2016

Microenvironmental control of epithelial cell fate

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

MICROENVIRONMENTAL CONTROL OF EPITHELIAL CELL FATE

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Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2016

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ABSTRACT

Cancer is a devastating condition, yet its prevalence is not surprising when one considers the possibility that growth and motility define the default state of epithelial cells. What is more surprising is that epithelial cells can be induced to a "fragile quiescent state" in multicellular organisms through constant inhibitory influences from extracellular sources. In other words, the immotile and growth-constrained behavior we associate with epithelia (quiescence) is not the default state cells in a multicellular organism, but rather must be exogenously induced by tissue-specific (and systemic) factors. Quiescence therefore, is a fragile existence for any cell, as the removal of differentiating signals should cause the cell to revert to a migratory, stem-like default state. It will be argued that cancer is better understood as a disease of tissues rather than individual cells, and the complexities of tissues cannot be inferred from the study of cells in isolation. *In-vitro* studies which have been used to explain carcinogenesis will be critically reviewed and their relevance to *in-vivo* conditions will be questioned. Complex signaling mechanisms define the relationship between the epithelium and other cells in a metazoan tissue. These signals originate both from the stromal/mesenchymal compartments and between epithelial cells. Studies have shown that carcinogenic insults which affect the stroma alone can turn an otherwise normal epithelium cancerous, while transplanting "cancerous" epithelial cells into an otherwise

normal stroma does not result in neoplasm formation. Experimental evidence has confirmed that the differentiation fate of any epithelial cell is malleable depending on its environmental context. Further, it was previously thought that epithelium had to dramatically change identity in order to be capable of migration. It will be shown that collective motility is an endogenous capability of epithelial cells, and multiple non-mammalian and mammalian *in-vivo* studies of collective epithelial motility will be reviewed to better understand epithelial motility in cancer. In fact, it will be shown that the creation of adhered epithelial sheets requires the same morphological changes necessary for motility. Lastly, evidence that heterogeneous cell populations contribute to epithelial cell migration in development and metastasis will be presented.

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LIST OF ABBREVIATIONS

A	Adenine
ACC	Adenoid Cystic Carcinoma
AJ	Adhering Junction
Alpi	Alkaline Phosphatase
α-SMA	Alpha Smooth Muscle Actin
APNG	Alkyl Purine DNA N-Glycosylase
ADAM	A Disintegrin and Metalloproteinase
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
Btl	Breathless (FGF-R)
BrdU	5-bromo-2'-Deoxyuridine
cAMP	Cyclic AMP
CAF	Cancer Associated Fibroblast
CBF1	Centromere Binding Protein 1
Cdc42	Cell Division Control Protein 42
c-Jun	Jun Proto Oncogene
c-met	HGF Receptor
CSF	Colony Stimulating Factor 1
CSL	CBF1, Suppressor of Hairless, Lag-1

COMMA-D	Mouse Mammary Epithelium Cell Line
CXCR4	CXC Chemokine Receptor Type 4
Dll	Delta-Like Ligand
Dkk	Dickkopf WNT Signaling Pathway Inhibitor
DNA	Deoxynucleic Acid
Dpp	Decapentaplegic
Dsrf	Drosophila Homolog of Mammalian Serum Response Factor
εA	1,N6-Ethenoadenine
εC	3,N4-Ethenocyostine
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
Elf5	E74-like ETS (E twenty-six) Transcription Factor 5
EM	Electron Microscopy
EMT	Epithelial to Mesenchymal Transition
ERK2	Extracellular Signal Related Kinase (aka MAPK)
ETS	E Twenty-Six
FAK	Focal Adhesion Kinase
FGF	Fibroblast Derived Growth Factor
FSTL1	Follistatin-Related Protein 1
Gap1	GTPase Activation Protein 1
GDNF	Glial Derived Neurotrophic Factor

GGF	Glial Growth Factor
HER2/erbB2/neu	Human Epidermal Growth Factor Receptor 2
HES1	Hair and Enhancer of Split-1
HGF	Hepatocyte Growth Factor/Scatter Factor
IGF	Insulin-like Growth Factor
IL-6	Interleukin 6
JAK	Janus Kinase
Krox20	Early Growth Response Protein 2
LAG3	Lymphocyte-activation Gene 3
Lgr5	Leucine-rich Repeat-containing G-protein Coupled Receptor 5
LIF	Leukemia Inhibitory Factor
Math1	Atonal Homolog 1
miR-21	MicroRNA 21
MAPK	Mitogen Activated Protein Kinase
MDA-MB-231	Human Epithelial Breast Cancer Cell Line
MECs	Mammary Epithelial Cells
MED8	Mediator Complex Subunit 8
MEE	Median Edge Epithelium
MES	Midline Epithelial Seam
MMP	Matrix Metalloproteinase
MRCK	Myotonic Dystrophy Kinase Related Cdc42 Binding Protein Kinase

MT1-MMP	Membrane Type 1 Matrix Metalloproteinase
MYPT1	Myosin Phosphatase Target Subunit 1
NCI	National Cancer Institute
NG	N-Methyl-N'-Nitro-N-Nistrosoguanidine
NGF	Nerve Growth Factor
NICD	Notch Intracellular Signaling Domain
NEXT	Notch Extracellular Truncation
NMU	N-Nitromethylurea
Notch	Neurogenic Locus Notch Homolog Protein 1
OLFM4	Olfactomedin 4
p53	Tumor Protein p53
p450	Cytochrome p450
pKA	Protein Kinase A
PDGF	Platelet Derived Growth Factor
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
PP1	Src Kinase Inhibitor
PP2	Src Kinase Inhibitor
RANKL	Receptor Activator of Nuclear Factor κ Ligand
Ras	Rat Sarcoma Small GTPase
Rac	Ras Related C3 Botulinum Toxin Substrate 1

Raf	Raf1 Proto-Oncogene, Serine/Threonine Kinase
RER	Rough Endoplasmic Reticulum
Rho	Ras Homolog
ROCK	Rho Kinase
RS	Retrorsine
RTK	Receptor Tyrosine Kinase
SCC	Squamous Carcinoma Cells
SDF1	Stromal Cell Derived Factor 1
snail1	Snail Family of Zinc Finger 1
Src	Rous Sarcoma Virus Non-receptor Tyrosine Kinase
SMT	Somatic Mutation Theory
STAT3	Signal Transducer and Activator of Transcription
TGF-β	Transforming Growth Factor Beta
TNFRSF19	Tumor Necrosis Factor Superfamily Member 19
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VASP	Vasodilator-stimulated Phosphoprotein
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless/Integrated
WT	Wild Type
Xpb1	DNA Repair Helicase XPB1
YAP	Yes-associated Protein

Y118	Tyrosine 118
Zn-finger	Zinc Finger

INTRODUCTION: Assumptions of the Somatic Mutation Theory

According to the National Cancer Institute (*NCI*), there will be 1.68 million newly diagnosed cases of cancer during 2016 in the United States. Nearly 600,000 people will die of cancer this year, making it the second leading cause of death ("Cancer Statistics - National Cancer Institute" 2016). In 2013 expenditures in the United States for cancer treatment totaled nearly $125 billon, and this number is predicted to rise by nearly 25% over the next 4-5 years. As a sign of hope for research efforts to date, nearly 14.5 million people are outliving their cancer diagnosis and this group is expected to reach 19 million over the next 8 years. Five year cancer survival rates have consistently trended upwards since the *NCI* started collecting detailed data in 1975, from 48.9% between 1975-77 to 68.7% between 2005-2011 (Howlader 2016).

Nearly 80% of dangerous human malignant cancers originate from epithelial tissues that proliferate and migrate uncontrollably. (Ye and Weinberg 2015). A prevailing theory of carcinogenesis is "somatic mutation theory" (*SMT*) which postulates that individual epithelial cells become cancerous (i.e. proliferative and migratory) due to an accumulation of somatic mutations which activate oncogenes and lead to loss of function in tumor-suppressor genes. *SMT* theorists have accepted the idea that intercellular signaling and cell-cell interactions are important in the progression of carcinogenesis. Yet, proponents of the *SMT* have maintained their focus on the transformed epithelial cell, assuming the mesenchyme to be an *accessory* to

carcinogenesis initiation and metastatic invasion; a blind follower of the program initiated by the carcinoma cell. (Douglas Hanahan and Coussens 2012; Douglas Hanahan and Weinberg 2011) However much experimental evidence challenges this notion and indicates the true tumor microenvironment is not arranged in such a hierarchical structure (i.e. epithelium controlling stroma). Studies outlined in this review will show that signals originating in the stroma have the ability to differentiate and normalize transformed and "cancerous" cells. Further, epithelial cells within tissues can also direct the differentiation of neighboring epithelial cells and inhibit "cancerous" epithelial behavior. Thus, intercellular signaling at the tissue level may regulate the initiation of cancer. (Carlos Sonnenschein and Soto 2013; C. Sonnenschein and Soto 1999)

It will be shown in this review that all epithelial cells are capable of motility, de-differentiation and limited transdifferentiation within the epithelial lineage. In fact, all cells require continuous tissue-derived factors to maintain their differentiation and inhibit endogenous capabilities for growth and motility. Upon removal of these differentiation signals, any epithelial cell should be capable of reverting to a less differentiated stem-like cell. A current theory of metastasis, the epithelial to mesenchymal transition (*EMT*), requires epithelial cells dramatically change lineage and behave as isolated mesenchymal cells in order to metastasize. Instead it will be shown that upon loss of differentiating signals, epithelial cells will readily become migratory without losing epithelial identity. Therefore, the cardinal characteristics of metastasizing epithelial cells (stem-like and migratory) are not *acquired* properties upon genetic

mutations but rather endogenous characteristics “uncovered” upon loss of differentiating pressures from the tissue signaling environment.

More attention has been paid to the study of oncogenes than to understanding cell-cell relationships within the tissue where particular neoplasms arise. (D. Hanahan and Weinberg 2000; Stratton, Campbell, and Futreal 2009) The focus on the genomics of the transformed cell (which is thought to have accumulated enough somatic mutations to begin acting autonomously) has overshadowed the interactions and signaling between cells in tissues where neoplasms arise. Recently, the stromal compartment has received more attention. (Douglas Hanahan and Weinberg 2011) *SMT* theorists however assume the stromal-epithelial and epithelial-epithelial interactions only become important once a cancer cell has transformed and acquired, through mutations, the ability to corrupt mesenchymal tissue cells. (Douglas Hanahan and Coussens 2012) Evidence presented below challenges this idea, and uncovers the striking influence of the tissue microenvironment on epithelial cells both during carcinogenesis and in the normal physiology. If differentiating inputs are removed, epithelial cells would exhibit properties of any autonomous or cancerous cell: uncontrolled proliferation and migration. Thus it is only through an imposed restraint by the environment that an epithelial cell does not exhibit a cancerous phenotype. Rather than properties acquired through the serendipity of somatic mutations, these characteristics are innate. A related question arises: what is a cancerous cell, and how is it different from a normal epithelial cell? What properties of a cancerous cell does an epithelial cell not possess? This review

will seek to argue that there are very little differences between normal and cancerous epithelial cells, and therefore the control of cancer progression occurs at the level of cell-cell interactions rather than within the genome of any one cell.

The first section of this review will examine the influence of the surrounding mesenchyme and neighboring epithelium on differentiated epithelial cells in the adult animal, and seeks to address whether cell-fate decisions and the initiation of cancer may be controlled by these cell-cell interactions rather than by the genome of any one cell.

How the Microenvironment Controls Epithelial Cell Fate

What influence does the stromal compartment have on terminally differentiated epithelial cells? Graft experiments from the early 1980's revealed that tissue microenvironments exert strong differentiating pressures on the epithelium of the adult animal. If bladder transitional epithelium is grafted onto the mesenchyme of the urogenital sinus in the adult Rat, the transplanted epithelium will transform its morphology to match the epithelium of the new environment. (Neubauer et al. 1983) It will form the branched secretory acinar glands characteristic of the prostate, as if the local environment dictated the identity of the cell rather than any genomic program of the epithelial cell itself. The grafted epithelium lost the morphology characteristic of bladder epithelium, membrane asymmetry and fusiform vesicles (used to increase or decrease membrane area as the bladder is stretched or relaxed), and gained a simple columnar morphology. These now columnar epithelial cells had gained features

consistent with secretory epithelium: nuclei and rough endoplasmic reticulum (RER) located basally and with Golgi complex and secretory granules located apically.

This result suggests a number of vitally important principles of relevance to cancer regarding the relationship between the epithelium and stroma. First, how plastic is the identity of terminally differentiated epithelia, is it free to gain any identity a local might pressure it to become? Did the surrounding cells of the bladder keep the epithelium differentiated? One must conclude that the prostate tissue continually exerts differentiating pressures on its epithelium, pressures so strong that morphologically and physiologically distinct epithelial cells can be transdifferentiated into parenchyma of the host tissue. Do all tissues have this capability or is it unique to bladder and prostate? The answer may reveal a misunderstanding we have regarding how fixed cellular identity is and how autonomous individual cells are.

Stromal influences on the epithelia of the adult breast are so strong that a wild-type mammary microenvironment is able to drive the differentiation of *male* testicular epithelial cells into breast epithelium. When male seminiferous tubule epithelial cells that do not express the progesterone receptor (*PR*) were mixed with *PR* knockout mammary epithelial cells and co-injected into a cleared fat pad of a female animal, the lobular mammary epithelium developed normally. (Bruno et al. 2014) This process requires a functional *PR* receptor, and when either cell type was injected into fat pads alone, the lobules did not develop normally. Yet, when the two cell-types were mixed before transplantation, the resultant chimeric epithelium curiously expressed the *PR*

receptor. Polymerase chain reaction (*PCR*) detection of the *Y-chromosome* revealed that the testicular epithelial cells were now *PR* receptor positive. These testicular-epithelium-derived *PR* positive mammary epithelial cells were also capable of self-renewal: when breast tissue from the first chimeric transplant were grafted onto the cleared fat pad of a second *PR* receptor knockout host, 40-50% of the luminal mammary epithelial cells were *PR* positive, maintaining the same *PR* receptor density as the original grafts. These effects were seen in both virgin and lactating mice.

