Wnt/β- Catenin Signaling Pathway Function in Uterine Leiomyoma: Potential for Therapeutic Intervention

A thesis

submitted by

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In partial fulfillment of the requirements for the degree of

Master of Science

in

Pharmacology and Experimental Therapeutics

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

Date

May, 2016

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Abstract

Uterine leiomyoma, frequently known as fibroid, is a benign smooth muscle tumor that occurs in women between the ages of 30-55 years old. Uterine leiomyoma is a collection of hyperplastic uterine smooth muscle, along with endothelial cells, fibroblast, collagen, and sulfate proteoglycan. The prevalence f uterine leiomyoma is clinically apparent in up to 25% of women, with nearly 80 % of black women suffering from this condition. Symptoms include heavy menstrual bleeding, along with pelvic pain. Uterine leiomyoma is also the greatest contributor to infertility in the US. Currently, the mainstream method of treatment is hysterectomy, which can limit pain and bleeding symptoms, but conversely results in infertility. Major factors that may contribute to the proliferation of the leiomyoma are the decrease in the protein CCN5 and the expression of the gene HBP1--both of which are components of the Wnt signaling pathway, a fundamental pathway in normal development and in disease. CCN5 is a matricelluar protein, which has a role in cell proliferation, angiogenesis, and progression of certain cancers. CCN5 is equivalent to WISP2 (Wnt-Inducible Secreted Protein 2). There is a marked decrease of CCN5 in uterine leiomyoma as compared to the normal myometrium from the same patient. HBP1, an HMG Box transcription factor, is a cell cycle inhibitor and a transcriptional repressor of the Wnt signaling pathway. The chromosomal locus for HBP1 (7q22.3) is deleted in many fibroid tumors and HBP1 is thus a potential fibroid suppressor gene. Our preliminary results using qRT-PCR in an analysis of patient matched uterine leiomyoma and myometrium show a decrease in both the HBP1 and CCN5 mRNAs. Further analyses seek to define the Wnt signaling mechanisms and are focused on our lab's unique organotypic model of leiomyoma, in which human fibroid tumors are grown in immune-compromised mice. We plan to pharmacologically target Wnt signaling and /or CCN5 and HBP1 to potentially suppress fibroid growth. Such studies are

important preludes to discovering new pharmacological interventions in a disease whose only complete “cure” is full hysterectomy.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisors Dr. Amy Yee and Dr. John Castellot for their continuous support of my study and related research, for their patience, motivation, and immense knowledge. Also, I would like to thank Dr. Eric Paulson for his guidance in research and help in lab. I could not have imagined having better advisors and mentors for my master study.

I thank my fellow lab and classmates for the stimulating discussions, for the sleepless nights we were working together before exams, and for all the fun we have had in the last two years. I take this opportunity to express gratitude to the Department of Pharmacology faculty members for their help and support.

Most importantly, none of this could have happened without my family. I would like to thank: Dr. Yasmeen Nazli, Dr. Syed Tariq Ibrahim, Khadeeja, and Bilal for their love and support throughout my life. Especially, my beautiful sister, Khadeeja, every time I was ready to quit, you did not let me and I am forever grateful. This thesis stands as a testament to your unconditional friendship and encouragement.

Alhamdulillah.

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Introduction

Uterine leiomyomas, also known as uterine fibroids, are the most common benign tumor in women of reproductive age. (Walker CL, Stewart EA 2005). Uterine leiomyomas are derived from pelvic smooth muscle cells and are benign monoclonal tumors. They appear to grow and develop through abnormal collagen growth and differences in extracellular matrix, as compared to the myometrium. Leiomyomas occur in about 70% of Causican women and 80% of African American women by the age of 50 (Cardozo ER, et al. 2011). Another statistic currently estimates that one in every two women, of reproductive age, in the United States has fibroids, thus making it the most common tumor effecting women. The pathophysiology of fibroids is still unknown, however studies have shown that the growth of fibroids is driven by reproductive hormones, specifically estrogen and progesterone (C. Benassayag, et. al., 1998). Estrogens play a role in the cell proliferation, by upregulating the abnormal growth of the fibroids. A study on estrogen receptors, ER-α and ER- β, found that mRNA and protein expression is higher in the leiomyomas, as compared to the myometrium. (K. A. Kovács et. al., 2001). Ishika and other authors suggest that estrogen can regulate progesterone receptors, thus progesterone can have a role in leiomyomas. (H. Ishikawa et. al., 2010). Fibroids are also characterized to have increase levels in CYP450 aromatase, and this enzyme leads to the synthesis of androgen to estrogen. Several growth factors like VEGF, EGF, and IGF have demonstrated to play a role in the formation of fibroids.

