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I, Alyssa L Gallas , hereby submit this original work as part of the requirements for the degree of Master of Science in Molecular & Developmental Biology.

It is entitled:

Lung tumors formed in the TGFΒRII conditional knockout mouse are the result of metastasis from the spontaneous tumor in the anorectal transition zone

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Lung tumors formed in the TGFβRII conditional knockout mouse are the result of metastasis from the spontaneous tumor in the anorectal transition zone

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By

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Abstract

Metastasis is the process by which a tumor migrates from its original position and travels to a distant location in the body through the bloodstream. It is a highly intricate process and accounts for 90% of all cancer deaths. This process can be studied through the use of the TGFβRII conditional knockout mouse. In this mouse, expression of transforming growth factor β receptor II (TGFβRII) is conditionally lost in Keratin 14+ cells of stratified squamous epithelium, which results in the formation of a spontaneous tumor in the anorectal transition zone as well as in the lungs; those cells deficient in TGFβRII permanently express the yellow fluorescent protein (YFP). We also induced a tumor by injecting YFP+ TGFβRII-deficient tumor-propagating cells that were sorted from a separate tumor into the anorectal transition zone of a syngeneic recipient mouse. Similarly, TGFβRII-deficient mice developing the spontaneous tumor and recipient mice with the induced tumor have YFP+ squamous cell carcinoma in their lungs. It was unknown whether the tumor in the lung of the spontaneous model formed as a result of metastasis or if it formed as a separate tumor from resident TGFβRII-deficient Keratin 14+ cells of the trachea. To test this, we first looked at the trachea of the TGFβRII conditional knockout mouse and found expression of Keratin 5 but not YFP, indicating that our knockout strategy is not active in the trachea. We then looked at various markers in the lungs to show that the lung tumor expresses the same markers of the spontaneous tumors but not the lung epithelia. We showed that the lung tumors express Keratin 5, Keratin 6 and Keratin 17, which are all coexpressed with the YFP+ tumor cells in both the spontaneous and induced tumors. We also showed that the lung marker thyroid transcription factor 1 (TTF1) is expressed around the tumor cells but does not coexpress with YFP. These data suggest that the tumor that forms in the lung is the result of a metastasis from the tumor in the anorectal transition zone. Further investigation into how this metastasis forms would potentially lead to the development of targeting strategies and better treatment options.

List of Abbreviations

ATZ	Anorectal transition zone
Bmpr1a	Bone morphogenetic protein receptor, type 1a
CD34	Cluster of differentiation 34
cKO	Conditional knockout
E15	Embryonic day 15
EMT	Epithelial-mesenchymal transition
EYFP	Enhanced yellow fluorescent protein
HPV	Human papillomavirus
K1	Keratin 1
K5	Keratin 5
K6	Keratin 6
K8	Keratin 8
K10	Keratin 10
K14	Keratin 14
K17	Keratin 17
K18	Keratin 18
NSCLC	Non-small cell lung cancer

PFA	Paraformaldehyde
SCC	Squamous cell carcinoma
SP-C	Surfactant protein C
TGFβ	Transforming growth factor beta
TGFβRI	Transforming growth factor beta, receptor type one
TGFβRII	Transforming growth factor beta, receptor type two
TTF1	Thyroid transcription factor 1
TZ	Transition zone
VEGF	Vascular endothelial growth factor
WT	Wildtype
YFP	Yellow fluorescent protein

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Introduction

Cancer and Metastasis

Greater than 90% of all cancers are epithelial in origin, which classifies them as carcinomas. Epithelium can be single- or multi-layered tissues and are essential for functions such as protection from infections, secretion, organelle transport, and tissue differentiation. All epithelia express keratins, which are intermediate filaments that protect the tissue from outside mechanical forces [1]. They are divided into two types: the acidic type I keratins, which include keratins 9-28 and the basic type II keratins, which include keratins 1-8 and 71-74 [2]. Basal cells are found closest to the basement membrane and are found throughout simple and stratified epithelia. These cells are able to differentiate as they move up into the suprabasal layers. As they differentiate, they lose expression of K5 and K14 and instead express K1 and K10 [1]. K5 and K14 expression is found in all stratified squamous epithelium [3], while K8 and K18 are found in all simple epithelia [4]. Keratin expression is cell-type specific and epithelial tumors mostly maintain keratin expression associated with the cell type of origin; therefore, keratin expression has been frequently used as markers for many human tumors and can even give insight as to how they should be treated [5]. Some recent evidence has suggested that keratins also have the ability to regulate epithelial tumorigenesis [5].

