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Identification of Molecular Mechanisms Underlying the Development of Barrett's Esophagus

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Abstract
Esophageal cancer is one of the deadliest cancers in the U.S and worldwide. Esophageal cancer is characterized by two subtypes: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). One of the major risk factors for the development of EAC is Barrett's esophagus (BE). BE is defined as incomplete intestinal metaplasia characterized by the presence of columnar and goblet cells in the formerly stratified squamous epithelium of the esophagus. Currently, the cell of origin for human BE has yet to identified. Using an innovative 3D organotypic culture system, we explored the role of inhibition of Notch signaling promotion of transdifferentiation of esophageal epithelial cells to BE. Our RNA microarray and tissue microarray (TMA) data support the premise that loss of Notch signaling is involved in BE. Inhibition of Notch signaling by dominant-negative-Mastermind-like (dnMAML), in concert with MYC and CDX1 overexpression, promoted transdifferentiation of esophageal epithelial cells towards BE as demonstrated by increased expression of columnar keratins and glandular mucins with decreased expression of squamous keratins. Our data show KLF4 and HATH1, as downstream effectors of the inhibition of Notch signaling, are involved in the initiation of BE. We investigated whether these findings translated into a genetically engineered mouse model. We addressed this by engineering transgenic mice to conditionally overexpress MYC specifically in the esophageal epithelium with the EBV-L2 (L2) promoter. We used a Tet-ON system to conditionally express MYC. To achieve this we created two new transgenic mice: *TetOp-Myc* and *L2-rtTA;TetOp-CreER*^{T2}. We bred *L2-rTta; TetOp-Myc* mice with *K14-Cdx2* and *TetOp-dnMAML* mice, in order to replicate our *in vivo* studies. These studies are ongoing. Invasion is a theme common to BE/EAC and ESCC. As a separate consideration, we investigated the mechanisms underlying invasion in ESCC. Using esophageal epithelial cells transformed by overexpression of EGFR and p53^{R175H}, we found a novel link between p53^{R175H} and c-Met, a receptor tyrosine kinase. These transformed cells show increased expression of the c-Met receptor mediated by p53^{R175H} overexpression. We show inhibition of c-Met expression in the transformed cells (EPC-hTERT-EGFR-p53^{R175H}) diminishes invasion. Our data suggest a new avenue of therapeutics for ESCC through the use of c-Met inhibitors.

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IDENTIFICATION OF MOLECULAR MECHANISMS UNDERLYING THE DEVELOPMENT OF BARRETT'S ESOPHAGUS

Maria Eugenia Vega

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Dedication

This work is dedicated to my grandfather, Roberto E. Vega-Laracuente, for inspiring me to pursue my love of scientific research and to my aunt and godmother, Carmen A. Vega, for her unconditional support and inspiration for pursuing cancer research. Foremost, I dedicate this thesis to my mother, Zulema Meregott, for her love, understanding, never-ending curiosity, and love of learning. Without her support and inspiration to live life to the fullest, this work would have not been possible.

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ABSTRACT

IDENTIFICATION OF MOLECULAR MECHANISMS UNDERLYING THE DEVELOPMENT OF BARRETT'S ESOPHAGUS

Maria Eugenia Vega

Anil K. Rustgi

Esophageal cancer is one of the deadliest cancers in the U.S and worldwide. Esophageal cancer is characterized by two subtypes: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). One of the major risk factors for the development of EAC is Barrett's esophagus (BE). BE is defined as incomplete intestinal metaplasia characterized by the presence of columnar and goblet cells in the formerly stratified squamous epithelium of the esophagus. Currently, the cell of origin for human BE has yet to identified.

Using an innovative 3D organotypic culture system, we explored the role of inhibition of Notch signaling promotion of transdifferentiation of esophageal epithelial cells to BE. Our RNA microarray and tissue microarray (TMA) data support the premise that loss of Notch signaling is involved in BE. Inhibition of Notch signaling by dominant-negative-Mastermind-like (dnMAML), in concert with MYC and CDX1 overexpression, promoted transdifferentiation of esophageal epithelial cells towards BE as demonstrated by increased expression of columnar keratins and glandular mucins with decreased expression of squamous keratins. Our data show KLF4 and HATH1, as downstream effectors of the inhibition of Notch signaling, are involved in the initiation of BE.

We investigated whether these findings translated into a genetically engineered mouse model. We addressed this by engineering transgenic mice to conditionally overexpress MYC specifically in the esophageal epithelium with the EBV-L2 (L2) promoter. We used a Tet-ON system to conditionally express MYC. To achieve this we created two new transgenic mice: *TetOp-Myc and L2-rtTA;TetOp-CreER*^{T2}. We bred *L2-rTta; TetOp-Myc* mice with *K14-Cdx2* and *TetOp-dnMAML* mice, in order to replicate our *in vivo* studies. These studies are ongoing.

Invasion is a theme common to BE/EAC and ESCC. As a separate consideration, we investigated the mechanisms underlying invasion in ESCC. Using esophageal epithelial cells transformed by overexpression of EGFR and p53^{R175H}, we found a novel link between p53^{R175H} and c-Met, a receptor tyrosine kinase. These transformed cells show increased expression of the c-Met receptor mediated by p53^{R175H} overexpression. We show inhibition of c-Met expression in the transformed cells (EPC-hTERT-EGFR-p53^{R175H}) diminishes invasion. Our data suggest a new avenue of therapeutics for ESCC through the use of c-Met inhibitors.

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Chapter I:

Introduction

Background and Significance

Barrett's esophagus (BE) is characterized by the switch of the normal squamous epithelium to a small intestinal-type epithelium (Shaheen and Richter 2009). Gastroesophageal reflux disease (GERD) is one of the most common risk factors for BE development. Both GERD and BE are major risk factors for esophageal adenocarcinoma (EAC) (Lagergren, Bergstrom et al. 1999; Gibson, Dhaliwal et al. 2013). Esophageal cancer can be classified into one of two major subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). In the United States there has been an increase in the cases of EAC by 600% since 1975 (Pohl and Welch 2005). The rise in cases of EAC is due to the increase in the prevalence of BE (de Jonge, van Blankenstein et al. 2014). Esophageal cancer is one of the deadliest cancers in the US, with a 5-year survival rate of 17.3% in 2013 (Howlader, Noone et al. 2013).

There are other risk factors associated with the development of BE, apart from GERD, including the following: obesity, cigarette-smoking and eradication of *H.pylori* infection (Badreddine and Wang 2010). The progression from BE to EAC occurs via a multistep process in which the metaplastic epithelium evolves to low-grade dysplasia, high-grade dysplasia and culminating in EAC. Some of the genetic alterations known to occur in BE are overexpression of cyclin D1 and EGFR oncogenes and loss of p53 and p16 tumor suppressor genes (Graham and McDonald 2010). Yet, the cell that gives rise to BE is still unknown. Therefore, the elucidation of the cell of origin for BE can provide not only an understanding of the mechanisms underlying metaplastic development, but also give insights into genetic factors that can drive progression to EAC.

The squamous esophageal epithelium

The esophageal epithelium functions to protect the underlying tissue from any external damage, such as that from microorganisms or toxic materials (Squier and Kremer, 2001). The esophageal epithelium is a stratified squamous type of epithelium, as opposed to the stomach and small intestine which consist of a simple epithelium. The esophageal epithelium can be characterized further into three distinct cellular layers: basal, suprabasal and superficial squamous (Figure 1). The esophageal basal cells are cuboidal in shape with a high nuclear to cytoplasmic ratio. Basal cells proliferate and give rise to differentiated suprabasal cells. There are various factors known to stimulate proliferation of the esophageal basal cells, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), platelet-derived growth factor (PDGF) and interleukin 1 (IL-1) (Feliciani, Gupta et al 1996; Squier and Kremer 2001). The rate of proliferation can also be modulated by other factors, such as diurnal variation, stress and inflammation. It has been shown that subepithelial inflammation can stimulate mitosis in basal cells. By contrast, severe inflammation can reduce proliferation (Kamer, Krebs et al. 1999; Squier and Kremer 2001).

The basal compartment is where putative stem/progenitor cells may reside. The identification and characterization of stem cells responsible for the maintenance of the esophageal epithelium remains elusive. Slow-cycling cells have been experimentally designated as label-retaining cells (LRCs). In the esophagus a small subset of epithelial cells can retain 3H-thymidine or BrdU for several months (Croagh, Phillips et al. 2007). Our group has used Hoechst dye exclusion to identify a subpopulation of esophageal epithelial cells, experimentally referred to as the side-population (SP), which have the

ability to self-renew and differentiate when grown in 3D organotypic cultures and in mouse injury models (Kalabis, Oyama et al. 2008).

Transgenic label-retaining cell assay has shown the presence of a single progenitor cell population in the basal layer of the mouse esophagus, which can contribute to tissue homeostasis and participate in the repair of the esophageal epithelium upon injury (Doupe, Alcolea et al. 2012). Similar studies, using transgenic lineage labeling assays, show loss of Notch signaling in the progenitor cells which leads to loss of differentiation of these progenitor cells (Alcolea, Greulich et al. 2014). Recently, Sox2 has been identified as a potential marker of putative stem/progenitor cells in the esophagus basal cells (Liu, Jiang et al. 2013). Moreover, whole mount staining of the human esophageal epithelium reveals that quiescent cells expressing putative stem cell markers (CD34+EpCAM-) reside in the tips of the esophageal papillae (Barbera, diPietro et al. 2014). Interestingly, single cell isolation using the stem cell markers CD34 and EpCAM show no significant differences in self-renewal in 2D cultures or differentiation in 3D cultures (Barbera, diPietro et al. 2014).

The differentiation of the esophageal epithelium can be mediated by the presence of various factors, such as extracellular calcium, phorbol esters, retinoic acid and vitamin D3 (Dotto 1999; Dotto 2008). As basal cells enter the differentiation phase, suprabasal cells become larger, begin to flatten and also start to accumulate keratins, which are cytoplasmic intermediate filaments (Figure 1). The proliferative basal esophageal epithelial cells express keratin 5 and keratin 14, and differentiated suprabasal cells express keratin 4 and keratin 13. Finally, in the terminal differentiation stage, the superficial squamous cells express involucrin, profilaggrin and desmocollins

(Squier and Kremer 2001). These cells desquamate into the lumen, and the process of epithelial renewal occurs continuously.

Regulation of the esophageal epithelium by developmental pathways

The development and differentiation of the esophageal epithelium are mediated through various signaling pathways, such as WNT, Hedgehog and Notch. During development both the esophagus and trachea arise from a same foregut tube. The separation of the foregut tube to form the esophagus (dorsal) and trachea (ventral) is mediated by certain signaling pathways. One of the pathways shown to be critical in this process is the WNT (Wingless) signaling pathway. The importance of WNT signaling during esophageal development has been established through certain mouse studies. For example, removal of Wnt2 and Wnt2b in mice leads to a loss of the separation of the esophagus and trachea (Goss, Tian et al. 2009). Furthermore, deletion of β-catenin, a downstream mediator of WNT signaling, during development leads to esophageal atresia and the formation of a tracheoesophageal fistula (Jacobs and Que 2013).

Hedgehog signaling has been shown to be critical during the development of the esophagus. Sonic Hedgehog (SHH) is expressed throughout the esophagus during development and later it is restricted to the distal esophagus (Ramalho-Santos, Melton et al 2000). Study of *Shh*$^{-/-}$ mice show a defect in the separation of the esophagus and trachea during development (Litingtung, Lei et al 1998; van den Brink 2007). Furthermore, knockout of specific SHH effector genes, Gli2 and Gli3 (*Gli2*$^{-/-}$*; Gli3*$^{-/-}$) results in complete atresia of the esophagus (Motoyama, Liu et al. 1998; van den Brink 2007).

The transcription factors Sox2 and Nkx2.1 have been shown to be critical in the separation of the anterior foregut into the trachea and esophagus. Nkx2.1 is normally expressed in the lung, thyroid and brain. Nkx2.1 expression in the foregut is restricted to the developing trachea. Studies with *Nkx2.1-/-* mice show inhibition the separation of the anterior foregut, leading to the formation of one common lumen connecting the pharynx to the stomach (Minoo, Su et al 1999; Que, Okubo et al. 2007). Furthermore, studies with conditional deletion of Sox2 in mice show defective separation of the foregut, and the mutant esophagus show increased levels of Nkx2.1 upon loss of Sox2. (Que, Okubo et al. 2007). Morphological analysis of the *Nkx2.1-/-* mutant esophagi has shown increased levels of Sox2 (Jacobs and Que 2013).

Notch signaling is active in the basal layer of the esophagus. Jag1, Jag2, Notch1 and Notch2 are highly expressed in the basal layer of the mouse esophagus and this expression decreases in the suprabasal layer (Sander and Powell 2004). Furthermore, Notch1 activation is observed in the onset of the stratification of the esophageal squamous epithelium. Inhibition of Notch signaling in the normal esophageal epithelium causes a loss of the normal differentiation of the epithelium, observed by loss of involucrin expression. This process has been shown to be mediated by Notch1 and Notch3 regulation of early differentiation genes (Ohashi, Natsuizaka et al 2010).

Metaplasia

Metaplasia is defined as the switch of one cell type with another cell type. The development of this new cell type is usually an adaptive response to some external or environmental insult. Metaplasia is known to occur in various tissues such as the lung, cervix, stomach and esophagus. For example, in lung, the injury caused by cigarette-

smoking promotes the replacement of the mucus-secreting ciliated pseudostratified columnar epithelium of the normal lung with a stratified squamous epithelium, referred to as squamous metaplasia. In the esophagus injury from GERD and cigarette-smoking lead to the replacement of the normal esophageal squamous epithelium with columnar cells or enterocytes and mucin-producing cells, reminiscent of the small intestinal epithelium, except for the lack of Paneth cells and enteroendocrine cells (Figure 2). This is referred to as intestinal metaplasia. Intestinal metaplasia occurs in the stomach as well. Gastric intestinal metaplasia is characterized by the appearance of an intestinal epithelium in the stomach, including the presence of enterocytes, goblet cells and Paneth cells (Correa, Piazuelo et al. 2010). In most cases metaplasia may be a desirable event. Yet, it is important to note that in same contexts, metaplasia can evolve to dysplasia and cancer, representing an undesirable event.

Cell of origin for Barrett's esophagus

There are several hypotheses for the cell of origin for BE, and these include: (1) transdifferentiation of cells from the esophageal basal layer or from the ducts of the esophageal submucosal glands, (2) migration of cells from the esophago-gastric junction (EGJ) or gastric cardia and (3) bone marrow derived progenitor cells.

Transdifferentiation is defined as a process by which one somatic cell change into another somatic cell, without undergoing a preceding pluripotent or progenitor stage (Orkin and Zon 2008). Potential evidence for transdifferentiation of the normal squamous epithelium to BE has been demonstrated in certain studies (Figure 2A). Studies of human BE biopsies using laser capture microdissection followed by DNA sequencing showed the same p16 mutation found in both the squamous epithelium and

BE, suggesting a common cell of origin (Leedham, Preston et al 2008). Moreover, *in vitro* studies using human immortalized esophageal epithelial cells overexpressing MYC and CDX1 showed a partial transdifferentiation to BE with expression of columnar keratins K8 and K19 as well as MUC5AC (Stairs, Nakagawa et al 2008). Furthermore, a study of human BE biopsies has shown that esophageal squamous epithelial cells express intestinal genes, namely CDX2 and MUC5AC, but have yet to acquire a columnar or goblet cell morphology (Hahn, Blount et al 2009). Overall, these studies provide potential evidence to support the plasticity of the esophageal squamous basal cells to transdifferentiate into the BE epithelium.

The second supposition for the potential cell of origin of Barrett's esophagus suggests the existence of residual embryonic cells at the EGJ or gastric cardia (Figure 2B). P63 global knockout mice show the expansion of residual embryonic cells upon the loss of the squamous epithelium. These residual embryonic cells replace the squamous epithelium with a columnar lined esophagus (Wang, Ouyang et al 2011). In addition, studies of the L2-Interleukin (IL)-1β transgenic mouse showed the development of esophagitis, BE and EAC (see later section). Lineage labeling of putative gastric cardia stem cells by Lgr5-GFP showed presence of gastric cardia derived cells in the BE lesions (Quante, Bhagat et al. 2012). Furthermore, recent studies comparing human BE to the gastric cardia epithelium have shown a similar proliferative and gene expression pattern between BE and the gastric cardia (Lavely, Nicholson et al. 2014).

Thirdly, it has been proposed that the cell of origin for BE may derive from the bone marrow (Figure 2C). Studies with rodents that undergo a surgical esophagojejunostomy, reveals the emergence of severe erosive esophagitis and BE. Female rats were lethally irradiated after which their bone marrow was reconstituted with

bone marrow of male rats. Following bone marrow transplantation the rats underwent esophagojejunostomy. Analysis of the BE lesions by FISH for the Y chromosome (male derived) showed the presence of male bone marrow derived cells (Sarosi, Brown et al 2008).

Genetic alterations in Barrett's esophagus

The study of BE has been limited mostly to the analysis of human BE biopsies. Yet, the histopathological analysis of these biopsies has led to the discovery of key genetic alterations that occur in BE metaplasia (Figure 3). One such genetic change is the expression of the homeobox transcription factors CDX1 and CDX2, which are expressed in BE. CDX1 and CDX2 are part of the caudal homeobox family of transcription factors, which are important for the development and differentiation of the small intestine and colon (Guo, Suh et al 2004). CDX2 has been found to be expressed in early esophagitis prior to the detection of BE, suggesting its expression may be an early event in the development of BE (Eda, Osawa et al 2003). Interestingly, conditional *Cdx2* knockout in the small intestine of the mouse results in squamous metaplasia (Gao, White et al. 2009). Moreover, ectopic expression of CDX2 specifically in the mouse esophagus (K14-Cdx2) showed reduced basal cell proliferation and altered cell morphology, indicative of early initiation of BE (Kong, Crissey et al. 2011).

Another gene that has been linked to BE is bone morphogenetic protein 4 (BMP4). BMP4 belongs to the transforming growth factor β (TGF-β) family, which is involved in controlling cellular differentiation, migration and proliferation. Studies of BE biopsies have found BMP4 and its downstream targets, phospho-SMAD 1/5/8 and ID2, are expressed in BE, but absent in the normal adjacent squamous epithelium (Milano,

van Baal et al 2007). Furthermore, studies of severe reflux esophagitis in rats showed activation of the BMP pathway in the inflamed squamous epithelium and in BE (Sarosi, Brown et al 2008).

Genetic alterations involving the silencing of tumor suppressor genes have been found to be present in BE metaplasia. One example is the loss of the *CDKN2A/p16*^{INK4A} (p16) tumor suppressor gene. *CDKN2A/p16*^{INK4A} is lost in 85% of BE samples (Wong, Paulson et al. 2001; Maley 2006). In the case of the *CDKN2A/p16*^{INK4A} locus most patients have either loss of heterozygocity (LOH) or methylation of the promoter. In BE patients LOH of the tumor suppressor TP53 has also been described, but importantly this event is rarely seen in the absence of LOH of p16 (Maley, Galipeau et al. 2004). This suggests TP53 LOH is a secondary event in the development of BE metaplasia. Aside from TP53 LOH, mutation of p53 has been reported as a prognostic marker for progression from BE to EAC (Sikkema, Kerkhof et al. 2009).

Genetic alterations in esophageal adenocarcinoma (EAC)

BE and gastroesophageal reflux disease (GERD) are the major risk factors for EAC. Another risk factor for EAC is obesity. Specifically, studies have shown a positive association between body mass index (BMI) and the risk of EAC (Kubo and Corley 2006). Alcohol and tobacco have been found to be associated only moderately with EAC risk (Freedman, Abnet et al 2007). Lastly, *Helicobater pylori* (*H.pylori*) infection, which in the distal stomach may act as a carcinogen, has been shown to have an inverse relationship with EAC. Therefore, *H.pylori* may be protective for the development EAC (Rokkas, Pistiolas et al 2007).

During the progression from BE to EAC, cells may accumulate a number of genetic alterations that confer a growth advantage (Figure 3). For example, in EAC the oncogenes cyclin D1, E, B1 and A are known to be activated (Arber, Lightdale et al 1996; Geddert, Heep et al. 2005; Zhang, Spechler et al. 2009). EAC also shows increased expression of transforming growth factor (TGF-α), epidermal growth factor (EGF) and EGFR receptor (EGFR) (Brien, Odze et al 2000; Brito, Filipe et al. 1995; Zhang, Spechler et al. 2009). Key tumor suppressor genes implicated in EAC include p16, p53, p14ARF, p27 and adenomatous polyposis coli (APC). In EAC, silencing of the p16 promoter has been reported in 45-54% of cases (Wong, Barrett et al. 1997; Zhang, Spechler et al. 2009). Furthermore, in EAC, TP53 LOH has been reported to be present in 50-90% of cases (Hamelin, Flejou et al. 1994; Zhang, Spechler et al. 2009). Similarly, 75-83% of EAC have no detectable p14ARF or p27 (Huang, Peters et al. 2009; Singh, Lipman et al. 1998; Zhang, Spechler et al. 2009). Lastly, methylation of *APC* has been found in 80% of BE patients with high-grade dysplasia or EAC (Kawakami, Brabender et al. 2000; Zhang, Spechler et al. 2009). Furthermore, during the progression of dysplasia in BE there is a decrease in membranous E-cadherin and β-catenin and an increase in nuclear localization of β-catenin (Bailey, Biddlestone et al. 1998; Washington, Chiappori et al. 1998; Zhang, Spechler et al. 2009).

Recently, studies of whole-genome sequencing of EAC have revealed chromosomal amplification at 18q11.2 in 21% of EAC, where the gene GATA6 resides. Further analysis reveals GATA6 amplification correlates with poor survival in EAC patients (Lin, Bass et al. 2012). A whole-exome sequencing study of EAC has identified 26 significantly mutated genes (Dulak, Stojanov et al. 2013). Novel mutations in ELMO1

and DOCK1, which are upstream modulators of the RAC1 GTPase, suggest the possible activation of the RAC1 signaling pathway in EAC (Dulak, Stojanov et al. 2013).

Epidemiology and genetic alterations of esophageal squamous cell carcinoma (ESCC)

Esophageal cancer can exist in two different forms: esophageal adenocarcinoma (EAC) and esophageal squamous cell cancer (ESCC). ESCC is mainly observed in African-American and Asian populations, while EAC mainly affects the white (Caucasian) population. Therefore, it is not surprising that the environmental risk factors for ESCC may differ from those for EAC (Figure 3). ESCC has linked to tobacco use, alcohol consumption, ingestion of salt-cured, salt-pickled and moldy foods, and the consumption of hot beverages (Zhang 2013). Some of the genetic changes found in ESCC include the following: EGFR and cyclin D1 amplification, as well as inactivation of $p16^{INK4A}$, p53 and p120-catenin tumor suppressor genes.

The epidermal growth factor receptor (EGFR) is part of the ErbB growth factor tyrosine kinase receptor family, which activates genes that can promote cell cycle progression, angiogenesis, survival, migration and invasion (Sibilia, Kroismayr et al. 2007). Overexpression of EGFR has been reported in 60-70% of ESCC tumors (Hanawa, Suzuki et al. 2006). Cyclin D1 amplification or overexpression leads to the constitutive activation of the cyclin D1/CDK4/6 pathway. Cyclin D1 amplification is found in 30-75% in cases of ESCC (Nakagawa, Zukerberg et al. 1995; Zhang 2013). Furthermore, increased CyclinD1 expression is correlated with poor prognosis (Hirai, Kuwahara et al. 1999; Zhang 2014).

Another critical genetic alteration in ESCC is the inactivation of the tumor suppressor $p16^{INK4A}$. In studies of primary ESCC 14 of 27 cases have point mutations or microdeletions of the p16 coding gene (Mori, Miura et al. 1994; Zhang 2013). Lastly, during the development of ESCC there are mutations of the tumor suppressor gene p53 (McCabe and Dlamini 2005). These mutations lead to impaired downstream function of the p53 tumor suppressor. Some of the most common of these mutations that occur in ESCC are the R175H, R248W and R273H (McCabe and Dlamini 2005). These mutations fall within the DNA binding domain of p53. Mutations in this region cause p53 to lose its specificity to DNA sequences either by causing global conformation changes or by interfering with promoter-specific contact (Joerger and Fersht 2007).

Recent genome-wide studies have investigated ESCC cases using whole-exome sequencing in order to identify significantly mutated genes (Song, Li et al.2014). Six tumor associated genes previously described to be involved in ESCC were identified: TP53, RB1, CDKN2A, PIK3CA, NOTCH1 and NFE2L2. Furthermore, two novel genes not previously described to be mutated in ESCC were identified: ADAM29 and FAM135B. Specifically, FAM135B mutant expression in ESCC cells was shown to promote malignancy (Song, Li et al.2014). Functional studies regarding the role of the ADAM29 mutation in ESCC have yet to be eluded. In addition, whole-genome sequencing was also able to identify significant amplification of the 11q13.3-13.4 region. Furthermore, this region was shown to contain MIR548K, which encodes a microRNA, which was shown to promote malignancy in ESCC cell lines (Song, Li et al. 2014).

3D organotypic culture model system

In order to study the progression of metaplasia to an invasive carcinoma, we have to make use of *in vitro* models to further analyze the underlying molecular mechanisms. Our lab has developed an 3D organotypic culture model for the study of ESCC. The use of this 3D organotypic cultures model allows for the investigation molecular mechanisms of epithelial-stromal interactions (Kalabis, Wong et al. 2012). The advantages of a 3D organotypic cultures, relate to the ability to recreate the normal cell polarization, differentiation cell patterns and expression of genes representative of those found in the *in vivo* tissues. By developing a human esophageal epithelial cells immortalized with telomerase (hTERT) overexpression, designated as EPC2-hTERT cells, we are able to study the esophageal epithelium. Our 3D organotypic cultures consist first of creating a "mesenchyme" layer composed of collagen I, matrigel and fetal embryonic fibroblast (FEF3) on which to grow the esophageal epithelial cells. After seeding the epithelial cells we are able constitute a complete stratified squamous epithelium by exposure to a liquid-air interface (Figure 4) (Harada, Nakagawa et al. 2003). Through these 3D organotypic cultures we are able to recapitulate the normal stratification normally found in the esophageal epithelium (Kalabis, Wong et al. 2012).

