), the similarity in phenotype of metastatic nodules to the parent tumor (171), and the dynamic EMT-MET processes known to be involved in bladder and colorectal cancer metastasis (172-175). These observations suggest that metastatic cells must shed enough of their epithelial nature to escape their tissue of origin, but retain the ability to become epithelioid once again to facilitate extravasation and survival in the new tissue.

Transcriptional control of EMT is complex, and in developmental settings typically begins with receipt of local molecular signals such as TGF-beta receptor ligands (141-143, 147) Wnts (176, 177), FGFs (178-181), and Notch ligands (149). TGF-beta and BMP signaling results both in broad chromatin remodeling via HMGA2 (182, 183)
and the assembly of numerous transcriptional complexes on pro-EMT gene promoter regions. These complexes include, but are not limited to Smad/LEF, Ets1, FoxC2, and MRTF-containing complexes, which drive expression of genes that suppress E-cadherin and other epithelial proteins while upregulating those associated with mesenchyme (137, 152, 184-187). The list of E-cadherin repressors is long and includes Snail family members, Twist, Zeb proteins, EZH2, and E47 proteins, as well as many others, which are thought to act in concert \textit{in vivo} to effect E-cadherin repression (127-129, 161, 182, 188). One or more of these proteins are the eventual targets of all EMT-promoting signaling pathways.

\textbf{1.6 Transforming growth factor (TGF)-beta signaling}

TGF-\(\beta\) signaling (Fig. 2) begins with the synthesis of one of 30 or more TGF-\(\beta\) superfamily preprotein dimers, all of which must be proteolytically processed to be secreted (189). The TGF-\(\beta\) superfamily includes bone morphogenetic proteins (BMPs), TGF-\(\beta\) s, activin beta-chains, growth and differentiation factors (GDFs), and the protein nodal of which are typically secreted as homodimers, although some members can form heterodimers (190, 191). While most TGF-\(\beta\) superfamily members can be proteolytically processed after secretion, only GDF8, GDF11, and the TGF-\(\beta\) s require post-secretion processing to initiate signal transduction, which is performed by proteases such as BMP1, MMP2, MMP14, plasmin, or elastase (192-195). TGF-\(\beta\) s are secreted in a form known as the large latent complex, in which TGF-\(\beta\) and its prodomain are bound by latent TGF-\(\beta\) binding proteins (LTBPs) and subsequently targeted to the ECM (193). After secretion
and in some cases proteolytic release of the mature, active ligand, TGF-β superfamily members bind their cognate receptors and induce formation of heterotetrameric
complexes that then activate through autophosphorylation (196-198). These tetrameric complexes form from preexisting dimers of type I and type II TGF-β receptors, whereupon the constitutively active type II receptor dimer activates the type I receptor via phosphorylation (197, 198). TGF-β pathway activation can be stifled at this level by ligand-sequestering antagonists such as Chordin, Noggin, and Cerebus, which can either modulate the TGF-β signal strength or block it altogether. Activated receptor complexes recruit and activate members of the SMAD family of transcription factors (SMADs 1, 2, 3, 5 and 8), which upon phosphorylation by the receptor complex are able to interact with SMAD4, the common binding partner of all receptor-regulated SMADs. Individual SMADs are recruited by specific type I TGF-β receptors; SMADs 1, 5, and 8 are phosphorylated by ALK1, 2, 3, and 6, while SMADs 2 and 3 are phosphorylated by ALK4, 5, and 7. SMAD complexes can act as transcriptional activators or repressors, depending upon phosphorylations and other modifications to SMADs that determine which proteins are recruited to target promoters. All SMADs with the exception of SMAD2 directly bind DNA, but are unable to recruit the basal transcriptional machinery, and act via chromatin remodeling enzymes such as SWI-SNF, p300, and CBP. In the absence of TGF-β pathway activation, SMAD response elements are often occupied by the transcriptional repressors SKI and SNON, which are rapidly degraded by the ubiquitin ligase Arkadia to allow for SMAD binding.

In addition to the receptor associated SMADs and the co-SMAD, SMAD4, the inhibitory SMADS (I-SMADS) SMAD6 and SMAD7 provide feedback inhibition for TGF-β signaling. I-SMADs are induced by R-SMAD transcriptional activity and
attenuate TGF-β pathway activation through several mechanisms such as competition with R-SMADs for receptor binding, recruitment of SMURF ubiquitin ligases to activated TGF-β receptors resulting in their degradation, recruitment of phosphatases to active receptors causing their deactivation, and competition R-SMADs for binding to SMAD response elements in target promoters. In addition to targeting genes involved in EMT such as E-cadherin and Vimentin, TGF-β receptor stimulation in untransformed cells elicits cell cycle arrest via SMAD/Sp mediated induction p15(Ink4b)(199, 200). This cytostatic induction of p15 is mediated by the transcription factor C/EBP-β and is required for the tumor suppressive effects of intact TGF-β pathway function (201, 202).

1.7 The Wnt / β-catenin pathway

Wnt glycoproteins are the ligands for Frizzled receptor complexes, which consist of frizzled receptors and low density lipoprotein receptors. While Wnt/Frizzled signal transduction (Fig. 3) has numerous downstream effectors that contribute to EMT, the majority of its output proceeds through either the canonical (β-catenin) or planar cell polarity (PCP) pathway. β-catenin normally resides at cell-cell junctions in a dynamic complex with other catenins and cadherins. When β-catenin is freed from these membrane-associated complexes, it ordinarily resides in a cytoplasmic pool that is targeted for destruction by a complex containing the scaffolding protein Axin and the Adenomatous polyposis coli (APC) tumor suppressor (203). Upon receipt of Wnt signals, β-catenin residing in this pool is stabilized by a mechanism that is poorly understood but appears to involve all 3 human homologs of the drosophila protein dishevelled in non-overlapping roles (204).
Figure 3. Wnt-frizzled signaling. Engagement of the Wnt ligand by Frizzled receptors results in stabilization of cytoplasmic β-catenin, which is a potent co-activator of LEF/TCF transcription factors. β-catenin normally exists in dynamic membrane-bound complexes with other catenins and cadherins, with some exiting to form a cytoplasmic pool that is targeted for proteasomal degradation by the Axin / APC / GSK-3β complex, which phosphorylates β-catenin priming it for ubiquitination. p68 RNA helicase promotes β-catenin stabilization by blocking this phosphorylation event. Wnt/Frizzled signaling also acts in β-catenin-independent signaling modes such as through promotion of PKC-mediated phosphorylation and activation of Snail.
The RNA helicase p68, which blocks phosphorylation of β-catenin in response to PDGF to promote EMT, may also play a role in Wnt-induced EMT or similar proteins may act in the fashion or p68 (205). Stabilized β-catenin translocates to the nucleus, where it acts as a potent co-activator for the LEF/TCF family of transcription factors, leading to EMT (206-210). Wnt signaling can also lead to EMT through β-catenin-independent pathways. Wnt5A induces EMT via PKC-dependent induction of Snail, even in the presence of dominant-negative TCF4, demonstrating that Wnt signaling promotes EMT via several pathways besides β-catenin (211). Similar to the action of the BMP antagonist Noggin in the TGF-β pathway, secreted inhibitors such as the Dickkopf proteins attenuate and modulate Wnt signal strength in both β-catenin-dependent and independent Wnt signaling modes (212-214). Wnt signaling also exhibits extensive cross-talk with other EMT-promoting pathways, most notably the Notch pathway (215-217).

1.8 Notch and EMT

Notch signaling (Fig. 4) is relatively simple compared to other EMT-related pathways such as TGF-β and Wnt / Frizzled signaling, and has considerable cross-talk with both, being required for TGF-β-induced EMT in some cases (218). Notch signaling, similar to the TGF-β and Wnt / Frizzled pathways, begins with engagement of a ligand - Delta, Lag, or Serrate - by the Notch receptor, triggering cleavage of Notch and release of its intracellular domain (219). Cleavage of membrane-bound Notch occurs in two steps, the first step is catalyzed by ADAMs family metalloproteases and results in release of the extracellular domain of Notch (220-222). Step two is carried out by the activity of the
presenilin-nicastrin-Aph1-Pen2 protein complex, which is more commonly referred to as 7-secretase, and releases the intracellular domain of Notch (NICD) which translocates to the nucleus (223). Freed NICD displaces co-repressors such as NcoR, SMRT and SHARP from their interaction with Notch target-gene bound CSL, clearing the way for recruitment of co-activators such as Mastermind (224-230). During EMT, the most important Notch targets appear to be the E-cadherin repressors snail and slug. Snail and slug are up-regulated directly via association of the NICD with their promoters and Snail indirectly via induction of lysyl oxidase, which stabilizes Snail via phosphorylation (149, 231, 232).

1.9 Snail

Snail promotes EMT in response to all the major pathways known to elicit EMT, such as the Wnt, Notch, TGF-β, and FGF signaling pathways (176, 182, 208, 211, 231, 233). Snail's function in EMT is centered around its role as a repressor of E-cadherin gene expression (234, 235), although it also targets other epithelial genes such as ERα, MUC1 and tight junction proteins (236-238). Snail effects repression of these genes by recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex and C-terminal binding protein (CtBP) (239-241). Snail also promotes cell survival via induction of cell cycle arrest (242-244), and suppression of the PTEN phosphatase (245). Consistent with the aggressive, metaplastic nature of many ERα- tumors, Snail expression is restricted by estrogen signaling (246), suggesting the existence of a Snail-ERα regulatory loop that governs cellular plasticity. ERα signaling represses Snail indirectly by activating transcription of MTA3, a component of the Mi-2 / NuRD
transcriptional repressor, which then targets Snail (246). Like many other factors involved in EMT, Snail is associated with metastasis, recurrence, and poor prognosis (247-249).