Metastasis, as opposed to the primary tumor itself, accounts for nearly 90% of all cancer deaths [6]. This indicates a fundamental need for treatments targeted towards preventing metastasis in addition to addressing the primary tumor itself. Metastasis is the spread of cancer cells from the cancer of origin to another organ or tissue type [6]. Despite the extensive research effort to determine how metastasis occurs, there is still much information to be uncovered. The general process of metastasis is thought to be well defined and contains a few crucial steps, classically characterized as local invasion, intravasation, survival in the circulation, extravasation and colonization [7]. First, the cancer cells within the tumor must locally invade the surrounding

stroma. To do this, they must break free from the basement membrane of the tumor epithelium, which serves as a barrier between the epithelium and the stroma [8]. It is widely suggested that the cancer cells can adopt genetic programs induced by certain transcription factors in order to invade the stroma. One of these genetic programs is epithelial-mesenchymal transition (EMT) in which the epithelial cells adopt a mesenchymal cell phenotype. The cells become more invasive through the dissociation of adherens and tight junctions and loss of cell polarity [9]. Once the invading cells invade the stroma, they then undergo intravasation, which is when they enter the lumen of the lymphatic vessels or blood vessels [8]. This step allows them to survive in the circulation and spread to various distant organ sites depending on the cancer type. It has been noted that many carcinomas only metastasize to a limited number of distant sites [10]. Two hypotheses have been proposed to address this phenomenon. The first is that some microvessels of some organs have too small a diameter to allow access to the invading cells. The second hypothesis is that the invading cells are predetermined to metastasize to certain tissues [8]. Extravasation occurs once the invading cells can no longer travel through the capillaries of an organ and pass through the endothelium. This process is followed by colonization; the invading cells settle in a distant organ and begin forming a secondary tumor [11]. Though much about these molecular mechanisms has been discovered, there is still much to learn so that new targeting strategies can be developed.

The TGFβ pathway in cancer and metastasis

The TGFβ superfamily consists of structurally related cytokines that are involved in many cellular processes, including differentiation, proliferation, adhesion and motility, among others [12]. The pathway is initiated when TGFβ ligand binds to TGFβRII, which then forms a complex with TGFβRI. In the canonical pathway, Smad2 and Smad3 are recruited, bind to TGFβRI and

are phosphorylated by the receptor complex [13]. Smad2 and Smad3 combine with Smad4, and this complex enters the nucleus where it can alter expression of many genes (Figure 1). This pathway has been shown to be important for inhibition of growth of epithelial cells because of its ability to activate cell cycle inhibitors [14]. Mutations of the essential components of this pathway can be found in many human cancers. Mutations or inactivation of TGFβRII have been observed in colon, pancreatic, lung and brain cancer [15]. The TGFβ pathway has the ability to transition from a tumor suppressor to a tumor promoter in later-stage tumors [16]. This occurs because cancer cells no longer respond to the growth inhibition from TGFβ and instead use TGFβ signaling to increase EMT [11]. TGFβ also has the ability to increase tumor vascularization by inducing VEGF; this can result in an increase in tumor growth as well as allow the tumor cells to migrate [17]. Overall, TGFβ remains an attractive therapeutic target because of its increased expression in tumor cells and tumor microenvironment. Though some therapies exist that target TGFβ signaling, its dual role as tumor suppressor and tumor promoter complicates the treatment process for patients and calls for further understanding of this dynamic pathway before pursuing clinical treatments [14].

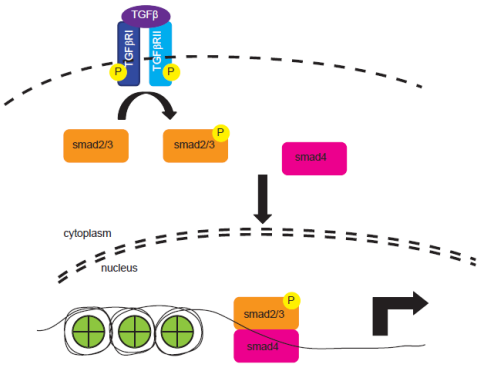

Figure 1. The TGFβ signaling pathway. TGFβ ligand binds to TGFβRII, which recruits a TGFβRI and forms a complex. Smad2/3 is phosphorylated by the receptor complex, forms a complex with Smad4 and enters the nucleus to cause transcription of the DNA.

Transition Zones

Transition zones (TZs) are defined as the junction between two different types of epithelia. These transition zones can be found throughout the body, including between the cornea and conjunctiva, between the esophagus and stomach, between the stomach and duodenum,

between the ectocervix and endocervix, and between the anal canal and rectum. The transition zone represents a unique microenvironment that is especially susceptible to tumor formation, possibly because it is receiving different signals from two epithelial subtypes [18]. Many reports of human cancer have confirmed the formation of squamous cell carcinoma (SCC) at various transition zones, including the anorectal transition zone (ATZ) [19], at the limbus between the cornea and conjunctiva [20], at the junction of the endocervix and ectocervix, and at the gastro-esophageal junction [21]. Tumors in transition zones have been shown to be more aggressive than others and cervical cancers are the second-most common cancer in women worldwide. They are triggered by human papillomavirus (HPV) and occur at the transition zone of the cervix [22]. Mouse models exist that develop spontaneous tumors in various transition zones. One example is a *Bmpr1a* conditional knockout mouse in which a Bmpr1a$^{fl/fl}$ mouse was bred to an *Mx1-Cre* transgenic mouse. This mouse developed neoplasia and polyps specifically at the gastrointestinal junctional zone [23]. Another example is a mouse deficient in *Smad3* that develops gastric tumors at the junction between the forestomach and glandular epithelium [24]. Both of these studies both show the susceptibility of transition zones to tumor formation and that TGFβ and its downstream effectors are frequently involved in tumor formation as well. These mice could be used to help gain further insight into how these tumors form.