Furthermore, our 3D organotypic culture system allows for the study of cell signaling pathways in esophageal epithelial cells, investigation of pharmacological inhibitors and study of the interactions between epithelial cells and fibroblasts. For examples, the expression of inducible AKT in esophageal epithelial cells shows an expansion of the proliferation of basal cells an impaired differentiation (Oyama, et al. 2007). Moreover, these epithelial cells can be transformed by the introduction of oncogenes (e.g., EGFR and CyclinD1) and inactivation of tumor suppressor genes

(mutant p53) (Grugan, Miller et al. 2010; Naganuma, Whelan et al. 2012). Furthermore, this 3D organotypic cultures system can be used to study BE. For example, our previous work showed overexpression of MYC and CDX1 genes lead to a partial transdifferentiation to BE (Stairs, Nakagawa et al. 2008). Furthermore, studies using BE cell lines grown in 3D organotypic cultures have demonstrated a significant difference in cell morphology between BE cell lines and esophageal epithelial cells (Kosoff, Gardiner et al. 2011).

Mouse models of ESCC, Barrett's esophagus and EAC

Studies of ESCC development *in vivo* provide critical insight into the molecular mechanism mediating tumorgenesis in the esophagus. Through the use of the Epstein-Barr virus L2 promoter Cre recombinase (L2-Cre), studies have shown tissue specific knockout of KLF4 in the esophagus epithelium. Moreover, these mice show increased basal cell proliferation and delays in differentiation. Interestingly, these mice show early hypertrophy, followed by subsequent dysplasia by 6 months of age (Tetreault, Yang et al. 2010). Furthermore, we have published a mouse model that can recapitulate the histopathology of ESCC. This is mediated by the loss of p120catenin, specifically in the esophagus and oral cavity by the use of the Epstein - Barr virus L2 promoter (L2) (Stairs, Bayne et al. 2011). Histological examination of *L2-Cre; p120*$^{LoxP/LoxP}$ mice reveal development of hyperplasia at 7-9 months of age, severe dysplasia at 9 months and ESCC by 12 months (Stairs, Bayne et al. 2011). Further study of the *L2-Cre ; p120ctn*$^{LoxP/LoxP}$ mice show the initiation of tumorgenesis for ESCC is in part mediated by the infiltration of myeloid derived suppressive cells (MDSCs) (Stairs, Bayne et al. 2011).

In vivo studies in rodents to investigate BE and EAC currently are an active area of research. One of the first *in vivo* models to investigate BE and EAC was in a surgical rodent model. This model involves a esophagojejunostomy, which lead to the induction of severe reflux in the esophagus of these animals. These rodents will develop severe esophagitis by 13 weeks, BE by 34 weeks and EAC by 5 months post-surgery (Pham, Genta et al. 2014). Recently, new studies have shown the development of a genetic mouse model for BE and EAC. In these studies the Interleukin-1β is express in the esophageal epithelium through the L2 promoter. These mice at baseline show an inflammatory response to the expression of IL-1β by 6 months, and a late incidence of BE 12-15 months. Furthermore, the addition of bile salts in the drinking water of the *L2-IL1-β* mice was shown to accelerate the appearance of BE and EAC, with tumors observed by 15 months of age (Quante, Bhagat et al. 2012).

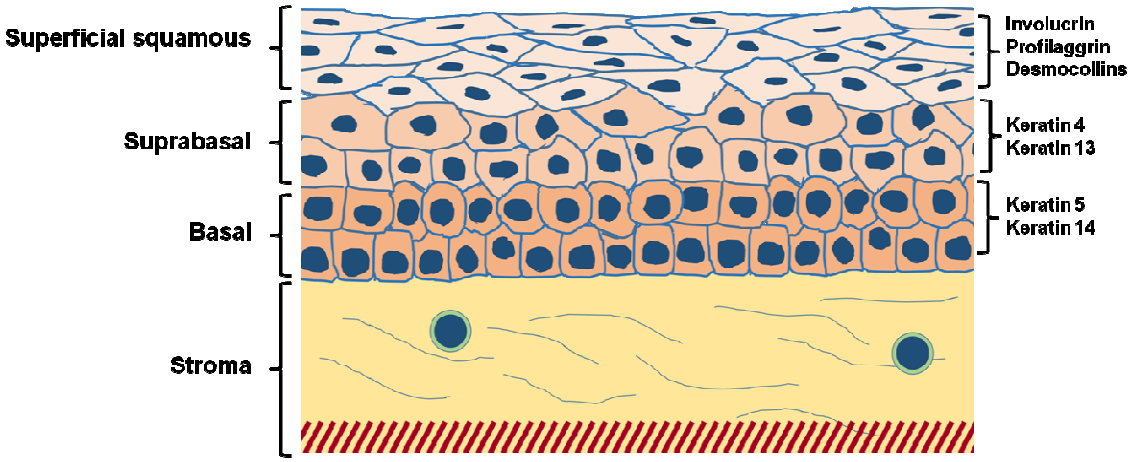


Figure 1: Normal esophageal squamous epithelium. Esophageal epithelium is characterized by three distinct cellular layers: basal, suprabasal and superficial squamous. Each layer can be identified by expression of differential cell markers. Basal cells express Keratin 5 and Keratin 14, suprabasal cells express Keratin 4 and Keratin 13, and superficial squamous express involucrin, profilaggrin and desmocollins.

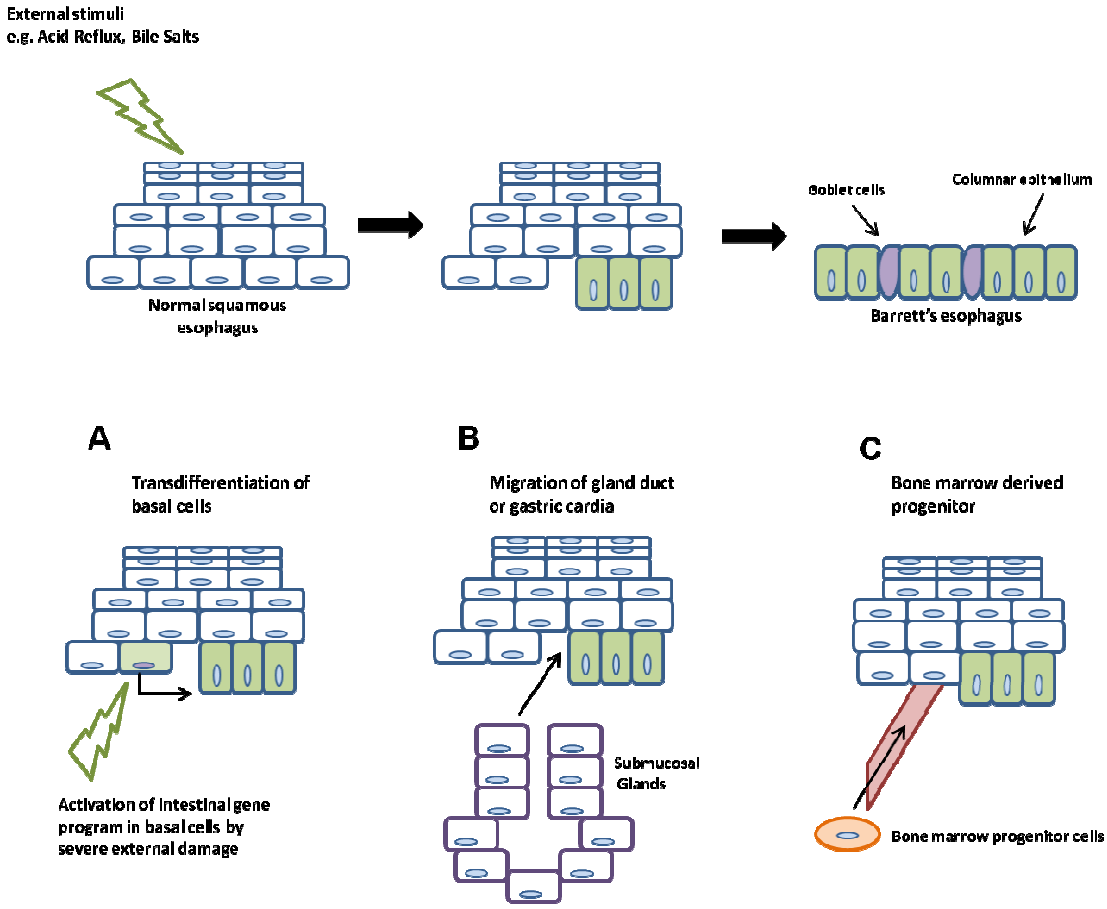


Figure 2: Cell of origin for Barrett's esophagus. The normal esophageal squamous epithelium undergoes damage due to external factors, such as acid reflux (GERD). Severe damage to the esophageal epithelium results in Barrett's esophagus metaplasia, which is composed of a columnar epithelium and mucin-producing goblet cells. There are three hypotheses for the cell of origin of Barrett's esophagus. (A) transdifferentiation of basal esophageal cells, (B) migration of submucosal esophageal gland duct cells or gastric cardia cells and (C) bone marrow progenitor cells.

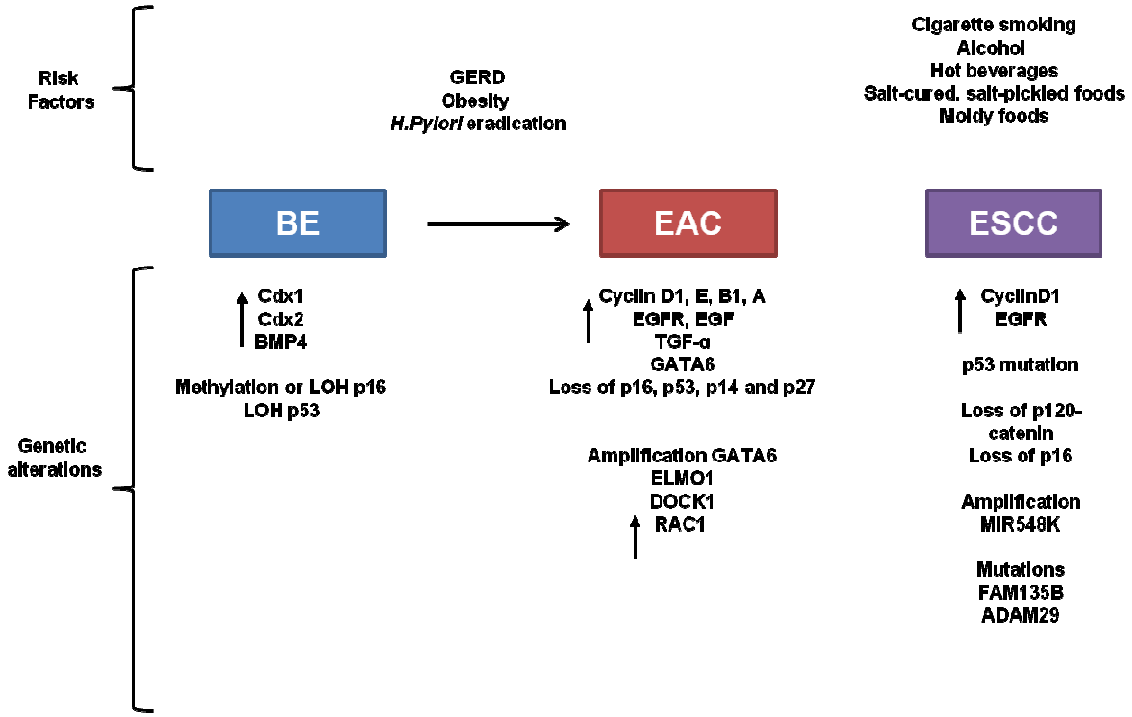


Figure 3: Risk factors and genetic changes in Barrett's esophagus, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). List of risk factors for BE and EAC, and ESCC (upper panel). Common genetic alternations found in BE, EAC and ESCC (lower panel).

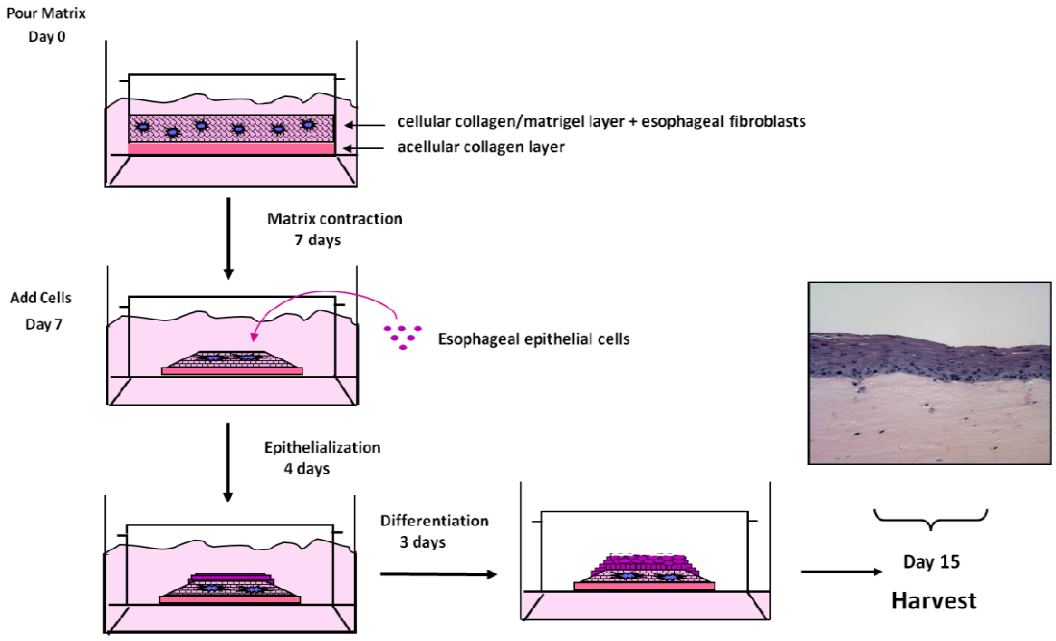


Figure 4: 3D organotypic culture model system. Day 0: placement of acellular collagen layer, followed by addition of collagen, matrigel and esophageal fibroblasts. After culture for 7 days, the epithelial cells are seeded on the surface of the contracted matrix. After culture for additional 4 days, cells are exposed to an air-liquid interface, promoting epithelial cell stratification and differentiation. Finally, on day 15 the 3D organotypic cultures are harvested for histology.

Chapter II:

Inhibition of Notch signaling enhances transdifferentiation of esophageal squamous epithelium toward Barrett's esophagus via HATH1 and KLF4

Abstract

Barrett's esophagus (BE) is defined as an incomplete intestinal metaplasia characterized by the presence of columnar and goblet cells in the formerly stratified squamous epithelium of the esophagus. BE is a documented precursor of esophageal adenocarcinoma. Currently, there is no clearly identified cell of origin for human BE and our interest is to understand how squamous epithelial cells may evolve to BE. To that end, we have investigated the role of Notch signaling in the development of BE. Notch signaling was inhibited in human esophageal epithelial cells by expression of dominant-negative-Mastermind-like (dnMAML) and potential cell transdifferentiation was assessed by 3D organotypic culture with evaluation of BE-lineage specific gene expression. Furthermore, KLF4 and HATH1 knockdown was in order to determine if the observed phenotype could be rescued following Notch inhibition. Data from our RNA microarray revealed that BE samples expressed lower levels of Notch receptors (NOTCH2 and NOTCH3) and the ligand (JAG1). Furthermore, a BE tissue microarray showed decreased NOTCH1 expression and the Notch signaling downstream target HES1. Notch inhibition promoted partial transdifferentiation of esophageal epithelial cells towards columnar-like cells as demonstrated by increased expression of columnar keratins and glandular mucins and decreased expression of squamous keratins. In 3D culture, elongated cells were observed in the basal layer of the epithelium with Notch inhibition. Importantly, knockdown of KLF4 reversed partially the effects of Notch inhibition. A partial reversal was observed as well with HATH1 knockdown. Thus, Notch signaling inhibition promotes transdifferentiation of esophageal cells towards BE-like metaplasia in part via upregulation of KLF4 and HATH1, representing a novel mechanism underlying the potential initiation of BE.

Introduction

Barrett's esophagus (BE) is defined as an incomplete intestinal metaplasia of the esophagus. BE is classically characterized by the presence of differentiated intestinal columnar cells and mucin-producing goblet cells. BE is estimated to have a prevalence of 5-6% in the US (Fitzgerald 2006; Hayeck, Kong et al. 2010). Gastroesophageal reflux disease (GERD), abdominal obesity, cigarette-smoking and *Helicobacter pylori* (*H.pylori*) infection eradication have been linked as risk factors associated with the development of BE (Fitzgerald, 2006). Furthermore, the local pro-inflammatory microenvironment plays a critical role in the initiation and maintenance of BE (Fitzgerald, 2006; Reid, Li et al. 2010). BE can progress to low-grade dysplasia, high-grade dysplasia and culminate in the development of esophageal adenocarcinoma (EAC). It is estimated that 0.5% of BE patients will develop EAC, and this risk increases to 10% in BE patients with high-grade dysplasia (Hayeck, Kong et al. 2010).

In 2013, an estimated 17,990 patients were diagnosed in the US with esophageal cancer, both EAC and esophageal squamous cell carcinoma (ESCC). Esophageal cancer is one of the deadliest cancers in the US with a 5-year survival rate of 17.3% in 2013 (Howlader, Noone et al. 2013). Therefore, studying the molecular mechanisms underlying the pathogenesis of BE could provide novel biomarkers or prognostic indicators for both BE and EAC patients. The study of BE has been limited historically to human biopsy samples, which have been used to analyze histopathological and genetic changes. Some of the known changes occurring in BE are the methylation and loss of heterozygosity (LOH) of p16 and also LOH of p53 (Graham and McDonald 2010). A mosaic pattern of genetic alterations can be found in human biopsy samples, complicating the identification of the initiating genetic changes that lead to BE.

There have been several hypotheses proposed for the cell of origin of BE (DeVault, McMahon et al. 2013). These include: (1) transdifferentiation of cells from the esophageal basal layer or from the ducts of esophageal submucosal glands, (2) migration of cells located at the esophago-gastric junction or of the gastric cardia cells and (3) bone marrow derived progenitor cells (Quante, Bhagat et al 2012; Sarosi, Brown et al. 2008; Wang, Ouyang et al. 2011).

Recent studies have undertaken a genetic approach to investigate the initiating events that lead to the development of BE. Through this approach, two genes, CDX1 and MYC (c-Myc), have been identified by our group to have a role in the potential development of BE (Stairs, Nakagawa et al. 2008). CDX1 is part of the caudal homeobox family of transcription factors (CDX1 and CDX2), which are important in the development and differentiation of the small intestine and colon (Guo, Suh et al. 2004). In fact, conditional knockout of *Cdx2* in the intestine of the mouse results in squamous metaplasia (Gao, White et al. 2009).

MYC is a transcription factor known to bind to E-box sequences and can activate approximately 15% of all genes in the human genome (Herold, Herkect et al. 2009; Meyer and Penn 2008). MYC is overexpressed in many cancers, including EAC, through gene amplification (Ruggero 2009; von Rahden and Stein 2006). MYC is involved in the activation and regulation of a variety of cellular processes such as cell cycle progression, cell differentiation, energy metabolism, angiogenesis and DNA damage repair (Meyer and Penn 2008). Our microarray analysis of BE samples revealed that negative regulators of MYC, namely MXI1 and MXD1, were downregulated, while certain MYC target genes ODC1 and CA2 were increased (Stairs, Nakagawa et al. 2008), suggesting that MYC signaling is active in BE. In addition, the

microarray data showed increased CDX1 and CDX2 expression in BE (Stairs, Nakagawa et al. 2008). Our previous studies using a human esophageal epithelial cell line immortalized with hTERT (EPC2-hTERT) have allowed us to study human esophageal epithelial biology (Harada, Nakagawa et al. 2003). Our previous data have shown that CDX1 overexpression together with MYC in the EPC2-hTERT cells can lead to a partial change towards BE (Stairs, Nakagawa et al. 2008). The partial change toward BE suggests the need to explore additional genetic changes in the context of MYC and CDX1.

Interestingly, loss of Notch signaling is required for the differentiation of the goblet cell lineage in the small intestine (van Es, van Gijn, et al. 2005; Fre, Huyghe et al. 2005). In addition, inhibition of Notch signaling in the small intestine by either γ-secretase inhibitor (GSI), or conditional knockout of *Rbpj*, can lead to goblet cell hyperplasia (van Es, van Glin et al. 2005; Fre, Huyghe et al. 2005; Menke, van Es et al. 2010). The Notch signaling pathway comprises four homologous transmembrane Notch receptors (NOTCH1-NOTCH4) that can be activated by transmembrane ligands, Delta or Jagged, generally expressed by neighboring cells (La Voie and Selkoe 2003, Benedito, Roca et al. 2009). Upon ligand binding, Notch receptors undergo cleavage by ADAM-family metalloproteases at the extracellular domain and by γ-secretase at the intracellular domain (La Voie and Selkoe 2003). These events lead to the release of the intracellular Notch domain (ICN) allowing its nuclear translocation, binding to RBPJ and activation of Notch target genes, such as HES1 and HES5 (La Voie and Selkoe 2003; Ntziachristos, Lim et al. 2014). Interestingly, Notch is known to be involved in cell fate decisions in several cell types including lymphocytes, neurons, skin and others (Ntziachristos, Lim et al. 2014).

Previous studies have shown Notch signaling can regulate intestinal cell differentiation, mediated through the negative regulation of KLF4 and HATH1. KLF4 and HATH1 are critical transcription factors required for proper differentiation of the secretory lineage in the context of the small intestine. Notch signaling can be a negative regulator of Krüppel-like factor 4 (KLF4) expression in the small intestine (Zheng, Pritchard et al. 2009). Indeed, it has been demonstrated previously that the KLF4 promoter contains ICN responsive elements, through which Notch can inhibit KLF4 expression (Zheng, Pritchard et al. 2009; Ghaleb, Aggarwal et al. 2008). KLF4 is part of a family of DNA-binding transcription factors that has been shown to play a role in multiple processes from proliferation, cell differentiation, inflammation and pluripotency (McConnell and Yang 2010). Recently, high KLF4 expression was reported in human BE biopsies; it was also shown that its promoter is activated by bile acid (Kazumori, Ishihara et al. 2011). In addition, KLF4 can increase the transcriptional activity of MUC2 and CDX2, suggesting a potential role in BE development (Kazumori, Ishihara et al. 2011).

HATH1 (Atonal homolog 1 or ATOH1) is a basic helix-loop-helix transcription factor that is known to be important for the differentiation of secretory cells in the small intestine (Shroyer, Helmrath et al. 2007). *Math1*$^{-/-}$ (mouse homolog of HATH1) mice lack goblet and other secretory cells in the small intestine and die shortly after birth (Yang, Bermingham et al. 2001). Previous studies have shown a possible inverse relationship between Math1 expression and Notch activity in the small intestine (Kazanjian, Noah et al. 2010). It is believed that HES1 can downregulate HATH1 expression (Kazanjian, Noah et al. 2010). This negative regulation of Math1 leads to a selection pressure towards the enterocyte lineage, while cells with low Notch and low Hes1 will have more

Math1 and differentiate towards the secretory lineage (Yang, Bermingham et al. 2001; Suzuki, Fukui et al. 2005).

In this study, we utilize an innovative three-dimensional (3D) organotypic culture model system to demonstrate that the cooperation of MYC, CDX1, and Notch signaling inhibition results in a switch of cell identity and lineage specification from the normal esophageal squamous epithelium to a BE-like metaplasia mediated through KLF4 and HATH1. Our data support the novel paradigm in which transdifferentiation of esophageal basal cells lead to initiation of BE.

Results

Notch signaling is downregulated in human Barrett's esophagus

In order to investigate the status of Notch signaling in Barrett's esophagus (BE), we performed a RNA microarray on human BE biopsies compared to adjacent normal squamous esophagus (GEO accession # GSE 13083) (Stairs, Nakagawa et al. 2008). A significant decrease in expression of NOTCH2 and NOTCH3 receptors by 4- and 2-fold respectively, was observed in BE versus paired normal squamous esophagus. Expression of the Notch ligand JAG1 was also decreased by 3-fold, thereby suggesting a potential downregulation of Notch signaling in BE (Figure 1A). We next performed IHC of human BE (n=15-23) and normal esophagus (n=25-27) tissue microarray (TMA) for NOTCH1 receptor, the active form of NOTCH1, ICN1 and its key downstream target the transcription factor HES1 (Figure 1B-C). We observed positive nuclear staining for HES1 and NOTCH1 restricted to the basal layer of normal esophageal epithelium, whereas ICN1 showed more diffused cytoplasmic staining (Figure 1B). In BE, we observed a loss of nuclear HES1 staining and loss of nuclear and membranous NOTCH1, and decreased ICN1 staining (Figure 1B). The TMA was scored for intensity of staining (Supplemental Table 1). HES1, NOTCH1 and ICN1 protein expression is decreased significantly in BE when compared to normal esophagus (Figure 1C). Thus, data from both RNA microarray and TMA suggest that Notch signaling is downregulated in BE compared to the normal esophageal epithelium, suggesting Notch signaling may be required as well for maintenance of the normal esophageal squamous epithelium.

Inhibition of Notch signaling induces morphological changes in esophageal epithelial cells

Next, we proceeded to inhibit Notch signaling in the context of MYC and CDX1. We infected EPC2-hTERT-MYC-CDX1 (MYC-CDX1) cells with a construct encoding for dominant-negative Mastermind-like (dnMAML) to inhibit Notch signaling (MYC-CDX1-dnMAML cells). We confirmed expression of dnMAML-GFP tagged protein by western blotting using a GFP specific antibody (Figure 2A). To verify Notch signaling abolition following dnMAML overexpression, we used an 8X-CSL-Luciferase reporter construct, which upon expression of ICN1 can activate luciferase expression. We observed a significant inhibition of nearly 10-fold in the presence of dnMAML (MYC-CDX1-dnMAML-ICN1 cells) compared to MYC-CDX1-ICN1 (Figure 2B). We confirmed also downregulation of Notch target gene expression by dnMAML via quantitative PCR (qPCR). Indeed, we observed a significant decrease of HES1 (3.3-fold) and HES5 (12.5-fold) expression in MYC-CDX1-dnMAML when compared to MYC-CDX1 cells (Figure 2C). These results support that dnMAML overexpression is sufficient to inhibit Notch signaling.

