Exploring Epithelial Communication to the Mesenchyme and its Impact on the Expression of Genes Related to Tumorigenesis

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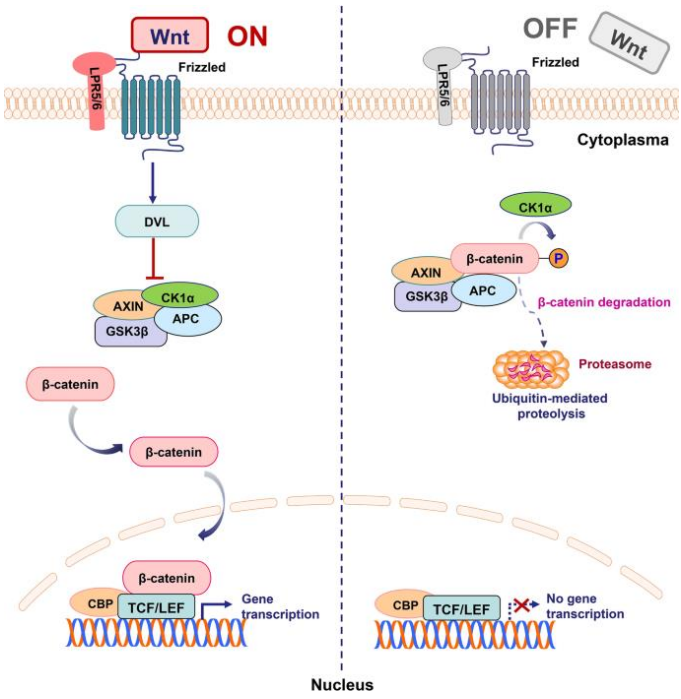
Abstract

Homeostasis of the epithelium is dependent on the Wnt/beta-catenin pathway, which regulates the proliferation of intestinal stem cells. The gain of function mutations in the beta-catenin gene leads to rapid cell proliferation and malignant growth in the epithelium. In addition, the maintenance of these stem cells appears to be linked to mesenchymal-derived factors. Although the communication between epithelial and mesenchyme cell populations remains uncharacterized, understanding this mechanism will help us further understand the various pathways involved in tumor initiation processes. Our results show that the communication between the mesenchyme and epithelium during transformation is influenced by varying levels of protein-expressing genes including *Wnt2b*, *Grem1*, and *Bmp6*.

1 Introduction

The Wnt/beta-catenin pathway regulates the transcription of Wnt target genes—genes which are highly deregulated in solid tumors (Fevr et al., 2007). In the absence of the Wnt ligand, beta-catenin, a protein normally sequestered in the cytoplasm, is constantly degraded and is prevented from entering the nucleus.

In the presence of the Wnt ligand, Wnt is able to bind to its receptor Frizzled, which activates downstream signaling and prevents the destruction of beta-catenin, thus enabling its entry into the nucleus. In the nucleus, beta-catenin promotes the transcription of Wnt target genes, causing rapid cell proliferation (Cleavers and Nusse, 2012). Cells dividing at an accelerated rate can become cancerous and eventually lead to the formation of a tumor.



The intestinal stem cell niche is primarily made up of several cell types, including: (1) differentiated cells responsible for the transport and absorption of nutrients, and (2) mesenchymal cells which supply signals to the epithelial cells to maintain homeostasis. Increased Wnt signaling can be induced by activating the beta-catenin gene, which allows for abnormal epithelial growth. Due to the expression of tamoxifen inducible Cre recombinase, Villin-Cre^{ERT2} transgenic mice have the ability to delete exon 3 of the beta-catenin allele in the intestinal epithelium (Marjou et al., 2004). Mice expressing Villin-Cre^{ERT2} can delete a portion of the beta-catenin gene in the presence of tamoxifen, resulting in a more transcriptionally active form of the gene. Mouse models have shown that mice with Villin-Cre^{ERT2} and injected with tamoxifen show visible malignant tissue transformation in the duodenum. But in organoid models, exposed to tamoxifen,

rapid cell proliferation can be seen almost immediately. However, epithelial transformation only becomes visible around Day 13 in mouse models. Although organoids mimic intestinal stem cells (crypts) in vitro, they do not provide a holistic view of a biological system. For example, they lack the presence of the mesenchyme. This suggests that the mesenchyme must play some role in the suppression of mutant growth. However, the way mesenchyme and epithelial cells influence one another is unknown. Yet, it is known that mesenchymal-derived factors are essential to maintain intestinal epithelial stem cells, so the reduction of these factors may be contributing to the suppression of intestinal transformation in the transgenic mouse (Stzepourginski et al., 2017).