Figure 4. Notch signaling. Binding of a Delta - Serrate - Lag2 family ligand by the Notch receptor triggers cleavage of the receptor and releases of the intracellular domain of Notch (NICD). Cleavage takes place in two steps, the first is an extracellular cleavage event mediated by TACE that primes the receptor for the second cleavage event in the transmembrane domain performed by the γ-secretase complex. Once released, the NICD translocates to the nucleus where it disrupts interactions between the transcription factor CBL and co-repressors such as NcoR and SMRT. Co-activators such as Mastermind and then free to bind to CBL, which constitutively resides on the promoter regions of Notch target genes.
1.10 Twist

Similar to Snail proteins (Fig. 5), Twist has been reported to promote EMT via suppression of E-cadherin (161, 182, 250, 251), and up-regulation of genes associated with mesenchyme such as N-cadherin, FGFR2, and cadherin 11 (187, 252, 253). While the mechanisms by which Twist activates or represses transcription has not been solved fully, it is clear that dimerization partner selection is the major determinant of Twist activity (252, 254). Twist homodimerization or dimerization with E2A E12 results in transactivation (252, 254), while partnering with Id proteins and other bHLH repressors results in repression (252). In addition to targets associated with EMT, twist has profound effects on cell survival and genome integrity (255, 256). Twist represses expression of p14 (ARF) while upregulating AKT2, leading to checkpoint failure, drug resistance, and genomic instability (257, 258). Twist is also a key mediator of drug resistance induced by NF-kB activation, and appears to regulate phosphorylation of Bcl-2 proteins (259).

Regulation of twist occurs mainly at the transcriptional level. Twist can be induced by Wnt, hypoxia, Notch, TGF-β, NF-kB, and VEGF, amongst other pro-EMT pathways and stimuli (162, 182, 259-261). Twist is a direct target of HIF1α and HIF2α and is associated with tumor vascularity, suggesting that Twist participates in angiogenesis and may promote metastasis via enabling access to the bloodstream as well as through EMT (162, 165, 262). In addition to its role in EMT and angiogenesis, Twist is associated with distant metastasis, recurrence, poor prognosis, and tumor vascularity (162, 165, 263-267), a common finding with transcription factors related to EMT.
Figure 5. Regulation of the E-cadherin repressors snail, slug, and twist in breast cancer. Snail, slug, and twist are induced by activation of multiple signaling pathways such as the Wnt/β-catenin, TGF-β, and notch pathways (black arrows), as well as by microenvironmental stimuli (hypoxia and inflammation, blue and red arrows, respectively). In breast tissue, estrogen signaling represses snail via MTA3 and is repressed by snail, consistent with the aggressive nature of ERα-negative tumors. The output that proceeds from these pro-EMT factors promotes motility and invasiveness through repression of cadherins and desmosomal proteins, as well as survival via repression of p14ARF and PTEN and up-regulation of AKT2.
1.11 Slug

The zinc finger transcriptional repressor slug (SNAI2) provides a link between EMT and acquisition of stem-cell like properties by tumor-initiating cells. Slug facilitates EMT downstream of multiple pathways such as the TGF-beta, notch, Wnt, and FGF pathways (182, 232, 268, 269), which promote maintenance of stem cell identity (270-276). Additionally, slug is a key player in stem cell biology, with important transcriptional targets unrelated to EMT such as PUMA and BRCA2 (124, 277-283). Consistent with the current thought on stem-cell like properties of tumors cells in basal-like carcinomas, slug is a driving force behind the basal phenotype (114). Slug also predicts poor prognosis and recurrence in multiple tumor types including breast cancer, which further suggests that slug is at the heart of the aggressive nature of basal-like carcinomas (127, 168, 169, 248). In the context of EMT, slug is best known as a repressor of E-cadherin expression (129). However, slug targets multiple epithelial genes such as high molecular weight keratins and desmosomal proteins to promoter cellular motility and suppression of the epithelial phenotype (158, 268, 284). Multiple studies have implicated slug in DNA damage responses, particularly those of stem/progenitor cells and transformed cells (124, 278, 279, 282, 283, 285). Slug promotes survival in the face of genotoxic stress, centered on its ability to stifle p53-mediated induction of the pro-apoptotic protein PUMA (278). This fact may explain the frequency of recurrence in basal-like carcinomas of the breast, which frequently display initial sensitivity to treatment but have high recurrence rates (122). Structurally, slug consists of a SNAG domain that effects transcriptional repression and five DNA-binding ZNF domains, and is the most conserved of all mammalian snail homologs (286). The SNAG domain, which is
common to all snail family proteins and multiple other oncogenes, functions as an interaction domain with scaffolding proteins to recruit macromolecular repression complexes to slug target genes (287). Examples of this include recruitment of Ajuba LIM proteins, which in turn recruit protein arginine methyltransferase 5 (PRMT5) to repress transcription (287-289), and recruitment of the Sin3A/Histone Deacetylase 1 (HDAC1)/HDAC2 repressor complex (239), both to the E-cadherin promoter. Several studies have shed light on the regulation of slug expression. The promoter region of SLUG contains multiple regulatory elements, including MyoD, TCF/LEF, AhR, E2A-HLF, and SMAD-responsive regions (184, 290-293), all of which facilitate transactivation of the SLUG gene. However, the factors that restrict inappropriate slug expression have thus far remained unidentified.

1.12 Comparison of pathological and developmental EMT

EMT in developmental contexts is tightly controlled in space and time (294). Often, likely always, multiple pathways associated with EMT induction are simultaneously activated, and crosstalk between them confers the precision needed for proper development of embryonic and mature tissues. An example of this precision can be found during gastrulation, which is tightly controlled by the BMP antagonist Noggin (138). Spatially and temporally controlled NOG expression brings gastrulation to an end in an appropriate time frame, while still allowing for EMT-like events to shape the tailbud. Mice mutant for NOG fail to bring a halt to the ingressive cell movements of gastrulation, and NOG null mice have severe skeletal defects and mesenchymal hyperplasias by the time of lethality on embryonic day 18.5 (295, 296). Additionally,
mice and humans haploinsufficient for NOG suffer from skeletal defects such as carpal and tarsal fusions, which highlights the necessity for an "off-switch" for non-pathologic EMT processes to function normally (296). In the developing heart, defects in NOG or the EMT "on-switch" BMP2 leads to defects in the endocardial cushions and derived structures, and proper functioning of both switches requires input from the notch, leptin, Wnt, and HGF signaling pathways to ensure appropriate expression of the downstream targets slug and snail (136, 148, 149, 296-298). This implies that one of the ways that EMT is suppressed in mature tissues is a requirement for convergence of multiple EMT-promoting pathways to initiate and sustain EMT, and may explain the rarity of EMT events observed in solid tumors, since activation of an EMT promoting pathway could be sufficient to drive tumor growth without reaching the signal threshold for EMT. That is, pro-EMT signals must hit a specific pitch before the anti-EMT signals are overridden and EMT occurs. Thus it appears one of three phenomena drive pathologic EMT: a relentless on-switch, a malfunctioning or absent off-switch, or the creation of new on-switches which results in enough "on" signal to drive EMT (Fig. 6).

Overexpression of EMT-promoting factors and loss of antagonistic factors has been reported in many cancers (173). An important example of a malfunctioning switch was recently elucidated by Gomis et al, when they discovered that loss of the LIP isoform of the transcription factor C/EBPβ eliminated the cytostatic effects of TGFβ in human breast tumors (202). This malfunctioning switch allows the cancer cells to receive the benefit of the pro-invasive properties of TGFβ and explains the biphasic action of TGFβ in tumors. As more intrinsic breaks and feedback loops on the EMT process are
uncovered, the apparently contradictory tissue specific roles of other EMT-related factors such as Notch may be understood more fully (299).

1.13 Pathologic vs. non-pathologic EMT: role of hypoxia

Two major EMT-promoting switches are present under pathologic conditions that are absent in developmental EMT: hypoxia and inflammation. Hypoxia is present even in microscopic tumors, and contributes to tumor progression, angiogenesis, and radioreistance (300-302). Hypoxia results in prolylhydroxylase-dependent stabilization, dissociation from the VHL ubiquitin ligase, and activation of hypoxia-inducible factor 1-\(\alpha\) (HIF1\(\alpha\)), as well as activation of HIF2\(\alpha\), both of which activate genes involved in angiogenesis as well as those involved in EMT (162, 303). HIF1\(\alpha\) directly binds the hypoxia response element (HRE) in the Twist1 proximal promoter region in response to hypoxia and induces EMT, an effect reversible by siRNA directed to Twist (162). HIF2\(\alpha\) has also been reported to transactivate Twist in response to hypoxia, but intriguingly HIF2\(\alpha\) targets intronic HREs that are not responsive to HIF1\(\alpha\) (262). This is particularly interesting in light of previous reports that HIF1\(\alpha\) and HIF2\(\alpha\) are differentially activated during acute and prolonged hypoxia in tumors, where high HIF2\(\alpha\) expression correlated with VEGF expression, advanced clinical stage, and poor prognosis (304). This would suggest that during initial acute hypoxia HIF1\(\alpha\) expression triggers angiogenesis and pro-survival Twist expression, while continued low-level hypoxia causes continued expression of Twist via HIF2\(\alpha\) and promotes EMT and metastasis. As HIF-driven EMT appears to be dependent upon Twist, arrival in a mature, oxygenated tissue may decrease Twist expression sufficiently to allow for MET and establishment of metastatic colonies.
Several other factors appear to be required for hypoxia driven EMT. The Notch signaling pathway is necessary to convert the hypoxic stimulus into a pro-EMT signal and promote Snail expression directly and via lysyl oxidase mediated phosphorylation of Snail (231). Additionally, increased expression of the urokinase-type plasminogen activator receptor (uPAR) and activation of cell signaling factors downstream of uPAR.
such as Akt and Rac1 have been shown to be required for full manifestation of the EMT phenotype in response to hypoxia (305). Blockage of either Notch or uPAR signaling in these studies was sufficient to halt hypoxia-induced EMT, and activation of Notch or overexpression of uPAR could substitute for hypoxia in EMT promotion. This is further evidence that EMT signaling must reach a threshold before EMT is initiated, and that tumor-associated hypoxia represents a pro-EMT signal that tilts the balances in favor of EMT but is not sufficient to initiate it alone, else nearly all solid tumors would display EMT. Hypoxia also contributes to pathogenic EMT in non-transformed cells, such as during kidney fibrosis (306, 307), suggesting that EMT and transformation are separate processes that can employ many of the same effectors.