We next used 3D organotypic cultures to analyze changes in cell morphology and differentiation (Kalabis, Wong et al. 2012). We observed that MYC-CDX1-dnMAML cells formed a thinner stratified epithelium than MYC-CDX1 cells, suggesting disruption of normal differentiation. We also noted that MYC-CDX1-dnMAML 3D cultures showed an altered cell morphology in the basal layer (Figure 2D), when compared to MYC-CDX1 cells. In order to further characterize these changes in the basal layer, we performed electron microscopy of MYC-CDX1 and MYC-CDX1-dnMAML cultures (Figure 2E). We observed an elongation of MYC-CDX1-dnMAML basal cells when compared to MYC-CDX1 cells, consistent with acquisition of a columnar-like morphology. Indeed, basal cellular height was significantly increased (1.4-fold) in 3D cultures overexpressing

dnMAML (Figure 2F) compared to MYC-CDX1. These changes in cell morphology in the basal layer in MYC-CDX1-dnMAML cells suggest that the inhibition of Notch signaling may promote transdifferentiation of normal esophageal epithelium towards a more columnar-like epithelium.

Inhibition of Notch signaling induces a switch from squamous to columnar gene expression

We analyzed further our 3D cells to investigate if morphological changes observed in MYC-CDX1-dnMAML cells reflect changes in lineages. We conducted immunohistochemistry (IHC) for the squamous keratin 13 (K13). In MYC-CDX1 cells, we observed strong staining for K13 in the suprabasal region, whereas staining was reduced significantly in MYC-CDX1-dnMAML cells. Conversely, we observed increased staining of columnar keratin 19 (K19) in both the basal and suprabasal compartment in MYC-CDX1-dnMAML cells compared to MYC-CDX1 cells (Figure 3B).

We next used qPCR to evaluate additional squamous and columnar lineage specific keratins in MYC-CDX1-dnMAML cells. Prior to this, cells were grown in the presence of calcium (0.6 mmol/L) for 48 hrs to allow squamous differentiation (Ohashi, Natsuizaka et al. 2010). We observed that MYC-CDX1-dnMAML cells expressed reduced levels of squamous keratins: K5 (5-fold) K13 (16.6-fold) and K14 (5-fold) (Figure 4A). Furthermore, MYC-CDX1-dnMAML cells expressed higher levels of columnar keratins: K8 (2.2-fold), K18 (2.8-fold), K19 (1.9-fold) and K20 (2.8-fold) compared to MYC-CDX1 cells (Figure 4B). These results suggest that inhibition of Notch signaling via dnMAML promotes a switch in gene expression from squamous to columnar keratins. Furthermore, since BE is often characterized by the presence of goblet cells in

the esophageal epithelium, we next investigated expression of mucins, the major protein family secreted by this cell type. Interestingly, we observed increased levels of MUC2 (10.4-fold), MUC3B (21.5-fold), MUC5B (305-fold) and MUC17 (116.3-fold) in MYC-CDX1-dnMAML cells when compared to MYC-CDX1 cells (Figure 4C). Thus, inhibition of Notch signaling fosters expression of goblet cell lineage specific genes. Finally, we quantified the expression of the squamous differentiation genes desmocollin1 and desmocollin3 (DSC1, DSC3). We observed that dnMAML overexpression in MYC-CDX1 cells decreased significantly DSC1 (20-fold) and DSC3 (3.7-fold) expression (Figure 4D). Taken together, these data support the premise that inhibition of Notch signaling in cooperation with MYC and CDX1 may orchestrate a genetic switch from a squamous cell lineage to an intestinal columnar cell lineage.

Inhibition of Notch signaling mediates transdifferentiation to a BE-like metaplasia via a HES1 independent manner

Classically, Notch signaling leads to activation of transcriptional factors such as HES1 and HES5. In the intestine, HES1 has been shown to be a negative regulator of HATH1, thereby promoting absorptive cell fate over secretory cell fate (Zheng, Shou et al. 2000; Kazanjian, Noah et al. 2010). Therefore, we investigated if downregulation of HES1 could mimic the results obtained with dnMAML overexpression in MYC-CDX1 cells. We performed stable knockdown of HES1 with two independent shRNA constructs (shHES1 #1 and shHES1 #2) in MYC-CDX1 cells (Supplemental Figure 1A). We did not observe any changes in squamous and columnar keratins or in mucin genes expression upon knockdown of HES1 suggesting that HES1 alone is not sufficient to transdifferentiate esophageal epithelial cells (Supplemental Figure 1B-D). We observed a trend of upregulation of HATH1 expression validating the functional HES1 knockdown

(Supplementary Figure 1E). Thus, HES1 knockdown could not recapitulate the effects of Notch signaling inhibition suggesting that Notch might act via other downstream targets to regulate transdifferentiation to BE.

HATH1 knockdown leads to partial reversal of the transcriptional changes following Notch signaling inhibition

Previous studies have shown a possible inverse relationship between Math1 expression and Notch activity in the context of the small intestine. It is believed that HES1 can downregulate HATH1 expression (Kazanjian, Noah et al. 2010). Interestingly, HATH1 expression is increased 58-fold in MYC-CDX1-dnMAML cells (Figure 5A) as observed by qPCR. We were unable to detect HATH1 via Western blotting (data not shown). Thereby, we investigated HATH1 as a possible mediator of the effects observed by Notch signaling inhibition in the MYC-CDX1-dnMAML cells. Using stable lentiviral infection, we achieved HATH1 knockdown of 2.6-fold in the MYC-CDX1-dnMAML cells (Figure 5B). Analysis of columnar keratins expression showed a significant decrease of K8, a decreased trend of K20 and no change for K18 and K19 in the MYC-CDX1-dnMAML-shHATH1 cells (Figure 5C). We observed a significant decrease for MUC2, but no changes in MUC3B, MUC5B and MUC17 (Figure 5D). Interestingly, we observed a significant increase of the squamous keratins K5, K13 and K14 in the MYC-CDX1-dnMAML-shHATH1 cells (Figure 5E). We also observed an increased expression of DSC1 and DSC3, upon HATH1 knockdown (Figure 5F). Furthermore, analysis of MYC-CDX1-dnMAML-shHATH1 cells in 3D cultures show no changes in cell morphology (data not shown). Taken together, these results demonstrate that HATH1 may partially mediates the transdifferentiation to a BE-like metaplasia promoted through Notch signaling inhibition.

KLF4 knockdown reverses the morphological and transcriptional changes following Notch signaling inhibition

Active Notch signaling mediated by ICN1 downregulates KLF4 expression in the intestinal epithelium (Ghaleb, Aggarwal et al. 2008). Conversely, inhibition of Notch signaling via GSI can cause upregulation of KLF4 expression (Zheng, Pritchard et al. 2009). Interestingly, KLF4 expression is increased in MYC-CDX1-dnMAML cells when compared to MYC-CDX1 cells (Figure 6A, 6B). Therefore, we investigated whether KLF4 knockdown in MYC-CDX1-dnMAML cells could reverse the morphological changes observed following dnMAML overexpression. Using stable lentiviral infection, we achieved significant knockdown of KLF4 using 2 independent shRNA sequences in MYC-CDX1-dnMAML cells (Figure 6C, 6D). We observed the highest degree of KLF4 knockdown in the MYC-CDX1-dnMAML-shKLF4 #3 (3.3-fold in RNA and 25-fold in protein) cells. Stable KLF4 knockdown results in a decrease of elongated (columnar-like) cells observed with Notch signaling inhibition in 3D culture (Figure 6E). Furthermore, cells at the basal layer of the epithelium have a more cuboidal shape, suggesting that inhibition of KLF4 in the MYC-CDX1-dnMAML cells may reverse morphological changes observed with Notch signaling inhibition.

Analysis of columnar lineage specific keratins showed significantly decreased K8 and K20 expression, but no changes in K18 and K19 in MYC-CDX1-dnMAML-shKLF4 cells (Figure 7A). We also observed a significant decrease in MUC2 and MUC5B expression, but no changes in MUC3B and MUC17 (Figure 7B). Furthermore, we evaluated expression of the squamous keratins K5, K13 and K14 in MYC-CDX1-dnMAML-shKLF4 cells and MYC-CDX1-dnMAML-shScramble cells. KLF4 knockdown significantly increased expression of K5, K13 and K14, supporting the premise that KLF4

knockdown can partially reverse the switch from squamous to columnar keratins observed with Notch signaling inhibition (Figure 7C). Moreover, expression of the squamous differentiation marker DSC1 is increased significantly in MYC-CDX1-dnMAML-shKLF4 cells (Figure 7D). Overall, these results demonstrate that dnMAML-induced transdifferentiation may be in part mediated by KLF4. Taken together, our data demonstrate a novel function for Notch signaling in BE development mediated by KLF4 and HATH1. Importantly, this supports the model in which esophageal epithelial basal cells might serve as a potential the cell of origin for Barrett's esophagus metaplasia in a 3D organotypic culture model system.

Discussion

Barrett's esophagus (BE) is defined as an incomplete intestinal metaplasia of the esophagus, and the biological mechanisms underlying its development remain to be clarified. Herein, we demonstrate that Notch signaling is downregulated in human BE, suggesting that development of intestinal metaplasia in the esophagus could require inhibition of Notch signaling. Indeed, inhibition of Notch promotes the initial phases of transdifferentiation in our model system. First, there is the appearance of elongated columnar-like cells in the basal layer of 3D organotypic cultures in response to Notch inhibition. Second, there is a switch in genes that denote the squamous versus BE lineages, characterized by a diminution of squamous keratins and differentiation markers in favor of an induction of columnar keratins and mucins. These results suggest that the combination of Notch inhibition and MYC and CDX1 overexpression may promote transdifferentiation of esophageal epithelial cells towards BE metaplasia.

Transdifferentiation may be viewed as lineage reprogramming, involving a process where one somatic cell switches into another somatic cell without undergoing an intermediate pluripotent or progenitor cell type (Orkin and Zon 2008; Graf and Enver 2009). The cell of origin for development of BE remains the subject of investigation, but transdifferentiation of esophageal stratified epithelium is one of the proposed models (DeVault, McMahon, et al. 2013). One study of human BE biopsies has revealed that esophageal cells undergo a transition of expression of intestinal markers like CDX2 and MUC5AC (Hahn, Blount et al. 2009). Interestingly, NOTCH1 activation is observed at the onset of squamous differentiation of the esophageal epithelium. NOTCH1 and NOTCH3 orchestrate transcriptional regulation of early differentiation markers in a CSL-dependent manner (Ohashi, Natsuizaka et al.2010). Perturbation of esophageal

squamous differentiation is notably observed following loss of Notch signaling in the esophageal epithelium (Ohashi, Natsuizaka et al.2010). Notch signaling orchestrates cell differentiation in several tissues. Furthermore, loss of Notch signaling is required for the differentiated goblet cells and other secretory cell lineages in the small intestine (van Es, van Gijn et al. 2005; Fre, Huyghe et al. 2005).

We show that the combination of Notch inhibition with MYC and CDX1 overexpression leads to characteristic features of BE, namely the production of mucin by goblet-like cells and the presence of columnar-like cells. It was suggested previously that HATH1 induction by Notch inhibition induces MUC2 via CDX2 expression in esophageal cancer cell lines (Tamagawa, Ishimura et al. 2012). Moreover, goblet cell differentiation was induced by Notch inhibition in the *L2-IL-1β* mouse model of Barrett's-like metaplasia (Quante, Bhagat et al. 2012). Hence, Notch signaling inhibition could be necessary for the initiation of the BE metaplasia program by orchestrating transcriptional regulation of key genes implicated in goblet cell terminal differentiation.

Previous studies have shown a possible inverse relationship between HATH1 expression and Notch activity in the small intestine. This regulation has been suggested to be mediated by HES1 (Kazanjian, Noah et al. 2010). Furthermore, studies of BE cell lines treated with bile acid show decrease Notch expression accompanied by HATH1 and CDX2 upregulation (Morrow, Avissar et al. 2009). Interestingly, our MYC-CDX1-dnMAML cells show increased HATH1 expression, supporting the premise that Notch is a negative regulator of HATH1. Yet, our HATH1 knockdown data only shows a partial reversal of the gene expression changes observed upon Notch signaling inhibition in the MYC-CDX1 cells. Moreover, HATH1 knockdown had no effect in the changes in cell morphology observed in the MYC-CDX1-dnMAML cells. Thus, our data suggest HATH1

is a limited mediator of the transdifferentiation process induced by Notch signaling inhibition.

KLF4 can be regulated negatively by Notch signaling (Zheng, Pritchard et al. 2009). Herein, we demonstrate that inhibition of Notch signaling causes an activation of KLF4 expression and that knockdown of KLF4 can reverse some of the genetic and morphological changes induced by Notch signaling inhibition. These data support KLF4 as a potential driver in the activation of intestinal cell lineage genes upon Notch signaling inhibition, suggesting a possible novel mechanism through which Notch signaling promotes initiation of BE development. Interestingly, KLF4 is strongly expressed in Barrett's esophagus and its expression is induced in response to bile acids. KLF4 and CDX2 also cooperate to induce production of MUC2. Interestingly, KLF4 has been linked to other models of transdifferentiation, including conversion of smooth muscle cells into osteogenic cells in the context of hyperphosphatemia and conversion of fibroblasts into neural progenitors or cardiomyocytes (Yoshida, Yamashita et al. 2012; Kim, Efe, et al. 2011; Efe, Hilcove et al. 2011). KLF4 is one of the key factors (OCT4, SOX2, MYC, NANOG and KLF4) that can reprogram the fate of somatic cells into inducible pluripotent stem cells (iPSC). KLF4 is also recognized for its capacity to maintain the pluripotent state of embryonic stem cells (ESC) (McConnell and Yang 2010). Therefore, KLF4 activation in response to Notch inhibition could facilitate transdifferentiation of esophageal squamous cells into intestinal-like cells by binding to promoters of columnar keratins and mucin genes to enhance their expression (Figure 8).

Herein, we provide evidence to support the notion that esophageal basal cells might serve as potential cells of origin for BE. Several models of the cell of origin for BE have been proposed. These are not necessarily mutually exclusive and may be context

dependent. One model suggests that cells may migrate from the esophago-gastric junction (EGJ) or from the gastric cardia to the distal esophagus (Quante, Abrams et al. 2012). By lineage-labeling gastric cardia stem cells (LGR5+) cells in *L2-IL-1β* transgenic mice that develop BE and EAC, it was demonstrated that migration of gastric cardia cells give rise to BE tissue (Quante, Bhagat et al. 2012). It is also possible that bone marrow derived progenitor cells give rise to BE. Indeed, male to female bone marrow transplants in a severe reflux esophagitis rat model showed that the developing BE epithelium was of male origin (Sarosi, Brown et al. 2008), suggesting that progenitor cells originating from the bone marrow can contribute to BE development.

Metaplasia may represent an adaptive response to a stressful local environment, and involve a complex interplay between epigenetic and genetic factors or alterations. It can occur in several tissues including esophagus (BE), stomach, pancreas, lung, cervix and skin. Metaplasia may be reversible or irreversible, and may progress to dysplasia and cancer. For example, BE can progress to low-grade and high-grade dysplasia and culminate in esophageal adenocarcinoma (EAC). Our studies suggest cell autonomous mechanisms involving Notch signaling and pivotal transcription factors: MYC, CDX1, HATH1 and KLF4, which promote a partial reprogramming of the esophageal cells toward BE. However, the complete emergence of BE, and certainly progression to a dysplastic state and EAC may involve cell non-autonomous mechanisms, such as inflammation and activation of Hedgehog signaling and Wnt signaling (Fang, Chen et al. 2013; Clement, Guilleret et al. 2008; Yang, Wang et al. 2012). In summary, we now demonstrate key mechanisms underlying the initiation of BE, which holds the potential for future biomarker studies for patients at risk for progression to EAC.

Methods

Cell lines

EPC2-hTERT-MYC-CDX1 cells and their derivatives: EPC2-hTERT-MYC-CDX1-dnMAML, EPC2-hTERT-MYC-CDX1-dnMAML-shKLF4, EPC2-hTERT-MYC-CDX1--dnMAML-shHATH1, EPC2-hTERT-MYC-CDX1-dnMAML-shScramble, EPC2-hTERT-MYC-CDX1-shHES1 and EPC2-hTERT-MYC-CDX1-shScramble were grown in KSFM (Keratinocyte Serum Free Medium, Invitrogen Carlsbad, CA) with Ca^{2++} and supplements: BPE (bovine pituitary extract), EGF and 1% Penicillin Streptomycin (Invitrogen), as described previously (Harada, Nakagawa et al. 2003). Cells were treated with 0.06 mmol/L calcium chloride (Ca^{2++}) to promote squamous differentiation for 48 hrs before harvesting RNA. Phoenix A cells were grown in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Sigma, St. Louis, MO) and 1% Penicillin Streptomycin. FEF3 cells (fetal embryonic fibroblasts) were grown in DMEM supplemented with 10% HyClone FBS (GE Healthcare Life Sciences, Piscataway, NJ) and 1% Penicillin Streptomycin, as described previously (Grugan, Miller et al. 2010).

Stable transduction

MYC-CDX1 cells were transduced with pBabe-puro or pBabe-dnMAML-GFP-puro or pBabe-zeo, pBabe-dnMAML-GFP-zeo virus. MYC-CDX1 cells were also transduced with pLKO.1 shScramble-puro or pLKO.1 TRC puro-shHES1 virus. MYC-CDX1-dnMAML (zeo) cells were transduced with pLKO.1 shScramble-puro, pLKO.1 shKLF4 or pLKO.1 shHATH1 virus. Transduced cells were selected with 1ug/ml puromycin (EMD-Millipore, Billerica, CA), or 10ug/ml zeocin (Invitrogen, Carlsbad, CA) for 7 days.

3D Organotypic culture

EPC2-hTERT-MYC-CDX1 cells and their derivatives were grown using the 3D organotypic culture system as described previously (Kalabis, Wong et al. 2012). Cultures were fixed overnight in 10% buffered formalin phosphate (Fisher, Waltham, MA) before paraffin embedding and sectioning.

Histology and Immunohistochemistry

Hematoxylin and eosin (H&E) staining as well as immunohistochemistry (IHC) were performed as described previously (Stairs, Nakagawa et al. 2008). The following antibodies were used for IHC: K13 (Abcam, Cambridge, MA) 1:500, K19 (BioLegend, San Diego, CA) 1:100, HES1 (Abcam, Cambridge, MA) 1:500, NOTCH1 (Epitomics, Burlingame, CA) 1:100 and ICN1 (Cell Signaling, Beverly, MA) 1:200. Biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and ABC avidin-biotin-DAB detection kit (Vector Labs, Burlingame, CA) were used for detection and visualization according to supplier's protocol.

Cell height measurement

Quantification of cell height at the basal layer of MYC-CDX1 and MYC-CDX1-dnMAML cells grown in 3D organotypic cultures was performed by measuring 15 cells per HPF (high power field) of H&E (n=360). We measured 4 independent 3D organotypic cultures for each cell line. Statistical analysis for significance was determined by student t-test with $p<0.05$ as statistically significant.

Tissue Microarray

Tissue microarray (TMA) of human biopsies of Barrett's esophagus (n=15-23), normal esophagus (n=25-27) and liver control were stained for status of Notch signaling. IHC staining of TMA was performed using the following antibodies: HES1, NOTCH1 and ICN1. Scoring for positive staining of HES1, ICN1 and NOTCH1 was analyzed by quantitative evaluation of staining intensity with a scale of 0-2 (0=none to 2=strong), by a pathologist (Andres Klein-Szanto) in a blind manner.

Statistical Analysis

For gene expression changes in qPCR studies, statistical significance of comparisons between MYC-CDX1 and MYC-CDX1-dnMAML cells and between MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shKLF4 cells were determined by the student *t-test* with $p<0.05$ as statistically significant. Error bars represent the mean ± SEM (Standard Error of the Mean) from at least triplicate experiments. Scoring data of TMA was analyzed for statistical significance by Fisher's Exact Test with $p<0.05$ as statistically significant.

Retroviral and lentiviral production

Retroviral productions for the following vectors (pBabe-puro and pBabe-dnMAML-GFP-puro, pBabe-zeo, pBabe-dnMAML-GFP-zeo) were performed using Phoenix A cells as described previously (Okawa, Michaylira, et al. 2007). Lentiviral production of 10µg pLK0.1 shRNA constructs was performed in 293T cells with pLKO.1 shScramble-puro, pLKO.1 TRC puro-shHES1 or pLKO.1 TRC puro-shKLF4 in combination with 6µg pPAX2 and 3µg pMD2.G. For both retroviral and lentiviral production, cells were

transfected using the Calcium Phosphate transfection protocol, as described previously (Pear, Scott, et al. 1997). Viral supernatants were harvested using a low serum DMEM containing 5% FBS at 48 and 72 hours post-transfection and stored at -80C.

shRNA constructs

Constructs were made following the AddGene protocol for the pLKO.1 TRC puro cloning strategy for adding shRNA to the lentiviral vector (Moffat, Grueneberg et al. 2006). Sequences used for oligos in Methods Table 1.

Methods Table 1: shRNA constructs oligos

shRNA CONSTRUCT	**FORWARD OLIGO**	**REVERSE OLIGO**
shHATH1 #1	CCGGAACTTCCAGCAAACAG GTGAATTCTCGAGTTCACCT GTTTGCTGGAAGTTTTTTTG	AATTCAAAAAAACTTCCAGC AAACAGGTGAATTCTCGAG TTCACCTGTTTGCTGGAAGT T
shKLF4 #1	CCGGAAGGACTTTATTCTCT CCAATTTCTCGAGATTGGAG AGAATAAAGTCCTTTTTTTG	AATTCAAAAAAAGGACTTTA TTCTCTCCAATTTCTCGAGA TTGGAGAGAATAAAGTCCTT
shKLF4 #3	CCGGAACCTTACACATGAAG AGGCATTCTCGAGTGCCTCT TCATGTGTAAGGTTTTTTTG	AATTCAAAAAAACCTTACAC ATGAAGAGGCATTCTCGAG TGCCTCTTCATGTGTAAGGT T
shHES1 #1	CCGGAACAACACGACACCG GATAAAAATTCTCGAGTTTAT CCGGTGTCGTGTTGTTTTTT TG	AATTCAAAAAAACAACACGA CACCGGATAAAAATTCTCGA GTTTATCCGGTGTCGTGTT GTT
shHES1 #2	CCGGAAGCTCTGAAGAAAGA TAGCTTTCTCGAGAGCTATC TTTCTTCAGAGCTTTTTTTG	AATTCAAAAAAAGCTCTGAA GAAAGATAGCTTTCTCGAG AGCTATCTTTCTTCAGAGCT T

Luciferase Assay

Cells were seeded into 24-well plates 24 hrs before transfection at a concentration of $1.5x10^5$ cells per well and transfected using Lipofectamine LTX with PLUS Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The following vectors were used for transfection: 0.2µg pGL3-8XCSL (Ohashi, Natsuizaka et al. 2010), 0.01µg pRL-SV40 and 0.2µg MigRI or MigRI-ICN1. Transfected cells were harvested 24 hrs post-transfection for measurement of luciferase activity using the Dual Luciferase Assay Kit (Promega, Madison, WI) and following the manufacturer's protocol.

RNA extraction and quantitative PCR

Total RNA was extracted using the RNeasy Kit (Qiagen, Germantown, MA) according to the manufacturer's protocol. First strand cDNA synthesis was performed using the SuperScript First Strand Synthesis Kit (Invitrogen, Carlsbad, CA). Real-Time PCR was performed and analyzed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Grand Island, NY) with TaqMan Universal PCR Master Mix (Applied Biosystems, Grand Island, NY) or Power SYBR Master Mix (Applied Biosystems, Grand Island, NY). qPCR was performed using human Taqman probes (Applied Biosystems, Grand Island, NY): HES1: Hs00172878_m1, HES5: Hs01387463_g1, MUC2: Hs03005094_m1, K13: Hs00999762_m1, K8: Hs01670053_m1, K20: Hs00300643_m1, HATH1: Hs00245453_s1, KLF4: Hs00358836_m1 and β-ACTIN: NM_001101.2. The following human SYBR green primers were used for: K5, K14, K18, K19, MUC3B, MUC5B, MUC17, DSC1, DSC3 and β-ACTIN, primers sequences in Methods Table 2.

Methods Table 2: SYBR green primers

GENE (HUMAN)	FORWARD PRIMER	REVERSE PRIMER
K5	TTCTTTGATGCGGAGCTGT	CATGGAGAGGACCACTGAGG
K14	GTCTGGCCGCGGATGAC	GATGTCGGCTTCCACACTCA
K18	AAAGGCCTACAAGCCCAGAT	CACTGTGGTGCTCTCCTCAA
K19	GATGAGCAGGTCCGAGGTTA	TCTTCCAAGGCAGCTTTCAT
MUC3B	AGGTGGGCATGGAAGTGTCT	CTGTAGGCCTGGGAAGTGTTG
MUC5B	GCTGCTGCTACTCCTGTGAGG	AGGTGATGTTGACCTCGGTCTC
MUC17	GGGCCAGCATAGCTTCGA	GCTACAGGAATTGTGGGAGTTCA
DSC1	TCTTCGAGTTCCTTCTCATCTTCAG	GATTAGGCTGGCCGACTTGA
DSC3	TTCTCAGGCGTGCCAAGAG	GAAAGGGCCCAAGGAATTCT
β-ACTIN	CCTGGCACCCAGGACAAT	GCCGATCCACACGGAGTACT

Western Blotting

Western Blot analysis was performed as described previously (Grugan, Vega et al. 2013; Stairs, Nakagawa et al. 2008). The following primary antibodies were used in 5% Milk in 1X PBS-T (0.1% Tween-20): MYC 1:250 (SantaCruz, Dallas, TX), CDX1 1:1000 (Abcam, Cambridge, MA), β-ACTIN (Sigma, St. Louis, MO) 1:15000, KLF4 1:15000 (Biosource/QCB graciously provided by Jonathan P. Katz, University of Pennsylvania), HES1 1:1000 (Abcam, Cambridge, MA). The GFP (Cell Signaling, Beverly, MA) primary antibody was used at 1:1000 in 5% BSA in 1X PBS-T (0.1%Tween-20). Secondary

antibodies (HRP-mouse, HRP-rabbit) were used at 1:5000 in 5% Milk in 1X PBS-T. Blots were developed using ECL Plus reagent (Invitrogen, Carlsbad, CA). Signals were quantified by densitometry.