To further investigate the extent of differentiation of the transplanted cells, the expression mammary epithelial markers, *receptor activator of nuclear factor κ ligand* (*RANKL*) and *E74-like ETS (E twenty-six) transcription factor 5* (*Elf5*), were tested. *RANKL* is a downstream target of the activated *PR* receptor and is thought to induce alveologenesis. *RANKL* stimulates the expression of the transcription factor *Elf5* in neighboring *PR* negative epithelia. Dual staining of *PR* and *RANKL*, and *PR* and *Elf5* showed overlapping staining of *PR* and *RANKL* and non-overlapping staining of *PR* and *Elf5*, as expected. Thus, testicular derived *PR* positive cells expressed other markers of differentiated mammary epithelium, and were able to signal through a functional *PR* receptor to produce ligands that maintained differentiation of neighboring cells. (Bruno et al. 2014) These results indicate that epithelial identity is not fixed but rather a product of local environmental cues.

Fibroblastic mammary mesenchyme seems to provide growth promoting cues required by the epithelium in order to form normal ducts, while the mammary fat pad

may provide inhibitory cues. Transplanting epithelial cells into fibroblastic mesenchyme without the fat pad leads to ductal hyperplasia. Further, epithelial cells transplanted into the fat pad without fibroblasts at the same age as the previous experiment (14-16 days) fail to develop. (Sakakura, Sakagami, and Nishizuka 1982) This suggests factors from the both fibroblastic and fatty mesenchyme induce epithelial differentiation. As additional evidence for the strength of this epithelial/stromal interaction in mammalian breast development, if mammary epithelial cells of the same age as the above experiments are transplanted into the mesenchyme of the salivary gland, they will differentiate into the morphology of salivary gland epithelium. (Sakakura, Nishizuka, and Dawe 1976) However, these transplants maintained some aspects of mammary epithelial differentiation, synthesizing milk proteins in response to a post-pregnancy hormonal load. The principle message: the mesenchyme exerts a strong influence on epithelial differentiation, morphology and behavior. Further, epithelial identity may be more fluid and flexible than we currently understand, able to functionally and morphologically adapt to new tissue environments after being thought of as "fully differentiated."

Convincing evidence has proven the tissue environment has enormous impact on the behavior of a cancerous epithelium. The first series of experiments utilized co-injection of transformed mammary epithelial cells and normal mammary epithelial cells into cleared fat pads. (Booth et al. 2011) Mouse mammary tumor virus promoter driven expression of *neu*- lacZ (the *neu* "oncogene" encodes *HER2* also known as *erbB2*, which

is a member of the *epidermal growth factor* (*EGF*) family of receptor tyrosine kinases) generated *neu* positive tumors when injected in cleared fat pads alone. When co-injected with wild-type *MECs* (mammary epithelial cells), the *lacZ* positive tumorigenic cells adopted a normal phenotype, interspersing within the epithelium along wild-type (*WT*) cells at all points along the ductal tree. These chimeric animals did not form tumors for the duration of the experiment (18 months), while animals injected with *HER2/erbB2/neu* cells alone formed tumors in 5-7 months. In chimeric animals Neu^+ cells participated in ductal morphogenesis alongside normal cells, adopting differentiation markers based on their final position appropriately. Basally located Neu^+ cells adopted a myoepithelial identity, as noted by expression of smooth muscle actin, while Neu^+ cells located at the lumen expressed *keratin8* as expected. In host animals that progressed to full term pregnancy, the putative "tumorigenic" cells were positioned among normal cells in mammary ducts and even localized to secretory lobules, adopting a functional secretory phenotype as noted by co-expression of the milk phosphoprotein *β-casein*. These formerly tumorigenic cells, induced to differentiate into morphologically and functionally normal epithelium in the wild-type mammary microenvironment, continued to express *Neu/ErbB2/HER2*. Importantly, *ErbB2* receptor activation was silenced through some unknown mechanism (phosphorylated *ErbB2* was not found). This mechanism to subdue the "oncogenic" signaling was epigenetic, as DNA sequencing did not reveal further DNA mutations or aneuploidy (which could have been caused by

cell-fusion with WT *MECs*). *ErbB2* is not thought to homodimerize, but rather heterodimerize with *epidermal growth factor receptor* (*EGFR*), and phosphorylated *EGFR* (*pEGFR*) was identified in the chimeric mice indicating *EGFR* signaled normally. As further evidence co-injected wild-type *MECs* repressed intact *ErbB2* signaling in the neighboring mutated cells, when chimeric breast tissue was dissected and sorted into individual cell populations, the $Erb2^{+}$ cells were able to form tumors when they alone were transplanted into a new host. This indicates that the tumorigenic potential of these cells was retained, but signals from adjacent *WT* epithelial cells had silenced this capacity to form tumors without altering their genome. These, along with other results reviewed by Goruppi and Dotto, indicate that the tumor microenvironment provides important, perhaps even rate-limiting, limitations on the development of carcinogenesis. (Goruppi and Dotto 2013)

The growth-constraining effects of surrounding tissue cells (stromal and epithelial) on the tumorigenic potential of individual epithelial cells is not confined to the breast. Carcinogen treated hepatocytes do not proliferate or colonize the liver when transplanted into a normal host. However, if the host liver was pretreated with the pyrrolizidine alkaloid retrorsine (*RS*), known to block cell-cycle progression, transplanted cells will progress to histologically confirmed hepatocellular carcinoma. (Laconi et al. 2001) At 4 months' post-transplantation, the livers of the *RS* pretreated hosts had doubled in weight. Yet if carcinogen treated hepatocytes were transplanted into normal hosts, livers did not change weight, nor did gross dissection reveal visible nodules.

Transplanted cells in normal host were found in clusters of no more than five cells, and this density did not increase from 2 to 4 months' post transplantation. This again suggested that the wild-type host exhibited a restraint on the tumorigenic potential of the transformed and transplanted cells. Neoplasms only formed when the resident cells within the putative tumor microenvironment were altered, and the neoplasms formed independently of the genotype of the transplanted cells. This throws some doubt into the face of the *SMT* hypothesis of carcinogenesis, revealing that carcinogen-treated epithelial cells will not form tumors unless the microenvironment is disrupted. An important follow up to this study would be to probe the intercellular signaling mechanisms affected by *RS* treatment to identify what is secreted by normal liver cells that restrain the growth of transplanted carcinogenic cells.

Is this result confined to the liver, or does an analogous mechanism exist in other tissues? Another group of investigators sought to define the target of carcinogens in mammalian breast tissue. (Maffini et al. 2004) Was it the mesenchyme, parenchyma, or both? The authors' transplanted mammary epithelial cells, pretreated with either the chemical carcinogen *N*-nitromethylurea (*NMU*) or sham into cleared fat pads of host animals pretreated with *NMU* or sham. It was discovered that only the animals with stroma previously exposed to *NMU* developed neoplasms. Tumor formation did not depend on whether transplanted epithelial cells were exposed to the carcinogen, as when transplanted to a *WT* animal, tumor incidence was not higher in epithelial cells exposed to the carcinogen than vehicle treated epithelial cells. When transplanted into

WT hosts, *NMU* treated epithelial cells exhibited normal ductal branching and no evidence of neoplasm formation and *WT* epithelial cells formed tumors when transplanted into *NMU*-treated hosts. Further, the authors found the incidence of "tumorigenic" *Ha-ras-1* mutations did not correlate with neoplasm development. The existence of *Ha-ras-1* mutations were not predictive of tumor growth and transplanted epithelial cells which did not generate neoplasms were just as likely to harbor the mutation. In fact, the likelihood of harboring an activating *Ras* mutation was just as high in vehicle treated animals, or even WT animals randomly selected from the authors' rat colony than in animals exposed to *NMU*. These results favor the hypothesis that mammalian neoplasms arise due to altered cell-cell interactions within a tissue, rather than the genetic status of any subgroup of epithelial cells. It also confirmed data presented previously that a wild-type tissue microenvironment can normalize "cancerous" epithelial cells.

Other groups have similarly generated data separating "oncogenic" mutations and the onset of carcinogenesis. One group discovered that although transplanted *Ha-ras-1* mutated epithelial cells had a survival advantage over *WT* (increasing *in-vivo* relative abundance five-fold compared to WT as animals aged from 50-570 days), they were not selectively abundant in spontaneously formed tumors, and never constituted more than .001% of the epithelial tumor cells. (Cha et al. 1996) Another study investigated how hemi-body irradiation influenced neoplasm development of transplanted mammary epithelial cells that harbored *p53* mutations in both alleles

(*COMMA-D*). (Barcellos-Hoff and Ravani 2000) When injected into non-irradiated hosts, the *COMMA-D* cell line differentiated normally into ductal outgrowths, produced milk proteins and did not form tumors. However, exposure to hemi-body ionizing radiation before transplantation preferentially increased the tumorigenic potential of these *p53* mutated epithelial cell line only on the side of the body exposed to the radiation. The study confirmed data from Maffini et al., Booth et al., and Laconi et al. that the tissue environment determines whether neoplasms developed, not the genetic makeup of the epithelial cells that will go on to populate the tumor. The above examples illustrate how the function and signaling of the tissue microenvironment may be an influential driver of neoplastic formation, rather than the genetic makeup of any particular epithelial cell.

The results above are similar to a study performed more than forty years ago. In a classic example of the power of the microenvironment to influence or normalize aberrant behavior of malignant embryonic cells, teratocarcinoma cells taken from an intraperitoneal tumor can be transplanted into a developing embryo at the blastocyst stage to generate completely normal animals. (Brinster 1974) Sixty offspring were raised to adulthood using this experimental protocol with no observable defects. No tumors formed in the hybrid animals, although one animal exhibited a patch of agouti hair that must have been caused by the chimerism.

If tissues control epithelial cell differentiation, changes in tissue requirements could lead to a reprogramming or modulation (transdifferentiation) of cell fate under *in-vivo* conditions. Indeed, following injury to ductal cells in the mammalian liver,

differentiated hepatocytes will switch lineage, downregulate hepatocyte markers, upregulate ductal markers and incorporate into biliary ducts actively being repaired. (Tarlow et al. 2014) Although most of the repairing cells were ductal in origin, a significant portion (up to 39%) were hepatocyte derived. Once the injury was resolved, the same hepatocytes that were driven by the injury to populate ducts, became exclusively induced into the hepatocyte lineage. This indicates that tissue injury can modulate the local signaling environment and dictate the differentiation of the cell. Once the injury resolved, cells were allowed to resume their normal differentiation program. Thus injury produced signals that drove the transdifferentiation of existing hepatocytes into ductal cells as they are needed. The data suggest that if ducts are functioning properly, hepatocytes will be made, while if ducts sustain injury, they will be repaired before additional hepatocytes are produced. These experiments were successfully reproduced using human hepatocytes. This adds evidence to the idea that cell-fate decisions are decided by cell-cell signaling within tissues, if these signals are interrupted, aberrant or incomplete differentiation could lead to carcinogenesis.

A commonly used mouse model of *Ras* based lung and liver tumorigenesis is urethane (ethyl carbamate) exposure. *Ras* gain-of-function mutations are thought to underlie many cancers, and it is thought that urethane exposure induces mutations in *Ras* leading to tumorigenesis. (Pylayeva-Gupta, Grabocka, and Bar-Sagi 2011) The mechanism is thought to be through a P450 metabolite (vinyl carbamate epoxide) that forms etheno-adducts in DNA. (Benigni and Bossa 2011) Presumably, amino acid

substitution follows alkylation at a conserved mutational site in codon 61 leading to H-*Ras* or K-*Ras* gene activation. However, the activation of the downstream targets of *Ras*, *MEK* and *ERK* only became apparent 30 days after urethane exposure. (Yano et al. 1999) In a different representative study of urethane induced tumorigenesis, tumors appeared 16 weeks after exposure. Ethenobases are repaired by base excision repair, involving *DNA N-Glycosylases*. The most common DNA adduct formed by urethane exposure, 7-(2-oxoethyl)guanine, does not lead to either thymine or adenine miscoding. (A. Barbin, Laib, and Bartsch 1985; Holt et al. 2000) Instead, the εA (1,N6-ethenoadenine) and εC (3,N4-ethenocyostine) exocyclic adducts formed are thought to be the initiators of DNA lesions, despite occurring at a much lower frequency. In a study by Barbin et al., the focus was on adenine alkylation because of its ability to form the AT→TA transversions within exon 61 of *Ras* that were observed in previous studies of urethane treated mice. The base excision enzyme *APNG* (Alkyl Purine DNA *N*-glycosylase) repairs adenine, so the authors investigated tumorigenesis in *APNG*$^{-/-}$ (null) mice. Not surprisingly, lung and liver tissue from *APNG*$^{-/-}$ mice possessed an increased number of alkylated adenine nucleotides, and these insults persisted longer than *APNG* competent mice. Lung tissue of APNG null mice exhibited a 2-fold increase in ratio of εA/A at 6 hours' post-exposure. By 96 hours' post-exposure, the εA/A ratio of *APNG*$^{-/-}$ null mice had dropped to 1/3rd of the 6-hour peak, while by this time almost all damaged DNA was repaired in *WT* mice. By extrapolation, *APNG*$^{-/-}$ null mice would have cleared lung mutations by 144 to 168 hours post treatment, almost twice the amount of time taken by *APNG* competent mice.

Despite clearing almost all DNA damage from the mutagen by 96 h (WT) or 168 h (*APNG*$^{-/-}$), liver tumors did not appear until a full year after mutagen exposure, even in the highest dose group. Significantly, the lack of DNA repair mechanisms did not increase the likelihood of liver tumor formation. No increase in tumor susceptibility was observed in the liver or lung in DNA-repair deficient mice. Even in the high dose group, lung tumors were not observed in any mice (*APNG* competent or not). Between 15 and 18 percent of hepatocytes were apoptotic 24 hours after urethane exposure, as measured by the *TUNEL* assay, but no difference was seen between groups. Cell proliferation, measured by *BrdU* incorporation, peaked 24-hours post-exposure, but again was not different between groups. (Alain Barbin et al. 2003) When these results are taken together with the time-course of *Ras* cascade activation, it provides some indirect evidence suggesting that in the urethane model of *Ras* based carcinogenesis, DNA damage leading to *Ras* hyperactivation may not the mechanism of tumor formation. It suggests that urethane may modulate other inhibitory signals imposed upon the epithelium by the tissue.