1.14 Pathologic vs. non-pathologic EMT: role of inflammation

The most well known link between inflammation and cancer is the transcription factor NF-κB, which activates transcription of genes that promote inflammation, cell survival, EMT, and cellular transformation, both initiating and feeding forward in these responses (308). NF-κB mediates EMTs initiated by the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNFα) via induction of the E-cadherin repressors ZEB-1 and ZEB-2, and up-regulates Twist and Snail (259, 309, 310). In addition to activation by inflammatory stimuli, NF-κB-driven EMT can be activated by genotoxicants such as 7,12-dimethylbenz(a)anthracene (DMBA), and by hypoxia (311, 312). NF-κB can also be activated by loss of E-cadherin via p38 MAPK, providing a feed-forward mechanism for sustained EMT signaling (313). NF-κB can also feed forward on itself (314), suggesting another possible mechanism by which NF-κB signaling can sustain EMT. Ectopic
expression of supraphysiological levels of active NF-κB is also capable of inducing EMT (309), indicating that NF-κB activity represents another EMT on-switch that initiates EMT upon reaching of a threshold signal. Physiological levels of cancer-related NF-κB activity do appear to be sufficient to drive EMT on their own, given the prevalence of aberrant NF-κB activation and rarity of EMT in breast cancers (116, 132, 315). EMT in breast cancer is often associated with ERα negative tumors, which have poorer prognosis than ERα positive tumors (117). Therefore, it is not surprising that ERα represses expression of the RelB subunit of NF-κB (316). ERα is in turn suppressed by the NF-κB target Snail (238), which is itself a target of suppression by MTA3, a downstream effector of ERα (246).

Inflammatory NF-κB-mediated signaling pathways also have extensive cross-talk with other EMT-promoting factors such as Wnt, Notch, HIF1α, and TGF-β (317-323). NF-κB transactivates the HIF1α promoter, and is required for hypoxia-induced HIF1α accumulation (321). HIF1α returns the favor by promoting NF-κB activation via phosphorylation of IKK and NF-κB itself, suggesting cooperation between hypoxia and inflammation in pathogenic EMT (324). Inflammatory signaling pathways can also crosstalk with HIF1α in an NF-κB-independent fashion, as LPS-induced HIF1α activation appears to be NF-κB-independent but is sensitive to antioxidants (325), and processes that generate reactive nitrogen species can stabilize HIF1α through S-nitrosylation of HIF1α's oxygen-dependent degradation domain, preventing interaction with VHL (326). Knowledge of the extensive entanglements between the various EMT-promoting pathways has provided numerous potential therapeutic targets, however,
relatively little work has been done to elucidate the factors that restrain EMT in normal tissues and less advanced cancers.

1.15 SIM2

In the developing Drosophila CNS, specification of the CNS midline is dependent upon the basic helix-loop-helix Per-Arnt-Sim transcription factor single-minded (sim), which serves as the master regulator of midline differentiation (327, 328). Singleminded-2 (SIM2) is one of two vertebrate orthologs of the Drosophila sim (dsim) protein, but it differs in functioning as a transcriptional repressor (329-332). SIM2 was initially identified by positional cloning around the DS (DS) critical region of chromosome 21 and is believed to contribute to many of the physiological abnormalities associated with trisomy 21 (333). SIM2 functions as a heterodimer with ARNT, similar to drosophila counterpart dsim that dimerizes with the ARNT ortholog Tango to control expression of midline genes in the central nervous system (CNS) (334, 335). SIM2 is expressed in multiple tissues including the kidneys, lungs, brain, and breast, in both adult and developing tissues (329, 336-338). Sim2 plays an important role in development, as Sim2 null mice die shortly after birth due to multiple abnormalities, including cleft palate, improper diaphragm development, and rib defects (337, 338). The molecular mechanisms controlling these processes are complex and involve the concerted actions of many factors, and suggesting a master regulatory role for SIM2 in the many developmental processes that are severely disrupted in Sim2 null mice. Indeed, SIM2s, a splice variant of SIM2, was recently identified as a regulator of MMP3 gene expression, which plays a critical role in the development of the palate, mammary gland, and neuromuscular
junctions (15, 339-342). As the phenotype of MMP3 null mice is significantly different from and less severe than that of Sim2 null mice, it is clear than many more important developmental targets of SIM2 remain to be elucidated.

1.16 The Down syndrome (DS) tumor profile

Individuals with DS have a markedly different tumor spectrum than the general population. Specifically, leukemias are greatly increased while solid tumors are decreased in DS patients (343-350). Most striking is the profound protection women with DS enjoy from breast cancer, with as much as 25-fold protection compared to the non-DS population (344, 347-350). Recently, this solid tumor protection was proposed to be due to at least in part to gene dosage-mediated increases in ETS2 levels (351). However, this cannot explain the protection that women with DS enjoy, as there is overwhelming evidence that ETS2 acts as an oncogene in breast tissue by promoting tamoxifen resistance and suppressing the tumor suppressor BRCA1 (352-355). Therefore, other mechanisms must be invoked to fully explain the tumor spectrum of DS patients.

Due to its position in the DSCR of chromosome 21, and its roles in regulating responses to xenobiotics and hypoxia, SIM2 is a strong candidate for contributing to trisomy 21-related solid tumor suppression. Other laboratories have reported that SIM2s is oncogenic in the pancreas, prostate, and colon (356-359), a conclusion based largely on the observation that antisense oligos targeted to the 3’ untranslated region of the SIM2s mRNA resulted in apoptosis. However, as loss of tumor suppressors such as VHL can
lead to senescence (360), this conclusion requires mechanistic support, especially in light of the frequency of SIM2s loss in breast tumors (336).

1.17 The mechanism of SIM2-mediated tumor suppression

To attempt to shed light on the true nature of SIM2s as relates to cancer, we used a retroviral shRNA system to silence SIM2s in the relatively non-invasive breast cancer cell line MCF-7, which expresses high amounts of SIM2s relative to more invasive cell lines (336). Based on previous studies from MDA-MB-435 cells, we hypothesized that loss of SIM2s would confer enhanced invasiveness and tumorigenic properties. As expected, silencing of SIM2s lead to enhanced invasiveness, and also promoted EMT, xenograft tumor growth, and metastasis. Investigation into the mechanism underlying these observations revealed that the oncogenic E-cadherin repressor slug and the invasion-associated metalloprotease MMP2 are direct targets of SIM2s-mediated transcriptional repression. Furthermore, SIM2s binds the slug promoter directly and represses transcription, making it the first known repressor of slug expression. Sim2-/- mammary glands also displayed hallmarks of EMT such as E-cadherin loss and increased nuclear accumulation of β-catenin, suggesting that Sim2s' roles in regulating slug is conserved amongst mammals. Silencing of SIM2 also severely disrupted acinar morphogenesis in untransformed MCF-10A cells, but was not sufficient to induce EMT.

As slug plays an important role in stem cell biology and SIM2i cells were much more tumorigenic than their control "scrambled" (SCR) counterparts, we asked whether SIM2 loss affected self-renewal capacity or expression of stem cell markers. When
grown under low attachment conditions in normal growth media, SIM2\textsubscript{i} cells had a
decided advantage in self-renewal as measured by mammosphere formation, but this
effect was masked by supplementation with growth factors. Using commonly used
markers for tumor-initiating cells in MCF-7 populations, we found that SIM2\textsubscript{i} cells had a
significantly larger number of putative tumor-initiating CD29\textsuperscript{+} CD24\textsuperscript{lo} cells, consistent
with their enhanced tumorigenicity. In both stem cells and cancer cells, slug has been
reported to enhance cell survival in the face of DNA damage, and therefore we asked
whether depletion of SIM2\textsubscript{s} enhanced chemoresistance. MCF-7 cells infected with the
SIM2-specific virus (SIM2\textsubscript{i}) were resistant to doxorubicin, but not ionizing radiation
when compared to SCR cells. Additionally, we found that the reported slug target PUMA
was not induced in response to DNA damage in SIM2\textsubscript{i} cells, consistent with their high
levels of slug expression. Interestingly, SIM2\textsubscript{s} deficient MCF-7 cells also failed to induce
the cyclin-dependent kinase inhibitor p21/ WAF1/CIP1 under conditions where it was
robustly induced in SCR cells, suggesting a role for SIM2 in DNA damage signaling.
Further examination of the DNA damage response in SIM2\textsubscript{i} cells revealed that they were
p53 positive, and had extremely elevated constitutive p53 levels, with no mutations in
p53. While apparently insufficient to drive expression of p21 or PUMA, other p53 targets
such as MDM2 and NOXA were induced similarly in both cell types in response to DNA
damaging agents. ChIP analysis of the p21 promoter confirmed that SIM2 binds the p21
promoter near the p53 response element in response to doxorubicin and ionizing radiation
(R. Metz, unpublished results), raising the question of whether SIM2 and p53 interact
either directly or in a complex. Indeed, we observed a DNA-damage dependent
interaction between SIM2 and p53, which occurred with 4 hours of DNA damage. We
also found that SIM2 is itself stabilized by DNA damage, while its RNA expression remains unchanged. Our studies suggest strongly that SIM2 is a tumor suppressor, although it is possible that SIM2 has tissue-specific activities that make tumor-promoting in other tissues. Based on our data presented here and published work on slug, EMT, and basal-like carcinomas of the breast, we hypothesize that SIM2 loss contributes to the basal phenotype and may be a useful diagnostic marker for these tumors.
2.1 Cell line maintenance and drug treatment

MCF7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. MCF10A cells were maintained in DMEM/F-12 supplemented with 5% fetal bovine serum, 1% penicillin and streptomycin as well as 20 ng/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin and 10 µg/ml insulin. All cells were grown in 5% CO₂ at 37°C.