Although some women are asymptomatic and undiagnosed, about 15-30% of women will have severe symptoms. The abnormal growth of fibroids in the uterus can cause excessive uterine bleeding, anemia, recurrent pregnancy loss, pre-tern labor, and pelvic discomfort.

Diagnosis of fibroids usually occurs through symptomatic presentation. Ultrasound detection of fibroids typically shows the uterus will be enlarged and irregular.

While treatments for fibroids can be surgical or medical, , the only curative treatment is a hysterectomy, which is a complete removal of the uterus. The less invasive procedures include myomectomies and selective uterine-artery embolization, for women who want to retain the option of having children. . However, recurrence of fibroids following these procedures is very common, greatly limiting their utility. None of the medical therapies are suitable for women who wish to bear children, although hormone replacement therapy is an alternative method for women who want symptomatic relief. Since fibroids are predominantly regulated by sex hormones, the hormone replacement therapy will reduce the size of the fibroids and relieve fibroid symptoms (Cornforth, 2015). Examples of some classes of hormone release therapies are selective estrogen receptor modulators (SERM), antiprogestrins, and birth control. However, in all cases when the hormone replacement therapy has been stopped the fibroids will begin to grow and sometimes return to their original size. The annual economic burden for uterine fibroids in women of reproductive age is about $5.9 billion and there is a need to discover a new treatment (Cowan, 2010).

The Wnt/β- catenin pathway

The Wnt/β-catenin pathway is important in embryonic development and maintenance of adult stem-cell niches such as colon crypts. Wnt signaling can activate cell cycle and maintain the undifferentiated or less differentiated phenotype. In the uterus, this pathway plays a role in the function of somatic cells between the myometrium and leiomyoma. The Wnt/β-catenin pathway has been shown to promote cell proliferation particularly in epithelial derived cancers

and active in stem cell populations. Frizzled is the major receptor for Wnt molecules, while the LRP5/6 proteins function as co-receptors. In the off state, when the Wnt molecule is not present, there is no dimerization of the Frizzle and LRP5/6 receptors, therefore no downstream effects of activation will occur. A destruction complex, that is composed of Glycogen Synthase Kinase 3 Beta (GSK-3 β), Axin II, Adenomatous polyposis coli (APC), and Casein Kinase 1 (CK1) is normally active in the absence of Wnt ligand binding. The destruction complex degrades β-catenin, thus preventing signal transduction by b-catenin. β-catenin destruction by the complex is initiated by GSK-3b, which phosphorylate four serine and threonine residues on β-catenin. The phosphorylated β-catenin, at serine residues 37 and 33, is ultimately recognized by β-transducin enzyme, which adds ubiquitin molecules. Ubiquitinated β-catenin is thus targeted for proteasomal degradation, as explained in Figure 1B.

During the activation phase of the Wnt/β-catenin pathway, there is an accumulation of β-catenin in the cell that will move into the nucleus. The frizzled receptor (FZ) bind to the Wnt ligand, leading Fzd/LRP5/6 interaction with a protein called Disheveled (DVL). Disheveled prevents the assembly of the β-catenin destruction complex. DVL binds to Axin II, allowing GSK-3b to phosphorylate the LRP5/6 receptor and causing an inhibitory feedback. The number of β-catenin molecules will increase due to decreased degradation. leading to accumulation of β-catenin in the cell and subsequent translocation to the nucleus. When β-catenin enters the nucleus, it will bind to TCF/LEF transcriptional factors that will activate a number of genes such as c-MYC and Cyclin D-1 (Sampson et. al., 2001.) as explained in Figure 1A.

One disease that occurs through the mutation of the destruction complex is familial adenomatous polyposis (FAP). The APC gene was originally discovered to be the culprit in the hereditary cancer. FAP patients, inheriting one defective APC allele, develop large numbers of

colon polyps, or adenomas, early in life. Loss of APC also leads to the inappropriate stabilization of β-catenin, implying that the absence of functional APC transforms epithelial cells through activation of the Wnt cascade. Colorectal cancer almost invariably initiates with an activating mutation in the Wnt cascade. (Kinzler et. al., 1996). This ties to the potential of Wnt- signaling in driving fibroid growth, because there could be a mutation in the Wnt cascade.

Wnt signaling is subject to significant inhibitory regulation. As one example the sFRP family members (soluble frizzled receptor proteins) are expressed extracellularly and bind to Wnt ligands, preventing Wnt receptor activation (Logan and Nusse, 2004). Another example is the Dickkopf (Dkk) small family of 24-29 kDa secreted glycoproteins (Niehrs, 2006). DKK1 acts as a Wnt antagonist indirectly, by preventing the Wnt -dependent interaction of LRP and frizzled (Bafico et al., 2001). In colon cancer, DKK1 is a downstream target gene of β- catenin and typically silenced in colon cancer by DNA hyper methylation (Gonzalez-Sancho *et al.*, 2005). Overexpressing DKK1 in colon cancer cells (Aguilera *et al.*, 2006) reduces colony formation and tumor growth in xenografts, suggesting a tumor-suppressor function for DKK1 (Aguilera *et al.*, 2006).