Because these transition zones are susceptible to tumor formation, many have postulated that transition zones serve as a stem cell niche. Recently they have been shown to express common stem cell markers such as CD34 and contain a population of slow-cycling cells, which is characteristic of stem cells [25]. One group discovered a stem cells niche in the transition zone of the mouse ovarian surface epithelium called the hilum. Cells of the hilum are slow-cycling, express stem and progenitor cell markers, and display long-term stem cells properties [26]. Overall, transition zones remain an intriguing system to study cancer but require further understanding into the mechanisms of why they are highly susceptible to tumor formation.

Lung airway epithelium

Although solid tumors can metastasize to one or multiple sites depending on the cancer type, one of the most common sites is the lung [27]. The human lung airway is comprised of pseudostratified epithelium, 30% of which are basal cells. These basal cells express *Trp-63*, K5 and K14. K5 has been shown to be expressed in nearly all basal cells; however, only a small portion of basal cells express K14 [28]. In the mouse, basal cells are limited to the trachea whereas in humans, basal cells are found throughout the large and small airways [29]. Many have suggested that the basal cells are responsible for airway repair, which implies that they function as a stem cell population [30-32]. In support of this, in naphthalene-induced injury, a small population of K14 cells expanded and K14 expression was upregulated at the site of injury [33]. Further studies are required to determine if this small population of basal cells is truly a stem cell population and whether this system could serve as a model system to treat human disease of the airways.

Using a conditional knockout mouse to study cancer and metastasis

Our lab utilizes the *Keratin14-Cre/TGFβRII*$^{fl/fl}$*/Rosa-YFP*$^{fl/fl}$ mouse (referred to as *TGFβRII cKO*) (Figure 2A). In this conditional knockout mouse, a loxP-flanked stop codon is placed in front of enhanced yellow fluorescent protein (EYFP) and is inserted into the Rosa26 locus. Also, exon 4 of TGFβRII is floxed out in Keratin14-positive (K14+) cells, thus causing YFP to label those cells deficient in TGFβRII. This model is sufficient to deplete the TGFβ pathway signaling in K14+ cells. The Keratin 14 (K14) promoter is active in proliferating stratified squamous and glandular epithelia as well as in oral, anal and genital stratified squamous epithelia; it is strongly active by embryonic day 15 (E15) [34]. These mice are phenotypically normal during early adult life, but

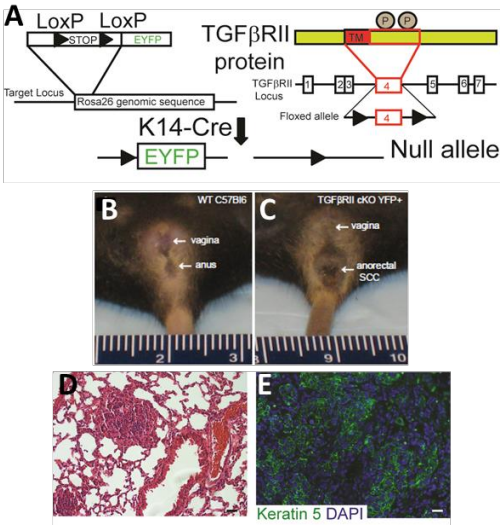

Figure 2. Conditional knockout strategy to induce spontaneous squamous cell carcinoma. (A) A loxP-flanked stop codon is placed in front of EYFP and is inserted into the Rosa26 locus. Exon 4 of TGFβRII is floxed out using a K14-Cre driver. This causes all cells that are deficient for TGFβRII to be labeled with YFP expression. (B) Image showing vagina and anus of WT C57Bl/6 mouse. (C) Image showing the spontaneous formation of SCC in the anorectal region of a TGFβRII cKO YFP+ mouse. (D) H&E stain of SCC found in the lung epithelium of TGFBRII cKO mouse. (E) Immunofluorescence of SCC in the lung epithelium of TGFβRII cKO mouse stained with Keratin 5.

go on to develop spontaneous SCC as early as four months of age [35]. The SCC develops specifically at the junction between the stratified squamous epithelium of the anal canal and the columnar epithelium of the rectum, otherwise referred to as the ATZ (Figure 2B, C). Interestingly, SCC does not occur in the skin unless additional mutations are applied or at the junction between the esophagus and stomach, which are both deficient in TGFβRII. This is probably because the K14-Cre used in these studies does not target the esophageal epithelium very efficiently and the skin requires an additional oncogenic effect to develop SCC. However, these mice also develop tumors in their lungs that are K5- and K14-positive (Figure 2D, E). It is unclear if these tumors arise because the spontaneous tumors metastasize to the lungs or if they are derived from the existing population of K14 cells in the lungs that could have lost expression of TGFβRII.

The mouse model described above will be used to answer the following questions. First, it is necessary to screen the entire lung for expression of YFP. This would tell us if any tumor cells have invaded the lung at the time of analysis. Second, we will examine the trachea and observe if our targeting strategy was successful in targeting the K5+ cells of the trachea, which would prove that the lung tumors did not form from those resident K5+ cells of the lung. We will also use various markers such as K5, K6, K17 and TTF1 to stain the lung tumor tissue and determine if the tumor is a result of metastasis. We hypothesize that these tumors are the result of metastasis from the primary anal tumor.