2.2 Plasmids

A 3,280 bp region of the human SLUG gene promoter region was amplified from human genomic DNA (Roche) using Accuprime Taq DNA polymerase (Invitrogen) and the primers F 5’-GCC TAT GCC ACA CTC TGG TT-3’ and R 5’-GGC GCC TCT GAA GTC ACC-3’ and cloned into the pGL3-basic luciferase reporter vector (Promega). The human SIM2s expression vector has been described previously (336). The human SIM2-specific shRNA constructs were generated by ligating a dsDNA insert designed by Ambion into their pSilencer U6-retro 5.1 shRNA vector. The dsDNA insert sequences were based upon Ambion pre-designed siRNA sequences #3116 (SIM2i #1), 114477 (SIM2i #2), and 114478 (SIM2i #3). The insert was created by annealing the oligos 5’-GAT CCG GTC GTT CTT TCT TCG AAT TTC AAG AGA ATT CGA AGA AAG AAC GAC CTC TTT TTT GGA AA-3’ (top) and 5’-AGC TTT TCC AAA AAA GAG GTC GTT CTT TCT TCG AAC TCT TT TCC AAT ATT CGA AGA AAG AAC GAC CG-
3' (bottom) for 3116; 5'-GAT CCG CAG TGA CCT TCT GTA CAC TTC AAG AGA
GTG TAC AGA AGG TCA CTG CTT TTT TGG AAA-3' (top) and AGC TTT TCC
AAA AAA GCA GTG ACC TTC TGT ACA CTC TCT TGA AGT GTA CAG AAG
GTC ACT GCG-3' (bottom) for 114477; and 5'-GAT CCG CGG GCA ACA GTA TTT
ATG ATT CAA GAG ATC ATA AAT ACT GTT GCC CGT GTT TTT TGG AAA-3'
(top) and 5'-AGC TTT TCC AAA AAA CAC GGG CAA CAG TAT TTA TGA TCT
CTT GAA TCA TAA ATA CTG TTG CCC GCG-3' (bottom) for 114478 to yield
overhangs compatible with the vector. The scrambled control vector was provided by
Ambion.

2.3 RNA isolation and real time RT-PCR

RNA was isolated using a Qiiaquick RNeasy Mini kit with Qiashredder columns
(Qiagen) and DNase digested (Roche). Two µg of RNA was reverse transcribed with
Superscript II Reverse Transcriptase (Invitrogen) with an oligo d(T)12-18 primer. Relative
quantitative PCR was performed using Sybr Green master mix and cDNA-specific
primers. TBP was used as the housekeeping gene. Data were collected using ABI 7500
system software, and analyzed by the △△C_T method. Primers used for real time RT-PCR
are shown in the table 3.
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All primers were designed based on the human NCBI reference mRNA sequence using primer express software from ABI and purchased from IDT.
2.4 Chromatin immunoprecipitation

ChIP assays were carried out as described by the manufacturer (Upstate Cell Signaling) with a few modifications. Briefly, cells were fixed with 1% formaldehyde for 10 minutes. Crosslinking was stopped by addition of 125 mM glycine. Cells were washed in the presence of protease inhibitors (Complete tablets, Roche), pelleted at 2000 rpm for 4 minutes at 4°C and resuspended in SDS lysis buffer with protease inhibitors (Complete tablets, Roche). Aliquots of 200 µl (1x10^6 cells) were sonicated for 10 pulses of 10 seconds each to shear chromatin to between 200 and 1500 bp. The supernatant was collected and diluted in ChIP dilution buffer to an appropriate amount. After pre-clearing twice with salmon sperm DNA/protein A agarose-50% slurry in TE/Na azide/0.1% BSA, the supernatant was incubated overnight with antibody at 4°C. Antibodies used were normal rabbit IgG (5 µg, Upstate), anti-Sim2 (Rb pAb, 5 µg, Chemicon), and anti-slug (Rb pAb, 5 µg, Santa Cruz). The salmon sperm DNA/protein A agarose-50% slurry was then used to collect the antibody complexes for 1 hour at 4°C. Agarose was pelleted at 1000 x g at 4°C for 1 minute and washed sequentially with low salt wash buffer, high salt wash buffer, lithium chloride wash buffer and TE buffer. The protein complex was eluted with 1% SDS, 0.1 M NaHCO₃, and the crosslinking was reversed in 200 mM NaCl at 65°C for 4 hours. Ethanol was added to precipitate the DNA, which was then resuspended in TE and digested with proteinase K for 1-2 hours at 45°C. DNA was purified using the Qiaquick PCR purification kit. PCR was performed with primers flanking the CME in the SLUG promoter region (5′- TGT GTC CAC GTG GCT CTA AG -3’ and 5′- CGC GTG CTA GCG AGT AAC A -3’), and sites 500-1000 bp upstream (5′-ATG CCC GCT CTG ACA ATT T-3’ and 5′-GGT GTG TGA AAA GCA GCA GTG).
CAA-3’), and downstream (5’-CCA GGT TTC CAG TTT GTG TG-3’ and 5’-TGT GTA TGG TCT TCA ATC TA-3’) of the CME primer set. Primers flanking the first two E-box elements in the E-cadherin promoter were: 5’- AAA AGC CCT TTC TGA TCC CA -3’ and 5’- TGG AGT CTG AAC TGA CTT CCG -3’.

2.5 Western blot

Protein was isolated using RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors and MG-132 added or with Nonidet-P40 (NP-40) buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 2 mM EDTA) with protease and phosphatase inhibitors. Protein was loaded and run on an 8% or 10% acrylamide gel at 110 V until the dye front reached the bottom of the gel. Protein was transferred to a PVDF membrane (Bio-Rad) at 100 mA for 2 hours or at 60 mA overnight in a cold room. Membranes were blocked in 5% milk and probed with the primary antibody for 2 hours at RT or overnight at 4°C. Membranes were washed in PBS, 0.1% Tween-20 and probed with the appropriate secondary antibody, anti-rabbit (Bio-Rad) or anti-mouse (Santa Cruz), at 1:5000 for 45 minutes at room temperature. Blots were again washed in PBS, 0.1% Tween-20 and developed with the ECL Plus Western Blotting Detection System (Amersham). All washing, blocking, and antibody incubation steps were performed with gentle rocking. Antibodies used for western blotting and immunostaining are summarized in table 4.
Table 4. Antibodies and conditions.

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<th>Antibody</th>
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N/A indicates that the antibody is suitable for the application but was not used or optimized.
2.6 Zymography

To determine MMP2 activity, conditioned medium from cells was concentrated ~20-fold using Centricon 10 spin concentrators (Amicon). Equal amounts of protein were mixed with Laemmli sample buffer without reducing agents, incubated for 15 minutes at 37°C, and separated on 8% polyacrylamide slab gels containing 1 mg/ml gelatin. Following electrophoresis, gels were placed in 2.5% Triton X-100 for 30 minutes then incubated at 37°C in 50 mM Tris-HCl, pH 7.4, containing 5 mM CaCl$_2$ for 18 hrs. MMP2 activity was visualized by Coomassie blue staining.

2.7 Stable transduction

HEK-293T amphotrophic Phoenix cells were transfected with 15 μg pSilencer U6-retro 5.1 retroviral vector (Ambion, Austin, TX) with SIM2-specific insert or a non-specific "scrambled" insert, or a non-retroviral plasmid (pcDNA3) for mock infection. After 24 hours, cells were placed at 32°C. Viral media was harvested 48 and 72 hours after transfection and used to infect cells after passage through a 0.45 μM filter. Infected cells were centrifuged at room temperature immediately after application of viral media, and housed at 32°C during infection. 24 hours after the second application of viral media, cells were rested for 24 hours in their respective normal growth media before initiation of puromycin selection. Selection was considered complete when all mock-infected cells had died.
2.8 Transient transfection

MCF-7 cells were used for all transient transfections. One hundred ng (0.1 μg) of plasmid containing transcription factor was mixed with 0.2 μg of plasmid containing promoter construct. Three μL of Genejuice (Novagen) was used per microgram of DNA. DNA and Genejuice were mixed in Opti-MEM media (Invitrogen). Protein was harvested 2 days after transfection, using Reporter Lysis Buffer (Promega). Luciferase activity and total protein were measured as described previously (336). Luciferase activities were normalized to total protein values and are represented as the means ± SE for three wells per condition.

2.9 Cell proliferation/death assay

Cells were seeded at 25,000 cells/well (MCF-7) or 10,000 cells/well (MCF-10A) in triplicate in 6-well plates containing selection medium. Cells were harvested and counted with a Coulter counter days 2-7 after plating. For drug-induced cell death assays, SCR and SIM2-depleted MCF-7 cells were seeded at 500,000 cells per well in 6-well plates. The next day cells were treated with 1 μM doxorubicin or 10Gy of ionizing radiation. Cells were counted, using a Coulter counter, in triplicate (3 wells) at the time of treatment and 24 hours intervals thereafter for 3 days.
2.10 Migration and invasion assays

Migration and invasion were measured using control and matrigel-coated invasion chambers (Falcon BD, Franklin Lakes, New Jersey). 12,500 cells were seeded in serum-free DMEM in the upper chamber with serum-containing medium in the lower chamber as a chemoattractant. After 20 hours at 37°C, cells were scraped from the upper chamber with a cotton swab, and the underside of the membranes were fixed in 100% MeOH, stained with DAPI, and counted. Percent invasion was calculated as per the manufacturer’s instructions.

2.11 Immunofluorescence

Cells were seeded on cover slips in 6-well plates. The following day the cells were fixed in 3.8% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 15 minutes at 4°C. The cells were blocked in 5% BSA for 45 minutes and then probed with primary antibody for 2 hours at room temperature. After washing in PBS, the secondary antibody was applied in blocking buffer for 45 minutes at room temperature. The secondary antibody was then washed off in PBS and 5μg/mL DAPI was applied for 5 minutes in the dark at room temperature. After thorough washing in PBS, Cells were mounted in Prolong Gold fluorescence mounting media without DAPI (Molecular Probes) and allowed to cure overnight before viewing. DAPI and fluorescent Alexa Fluor-conjugated secondary antibodies and phalloidin were purchased from Molecular Probes and applied according to the manufacturer’s instructions.
2.12 Immunohistochemistry

Mammary gland and xenograft tumor sections on Superfrost plus microscope slides were baked at 62°C for 30 minutes in an upright position then rehydrated by sequential washes in xylene and a series of graded ethanol washes. Antigen retrieval was performed for 10 minutes at 98°C in 0.01 mol/L sodium citrate buffer, pH 6.0, in a microwave oven. When appropriate, sections were incubated in 3% hydrogen peroxide for 6 minutes to block endogenous peroxidase activity. After a 45 minute block in 10% serum, the sections were incubated with the primary antibody overnight at 4°C or for two hours at room temperature. Secondary detection was performed with a fluorescent secondary antibody or the appropriate biotinylated secondary antibody, Vectastain ABC elite kit, and DAB (Vector Labs, Burlingame, CA).