Given the evidence above, it is reasonable to assume that the surrounding tissue has a profound influence on the differentiation and thus on the tumorigenic potential of epithelial cells. As discussed, this effect is so strong that “cancerous” epithelial cells can be induced to differentiate into phenotypes indistinguishable from normal tissue. What about the induction of carcinogenesis? Some proponents of the *SMT* may dismiss the value of non-mutagenic cell-exogenous influences on neoplasm development, but these

studies give evidence to support the stroma as capable of transforming genetically normal epithelium into a cancerous phenotype. As carcinogen exposure appears to initiate neoplasm formation only when the entire tissue was treated, not when only epithelial cells are treated and transplanted into normal hosts, do we need to redefine what the mechanism of action of a carcinogen is? To wit, it was known that the iris epithelium of newts could transform into a new lens after injury. However, only the dorsal portion of the iris epithelium was capable of this regeneration, not the ventral. Yet, when the ventral cells were treated with a known mutagen *N-methyl-N'-nitro-N-nistrosoguanidine* (*NG*), they became capable of forming lenses instead of forming tumors. (Eguchi and Watanabe 1973) Treatment with a known carcinogen re-programmed this portion of the iris epithelium to allow it to induce a differentiation program it had previously lost the ability to evoke. Should we rethink our understanding of how carcinogens operate, beyond somatic mutations and into reactivating signaling processes potentially akin to development?

What then is a Cancer Cell?

If normal epithelial cells can be induced to form tumors by an activated microenvironment and "oncogene" activated epithelial cells can be normalized by a wild-type microenvironment, maybe "cancerous" epithelial cells are not much different than wild-type epithelial cells. Further, if the existence of DNA mutations within an epithelial cell can be masked by inhibitory signaling within a tissue, maybe DNA

mutations within epithelial cells are not the rate-limiting cause of cancer. Perhaps "cancerous" epithelial cells are no different than normal epithelial cells. Data presented above suggests the true driver of carcinogenesis may be the tissue microenvironment, independent of tumor cell genetics. Further, it has been shown that somatic mutations are common in *WT* epithelial cells, and cancer derived epithelia only show a 2-fold increase in mutation rate. (Martin et al. 1996) Evidence presented so far and in upcoming sections may indicate that all epithelial cells are all potentially "carcinogenic" (endowed with the abilities to migrate and grow/divide) and neoplasm occurrence may be determined by growth and motility limiting pressures originating from the tissue environment. Therefore, the question remains: what is unique about cancer cells?

The machinery for cell division, and cell cycle control is conserved across all eukaryotic cells, from human cells to single cell protozoa, let alone between normal and cancerous human cells. (Alberts 2008) The main difference in transcriptional profiles between single celled organisms and multicellular eukaryotes is the expression of factors that induce differentiation, sub-specialization within multicellular organisms and the concomitant inhibition of proliferation and motility. (Xia et al. 2006) Both "cancerous" and normal epithelial cells have the capacity for proliferation, growth and migration. Thus, a unique proliferative and motile capacity that defines cancer cells as radically different from their normal counterparts should be revisited. Motility does not differentiate cancerous and normal epithelial cells. Differentiated epithelia are assumed to be stationary, but it will be shown that epithelia are capable of collective, adhered

migration. (Matsubayashi et al. 2004; Friedl and Gilmour 2009) Furthermore, the mechanism of this endogenous ability is conserved within metastasizing epithelial cells. (Friedl et al. 2012)

The profound adaptation of multi-cellular organisms is *inhibition* of the basic cellular processes of division, growth and motility. *In-vitro* experiments discussed later by Hay and later Weinberg taught us much about how cells grow, move and proliferate, but not why. Cancer cells proliferate and move, not because they are unique, but because they are historically programmed to behave this way, like all eukaryotic cells. (Binamé et al. 2010) Experiments regarding the "reversibility of the malignant phenotype" give strong evidence that cell phenotypes are context-dependent, and the cancer cell is again not unique in that aspect (Hendrix et al. 2007; Carlos Sonnenschein and Soto 2011; Kenny and Bissell 2003) Understanding carcinogenesis requires an investigation into what signaling context has driven a particular cell to be "cancerous." Furthermore, the "cancerous" epithelial phenotype, as will be outlined below, is not dramatically different from a normal epithelial phenotype. It follows that we should supplement the investigation into what exogenous factors induce motility and proliferation (almost everything), with what factors once removed release the epithelial cell from inhibition, across the fine line between growth and malignancy.

It may be a mistake to accept the notion that differentiation and quiescence is the default state of cells. The reality is that human cells are genetically programmed to move and divide, similar to our single-celled amoeboid ancestors. The emergence of

metazoan from solitary, wandering single cells *must* have involved inhibition of characteristic behaviors of single cells: unchecked growth in ample nutrients and motility to find new nutrients. (Sebé-Pedrós et al. 2013) Analysis of stem cells has bolstered this line of reasoning: they were shown to carry an innate program for division, while differentiation required exogenous factors. (Ying et al. 2008) What is understood as the default behavioral state of human epithelial cells, immobility and quiescence, does not follow from evolutionarily logic, but must be something exogenously induced in order to prevent chaos in a multi-cell, multi-tissue organism.

Is Cancer the Result of an Interruption of Microenvironmental Differentiation Cues?

Consistent with the idea that differentiation and quiescence must be exogenously maintained, recent work in the mammalian airway epithelium revealed that selective ablation of basal stem cells led to increased proliferation and subsequent de-differentiation of neighboring luminal secretory cells *in-vivo*. The secretory cell population induced to de-differentiate (now basal-cell-like) persisted in its new phenotype over a 2-month experiment. These cells were functionally and morphologically equivalent to endogenous basal stem cells, able to differentiate into all three airway epithelial cell types (basal, secretory, ciliated) and repopulate them appropriately after injury. (Tata et al. 2013) Direct, or near-direct contact between the basal cells and the differentiated cells was required to prevent de-differentiation in an *ex-vivo* sphere forming assay of stemness. Further, loss of basal cell contact led to

secretory cell proliferation. The factor provided by the basal cells to the secretory and ciliated cells to maintain differentiation may signal through *hippo*, a family of serine/threonine kinases that phosphorylate and inactivate *YAP* signaling. While the factor provided by the differentiated cells to the basal cells may activate *YAP* signaling, as *YAP* was required for basal stem cell maintenance, and *YAP* loss led to the differentiation of basal cells into secretory cells. Conversely, *YAP* overexpression led to basal cell proliferation and inhibition of secretory/ciliated cell differentiation. (Zhao et al. 2014) These studies provide supporting data to the tenet of this thesis: without restraining influences provided by a tissue microenvironment, cells will revert to their default state: uncontrolled growth and replication. Thus, in normal airway physiology, factors secreted by differentiated cells (ciliated and secretory) maintain the stem-like state of the basal cells, and factors from the basal cells maintain the differentiation state of the ciliated and secretory cells. This is entirely consistent with the local environment (in this case neighboring epithelial cells) determining the identity of any individual epithelial cell within a tissue. Further, the true state of cells in isolation may lie closer to stemness rather than quiescent differentiation as assumed by *SMT* theorists. (Douglas Hanahan and Weinberg 2011) Growth and motility are not acquired properties after insults to the genome, they are endogenous properties of all cells that must be inhibited within multicellular tissues. Cell specialization is exogenously driven by tissue-derived factors external to any one cell, and in order to maintain differentiation and inhibit carcinogenesis, signals must be continually provided.

Intercellular signaling in the mammalian intestinal epithelium provides another example of the external signals required to maintain the differentiation state of epithelial cells. (Tetteh et al. 2016; van Es et al. 2012) *Paneth* cells have a relatively long life-span and act to nourish *Lgr5*$^{+}$ stem cells, providing *wingless/integrated* (*Wnt*), *Notch* and *EGF* signaling. *Lgr5*$^{+}$ stem cells differentiate into enterocytes or secretory cells as they migrate up the crypt toward the intestinal villus. (Tetteh et al. 2016) *Notch* signaling is crucial to this lineage selection within the intestinal epithelium. Upon ligand binding to a *Notch* receptor, sequential proteolytic cleavage occurs to initiate receptor activation. *A disintegrin and metalloproteinase* (*ADAM*) first cleaves the *S2* site to release an extracellular domain, while the *Notch extracellular truncation domain* (*NEXT*) remains membrane-adhered and contains an intracellular signaling domain *Notch intracellular signaling domain* (*NICD*). (Kopan and Ilagan 2009) Subsequent *γ-secretase* cleavage at the *S3/S4* sites of the transmembrane domain release *NICD* and allow it to translocate to the nucleus. *NICD* binds the DNA-interacting protein *centromere binding protein 1* (*CBF1*)*, suppressor of hairless, Lag-1* (*CSL*) stimulating the recruitment of other factors (*Mastermind/Lag-3* and *mediator complex subunit 8-MED8*), which together activate expression of downstream target genes. If *Notch1/2* receptors are activated in the stem cells, *Hes1* expression increases, which in turn inhibits the expression of the *Math1* transcription factor that is vitally important for secretory lineage differentiation. Blockade of *Notch* allows *Math1* expression and routes stem cells into the secretory lineage. (van Es et al. 2012) Notch activation through either *Dll1* or *Dll4* inhibits markers

of cell stemness in the crypt such as *olfactomedin 4* (*Olfm4*), *follistatin-related protein 1* (*Fstl1*) and *tumor necrosis factor superfamily member 19* (*Tnfsrf19*). Radiation damage to the crypt induced apoptosis and depleted *Lgr5*$^{+}$ stem cells. (Tetteh et al. 2016) Interestingly this reverted *Dll*$^{+}$ secretory precursor cells to *Lgr5*$^{+}$ stem cells. This ability of intestinal epithelial cells to return to a de-differentiated state was thought to be restricted to the relatively rare secretory cell types. Yet, evidence from Tetteh et al. reveals this property can be generalized to committed *alkaline phosphatase* (*Alpi*$^{+}$) enterocyte precursors as well, as would be predicted from the hypothesis that tissue-specific signals determine differentiation state. Similar to secretory precursors, these *Alpi*$^{+}$ cells dedifferentiated into *Lgr5*$^{+}$ stem cells when the endogenous stem cells were ablated. These putative stem cells were able to repopulate all epithelial cells of the intestinal villi, including enterocyte and secretory cell lineages. Interestingly, 3 days after exiting the crypt the enterocytes were no longer able to dedifferentiate, suggesting a signal emanating from the crypt may locally retard the differentiation program and facilitate dedifferentiation. The results also suggest, similar to the mammalian lung, that signals from a tissue stem cell compartment drive differentiation of nearby epithelial cells. Further, selective loss of stem cells leads to dedifferentiation of mature epithelial cells until the stem cell progenitor pool is successfully replaced.

Of fundamental interest to the thesis of this review, which challenges the notion that mutations in “oncogenes” must occur in order to activate fundamental cellular behaviors, the authors induced mutations in the *APC* (adenomatous polyposis coli) and

Ras pathways. (Tetteh et al. 2016) These mutations are thought to act synergistically during colorectal cancer formation. Epithelial cells harboring these mutations were only able to form tumorigenic organoids *in-vitro* but did not yield any neoplasms/adenomas *in-vivo*, where signals emanating from the surrounding tissue would be predicted to inhibit neoplasm formation while driving differentiation and limiting the size of the progenitor pool. Mutations in the *APC* "tumor suppressor" gene and related *Wnt/β-catenin* "oncogene" are thought to initiate progression to neoplasia and colon polyp formation. Loss of function mutations in *APC* have been observed in 60% of colonic adenomas and carcinomas. (Janssen et al. 2006) Constitutively active *Ras* (*KRAS*) "oncogene" mutations are thought to drive tumorigenic progression and growth, synergistically enhancing *Wnt* hyperactivation in *APC* null mutants. Mice with mutations in both of these genes (*KRAS* and *APC*) are observed to accumulate 10-fold increase in tumor burden and a corresponding increase in tumor invasiveness. However, in the Tetteh et al. study, dual mutations in *APC* and *KRAS* only resulted in *in-vitro* tumor formation, whereas *in-vivo* adenomas did not form. (Tetteh et al. 2016) The crucial distinction between studies from Janssen et al. and Tetteh et al. could be the presence of potential off-target effects of the *KRAS* and *APC* mutations. This is a vital distinction, as mutations in multiple cell types could lead to disruptions in cell-cell interactions which normally inhibit neoplasm formation; i.e. factors exogenous to the cell population being studied. The study from Janssen et al., which reported *APC* and *KRAS* mutation drove *in-vivo* tumorigenesis, used markers to target mutations that are not exclusive to

enterocytes (*DNA repair helicase Xpb1* and *Villin*). (Janssen et al. 2006) While the study which did not show *in-vivo* tumorigenesis, the enterocyte-specific protein *Alpi* was used, which unlike *Xpb1* and *Villin* was not expressed in $Lgr5^{+}$ stem cells or secretory precursor Dll^{+} cells. (Tetteh et al. 2016; Kim, Escudero, and Shivdasani 2012) Thus in Janssen's study, *Wnt* and *Ras* hyperactivation was not restricted to the enterocyte lineage, but rather all intestinal epithelial lineages. Therefore, the study showing overactive *Wnt* and *Ras* led to tumorigenesis could have resulted in signaling dysfunction at the tissue level, leading to removal of differentiating pressures or loss of inhibitors of proliferation and motility that emanate from neighboring epithelial or mesenchymal cell types. However, this must be reproduced in other tissues and models, as other studies have shown cooperativity between *APC* and *Ras* mutations led to increased tumorigenesis. (Davies et al. 2014; Sánchez-Rivera et al. 2014)

Interactions between mammalian *Schwann* cells and the neurons they support provide another example of the requirement for continual exogenous pressures to maintain differentiation state. In myelinating *Schwann* cells, *cyclic AMP* (*cAMP*) signaling is required to differentiate and induce a myelinating phenotype. During neuronal injury, signals mediated by *protein kinase A* (*pKA*) from the neuron to the *Schwann* cell are lost, resulting in reduced *cAMP* levels in the *Schwann* cell. This reduction allows *Schwann* cell dedifferentiation toward a state that is thought to promote regeneration of the injured neuron. (Merrell and Stanger 2016; Monje et al. 2010) Interestingly, the high levels of *cAMP* required for differentiation blocked the ability of *mitogens/RTK* (receptor tyrosine

kinase) ligands such as *Neuroregulin*, platelet derived growth factor (*PDGF*), insulin like growth factor (*IGF*), and fibroblast derived growth factor (*FGF*) to induce cell-cycle progression or proliferation of the *Schwann* cell. If *cAMP* levels were exogenously reduced in the *Schwann* cell, its ability to progress through the cell cycle was restored and the cell-cycle and growth arrest phenotype induced by *cAMP* was lost. Thus *cAMP* levels determined the proliferative capacity of the cell, not the presence or absence of "growth factors." This distinction is raised to contract the view propagated by *SMT* theorists that neoplasm formation occurs as the result of activating mutations in "growth factors," which implies the default state of cells is quiescence. *Schwann*/neuron interactions give further evidence that quiescence is exogenously maintained, in this case through *cAMP* levels, while no amount of growth factor activation could overcome the inhibition provided by a neighboring cell. The Schwann cells did not lose all differentiation marker expression upon dedifferentiation, suggesting *cAMP* levels represented a fine-tuning of differentiation status, rather than a drastic change into a progenitor cell. Dedifferentiation did coincide with loss of intracellular vacuoles, a reduction in size and the appearance of cytoplasmic processes (perhaps used to drive motility to find another neuron if the injured neuron did not recover). These cells did not automatically initiate cell-cycle entry under *in-vitro* conditions of nutrient starvation. Instead, the Schwann cells became "competent to resume proliferation" when nutrients or mitogens were added to the media.