AREG, a ligand of EGFR (epidermal growth factor receptor), can trigger signaling cascades that mediate cell survival, proliferation, and motility. Thus, AREG may be a candidate ligand that allows the epithelium to communicate with the mesenchyme (Wang et al., 2020). Transformed epithelium expresses high levels of AREG, which may be sensed by mesenchyme cells. Mesenchyme cells like PDGFRA^{hi} are enriched near the crypt-villus junction where stem cells reside and promote BMP ligands (promotes differentiation/blocks proliferation). A subset of PDGFRA^{lo} cells can be found at the bottom of crypts and provide growth factors for stem cells (McCarthy et al., 2020). If AREG is communicating with PDGFRA^{lo} cells in the mesenchyme, the PDGFRA^{lo} cells would express decreased levels of GREM1, a protein that functions to suppress BMP and permits proliferation. Subsequently, this may lead to an upregulation in expression levels of BMP. Early intestinal tumorigenesis reflects the deregulation of Wnt and BMP signals (Davis et al; 2015). It has also been recognized that BMP signaling restricts intestinal epithelium hyperproliferation (Qi et al., 2017). If we could understand how the mesenchyme is communicating with the epithelium, it would give insight to how it is able to suppress the abnormal growth, allowing for novel therapeutic interventions during initial tumor development. The goal of this project is to learn how epithelial cells are communicating with mesenchyme cells in an elevated Wnt activity background. Elevated levels of Wnt prevents beta-catenin from being degraded, which promotes expression of Wnt target genes, resulting in the mutant phenotype. Through signals (e.g. AREG) sent by the epithelium, the mesenchyme may detect the cellular transformation and respond in hopes of preventing the abnormal growth. If mesenchyme cells are treated with conditioned media from mutant organoids (crypts), then the genes which are altered are communicating with the mesenchyme. If epithelial cells undergo abnormal growth, the ligands produced could be sensed by PDGFRA^{lo} cells in the mesenchyme compartment, which may respond by increasing Bmp genes and decreasing *Grem1* and *Wnt2b* production.

2 Materials and Methods

Animal and Tissue Processing

Animal experiments are conducted in accordance with Rutgers University Institutional Animal Care and Use Committee (IACUC).. The transgenic mice in this study are engineered to conditionally express the beta-catenin exon3 deletion. To induce recombination, tamoxifen (1mg/20g) is injected intraperitoneally for 4 days daily. Day 1 is considered the beginning of treatment, and mice are sacrificed and investigated at Day 10. This model is ideal for studying tumorigenesis *in-vivo* because of similarities in mouse and human gastrointestinal tracts. At Day 10, mouse intestines (duodenum) are collected and fixed in 4% paraformaldehyde solution overnight at 4°C. Tissues are then washed with phosphate-buffered saline (PBS) and dehydrated in increasing concentrations of ethanol. Tissues are then transferred to xylene and paraffin mixture for one hour, then 100% paraffin for another hour. Tissues are embedded for sectioning.

Organoid Forming Assay

Crypt-derived organoids are isolated from mouse duodenum on Day 10 after 4 consecutive treatment days of tamoxifen administered at 1mg/20g mice. Collected duodenum samples are washed in PBS, cut into ¼ inch pieces, and rotated in 3mM EDTA for 5, 10, and 20 minutes (each time

replace the EDTA). Tissues are then agitated and filtered through a 70μm filter for crypt enrichment. Crypts are then washed in PBS and the pellet is resuspended in BME-R1. Media for the crypts include advanced DMEM supplemented with GlutaMAX (auxiliary energy source for rapidly dividing cells), HEPES (used to maintain pH), EGF (epidermal growth factor: involved with cell signaling pathway controlling cell division), Noggin (prevents cell differentiation), NAC (N-acetylcysteine: protects against rise of internal oxidant levels), N2 (promotes *in vitro* differentiation of stem cells), B27 (promotes growth and proliferation, but not differentiation), RSPO (positively regulates Wnt/beta-catenin signaling, which induces proliferation), and Pen-Strep (prevents bacterial contamination). After 3 days of organoids being treated with this media, it becomes conditioned media (contains ligands excreted by epithelium).