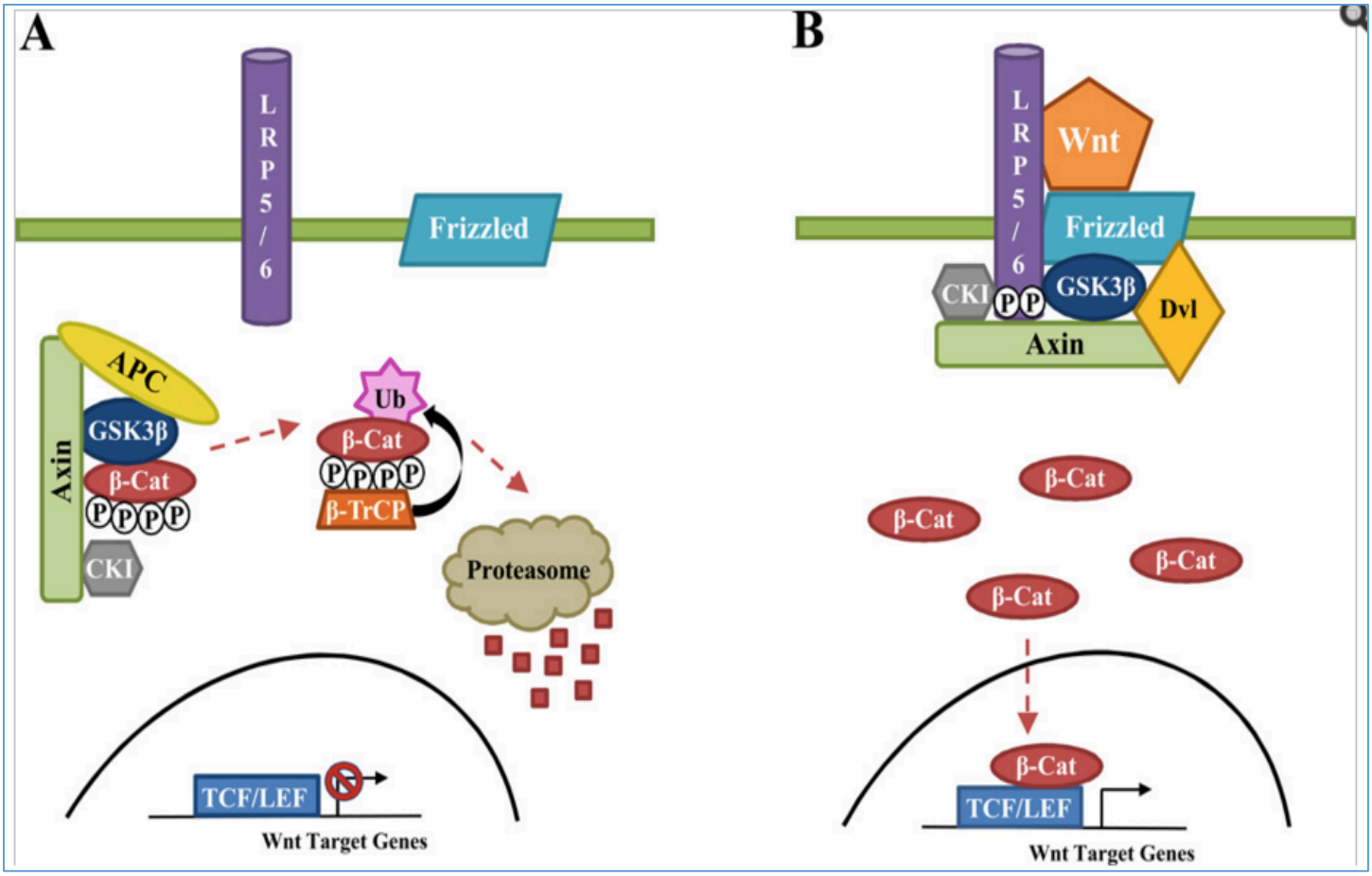

Figure 1: Wnt/ β- catenin pathway. *A. Represents the absence of the Wnt ligand leads to the destruction complex phosphorylating and ubiquitin β- catenin for degradation. B. In the presence of the Wnt ligand, the destruction complex will interact with the surface molecules. Therefore, β- catenin is stabilized and translocate the nucleus, where is works as a co activators and promotes the transcription of Wnt genes. (*Brain Res. 2013 Jun 13; 1514: 63–74. Published online 2012 Dec 19.)