The ability of a *Schwann* cell to myelinate is determined through an antagonistic relationship between a transcriptional regulator that promotes myelination *Krox-20* and an inhibitory transcriptional regulator *c-jun*. Data revealed increased intracellular levels of *cAMP* repressed *c-jun* and activated *Krox-20*, providing a mechanism of *cAMP* regulation of *Schwann* cell differentiation. (Monje et al. 2010) Presumably an inhibitory signal constitutively released from nearby neurons acted through *cAMP* and *Krox-20* to maintain the differentiated state of the *Schwann* cell. Upon injury to the neuron, this signal was eliminated, leading to reduced *cAMP* levels and *Krox20* levels while disinhibiting *c-jun*, leading to a dramatic change in *Schwann* cell morphology and function. This transition is thought to promote the regeneration of the neuron, providing another example of how cell-mediated inhibition of growth and stemness characterizes tissue function. (Merrell and Stanger 2016) This gives further support to the thesis that in the default state, cells are nothing like the quiescent well-behaved static entities we believe they are, but instead the dormant phenotype must be exogenously maintained. Considering the possibility that tissue-specific terminal differentiation and growth-arrest is an imposed phenotype, the identification of factors that mediate this signaling could be used to develop more effective cancer treatments.

Coordinated migration is another behavior within the normal repertoire of epithelial cells that must be inhibited by differentiating factors in order to maintain tissue order. E74-like factor 5 (*Elf5*), of the *ETS* family of transcription factors, could be one such factor. In the mammary epithelium it acts to maintain epithelial phenotype

and suppress epithelial migration. Loss of *Elf5* resulted in a disorganized mammary epithelium and prevented alveologenesis and lactation. *Elf5* knockout in luminal epithelial cells resulted in downregulation of the epithelial differentiation marker *E-cadherin* and upregulation of migratory markers like *vimentin* and nuclear *snail2*. Other markers that indicated a loss of epithelial phenotype were transcriptionally upregulated like *Twist* and *Zeb* following *Elf5* knockdown. (Chakrabarti et al. 2012) *Snail2* expression is activated in other motile cells like macrophages infiltrating an inflamed colon or during an acute tissue injury, in a transforming growth factor beta 1 (*TGF-β1*) dependent manner. SiRNA knockdown of *snail* also repressed invasion in a macrophage cell line *in-vitro*. (Hotz et al. 2010) *Twist* binds to the *snail* promoter and induces its expression, and *snail* induction is required for *twist* induced metastasis and motility. (Casas et al. 2011) As further evidence that *Elf5* is required for epithelial phenotype maintenance and differentiation, *Elf5* levels were reduced in epithelial breast cancer cell lines that had a more invasive and migratory phenotype, and overexpression of *Elf5* suppressed a *TGF-β* induced migratory transcription profile in normal mammary epithelial cells. Enforced stable expression of *Elf5* in the highly invasive and motile *MDA-231* breast cancer cell line induced markers of differentiated epithelium, including a reversion to cuboidal morphology (compared to *MDA-231's* characteristic spindle-like appearance), upregulation of *β-catenin* localized to cell-cell junctions, and increased appearance of *F-actin* cables and tight junctions' characteristic of epithelia. Using chromatin immunoprecipitation, it appeared *Elf5* directly bound to a conserved region within the

snail2 promoter, which indeed contained a putative *Elf5* binding motif, suppressing its expression. Importantly, *Elf5* overexpression greatly decreased lung metastasis nodules in a xenograph model using the highly lung-invasive epithelial breast cancer cell *LM2* subline of *MDA-231*. (Chakrabarti et al. 2012)

Activating or inducing positive regulators of *Elf5* expression could lead to anti-metastasis treatments for mammary epithelial cancers. As noted previously, *Elf5* expression is not generated cell-autonomously, but rather induced by local cues (Progesterone receptor signaling stimulates *RANKL* expression, and *RANKL* induces *Elf5* in neighboring epithelial cells). Interrupting *RANKL* signaling released the inhibition on epithelial cell migration. A proposed crucial differentiating feature between cancerous epithelial cells and normal epithelial cell is migratory ability. If interrupting a single signaling pathway, in the absence of somatic mutation, causes WT epithelial cells to behave like cancer cells, again how different are cancer cells from normal cells? Could it be that many or all the phenotypic characteristics we assign to cancerous epithelial cells are actually inherent in normal epithelial cells? According to the experiment results reviewed above, these characteristics needn't be generated *de-novo* by a cancerous epithelial cell, but rather their existence is *revealed* upon *removal* of differentiating factors.

Intercellular signaling in the mammalian airway epithelium, intestinal crypts, mammary epithelium and between the *Schwann* cell and peripheral neurons indicate that local signals provide constant inhibitory cues to maintain differentiation and

prevent proliferation and motility. Is it any wonder that if these local cues become impaired, excessive epithelial growth or metastatic dissemination could occur, perhaps without the need to invoke genomic alterations? Like growth inhibition, motility arrest is assumed to be a property fundamental to most epithelial cells. The next section will argue that epithelial motility is a default cellular program, not something that must be induced or acquired through genetic alterations.

The Morphological Plasticity of Epithelial Cells-Migrating Groups

As argued above the default state of epithelial cells is profoundly misunderstood, but so is the range of normal behaviors these cells are capable of. In the study of cancer, cell-type conversions (into dramatically different lineages) are cited to explain "newly acquired" behaviors of epithelial cells, such migratory ability. (Ye and Weinberg 2015) This section serves to broaden the range of morphologies and actions that are within the behavioral range of non-transformed epithelial cells. It will be shown that common observations assigned solely to "cancerous" cells, like the migratory ability required for metastasis, behaviors within the repertoire of normal epithelial cells.

The morphological variability of differentiated epithelium is readily observed during wound healing. In the initial response to tissue injury, epithelial cells migrate as a collective sheet to close wounds. (Friedl and Gilmour 2009) They behave as a supracellular "micro-tissue" that exhibits collective polarity, coordination, intercellular contact and communication, and the ability to migrate directionally. Similar to a

physiological tissue, epithelial cells in this arrangement develop sub-specializations: pathfinder cells (aka *tip cells*-to be discussed later) or following cells (aka *stalk cells*). The *tip cells* exhibit bipolarity: the leading edge of the cell explores the environment, responds toward nutrient gradients and extends *lamellipodia*, *filopodia* or *pseudopodia* (finger-like cytoskeletal membrane-bound projections) to create focalized adhesions to the substratum and propel the cell forward. These *tip cells* use actinomyosin contraction to generate force and movement, all while staying adhered to subsequent *stalk cells* creating an epithelial sheet. Previously thought to be passive bystanders, *stalk cells* have been shown to aid forward progression and exhibit "cryptic" *lamellipodia* which are hidden from view using conventional top-down imaging, but are readily apparent when the cell sheet is observed laterally or when forces produced by the migrating cohort are calculated (this review will return to data supporting this topic shortly). The forward movement of *tip cells* leading an epithelial collective and the motility of single cells utilize conserved systems. These include actinomyosin rearrangements using small GTPases *Ras homolog* (*Rho*), *Ras Related C3 Botulinum Toxin Substrate 1* (*Rac*) and *cell division control protein 42* (*Cdc42*), each part of the *Ras* superfamily of *guanosine triphosphate* (*GTP*) hydrolases, and their effector kinases such as *Rho kinase* (*ROCK*). (Khalil and Friedl 2010; Amano, Nakayama, and Kaibuchi 2010; Vaezi et al. 2002; Bustelo, Sauzeau, and Berenjeno 2007) Leading cells seem to utilize a greater diversity of *extracellular matrix* (*ECM*) interacting *integrins* to migrate the novel environment. They also modify *ECM* components with pericellular proteolysis using membrane bound

matrix metalloproteinases such as *MT1-MMP/MMP14*. This may serve to homogenize the *ECM* substrate and ease the forward progression of following *stalk cells*, allowing them to express a more restricted array of ECM interacting *integrins*. (Friedl and Gilmour 2009) In order to move as a unit, cell-cell communication is vital to coordinate cell behavior within the group. Studies have shown that mitogen activated protein kinase activation (*MAPK*), starting at the leading edge, can proceed rearward in a wave-like manner passing from cell to cell. (Nikolić et al. 2006) Further examination revealed that the *MAPK* signal activation swept rearward in two waves. The first wave was fast and brief. Activation peaked in under 5 minutes and dissipated by 20 minutes, save for the first row of migrating cells. The second wave gradually built strength and distance from the wound margin, reaching 600 µm rearward by 4 hours. Presumably the first wave was a priming step, as it occurred much too fast to be associated with movement, while the second wave corresponds with frank epithelial sheet migration. Specific inhibition of extracellular signal regulated kinase (*ERK2* aka *MAPK1*) using a kinase-dead dominant negative mutant, significantly reduced post-wounding migration. Furthermore, as the wound closed, and cells stopped moving, *ERK* was inactivated. Interestingly, serum was not required after wounding for *ERK1/2* activation or migration, indicating that *ERK1/2* was activated independent of growth-factor stimulated signaling. *ERK1/2* activation also seemed to occur without protein synthesis or transcription, as *cyclohexamide* nor *actinomycin-D* treatment did not disrupt motility. *Src kinase*-mediated activation of *ERK* in epithelial cells has been found during studies of

the hepatocyte growth factor and epidermal growth factor receptor tyrosine kinase (ligand-receptor: *HGF-cMET*, *EGF-EGFR*)-mediated formation of focal complexes (adhesion precursors) and focal adhesions (sites of mechanical force transduction connecting the actin cytoskeleton to the *ECM*) within *lamellipodia* required for movement. *Src kinase* inhibitors *PP1* and *PP2* were effective in blocking the movement of the cell sheet and the second wave of *MAPK* activation during the wound healing studies, but did not affect the first wave of activation, suggesting each wave is controlled independently. (Matsubayashi et al. 2004) In that same study, activated *ERK* was not only found in the nucleus, consistent with its defined role as a transcription modulator, but also within the leading edge of *lamellipodia*. Inside *lamellipodia ERK* associated with *paxillin*, a docking site for multiple proteins at focal complexes/adhesions, including *vinculin* and *FAK* (focal adhesion kinase). The *ERK/paxillin* association was precipitated through *Src kinase* phosphorylation of *paxillin*. Interestingly, activated *ERK-paxillin* association was most strongly noted within 10 minutes of *RTK* activation. By 30 minutes, focal complexes matured to focal adhesions, yet *ERK* activation and *ERK-paxillin* co-localization was much reduced. However, *paxillin* (without *ERK*) remained localized at focal adhesions through 180 minutes after *RTK* stimulation, suggesting *ERK* association is a priming step. Interestingly, mere plating of epithelial cells on a *fibronectin* medium resulted in *paxillin* incorporation into adhesion complexes but resulted in less sustained *ERK* activation. Through a series of convincing experiments, the authors concluded that *Src* acted to phosphorylate *paxillin* at *Y118* and

this residue served as the site for *ERK* association. *Paxillin* was proposed to serve as a scaffold for *ERK* activation by binding its activators *Raf* and *MEK*. Mutating the *Y118 ERK* binding site on *paxillin* abolished *RTK* mediated cell motility *in-vitro* and disrupted the formation of focal adhesions necessary for force transduction and motility. (Ishibe et al. 2003) Identification of these signaling cascades provides an array of targets to inhibit collective epithelial cell migration and is supremely relevant to metastasis treatment by generating targets for drug development.

As noted above, in a migrating epithelium there are leading cells and following cells. A misunderstanding is that leading cells are uniquely migratory, and the following cells are passively pulled along. (Friedl et al. 2012) A fascinating study in *Nature Physics* showed that nearly all cells in a migrating epithelial sheet contribute to traction forces. (Trepat et al. 2009) When they grouped cells by distance from the leading edge, and plotted the forces generated by each of these groups on the underlying substrate, all groups of cells fit in a single Gaussian curve. This indicates that leading edge cells do not generate more forces than cells away from the leading edge, and all cells contribute to collective movement.