Materials and Methods

Mice and genotyping

The conditional knockout TGFβRII$^{flox/flox}$ x K14-Cre mouse model [35, 36] has been derived in a pure C57BL/6 background and backcrossed [37] into a mouse reporter containing an Enhanced Yellow Fluorescent Protein gene (EYFP) inserted into the Gt(ROSA)26Sor locus [38] and called Rosa-Flox-STOP-Flox-EYFP (Jackson Laboratory). Specifically, a LoxP-flanked stop codon was placed in front of the EYFP gene, which was then inserted into the Rosa26 locus. Also, exon 4 of TGFβRII was floxed out using a K14-Cre driver. This strategy causes all cells that are deficient in TGFβRII to be labeled with YFP expression.

Control mice were either TGFβRII$^{flox/flox}$ x Rosa-Flox-STOP-Flox-EYFP in a C57BL/6 background (TGFβRII cKO asymptomatic) or C57BL/6 mice (WT). The WT mice were used to induce a tumor in the anorectal region according to the protocol that was previously published [37]. YFP+ cells were sorted from TGFβRII cKO mice and placed in cell culture. These cells were then injected into the ATZ; for this injection, one million cells were used. Wildtype, wildtype with an induced tumor, and asymptomatic cKO mice were sacrificed at 12 weeks of age and the spontaneous cKO mouse was sacrificed at almost 17 months of age. The lungs and ATZ were subsequently collected. The lung was screened for the presence of YFP+ tumor cells by the procedure detailed below.

All experiments were approved by the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee (IACUC) and carried out using standard procedures. Genotyping was conducted by PCR of tail skin DNA using mouse Cre, TGFβRII and EYFP primers as described previously [35, 39] Cre primers were created using the following sequences: TTGCCCTGTTTCACTATCCAG and TGCTGTTTCACTGGTTATGCGG. TGFβRII primers were created using the following sequences:

TATGGACTGGCTGCTTTTGTATTC and TGGGGATAGAGGTAGAAAGACATA. EYFP primers were created using the following sequences: AAAGTCGCTCTGAGTTGTTAT, GCGAAGAGTTTGTCCTCAACC, and GGAGCGGGAGAAATGGATATG.

Tissue collection and OCT processing

Wildtype, wildtype with an induced tumor, and asymptomatic cKO mice were all sacrificed at 12 weeks of age. The spontaneous cKO mouse was sacrificed at almost 17 months of age. All mice were sacrificed using standard procedures approved by the Cincinnati Children's Hospital Research Foundation IACUC. The lungs were first fixed by injecting 4% paraformaldehyde (PFA) into the lungs through the trachea. The lungs and anorectal region were harvested and placed in 4% PFA overnight. To preserve the YFP expression, a four-day OCT processing protocol was used. Day 1 involved placing the tissue in the 4% PFA. Day 2 involves placing the tissue in a 30% sucrose in 1X PBS solution followed by a day of 2 parts 30% sucrose and 1 part OCT. The last day involves placing the tissue in OCT. The tissue was then embedded in OCT and stored at -80C.

Immunohistochemistry

A cryostat was used to section the samples preserved in OCT (10μm thickness). Lung sections were screened for YFP expression using every tenth slide after applying a 0.1% Triton in PBS stain for 10 minutes to permeabilize the tissue and allow for YFP detection. Antifade was applied to the slides prior to mounting with a coverslip in order to preserve the fluorescence. Primary antibodies against the following proteins were applied using the indicated dilutions: TTF1 (a generous gift from Dr. Jeffrey Whitsett; 1:500), Keratin 5 (Seven Hills Bioreagents, Cincinnati, OH; 1:2000), Keratin 17 (a generous gift from Dr. Pierre Coulombe; 1:2000), CD49f (BD Biosciences, San Jose, CA; 1:500), and Keratin 6 (a generous gift from Dr. Elaine Fuchs; 1:500). 4',6-diamidino-2-phenylindole (DAPI) was used as a marker of cell nuclei (Sigma

Chemical Co., St. Louis, MO; 1:2000). Secondary antibody Alexa-Fluor-555 (Invitrogen Corporation, Carlsbad, CA) was used at a dilution of 1:1000. Immunostained sections were analyzed using a fluorescent microscope AxioImager M1 (Zeiss) and pictures were taken with an axioCam MRm camera (Zeiss). Images in different focal planes were combined using the Extended Focus Module within the Axiovision software suite (Zeiss).