HBP1 in Uterine Leiomyomas

HBP1 (HMG box transcription factor 1) is a sequence specific transcriptional regulator containing DNA binding domain called HMG box. HBP1 is a Wnt inhibitor, however, it was first identified through its interact with retinoblastoma protein (RB) and acts as a transcriptional repressor. HBP1 has been shown to repress gene expression both by preventing transcriptional activators from binding their target genes as well as by its direct, sequence-specific DNA binding activity (Escamilla-powers J.R et. al., 2010)

The cell cycle pathway is regulated by the retinoblastoma protein family that causes growth suppression through arrest in G_1 phase of the cell cycle. The regulation of cell cycle progression involved complex interaction of cyclin D kinases. During the cell cycle, phosphorylation of RB by cyclin and CDK kinases makes RB dissociated from E2F. E2Fs are required for the activation of numerous genes that are necessary for G1-to-S progression. The cell cycle then progresses through S phase and ultimately leads to increased cell proliferation (Tevosian S.G, et. al., 1997). Upregulation of HBP1 has been shown to promote cell cycle arrest (Shih H.H et. al., 1998).

HBP1 acts as a tumor suppressor gene by inhibiting β-catenin from binding to LEF/TCF and preventing expression of target genes. HBP1 represses Wnt/β-catenin growth regulatory genes, including cyclin D1, which is involved through cell cycle, and C- MYC, which is involved in cell cycle progression, apoptosis and cellular transformation. the interactions of HBP1 and RB are critical for premature senescence. As a transcriptional inhibitor, HBP1 has three mechanisms: direct repression through sequence-specific DNA binding, inhibition of transcriptional activators, or induction of heterochromatic regions. HBP1 directly represses

through a high-affinity element on target genes, N-MYC, and Macrophage Migration Inhibitory Factor (MIF) genes (Pan et. al., 2013).