Additional studies have given morphological support to this concept of uniform traction generation. One such study showed that cells on the interior of the migrating epithelium (*stalk cells*) extended *lamellipodia* to interface the substratum while also maintaining cell-cell adhesions. Further, there seem to exist behavioral plasticity within the migrating collective. Cells following the leading edge of an invading cluster

proteolytically degraded the walls of tubes formed by leading cells to facilitate passage of subsequent cells of the epithelial collective. (Friedl and Wolf 2008) Morphological plasticity was also characteristic of the leading cells. In a modified wound healing assay, leader cells were able quickly switch between different phenotypes. When presented with a free surface, the epithelium behaves as if there has been a wound, collectively migrating over the free substratum. As soon as leader cells at the front edge of migrating collective come in contact with another group of epithelial cells, they modify their morphology, reduced membrane *ruffling* and retracted *lamellipodia*, becoming indistinguishable from the other cells in the collective epithelium. Elevated extracellular calcium was required to form these adhesive contacts between two migrating epithelial sheets. (Poujade et al. 2007) This assay also provided evidence that an actual wound or a chemokine/growth factor gradient was not needed to induce epithelial migration, but merely a free surface upon which to migrate. It revealed that epithelial cells can undergo dynamic morphological changes, cycling between stationary to migratory in short periods of time. Further, video microscopy revealed that the development of cell-cell adhesions during epithelial tissue formation used transient and searching *filopodia/lamellipodia* to contact neighboring cells and to ultimately form adhering junctions at the precise locations where the membrane extensions contacted neighboring cells. (Vaezi et al. 2002) So, not only do all cells in a migrating epithelium contribute to the propulsion of the group, epithelial cells do not require exogenous growth factor stimulation or genetic mutations in order to exhibit migratory behavior.

Of interest, these studies illustrate that in order to create the cell-cell contacts required by an epithelium, individual epithelial cells must utilize the exact same intracellular signaling mechanisms and cell-shape changes characteristic of *motility* and *migration*, as will be discussed in further detail below.

The mechanism of cell-cell adhesion that characterizes the formation of a mammalian epithelium has been investigated *in-vitro* using mouse epidermal cells and canine and marsupial kidney epithelial cells. (Vasioukhin et al. 2000) Unique morphological changes observed during this process were verified in *in-vivo* using mouse epidermal cells. When adjacent mouse epidermal cells were stimulated with calcium, they produce *filopodia* through *actin* polymerization which penetrate and embed within adjacent cells. At the tip of each extending *filapodium* are clusters of *adhering junction* (*AJ*) related proteins such as *E-Cadherin*, *α,β catenins*, *VASP*, *Mena*, *vinculin* and *zyxin*. Under low calcium conditions, neither *AJ*'s nor *desmosomes* form (*desmosomes*-less dynamic and more robust cell-cell adhesions that associate with the intermediate filament network). Although keratinocytes were studied primarily, kidney epithelial cells also displayed similar behavior. Utilizing scanning and transmission electron microscopy (*EM*), the authors noted that high calcium increased the length and quantity of *filopodial* extensions. *Filopodial* formation increased most robustly at intercellular contacts. These intercellular junction *filopodia* appeared to protrude into the neighboring cell membrane without penetrating, forming a series of interdigitating contact points. At the terminal end of each *filopodial* process *AJs* seemed to form, as

evidenced by an increase in electron density and positive staining with *AJ* specific antibodies, as well as punctate *actin* filament bundling. These *EM* visualized structures observed *in-vitro* resembled interdigitating *filopodial* structures seen in mouse skin epidermal structures *in-vivo*, suggesting that the *in-vitro* results accurately represent the *in-vivo* process.

The authors also introduced the concept of an "adhesion zipper," consisting of a two-rowed *AJ* puncta between nascent adhering cells (each cell contributed a single row) during the process of epithelial adhesion and sheet formation. (Vasioukhin et al. 2000) Within 7 hours of calcium signal initiation, the two-rowed puncta had begun to merge into one. As these contact sites matured, the intercellular interface became highly undulated: shallow depressions in one cell were filled with extensions from the neighboring cell. *AJs* lined contact points between cells. Further along in time, 16 hours past the initial calcium stimulus, the offset wave-and trough interdigitation had flattened to create continuous contacts of alternating *AJs* and *desmosomes* and by 20 hours they had formed a single continuous line. Although the authors reported this alternating pattern was often observed *in-vivo*, the classic structural picture of epithelia places *desmosomes* and *AJs* physically separated, whereby *AJs* are located more apically, forming a transcellular network of parallel bundles of *actin* microfilaments capable of transmitting force laterally, while *desmosome* plaques locate subjacent to the *AJs*. Considering the dynamic nature of *AJs*, it is possible that once cells were stably adhered, subsequent differentiation and maturation modified the precise location of

the *AJs* to fit the structural needs of the epithelial sheet and tissue. Nevertheless, this study, and others, unequivocally indicate that forming cell-cell contacts within an epithelium required the morphological plasticity utilized by migrating cells. (Green et al. 2010; Vasioukhin et al. 2000) In other words, every cell that is capable of forming an epithelium *must* be capable of migration.

In-vitro studies are the least convincing method for studying the existence of collective epithelial migration in living tissues. Non-mammalian models provide the most studied *in-vivo* examples of collective epithelial cell migration and corresponding intercellular signaling networks. The phylogenetic gap between non-mammals and humans limits direct application of the results gleaned from non-mammalian studies, but the strong genetic and physiological conservation between non-mammals and humans allows for their use as homologous systems for studying the cell-cell signaling networks within migrating epithelia in humans. (Peterson et al. 2008) One such example is posterior lateral line formation in *zebrafish*. (David et al. 2002) When fully differentiated, the posterior lateral line is a sensory organ that allows fish to localize movement in their environment. Individual organs called *neuromasts*, innervated by bipolar sensory neurons, are arranged in series along the flank of the animal. During development more than 100 cells directionally migrate in a cohesive unit along a previously deposited *stromal cell derived factor 1* (*SDF1*) chemokine strip. Migration along the chemokine strip required the expression of the *SDF1* receptor *CXC chemokine receptor type 4* (*CXCR4*) in the migrating cells. However, not all cells need to express the

receptor in order for the collective to move, and individual cells that lack the receptor migrated normally when normal cells are present. (Haas and Gilmour 2006) As little as 4 cells, close to the leading edge (within 20 μm), out of more than 100 needed to express the chemokine receptor in order for the whole collective to migrate normally. Furthermore, under normal conditions, the migrating collective of epithelial cells directed the formation and extension of the lateral line nerve, which in turn guided the migration of glial cell precursors. Thus, the *SDF1*/*CXCR4* signaling system in a small group of epithelial cells controlled the migration of multiple cell types required to form the lateral line sensory organ. Indeed, *CXCR4* expression in a small group of cells near the leading edge of the collective was able to rescue the migration of all three cell types (neural, glial, and epithelial). These cells did not respond to a concentration gradient of the chemokine, and did not migrate cell-autonomously once in the chemokine stripe. When the strip of *SDF1* was cut short, the migrating cells made a U-turn and continued to deposit sensory organs along the strip but in the opposite direction. Interior cells of the migrating cohort exhibited *lamellipodia* polarized in the direction of collective movement. Of interest, *CXCR4* deficient cells exhibit this same *lamellipodia* morphology when migrating in the interior of the collective, suggesting the *lamellipodia* formation is due to intercellular signaling rather than individual response to the chemokine.

Discrete and restricted fibroblast growth factor (*FGF*) signaling from neighboring cells was required in order to induce differentiation and associated morphological changes (apical constriction and formation of rosette structures) within the migrating

epithelial cells. (Lecaudey et al. 2008) The migrating cells were sequentially selected to form neuromast epithelial mechanosensory hair cells. A fundamental question regarding migrating collectives is what maintains the polarity within the group and allows directional movement. *Wnt/β-Catenin* signaling seems to be involved. (Aman and Piotrowski 2008) *APC* is a necessary component of the complex that targets *β-Catenin* for destruction in the absence of *Wnt* signaling. *Wnt* signaling is critical for stem cell maintenance and homeostasis in mammalian tissues. For example, *Wnt* is necessary for epithelial differentiation/progenitor homeostasis in epithelial crypts of the small intestine, such that disruption of *Wnt* signaling leads to absence of crypt progenitor cells. Constitutive activation of the *Wnt/β-Catenin* pathway is a hallmark of colon cancer. (Reya and Clevers 2005) In *zebrafish*, *FGF-3/10* ligands seemed to be produced by leading cells in the migrating cohort which acted on the trailing cells, as *FGF* receptor activation was restricted to non-leading cells. (Aman and Piotrowski 2008) Interestingly, *Wnt/β-Catenin* activates transcription of both the *FGF* ligand and a membrane bound *FGF* signaling inhibitor only in the leading cells. Thus *Wnt/β-Catenin* induced ligand production in the leading cells but restricted signal transduction to the non-leading cells portion. Forcing W*nt/β-Catenin* activation throughout the migrating epithelial cohort slowed migration, particularly because lagging cells seemed to migrate randomly. Thus *Wnt/β-Catenin* signaling seems to facilitate independently oriented motion, and the restriction of this signaling to the leading edge allowed those cells to determine the direction of motion. Further, *FGF* signaling was required in the trailing region to

differentiate deposited epithelial cells into the radial sensory structures of the lateral line organ. Additionally, *FGF* receptor activation produced a factor *Dickkopf Wnt signaling pathway inhibitor* (*dkk*) that acted to limit *Wnt/β-Catenin* signaling. Thus *Wnt/β-Catenin* and *FGF* signaling between discrete groups of cells within the migrating cohort acted to maintain polarity of the migrating epithelium through complex signal activation/inhibition.

Tracheal branching morphogenesis in *D. melanogaster* provides another non-mammalian *in-vivo* model of collective epithelial cell migration, and offers molecular mechanisms of inter-epithelial cell-cell communication and differentiation potentially conserved in humans. Instead of lungs, *drosophila* utilize a network of branched and interconnected epithelial lined tubules that oxygenate tissues through passive diffusion from a small number of external openings (spiracles). This tubular network begins as twenty clusters of ≈80 cells (placodes) that invaginate from the ectoderm to form a bag-like epithelium. (Steneberg, Hemphälä, and Samakovlis 1999; Affolter and Caussinus 2008) Tracheal placodes are determined in part by the action of two transcription factors *trachealess* and *drifter/ventral veinless*, which induce expression of *FGF-R* (*FGF* receptor) and other tracheal cell specific markers. Invagination does not occur in *trachealess* mutants. Main branches are formed when 1-2 cells within the newly formed sac begin to migrate in pre-determined directions. (Sutherland, Samakovlis, and Krasnow 1996) A different subset of cells (*stalk* cells-similar to mammalian collective migration) remain attached to and follow the leader cells. The whole structure forms a

hollow tube as the leader cells intravasate into the embryo. Primary, secondary and terminal branches sequentially form smaller structures and at the conclusions of the respiratory network are specialized epithelial cells called *terminal cells* which send out hollow cytoplasmic protrusions to deliver air to specific tissues. A single ectodermal placode creates one arborized network of air delivery cells, and multiple arborizations are interconnected with specialized cells known as *fusion cells* to form an animal wide network. (Zelzer and Shilo 2000) The migration guidance cue for branch formation is *branchless*, an *FGF* homolog, which binds to its cognate *RTK, breathless* (*FGF-R*) on the migrating epithelial cells to activate the *MAPK* signaling cascade. (Sutherland, Samakovlis, and Krasnow 1996) The *FGF*-like ligand (referred to as *FGF* hereafter) is secreted in a spatially and temporally restricted manner, originating from epithelial and mesodermal cell clusters external to the invaginating sac. *FGF* expression immediately precedes formation of individual epithelial outgrowths, and as sequential outgrowths mature, *FGF* expression is turned off. The main effect of *FGF* is to regulate primary branching and migration of the tracheal system, but *FGF* may be involved in secondary and terminal branch formation as well. *FGF* is expressed close to positions where secondary branches formed and *FGF* mutants fail to express required factors for secondary and terminal branches: the transcription factors *pointed* and *pruned/blistered/Dsrf* which act downstream of *Ras*. *FGFR* (*btl*) mediated signaling is required for *filopodia* extension in migrating tracheal epithelial cells (similar to *TGF-B* effects in *MEE* migration discussed on page 49), as visualized using *3D* reconstructions of

time-lapse confocal imaging of an *actin*-GFP marker driven by *FGF-R* (*btl*) expression. Genetic screens uncovered a novel cytoplasmic protein that can differentiate the guidance effects of *FGF* from the motility effects, suggesting a bifurcation in the downstream *MAPK* pathway, as mutations in this protein (*stumps*) cause defects in homing/guidance but not random motility. (Imam et al. 1999) *FGF-R* (*btl*) mutant experiments seem to reveal a competition within the migrating cohort for activation through *FGF* signaling, such that cells with the strongest response to *FGF* become leader cells by migrating to the leading portion of the collective. Cells with a kinase domain mutation within *FGF-R* that weakens but does not abolish responsiveness to *FGF* almost never adopted a leader cell fate. Complete loss of *FGF-R* activity reduced the number of leader cells, such that the ratio of leader cell to *stalk cell* was altered from 1:3 to 1:51. Thus it seems in the selection of cells for leader cell fate, there is a competition amongst the invaginated tracheal cell pool. Cells with greater responsiveness to a guidance cue can even change position within the adhered migrating cohort and navigate to the lead, perhaps with cooperation from the other cells in the cohort. Once leader position is established, lateral inhibition through production of the *Notch* ligand *Delta* and subsequent *Notch* activation in neighboring cells seems to limit the number of leader cells that are formed, presumably in order to maintain coherent migration. (Ghabrial and Krasnow 2006) This restriction of leader cell differentiation, through lateral inhibition of *FGF-R* by *Notch* in *D. melanogaster* is similar to the *dkk* mediated inhibition of *Wnt/B-catenin* signaling in *zebrafish*.