RESULTS

No lesions are present in the anorectal region of TGFβRII cKO asymptomatic mouse

We first compared the wildtype (WT) C57BL/6 mouse to the TGFβRII cKO asymptomatic mouse to ensure that there were no lesions present. This experiment was necessary because the entire lung was sectioned and screened for any presence of metastasis by expression of YFP+ cells. The best way to know if the mouse is truly asymptomatic is to analyze the ATZ before processing the whole lung. If there are no YFP+ cells in the lung, that implies that the tumor cells from the ATZ have not metastasized to the lung or that there are no resident K14+ cells in the lung that would express YFP due to loss of TGFβRII in those cells. The WT and asymptomatic mice were age-matched at 13 weeks old, which is too early of an age for the TGFβRII cKO mice to develop SCC in the anorectal region. As previously mentioned, the TGFβRII cKO asymptomatic mice don't develop tumors until at least 8 months of age. The ATZ of both mice were dissected and processed using the

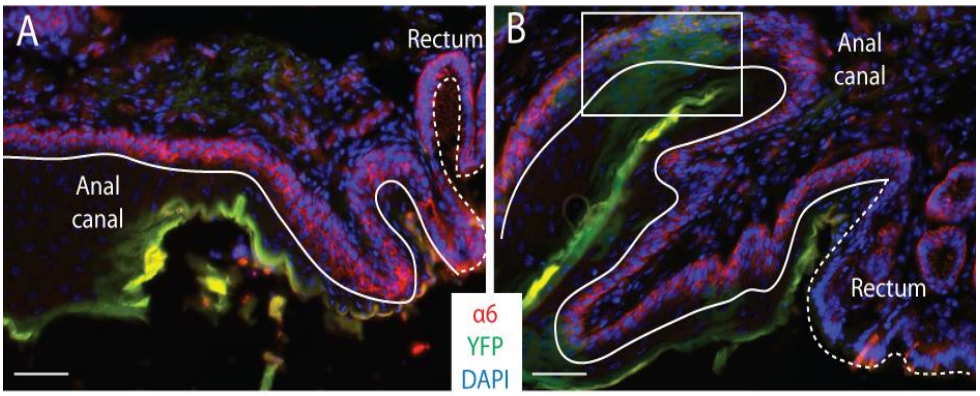

Figure 3. α6-integrin expression in mouse anal canal confirming no lesions are present in the ATZ. 10μm sections of a WT C57BL/6 mouse (A) and a TGFβRII cKO asymptomatic mouse (B) anal canal and rectum stained with α6-integrin, YFP and DAPI. The solid white indicates the stratified epithelium of the anal canal and the dashed white line indicates the simple epithelium of the rectum. The boxed area (B) denotes a portion of the anal canal that is YFP+. Scale bar=50μm.

paraformaldehyde (PFA) and sucrose method of fixation. After sectioning through the entire tissue, the sections were stained with α6-integrin and DAPI. The ATZs of both the WT (Figure 3A) and asymptomatic mouse (Figure 3B) are shown and appear to be normal, as indicated by α6-integrin expression. There is no break in the basement membrane and there is continuous expression of α6-integrin throughout the basal layer. We would only expect a break in the expression of α6-integrin if the cells became invasive and had to break through the basement membrane in order to migrate. The boxed area in Figure 3B denotes YFP expression, which indicates loss of TGFβRII in these cells. It is common to see mosaicism in our TGFβRII cKO mice because the K14-Cre promoter is known to drive mosaic Cre expression. These results show that there was no tumor formation in the ATZ of the asymptomatic mice and that they could be further analyzed by expression of various markers.

Analysis of K5 and YFP expression in the murine trachea

As previously mentioned, K5 is not expressed in the lower airways of the mouse but is instead expressed in the trachea. The goal of this experiment was to determine if our targeting strategy would also target the trachea. To determine this, we performed an immunostain on the trachea of a WT mouse (Figure 4A and B) as well as the TGFβRII cKO

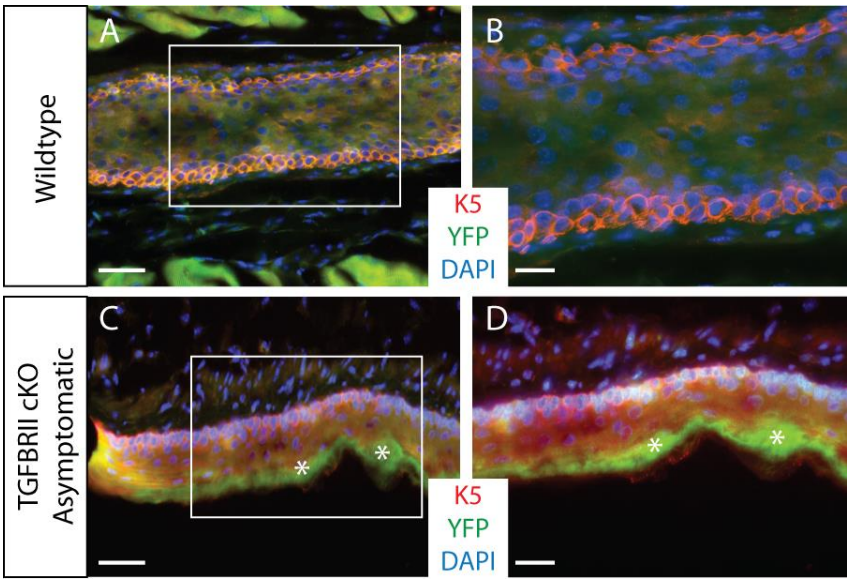

Figure 4. Our targeting strategy was unsuccessful in the mouse trachea. 10μm sections of a WT mouse (A, B) and a TGFβRII cKO asymptomatic mouse trachea (C, D) were stained with K5, YFP and DAPI. Scale bar=50μm. Boxed areas show higher magnification of WT (B) and asymptomatic (D) mice. Scale bar=20μm. Asterisks denote areas of autofluorescence.

asymptomatic mouse (Figure 4C and D) and stained with K5 and YFP. As expected, K5 stained the basal cells of the tracheal epithelium, but there was no YFP expression present (Figure 4A and C). K5 expression surrounding the basal cells can be better observed in the 40X magnification (Figure 4B and D). Lack of expression of YFP suggests that the trachea was not successfully targeted for deletion of TGFβRII in the K5+ cells. This could be because our K14-Cre promoter does not efficiently recombine in the trachea. Overall, K5 was expressed in the basal cells of the tracheal epithelium, but TGFβRII was not successfully deleted in these cells.