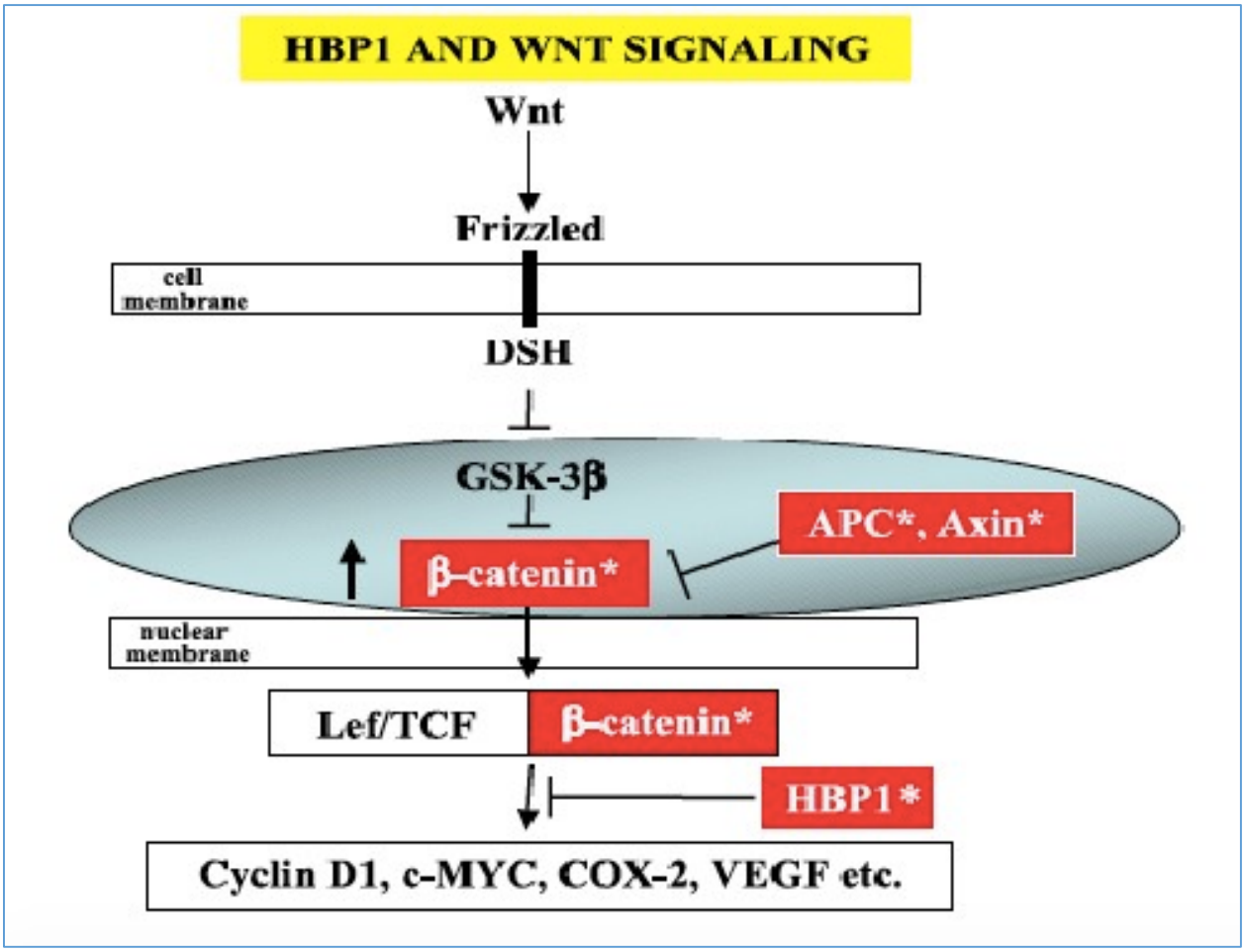

Figure 2: HBP1 in Wnt Signaling Pathway. *This figure shows that HBP1 is a negative regulator of Wnt signaling pathway and prevents the expression of downstream Wnt targets through interactions with TCF/LEF. (Yee Lab Figure)*

Even though fibroids are a common benign tumor disease that affects a significant percentage of women, the disease has no common genetic mechanism. However, cytogenetic mapping has suggested that several common chromosomal alterations occur in somatic cells in the myometrium of affected women. Approximately 40% of UL have non-random and tumor-specific chromosome abnormalities. This has allowed classification of some UL into well-defined subgroups which include deletion of portions of 7q and trisomy 12. Inactivation of one X-chromosome in normal female cells, have demonstrated that leiomyomas develop as clonal lesions. Initially, glucose-6-phosphate dehydrogenase (G6 PD) isoenzyme analysis was used to demonstrate the independent clonal origin of multiple tumors in a single uterus (Sandberg 2005).

Deletions involving X-chromosome, 7q22 -32, comprising a 30 Mbp, is one of the most common chromosomal aberrations associated with sporadic uterine fibroids. (Ptacek T. et. al., 2007).These deletions are present in about 35% of all uterine fibroids. In some leiomyomas, 7q deletion was the only cytogenetic abnormality observed. This suggests the existence of a tumor suppressor gene within the 7q21-32 region. In a recent study, loss of heterozygosity was observed in 57.5% of fibroids with 7q deletion (Hodge JC et. al., 2009). *HBP1* at 7q22.3 is of interest as loss of expression of this proliferation repressor has been associated with invasive breast cancer, suggesting the decreased expression found in del(7q) leiomyoma may contribute to the proliferative capacity of these tumors (Paulson et al., 2007), as explained in Figure 3 and 4.