Individual tracheal branches require inputs beyond *FGF* in order to differentiate properly. A member of the *TGF-β* superfamily, *dpp* is required for dorsal branch formation (a primary branch). *Dpp* controls both the number of migrating cells in the dorsal branch and the differentiation of the specialized doughnut-like *fusion cell* that mediates the formation of connecting junctions between arborized placodes within the animal. The *fusion cell* is identified by expression of the *Zn-finger* protein *escargot*. One effect of *dpp* signaling in the selection and differentiation of the *fusion cell* is the induction of *Delta* expression. *Escargot* led to expression of the cell-cell adhesion protein *E-Cadherin* in the fusion cell while reductions in *dpp* signaling prevented the fusion cell from adhering to its cognate partner. (Zelzer and Shilo 2000) A series of imaging experiments utilizing ectopic expression revealed that *Delta* expression from the fusion cell activated *Notch* signaling in surrounding cells to restrict their fate away from a *fusion cell*, in an identical mechanism as leader cells restricted the fate of neighboring cells to stalk cells in both zebrafish and *D. melanogaster*. (Steneberg, Hemphälä, and Samakovlis 1999) *FGF* signaling was shown to induce *Delta* expression, and although all tracheal cells expressed *FGF-R*, *Notch* activation in cells outside of the migrating tip cells restricted *MAPK* activation downstream of *FGF* to the tip cells only, thereby selecting *FGF* signaling in a subgroup of tracheal cells to promote motility and coordinate guidance of the entire group. (Ikeya and Hayashi 1999)

Continuing with a discussion of receptor tyrosine kinases, and revealing further complicating factors of signaling cascades, the *RTK EGFR* is known to control cell

proliferation, survival and differentiation in multiple different tissues and species. (Shilo 2005) In drosophila *EGFR* primarily signals through the *Ras1/Raf/MEK/MAPK (ERK1/2)* pathway, and controls the ultimate lineage determination of multiple cell types. Modulation of the pathway seems to occur through alternate mechanisms besides ligand binding and a number of mechanisms have been identified. The principle ligand of *EGFR* is *Spitz*, which is expressed in membrane-bound form and is only able to activate its receptor upon proteolytic cleavage, similar to *Notch*. *Spitz* appears to be most similar to *TGF-β* in structure, but possesses functional *EGF* domains and signals through *EGFR*. Second, a multi-pass transmembrane protein *Rhomboid* (perhaps the *Spitz* protease) seems to potentiate *EGFR* signaling, and its expression is regulated more stringently than the *EGF* receptor or ligand. Additional ligands exist for *EGF-R* that also show a more restricted expression pattern than *Spitz*, such as *vein* and *gurken*. As a further modulatory mechanism, there exists a secreted *EGFR* inhibitor, *Argos*, which does not interact with the *EGF* receptor but rather sequesters and inactivates its ligand in the extracellular space. (Klein et al. 2004) Of interest, *Argos* is produced in cells with high levels of *EGFR* activation, and is thought to inhibit *Ras1/Raf/MEK/MAPK (ERK1/2)* in neighboring cells, thereby restricting *ERK1/2* activation to the leading cells. (Wasserman and Freeman 1997) Interestingly, *Argos* seems to diffuse faster than *spitz* despite being physically larger, suggesting *Argos* activity requires inter-epithelial cooperation.

As a confirmation of this pathway's relevance to human diseases, *ERK1/2* is known to drive coordinated epithelial migration during wound healing in mammalian

cells (reviewed in an earlier section of this review). Lastly, genetic screens identified an intracellular inhibitory factor for *Ras1-MAPK (ERK1/2)* named *sprouty*, from the observation that its knockout caused excess tracheal branching. Loss of function mutants for *sprouty* also exhibit excessive accumulation of photoreceptor and pigment epithelial cells in the developing eye, suggesting *sprouty* limits cell accumulation or migration. *Sprouty* is associated with the intracellular membrane and seems to interact with multiple RTKs (*FGF*, *EGF*, *Torso*, *Sevenless*). It immunoprecipitates with *Drk* (downstream of receptor kinase), a homolog of mammalian *Grb2*, and *GTPase activated protein 1* (*Gap1*). *Drk* and *Gap1* are signal adaptors between the *EGF* receptor and *Ras1*. (Casci, Vinós, and Freeman 1999) Mammalian homologs to *sprouty* which are amenable to drug-induced activation (or disinhibition if endogenous inhibitors are found) could have utility for restricting *RTK* activation and limiting migration in cancerous cells.

Four homologs of *sprouty (1-4)* have been identified in mammalian systems. They have been shown to antagonize *RTK* mediated *Ras* signaling from a wide range of receptors: *EGFR*, *FGF-R*, *vascular endothelial growth factor* (*VEGF-R*), *platelet derived growth factor* (*PDGF*), *nerve growth factor* (*NGF*), *brain derived neurotrophic factor* (*BDNF*), *glial derived neurotrophic factor* (*GDNF*) and *hepatocyte growth factor receptor* (*c-MET*). In the failing mammalian heart, *sprouty1* is downregulated in cardiac fibroblasts through increased expression of the *sprouty1* targeting microRNA *miR-21*. Thus *miR-21* seems to activate the *Ras1-MAPK (ERK1/2)* pathway in the face of local tissue inhibition, promoting the survival of fibroblasts. Increased fibroblast survival leads

to enhanced fibrosis and architectural support for the poorly performing heart. However, modern cardiac care has circumvented the need for this endogenous but imperfect fibrotic protective mechanism. To this end, the authors found inhibiting *miR-21* reduced *Ras1-MAPK (ERK1/2)* signaling, reducing fibrosis and hypertrophy while normalizing contractility. (Thum et al. 2008) In another study, *sprouty2* overexpression reduced tumor frequency by nearly 30%, and tumor volume by 40%. (Minowada and Miller 2009) Thus *sprouty* proteins are plausible targets to retard *Ras1-MAPK (ERK1/2)* overactivation and the excessive cell accumulation and migration in tumorigenesis.

Questions regarding the Epithelial to Mesenchymal Transition

Epithelial migration during development and metastasis was previously thought to require a dramatic transdifferentiation of an epithelial cell into an entirely different state: the epithelial to mesenchymal transition (*EMT*). (Kalluri and Weinberg 2009) However, a critical review of the supporting data suggests that this transdifferentiation program is an *in-vitro* artifact rather than a physiological reality. The *in-vitro* studies that supported this theory lacked the differentiating and inhibitory pressures supplied by tissues *in-vivo*. Well-studied examples of collective epithelial migration during developmental or physiological processes (wound healing, *MES*, *drosophila* trachea and *zebrafish* lateral line formation described above) are conserved across evolution. Furthermore, utilizing both clinical data and animal models, it will be proposed that

collective migration is the main mechanism by which epithelial cells metastasize, obviating the need for an *EMT* program.

Confusion regarding the prevalence of *EMT* will be discussed, starting with a proposed *EMT* marker *snail*. The *snail* family of transcriptional repressors (mentioned previously during the discussion of *Elf5*) have defined functions in cell-survival and motility, but are also expressed in cases of collective or adhered migration where epithelial morphology is maintained, and therefore cannot be a true marker of *EMT*. Nevertheless, *snail* expression has been firmly linked to *EMT*. (Wang et al. 2013) Historically the role of the *snail* superfamily was thought to be restricted to mesodermal development and the induction of motility, and only later were its capabilities stretched to also define *EMT*. *EMT* is amorphously defined as the process by which differentiated epithelial cells lose neighboring cell-cell contacts and adopt a mesenchymal and migratory phenotype and morphology similar to fibroblasts (spindle-like with cellular processes). The *EMT* transitioned cell is thought to disseminate from its previous residence in a stationary epithelium using individual migration. This process has been accepted as dogma in development and metastasis, yet it deserves a critical review. (Thiery et al. 2009) *EMT* induction is purportedly initiated by receptor tyrosine kinase activation, through such ligands as *EGF*, *hepatocyte growth factor* (*HGF*), *FGF*, *TGF-β* and *bone morphogenic protein* (*BMP*). (Barrallo-Gimeno and Nieto 2005; Nieto 2002) In the context of carcinogenesis, it was thought that *E-Cadherin* was downregulated in invasive carcinomas, and *snail* was identified as a transcriptional regulator *of E-Cadherin*. (Perl et

al. 1998) This *snail/E-Cadherin* relationship alongside the notion that *snail's* broader function was to induce *EMT* led to the idea that *EMT* was the ultimate regulator and initiator of the metastatic cascade. So widespread was the idea that an *EMT* transition was required for successful metastasis, that in the 2011 reboot of the supremely influential Hallmarks of Cancer, *EMT* was reported to "Broadly regulate invasion…" and be *the mechanism* by which transformed epithelial cells became invasive. (Douglas Hanahan and Weinberg 2011) However, as outlined in a thoughtful review by Barrallo-Gimeno and Nieto, *snail* may be implicated in all studied *EMT* processes, but that does not mean the role of *snail* is to induce *EMT*. (Barrallo-Gimeno and Nieto 2005) In the following example regarding the development of the mammalian hard palate epithelium, long-range migration and *snail* activation will be shown to occur without loss of epithelial identity.

Palatal Shelves in mammals migrate as an adhered epithelial unit, the so called median edge epithelium (*MEE*). Eventually the leading edge cells (similar to *tip cells*) from opposing right and left migrating units adhere to form a thickened midline epithelial seam (*MES*). When these two groups of migrating epithelia come together, the leading cells intercalate and bind firmly using *filopodia* and *lamellipodia*, again suggesting the formation of a coherent epithelium required morphological changes characteristic of migration. *Snail* is expressed at the cells of the leading edge despite their firm adherence to neighboring epithelial cells located laterally and to the rear. *TGF-B3* signaling from the mesenchyme is crucial for palatal fusion and *filopodia*

formation and is thought to act upstream of *snail*. Without *TGF-B3*, soon-to-be adhered *MEE* cells have dramatically reduced *filopodia* on their apical membrane, but maintain strong *E-Cadherin* expression at their basal surface. *Snail1 and snail2* null animals show defects in *MEE* migration, but exhibit ample *filopodial* formation. (Murray, Oram, and Gridley 2007) *TGF-B3* null mice maintain *snail* expression, perhaps through overlapping roles of related *TGF-B* family members. Mesenchymal sources of *TGF-B1* upregulate expression in the absence of *TGF-B3*. *TGF-B3* null mice do have defects in *MEE filopodial* formation and cell-cell intercalation but migrate normally. Thus it seems there are different roles for *snail* family members and *TGF-B3* in palatal shelf fusion, whereby *snail* regulates migration and *TGF-B3* regulates formation of the *filopodia* necessary for the adhesion of the migrating shelves once they come in contact. (Taya, O'Kane, and Ferguson 1999; Martínez-Alvarez et al. 2004) Without *MES* epithelium fusion, the nasal and oral cavities do not separate, resulting in a so-called cleft palate. (Martínez-Alvarez et al. 2004; Barrallo-Gimeno and Nieto 2005) This provides an example of collective epithelial cell migration in development where a "marker" of *EMT* is expressed without the loss of intercellular adhesion. It also questions the tightly held assertion that *EMT* is required for epithelial cell migration in development. (Thiery et al. 2009; Kalluri and Weinberg 2009)

The *snail* family member *slug* shows increased expression without induction of a mesenchymal phenotype in cutaneous wound healing. As reviewed above, migrating mammalian keratinocytes retain cell-cell junctions and move as a cohesive sheet during

re-epithelialization in response to tissue injury. *Slug* was expressed in leading edge cells as they migrated over the wound area, even as these cells maintained contact with the sheet of epithelial cells. (Savagner et al. 2005)Inhibiting cell division and DNA synthesis with *mitomycin-C* did not change the wound re-epithelialization process, suggesting that *slug* expression at the wound margins was associated with migration without proliferation.

One might argue that, in the discussion of the functions of *snail* family member genes, the role of *EMT* transitions are overstated. As outlined above, there are multiple examples *snail* family expression in the context of motility with retained epithelial integrity. A close reading of the *EMT* literature reveals that the phenomenon would be more aptly named "collective epithelial migratory phenotype." In opposition to the *SMT* theory, mounting evidence suggests the epithelial cell is a morphologically plastic cell with an inherent ability to migrate in groups. Rather than a default state characterized by senescence, differentiation and inhibition of motility, these are phenotypes imposed by signals from the environment. Reversion to a motile state is an expression of an innate characteristic, not the acquisition of a new ability. It follows that this program must come from a *removal* of differentiating factors, rather than the *addition* of an *EMT* inducer or genetic mutation.

The confusion regarding the *EMT* transition is easy to understand given the experimental conditions used to generate the observation. Induction of migratory behavior from individual epithelial cells is readily observable under *in-vitro* culture

conditions. (Casas et al. 2011) Data rigorously presented by Hay in the 1960s-2000s using this type of experiment supported the concept of *EMT*. Importantly, *EMT* has always lacked strong support from *in-vivo* data. (Greenburg and Hay 1988; Greenburg and Hay 1986) Interestingly, a closer review of the gastrulation/mesodermal invasion studies performed in the 1980's readily admit that single-cell movements were rare, and instead movement with retained cell-cell contacts was seen much more often. (Nakatsuji, Snow, and Wylie 1986) If we admit the possibility that the specialized morphology of a fully differentiated epithelium is exogenously imposed rather than generated by individual cells through genetic pre-determination, the observation that epithelial cells acquire motility *in-vitro* is not surprising. Furthermore, not only do culture conditions remove inhibitory signals, they also supply activating signals. *In-vitro* studies developed by Hay to link *EMT* to development or more recently performed by Weinberg's group to link *EMT* to the metastatic process utilize a wholly unnatural microenvironment in part through growth factor enrichment. (Greenburg and Hay 1986; Yang et al. 2004) Analysis of calf serum similar to that utilized by both groups include *bFGF*, *TGF-β1*, *IGF* and *glial growth factor* (*GGF*) among others. (Zheng et al. 2006) Given that a tissue-imposed epithelial phenotype is a possibility, it follows that the microenvironment could severely regulate the availability of nutrients and other signaling pathways activators, a situation wholly unlike *in-vitro* conditions which provide nutrients in overabundance and lack differentiating pressures. For these reasons, it can be argued that conclusions drawn from *in-vitro* studies have less relevance to the

understanding of tumor progression *in-vivo* than what is commonly assumed. *In-vitro* studies have yielded a wealth of information on signaling cascades and the biology of individual cells, but very likely miss key events in the complete pathway of carcinogenesis.

What Does Metastasis Look Like in the Clinic (*in-vivo*)?

A good place to truly examine the relevance of *EMT*, and investigate how cells actually migrate in cancer is through examination of human tissue samples. Is there abundant clinical evidence to indicate that *EMT* is part of the metastatic cascade, as a majority of the cancer biology field would argue? (Douglas Hanahan and Weinberg 2011) A recent review admitted the lack of evidence for a binary switch during *EMT* transition and indicated that epithelial markers were retained in these supposedly "mesenchymal" cells. (Ye and Weinberg 2015) Furthermore, data on which they base their argument only utilized "markers" of mesenchymal cells without much consideration of actual cell morphology. Their "rigorous" criteria for *EMT* existence in an *in-vivo* model of pancreatic ductal carcinoma (overexpressing *Ras* and lacking *p53*) included *one cell* showing both mesenchymal and epithelial markers in less than 5% of the induced pre-malignant lesions. (Rhim et al. 2012) Furthermore, the cells they chose to best illustrate this process in their report (i.e. used in figures) were clearly not migrating as individuals, but were instead closely associated and in contact with their neighbors.