Analysis of various markers within normal and metastatic lungs

To determine if the cancer cells arise from a population of K14+ cells within the lung, various markers were used to immunostain lung sections from different mice. The mice analyzed included a TGFβRII cKO asymptomatic mouse that has no metastasis, a mouse with an induced tumor and a TGFβRII cKO mouse with a spontaneous tumor. We were able to induce a tumor by injecting the YFP+ cells collected from another TGFβRII cKO spontaneous tumor into the ATZ of the recipient mouse [37]. These cells take up residence in the ATZ and eventually metastasize into the lung. K5 in the mouse is expressed in the spontaneous tumors of the TGFβRII cKO mice. Our results show a lack of expression of K5 in the asymptomatic mouse lung (Figure 5A). However, K5 is expressed in both the induced and spontaneous lung tumors and is coexpressed with YFP, suggesting that the lung tumors originated from the tumors of the ATZ (Figure 5E and I). The lung marker TTF1, which marks the type II pneumocytes and Clara cells, was expressed in the small airway of the asymptomatic lung as well as in the lung tumors (Figure 5B, F and J). Importantly, the TTF1+ cells were not coexpressed with the YFP+ tumor cells and instead are expressed around the tumor cells. K6 is expressed by bronchial epithelium in the lung, in the ATZ, and in the anal canal of mice. Our results show a low level of expression of K6 in the small airway of the asymptomatic lung (Figure 5C). It was also found to be

expressed at low levels in the lung tumors and is coexpressed with YFP (Figure 5G and K). K17 is expressed in the ATZ, as well as during injury, inflammation and in squamous cell carcinoma. There was no expression found in the small airway of the asymptomatic lung (Figure 5D). K17 had greater expression in the spontaneous tumor compared to the induced tumor (Figure 5H and L). Taken together, these results show that the formation of a tumor in the mouse lung is a result of metastasis from the primary tumor in the ATZ and not the result of a tumor that formed in the lung separately from the tumor in the ATZ.