Genotyping

Mice toes are clipped and used as a source for DNA genotyping analysis. DNA is extracted using the Kappa Buffer QIAgen Kit followed by PCR for amplification. Genotypes are confirmed by DNA electrophoresis.

Hematoxylin and Eosin Staining

To understand the changes in the epithelial architecture in terms of the formation of crypt progenitor cell phenotype (CPC), we conducted H&E histological analysis. Tissues are initially washed in xylene twice (5 min each). Then tissues go through two consecutive 100% EtOH washes (5 min each), followed by a 3 min 95% EtOH wash, a 3 min 85% EtOH wash, a 3 min 70% EtOH wash, and a 5 min double-distilled water wash. Unstained sections of mouse duodenum are stained with Hematoxylin (30 seconds) and dehydrated by dipping in water, followed by increasing alcohol percentages. Tissues are then counterstained with eosin for 1 minute (eosin in counterstain, which distinguishes between cytoplasm and nuclei).

RNA/DNA Preparation and qPCR

Mesenchyme cells are dissolved in Trizol. RNA is prepared according to the manufacturer's protocols. The RNA is then reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System. SYBR Green is used to amplify the cDNA through qPCR analysis. Vimentin is used for normalization.

Mesenchyme Treatment with Conditioned Media

The main experiment consists of using mesenchyme cells and treating them with (1) conditioned media from mutant organoids treated with CCM, (2) mutant organoids treated with mesenchyme media, (3) wild type organoids treated with CCM, and (4) mesenchyme media as a control. To demonstrate the response by the mesenchyme, changes in ligand expression are examined among treatment groups. After passaging mesenchyme cells, they are plated, and are treated with mesenchyme media on Day 1. Pictures of the cells are taken on Day 4, and the media is aspirated and replaced with conditioned media or mesenchyme media for control. The plate consists of 4 rows and 3 columns; rows 1 and 2 are made up of a confluent wild-type mesenchyme cells, and row 3 consists of sub-confluent wild type mesenchyme cells. Samples in the first column serve as controls and are treated with mesenchyme media, samples in the second column are treated with conditioned CCM from mutant organoids, samples in the third column are treated with conditioned mesenchyme media from mutant organoids, and samples in the fourth column are treated with conditioned CCM from wild type organoids. The fourth column is a control because the wild type organoids do not express mutant growth, so we expect there to be no change in Grem1 and Wnt2b expression. On Day 6, the cells are harvested with Trizol for RNA isolation, which is then converted to cDNA. For this experiment we measure 11 protein coding genes (*Bmp2*, *Bmp3*, *Bmp4*, *Bmp5*, *Bmp6*, *Grem1*, *Wnt2b*, *Rspo1*, *Rspo3*, *Wnt5a*, and *Wnt4*) using qPCR and compare their relative abundances.

3 Results

Tissue images are taken at Day 10 post-tamoxifen treatment and stained using hematoxylin and eosin. Figure 1 shows the histology of the control and mutant epithelium. The control and mutant epithelium for replicates 1 and 2 show little difference in terms of transformation. This demonstrates the delay stated before, suggesting that the mesenchyme is playing a role to suppress mutant epithelial growth.

To understand the mesenchyme response to ligands secreted by mutant epithelium, gene expression changes are examined following treatment with conditioned media derived from organoid cultures. qPCR (Figure 2A and Figure 2B) analysis demonstrated that confluent and sub-confluent mesenchyme cells do not respond in the same way. For the confluent cells, *Grem1* and *Wnt2b* tend to be downregulated. *Grem1* is a *Bmp* antagonist; therefore, by downregulating the gene *Bmp* can have a larger effect on the epithelium which would lead to a decrease in activity of the Wnt/beta-catenin pathway. For the sub-confluent mesenchyme cells, *Grem1* is downregulated in all experimental groups while *Wnt2b* is also downregulated except in cells treated with conditioned CCM from wild type organoids. Again, the conditioned media from mutant organoids are causing *Grem1* and *Wnt2b* to be downregulated in order to suppress mutant epithelial growth.