Nonetheless, does a comprehensive histological analysis of clinical samples confirm the existence of *EMT*? Noted pathologist David Tarin has studied many thousands of patient tissue samples without observing a single convincing *EMT* occurrence. He believes the existence of poor and disordered differentiation characteristic of carcinomas fools researchers into believing in the existence of *EMT*. But he affirms that such morphological disorder does not call for invoking the drastic change in cell lineage necessitated by an *EMT* transition. As an example, infiltrating lobular carcinomas of the breast do not migrate as scattered individual cells, but rather in a sequential daisy chain, firmly adhered to their front and back neighbors, reminiscent of collective epithelial cell migration utilized during development in mammalian and non-mammalian systems. Certainly the polarity of these cells has changed, and they have re-expressed migratory characteristics, but they have not lost their epithelial identity. Furthermore, adenocarcinomas invade as glandular structures, and squamous cell carcinomas invade as clumps and maintain differentiation and the phenotype of secretory epithelium, even producing *keratin*. Even the occasional spindle cell appearance of squamous and adenocarcinomas are rarely seen migrating individually. (Tarin 2005) Furthermore, these cells are most likely myoepithelial cell in origin rather than the result of a dramatic transdifferentiation program into an entirely different cell lineages. (Zarbo 2002; Tarin 2005) At some point after arrival at the metastatic niche site, these cells should finish their differentiation program and adopt a fully mesenchymal identity, such as adipose tissue, muscle or bone. Yet this scenario is not

observed clinically. Instead the origin of distant metastatic sites is commonly identified by the metastasizing cells consistency with features from the original neoplasm, arguing that these cells never lost their identity in the first place. (Tarin 2005) Thus *EMT* theory seems to indicate that cancer cells change their gene expression pattern to become an different cell type, and is able to run this program in reverse at the new metastatic niche. Differentiation cues at that distant niche presumably would drive the newly mesenchymal cell to form structures supporting the new tissues, rather than the site of origin. That differentiating signal at the metastatic niche presumably would be ignored while other signals are integrated, so as to allow expression of *integrins* corresponding to the new *ECM* for example. Thus the cancer cell must ignore the strong cues to differentiate into a functionally useful cell for tissue, but "listen" to the cues that recognize that they have in fact arrived to a suitable niche.

Clinically many examples exist of metastatic and invasive carcinomas retaining a well-differentiated epithelial morphology, indicating *EMT* is not required for metastasis. Prostate metastatic lesions in lymph nodes retain epithelial features and a polarity characteristic of prostate epithelium. They form acinar glandular structures with normal apical/basolateral polarity. Their secretory phenotype is recognizable; nuclei are located in the basolateral portion of the cell while secretory products were confined to the apical portion. Apical specific markers were retained in their usual polarized location. (Christiansen et al. 2005) Furthermore, in a study of 149 patient samples of invasive ductal breast carcinomas, less than 10% of the invasive tumors did not form

recognizable epithelial tubules and most cells were polarized normally. (Tan et al. 1999) An additional study of 208 tumor biopsy samples found 72% stained positive for *E-Cadherin*, a marker of cell-cell contact. The expression of this adhesion marker was unrelated to invasion of lymph nodes or appearance of other metastatic lesions. (Lipponen et al. 1994) Further, elegant 3D reconstruction of tumor buds at the invasive fronts of adenocarcinoma tumors, where cancer cells are exiting the main tumor and entering the stroma, in the lung, pancreas, colon and beast revealed it was extremely unlikely to have a single cell invading the stroma. Their analysis of 5000 invasive buds across 15 tumor samples indicated the chance of finding a single invasive cancer cell was .003%, at 99.9% statistical power. Instead most buds were multi-cellular and retained connection to the primary tumor. (Bronsert et al. 2014) These data give further evidence that metastasis occurs without the need to invoke a radical change in cell lineage. They reveal that infiltrating epithelial cancer cells migrate adhered to one other while retaining much of their original morphology. It also indicates that we should explore collective cell migration in greater detail, utilizing progress made in mammalian and non-mammalian models discussed above, if we aim to dissect the true program of metastatic progression. We have been easily led astray when trying to answer *in-vivo* questions using *in-vitro* artificial environments. If instead we ground our observations on clinical data that reveal epithelial features are retained within invading groups of cells, it becomes unnecessary to invoke a radical change in gene expression and lineage in order to explain the “acquired” motility of epithelial cells. Single cell “mesenchymal”

migration, so beautifully described in 2D collagen preparations, does not accurately recapitulate the nature of metastasis *in-vivo*. If we accept that epithelial cells can both be motile and maintain cell-cell adhesions, then the process of unraveling the mechanisms of invasion will be greatly simplified.

Further evidence that the re-expression of a motile phenotype in epithelial cells does not require a dramatic and global change in gene expression comes from the fact that nearly every stimulus (even non-mutagenic) can induce "*EMT*." Almost all peptide growth factors (*EGF*, *HGF*, *TGF-β*), cytokines, transcription factors, hypoxia, oxidative or metabolic stress, or collagen sheets can induce *EMT*. (Savagner 2015) It seems as soon as an epithelial cell is placed *in-vitro*, thereby removing the microenvironmental factors maintaining its differentiation, any stimulus can induce motility. This bolsters the idea that migration is an endowed capability rather than acquired one in epithelial cell types.

Cell-Cell Cooperativity of Heterogeneous Cell Populations Facilitate Metastatic Invasion

Migratory macrophages and eosinophils seem to play a role in development of the mammary gland. (Gouon-Evans, Rothenberg, and Pollard 2000) Both cells preferentially accumulate near terminal end buds, the mitotically active migratory glandular structures that serve as precursors for ductal development. Depleting leukocytes through sublethal γ-irradiation inhibited ductal outgrowths, without affecting proliferation of mammary epithelial or stromal cells, or increasing estrogen levels (the

major hormone for mammary gland development). Bone marrow transplant immediately after irradiation rescued ductal development, and this rescue coincided with the re-emergence of leukocytes in the blood and accumulation of macrophages and eosinophils at terminal end buds. Mice deficient in the macrophage differentiating factor *colony stimulating factor 1* (*CSF1*) are depleted of macrophages. These transgenic animals exhibit disorganized and under-developed mammary gland ductal trees. This phenotype was completely rescued upon exogenous *CSF1* supplementation.

In a murine model of breast cancer, macrophage depletion through *CSF1* knockout significantly delayed and reduced lung metastasis, but did not alter primary tumor growth. Correspondingly, loss of macrophages also retarded tumor histological progression to late-stage invasive carcinoma. Tumor advancement to the invasive stage was correlated with macrophage infiltration to the tumor site. However, in the *CSF1* null mutants, although tumors took a significantly longer time to progress to the invasive carcinoma stage, they did ultimately show a metastatic phenotype without peritumoral macrophage accumulation. Yet *CSF1* overexpression in the mammary epithelium significantly advanced tumor progression to the invasive stage, suggesting macrophages promote tumor progression but has functional redundancies. (Lin et al. 2001) The mechanism of this macrophages and tumor cell interaction has been proposed to be a paracrine signaling loop, whereby cancer cells release the macrophage attractant *CSF1*, while in turn macrophages secrete *EGF* which activates the tumor cell. This signaling mechanism may create a positive feedback loop, as *CSF1* binding on macrophages led to

increased production of *EGF*, and *EGF* binding on carcinoma cells promoted *CSF1* production. These two cell types were observed to migrate together toward an artificial chemotactic gradient and inhibition of either *CSF1* or *EGF* signaling was able to block migration of both cells *in-vitro*. (Goswami et al. 2005)

Macrophages are thought to accumulate specifically at the tumor margin and along the tumor vasculature. Metastasizing tumor cells are observed to preferentially intravasate into vessels near perivascular macrophages. *CSF1* depletion, confirmed by a 6-fold reduction of perivascular macrophages, led to a 16-fold reduction in viable tumor cells in the systemic circulation. Intravenous injection of anti-*CSF1* antibody reduced tumor cell levels in blood by 6-fold, suggesting this process is *CSF1* dependent. (Wyckoff et al. 2007) Although no mechanism was suggested by which the macrophages signal endothelial cells to improve cancer cell dissemination efficiency, these data suggesting that perivascular macrophages do aid in the vascular intravasation of disseminating tumor cells.

Fibroblasts may play an integral role in cancer cell dissemination. Fibroblasts are endogenously capable of modifying the *ECM*, which metastasizing epithelial cells must navigate *en route* the vasculature. A fascinating study using a modified culture system to recapitulate *in-vivo* conditions *in-vitro* has shown that fibroblasts may lead groups of collectively migrating squamous carcinoma cells (*SCC*), a phenomenon that has also been identified in subsequent studies using adenoid cystic carcinoma (*ACC*) cells and cancer associated fibroblasts (*CAFs*) derived from the salivary gland. (Li et al. 2016)

When *SCC* epithelial cells were cultured alone upon a dense matrix consisting of predominantly *collagen I*, with a lesser density of *laminins* and *collagen IV*, they did not invade. But if fibroblasts isolated from either oral or vulval squamous cell carcinoma were mixed into the underlying matrix, the overlaid *SCC* cells invaded the matrix as adhered cohorts of cells, while maintaining expression of the epithelial markers *E-Cadherin* and *p120* catenin. Interestingly, the migration speed of *SCC* epithelial cells was not significantly enhanced by the presence of fibroblasts, confirming that cohorts of epithelial cells were capable of collective invasion autonomously. Adding an 80 μm layer of matrix between the *SCC* cells and the fibroblasts abolished *SCC* invasion into the matrix, suggesting that it was not a long range diffusible factor secreted by the fibroblasts that promoted invasion. However, if the underlying matrix was first cultured with fibroblasts, then fibroblasts depleted, *SCC* cells were able to invade. This, along with co-culture barrier experiments, suggested it was fibroblasts-mediated remodeling of the matrix that permitted the *SCC* invasion rather than intercellular signaling. (Gaggioli et al. 2007) Although the presence of a short range signaling factor could not be ruled out. Another group found fibroblast mediated remodeling and alignment of collagen fibers at the tumor/stromal junction, elegantly detected by plane-polarized imaging, as well the fact that cancer cell migration velocity and directional coordination increased over this area of modified *ECM*. (Lee et al. 2011) However, in the enhanced *in-vitro* co-culture study using *SCC* cells, the most successful invasion occurred when

fibroblasts and *SCC* cells were first mixed then deposited on top of the matrix together, suggesting the fibroblasts acted in an manner beyond *ECM* modification.

Closer analysis of invading chains of epithelial cells revealed that fibroblasts always led the invading epithelial cohort, although subsequent epithelial cells were able to change position within the migrating cohort, as noted in other studies. It was not investigated whether this reorganization depended on intracellular signaling strength, akin to what is observed in *drosophila* tracheal cell migration. Transmission electron microscopy revealed the presence of holes in the matrix behind migrating fibroblasts, and *SCC* cells within these holes. The remodeled matrix behind fibroblasts consisted of thickened bundles of *collagen* along the sides of the formed tubes, and deposition of *ECM* components *fibronectin* and *tenascin* C, although inhibition of *fibronectin* and *tenascin C* deposition did not alter *SCC* invasion. Further analysis of the fibroblast migration revealed a dependence on *Rho/ROCK* signaling. Blockade of *RhoA* (but not *RhoB*), *ROCK1* or *ROCK2* inhibited the formation of holes and the ability of fibroblasts to contract matrix. Blocking *non-muscle myosin*, *ECM* interacting *Integrin α3* or *α5* or *MMP* function also inhibited fibroblast dependent matrix remodeling and subsequent *SCC* cell invasion. Only one downstream target of *ROCK* was correlated with the *ECM* remodeling ability of fibroblasts, the phosphorylation of *myosin light chain*. Interestingly, inhibition of *Rho*, *ROCK* or *Integrin α3* or *α5* function in *SCC* cells did not affect their invasive ability, suggesting they used alternate signaling pathways than the fibroblasts to generate motility in this assay. Indeed, inhibition of *cdc42* (akin to mammalian epithelial

migration during wound healing) and myotonic dystrophy kinase-related *CDC42*-binding protein kinases (*MRCK*) in *SCC* cells abolished their ability to follow fibroblasts, and disrupted co-localization of *phosphorylated myosin light chain* at the *actin* cortex in these cells. Further, histological analysis of clinical tissue samples of head and neck squamous cell carcinoma revealed fibroblast led collective invasion of *SCC* cells, and activation of *Rho* within the fibroblasts. (Gaggioli et al. 2007) This work was bolstered by the observation that collagen fibers surrounding human tumors were reorganized to align normal to the tumor/stromal interface, similar to what was seen in the model, and this seemed to enhance cancer cell dissemination from the tumor site. (Provenzano et al. 2006)

The data regarding separate intracellular mechanisms of cell-shape change and migration within the fibroblasts and *SCC* cells is interesting. Other studies have indicated that *ROCK* and *MRCK* signaling cooperate to mediate actinomyosin contractility, as they both ultimately modulate myosin light chain 2 (*MLC2*) phosphorylation through *T696* phosphorylation on *MYPT1* (myosin phosphatase target subunit 1), upstream of *MLC2*. It seemed *ROCK* and *MRCK* function could overlap during an *in-vitro* invasion study using colorectal carcinoma cells. Both *ROCK* and *MRCK* mediated morphological modifications of *BE* cells into a rounded (more dependent on *ROCK*) or elongated form, either of which could be used to drive motility. (Wilkinson, Paterson, and Marshall 2005) Identification of overlapping pathways of motility is vital, at least in *CAFs* as a study on a limited number of cancer cell types has shown that *CAFs* (and less convincingly, the epithelial

MDA-MB231 cell line) could modify their method of motility when their "preferred" method was blocked. In a fibrosarcoma cell line which usually migrated in a protease dependent mechanism and maintained a spindle-like morphology, *Integrins* and *MMPs* co-localized at the cell membrane which allowed adherence to and degradation of the matrix. However, when this method of migration was blocked using a protease cocktail, the cells adopted a more rounded, "amoeboid" morphology, but did not change overall migration speed. (Wolf et al. 2003)