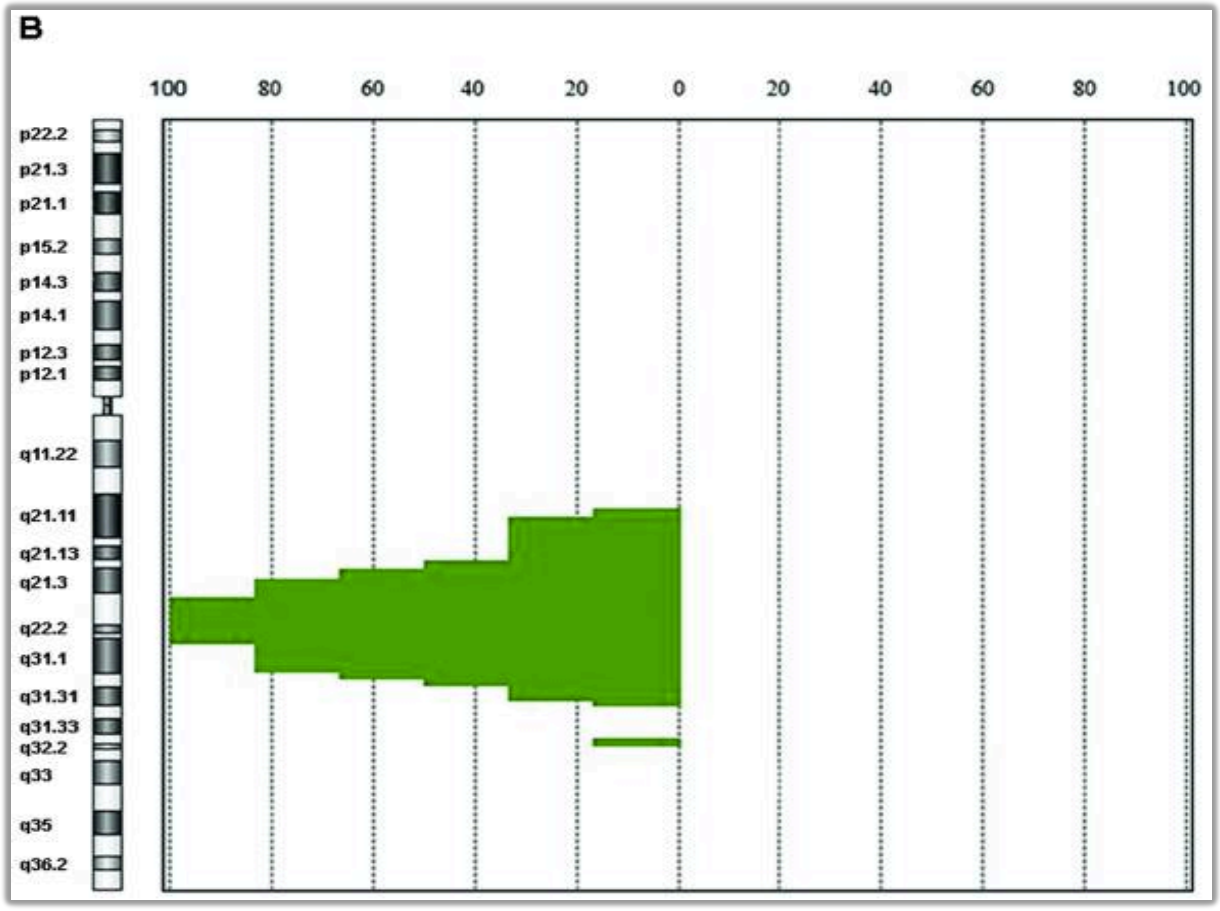

Figure 3: The 7q22.3 Region Containing the Wnt Signaling Inhibitor HBP1 is Commonly Deleted in Fibroid Tumor. *(B) Genomic penetrance summary of chromosome 7 showing the affected regions and in what percentage of the six cases they were found to be abnormal*. From the Morton Lab (Genes Chromosomes Cancer. 2009 Oct;48(10):865-85. doi: 10.1002/gcc.20692.)

Gene name	Protein	Accession number
SRPK2	SFRS protein kinase 2	NM_182691.1
PUS7	Pseudouridylate synthase 7 homolog (S. cerevisiae)	NM_019042.3
RINT1	Rad50-interacting protein 1	NM_021930.3
SYPL	Synaptophysin-like protein	NM_006754.2
PBEF1	Pre-B-cell colony enhancing factor 1	NM_005746.1
PIK3CG	Phosphoinositide-3-kinase, catalytic, γ polypeptide	NM_002649.2
PRKAR2B	Protein kinase, camp-dependent regulatory, type II, β	NM_002736.3
HBP1	HMG-box transcription factor 1	NM_012257.3
COG5	Component of oligomeric golgi complex 5	NM_181733.1
GPR22	G-protein-coupled receptor 22	NM_005295.1
DLD	Dihydrolipoamide dehydrogenase	NM_000108.2