2.13 Co-immunoprecipitation

Protein samples from MCF-7 cells were harvested in non-denaturing protein lysis buffer supplemented with protease and phosphostase inhibitors. 100 were pre-cleared twice with salmon sperm DNA/protein A agarose-50% slurry in TE/Na azide/0.1%BSA, the supernatant was incubated overnight with antibody at 4°C. Antibodies used were anti-Sim2 (1:100, Chemicon), normal rabbit IgG (1:1000, Upstate), and anti-NFκB p65 (1:100, Abcam). The salmon sperm DNA/protein A agarose-50% slurry was then used to collect the antibody complexes for 1 hour at 4°C. Agarose was pelleted at 1000 x g at 4°C for 1 minute and washed consecutively with low salt immune complex wash buffer, high salt immune complex wash buffer, lithium chloride immune complex wash buffer and TE buffer. The pellet was resuspended in 50 μl of 1X SDS-PAGE loading buffer,
boiled for 5 minutes and 10 μl of sample was run by SDS-PAGE. Western blotting was performed as described above, with anti-Sim2 and rabbit anti-NFκB p65 (1:1000, Abcam) primary antibodies.

2.14 Flow cytometry

MCF-7 cells bearing either a control (SCR) or SIM2-specific (SIM2i) shRNA expression construct were analysed by flow cytometry according to the protocol recommended by BD. Briefly, cells were washed, trypsinized, counted on a Coulter counter, and resuspended in PBS, 1% FBS at 2.5 million cells per ml. 250,000 cell aliquots of this suspension were incubated with 20 μl of experimental antibodies or isotype controls for 30 minutes at RT in the dark as recommended by BD. Cells were again washed, resuspended in PBS 1% FBS and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems) flow cytometer, equipped with a 15 mW air-cooled argon laser, using CellQuest (Becton Dickinson) acquisition software. List mode data were acquired on a minimum of 10,000 events falling within light scatter gates set to include cells while excluding dead cells and clumps of cells. Data analysis was performed in FlowJo (Treestar, Inc.) using forward and side light scatter to gate the cells. Antibodies and isotype controls used in flow cytometry experiments were purchased from Becton Dickinson and used according to the manufacturer's protocol.
CHAPTER III
LOSS OF SIM2s INDUCES EMT IN MCF-7 CELLS*

3.1 shRNA directed to SIM2s promotes EMT

Previously, we have shown that SIM2s expression is lost in human breast tumors and correlates inversely with breast cancer cell invasiveness (336). Furthermore, reintroduction of SIM2s into highly invasive cancer cells resulted in decreased proliferation, migration and invasion. To determine if loss of SIM2s is sufficient to cause progression, MCF-7 cells were transduced with either a non-specific “scrambled” control (SCR) shRNA retroviral construct, or one of three SIM2-specific shRNA constructs targeting different regions of the SIM2 mRNA (SIM2i_{114478}, and SIM2i_{3116} and SIM2i_{114477}, Fig. 7A). Significant reduction in SIM2s protein was observed in the SIM2i_{114478}, and SIM2i_{3116} cell lines (Fig. 7B), but not in SIM2i_{114477} (data not shown). In three independent infections, MCF-7 SIM2i_{3116} and SIM2i_{114478} cells showed significantly enhanced invasive ability (Fig. 6C) and underwent a morphological change from the cobblestone epithelial shape typical of MCF-7 cells to a spindly mesenchymal cell-like morphology (Fig. 7D), suggesting that loss of SIM2s expression had induced EMT.

*Part of the data reported in this chapter is reprinted with permission from “Loss of singleminded-2s in the mouse mammary gland induces an epithelial-mesenchymal transition associated with up-regulation of slug and matrix metalloprotease 2” by Laffin et al., 2008. *Molecular and Cellular Biology*, 2008 Mar;28(6):1936-46. Copyright 2008 American Society for Microbiology.
To confirm that the morphological changes observed with the loss of SIM2s was indeed an EMT, we utilized the SIM2i_{3116} MCF-7 cell line, which will be referred to from here on as SIM2i, to assay the expression of additional epithelial and mesenchymal cell markers. Western blots (Fig. 7E), differential interference contrast microscopy (Fig. 8A) and immunofluorescence analysis (Fig. 8B) confirmed that SIM2i cells lost expression of keratin 18 and E-cadherin and increased expression of N-cadherin and vimentin. Although SIM2i MCF-7 cells express mesenchymal markers and do not express E-cadherin, increased nuclear accumulation of β-catenin was not detectable by antibodies against the active, non-phosphorylated (S37, T41) form (data not shown). There was, however, a marked increase in nuclear accumulation of the phospho-S33, S37, T41 form of β-catenin (Fig. 8B), a phenomenon recently shown to be associated with poor prognosis in aggressive breast carcinomas (361). Changes in SIM2i protein levels were accompanied by dramatic decreases in keratin 18 and E-cadherin message and increased N-cadherin and vimentin mRNA, as measured by real-time RT-PCR (Fig. 8C). Together, these data indicate that loss of SIM2s in MCF-7 cells results in a morphological, functional and biochemical switch from an epithelial to a more mesenchymal-like phenotype.
3.2 SIM2i MCF-7 cells form rapidly growing ERα− tumors in nude mice

To assess the tumorigenic effects of SIM2s loss, we compared tumor forming ability of SIM2i cells to control cells using a nude mouse xenograft assay. SIM2i cells rapidly developed into tumors that were three times larger than controls by day 10 (Fig. 9A). SIM2i cell derived tumors maintained this size advantage throughout the experiment and were 6-fold larger than SCR-derived tumors with an average weight 7-fold higher than control tumors at the conclusion of the study seventeen days after injection (Fig. 9B). SIM2i tumors appeared to be more vascularized than control tumors (Fig. 9C), an observation that was confirmed by increased CD31 and VEGF immunoreactivity of SIM2i-derived tumor sections (Fig. 9D). Quantification of BrdU-positive cells from
xenograft tumor sections showed a 35-fold increase in SIM2i cell proliferation (Fig. 9D; SCR = 1.1% ± 0.543%, SIM2i = 35.1% ± 2.24%), suggesting a link between increased vascularity, cell proliferation and tumor growth in SIM2i-derived tumors. Similar to our in vitro studies (Fig. 8), SIM2i-derived tumors displayed signs of EMT with decreased E-cadherin and increased vimentin staining (Fig. 9D).

Figure 8. Conformation of EMT in SIM2i MCF-7 cells. (A) Differential interference contrast images showing detailed morphology of SCR and SIM2i MCF-7 cells. (B) Immunofluorescent staining of control (SCR, top panels) and SIM2i (bottom panels) MCF-7 cells corroborated the Western blot data showing that loss of SIM2s expression results in a switch from an epithelial to a more mesenchymal phenotype. In addition, increased nuclear staining of phosphorylated β-catenin, a phenomenon recently associated with poor breast cancer prognosis, can be seen in SIM2i cells. (C) Real time RT-PCR analyses of SCR and SIM2i MCF-7 cells for expression of Keratin 18, E-cadherin, N-cadherin and Vimentin mRNA. Data were obtained from three wells per group, analyzed by the ΔCT method and are expressed as the average fold difference ± S.E.M. * p>0.05.
Figure 9. Loss of SIM2s enhances in vivo tumorigenicity. SCR and SIM2i MCF-7 cells were mixed with Matrigel and injected into the flanks of nude mice to determine the effects of SIM2s loss on tumor growth (A) SIM2i MCF-7 tumors were significantly larger than control tumors by ten days post-injection (B) The average weight of SIM2i derived tumors was significantly higher than control tumors. (C) Representative tumors from SCR and SIM2i MCF-7 cells. Note the apparent increased vascularization in SIM2i-derived tumors, supported by CD31 and VEGF protein staining (D). BrdU staining indicated that SIM2i tumor cells divide at a significantly higher rate than SCR control tumors, which maintained their EMT phenotype in vivo (D). The lack of an exogenous estrogen requirement, corresponded with loss of ERα expression. Bar = 100μm. Data are represented as mean ± SEM, * indicates p<0.05, ** indicates p<0.005.
Normally, tumor development in the MCF-7 xenograft model requires exogenous estrogen to sustain growth. Therefore, we were surprised to see tumors develop so rapidly in the absence of estrogen. The accelerated rate of SIM2i tumor growth coupled with the apparent estrogen-independent nature of this growth prompted us to look at ERα expression. Indeed, ERα was undetectable in SIM2i-derived tumors by immunohistochemistry (Fig. 9D), or in SIM2i MCF-7 cells by either real-time RT-PCR (Fig. 10A) or western blot (Fig. 10B). Furthermore, SIM2i cells no longer responded to estrogen treatment as measured by induction of the estrogen-responsive genes PR and PS2 (Fig. 10C and 9D). The striking increase in SIM2i MCF-7 cell tumorigenesis confirms that loss of SIM2 has functional consequences in vivo and provides further evidence that SIM2s may represent an important hurdle to breast cancer progression.

Figure 10. Loss of ERα expression and estrogen responsiveness in SIM2i MCF-7 cells. (A) Quantitative real time PCR analysis of control (SCR) and SIM2i MCF-7 cells for ERα, RNA. Data were obtained from three wells per group, analyzed by the ΔΔCT method and are expressed as the average fold difference ± S.E.M. * p>0.05. (B) Western analysis of SCR and SIM2i MCF-7 cells corroborated the mRNA data and confirmed that loss of SIM2s expression results in loss of ERα. (C-D) Quantitative real time PCR analysis of vehicle (DMSO) and estradiol (E2) treated SCR and SIM2i MCF-7 cells indicated that estrogen-responsiveness is lost in SIM2i cells. Both progesterone receptor (C) and PS2 (D) mRNA induction following estradiol treatment was lost in SIM2i cells. Data are presented as the average fold difference ± S.E.M. * p>0.05.
3.3 MMP3 and Rac1b are not induced in MCF-7 cells upon loss of SIM2s and MMP inhibition does not affect the EMT phenotype

In previous studies utilizing MDA-MB-435 cells, we reported that MMP3 is a direct target of SIM2s-mediated transcriptional repression (362). MMP3 has also been
reported to be sufficient to initiate EMT via generation of reactive oxygen species and a splice variant of Rac1, Rac1b (363). Therefore, we hypothesized that up-regulation of MMP3 may contribute to the aggressive, invasive phenotype observed in SIM2s-depleted cells. We observed an increase in MMP3 promoter activity in the SIM2i cells, and SIM2s retained its ability to repress the MMP3 promoter in a dose-dependent fashion in MCF-7 cells (Fig. 11A and 10B), consistent with previous results. However, in contrast to our
findings in MDA-MB-435 cells, MMP3 mRNA expression was not significantly altered (Fig. 11C), and MMP3 protein expression and activity were unchanged (Fig. 11D), suggesting that this apparently inconsistent result is due to intrinsic differences in basal MMP3 expression between MDA-MB-435 and MCF-7 cells.