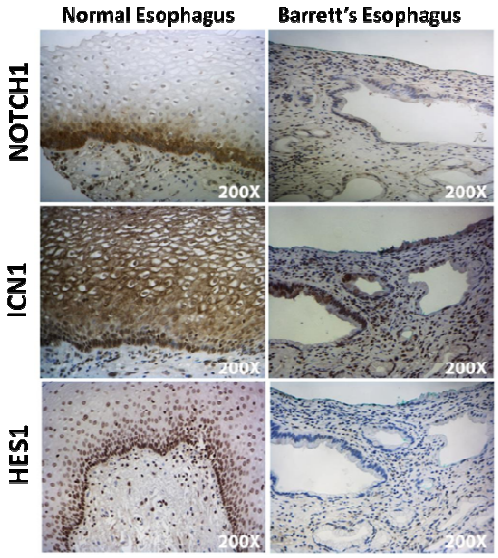
Chapter II:

Figures and Figure Legends

A

Gene Symbol	Fold (BE vs. norm.)	p-value (BE vs. norm.)
NOTCH2	-4.41726	4.62E-06
NOTCH2	4.80363	7.95E 07
NOTCH3	-6.3073	2.94E-03
JAG1	-2.66073	1.02E-04
JAG1	-2.80861	1.50E-04
JAG1	-3.72515	7.20E-04

B



C

Staining/IHC	Average Score		p-value
	Normal Esophagus	Barrett's Esophagus (BE)	BE vs norm.
NOTCH1	1.8	0.5	<0.0001
ICN1	0.8	0.14	0.00017
HES1	1.1	0.4	0.018

Figure 1: Notch signaling is decreased in human Barrett's esophagus. (A) Microarray analysis from seven samples of Barrett's esophagus (BE) and their adjacent normal squamous esophagus for status of Notch signaling pathway, GEO accession #GSE13083. (B) Representative image of normal esophagus and Barrett's esophagus tissue from tissue microarray (TMA) stained for NOTCH1, ICN1 and HES1 (200X Magnification). (C) Average scoring for positive staining in the TMA and statistical analysis using Fisher's exact t-test.

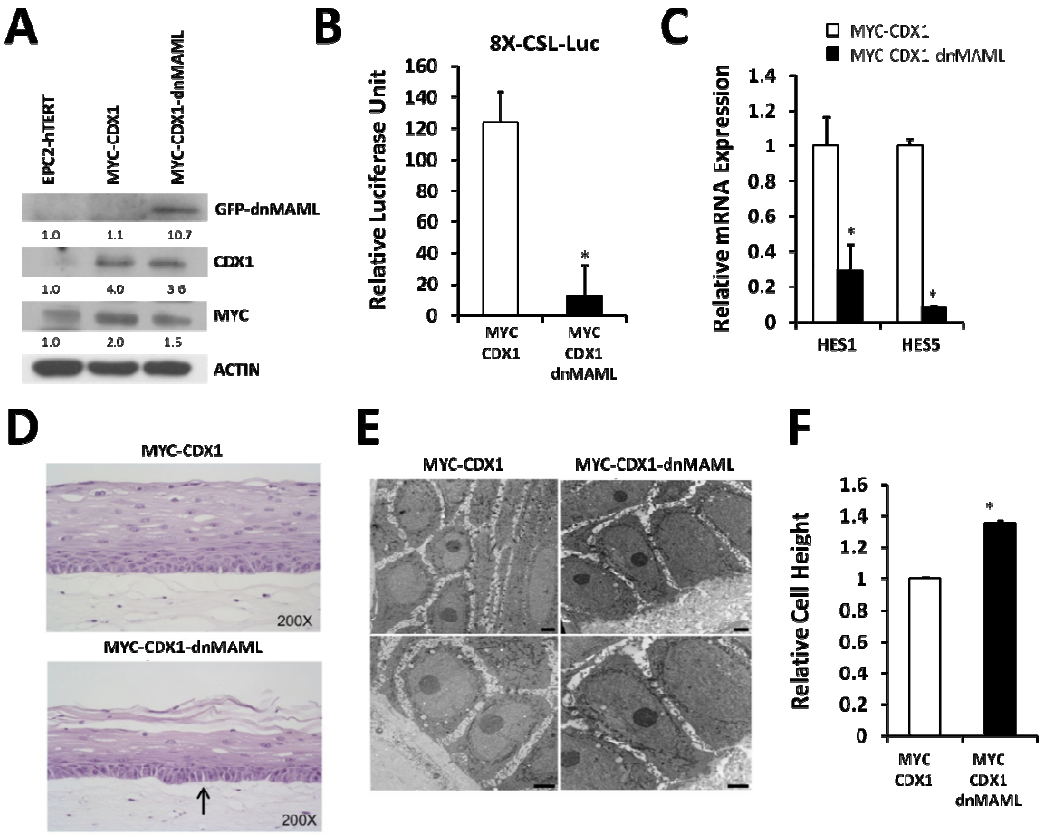


Figure 2: Inhibition of Notch signaling in esophageal epithelial cells changes basal cell morphology in 3D cultures. (A) Western blotting for GFP (dnMAML), MYC and CDX1 in EPC2-hTERT, MYC-CDX1 and MYC-CDX1-dnMAML cells. (B) Luciferase assay with Notch-responsive pGL3-8XCSL reporter vector in MYC-CDX1-ICN1 and MYC-CDX1-ICN1-dnMAML cells, graph represents mean ± SEM (n=3). Student t-test was performed to determine significance, *p≤0.05. (C) Quantitative PCR (qPCR) for Notch downstream targets HES1 and HES5 in MYC-CDX1 and MYC-CDX1-dnMAML cells. Graph represents mean ± SEM (n=3) and student t-test was performed to determine significance, *p≤0.05. (D) H&E staining of representative 3D organotypic cultures of MYC-CDX1 and MYC-CDX1-dnMAML cells, arrow indicates elongated cells, (200X Magnification). (E) Electron microscopy of MYC-CDX1 and MYC-CDX1-dnMAML 3D organotypic cultures, scale bars=0.2µm. (F) Graph represents relative height of MYC-CDX1 and MYC-CDX1-dnMAML basal layer cells mean ± SEM (n=4). Student t-test was performed to determine significance, *p≤0.0001.

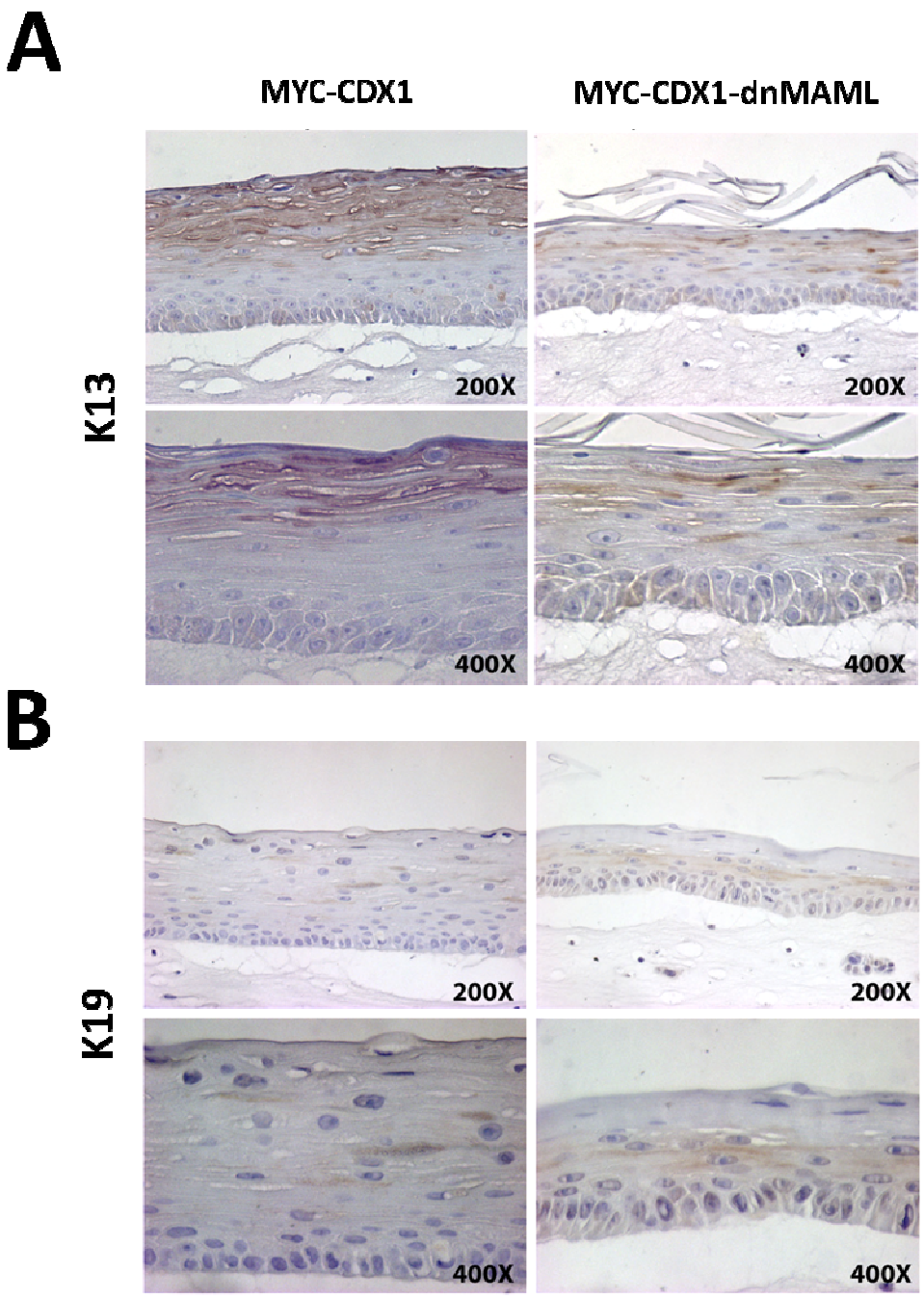


Figure 3: Inhibition of Notch signaling in esophageal epithelial cells decreases squamous K13+ cells and increases columnar K19+ cells in 3D organotypic culture. IHC staining of 3D organotypic cultures for squamous keratin K13 (A) and columnar keratin K19 (B) in MYC-CDX1 (left panel) and MYC-CDX1-dnMAML cultures (right panel) (200X and 400X Magnification).

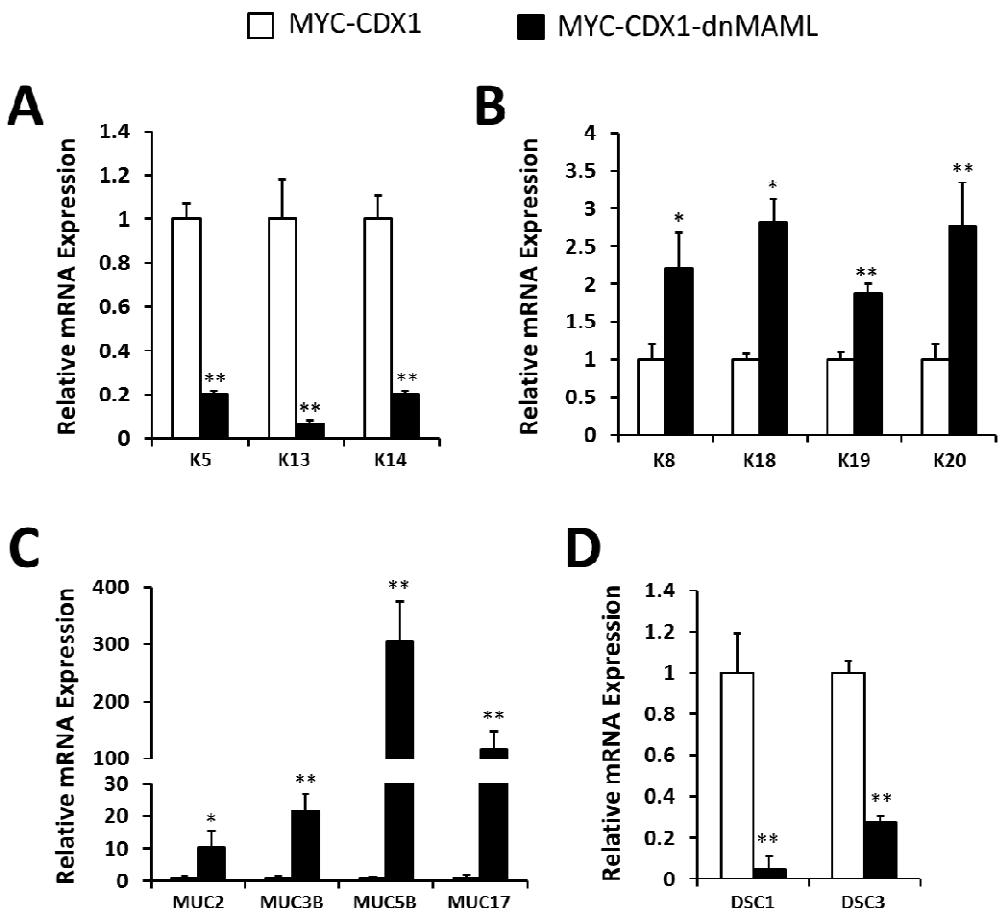


Figure 4: Inhibition of Notch signaling in esophageal epithelial cells promotes a switch from the squamous lineage to a columnar lineage. qPCR of (A) squamous keratins K5, K13 and K14; (B) columnar keratins K8, K18, K19 and K20; (C) mucin genes MUC2, MUC3B, MUC5B and MUC17; (D) and differentiation genes DSC1 and DSC3 in MYC-CDX1 and MYC-CDX1-dnMAML cells. Graph represents mean ± SEM (n=6). Student t-test was performed to determine significance, *p≤0.05, **p≤0.001.

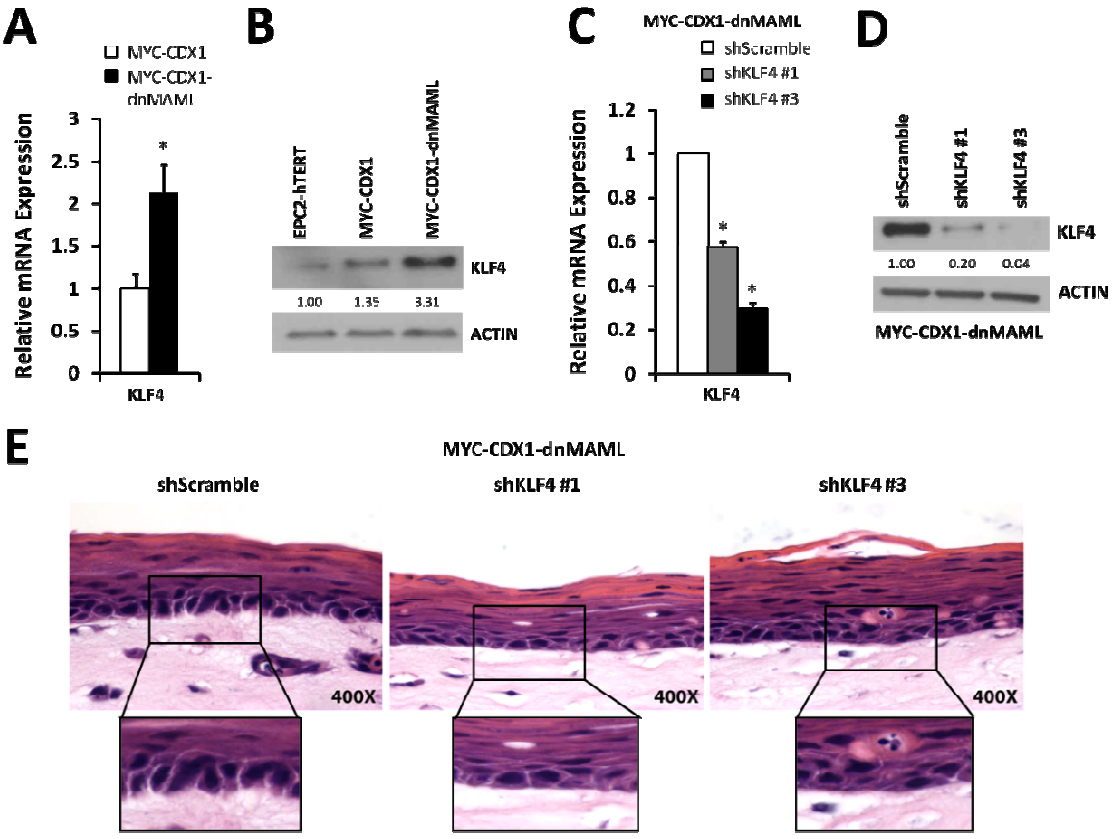


Figure 5: KLF4 knockdown reverses partially the morphological changes induced by Notch signaling inhibition in 3D cultures. (A) qPCR of KLF4 expression in MYC-CDX1 and MYC-CDX1-dnMAML cells. (B) Western blotting for KLF4 in EPC2-hTERT, MYC-CDX1 and MYC-CDX1-dnMAML cells. (C) qPCR of KLF4 in MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shKLF4 cells. (D) Western blotting for KLF4 in MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shKLF4 cells. (E) H&E staining of MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shKLF4 3D organotypic cultures (400X Magnification). Graph represents mean ± SEM (n=6). Student t-test was performed to determine significance, *p≤0.01.

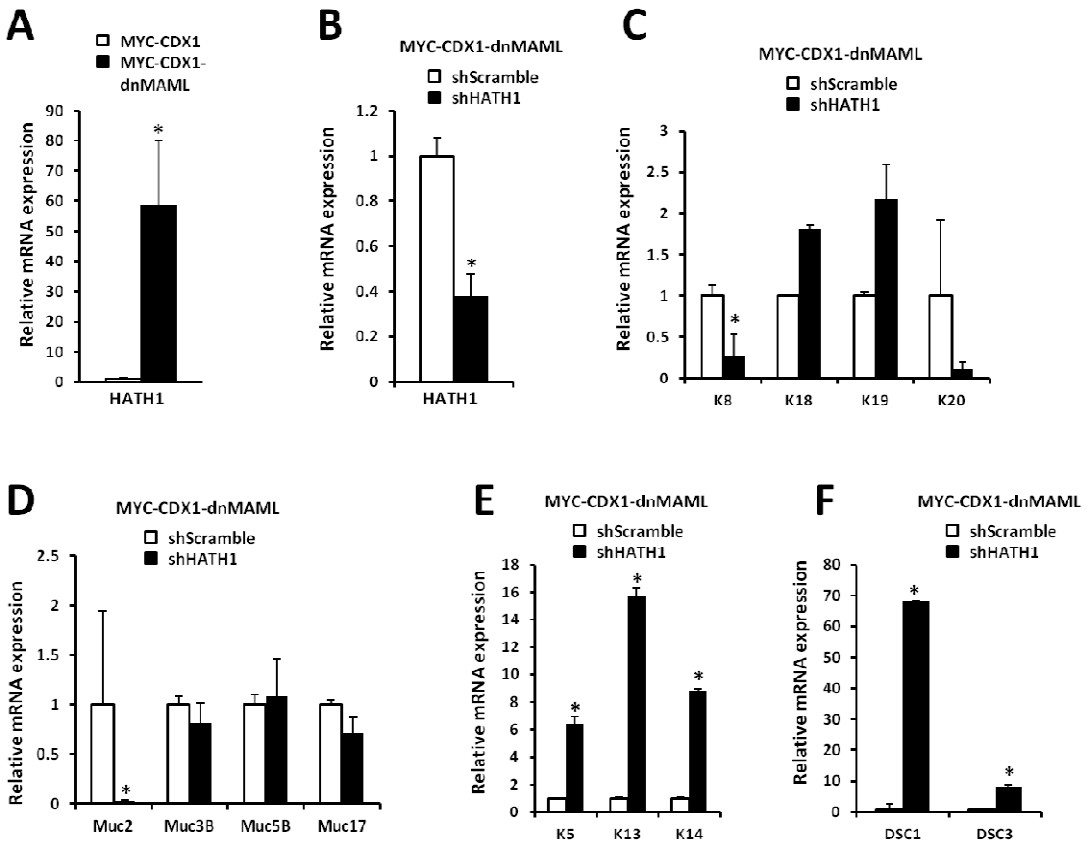


Figure 6: HATH1 knockdown partially reverses changes induced by Notch signaling inhibition in esophageal epithelial cells. (A) qPCR of HATH1 expression in MYC-CDX1 and MYC-CDX1-dnMAML cells. (B) qPCR of HATH1 in MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shHATH1 cells. qPCR of (C) columnar keratins K8, K18, K19 and K20; (D) mucin genes MUC2, MUC3B, MUC5B and MUC17; (E) squamous keratins K5, K13, K14; (F) and squamous differentiation markers DSC1 and DSC3 in MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shHATH1 cells. Graph represents mean ± SEM (n=6). Student t-test was performed to determine significance, *p≤0.05.

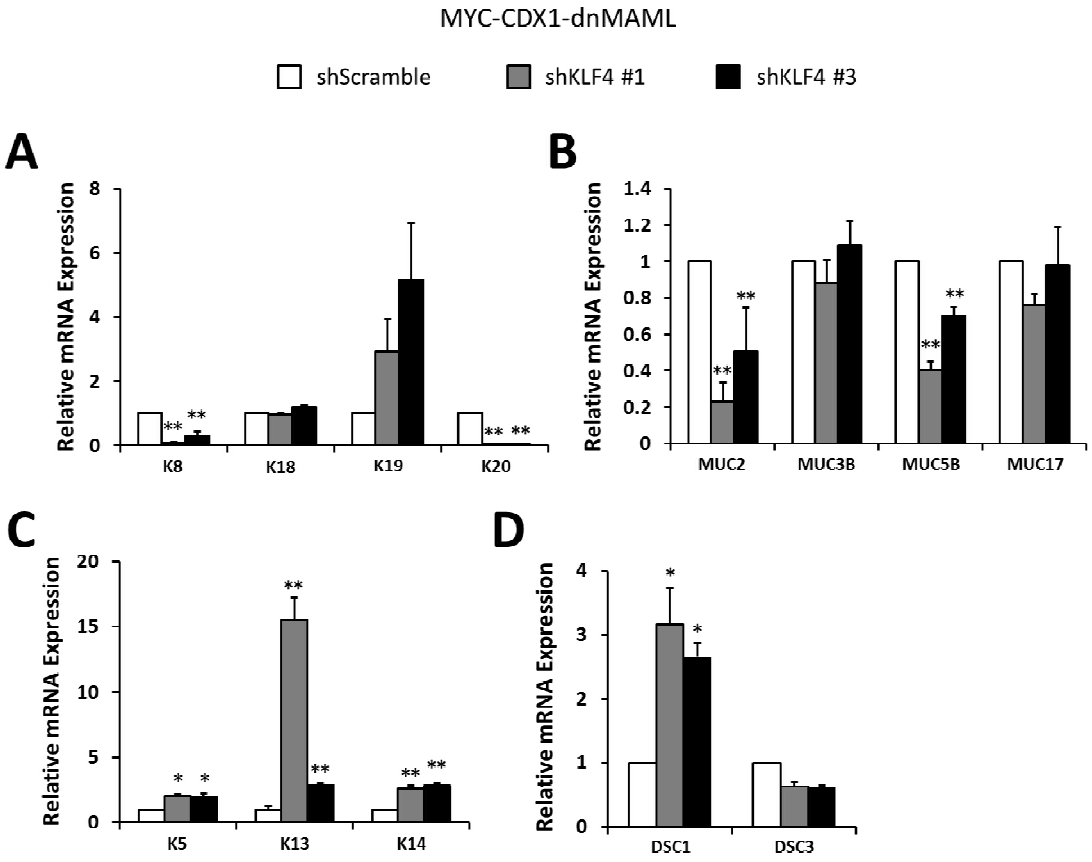


Figure 7: KLF4 knockdown reverses lineage changes induced by Notch signaling inhibition in esophageal epithelial cells. qPCR of (A) columnar keratins K8, K18, K19 and K20; (B) mucin genes MUC2, MUC3B, MUC5B and MUC17; (C) squamous keratins K5, K13 and K14; (D) and squamous differentiation markers DSC1 and DSC3 in MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shKLF4 cells. Graph represents mean ± SEM (n=6). Student t-test was performed to determine significance, *p≤0.01, **p≤0.001.