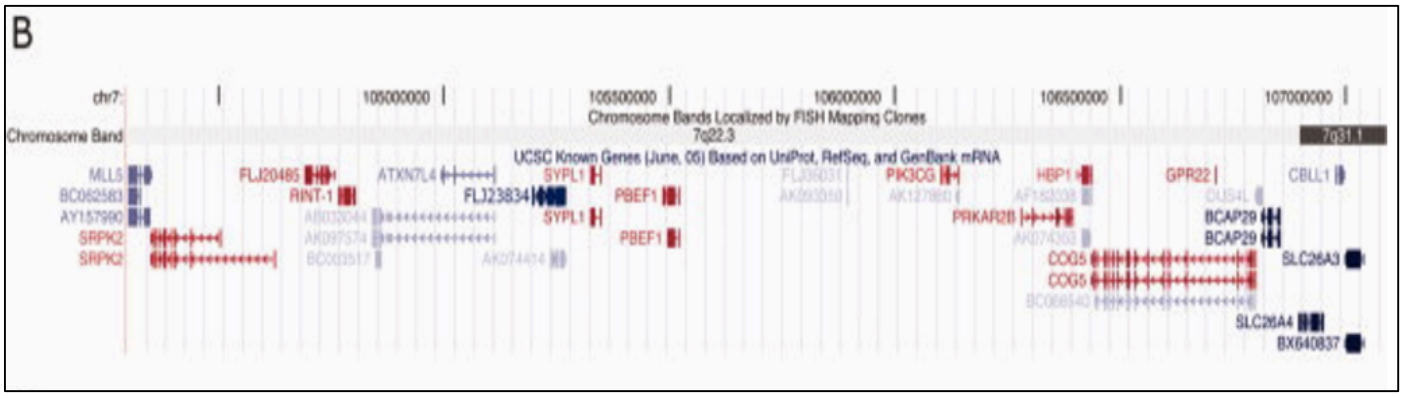

Figure 4: *(A)* ***Genes Screened in the Minimal Deleted Region.*** *Within this region, 10 manually annotated known protein coding genes. (B)* ***Delimiting plots and minimal regions of deletion on 7q22.3–q31.1 and 7q34.*** *Minimum deleted region of 7q22.2–q31. Genes highlighted in red have been further annotated by the VEGA project and were sequenced in this study. This figure shows the HBP1 is one of the 10 genes that is in the minimal deletion region of chromosome 7 (*Vanharanta et. al., 2007).

Loss of CCN5 Expression in Uterine Leiomyomas

CCN5 is a member of the CCN (**c**ysteine-rich 61-**c**onnective tissue growth factor-**n**ephroblastoma-overexpressed) family of genes, which has been implicated in many different cell functions including cell proliferation, migration, differentiation, apoptosis, angiogenesis, as well as tumorigenesis and fibrotic disease. The CCN5 protein, unlike the other CCN family members, lacks a carboxy-terminal domain and is an inhibitor of smooth muscle cell proliferation. These observations led the Castelot lab to hypothesize that CCN5 would have a biological activity profile opposite of the four-domain CCN members (Mason et al., 2004). CCN5 is a matricelluar protein, which has a role in cell proliferation, angiogenesis, and progression of certain cancers. CCN5 is also known as WISP2 (Wnt-Inducible Secreted Protein 2). CCN5 lacks the C-terminal domain thought to be important for the mitogenic activity of CCN2 (Brigstock *et al*., 1997); therefore, CCN5 inhibits rather than stimulates cell proliferation. Consistent with this idea, we have demonstrated that CCN5 inhibits vascular smooth muscle cell (VSMC) proliferation and motility. A study conducted by Castellot, showed that CCN5 was down-regulated in leiomyoma compared to matched myometrium in 10 of 10 uteri, through RT-PCR (Mason et al., 2004).

Med12 Mutation in Uterine Leiomyomas

MED12 is known as a mediator complex subunit 12 (*MED12*) gene, which consists of 26 subunit transcriptional regulators that bridge the DNA regulatory sequences to the RNA polymerase II initiation complex. (Taatjes 2010). The MED12 gene has been known to play roles in gene-specific transcription processes, and it is also involved in many developmental processes (Taatjes 2010). MED12 is localized on chromosome Xq13.1. Studies have shown that

the MED12 gene somatic mutations are associated with uterine fibroids in women from Finland, South Africa, North America, and Korea (Je et al. 2012). Moreover, it has been shown that MED12 is implicated in the transcription activation of WNT target genes by interacting with β-catenin (Kim et al. 2006). Together with MED13, CDK8, and Cyclin C, MED12 forms a Mediator sub complex known as a kinase or CDK8 module. This complex is suggested to have a role in transcriptional repression, but it also seems to act as a positive coregulatory of p53 target genes. MED12 also directly interacts with β-catenin and is required for the cellular response to Wnt signals (Makinen et al. 2011). Reduced expression of direct Wnt/β-catenin signaling target genes cyclin D1, Axin II, and MYC has been shown in MED 12 hypomorphic mouse embryos (Makinen et al. 2001).