3.4 SIM2s represses MMP2 expression and activity

As previous work had suggested that SIM2s may regulate several MMPs, we measured expression of several MMPs and related factors in SCR and SIM2i cells by real time RT-PCR to determine if another MMP could be playing a similar role to MMP3 in EMT promotion. We found MMP2 to be up regulated >4,500-fold in SIM2i cells, while others including MMP9 were unchanged (Fig. 12A and not shown). MMP14 and TIMP2, which are required for MMP2 activation, were also up regulated approximately 6- and 7-fold, respectively, in SIM2i cells (Fig. 12A). The MMP2 mRNA increase observed in SIM2i cells was corroborated at the protein and activity levels (Fig. 12B). In addition, MMP2 levels were markedly elevated in xenograft tumor sections derived from SIM2i cells in comparison to those derived from control cells (Fig. 12C), confirming that loss of SIM2s lead to increased MMP2 expression.
Increased MMP2 expression was attributable to de-repression of the MMP2 promoter, as expression of an MMP2-controlled luciferase reporter was significantly higher in SIM2i cells (Fig. 12D). Co-transfection of the MMP2 reporter and increasing amounts of SIM2s expression vector into MCF-7 cells resulted in dose-dependent inhibition of luciferase activity (Fig. 12E). These results suggested that MMP2 could be playing a role in EMT regulation similar to MMP3, however, we could not modulate expression of any EMT-related factors in MCF-7 with MMP inhibitors or free radical scavengers, and Rac1b expression was lower after the onset of EMT (data not shown). This implies that MMP2 does not regulate EMT in MCF-7 cells, and that protease-mediated mechanisms are not involved in the EMT observed after SIM2 depletion. Recently, other studies have also shown that MMP activity is not required for EMT (136). These findings suggest that regulation of MMPs by SIM2s is important for its tumor suppressor function but is not the mechanism by which SIM2s regulates EMT-like processes.
Figure 13. SIM2i binds and represses expression from the Slug promoter. (A) Slug, but not Snail, mRNA levels are significantly reduced following loss of SIM2i expression in MCF-7 cells. (B) Increased Slug mRNA observed in SIM2i cells corresponded to significantly elevated Slug protein levels, as measured by Western blot. (C) Expression of a Slug promoter-controlled reporter was significantly increased in SIM2i/MCF-7 cells. Data are represented as mean ±SEM, * indicates p<0.05. (D) SIM2i-mediated repression of Slug is dose-dependent as increasing amounts of SIM2i expression vector repressed expression of the Slug reporter. Data are represented as mean ±SEM, * indicates p<0.05, ** indicates p<0.005. (E) Several putative regulatory regions are present in the first 3,280 bp of the Slug promoter (top). ChIP analysis using an antibody to SIM2 was used to ascertain SIM2i binding to the Slug promoter in SCR and SIM2i MCF-7 cells. Precopterted chromatin was assayed for the presence of Slug promoter DNA surrounding a region containing a CME and multiple XRE consensus sequences using primers indicated by arrows in the promoter schematic. SIM2i protein was detectable in this region of the Slug promoter in SCR cells only. Binding of SIM2i was not observed in regions 0-1000 bp upstream or downstream of the CME region. XRE: xenobiotic response element, CME: central midline response element, LEF-TCF: lymphoid enhancer factor / T-cell factor response element. (F) Slug and SIM2i binding to the E-cadherin promoter was analyzed in SCR and SIM2i MCF-7 cells by ChIP. A schematic representation of the human E-cadherin promoter from -287 to +56 with respect to the transcriptional start point shows putative E-box, CCAAT enhancer binding protein and SP1 regulatory regions as well as the relative locations of primers used to assay binding. SIM2i binding to the E-cadherin promoter was detectable in SIM2i cells, but not in SCR cells. In contrast, SIM2i binding to the E-cadherin promoter could not be detected in either cell line. Data are represented as mean ±SEM, * indicates p<0.05, ** indicates p<0.005.
3.5 SIM2s participates in maintenance of E-cadherin expression via repression of slug

We next explored the possibility that SIM2s may regulate other factors involved in EMT. Members of the SNAIL family of transcription factors have been implicated in regulation of both normal and pathological EMT events (242, 364-366). During cancer progression, increased SNAIL binding to E-box regulatory DNA elements causes transcriptional repression of E-cad, which contributes to transformation, angiogenesis and EMT (242, 364-366). To determine if members of the SNAIL family are involved in the EMT induced by SIM2s loss, SCR and SIM2i cells were analyzed for snail and slug mRNA levels by real time RT-PCR. SNAIL mRNA levels were not significantly affected by SIM2 loss in MCF-7 cells (Fig. 13A). In contrast, slug expression was up regulated more than 250-fold in SIM2i cells (Fig. 13A), which corresponded to an increase in slug protein levels as determined by Western analysis (Fig. 13B).

Since SIM2s functions as a transcriptional repressor (18, 20, 25), we hypothesized that SIM2s directly inhibits slug expression at the promoter level. A luciferase reporter under control of the human SLUG promoter was transfected into SCR and SIM2i MCF7 cells and analyzed for activity. In SIM2i MCF-7 cells, basal reporter expression was increased 10-fold over control cells (Fig. 13C). In addition, we observed concentration-dependent repression of SLUG promoter-controlled gene expression with increasing amounts of SIM2s in MCF-7 cells (Fig. 13D). The human SLUG promoter contains multiple putative regulatory elements including E-boxes, xenobiotic response elements (XREs), and central midline element (CME) core sequences (Fig. 13E). Using an
antibody to SIM2 in ChIP analyses, we found that SIM2s binds a region of the SLUG promoter containing the CME and two XRE core sequences in SCR, but not SIM2i MCF-7 cells (Fig. 13E). Analysis with primer sets 500 bp upstream and 500 bp downstream of the CME demonstrate that the binding is specific to the CME region (Fig 12E). SIM2s was unable to bind a region of the E-cadherin promoter containing E-boxes that are targeted by SNAIL protein family members; however, increased binding of SLUG to this region was apparent in SIM2i MCF-7 cells (Fig. 13F). As the ChIP primers used in our study flank the putative CME and two XREs, it seems likely that SIM2s-mediated repression of SLUG involves binding of SIM2s to one or more of these elements. Taken together, these data suggest that loss of SIM2s leads to de-repression of SLUG expression and increased slug binding to the E-cadherin promoter where it potentially plays a role in repressing E-cadherin transcription.

3.6 Sim2<sup>-/-</sup> mammary glands display hallmarks of EMT

Our initial analyses of Sim2<sup>-/-</sup> mammary outgrowths, coupled with in vitro MCF-7 cell studies, strongly suggest that Sim2s restricts tumor progression associated with EMT-like events. To confirm this in vivo, Sim2<sup>-/-</sup> mammary sections were analyzed for various epithelial and mesenchymal markers. Uniform polarity (measured by Aquaporin-5 staining) and robust E-cadherin staining was observed throughout wild-type mammary epithelium, but was totally absent in Sim2<sup>-/-</sup> glands (Fig. 14A). Not surprisingly, the increased invasive ability of Sim2<sup>-/-</sup> epithelial cells was associated with increased Mmp2 protein levels (Fig. 14A). These observations strongly suggest that an EMT similar to that observed in SIM2i MCF-7 cells had occurred in Sim2<sup>-/-</sup> glands. This was further
supported by increased nuclear accumulation of β-catenin in Sim2−/− ductal epithelium (Fig. 14A). Consistent with our model that SIM2s-mediated repression of SLUG suppresses EMT, we found increased slug staining in Sim2−/− glands (Fig. 14A). Taken together, these data show that loss of Sim2s during mouse mammary gland ductal development results in slug up regulation, loss of epithelial cell characteristics, increased Mmp2 expression and invasion into the surrounding stroma, reminiscent of an EMT.

![Figure 14. Loss of Sim2s in the mouse mammary gland results in a phenotype consistent with EMT. Analysis of mouse mammary outgrowths from WT and Sim2−/− mice revealed that loss of Sim2s expression is associated with events including disrupted polarity as measured by aquaporin5 expression, loss of E-cadherin, up regulation of Mmp2 and increased nuclear accumulation of β- catenin. Consistent with our hypothesis that Sim2s-mediated down regulation of Slug represses EMT, we found that slug protein levels were significantly elevated in Sim2−/− mammary glands.]

3.7 Silencing of SIM2 in normal breast-derived cells results in increased motility, invasiveness, and abnormal 3D acinar morphogenesis