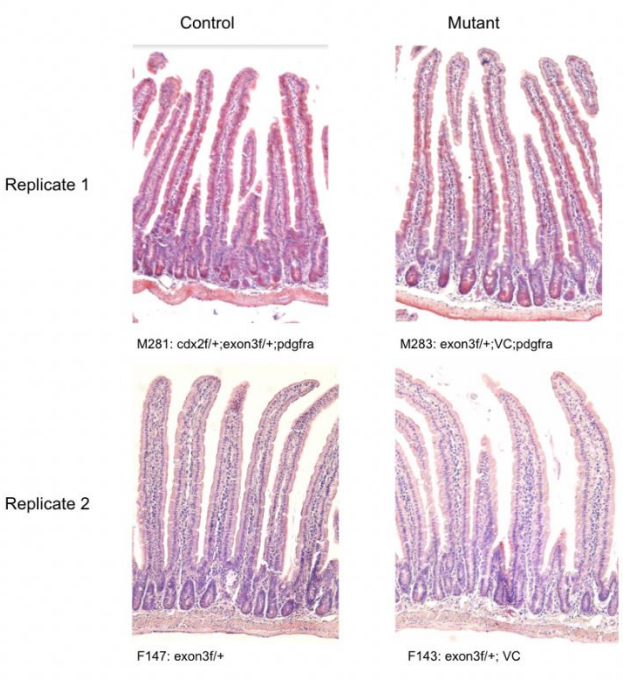


Figure 1: *H&E staining of two independent replicates showing the difference between the control and mutant epithelium.*

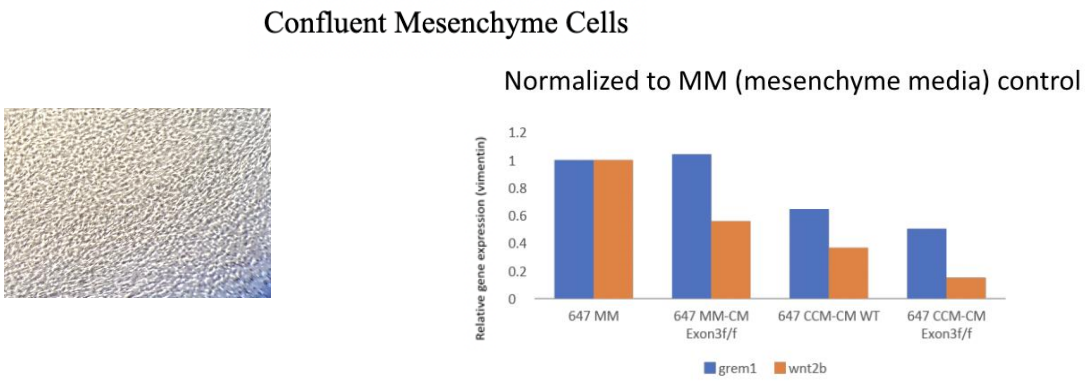


Figure 2A: *The image shows the density of the cells; cells are confluent. 647 is the confluent mesenchyme cell line. The leftmost bars represent Grem1 and Wnt2b expression in the control: mesenchyme cells treated with mesenchyme media. To the right of that is mesenchyme cells treated with mesenchyme conditioned media from mutant organoids (647 MM-CM exon3f/f). To the right of that is mesenchyme cells treated with CCM condition media from wild type organoids (647 CCM-CM WT), and the rightmost graph shows mesenchyme cells treated with CCM conditioned media from mutant organoids (647 CCM-CM Exon3f/f). The graph shows relative gene expression (n=3), compared to cells treated with mesenchyme media.*