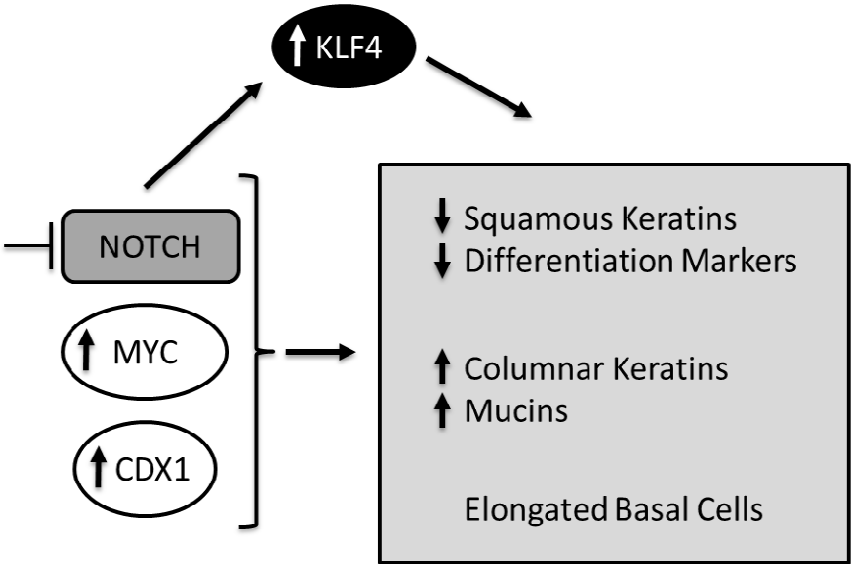


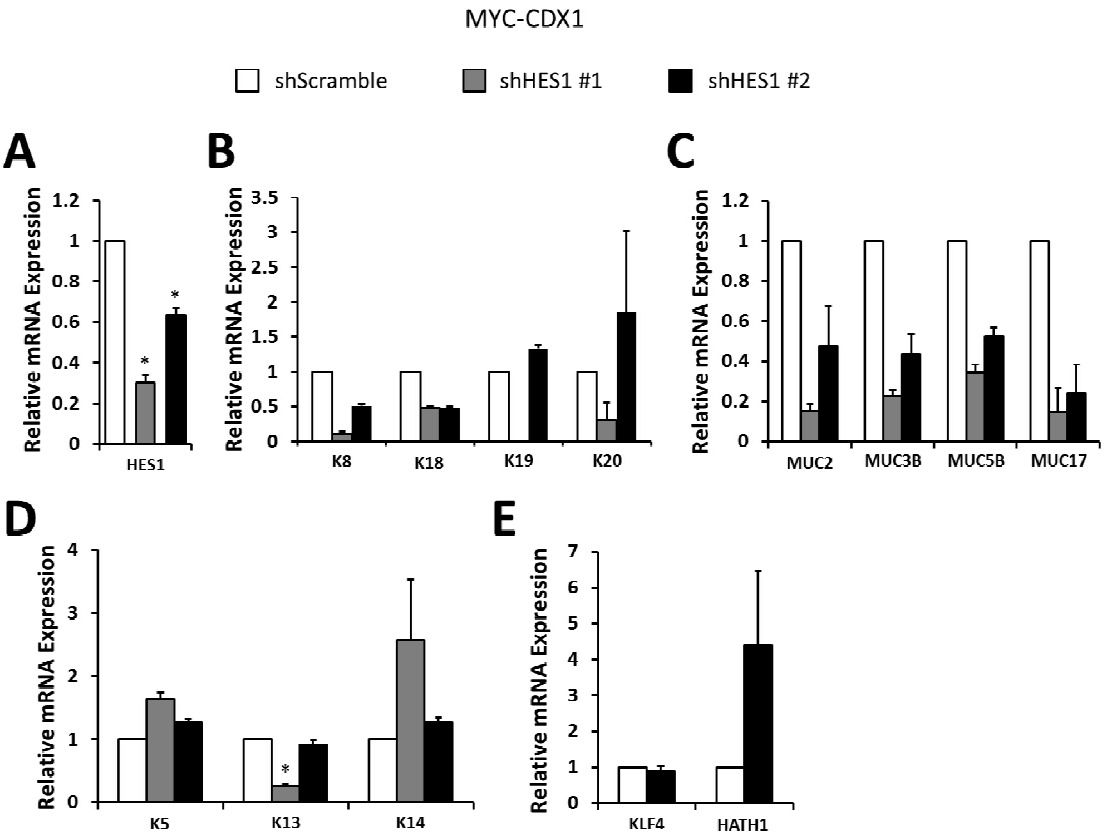
Figure 8: Model. Inhibition of Notch signaling in conjunction with MYC and CDX1 expression promotes increased expression of columnar keratins and mucin genes as well as decreased expression of squamous keratins and other markers of differentiation. Inhibition of Notch also triggers changes in cell morphology in the basal layer. Inhibition of Notch signaling promotes KLF4 expression and the initiation of a transdifferentiation program towards a BE-like metaplasia.

Chapter II:

Supplementary Figures

NOTCH1	Normal Esophagus	Barrett's esophagus
Positive	19	0
Negative	8	15
Total Number	27	15
Average Score	1.8	0.5
Normal vs. BE p-value	<0.0001	
ICN1	**Normal Esophagus**	**Barrett's esophagus**
Positive	20	6
Negative	5	19
Total Number	25	25
Average Score	0.8	0.14
Normal vs. BE p-value	0.00017	
HES1	**Normal Esophagus**	**Barrett's esophagus**
Positive	19	9
Negative	6	14
Total Number	25	23
Average Score	1.1	0.4
Normal vs. BE p-value	0.018	

Supplemental Table 1: Tissue Microarray scoring for Notch signaling IHC positive staining in human Barrett's esophagus. TMA staining for HES1, NOTCH1 and ICN1 was scored for positive staining in a scale from 0-2. Statistical analysis was performed with Fisher's exact t-test comparing normal squamous to Barrett's esophagus.



Supplemental Figure 1: Knockdown of HES1 in MYC-CDX1 cells does not cause transdifferentiation. qPCR of (A) HES1; (B) columnar keratins K8, K18, K19 and K20; (C) mucins MUC2, MUC3B, MUC5B and MUC17; (D) squamous keratins K5 ,K13 and K14; (E) KLF4 and Hath1 in MYC-CDX1-shScramble and MYC-CDX1-shHES1 cells. Graph represents mean ± SEM (n=6), student t-test was performed to determine statistical significance, *p≤0.01.

Chapter III:

Development of a genetic mouse model of Barrett's esophagus

Abstract

BE is characterized by the presence of an intestinal columnar epithelium and mucin-producing goblet cells. BE is the major risk factor for the development of esophageal adenocarcinoma (EAC). Esophageal cancer is one of the deadliest cancers in the US, with a 5- year survival rate of 17.3% in 2013. In the US, there has been an increase in the cases of EAC. The rise of EAC can be associated with the increased prevalence of BE. It is critical to develop *in vivo* models for BE and EAC. Therefore, we sought to develop a genetic mouse model for BE by overexpressing MYC and CDX2, specifically in the esophageal squamous epithelium. We first designed a mouse with conditional expression of MYC in the esophageal epithelium. In order to achieve this we created a Tet-ON system using the Epstein-Barr virus L2 (EBV-L2) promoter (designated *L2-rtTA*), which is specifically active in the esophageal epithelium. Our system allows us to activate MYC overexpression post-weaning by feeding the mice Dox (doxycycline) chow. Secondly, we bred our *L2-rTta; TetOp-Myc* mice with Keratin14-Cdx2 (*K14-Cdx2)* mice. Herein, we demonstrate the activation of MYC and CDX2 specifically in the mouse esophagus. To date, we have observed no significant changes in the histology of the esophagus of *the L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice with or without 0.3% deoxycholic acid (DCA) treatment. Current and future experiments involving aging the mice, as well as crossing with mice in which Notch signaling is inhibited.

Introduction

BE is characterized by the presence of differentiated intestinal columnar cells and mucin-producing goblet cells. Classically, BE studies focus on analysis of genetic alterations found in human BE biopsies. Interestingly, BE is characterized by a mosaic pattern of genetic alterations, complicating the identification of initiating genetic changes that lead to BE. In order to study the mechanisms leading to the development of BE, certain *in vitro* and *in vivo* models have been generated. Current *in vitro* models for the study of BE include: 2D cell culture, 3D organotypic cultures and *ex vivo* transplantation models. Cell culture models for BE include the use of the immortalized esophageal epithelial cell lines (EPC2) and immortalized BE cell lines (CP-A, CP-B, CP-C and CP-D) (Garman, Orlando et al. 2012). Interestingly, the use of 3D organotypic cultures allows the evaluation of the interactions between epithelial cells and the stroma (Kalabis, Wong et al. 2012). Recent studies using 3D organotypic cultures to grow the BE immortalized cell lines (CP-A, CP-B, CP-C and CP-D) show heretogeneity between all four cell lines when grown in 3D cultures (Kosoff, Gardiner et al. 2012). An alternative system to 3D culture system is the use of an *ex vivo* system, namely a heterotypic rat trachea transplantation model (Croagh, Redvers et al. 2012). Recent studies using this system have shown that Sox9 expression in mouse esophageal epithelial cells promotes K8 expression and columnar differentiation (Clemons, Wang et al. 2012). The use of these cell culture models all allow for the evaluation of gene regulation in a controlled environment, but the environment can differ greatly from that found *in vivo*.

Limited *in vivo* models have been developed for the study of BE and EAC. In one such model, rats undergo an esophagojejunostomy that results in massive bile reflux. Post-surgery these rats develop BE and EAC (Gibson, Zaidi et al. 2013). This

surgical model allows for the evaluation of potential therapeutics. Treatment with a Smoothened inhibitor leads to a decreased incidence of BE and EAC in rats (Gibson, Zaidi et al. 2013). However, this rat surgical model has limitations due to technical difficulty and high mortality rate post-surgery. Moreover, the development of tumors in this model relies excessively upon bile reflux, which may not be physiologic.

Recent work has focused on the development of genetic models in mice to study BE and EAC. Studies of a p63 global knockout mice show the expansion of residual embryonic cells upon the loss of the esophageal squamous epithelium. These residual embryonic cells replace the squamous epithelium with a columnar lined esophagus (Wang, Ouyang et al 2011). One caveat of this model is that the p63 knockout mice are embryonic lethal and survive minimally in the post-natal period. Another study with transgenic mice engineered to overexpress IL-1β and treated with bile in the drinking water revealed that the mice develop esophagitis, BE and EAC. Furthermore, lineage labeling of the gastric cardia stem cells by Lgr5-GFP showed the presence of gastric cardia derived cells in the BE lesions (Quante, Bhagat et al. 2012). Findings from the *L2-IL1β* mice model have shown the important role inflammation plays for the development of BE. Given our previous *in vitro* studies that show MYC and CDX1 overexpression in esophageal epithelial cells leads to transdifferentiation toward BE (Stairs, Nakagawa 2008). We sought to develop a genetic mouse model for BE by overexpressing MYC and CDX2, specifically in the esophageal squamous epithelium, to test if transdifferentiation can lead to BE.

Results

Vector design and generation of new transgenic mice: *L2-rtTA; Tet-Op-CreER^{T2}* and *Tet-Op-Myc*

In order to achieve the conditional expression of MYC, we designed a Tet-ON system, driven by the EBV-L2 promoter. The EBV-L2 promoter has been shown to be active by our group in the mouse oral cavity (Nakagawa, Wang et al. 1997). We first designed a vector (pRIL) with the EBV-L2 promoter driving expression of rtTA (tetracycline transactivator) (Figure 1A). Furthermore, we inserted an HA-tagged luciferase, in order to optimize the detection of cells with EBV-L2 promoter activity (Figure 1A). Next, we sought to add the ability to induce expression of the Cre recombinase specifically by the EBV-L2 promoter. Therefore, we created a vector (pG-CreER^{T2}) with the Tet-Op promoter driving expression of CreER^{T2}, a modified Cre recombinase fused to the estrogen receptor (ER^{T2}) (Figure 1B), which allows for activation of the Cre by treatment with tamoxifen. Next, we designed a vector (pGIT-Myc) expressing the Tet-Op promoter driving expression of MYC; we also labeled the expression of this vector with FLAG-tagged TdTomato downstream of the Tet-Op promoter (Figure 1C).

We proceeded to prepare the vectors for DNA microinjection into mice. Therefore, we digested our DNA constructs with the *Pac I* restriction enzyme, which led to the linearization of the DNA construct. This also allowed for the removal of the DNA vector backbone. We confirmed proper quantification and purification of the linearized DNA (Supplemental Figure 1). DNA microinjection was performed by the Transgenic and Chimeric Mouse Facility into C57BL/6 fertilized eggs. The injections were done in

the following combinations: (A) pRIL+ pG-CreER^{T2} and (B) pGIT-Myc (Figure 2). Subsequent to the DNA microinjection, we received two founder (F1) mice for each mouse line: *L2-rtTA; Tet-Op-CreER^{T2}* and *Tet-Op-Myc* (Figure 2A-B). We confirmed the genotyping for each of the mouse lines in the F2 generations (Supplemental Figure 2A-B). Thus, our *L2-rtTa; Tet-Op-CreER^{T2}* mice express the rtTA promoter in the squamous oral cavity, esophagus and forestomach. Furthermore, treatment with doxycyline (dox) activates the binding of the rtTA transcription factor to the Tet-Op promoter and promotes the activation of CreER^{T2} or MYC (Figure 2A-B).

L2-rtTA; Tet-Op-CreER^{T2} mice show specific expression of luciferase in esophagus and squamous forestomach

Next, we proceeded to confirm the expression of luciferase in the *L2-rtTA; Tet-Op-CreER^{T2}* mice. We isolated tissue from the esophagi and squamous forestomachs of the mice. We observed significant luciferase activity in the esophagus (Figure 3A) and squamous forestomach (Figure 3B) in both founders of the *L2-rtTA; Tet-Op-CreER^{T2}* mice, compared to wild type control littermates. Furthermore, we observed no luciferase activity in our negative control tissue (liver) (data not shown). These results confirm the specific expression of our *L2-rtTA; Tet-Op-CreER^{T2}* double transgenic vectors restricted to the esophagus and squamous forestomach in both founder mouse lines.

K14-rtTA; TetOp-Myc mice show activation of MYC expression in mouse esophagus

We proceeded to verify the function of the Tet-Op promoter in the *Tet-Op-Myc* mice. To activate the expression of the promoter, we bred our *Tet-Op-Myc* mice to the *K14-rtTA* mice (Nguyen, Rendl et al. 2006). The K14 promoter is expressed in the skin,

salivary gland and mammary-gland epithelium. In addition, it is expressed in epithelial cells of other tissues, including tongue, esophagus, forestomach and thymus (Wang, Zinkel et al. 1997). In order to activate the Tet-ON system, the experimental *K14-rtTA; Tet-Op-Myc* mice and control mice were treated with 2g/L doxycycline in 5% sucrose for a period of 2 weeks. Mice were sacrificed at the end of the treatment period. We collected the esophagus and liver (negative control). We performed immunohistochemistry (IHC) for Myc protein expression in the esophagi of the *K14-rtTA; Tet-Op-Myc* and wild type mouse tissue (Figure 4). We observed significant positive staining for Myc protein in the *K14-rtTA; Tet-Op-Myc* esophagi compared to the wild type esophagi (Figure 4). Our findings confirm that the *Tet-Op-Myc* transgenic mice can be activated by the K14 promoter expressing rtTA with the additional treatment of doxycycline.

L2-rtTA; Tet-Op-Myc; K14-Cdx2 **mice show no significant histological changes in the esophagus and squamous forestomach 6 or 12 months post-treatment with doxycycline**

We proceed to breed the *L2-rtTA; Tet-Op-CreER*^{T2} mice with the *Tet-Op-Myc* mice (Figure 5A). From this breeding strategy we were able to generate the *L2-rtTA; Tet-Op-Myc* mice. These mice are able to overexpress MYC specifically in the esophagus and forestomach when treated with doxycycline. Next, we bred the *L2-rtTA; Tet-Op-Myc* mice with the *K14-Cdx2* mice (Figure 5B). The *K14-Cdx2* mice have been described previously to overexpress Cdx2 in the mouse esophageal and squamous forestomach epithelia (Kong, Crissey et al. 2011). From this breeding we were able to generate the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* experimental mice (Figure 5B).

Next, we treated the *L2-rtTA; Tet-Op-Myc* and *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice, and wild type control littermates, with doxycycline-diet chow starting at 1 month of age. For our preliminary studies we sacrificed mice at 6 and 12 months post-treatment with doxycycline-diet. Hematoxylin and eosin (H&E) staining of sections of the esophagi and squamous forestomachs, showed no significant histological changes at either 6 (n=1) or 12 (n=3) months of age (data not shown). Furthermore, we analyzed the esophagi for MYC and Cdx2 expression by IHC (Figure 6A-B). We observed positive staining for MYC in the *L2-rtTA; Tet-Op-Myc* and *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice and weak background staining in the wild type mice (Figure 6A). Moreover, we observed positive staining for Cdx2 in the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* and *K14-Cdx2* mice (Figure 6B, Supplemental Figure 3A). We confirmed Cdx2 overexpression by the *K14-Cdx2* mice by western blotting of the esophagus, forestomach and tongue tissues (Supplemental Figure 3B).

L2-rtTA; Tet-Op-Myc; K14-Cdx2 **mice treatment with 0.3% deoxycholic acid (DCA) did not show significant alteration of the esophagus or squamous forestomach epithelium**

Previous studies by Quante et al. have shown that treatment of mice with 0.3% deoxycholic acid (DCA), starting at 3 months of age, accelerates the appearance of BE lesions in their *L2-IL1β* (Interleukin 1β) mice (Quante, Bhagat et al. 2012). Therefore, we sought to investigate whether in *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice the additional treatment with 0.3% DCA in the drinking water would promote changes towards BE. We started treatment of our *L2-rtTA; Tet-Op-Myc* and *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice and wild type control mice with doxycycline-diet at 1 month of age and then 0.3% DCA in the drinking water at 3 months of age. We sacrificed mice at 12 months (n=2 for each

group) after the start of the doxycycline treatment and analyzed the esophagus and squamous forestomach by H&E staining. We did not observe any significant histological changes (data not shown)

Discussion

We have designed and generated a mouse model for the inducible expression of MYC and stable Cdx2 expression in the esophagus and squamous forestomach. In order to achieve this we created a Tet-ON system using the EBV-L2 promoter. Our system allows us to activate MYC overexpression post weaning by feeding the mice Dox chow (doxycycline). Here, we show the activation of the Tet-Op-Myc promoter by in the *K14-rtTA* mice and our double transgenic *L2-rtTA; Tet-Op-CreER*^{T2} mice, upon treatment with doxycycline.

Following the successful overexpression of MYC in the mouse esophagus we sought to activate the expression of Cdx2. Therefore, we bred our *L2-rTta; TetOp-Myc* mice with the previously described *K14-Cdx2* mice (Kong, Crissey et al. 2011). We confirmed the expression of Cdx2 in the esophagus under the regulation of the K14 promoter. Previous studies of the *K14-Cdx2* mice have shown that expression of Cdx2 in the esophageal epithelium leads to a decrease in proliferation of basal cells and decreased cell to cell adhesion due in part by decreased expression of Desmocollin 3. Interestingly, we did not observe any changes the histology of the esophagi and forestomachs of the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice at either 6 or 12-months of age. In preliminary studies, analysis for changes in expression of squamous keratin 13 (K13) and columnar keratin 8 (K8) showed no changes (data not shown). Treatment of mice with 0.3% DCA for 12-months caused no significant changes in the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice in preliminary results.

While, our preliminary studies show a lack of histological changes in the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice, we will plan to further analyze these for changes towards a

BE phenotype by aging the mice. First, we will analyze the esophagi of *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice treated with 0.3% DCA for changes in gene expression of columnar (i.e. K8, K19, K20) and squamous keratins (i.e. K5, K13, K14) by IHC staining. Secondly, given previous results found in the *K14-Cdx2* mice, we will analyze for changes in basal cell proliferation (i.e. Ki67) and cell to cell adhesion loss (i.e. Desmocollin-3 and electron microscopy).

Lastly, our *in vitro* studies suggest that inhibition of Notch signaling (via dnMAML) in conjunction with MYC and CDX1 expression further promotes a BE-like metaplasia (Vega, Giroux et al. 2014). Therefore, we propose to breed our *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice with the *Tet-Op-dnMAML/GFP* mice (Fu, Chang et al. 2009) in order to inhibit Notch signaling in the mouse esophagus and forestomach, in conjunction with MYC and Cdx2 overexpression. The Tet-Op-dnMAML/GFP mice express dominant negative Mastermind-like (dnMAML) fused to GFP driven by the Tet-Op promoter. Breeding of these mice expressing rtTA together with treatment with doxycycline leads to expression of dnMAML-GFP. Currently, we are breeding of the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice with the *Tet-Op-dnMAML/GFP* mice.

In our future studies we will treat the *L2-rtTA; Tet-Op-Myc; K14-Cdx2; Tet-Op-dnMAML/GFP* mice with doxycyline to induce expression of MYC and dnMAML in the esophagus. We expect inhibition of Notch signaling with MYC and Cdx2 may promote changes towards BE *in vivo*. We plan to analyze the *L2-rtTA; Tet-Op-Myc; K14-Cdx2; Tet-Op-dnMAML/GFP* mice at 6 and 12 months of age post-treatment with doxycycline ± 0.3% DCA. We will analyze the esophagi for changes in cell morphology by H&E and electron microscopy. Finally, we will also analyze these mice for changes in gene

expression of columnar (i.e. K8, K19, K20) and squamous keratins (i.e. K5, K13, K14) by qPCR and IHC staining.

Methods

Vector subcloning

Subcloning of CreER^{T2} and Myc into the pGIT vector was mediated by an initial subcloning to the pBluescript FS+ vector to add the *Fse I* and *Sbf I* sites for insertion to the pGIT vector. First for CreER^{T2}, we digested the pCAG-CreER^{T2} vector with *Not I*, followed by Klenow digest for blunting of *Not I* site, after which we cut with *EcoR I*. We subcloned the CreER^{T2} into the pBluescript FS+ cut with *EcoR I*/*EcoR V*. Screening for positive clones was done by double digest with *Bgl II* and *EcoR I*, with a 2kb band as the positive clone. Before subcloning the CreER^{T2} to the pGIT vector we removed the tdTomato by Swa I digest followed by blunt ligation. After removal of tdTomato in the pGIT vector, we cloned CreER^{T2} by *Fse I* and *Sbf I* sites. For the pGIT-Myc subcloning, we first digested Myc from the Tet-O-Myc vector by *EcoR I* single digest, followed by ligation to the pBluescript FS+ vector to the *EcoR I* site. Screening for correct direction of the Myc transcription start site was done by digest with *EcoR V*, with a 1.24kb band for the correct direction and *Sbf I* digest with 866bp band for the correct direction. Following digestion we sequenced positive colonies for confirmation before subcloning into the pGIT vector using *Fse I* and *Sbf I* sites. All digests were performed using restriction enzymes from New England Biolabs (Ipswich, MA) following manufacturer's protocols. Sequencing of CreER^{T2} and Myc was performed after insertion to the pBluescript FS+ vector using the M13 Forward and M13 Reverse primers (Stratagene/Aligent, Santa Clara, CA).

Preparation of DNA for microinjection into oocytes

We first digested 20µg of each pRIL, pGIT-Myc and pG-CreER^{T2} vector with *Pac I* (New England Biolabs, Ipswich, MA) at 37℃ overnight, following the manufacturer's instructions. Next, we separated the linearized DNA by running the fragment on an agarose gel in 1X TAE, after which we excised the fragment from the gel. We processed the gel fragments through Qiaex gel extraction kit (Qiagen, Germantown, MA), according to the manufacturer's instructions. We next ethanol precipitated the recovered DNA from this initial purification step. Next, we passed the DNA through a Millipore Ultrafiltration Spin-Columns, 0.45µm cutoff (Millipore, Billerica, MA). DNA recovered from this secondary purification step was resuspended in an injection buffer (10 mM Tris/0.1 mM EDTA, pH 7.5 prepared with distilled water). Lastly, we estimated the DNA concentration by comparing the ethidium bromide staining of a sample run on an agarose gel next to a standard of known concentration, before submitting the samples for DNA microinjection.

Mice

All mouse studies and breeding were carried out under the approval of the Institutional Animal Care and Use Committee at the University Pennsylvania. For short-term treatment with doxycycline, mice were placed on drinking water containing 2g/L doxycycline in 5% sucrose (protected from light) for a period of 2 weeks. For long-term doxycycline treatment mice were administered a doxycycline by dox-diet chow 200mg/kg at one month of age (Bio-Serv, Flemigton, NJ). For bile treatment mice were placed on drinking water containing bile acid (0.3% DCA, pH 7.0) at the age of three months.

Genotyping

The following primers were designed for detection of trangene for the *L2-rtTA; Tet-Op-CreER*^{T2} and *Tet-Op-Myc* mice. For genotyping the *L2-rtTA; Tet-Op-CreER*^{T2} mice we designed the iCre primers that detect the junction between Tet-Op promoter and CreER^{T2} and the rtTA primers that detect the junction between the EBV-L2 promoter and rtTA. For the genotyping the *Tet-Op-Myc* we designed two sets of primers: TMyc which detects the junction between the Tet-Op promoter and Myc and TdTomato which detects the junction between the IRES and TdTomato. All four primers sets have an annealing temperature of 60℃ for 30 seconds.

PRIMERS SET	FORWARD	REVERSE	EXPECTED BAND SIZE
iCre	TTT CCC TGC CAC AGC TTG ATA GC	TGT AAT TGA ACT GGG AGT GGA CAC C	~ 250 bp
rtTA	GGG ACC ACA TAC ATC CCT GTC TCC	TGG GCC AGC TTT CTG GTG G	~ 250 bp
TMyc	GGT GGG AGG CCT ATA TAA GCA GAG C	GGG CAG CAG CTC GAA TTT CTT CC	~ 300 bp
TdTomato	GCC ACA ACG AGG ACT ACA CCA TCG	GCT TCA CGT AAG CCT TGG ATC CG	~ 300 bp

Histology and Immunohistochemistry

Hematoxylin and eosin (H&E) staining as well as immunohistochemistry (IHC) were performed as described previously (Stairs, Nakagawa et al. 2008). The following antibodies were used for IHC: 1:500, Cdx2 (Abcam, Cambridge, MA) 1:500, MYC (Leica Biosystems, Richmond, VA). Biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and ABC avidin-biotin-DAB

detection kit (Vector Labs, Burlingame, CA) were used for detection and visualization according to supplier's protocol.

Statistical Analysis

For relative luciferase expression changes in luciferase assay studies, statistical significance of comparisons between wild type and *L2-rtTA; Tet-Op-CreER^{T2}* founders F1 and F2 esophagus and squamous forestomach were determined by the student *t-test* with $p<0.05$ as statistically significant. Error bars represent the mean ± SEM (Standard Error of the Mean) from at least triplicate experiments.

Luciferase Assay

Esophagi, forestomachs and livers (negative control) were harvested from wild type and *L2-rtTA; Tet-Op-CreER^{T2}* mice, and flash frozen to -80℃ in dry ice. Frozen tissues were harvested for the luciferase assay following the "Preparation for Plant Tissue Lysate" protocol provided by the manufacturer's protocol for lysate preparation. The Luciferase Assay Kit (Promega, Madison, WI) was used for detection of luciferase activity following the manufacturer's protocol.