Another study sought to investigate the signaling cascades by which normal fibroblasts conferred the pro-invasive *CAF* phenotype. *TGF-B1* treatment was sufficient to convert dermal fibroblasts, who had previously been unable to facilitate *SCC12* invasion nor contract collagen gels, into a phenotype that could promote invasion of these activated epithelial cells. Interestingly while *TGF-B1* was found necessary to promote *CAF* conversion, while *Janus kinase/signal transducer and activator of transcription* (*JAK/STAT3*) was required to maintain the *CAF* phenotype. Further investigation revealed that *TGF-B1* mediated a 100-fold increase in the expression of the cytokine leukemia inhibitory factor (*LIF*) and a 5-fold increase in *mRNA* of the interleukin 6 (*IL-6*) cytokine. Together these cytokines appear to mediate *STAT3* phosphorylation and activation downstream of *JAK1*. As confirmation, *LIF* blocking antibodies abrogated the ability of *TGF-B1* to induce collagen gel contraction by fibroblasts and promote *SCC12* cell invasion. Furthermore, fibroblasts treated with *LIF* alone were able to recapitulate the *TGF-B1* actions to promote the *CAF* phenotype. SiRNA mediated

knockdown of *RhoA* or blockade of *ROCK* was able to abolish both *TGF-B1* and *LIF* mediated effects. Taken together, the results show that the *LIF* cytokine is actually responsible for *TGF-B1* induced actinomyosin activation and contractility underlying the *ECM* remodeling by fibroblasts used to enhance epithelial cancer cell invasion. Furthermore, media conditioned by two related *SCC* cell lines was able to transform WT dermal fibroblasts into *CAFs*. Only antibody blockade of *LIF*, not *TGF-B1*, was able to stop this transformation. This gives a mechanism by which carcinoma cells could induce *CAF* formation in the tumor microenvironment, and also reveals the dependence of this process on *LIF* rather than *TGF-B1*, which shows ubiquitous signaling molecules may have more specific mediators downstream outside of their signaling cascade. This process was also revealed to be independent of *TGF-B1* induced *α-SMA* expression. This is an important point, as *CAFs* are traditionally defined by *α-SMA* expression, yet this study revealed that *LIF* stimulated fibroblasts can promote invasion of carcinoma cells without expressing *α-SMA* as commonly assumed. (Albrengues et al. 2014; Kalluri and Zeisberg 2006)

To investigate further *in-vivo*, two breast carcinoma cell lines that dramatically differed in their *LIF* expression were transplanted into fat pads of mice. Peritumoral collagen fibers were remodeled and aligned in the tumors produced by high *LIF* producing cells. Inhibiting *JAK1/2* with *Ruxolitinib* abolished *STAT3* activation, collagen fiber realignment, and also significantly reduced tumor cell invasion into the stroma, but did not change the tumor size. Furthermore, *Ruxolitinib* treatment abolished the ability

of activated fibroblasts (*CAFs*) treated with *LIF* or *TGFB1* to promote invasion of squamous carcinoma cells, contract collagen gels or realign collagen fibers. *LIF* staining was high in biopsies from lung, skin and head and neck carcinoma samples. Further analysis of these carcinoma types revealed that collagen reorganization and alignment, and worse clinical outcomes were associated in carcinomas of relatively high *LIF* expression. (Albrengues et al. 2014) *LIF* inhibition therefore could be an attractive target to reduce cancer cell metastasis.

A hallmark of the tumor microenvironment is angiogenesis. A *de-novo* vasculature is required to nourish a growing tumor mass, and tumors without angiogenesis show increased cancer cell apoptosis or necrosis prohibiting additional tumor growth. Further, the ability of cancer cells to metastasize to distant sites is enhanced by the additional routes created by new vessels to access the systemic circulation. Vascularization is stimulated by the *VEGF* family of polypeptides, originally identified by Harold Dvorak as factors promoting vascular permeability secreted by cancer cells. Separately *VEGF* was identified by other groups studying angiogenic *FGF* signaling initiated by Judah Folkman's work on cancer cells. (Douglas Hanahan and Weinberg 2008; Senger et al. 1986; Senger et al. 1983) *VEGF-C* is thought to contribute to angiogenesis in the tumor microenvironment by binding *VEGF-R3* expressed on migrating endothelial *tip cells* at the leading edge of growing vessels. (Baeriswyl and Christofori 2009) *VEGF* induced *filopodia* formation in endothelial *tip cells*. *Tip cells* migrate with *stalk cells* as part of an adhered endothelial collective toward *VEGF*

gradients, similar to *tip/stalk cell* behavior in response to *FGF* in the developing trachea in *D. melanogaster*, or *Wnt/β-Catenin* in *zebrafish* (Gerhardt et al. 2003) *Notch* signaling between *tip* and *stalk* endothelial cells further recapitulates *D. melanogaster* trachea development, whereby lateral inhibition through *Notch* receptor activation restricts cells away from a *tip cell* fate. Heterozygous *dll4* (delta like ligand for *Notch*) null mutant mice display increased *filopodia* formation and an increased number of *tip cells*, while, *Lox/Cre* inactivation of *Notch1* selectively produces tip cells. Nearly all tip cells showed *dll4* expression, akin with tip cell production of similar *Notch* ligands in *D. melanogaster*. (Hellström et al. 2007) Further, just as *FGF* induces *dll* expression in *D. melanogaster tip cells*, *VEGF-A* induces *dll4* expression in endothelial cells, most appreciably at the leading front of vascular sprouts (the *tip cell* region). (Lobov et al. 2007; Hainaud et al. 2006) Exogenous *Notch* activation, using the Notch signaling peptide *jagged1*, decreased *filopodial* density by one third, decreased vessel density by nearly half and significantly reduced branching. (Hellström et al. 2007) In terms of cancer therapy, targeted activation of *Notch* signaling, restricted to endothelial cells using an *ADC* (Antibody-Drug Conjugate) or similar targeted drug delivery system, would be an intuitive mechanism to disrupt tumor vasculogenesis. It would reduce *lamellipodia* formation, vessel sprouting, and branching to limit the ability of the *de-novo* vascular tree to supply nutrients for tumor growth.

Curiously the reverse strategy has moved into the clinic as a potential cancer therapy. Inhibitors of *Notch* signaling, such as *GSIs* (*γ-secretase* inhibitors), or antibodies

against the notch ligand *dll4*, are currently being tested in clinical trials. (Khan and Bicknell 2016) The rationale is that *Notch* inactivation would lead to inappropriate and excessive tip cell formation and overabundant branching thereby reducing the perfusion efficiency of the newly formed vessels. Unfortunately, intestinal toxicity has been associated with *GSIs*. *GSI* treatment led to excessive production of secretory cells and a relative paucity of absorptive cells in the intestine, as *Notch* signaling cooperates with *Wnt/β-Catenin* direct differentiation in the intestinal crypt.

Some investigators have utilized *Notch* inhibition to drive differentiation programs thereby slowing tumor growth and inhibiting tumor mitosis. They have noted that *APC* null proliferative adenoma cancer cells are converted to post-mitotic goblet cells upon *Notch* inhibition, providing a viable treatment option if gastrointestinal side effects could be reduced. (van Es et al. 2005) In fact, selective targeting of individual *Notch* receptor subtypes (e.g. *Notch1* selective) has shown potent anti-tumor and anti-angiogenic effects while eliminating the over-representation of secretory cell types in the intestine, thereby potently reducing or eliminating intestinal toxicity. (Wu et al. 2010)

Modulating *Notch* signaling may provide a powerful method to disrupt the migration of groups of epithelial and endothelial cells. This in turn could profoundly impact cancer treatment by providing a series of drug development targets which reduce metastasis and angiogenesis, crucial processes underlying the progression of cancer.

Conclusion

The assumption that epithelial cells at steady-state are well-behaved and inflexibly differentiated (rather than primed to divide, grow and move) has so far limited our conceptual understanding of carcinogenesis and metastasis. When viewed from the perspective that the default state of cells is division and motility, multicellular tissues are highly unstable structures. Yet somehow, through complex signaling mechanisms such as *progesterone* receptor mediated induction of the anti-migratory *Elf5*, a differentiated phenotype is maintained. It comes to reason that differentiated cells in the body must receive continual cues to maintain that differentiation, as outlined previously in the breast, lung, intestine, prostate and salivary gland. An interruption of these signals, rather than serendipitous genetic mutations, could lead to the unrestricted growth and motility that defines carcinogenesis and metastasis.

Caught up in the recent pace of technological change for studying DNA and the genome, we have become satisfied that a reductionist philosophy and studying the nucleus of single cells will sufficiently explain emergent properties of tissues and the interactions between cells therein. Our confidence in cracking the genetic code, despite the fact that we still do not know the function of over 97% of the genome! (Cavalli-Sforza 2005) This “non-coding” DNA is almost as likely to be conserved as coding regions between species, and such intolerance to mutations over evolutionary time suggests a function. (Meisler 2001) Furthermore, it is well known that the DNA sequences of *homo sapiens* and *mus musculus* are almost identical. Additionally, when one considers

functional similarity of proteins rather than identical genetic sequences, the amino acid sequence for the ribosomal protein *L36* is unquestionably homologous and easily aligned between *homo sapiens*, yeast, *Escherichia coli*, and even the cereal grain rice (*oryza sativa*). (Koonin and Galperin 2003) The answers to the open questions regarding cancer cell behavior, which is more thoughtfully understood as the study of normal epithelial cells in the absence of differentiation cues, are not in the genome. It is in the complex cell-cell interactions that take place within the tissue. Aspects of this signaling can be understood by studying the genome and cells in isolation, but the result of cell-cell interactions within a tissue cannot be predicted from DNA sequences. Only through thoughtful study of cell-cell signaling mechanisms, from a variety of *in-vivo* and *ex-vivo* model systems, will it be possible to reconstruct the complex intercellular interactions within actual tissues. As this review hopefully indicates, there exists a wealth of data from a variety of model systems, including non-mammalian ones, that could be used to develop novel cancer treatments. Tissue-specific cell-cell signaling pathways offer specificity light years beyond some of the carpet-bomb chemotherapy treatments still being utilized in the clinic. A first step towards this currently unpopular paradigm is admitting that our understanding of epithelial cell behavior, the origin of carcinogenesis, and the metastatic process, all require revision.

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CURRICULUM VITAE

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Education and Test Scores

MCAT- 37, January 2014
Master of Science- Medical Science, *Boston University,* September 2016
GPA 4.00 (*anticipated)*
Master of Science- Neuroscience, *Tufts University,* August 2012
GPA 3.54
Bachelor of Science- Neuroscience, *University of Pittsburgh,* May 2007
GPA 3.12, Minor in Chemistry

Publications:

O'Toole KK, Hooper A, **Wakefield S**, Maguire J. Seizure Induced disinhibition of the HPA Axis increases seizure susceptibility." Epilepsy Research. 2014 Jan.

Sarkar J, **Wakefield S**, Mackenzie G, Moss SJ, Maguire J. Neurosteroidogenesis is Required for the Physiological Response to Stress: Role of Neurosteroid Sensitive GABA-A Receptors." Journal of Neuroscience. 2011 Dec 14.

Wakefield SE, Dimberg EL, Moore SA, Tseng BS. Dystrophinopathy presenting with Arrythmia in an Asymptomatic 34 year-old: A Case Report." Journal of Medical Case Reports. 2009 July 24.

Experience

Clinical Research Assistant II, Beth Israel Deaconess Medical Center, Boston, MA, January 2014-Present

- Successfully coordinating a 20 subject clinical trial for Spino-Cerebellar Ataxia, working closely with physicians, nurses and administrative staff
- Conducts baseline, follow up examinations of mobility and motor network function
- Assists study physician conduct patient examinations, analyzes trial data using Matlab, excel, JMP; maintains regulatory documentation

- Conducted assessments of gait, balance and cognitive reserve in an outpatient clinic, the Brain Fit Club
- Earned bonus, raise and promotion after 1 year

Academic Tutor, Worcester, MA, December 2013-Present

- Biology, Chemistry and English tutor for high school students
- Developed positive relationships and achieved demonstrable results from underachieving students

Account Manager, Abpro, Lexington, MA, October 2012-May 2013

- Responsible for all aspects of account management: finding accounts, understanding customer needs, assessing feasibility of project success, writing proposals, negotiating contracts and ensuring deliverables are met
- Fostered team development, mentored new hires in sales/marketing

Graduate Student, Tufts University, Sackler School of Graduate Biomedical Sciences, Boston, MA September 2010 - May 2012

- Successfully completed MS thesis "GABAergic control of the HPA axis as a treatment target for Epilepsy"
- Attended and presented work at two Gordon Conferences (July 2010, Epilepsy; August 2011, Inhibition)
- Performed western blot, ELISA, small animal surgery, animal dosing, sacrificing, tissue processing and analysis, Immuno-staining

Volunteer Tutor, TutoringPlus, Cambridge, MA, Jan 2009- June 2009

- Helped prepare a high school junior for college level study habits on a one-on-one weekly basis through the school year

Research Assistant, Massachusetts General Hospital (MGH), Department of Neurology, Boston, MA October 2007- July 2010

- Presented poster: "Mechanism of Corticosteroid action in Muscular Dystrophy" at Massachusetts General Hospital for Children research symposium
- Shadowed a Neurologist in a weekly Muscular Dystrophy clinic at the MGH Hospital for Children
- Gathered data, conducted interviews and authored a case report entitled, "Dystrophinopathy Presenting with Arrhythmia in an Asymptomatic 34 year old man: a case report"
- Performed Western blot, animal sacrificing and tissue processing, Immuno-staining, rodent behavioral testing

- Coordinated move of lab from Denver, CO to Boston, MA and established Neurology Lab in Boston

Volunteer, Massachusetts General Hospital, Boston, MA, December 2007-March 2010

- Served as Appointment Buddy to Muscular Dystrophy patients to help them navigate the hospital campus (Dec. 2007-March 2010), acted as patient escort from outpatient surgery, Emergency Department Family Liaison

Leadership

Graduate Student Council Representative
Neuroscience Department, Tufts Sackler School, August 2011-May 2012

- Co-Editor of the Sackler Insight newsletter, also served on Sackler relays committee and social committee
- Instituted culture change within the Sackler School to further develop graduate student community cohesiveness

Certifications:

AHA Basic Life Support for Healthcare Providers