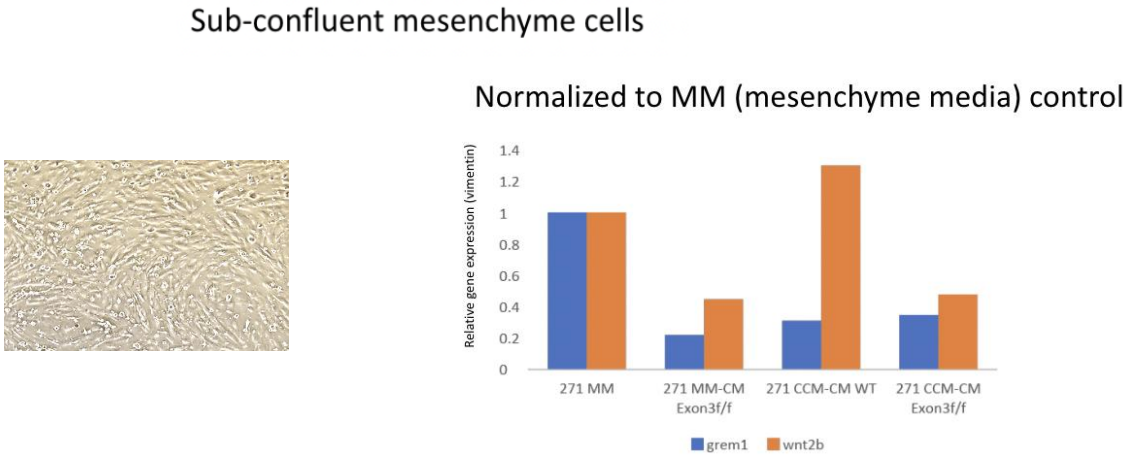


Figure 2b: *Image shows the density of cells; cells are sub-confluent. Graph shows relative gene expression of the three samples treated with conditioned media to the mesenchyme media for sub-confluent cells. Graph portrays same conditions as graph above except for sub-confluent cells.*

The graphs in Figure 3 demonstrate the relative gene expression seen across all 11 protein coding genes that were tested. The graph focuses on the mesenchyme conditioned media from mutant organoids vs. the control. The Bmp profile between the confluent and sub-confluent cells shows many similarities including *Bmp2* and *Bmp3* being downregulated, *Bmp4* staying constant, and *Bmp6* being upregulated. The upregulation in sub-confluent cells is much more profound. The Wnt profile in confluent and sub-confluent cells contain some differences like *Rspo3* being upregulated in confluent cells while being downregulated in sub-confluent cells. While *Wnt5a* is upregulated in both confluent and sub-confluent, *Wnt4* is downregulated in confluent, while profoundly upregulated in sub-confluent cells.

4 Discussion

The goal of the experiment is to identify possible signaling pathways that mesenchymal cells could use to communicate with epithelial cells. While the multitude of cells in the small intestine and their functions are known, like trophocytes, telocytes, and enterocytes (McCarthy et al., 2020), their ability to communicate with the microenvironment is understudied. The signaling crosstalk between the stroma cell population and the epithelium remains unknown. Understanding how these two types of cells may communicate can provide novel therapeutic interventions during initial tumor development. Histological review of H&E stained slides reported a lack of altered or abnormal tissue structures, suggesting that the mesenchyme is responsible for suppressing mutant epithelial growth. Protein expressing genes such as *Wnt2b*, *Grem1*, and *Bmp6* may be responsible for the cross talk between these two cell types. When conditioned media from mutant organoids is introduced to mesenchyme cells *Grem1* and *Wnt2b* are downregulated. The downregulation of Wnt prevents the expression of Wnt target genes, therefore controlling mutant transformation. *Grem1* in these cell lines is seen to be downregulated, which directly and indirectly (by inhibiting Bmp genes) prevents epithelial transformation. What is unusual is why most of the Bmp genes are either downregulated or stay constant while *Bmp6* is upregulated. Bmp signaling can be seen as the primary suppressor of dedifferentiation in the intestinal epithelium, and better understanding of its ligands will provide important steps to how the epithelium protects against oncogenesis (Perekatt et al., 2018). Although the Bmp gene expression is found to