EMT is rarely observed in breast tumors, typically in the most advanced metastatic cancers. Therefore, we asked whether silencing SIM2 in non-tumorigenic MCF-10A cells was sufficient to cause cellular transformation and / or EMT. SIM2
Figure 15. Silencing of SIM2 in MCF-10A Cells Disrupts Acinar Morphogenesis. (A) Western blot analysis of SIM2, E-cadherin, and Vimentin in Scr and SIM2i MCF-10A cells, demonstrating efficacy of knockdown and modest Vimentin expression. (B) Boyden invasion chamber assay comparing Scr and SIM2i MCF-10A cells. (C) Real-time RT-PCR did not reveal significant differences in expression of E-cadherin and Vimentin. (D) Immunofluorescence and DIC images of Scr and SIM2i MCF-10A acini grown on a matrigel basement showing loss of polarity and basement membrane integrity, malformed lumens, and multilobular appearance upon loss of SIM2. Data presented as mean ± SEM * indicates p<0.05.
depletion had no effect on *in vitro* proliferation rate, consistent with results from MCF-7 cells (Fig. 15A and not shown). However, SIM2 loss did increase invasiveness and cause up-regulation of mesenchymal markers in cells grown as monolayers (Fig. 15A and 15B). Gene expression changes were modest, and not statistically significant (Fig. 15C). Thus, as expected, silencing of SIM2 was not sufficient to cause EMT in MCF-10A cells. This likely reflects that in the tumor-derived MCF-7 cells, other barriers to EMT have been lost, or that MCF-7 cells could overcome the cell cycle arrest often seen during EMT (30, 243, 367, 368). MCF-10A cells can be grown in a matrigel basement as three-dimensional spheroids, which more closely recreate the natural microenvironment of epithelial cells than does monolayer culture (369, 370). As in the mouse or human mammary gland, cells in these spheroids growth arrest and form a hollow lumen through an apoptotic mechanism, and in some cases can be induced to differentiate and produce milk (30, 371-374). Due to the highly disrupted structure we had observed in Sim2 -/- mammary glands, we expected that depletion of SIM2 would disrupt acinar structure. By day 15, the control SCR MCF-10A cells had formed regular spheres with a single layer of polarized cells and a hollow lumen (Fig. 15D). In contrast, SIM2i MCF-10A cells were irregular and in some cases multi-lobular, and had small, misshapen, or absent lumens (Fig. 15D). Individual cells could be observed delaminating from the acinar structures and invading into the matrigel basement (Fig. 15D), suggestive of an EMT like event. However, E-cadherin expression was maintained in the SIM2i acini and no mesenchymal markers assayed were seen to be upregulated (Fig. 15D). As in Sim2 -/- mammary glands, the cells surrounding the lumens of SIM2i acini were poorly polarized and disorganized (Fig. 15D). Although highly abnormal, the SIM2i acini did undergo growth
arrest and remained growth factor dependent (not shown), suggesting that loss of SIM2
to expression caused at most a partial transformation.
CHAPTER IV

EFFECT OF DEPLETION OF SIM2s ON SELF RENEWAL AND DNA DAMAGE RESPONSES IN MCF-7 CELLS

4.1 Loss of SIM2s increases mammosphere formation in MCF-7 cells

Multiple reports have established that a subpopulation of cells within tumors and cell lines is the reservoir of the vast majority of self-renewal and tumorigenic capacities possessed by the population as a whole (3, 375-379), and have termed these cells cancer stem cells or tumor-initiating cells. The SLUG oncogene, which we have previously shown to be a direct target of SIM2s in breast cancer cells (Fig. 13), has been shown to play a role in DNA damage responses and the biology of normal and transformed stem cells (114, 124, 277-280, 380). Therefore, we assessed in vitro self-renewal activity and DNA damage signaling in SCR and SIM2i MCF-7 cells. When plated under ultra-low attachment conditions in standard growth media, SIM2i MCF-7 cells had a 6-fold greater rate of mammosphere formation (Fig. 15), suggesting that depletion of SIM2s resulted in enhanced self-renewal capacity. However, when plated in serum free media supplemented with EGF and bFGF, there was no significant advantage for either cell line (not shown). This may indicate that loss of SIM2 did not increase self-renewal capacity per se, but conferred resistantance to the pro-differentiation effects of serum. Alternatively, the mammosphere forming population in the SCR MCF-7 line may be more growth factor-dependent than their counterparts in the SIM2i cells, which is masked by excess growth factors in the media.
Several reports suggest that expression of CD24, in combination with either CD29 or CD44, can be used in FACS experiments to sort out stem cell-like tumor initiating cells in MCF-7 cells (270, 381, 382), which are generally CD24lo and CD29 or CD44 positive.
Comparison of these markers in SCR and SIM2i MCF-7 revealed that nearly all SCR MCF-7 cells were CD24+ CD44+, and the putative tumor-initiating cell enriched CD24low CD44+ population comprised approximately 5% of the total population, consistent with previous results from other laboratories (Fig. 16). SIM2i cells had largely ceased to express CD44, consistent with an EMT (Fig. 16). CD24 expression was also
greatly reduced, but a significant population of CD24low cells could still be observed (Fig. 16). These results were difficult to interpret due to the complete loss of CD44 in the SIM2i cells, so we performed a similar experiment using antibodies to CD24 and CD29. As shown in Fig 16, the SCR cells were largely double positive for the markers assayed, but contained a CD24low CD29+ subpopulation which accounted for approximately 5% of the cells. The majority of SIM2i MCF-7 cells were also CD29+, but only a small fraction were CD24+, and these expressed low amounts of CD24 (Fig. 16). There appeared to greater numbers of CD24low CD29+ cells in the SIM2i cells, suggesting that they contained more tumor-initiating cells, which is consistent with their greater tumorigenicity (Fig. 16 and 8). It has been observed that treatments with ionizing radiation can enrich for stem-like cells, which are more resistant to cellular stress and DNA damage (278, 381). In order to determine if the expanded CD24low CD29+ population in the SIM2i MCF-7 cells could be stem-cell like, we analyzed sham exposed and 10Gy - exposed cells for expression of CD24 and CD29. As expected, treatment with ionizing radiation enriched for CD24low CD29+ cells, suggesting that expansion of this cell type in the SIM2i MCF-7 cells may explain their greater tumorigenicity and in vitro self-renewal (Fig. 17).

4.2 Loss of SIM2s increases DNA damage resistance and alters DNA damage responses

Expression of slug and stem cell-like behavior are associated with drug resistance, and therefore we expected that SIM2i MCF-7 cells would be more resistant to DNA
Figure 18. Effect of ionizing radiation on tumor-initiating cell marker expression in SCR and SIM2/MCF-7 cells. Treatment with 10 Gy of IR significantly increased the proportion of CD20+ CD24lo and CD44+ CD24lo cells in SIM2 cells and to a lesser degree in the SCR MCF-7 cell line, suggesting that these cells are the self-renewing portion of the population.
damaging agents than their SCR counterparts. As expected, SIM2i MCF-7 cells were resistant to the anthracycline radiomimetic doxorubicin (Fig. 18A). However, the SIM2i MCF-7 cells were at least as sensitive to ionizing radiation as the SCR cells (Fig. 18A), raising the possibility that the resistance to doxorubicin was due to stem cell associated drug pumps (383).

In order to test this, we performed immunofluorescent staining of gH2AX foci, which localized to sites of DNA damage under repair. Both SCR and SIM2i MCF-7 cells had numerous gH2AX foci after doxorubicin treatment, and there was no observable...
Figure 20. Aberrant p53 signaling in the absence of SIM2. (A) Basal p53 levels are elevated in SIM2i MCF-7 cells, but is not inducible by DNA damage, whereas p53 is strongly induced by doxorubicin in SCR cells. Studies on p53 target kinetics revealed that while PUMA is induced normally by both SCR and SIM2i cells, p21 and NOXA are inducible only in SCR cells, a pattern that held true in cells treated with ionizing radiation (B). Lack of p21 induction was confirmed by immunofluorescence analysis (C), and co-staining with γH2AX demonstrates that even in cells that sustain massive DNA damage p21 induction does not occur. Quantification of p21 positive cells was performed by counting at least 5 random fields containing 10 or more cells for each group. Data is presented as mean ± S.E.M. * p<0.05
Figure 21. Expression of cell death, DNA damage response, and cell cycle genes in the presence and absence of SIM2. SCR and SIM2/MCF-7 were treated with doxorubicin or 10 Gy of ionizing radiation and harvested at the indicated time points post treatment. Consistent with Western blot data, p21 is not induced by DNA damage in the absence of SIM2. Data obtained from three wells per group, analyzed by the ΔΔCT method and are expressed as the average fold difference ± SEM. * indicates p<0.05.
Figure 21, Continued.
difference the number of foci or repair kinetics (Fig. 18B and not shown), indicating that the SIM2i MCF-7 cells resistance to doxorubicin was unlikely to be due to enhanced drug efflux. MCF-7 cells have a functional p53 response that closely resembles that found in tumors with wild-type p53 (384).

To ensure that the differences in survival were not due to alterations in p53 status, we performed western blot analysis of p53 and sequenced the p53 mRNA in SCR and SIM2i MCF-7 cells. Sequence analysis revealed no mutations in the mRNA. However, we observed that p53 levels in the SIM2i MCF-7 cells were dramatically higher than in the SCR cells, in the absence of any stress treatment (Fig. 19A and 19B). This caused us to ask how p53 target gene expression was affected in SCR and SIM2i MCF-7 cells. The majority of p53 target proteins such as NOXA and MDM2 were induced normally by ionizing radiation and doxorubicin, with most genes induced more rapidly by ionizing radiation (Fig. 19A and 19B). However, p21 and the pro-apoptotic protein NOXA were not induced by either treatment in the absence of SIM2 (Fig. 19A and 19B). Immunofluorescence analysis confirmed that even in cells that had sustained massive DNA damage as measured by formation of H2AX repair foci, p21 was uninducible in SIM2i MCF-7 cells (Fig. 19C). Analysis of p53 target gene mRNA expression suggested that the defect in induction of p21 was at the level of transcription, while the effect on NOXA was unclear (Fig. 20). Other p53 targets such as MDM2 were induced normally in the absence of SIM2 (Fig. 20), suggesting that SIM2 may be a promoter-specific p53 co-regulator. ChIP experiments confirmed that SIM2s binds to the p21 promoter after doxorubicin treatment (R. Metz, unpublished observations), raising the possibility that
transcriptional complexes containing SIM2 and p53 regulate p21 expression. To begin to address this, we asked if SIM2 is inducible by DNA damage and performed co-immunoprecipitation experiments with p53 and SIM2. We found that SIM2 is not induced by DNA damage at the RNA level (not shown).