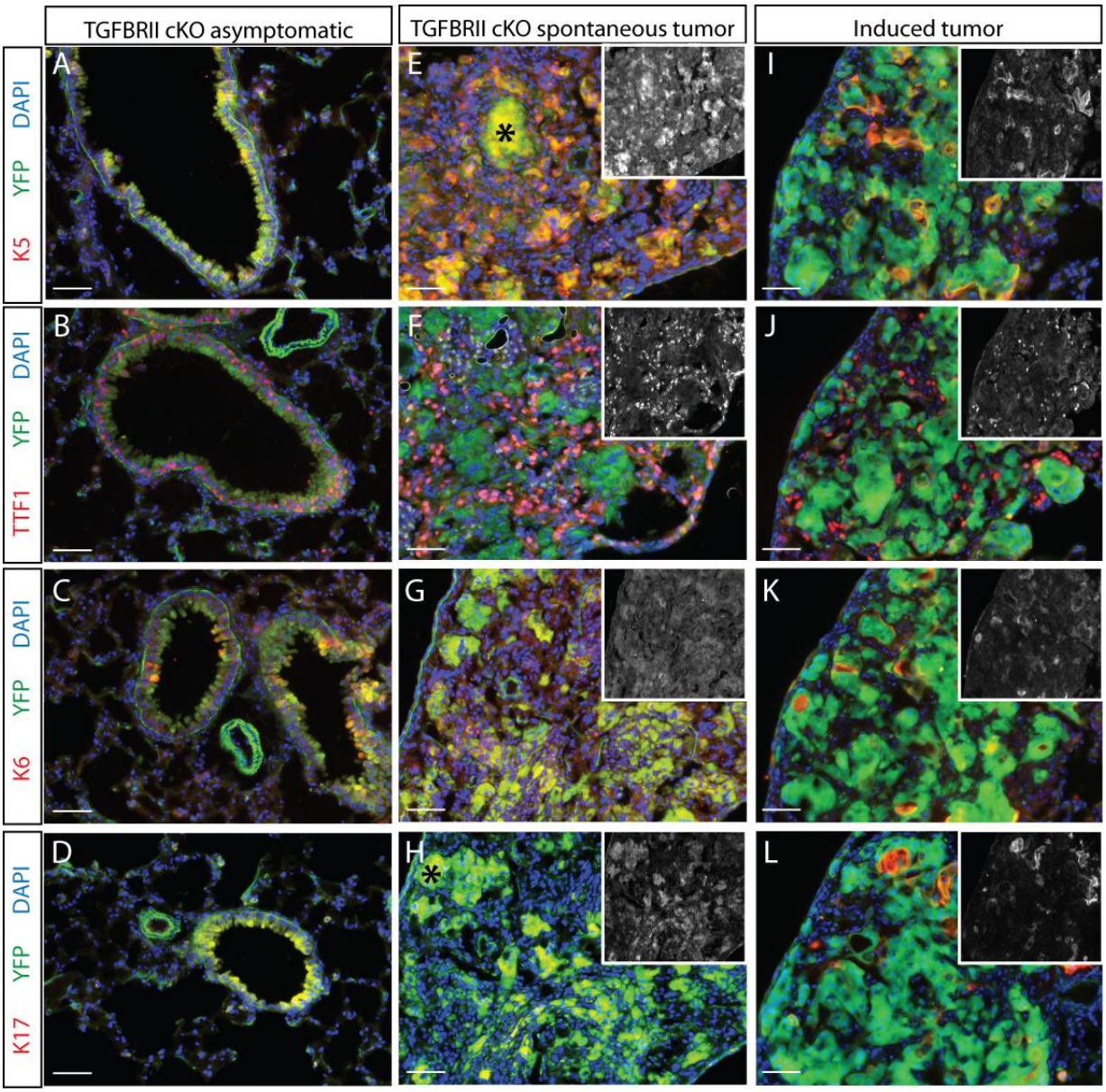

Figure 5. K5, K6 and K17 are expressed at varying levels in both the induced and spontaneous tumors, but the lung marker TTF1 is not expressed in the tumor cells. (A-D) Immunofluorescence analysis of lung tissue of asymptomatic mice stained with YFP (green) and the indicated markers in red. (E-H) Immunofluorescence analysis of lung tumors in spontaneous mice stained with YFP (green) and the indicated markers in red. (I-L) Immunofluorescence analysis of induced lung tumors stained with YFP (green) and the indicated markers in red. All boxed areas indicate the specified red marker in grayscale. All cells are counterstained with DAPI (blue). Scale bar=50μm. Asterisks denote areas of autofluorescence.

Discussion

Studying metastasis in a mouse model of anal tumor formation

Despite the extensive work dedicated to uncovering its causes and potential cures, cancer continues to affect about 13 million people every year, according to the National Cancer Institute [40]. Of the deaths caused by cancer, 90% of them can be contributed to metastasis to distant organs. The understanding of how tumors metastasize to distant organs would greatly benefit the medical field and how such tumors are treated.

In our mouse model, TGFβRII expression is conditionally lost in K14+ stratified squamous epithelial cells through the use of K14-Cre and TGFβRII-deficient expression is observed by permanent expression of YFP. These mice spontaneously form tumors at the ATZ, which is the transition between the columnar epithelium of the rectum and the stratified squamous epithelium of the anal canal. Intriguingly, only the ATZ and the genital area develop squamous cell carcinoma. The skin is phenotypically normal but can become tumorigenic through oncogenic exposure using H-Ras [35]. In other non-transitional epithelia such as the mammary gland, intestine, esophagus, pancreas and oral mucosa, conditional deletion of TGFβRII has no effect on these tissues and they develop normally [41-45]. However, like the skin, these tissues develop cancer if exposed to activated oncogenes such as K- or H-Ras or have suffered an additional loss of a tumor suppressor [35]. These data imply that TZs are intrinsically different compared to the non-transitional epithelia since tumors can form there without the need for an additional insult.

It was previously shown that loss of TGFβRII in K14+ cells of the mouse results in the formation of a tumor at the ATZ. It was also shown that K14+ tumors formed in the lungs, indicating metastasis [35]. Prior to this study, it was not fully known whether the tumors formed as a result of metastasis or because our targeting strategy caused loss of TGFβRII in the K14+ cells of the

lung and formed a separate tumor. Our results show that the former is true because we do not see the lung marker TTF1 expression within the YFP+ tumor cells. We observed that K5 expression is limited to the trachea and that these K5+ cells do not express YFP, proving that our targeting strategy does not affect tracheal cells. K5 is also expressed in the lung tumors, which indicates the presence of squamous cell carcinoma as a result of metastasis. These results clearly show that the tumor that originally forms in the ATZ metastasizes over time and takes up residence in the lung.

Transition zones have been proposed to be an ideal location for tumor formation, possibly because this area is receiving signals from two different types of epithelia [18]. This mouse model also gives us the opportunity to study the tumor microenvironment, which has been suggested to contain stromal factors that could contribute to the migration of the tumor cells. Our results show that the tumors in the lung were formed because of metastasis, but the potential role of stromal factors such as endothelial cells or immune cells has yet to be discovered. Overall, this specific mouse model allows us to study various aspects of tumor formation, such as the role of TGFβ in the process, the stromal factors in the tumor microenvironment and how to possibly prevent tumor formation and its subsequent metastasis.

Though our results argue for metastasis, there is one study showing that tumors could form in the mouse lung under the control of the K5 promoter. This study induced ectopic expression of the HPV-16 E6/E7 transgene under the control of the K5 promoter, which ultimately causes overexpression of these genes in basal cells of stratified squamous epithelia. Adenocarcinomas in the lungs were observed after about 6 months [46]. These tumors were described as being composed of small cells growing in lobules with an expansion of the lung parenchyma. The study was successful in showing how to drive tumor formation by causing overexpression of an oncogene, but there are a few key differences between this study and our results. First, they were able to observe tumors in the conducting airways of the lung, which is different from our

results because we only observed K5 expression in the trachea. The literature has been conflicting in this area of K5 expression in the lung, but more recently the literature has argued that K5 is only expressed in the trachea and not the conducting airways of the lower lung.