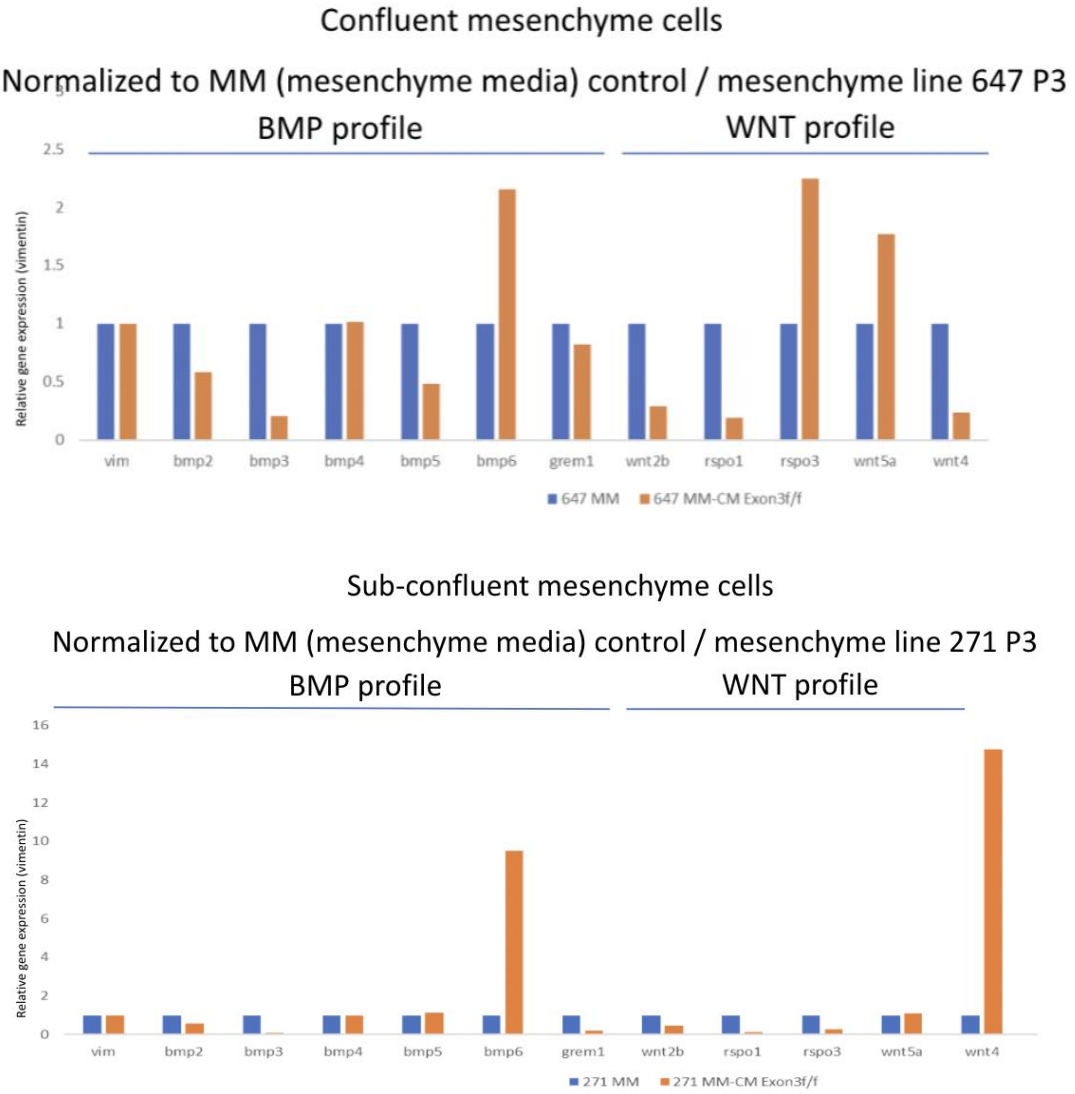


Figure 3: *Graphs show relative gene expression of the aforementioned 11 genes in sub-confluent and confluent mesenchyme cells. Graph compares the major experimental group to the control group. Because CCM is the primary media used to grow and sustain organoids, its data would be most resembled in-vivo conditions. This group would have organoids function normally so that the conditioned media would mimic in-vivo conditions, and then the mesenchyme response could be observed.*

not increase, this could be because *Grem1* expression is downregulated, leading to the translation of more Bmp proteins. The up and down regulation of Bmp, *Grem1*, and *Wnt2b* ligands may be, in part, how the mesenchyme is influencing epithelial cells, preventing early tumorigenesis.

The experiment demonstrated the differences between confluent and sub-confluent mesenchyme cells, as well as how different conditioned media affects mesenchyme cells. These differences are most profoundly seen in the genes *Bmp6* and *Wnt4*. Confluent cells have the ability to sense when they are running out of space, which can cause the cells to become less proliferative. Sub-confluent cells are rather in a more active growth phase. This could be affecting the signals the mesenchyme releases to the

epithelium, and responsible for the difference seen in response between confluent and subconfluent cells. These results come from a single experimental design and provide a basis for future experiments.

Bmp, *Grem1*, and *Wnt2b* may be signals that allow the mesenchyme to communicate with the transformed epithelium. These genes could be responsible for early prevention of initial tumorigenesis. These genes are potential ligands that suppress mutant epithelial growth. From here, geneticists could design experiments involving blocking these ligands with antibodies or treatment with these ligands to see the epithelial response from the mesenchyme■

5 References

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