Western Blotting

Western Blot analysis was performed as described previously (Grugan, Vega et al. 2013; Stairs, Nakagawa et al. 2008). The following primary antibodies were used in 5% Milk in 1X PBS-T (0.1% Tween-20): CDX2 1:1000 (Abcam, Cambridge, MA) and GAPDH 1:15,000 (Chemicon/Millipore, Billerica, MA). Secondary antibodies (HRP-mouse, HRP-rabbit) were used at 1:5000 in 5% Milk in 1X PBS-T. Blots were developed using ECL Plus reagent (Invitrogen, Carlsbad, CA).

Chapter III:

Figures and Figure Legends

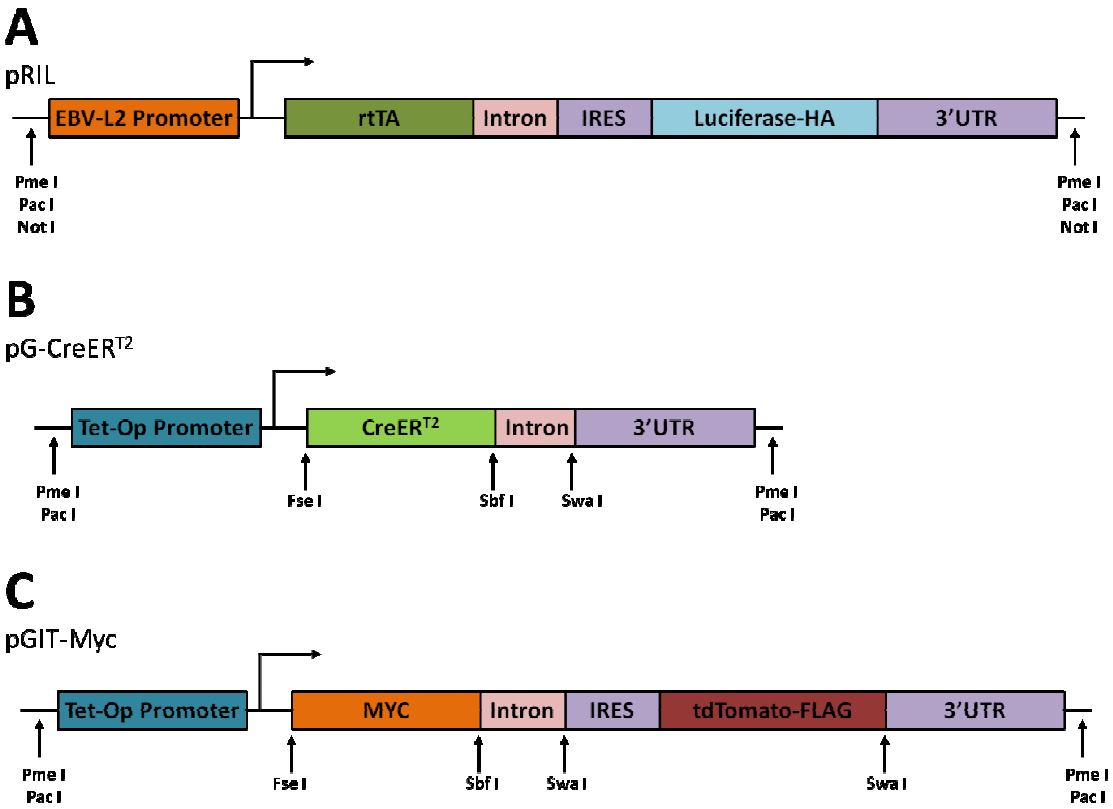


Figure 1: DNA vector design for generation of *L2-rtTA; TetOp-CreER^{T2}* and *Tet-Op-Myc* transgenic mice. The following vectors were designed to be used for the generation of novel transgenic mice (A) pRIL vector (B) pG-CreER^{T2} vector and (C) pGIT-Myc vector.

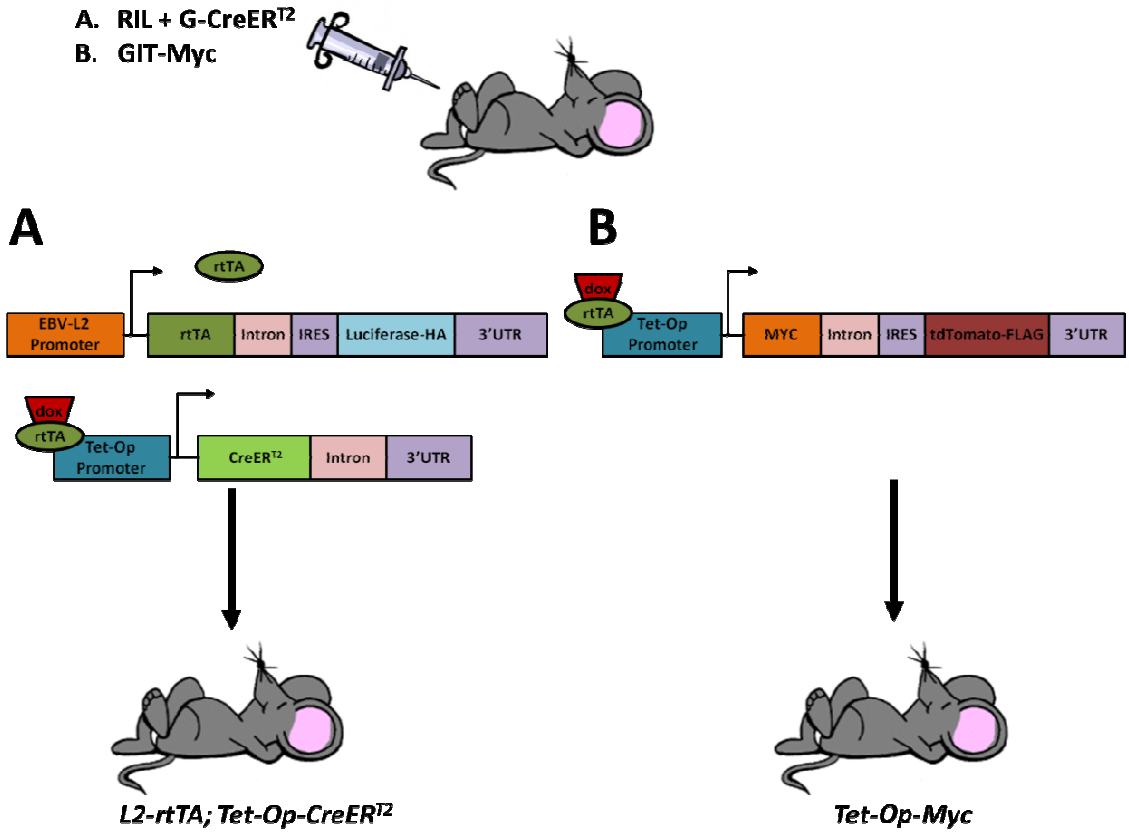


Figure 2: DNA microinjection for the generation of *L2-rtTA; Tet-Op-CreER^{T2}* and *Tet-Op-Myc* mice. Linearized DNA (A) RIL+G-CreER^{T2} and (B) GIT-Myc were microinjected into fertilized eggs by Transgenic and Chimera mouse facility. Following this injection we generated two founder lines (A) *L2-rtTA; Tet-Op-CreER^{T2}* and (B) *Tet-Op-Myc*.

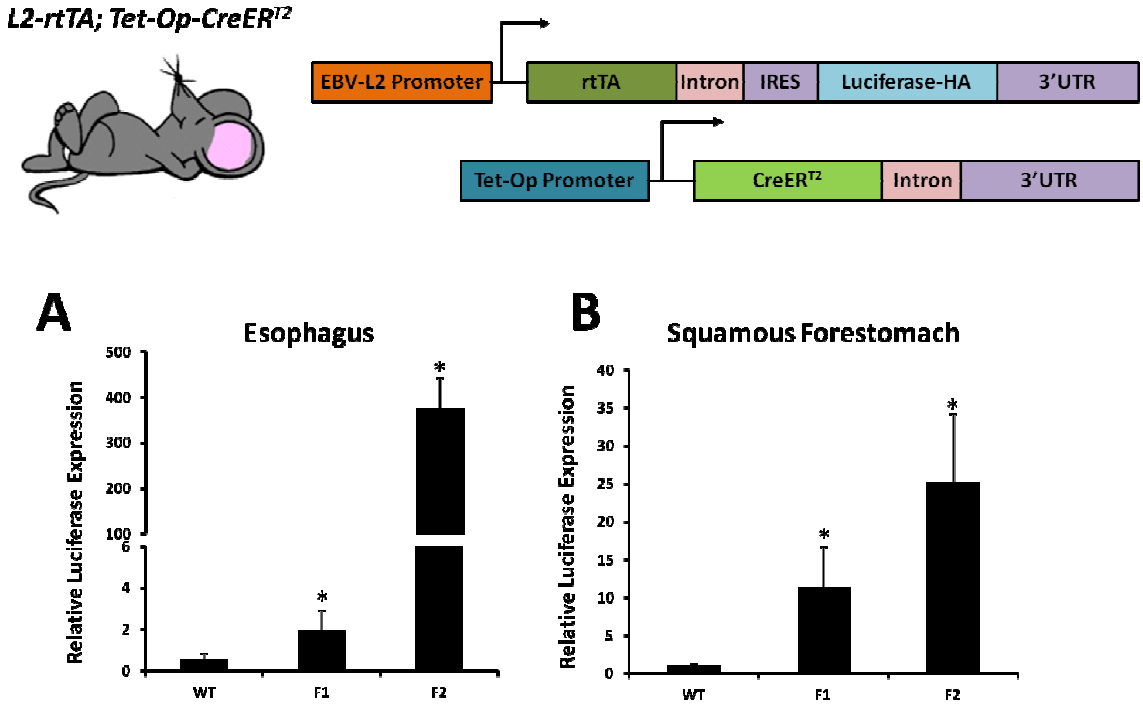


Figure 3: *L2-rtTA; Tet-Op-CreER^{T2}* mice show specific expression of luciferase in the esophagus and squamous forestomach. Luciferase assay activity of (A) esophagus and (B) squamous forestomach tissue of wild-type and two founder lines of the *L2-rtTA; Tet-Op-CreER^{T2}* mice (F1 and F2), graph represents mean ± SEM (n=3). Student t-test was performed to determine statistical significance, *p≤0.001.

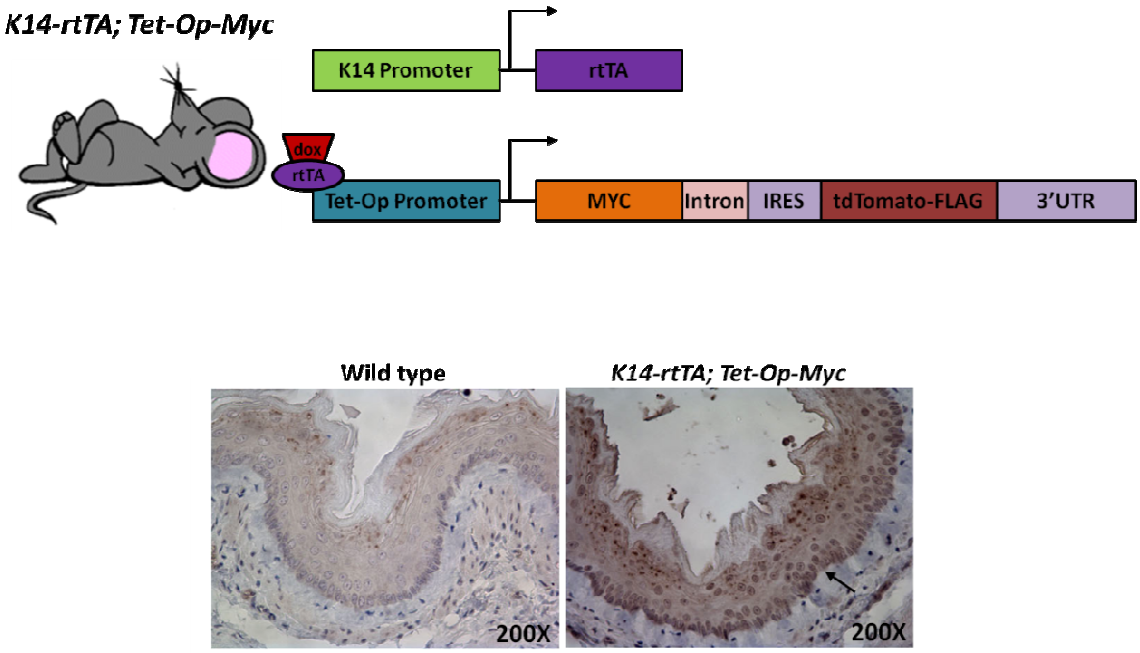


Figure 4: *K14-rtTA; TetOp-Myc* mice show activation of MYC expression in mouse esophagus. Mice treated for 2 week with doxycyline in drinking water to activate the Tet-Op promoter expression of MYC. IHC staining of esophagus for MYC expression in wild type (left panel) and *K14-rtTA; Tet-Op-Myc* mice (right panel) (200X).

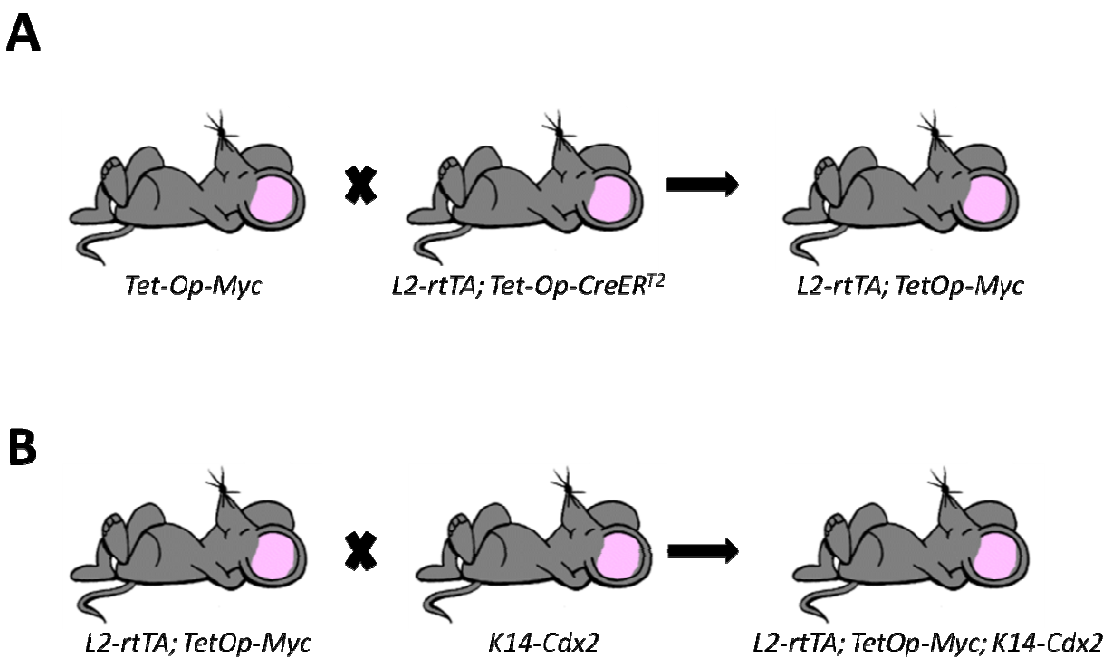


Figure 5: Breeding strategy for development of a potential model of Barrett's esophagus by overexpression of MYC and CDX2 in the mouse esophagus. (A) We bred the *Tet-Op-Myc* mice to the *L2-rtTA; Tet-Op-CreER^{T2}* mice to generate the *L2-rtTA; Tet-Op-Myc* mice. (B) Following the generation of the *Tet-Op-Myc* mice we bred these to the *K14-Cdx2* mice to generate the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice.

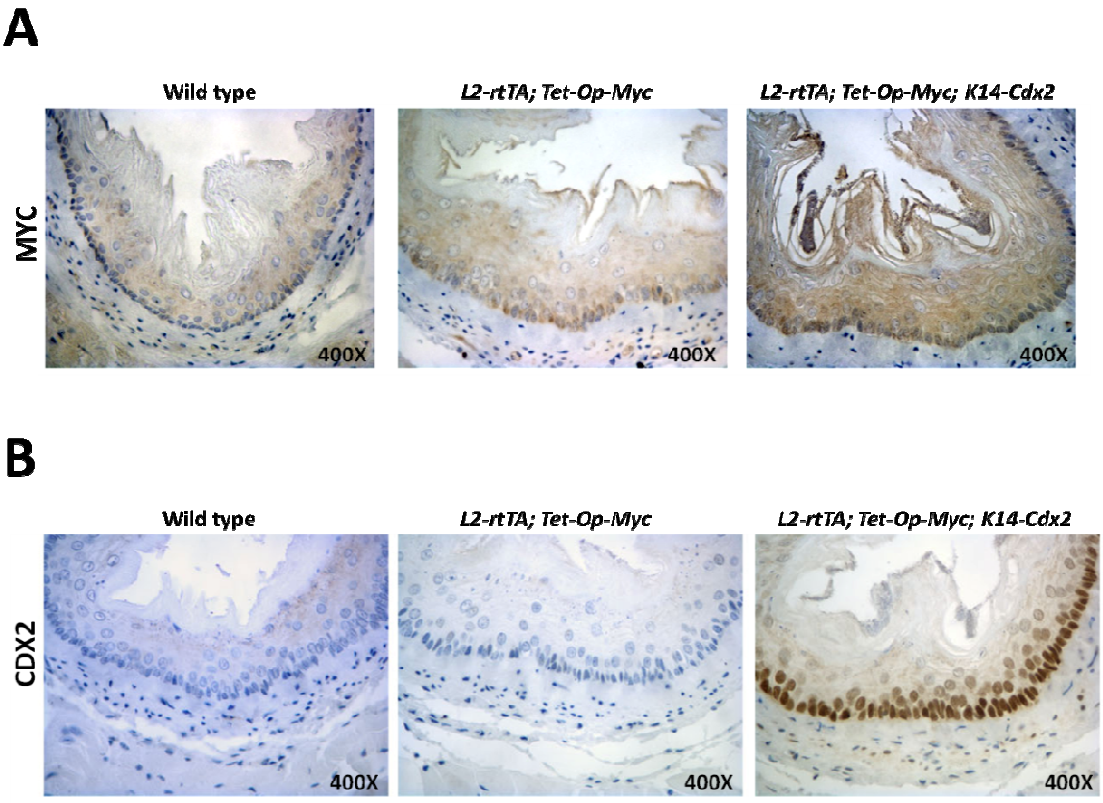
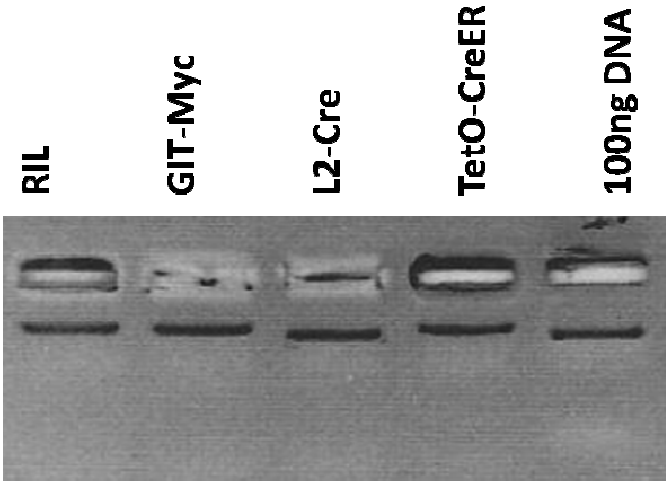


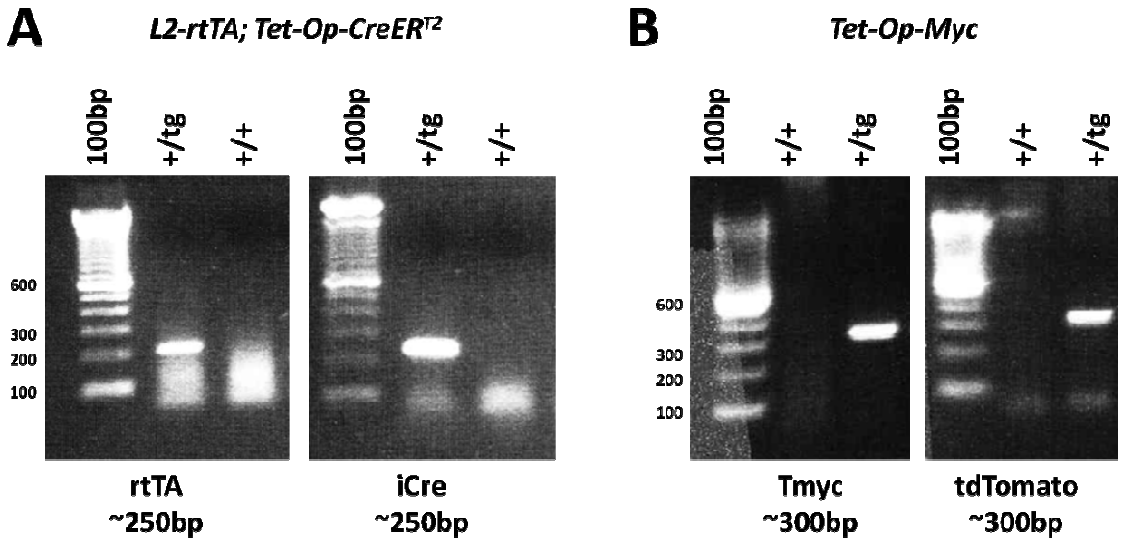
Figure 6: *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mouse esophagus show overexpression of MYC and CDX2. IHC staining of esophagus of wild type; *L2-rtTA; Tet-Op-Myc* and *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice for (A) MYC and (B) CDX2 (400X).

Chapter III:

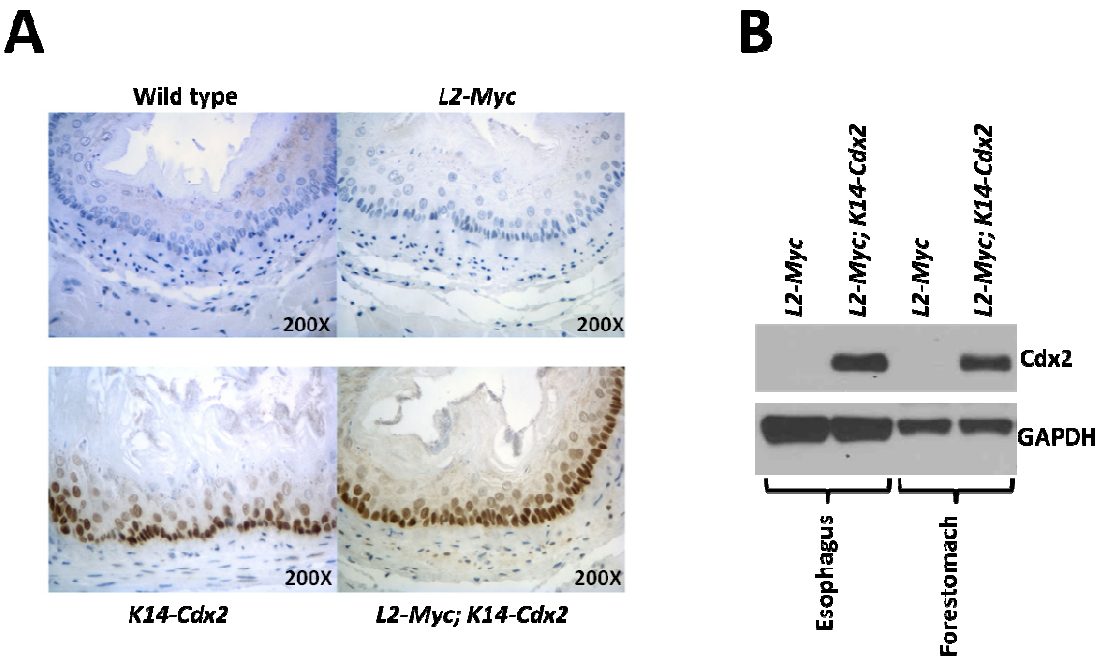
Supplemental Figures



Supplemental Figure 1: Linearized DNA quantification for microinjection into mice fertilized eggs. Agarose gel picture of DNA after two step purification steps. Quantification was verified by running DNA next to standard DNA amount of 100 ng. After correct quantification DNA was submitted to the Transgenic and Chimeric mouse facility.



Supplemental Figure 2: Genotyping of *L2-rtTA; Tet-Op-CreER^{T2}* and *Tet-Op-Myc* mice. Representative agarose gel pictures for genotyping of (A) *L2-rtTA; Tet-Op-CreER^{T2}* and (B) *Tet-Op-Myc* mice. Positive mice +/tg and wild type mice +/+.



Supplemental Figure 3: *L2-rtTA; Tet-Op-Myc; K14-Cdx2* and *K14-Cdx2* mouse esophagus, forestomach and tongue show expression of CDX2. (A) IHC staining of CDX2 in esophagus of wild type, *L2-Myc, K14-Cdx2* and *L2-Myc/K14-Cdx2* mice. (B) Western blotting for Cdx2 of *L2-Myc* and *L2-Myc; K14-Cdx2* from esophagus and forestomach.

Chapter IV:

A common p53 mutation (R175H) activates the c-Met receptor tyrosine kinase to enhance tumor invasion in ESCC

Abstract

There are two types of esophageal cancers: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). ESCC is known to have a poor prognosis due to its late diagnosis and early metastasis. Common genetic alterations found in ESCC include p53 mutations, p120catenin and p16 inactivation, as well as overexpression of oncogenes such as cyclin D1 and EGFR. In our study using primary esophageal epithelial cells genetically transformed by the overexpression of EGFR and $p53^{R175H}$ (EPC-hTERT-EGFR-$p53^{R175H}$), we find novel evidence of a link between $p53^{R175H}$ and the c-Met receptor tyrosine kinase to mediate tumor cell invasion. We observed increased c-Met receptor activation (phosphorylation) specifically in the cells expressing the $p53^{R175H}$ mutation; this activation was further enhanced by EGFR overexpression. Furthermore, c-Met phosphorylation was inhibited by using two small molecule compounds (CP31398 and 5-iminodaunorubicin) that have been demonstrated to activate wild-type p53 signaling. Furthermore, both compounds inhibited invasion in the EPC-hTERT-EGFR-$p53^{R175H}$ cells, suggesting that the mechanism of increased invasivion upon EGFR and $p53^{R175H}$ expression may be the result of increased c-Met activation. Taken together, mutant $p53^{R175H}$ and c-Met cooperate to foster tumor invasion in ESCC. Therefore, our data suggest that targeting c-Met and $p53^{R175H}$ may prove useful for the treatment of ESCC.