Second, the study showed that adenocarcinomas formed in the lung, whereas our data showed the formation of squamous cell carcinoma. Both are categorized as non-small cell lung cancer (NSCLC); about 48% of the cases are adenocarcinomas and about 28% are squamous cell carcinomas [47, 48]. They differ in expression profiles because adenocarcinomas express pro-surfactant protein C (SP-C) while squamous cell carcinomas express K5 and K14 [49, 50]. These criteria support our finding of squamous cell carcinoma in the lung because the tumor expresses K5. K6, which is expressed in bronchial epithelium in the lung, in the ATZ, and in the anal canal, was coexpressed with YFP in the lung tumor. Finally, K17, which is expressed during injury, inflammation as well as in squamous cell carcinoma, was expressed in the lung tumors of the spontaneous and induced tumors [51]. As mentioned in the results, the induced tumor forms as a result of direct injection of tumor-initiating YFP+ cells collected from a TGFβRII cKO mouse into the ATZ of the recipient mouse [37]. Our laboratory has extensively characterized these secondary tumors and show that they are very similar to the tumors arising spontaneously in terms of histology, the presence of different cell populations [28] and the formation of metastasis (McCauley and Guasch, in preparation). However, not surprisingly, these induced tumors form much quicker as we transplanted directly tumor-initiating cells directly into their niche of origin. A spontaneous tumor could take anywhere from four months to a year to develop, while an induced tumor could form in a matter of weeks. The time it takes for an induced tumor to form can be manipulated by how many cells are injected into the recipient: the more cells injected, the faster a tumor will form. Overall, the results obtained from the immunofluorescence analysis of the lung tumors from the spontaneously-formed tumor and from the induced tumor are very similar. Both K5 and K6 expression are used as markers of

squamous cell carcinoma in tumors of unknown origin or in metastatic tumors [51]. Their expression patterns between the two different tumor types are very similar. K17 differs slightly between the lung tumors from the spontaneous tumor and induced tumor, which can be explained by a possible difference in severity and aggressiveness. Since the lung tumor from the induced tumor has less K17 expression, the tumor may be more aggressive. This correlates with our data showing that the induced tumor is more aggressive because it can form faster than the spontaneous tumor. TTF1 is expressed in both lungs, but its expression is not coupled to the YFP+ tumor cells. This argues that the tumor did not originate from cells within the lung. Taken together, these data support our findings of squamous cell carcinoma in the lungs caused by metastasis.

Future directions

As previously mentioned, the mouse lung consists of a small population of basal cells that all express K5; some, but not all, of the basal cells that express K5 also express K14 [28]. It has been suggested that the function of these resident basal cells is to repair injured lung tissue; this also insinuates that these cells are a population of stem cells. After injury to the lung, these cells increase their proliferation and migrate to the site of injury to repair and replenish the injured tissue [33]. Since it has been shown that only a subset of K5-expressing basal cells also express K14 in the mouse lung [28], it would be relevant to create nearly the same TGFβRII cKO mouse but instead use the K5-Cre mouse. Many results could be conceivable using this mouse: we might expect to see the same tumor formation in these mice or we could observe additional tumor formation in a different location. It would be an interesting study to observe the differential expression patterns of K5 versus K14 and how loss of TGFβRII would affect K5+ tissues. One group has deleted TGFβRII specifically in the mouse airways through use of an inducible Keratin 5-Cre. They found that this deletion by itself is not sufficient to drive tumorigenesis in the lung but caused the formation of both adenocarcinomas and squamous cell

carcinomas upon oncogenic KRas activation [52]. These data are evidence that loss of TGFβRII is not sufficient to form tumors in the lung and support our findings of metastatic squamous cell carcinoma in the lungs.

Our results also beg the question of how and why TGFβ signaling is involved in the process of tumor formation at transition zones. As previously mentioned, TGFβ signaling is involved in many important processes including differentiation and proliferation [12]. Mutations in a number of its components can be observed in many human cancers and its regulation of various factors such as VEGF could provide a means by which tumor cells can escape the tumor microenvironment and migrate to distant locations [15, 17]. It is a unique pathway because it can act as both a tumor suppressor and tumor promoter [16]. It is also a complicated pathway because both TGFβ ligands and their receptors are expressed in every tissue type and can function differently in all of them [53]. It seems that the ATZ and TGFβ signaling have a unique relationship that allows for both tumor formation and metastasis. The results indicate a fundamental need for the discovery of therapies targeted toward loss of TGFβ and tumor formation.

Quite possibly the most powerful aspect of this research is the use of our mouse model to study both the formation of tumors as well as how these tumors metastasize to the lungs. As previously mentioned, this would be best studied through manipulation of the stromal factors in the tumor microenvironment and by determining how they contribute to the metastatic process. Further investigation of the TGFβ pathway would also give reasons as to how the tumor within the ATZ forms initially. These and many other studies would help determine the best treatment strategies for those patients affected by these types of tumors.

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