Figure 22. DNA damage triggers stabilization of SIM2 and interaction with p53 complexes. (A) Western blot analysis of SIM2 expression at the indicated times after treatment with either Doxorubicin or ionizing radiation. (B) Co-immunoprecipitation of SIM2 and p53. Antibodies used for IP are indicated above lanes. IP samples were blotted with anti-p53 antibody.
Intriguingly, SIM2 protein levels were robustly increased by both ionizing radiation and by doxorubicin (Fig. 22A), and as SIM2 is regulated by ubiquitin ligases (385, 386), this data suggests a stabilization mechanism similar to those known to mediate p53 and HIF1α responses (387-391). Additionally, we found that SIM2 and p53 appear to interact after treatment with DNA damaging agents (Fig. 22B), leading to hypothesize that SIM2 is a promoter-specific co-regulator of p53 target genes during DNA damage response signaling. These studies demonstrate that the molecular basis of tumor suppression by SIM2s is highly complex and multi-faceted, involving at least 3 distinct pathways: MMPs, EMT, and DNA damage response.
CHAPTER V

CONCLUSIONS

5.1 SIM2 is a transcriptional barrier to EMT

EMT has been studied in the context of development for several decades. However, only recently has the relevance of EMT to carcinogenesis and metastasis become widely accepted. Despite the rarity of full-blown EMT in human tumors, hundreds of published articles have identified dozens of transcription factors that initiate or promote EMT. Many of these factors such as twist, slug, and snail have been shown to be oncogenic in vivo, despite a lack of association in tumors with the clear EMTs they elicit in some contexts in vitro. The majority of studies on EMT involve ectopic expression techniques that raise the expression levels of these factors to hundreds or thousands of times beyond physiologic levels, which taken together with data from tumor samples suggests that a relatively high threshold of pro-EMT signalling must be reached before a clear phenotypic change results. This implies the existence of multiple factors that coordinately oppose EMT, though these factors have received little attention compared to pro-EMT pathways and transcription factors. In this study, we demonstrated that loss of SIM2 promotes EMT-like changes in vivo and in vitro (Fig. 8 and Fig.13). SIM2 is to our knowledge the first factor shown to repress the SLUG oncogene, and with the exception of work showing that estrogen signaling suppresses Snail expression via MTA3 (246), the first to elucidate a factor that opposes EMT at the level of transcription. SIM2 has been shown previously to stifle xenobiotic and hypoxia-driven gene expression (329, 330), suggesting that SIM2 stands astride and stifles several paths to EMT. Loss of
SIM2 was insufficient to stimulate EMT in non-transformed MCF-10A cells (Fig. 15), implying that multiple redundant factors block EMT in mature tissues and that these factors tend to be lost during transformation, whether the tumor cells display EMT-like features or not. SIM2-/- mammary glands displayed some features of EMT such as loss of E-cadherin, overexpression of slug, and nuclear accumulation of β-catenin (Fig. 13). This apparent discrepancy between the untransformed cells in the gland and the MCF-10A cells which did not undergo EMT may be explained by the difference in effectiveness between a gene knockout and shRNA-mediated silencing. If SIM2 were to be fully deleted in MCF-10A cells, it is possible that the anti-EMT barrier would be lowered sufficiently to allow for EMT to occur.

EMT is central to developmental processes in the early embryo and structural assembly of tissues such as the heart, palate, muscle, and bone (392, 393), although uncontrolled EMT can lead to defects due to mesenchymal hyperplasia (297). Development of many tissues such as the kidneys also requires MET, which suggests a critical role for proteins that restrict EMT. Defects in palate fusion, diaphragm development, and bone formation in Sim2-/- mice imply that SIM2-mediated cessation of EMT is crucial for development of multiple tissues (337). Considering that EMT-like changes tend to occur in advanced tumors and EMT-promoting factors are tumor-promoting without regard to EMT, strategies aimed at re-establishing expression of SIM2 or other barriers to EMT identified in the future have great therapeutic potential.
5.2 SIM2 has important tumor suppressor function independent of slug regulation

Previously, MMP3 was identified as a direct target of SIM2-mediated repression (336). In this study, we showed that SIM2 represses MMP2 at the expression and activation levels. Both MMP2 and MMP3 are associated with poor outcomes multiple tumor types, with one of the consistent associations being MMP2 and poor outcome in breast tumors (394-400). MMP2 is associated with metastasis, angiogenesis, tumor grade, size, and relapse free survival (401-403), consistent with the rapid metastasis and large vascular tumors formed by SIM2i MCF-7 cells in our study (Fig. 9). While the mechanism by which SIM2 represses MMP2 is unclear, we hypothesize it involves direct binding and repression of the MMP2 promoter. How SIM2 loss resulted in MMP2 activation is likely to be more complicated and indirect. NF-κB has been reported to promote MMP2 activation via induction of MMP14, which processes the MMP2 proenzyme (404, 405). Additionally, preliminary results suggest that SIM2 can antagonize NF-κB in some cases (T. Gustafson and R. Metz, unpublished observations), and MMP14 expression is elevated in SIM2i MCF-7 cells (Fig. 12), suggesting that this pathway may explain the effect of SIM2 on MMP2 activation. These results strongly suggest that SIM2 has multiple oncogenic targets and provide a rationale for therapeutic strategies based on re-establishing SIM2 expression in tumors without EMT-like features.

5.3 p53 responses are modulated by SIM2

We show in this study that SIM2 modulates p53 signaling directly through target genes such as p21, and likely indirectly through regulation of slug (Fig. 13). As p53 is central to DNA damage responses and suppression of tumorigenesis in virtually every, if
not all tissues, this implies that SIM2 is crucial for tumor suppression in a variety of
tissues. By co-regulating genes such as p21 and PUMA, and through suppressing slug,
SIM2 may function to fine tune the output of p53 activation, which can lead either to cell
cycle arrest or apoptosis. Given the tumor suppressive potency of p53 and the frequency
with which it is deleted or mutated in human cancers, it seems possible that those tumors
with no overt defects in p53 itself may have found other ways to neutralize p53 pathway
output, that is, all tumors are to one degree or another aberrant in p53 signaling. SIM2, as
a promoter-specific modulator of p53 function, may represent a path for evasion of p53.
It is noteworthy that the p53 targets whose transcription is apparently promoted by SIM2
are generally tumor suppressive (p21, PUMA), while some of those unaffected are
oncogenic (MDM2). SIM2 loss may even in some cases act as a gain-of function p53
mutation, and allow hijacking of p53 signaling to benefit the tumor, which can
differentially effect treatment response (406). As SIM2i cells displayed altered responses
to ionizing radiation and the radiomimetic doxorubicin (Fig. 16), SIM2 may therefore
have value not only as a prognostic indicator, but assist in selection of appropriate
therapies.

5.4 Implications of SIM2 loss in breast cancer

Given the known oncongenic potency of the SIM2 target genes SLUG and
MMP2, we hypothesize that SIM2 loss associates with poor prognosis in breast cancer.
Slug and MMP2 promote angiogenesis, metastasis, and recurrence, suggesting that loss
of SIM2 would also be associated with these processes. SIM2s-mediated antagonism of
HIF1α is another factor that could influence the effect of SIM2 loss in breast tumors. In
addition to promoting EMT, angiogenesis and metastasis, hypoxia interfaces with p53 signaling in the presence and absence of DNA damage, resulting in unique gene expression patterns in each case (407). It is possible that SIM2 loss could act as a hypoxia mimic, or simply compound the hypoxia that is found in even the smallest tumors (301). SIM2 is also likely to function in the interface between hypoxia and p53, and play a role in the cell's decision to survive and initiate angiogenesis or undergo apoptosis.

We also demonstrated that loss of SIM2 increased self-renewal and putative stem-cell-like tumor initiating cell populations in MCF-7 cells (Fig. 15). These cells are thought to be the root of tumor recurrence, and to play roles in angiogenesis(1). This implies that SIM2 promotes differentiation, and that reestablishment of SIM2 expression would synergize with many treatments in use and under investigation. This is supported by the ability of Singleminded proteins to promote differentiation of isotocin cells, CNS midline cells, and possibly TRH and somatostatin-secreting neurons (408-410). The potential role of SIM2 in suppression of stem cell identity will require in vivo validation, and studies underway to generate a conditional SIM2 allele that will clarify this issue.

5.5 SIM2 and basal-like breast carcinoma

Basal-like carcinomas are associated with poor prognosis, EMT, recurrence, drug resistance, aggressive clinical course, and are hypothesized to contain greater numbers of tumor-initiating cells (108, 110, 117, 121). Recently, it was reported that the basal phenotype is regulated by slug and hypoxia (114), suggesting that loss of SIM2 might also form part of the molecular definition of basal-like tumors via permission of slug...
expression and HIF1α activity. Consistent with this theory, SIM2 is not expressed in basal / myoepithelial cells in the mouse mammary gland, indicating its expression may suppress basal identity, or that basal-like carcinomas arise from cells that do not express SIM2. SIM2 loss is not likely to be sufficient to permit manifestation of the basal phenotype, as SIM2 is lost or reduced in up to 70% of tumors while basal-like carcinomas comprise less than 15% of invasive ductal carcinomas (113, 336). Whether SIM2 loss promotes the basal phenotype or basal carcinomas arise from cells without SIM2, SIM2 is a potential diagnostic marker to distinguish basal-like carcinomas.

5.6 SIM2 and Down syndrome

Individuals with DS have a spectrum of health disorders that is significantly different from the general population. In addition to differential risk for development of many cancers, DS patients have increased incidence of palatal abnormalities and atrioventricular (AV) septum defects, all of which involve EMT or genes that control EMT (136, 137, 151, 411). DS patients have greatly increased risk for development of leukemias, but we are unable to detect SIM2 in white blood cells and believe SIM2 does not play a role in DS-related leukemias. We hypothesize that regulation of EMT and tissue remodeling factors by SIM2 contributes both to the physical abnormalities and unique solid tumor protection experienced by DS patients.
5.7 Proposed model of SIM2 function in epithelia

Our results in mouse mammary epithelium and breast cancer cells suggest the function of SIM2 in mature tissue is to preserve epithelial identity within individual cells and in the tissue as a whole (Fig. 23), and does so along 3 main paths: Suppression of slug and possibly other EMT-promoting factors, maintenance of tissue integrity by suppression of MMPs, and by ensuring an appropriate response to DNA damage. We hypothesize that SIM2 expression is low under normal conditions, perhaps via E3 ubiquitin ligase-mediated degradation. Upon a challenge such as hypoxia or DNA
damage, SIM2 is rapidly stabilized, perhaps under the control of DNA-damage activated kinases such as CHK1. SIM2 would then suppress genes that might perturb the epithelium such as slug, while promoting cell cycle arrest via p21, thereby maintaining the tissue. As progenitor cells rely upon slug for protection from radiation-induced death (124, 278), SIM2 may be an important difference between the DNA damage responses of stem cells and differentiated cells. This also suggests that lack of SIM2 might be a useful stem cell marker, as based upon its apparently pro-differentiation effects SIM2 seems highly unlikely to be expressed in stem cells, and it is restricted to the epithelial compartment where stem cells are thought to reside. While it remains to be elucidated whether SIM2 is a cause or effect of differentiation, this study strongly suggests that SIM2 has a key role in safeguarding the structural and genomic integrity of epithelial tissues.
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