Introduction

Worldwide, esophageal cancer is the eighth most common malignancy and the sixth most common cause of cancer-related death. Esophageal cancer can be classified into two subtypes: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). The incidence of EAC and ESCC varies by epidemiological features specifically ESCC is mainly seen in African-American and Asian populations. Furthermore, human squamous cell carcinomas are the most common type of epithelial cancers. ESCC is an aggressive cancer with poor prognosis due to late diagnosis, local and distant metastases, and limited therapeutic options. Globally, ESCC is the most common type of esophageal cancer diagnosed world-wide. Risk factors for ESCC development include tobacco use, alcohol consumption, consumption of salt-cured, salt-picked and moldy foods and consumption of hot beverages (Prabhu, Obi et al. 2014).

The development of ESCC is a multistep, progressive process, and a number of genetic alterations in the tumor cells have been identified (Lehrbach, Nita et al. 2003; Okawa, Michaylira et al. 2007). Some of the genetic alterations found in ESCC include activation of oncogenes such as EGFR and CyclinD1, inactivation of tumor suppressor genes p16, p120catenin and mutation p53 (Okano, Snyder et al. 2006). Overexpression of EGFR has been reported in 60-70% of ESCC tumors (Hanawa, Suzuki et al. 2006). EGFR overexpression and p53 mutations are particularly common in premalignant lesions of ESCC (Volant, Nousbaum et al. 1995; Zhang 2013).

P53 is a critical tumor suppressor gene that acts as a gatekeeper by maintaining cellular genomic stability and regulating cell growth. In fact, loss of wild-type p53 or mutation in p53 is commonly found in many human cancers. Most p53 mutations are

missense mutations, which are single base pair substitutions that occur in the DNA binding domain (DBD). These mutations result in the loss of wild-type p53 function or a "dominant-negative" effect on wild-type p53 (Vousden and Prives 2009). Among these mutations there are six "hot-spot" residues most commonly found in human cancers; these are R175H, G245S, R248W, R273H, R249S and R282W (Freed-Pastor and Prives 2012). All these p53 mutations result in the stabilization and accumulation of the mutant p53 protein. In ESCC the loss of function of p53 in ESCC is usually mediated by missense mutations at the DNA binding site. Mutations R175H, R248W and R273H are commonly found in ESCC and are found to confer a selective growth advantage to increasingly malignant carcinomas (McCabe and Dlamini 2005). These mutations can be separated into two categories: 1) mutations that directly block DNA binding by p53 (R273H and R248W mutations – archetype) and 2) mutations that alter the global conformation of the p53 protein (R175H mutation – archetype).

Mutations of p53 have been shown to influence tumorigenesis in multiple ways, including loss of the gatekeeper functions of wild type (WT) p53 by acting as a dominant negative directly on WT p53, and more recently appreciated, through WT p53-independent gain of function (GOF) mechanisms. In the latter context, the putative mechanisms involve abrogation of the effects of p63, an ortholog of p53, and direct DNA binding to a different repertoire of genes leading to activation by mutant p53. Thereby, mutations of p53 could promote expression of genes not normally activated by the wild-type p53.

Clinically, tumors with p53 mutations carry a worse prognosis than those that are p53 null (Alsner, Sorensen et al. 2001; Poeta, Manola et al. 2007). P53 null cells can be transformed by expression of mutant p53 (Olive, Tuveson et al. 2004). Studies of mice

lacking one or both alleles of p53 show a predisposition towards development of osteosarcomas, lymphomas and lung cancers. Conversely, mice models targeting specific p53 mutations have been generated to address the effect of p53 mutation in tumor development (Olive, Tuveson et al. 2004). Interestingly, tumors in $p53^{R172H/+}$ transgenic mice tend to be of more epithelial origin compared to p53-/- or p53+/- murine tumors and metastasize more than those from $p53^{+/-}$ mice (Olive, Tuveson et al. 2004; Lang, Iwakuma et al. 2004).

The c-Met tyrosine kinase receptor activates a signaling response program termed “invasive growth” that is necessary both during normal embryonic development and adult tissue repair (Comoglio, Giordano et al. 2008; Comoglio and Trusolino 2002). C-Met receptor is normally expressed in epithelial tissue and can be activated by binding its ligand, namely hepatocyte growth factor (HGF) expressed by the mesenchymal cells. Specifically, upon activation there is trans-phosphorylation of Tyr 1234 and Tyr 1235 on the intracellular domain of c-Met, induction of docking molecules, and the activation of diverse signaling pathways, such as Ras, PI3K, STAT, β-catenin and Notch pathways (Trusolino, Bertorri et al. 2010).

Perturbed activation of c-Met signaling has been shown to be important for neoplastic transformation in a wide variety of tumor types, including ESCC. Activation of c-Met expression in cancer can trigger tumor growth, angiogenesis and promote metastasis. These oncogenic activities are largely mediated through c-Met amplification or overexpression, although rare receptor mutations have been detected (Knudsen, Vande Woude et al. 2008; Joffre, Barrow et al. 2011). Recent studies of genomic amplifications have identified that 1% of ESCC patients have c-Met gene amplification (Kato, Arao et al. 2013). Studies of tissue microarrays of ESCC have shown an

increased expression of c-Met by 92%, compared to the normal esophageal epithelium (Hu, Lam et al. 2001). Furthermore, clinical studies of ESCC patients have shown significant correlation between increased HGF serum levels and advanced tumor metastasis stage and decreased survival (Ren, Cao et al. 2005).

There is limited evidence linking p53 mutation and c-Met signaling in cancer. Sarcomas arising in Li-Fraumeni patients have increased c-Met protein levels (Rong, Donehower et al. 1995). WT p53 was shown recently to regulate c-Met expression and influence cell migration and invasion in normal and transformed ovarian epithelium (Hwang, Matoso et al. 2011). We have reported previously, using a model that recapitulates early genetic alternations in ESCC, that expression of mutant $p53^{R175H}$ and EGFR could transform immortalized human primary esophageal epithelial keratinocytes (EPC-hTERT), thereby dramatically increasing their migratory and invasive capabilities (Okawa, Michaylira et al. 2007). In this study, we report a previously unidentified direct connection between $p53^{R175H}$ and increased c-Met receptor activity that is linked directly to tumor invasion.

Results

Mutant p53^{R175H} expression leads to c-Met activation in a HGF-ligand independent manner

Our previous studies using EPC2-hTERT cells retrovirally infected to express mutant p53^{R175H}, show activation of c-Met pathway observed by increased c-Met phosphorylation. Furthermore, c-Met phosphorylation is further upregulated upon EGFR overxpression (Grugan, Miller et al. 2010). This specific pattern of activation was not shared with other selected receptor tyrosine kinases as IGF1Rβ and EGFR phosphorylation (data not shown). Therefore, we sought to investigate mediators of this activation of c-Met phosphorylation in the EPC2-hTERT-EGFR-p53^{R175H} cells. In a certain context, EGF and HGF cooperate to promote cell proliferation, scatter and invasion in mouse mammary cells (Accornero, Miretti et al. 2010). Recently, EGFR inhibition has led to the evaluation of targeting c-Met for EGFR inhibitor resistant tumors (Karamouzis, Konstantinopoulos et al. 2009; Jo, Stolz et al. 2000). Thereby, we investigated whether the activation of c-Met was dependent on EGFR kinase activity. Interestingly, our inhibition of EGFR tyrosine kinase activity by pharmacological inhibitor (AG11478) did not affect c-Met activation (Figure 1A). That being said, our results are not consistent with reports of the lack of cross-talk between EGFR and c-Met in mouse adult liver oval cells (Martinez-Palacian, del Castillo et al. 2012).

The c-Met tyrosine kinase receptor is classically activated by binding of its ligand HGF. Therefore, we next investigated whether the c-Met activation observed in the EPC2-hTERT-p53^{R175H}±EGFR was dependent on HGF. In our experimental conditions, a c-Met inhibitor (PHA665752) decreased c-Met phosphorylation in the EPC2-hTERT-

EGFR-p53^{R175H} cells (Figure 1B). But interestingly, c-Met phosphorylation was not affected by an HGF blocking antibody (previously optimized for blocking efficiency (Grugan, Miller et al. 2010)) in EPC2-hTERT-p53^{R175H}±EGFR cells (Figure 1B-C). Thereby, our data suggests c-Met activation is not mediated by HGF ligand binding under these experimental conditions. Further support of this conclusion emerges from the finding that HGF was undetectable in cell culture media (keratinocyte serum free medium, KSFM) or in conditioned media collected from EPC2-hTERT-EGFR-p53^{R175H} cells (data not shown).

We observed that c-Met phosphorylation was reduced when EPC2-hTERT-EGFR-p53^{R175H} cells were cultured in keratinocyte basal medium (KBM) or in KSFM without the added bovine pituitary extract (BPE) (Figure 1D), but was constitutively phosphorylated in KSFM with BPE ± additional EGF (normal culture conditions). Moreover, c-Met phosphorylation was not affected by eliminating the supplement of EGF. These data suggest that there is a factor(s) in BPE that influence c-Met phosphorylation. In order to determine if the p53^{R175H} mediated c-Met phosphorylation was the result of the potential expression and autocrine secretion of an unidentified ligand capable of activating c-Met, cells with low/absent c-Met phosphorylation (EPC2-hTERT-EGFR and EPC2-hTERT, respectively) were treated with conditioned media collected from EPC2-hTERT-EGFR-p53^{R175H} cells (Figure 1E). Our treatment with the condition media showed no increase in c-Met phosphorylation in either EPC2-hTERT-EGFR or EPC2-hTERT cells, compared to the baseline c-Met phosphorylation in KSFM. Thereby these data lead us to conclude that c-Met activation upon p53^{R175H} expression is ligand-independent, either HGF or some other ligand.

Phospho-Met expression is specific to the p53^{R175H} mutation

Different mutations of p53 have been described to be found in ESCC. Therefore, we sought to investigate whether other mutations of p53 could activate c-Met phosphorylation. We analyzed a survey of primary esophageal keratinocytes expressing additional p53 mutations (R273H, V143A, or R248W) described previously (Okawa, Michaylira et al. 2007; Michaylira, Wong et al. 2010). Our analysis for c-Met phosphorylation in EPC2-hTERT-p53(mutant)±EGFR with the following p53 mutations: R175H, R273H, V143A and R248W showed that the activation of the c-Met receptor upon p53 mutation was exclusive to R175H (Figure 2A).

Furthermore, we analyzed mouse esophageal keratinocytes (MEK) cells isolated from p53 null and WT mice with overexpression of the human p53^{R175H} mutant (previously described) (Andl, Mizushima et al. 2003), for activation of c-Met pathway. Interestingly, we did not have similar patterns of c-Met activation (Figure 2B). Overall, these cells showed no increased c-Met activation upon overexpression of the p53^{R175H} mutation. These differences between the human and mouse cell data might point to species differences due to the origin of the esophageal keratinocytes or the potential combinatorial effects of EGFR and p53^{R175H}.

Restoration of WT p53 function reverses p53^{R175H} mutant activation of phospho-Met and cell invasion

In order to determine if the increased c-Met phosphorylation was linked directly to p53 mutation, we used three pharmacologic activators of WT p53 signaling in order to restore normal p53 signaling. First we used 5-iminodaunorubicin (5-ID) which has been identified for its cytotoxicity independent of the cellular p53 status and does so by

activating p53 family member p73 and downstream p21 (Wang, Kim et al. 2006). Our treatment of ECP2-hTERT-EGFR-p53^{R175H} with 5-ID shows decreased expression of c-Met phosphorylation at 1.0μM and complete loss at 5.0μM (Figure 3A). Secondly we used CP-31398 and PRIMA-1 which have been shown to bind to mutant p53 and alter its conformation so as to restore proper DNA binding (Lambert, Gorzov et al. 2009; Wischhusen, Naumann et al. 2003). Treatment with CP-31398 compound resulted in a loss of c-Met phosphorylation in EPC-hTERT-EGFR-p53^{R175H} cells with a concomitant upregulation of p21 (Figure 3B). Moreover, PRIMA-1 treatment did not show an effect on phospho-Met or activation of p21 at 1μM or 10μM concentrations; treatments at higher doses proved to be toxic to the cells (Figure 3B).

We next sought to investigate whether the loss of c-Met phosphorylation in the EPC2-hTERT-EGFR-p53^{R175H} by treatment with 5-ID and CP-31398 could affect cell proliferation and invasion. Our analysis showed a decrease in cell proliferation with both 5-ID and CP-31398 (Figure 3C). Furthermore, both these compounds lead to a significant decrease in cell invasion in a boyden-chamber invasion assay (Figure 3D). Lastly, we used our three-dimensional (3D) organotypic culture system by which we can recreate the stratification of the esophageal epithelium in order to address the effects of these compounds on invasion. Interestingly, we observed that the EPC2-hTERT-EGFR-p53^{R175H} (DMSO) show invasion into the matrix. This invasion is loss significantly with the treatment with both 5-ID and CP-31398 (Figure 3E). These experiments give credence to the premise that the increased tumor cell invasion apparent with p53^{R175H} mutation is mediated in part by c-Met phosphorylation.

Discussion

Human squamous cell carcinomas are the most common type of epithelial cancers. One subtype, esophageal squamous cell carcinoma (ESCC), is an aggressive cancer with poor prognosis due in large by late diagnosis and limited therapeutic options. Specifically, ESCC is typically inoperable and fatal when detected due to early metastasis of tumor cells. Therefore, we have studied the genetic influences on esophageal tumor cell invasion, one of the initial critical steps of tumor dissemination and metastasis. In our previous studies we have modeled ESCC, using immortalized human esophageal epithelial cells overexpressing EGFR and $p53^{R175H}$ (EPC2-hTERT-EGFR-$p53^{R175H}$), which when grown in 3D organotypic cultures result in invasion into the underlying extracellular matrix (Okawa, Michaylira et al. 2007). Thereby, through this model system we can investigate the early invasion events characteristic of ESCC.

Here, we report a novel connection between mutant $p53^{R175H}$ expression and activation of the c-Met receptor tyrosine kinase in a HGF ligand independent manner and potentially independent of other ligands. One of the potential mechanisms to explain the link between $p53^{R175H}$ mutation and increased c-Met signaling in transformed esophageal keratinocytes could be due to direct transcriptional effect of the $p53^{R175H}$ on c-Met. It has been shown that mutant p53 can activate a distinct gene transcription profile than that of wild type p53, which can vary depending on the particular p53 mutation (Freed-Pastor, Prives et al. 2012). Thereby, mutant p53 may have a direct transcriptional effect upon c-Met by virtue of its GOF properties (Lu, Liu et al. 2013). Interestingly, our data showed no significant changes in the c-Met RNA (data not shown) or protein expression in our cells overexpressing mutant $p53^{R175H}$.

Another potential mechanism for c-Met tyrosine activation by $p53^{R175H}$ could be due to regulation of receptor trafficking. Mutant p53 has been demonstrated to result in enhanced integrin and epidermal growth factor receptor (EGFR) trafficking, which depends upon Rab-coupling protein (RCP) and results in constitutive activation of EGFR/integrin signaling (Muller, Caswell et al. 2009). To that end, mutant p53 may enhance c-Met signaling to promote cell scattering and invasion through both TAp63-dependent and -independent mechanisms (Muller, Caswell et al. 2009; Muller, Trinidad et al. 2013), however, this is dependent upon HGF ligand binding the c-Met receptor, a scenario not present in our results, suggesting that our specific interrelationship between mutant p53 and c-Met is mediated through another mechanism.

Recently, the miR34 family of microRNA was identified as a p53 transcriptional product and c-Met was confirmed to be a target of the miR34 family (Migliore, Petrelli et al. 2008; He, He et al. 2007; Siemens, Neumann et al. 2013). We sought to investigate whether the any of the mir34 family member expression could correlate with the phosphorylation of c-Met we observed in our system. Interestingly, we did find that mir34a is decreased in the $p53^{R175H}$ cells (data not shown). However, we do not find that overexpression or knockdown of mir34a had an effect on either c-Met expression levels or phosphorylation of c-Met levels (data not shown). Thereby, our data suggest the activation of c-Met signaling in our system is independent of $p53^{R175H}$ regulation of mir34a (Figure 4).

Our findings suggest a HGF-independent activation of c-Met signaling by mutant $p53^{R175H}$ (Figure 4). ESCC is a difficult cancer to treat. Currently, neo-adjuvant or adjuvant therapy involves chemotherapy and radiation. Biologics have gained some attention, although EGFR inhibition is not standard. Since $p53^{R175H}$ is present in a

subset of ESCC (Soussi, Asselain et al. 2006) we propose that it would be intriguing to consider c-Met inhibition therapy in this context of a future pre-clinical study.

Methods

Cell Culture

Primary human esophageal keratinocytes, designated EPC2-hTERT, and their derivatives are described previously (Harada, Nakagawa et al. 2003). The following mediums where used keratinocyte basal medium (KBM) from Lonza (Allendale, NJ) or using keratinocyte serum free medium (KSFM) (Invitrogen, Carlsbad, CA) without BPE (bovine pituitary extract) and EGFR supplements. Stable transduction of primary esophageal cells with retroviral vectors was described previously (Michaylira, Wong et al. 2010; Harada, Nakagawa et al. 2003; Takaoka, Harada et al. 2004). Briefly, pFB-neo retroviral vectors (Stratagene/Aligent, Santa Clara, CA) containing the entire coding sequence for the human EGFR (pFB-neo-WT-hEGFR), pBabe-puro-p53^{R175H} or pBabe-puro-p53^{R273H} or pBabe-puro-p53^{R248W} or pBabe-puro-p53^{V143A} were transfected into Phoenix-Ampho packaging cells (gift of Dr. Garry Nolan, Stanford University, Palo Alto, CA) using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Culture supernatants from individual Phoenix-Ampho cells were used to infect EPC2–hTERT cells. Cells were passaged 48 hrs after infection and selected with G418 (Invitrogen, Carlsbad,CA) (300 μg/mL), puromycin (EMD-Millipore, Billerica, MA) (0.5 μg/mL), for a period of 7 days, resulting in generation of control EPC2-hTERT-EGFR-p53^{R175H}, EPC2-hTERT-EGFR-p53^{R273H}, EPC2-hTERT-EGFR-p53^{R248W}, EPC2-hTERT-EGFR-p53^{V143A}, EPC2-hTERT-neo-p53^{R273H} and EPC2-hTERT-neo-p53^{R248W} cells. Independent infections and selections were performed to generate two additional cell lines of each genotype. Mouse esophageal keratinocytes (MEK) isolated from p53 null mice (MEK3N p53K puro, MEK2N p53K) and wild type p53 mice (MEK3N, MEK3N puro) previously decribed [38], were transfected with human p53^{R175H}, in both the

p53 null (MEK2 p53K $p53^{R175H}$) and wt p53 (MEK3N $p53^{R175H}$). The following reagents were used in cell culture studies: AG1478 (Sigma, St. Louis, MO) EGFR tyrosine kinase inhibitor (1uM) for 120min; PHA665752 phospho-Met inhibitor (Tocris Bioscience, Minneapolis, MN); 5-iminodaunorubicin (5-ID) (gift of Dr. Wafik El-Deiry, Hershey, PA) range from 0.1-10uM; CP-31398 (50ng/mL-5ug/, mL) (Tocris Biosciences, Minneapolis, MN) and PRIMA1 (1uM-10uM) (Cayman Chemical, Ann Arbor, MI).

Conditioned Media Treatment

Condition media of EPC2-hTERT-EGFR-$p53^{R175H}$ cells grown in 2D cultures in KSFM medium, was collected after 48 hrs, collected medium was spun down for 5min at 1000rpm to remove any cellular debris. EPC2-hTERT-neo-puro or EPC2-hTERT-EGFR-puro cells ($0.5x10^6$ per plate) where plated 24 hrs before treatment. Condition medium was added after 24 hrs of growth, cells where harvested 24 hrs after for protein lysis previously described (Grugan, Miller et al. 2010).

Western Blot Analysis

Cells were harvested in lysis buffer [50 mM Tris·HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, protease inhibitor tablet (Roche, Indianapolis, IN). Thirty micrograms of protein were run on a 4–12% SDS/PAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a poly (vinylidene difluoride) membrane (Immobilon-P; Millipore, Billerica, MA). Membranes were blocked in 5% nonfat milk (Bio-Rad Life Science, Hercules, CA) in PBS-T [1× PBS without Ca+2 and Mg+2 (Invitrogen, Carlsbad, CA) and 0.1% Tween 20] for 1 hr at room temperature. Membranes were then probed with primary antibody diluted in 5% milk in PBS-T overnight at 4 ℃, washed

with PBS-T, and incubated with anti-mouse or anti-rabbit secondary antibodies (1:5000 in PBS-T) for 1 hr at room temperature and washed in PBS-T. The signal was visualized using an enhanced chemiluminescence solution (ECL Plus; GE Healthcare Life Sciences, Piscataway, NJ) and exposed to Blue Lite Autorad film (ISC-BioExpress, Kaysville, UT).

Antibodies

Antibodies were obtained from the following sources: EGFR(Ab-12) (1:1000) NeoMarkers/Thermo Fisher Scientific (Waltham, MA), phospho-EGFR (Tyr1068) (1:500) and phospho-Met (Tyr1234/1235) (1:500) Cell Signaling Technology (Beverly, MA), Met antibody (C-28) (1:1000) Santa Cruz Biotechnology (Dallas, TX), p53 (Ab-6) (1:1000) Calbiochem (Billerica, MA), GAPDH (1:15000) Chemicon/Millipore (Billerica, MA) and p21 (WAF1 Ab-1) (1:1000) Calbiochem (Billerica, MA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies were from GE Healthcare Life Sciences (Piscataway, NJ). Mouse IgG1 control antibody was from R&D Systems (Minneapolis, MN). 2B8 mouse anti-human HGF IgG1 generated from hybridoma (AVEO Pharmaceuticals, Cambridge, MA) and mouse IgG1 control antibody from R&D Systems (Minneapolis, MN), where used in HGF blocking antibody experiment, as previously described (Grugan, Miller et al. 2010).

Cell proliferation WST-1 assay

5000 cells where plated in 100ul of KSFM in a 96-well plate for 72 hrs before reading with WST-1 reagent (Roche, Indianapolis, IN). After 48 hrs of growth, we added 5-ID and CP-31398 reagents at described concentrations. After 24 hrs 10ul of WST-1 reagent and incubated at 37℃ for 2 hrs. Following incubation plate was read using

ELISA plate reader to measure absorbency. All experiments were performed in triplicate on three independent days.

Boyden Chamber Invasion assay

For invasion assays, insert plates (8-μm pore size, 24-well insert) coated with growth factor reduced Matrigel matrix were used (BD Biosciences, San Jose, CA). Inserts were placed in a 24-well plate containing DMEM + 10% serum to stimulate cell invasion. 1×10^5 cells in serum-free medium were placed in each insert (plus the 5-ID or CP-31398). Twenty hours later, the cells remaining inside the insert were removed with a cotton swab and the invading cells on the insert bottom were labeled with 4 μg/mL Calcein AM dye (Invitrogen, Carlsbad, CA) in Hanks's Balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA) for 30 min at 37℃. The labeled cells were then read on a Biotek FLX800 multidetection microplate reader (BioTek, Winooski, VT) at 485 nm excitation and 528 nm detection. All experiments were performed in triplicate on three independent days.

Organotypic culture

H&E staining of organotypic 3D cultures show 5-ID and CP-31398 treatment can reduce invasion. The employment of EPC cells and their derivative cell lines in organotypic culture was done as described previously (Kalabis, Wong et al. 2012). The cultures were harvested and fixed for 1 hr or overnight in 10% buffered formalin phosphate (Fisher, Waltham, MA) before being paraffin-embedded and sectioned (Leica RM2155 microtome; Leica Microsystems, Buffalo Grove, IL) for hematoxylin and eosin (H&E) staining.

Statistical Analysis

Boyden-chamber invasion assay were analyzed for statistical significance comparing cells treated with vehicle versus CP-31398 or 5-ID using the Student t-test, with $p \leq 0.05$ as statistically significant. Error bars represent the mean ± SEM (Standard Error of the Mean) from at least triplicate experiments.

Chapter IV:

Figures and Figure Legends

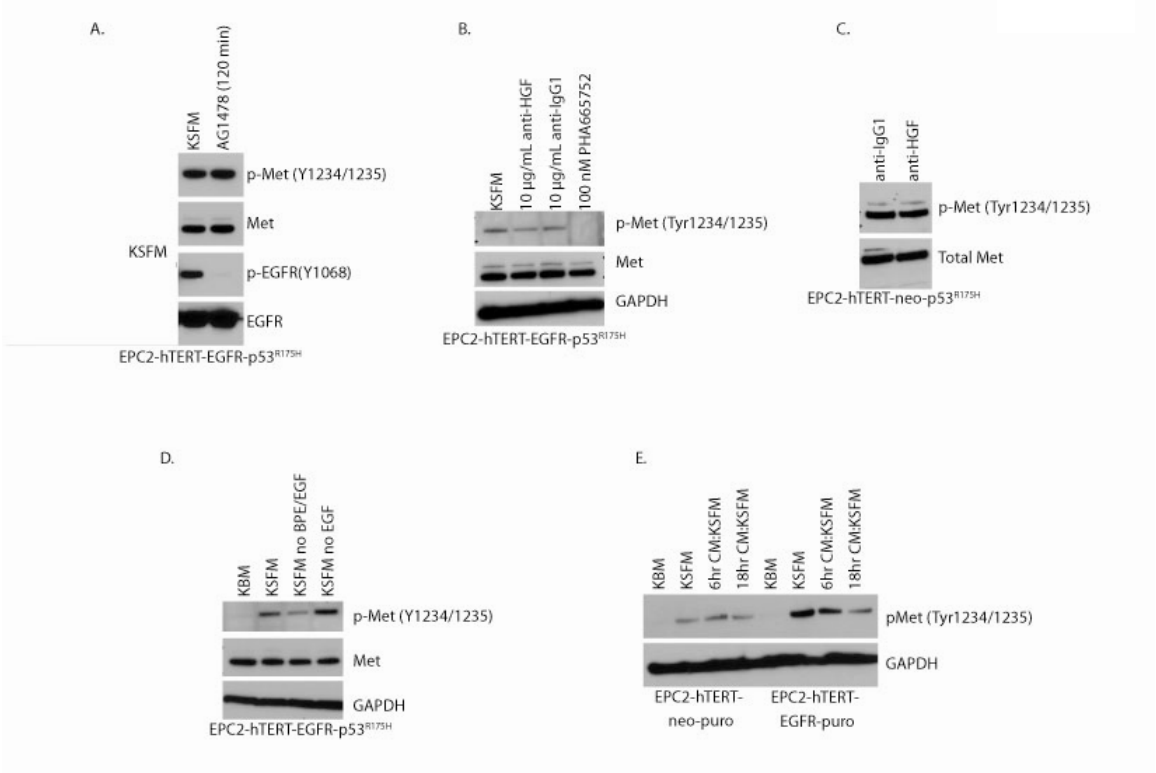


Figure 1: p53^{R175H} expression leads to c-Met activation in an HGF-independent manner. (A) Inhibition with AG11478 EGFR tyrosine kinase inhibitor (1uM) for 120 mins. **(B-C)** Inhibition with anti-HGF antibody or the PHA665752 phospho-Met inhibitor. **(D)** Activation of phospho-Met in EPC2-hTERT-EGFR-p53^{R175H} can be attenuated by using keratinocyte basal medium (KBM) or using keratinocyte serum free medium (KSFM) without BPE (bovine pituitary extract) and EGFR supplements. **(E)** Treatment of cells with condition media from EPC2-hTERT-EGFR-p53^{R175H} cells does not activate phospho-Met in EPC2-hTERT-neo-puro or EPC2-hTERT-EGFR-puro.

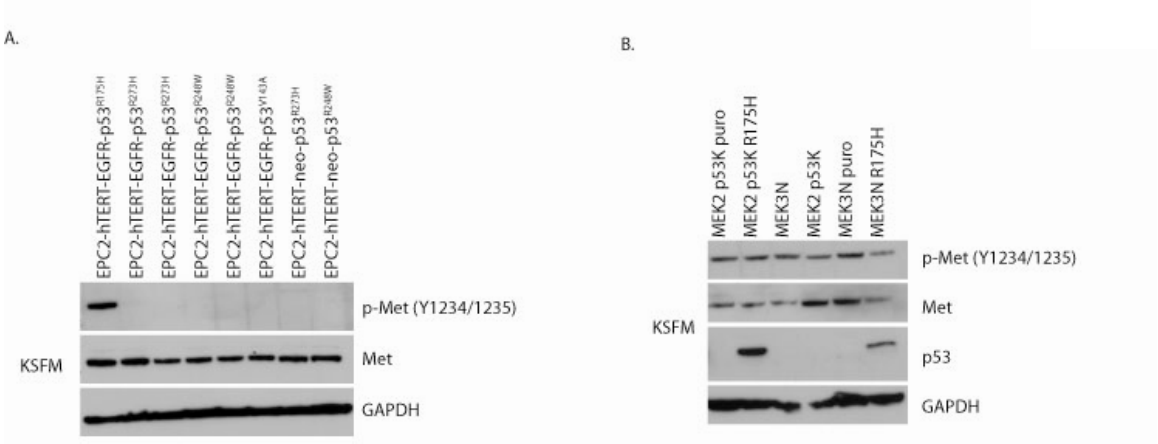


Figure 2: Phospho-Met expression is specific to the p53^{R175H} mutation. (A) Panel of four different p53 mutations R175H, R273H, R248W and V143A were analyzed by Western Blotting for phospho-Met expression. Only the cells expressing the p53^{R175H} mutant have increased phospho-Met expression. **(B)** Mouse esophageal keratinocytes (MEK) isolated from p53 null mice (MEK3N p53K puro, MEK2N p53K) and wild type p53 mice (MEK3N, MEK3N puro) were transfected with human p53^{R175H}, in both the p53 null (MEK2 p53K p53^{R175H}) and wt p53 (MEK3N p53^{R175H}). The p53^{R175H} did not have the same effect on activation of phospho-Met as in human keratinocytes.

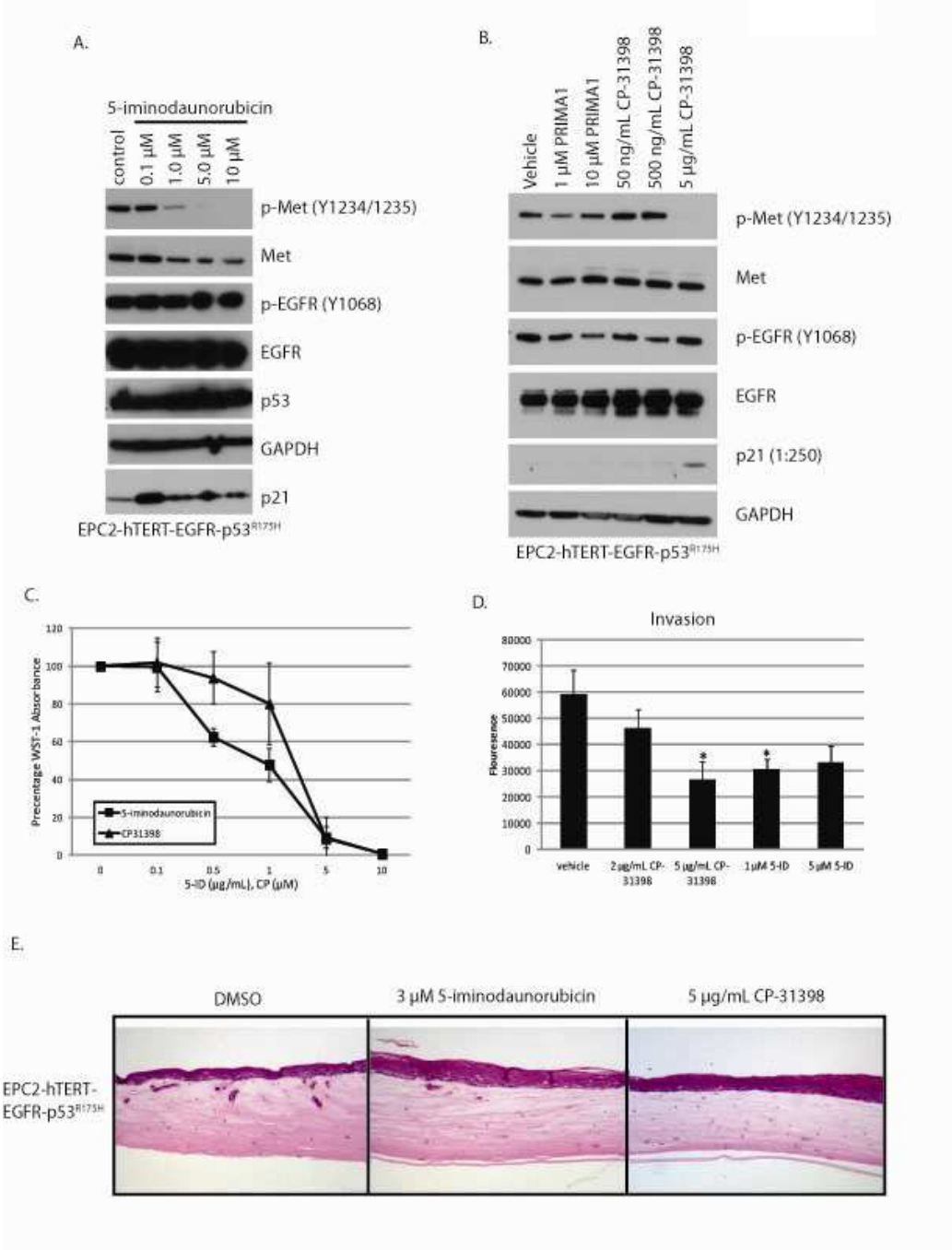


Figure 3: Restoration of WT p53 function reverses p53^{R175H} mutant activation of phospho-Met. (A-B) EPC2-hTERT-EGFR-p53^{R175H} cells treated for 24 hrs with 5-iminodaunorubicin (5-ID) in a range from 0.1-10uM **(A)**, CP-31398 (50ng/mL-5ug/mL) **(B)** show decrease in phospho-Met levels by western blotting; treatment with PRIMA1 (1uM-10uM) **(B)** had no effect in the levels of phopho-Met. **(C)** Treatment with 5-ID

(range 0.1uM-10uM) and CP-31398 (range (0.01ug/mL-10ug/mL) inhibited cells growth of EPC2-hTERT-EGFR-p53^{R175H} in WST-1 assay **(C)** Error bars represent ± SEM, and Student's t-test was used to determine significance (*, P ≤ 0.05). (D) Treatment of EPC2-hTERT-EGFR-p53^{R175H} with 5-ID (1uM and 5uM) and CP-31398 (2ug/mL and 5ug/mL) caused a decrease in boyden-chamber invasion assay. **(C-D)** Error bars represent ± SEM, and Student's t-test was used to determine significance (*, P ≤ 0.05). **(E)** H&E staining of organotypic 3D cultures show 5-ID (3uM) and CP-31398 (5ug/mL) treatment can reduce invasion, Magnification 100X.

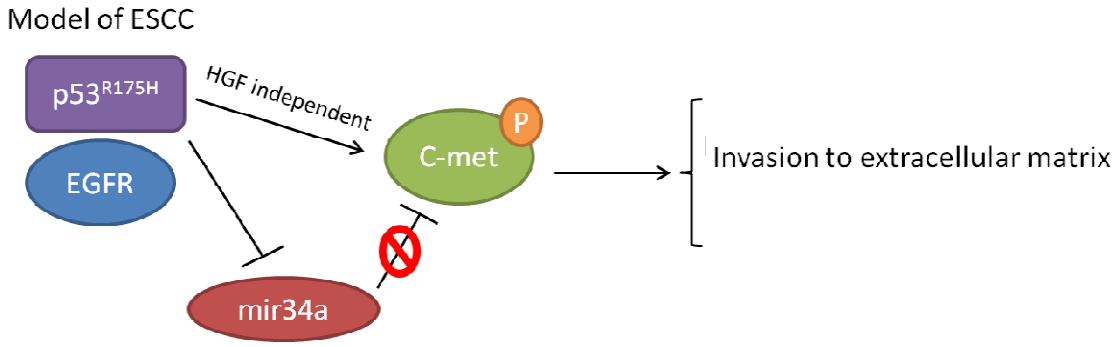


Figure 4: Model. Through an in vitro model of ESCC using human esophageal epithelial cells transformed by overexpression of EGFR and $p53^{R175H}$, we find novel evidence of a functional link between $p53^{R175H}$ and the c-Met receptor tyrosine kinase to mediate tumor invasion. Our data shows activation of c-Met receptor (by phosphorylation) by $p53^{R175H}$ in a HGF ligand independent manner. Furthermore, the activation of c-Met signaling is not mediated by $p53^{R175H}$ negative regulation of mir34a. Overall, our model support c-Met inhibition for the treatment of invasive ESCC.

Chapter V:

Thesis Summary and Discussion

Role of inhibition of Notch signaling in initiation of Barrett's esophagus

Throughout our study in Chapter II, we focus on Barrett's esophagus (BE), an incomplete intestinal metaplasia of the esophagus, a known precursor of esophageal adenocarcinoma (EAC). The biological mechanisms leading to the development of BE remain to be clarified. Several hypotheses have been proposed as to the cell of origin for BE (DeVault, McMahon et al. 2013). Currently, these hypotheses remain the subject of investigation. One such model is the transdifferentiation of the esophageal squamous epithelium, which served as the focus of our investigation. Through our study in Chapter II we sought to provide evidence in support of the premise of transdifferentiation of esophageal epithelial cells as the potential cell of origin for the initiation of BE. We did not seek to prove or disprove other premises. We believe also that it is possible that more than one cell type might serve as the origin of BE, and this may be very context dependent.

In Chapter II, we demonstrate that Notch signaling is downregulated in human BE, suggesting that development of intestinal metaplasia in the esophagus could require inhibition of Notch signaling. Furthermore, Notch inhibition via dominant-negative Mastermind (dnMAML), in conjunction with MYC and CDX1 expression, promotes transdifferentiation in our immortalized esophageal epithelial cells. We observed the appearance of elongated columnar-like cells in the basal layer of 3D organotypic cultures of the MYC-CDX1 cells with Notch inhibition (dnMAML). Moreover, the MYC-CDX1-dnMAML cells show a switch in genes that denote the BE lineage, characterized by a robust diminution of squamous keratins and differentiation markers in favor of a strong induction of columnar keratins and mucins. Taken together, these data suggest

during the development of BE the activation of MYC and CDX1, and the negative regulation of the Notch signaling pathway, can cause changes in the esophageal epithelial cells associated with transdifferentiation towards BE.

Since our 3D organotypic culture system limits us to the study of esophageal basal cells, we could potentially test other hypotheses independently of what has been published already. Work from Dr. Tim Wang's lab has shown the successful culture mouse gastric cardia cells (personal communication). These cells could be modified with MYC, CDX1 and dnMAML to explore if a BE phenotype is present, at least in 3D organotypic culture. Esophageal submucosal glands are absent in the mouse and only present in humans. This is an impediment but one could evaluate potential stem cell genes for expression in this compartment.

Yet, it is important to note that despite the alterations observed in the MYC-CDX1-dnMAML cells, they were still able to form a stratified epithelium in our 3D organotypic cultures. Since our 3D organotypic culture system is designed to promote the stratification of esophageal epithelial cells, it would be useful to further investigate alternative 3D organoid culture systems. Given, that these MYC-CDX1-dnMAML cells show the initiation of changes towards BE, we would expect the use of 3D system which promotes the growth of an intestinal-like epithelium, we might observe further evidence of transdifferentiation towards BE. In addition, our current findings suggest the need for additional genetic alterations may be required for the complete development of BE in our model system. Therefore, it would be interesting to address whether p16 LOH in addition to MYC, CDX1 and Notch inhibition will further promote transdifferentiation to BE. Genetic studies of human BE have shown that p16 LOH as an early event in the development of BE (Maley, Galipeau et al. 2004).

We sought next to investigate the mechanism through which the inhibition of Notch signaling in MYC-CDX1 cells promotes potential for transdifferentiation. Previous studies have shown Notch signaling can be a negative regulator of both KLF4 and HATH1 transcription factors (Zheng, Pritchard et al. 2009; Kazanjian, Noah et al. 2010). Interestingly, we observed the upregulation of both KLF4 and HATH1 upon Notch signaling inhibition in our system. Furthermore, we showed knockdown of either KLF4 or HATH1 in the MYC-CDX1-dnMAML cells partially reversed the changes observed upon Notch inhibition. Overall, our findings suggest that KLF4 and HATH1 might be mediators of the changes in gene expression and cell morphology observed in our MYC-CDX1-dnMAML cells. It would be interesting to investigate further whether knockdown of both KLF4 and HATH1 together would lead to a complete reversal of the changes observed upon Notch signaling inhibition. Moreover, it would be interesting to investigate whether KLF4 and HATH1 can directly bind the promoters of BE lineage specific genes to promote their activation by ChIP. In addition, we could investigate for novel targets for KLF4 and HATH1 in the context of transdifferentiation towards BE by ChIP-seq. Furthermore, we could investigate further whether the decreased expression of Notch signaling expression correlates with upregulation of KLF4 and HATH1 in human BE. If so, KLF4 and HATH1 activation may serve as potential early biomarkers for the detection of BE.

Our findings in Chapter II suggest collaboration between MYC, CDX1 and Notch signaling inhibition in promoting transdifferentiation towards BE and this is mediated in part by KLF4 and HATH1. How do these transcriptional factors interact, if at all? Our preliminary studies with both CDX1 and CDX2 overexpression, in conjunction with MYC, in esophageal epithelial cells; demonstrate increased K8 and K20 expression by qPCR

(data not shown). Furthermore, inhibition of Notch signaling in these CDX1-CDX2-MYC cells showed a further increase of both K8 and K20 (data not shown). It is conceivable that CDX1 and CDX2 cooperate through trans-activation of the other's gene promoter (Grainger, Hryniuk et al. 2013).

A global and unbiased approach would be to determine if there is overlapping binding of gene promoters involving CDX1, CDX2, MYC, KLF4 and HATH1. This would involve ChIP-Seq, recognizing the complexity of this approach. Additionally, we could analyze whether MYC, CDX1/CDX2, KLF4 and/or HATH1 bind to the promoters of BE lineage genes (columnar keratins, mucins) by ChIP. If there is overlap, we could determine if there is protein-protein interaction between specific transcriptional factors, performing ChIP-on-ChIP. Since inhibition of Notch signaling is critical, there may be some direct or indirect effect upon one or more of these transcriptional factors.

Recent studies have shown that activation of Notch signaling occurs in EAC. Interestingly, our tissue microarray (TMA) show increased NOTCH1, ICN1 and HES1 expression in low-grade dysplasia and high-grade dysplasia (data not shown). Furthermore, our preliminary studies for the gene expression status in BE cell lines for Notch signaling show decreased expression of HES1 and HES5 in the non-dysplastic CP-A cells, and show increased HES1 and HES5 in the high-grade dysplasia CP-D cells (data not shown). These preliminary findings suggest that re-activation of Notch signaling occurs during the progression from BE to EAC. Therefore, it would be interesting to investigate in future experiments the role of Notch signaling in the progression of BE to EAC.

Design and development of a genetic mouse model of Barrett's esophagus

The modeling of diseases through the use of *in vivo* mouse models can provide significant information regarding not only the mechanism leading to a disease, but also provide a physiological system in which to test potential therapies. Therefore, through our study in Chapter III, we sought to design a potential genetic mouse model for BE. First, we designed a mouse model that would allow us to overexpress MYC in the esophagus. In Chapter III, we show the successful generation of an inducible Tet-ON mouse system that leads to the overexpression of MYC in the esophagus upon treatment with doxycycline. Second, we bred the *L2-rtTA; Tet-Op-Myc* mice with the *K14-Cdx2* mice (Kong, Crissey et al. 2011) in order to achieve overexpression of MYC and Cdx2 in the esophagus. Our preliminary findings show the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* show no significant histological changes at 6 or 12 months of age with or without treatment with 0.3% DCA. Therefore, we plan to analyze further our *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice treated with 0.3% DCA for gene expression changes in columnar keratins (i.e. K8, K19, K20) and squamous keratins (i.e. K5, K13, K14) by qPCR and IHC.

The lack of histological changes in the esophagi and forestomach of the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* could be due to the requirement of Notch signaling inhibition to promote changes in cell morphology. Thus, we propose to breed the *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice with the *Tet-Op-dnMAML/GFP* mice. Through this breeding strategy we expect to acquire mice with overexpression of MYC and Cdx2, and Notch signaling inhibition specifically in the esophagus and forestomach. We propose to analyze the esophagi and forestomach of the *L2-rtTA; Tet-Op-Myc; K14-Cdx2; Tet-Op-*

dnMAML/GFP mice 9, 12 and 15 months post-treatment with doxycycline chow ± 0.3% DCA. We expect Notch signaling inhibition in conjunction with MYC and Cdx2 overexpression may lead to changes in the cell morphology of the basal cells, similar to those observed in 3D organotypic culture. We predict our mice will show increased expression of columnar keratins, and mucins genes. Moreover, we plan to make use of the tdTomato (*Tet-Op-Myc* mice) and GFP (*Tet-Op-dnMAML/GFP* mice) to isolate and sort the esophageal epithelial cells for further analysis through spheroid and organoid assays.

Our findings in Chapter II suggest KLF4 as a potential mediator of Notch signaling inhibition promoting the transdifferentiation to BE. Therefore, an alternative to breeding with the *Tet-Op-dnMAML* mice, we could breed our *L2-rtTA; Tet-Op-Myc; K14-Cdx2* mice with the *L2-KLF4* mice (Tetreault, Wang et al. 2010). The *L2-KLF4* mice have been show to have increased basal esophageal cell proliferation, increased expression of inflammatory cytokines. Furthermore, *L2-KLF4* mice show dysplasia by 6 month of age, and develop ESCC by 2 years. Therefore, we could investigate the role of KLF4 overexpression in the context of MYC and Cdx2 and whether it may promote transdifferentiation towards BE. Our study of the *L2-rtTA; Tet-Op-Myc; K14-Cdx2; Tet-Op-dnMAML/GFP* mice will allow us to pursue the hypothesis of transdifferentiation of esophageal basal cells to BE. We expect our mice will acquire changes in gene expression towards BE lineage markers and decreased expression of squamous lineage markers.

Our mouse model may have potential utility in the prevention, detection and therapy of human BE, all areas that are filled with challenges. First, it is believed that certain drugs, such as aspirin, proton pump inhibitors or statins, might be

chemopreventive agents in human BE (Falk, Buttar et al. 2012; Tsibouris, Vlachou et al. 2014). These could be tested directly in our mouse model, recognizing such studies would entail a great deal of time. Second, if we lineage-labeled our mice with YFP, then we could determine if such cells circulate in blood, and interrogate these cells for potential biomarkers that may be applicable to patients with various stages of BE. We could conduct genomic studies, either genomic or whole-exome sequencing, to determine what the profile of mutations in genes is, and compare to human BE. This has not been done to date in human BE. Such a comparison may also contribute to the development and application of biomarkers in human BE.

We cannot assume that our mice will develop EAC. Aging of mice will help to evaluate that possibility. Furthermore, we could breed our mice to the *L2-IL1β* mice. The *L2-IL1β* mice develop esophagitis, BE and EAC (Quante, Bhagat et al. 2012). Therefore, we expect expression of IL-1β in conjunction with MYC and Cdx2 overexpression and Notch inhibition may promote to the acceleration of BE to EAC. Such compound mice may prove to be attractive models for the investigation of novel therapeutics. Currently, early stage EAC is subjected to neoadjuvant chemoradiation therapy followed by surgery. Otherwise, therapy tends to be restricted to palliative measures for advanced stages of EAC. To that end, targeted therapeutics, linked to genomic alterations in BE and/or EAC, as well as the tumor microenvironment, might be feasible in our mouse model

Activation of c-Met receptor tyrosine kinase by a common p53 mutation (R175H) enhances tumor invasion in ESCC

Esophageal cancer is one of the deadliest cancers in the US. Esophageal cancer is subdivided into two major subtypes: esophageal adenocarcinoma (EAC) and

esophageal squamous cell carcinoma (ESCC). In Chapter IV, we investigated the role of a common p53 mutation R175H in enhancing ESCC invasion, mediated by activation of the c-Met receptor. In our study we used immortalized esophageal epithelial cells overexpressing EGFR and $p53^{R175H}$ (EPC2-hTERT-EGFR-53^{R175H}) and grew in 3D organotypic cultures. In Chapter IV, we show EPC2-hTERT-EGFR-$p53^{R175H}$ cells harbor activation of the c-Met, with increased c-Met phosphorylation.

Interestingly, our findings show the activation of c-Met is not mediated by increased expression of its ligand HGF. Furthermore, our data show activation of c-Met is specific to the $p53^{R175H}$ mutation. Therefore, our data suggest a novel connection between mutant $p53^{R175H}$ expression and activation of the c-Met receptor. Throughout our study in Chapter IV, we sought to investigate potential mechanisms to explain the link between $p53^{R175H}$ and c-Met activation. Through our studies using pharmacological reagents which lead to the restoration of wild-type p53, we demonstrate the activation of c-Met is dependent upon the presence of the mutant p53 function. Interestingly, our data showed no significant changes in RNA or protein levels of c-Met in the EPC2-hTERT-EGFR-$p53^{R175H}$ cells. Another potential mechanism $p53^{R175H}$ mediated activation of c-Met is through p53 regulation of the miR34 family. Our findings demonstrate decreased expression of mir34a in the EPC2-hTERT-EGFR-$p53^{R175H}$ cells. Yet, knockdown or overexpression of mir34a had no effect on activation of c-Met. Therefore, our data suggest c-Met activation by $p53^{R175H}$ is mediated in a mir34a independent mechanism.

Our findings in Chapter IV suggest the need to further investigate the mechanism through which $p53^{R175H}$ mediates activation of the c-Met receptor. Interestingly, recent studies have shown PAK1 can mediate activation of c-Met receptor in breast cancer

(Shrestha, Schafer et al. 2012). Therefore, it would be interesting to investigate whether $p53^{R175H}$ can activate PAK1 to mediate activation of c-Met. Interestingly, our preliminary studies show increased active PAK1 in the EPC2-hTERT-EGFR-$p53^{R175H}$ cells, as observed by phosphorylation of PAK1. Moreover, studies of p53 mediated apoptosis in rat kidney cells show PAK1 can act downstream of wild-type p53 (Thomas, Giesler et al. 2000). Thus, we propose further investigation of PAK1 signaling in the context of our EPC2-hTERT-EGFR-$p53^{R175H}$ cells.

Overall our findings in Chapter IV demonstrate an HGF independent activation of c-Met by mutant $p53^{R175H}$. Interestingly, in our model of ESCC, the restoration of wild-type p53 function led to decreased invasion in 3D organotypic cultures. Currently, treatment of ESCC is limited due to late diagnosis where invasion and metastasis are present. Therefore, our findings provide potential evidence for the development of novel therapeutic treatments targeted to restoration of p53 wild-type function or c-Met inhibition.

Concluding Remarks

Overall, our collective data in Chapter II highlight the potential mechanism of transdifferentiation as the possible initiation of BE in 3D organotypic culture. Our data suggest that overexpression of MYC and CDX1 in conjunction with inhibition of Notch signaling may foster transdifferentiation and this may be mediated through KLF4 and HATH1. Furthermore, our findings provide the basis to investigate these pathways in an *in vivo* context. We expect that the future studies of Chapter III may provide further novel insights to support transdifferentiation of the esophageal basal cells as a potential cell of origin for BE.

Lastly, our studies in Chapter IV we demonstrate a novel link between $p53^{R175H}$ and c-Met in mediating cell invasion in ESCC. Thus, our data support the need to explore new targeted therapeutic treatments for ESCC with a focus on p53 and c-Met.

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