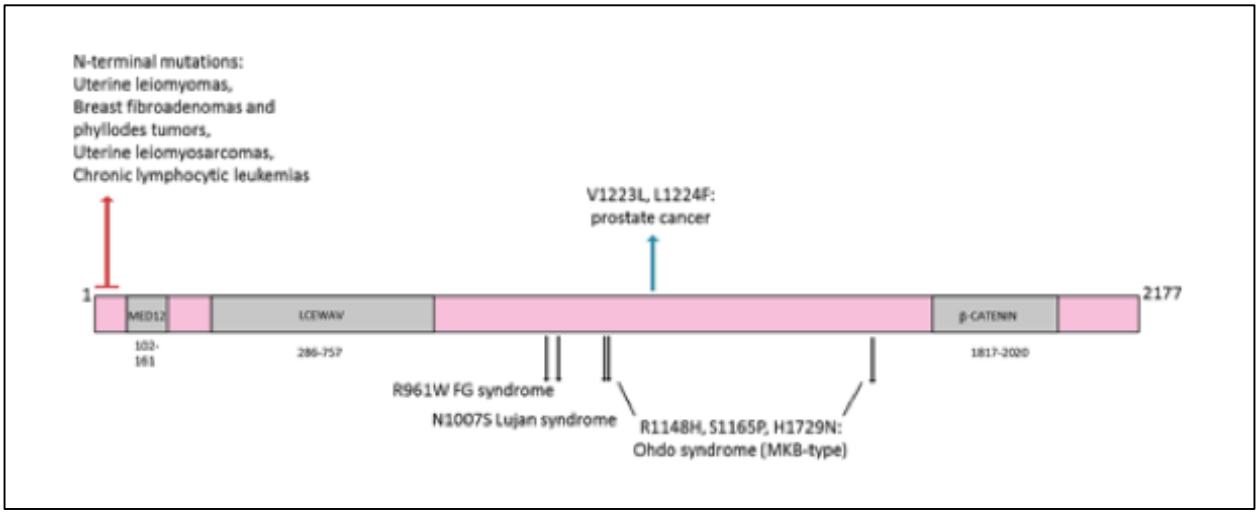

Figure 5: Somatic MED12 mutations in prostate cancer and uterine leiomyomas promote tumorigenesis through distinct mechanisms*. Somatic MED12 mutations are recurrently observed in various tumor types (above the amino acid alignment). Germline mutations in the gene cause X-linked intellectual disability syndromes* (The Prostate 2015).

Based on the observations described above, we hypothesize a decrease in both the HBP1 and CCN5 mRNA, as compared to the myometrium. Also, we predict an increase in the Wnt target genes like β-catenin and the mechanism of action would be a loss of HBP1 gene expression. The cytogenetic variation in the fibroids, compared to the myometrium, we hypothesize a decrease in MED 12 and estrogen receptors in the fibroids. To test these hypotheses, we performed the experiments detailed in the remainder of this thesis.

Materials and Methods

Myometrium and leiomyoma patient samples

Human myometrium and leiomyoma cells were obtained women under going a hysterectomy surgery from the removal of fibroids, at the Brigham and Women Hospital. For each patient multiple fibroids samples where removed, along with a normal myometrium sample. This allows for each patient to have a control sample, because the myometrium has no genetic and environmental changes. Each patient's fibroids were compared to the match myometrium that was received. Solid tumor masses were frozen in a -80^0 C. The myometrium and leiomyoma removed from patient 16 were provided in a small amount, there protein extracts could not be made for Western blots.

Protein Extraction and Western Blots

Leiomyoma and myometrium cells were lysated by using Ripa buffer including phosphatase and protease inhibitor (1:100 dilution). Protein quantification was done with Quant-it protein assay kit from Thermo Scientific. 40 μg of protein sample was added to 20 μl of a 1x BioRad Laemmli sample buffer, with β--mercaptoethanol, and RNase free water to have a total volume of 40μl. Protein extract was separated on BioRad gels with 4-15% gradient polyacrylamide gel, using gel electrophoresis and SDS-page. The gel was transferred to a polyvinylidene fluoride membrane and it was washed with PBS- Tween (1X PBS and 0.1%Tween) three times for 10 minutes each. The membrane was incubated with 5% bovine serum albumen (BSA) for at least an hour and then the primary antibody was placed on the membrane overnight, in the cold room. Membrane was washed with PBS and then incubated

with the secondary anti-rabbit antibody, with a dilution of 1:2000. The membrane was prepared for developing by using the Thermo Scientific West Femto Maximum Sensitivity Substrate; the two solutions were mixed in equal volumes of a 1000 ml. To develop the film, the membrane was exposed to film in the dark room for various times, depending on the antibody. After development, the membrane was stripped with 20 ml of stripping buffer containing 10% SDS, Tris-Cl, β-mercaptoethanol and RNase free water. The membrane was placed in a 53^0C water bath for 20 minutes and then washed with PBS Tween three times. The membrane can be stripped a total of three times before it has to be discarded.

Antibody	**Dilution**
Actin	1:20000
β- catenin	1:2000
P-GSK3 Beta	1:4000
T- GSK3 Beta	1:4000

Table 1: Antibody and dilution concentration used.

RNA extraction and purification

RNA was extracted from the cells by using the Trizol method. For each sample, about 20 μg of cell was added with 1000ml of Trizol in a glass daunce. The lysated cells were placed in a RNA free tube with 200 ml of Choloroform and centrifuged, in the cold room, at 10000rpm for 15 minutes. The clear aqueous solution was removed, which contains all the RNA and DNA, and placed in a clean RNA free tube with another 200ml of Cholorform and placed in the centrifuge.

The remain steps of the TriZol method was followed to obtain pure RNA. The RNA concentration was measured using a nano--drop spectrophotometer. An optimal OD 260/280 was required to get pure RNA with remaining protein extract.

cDNA Preparation and qRT-PCR Reaction:

cDNA was prepared using 400ng of purified RNA with the iScript Reaction mix, reverse transcriptase and RNase free water to a total volume of 20 µL. RT-PCR was performed using SYBR green Supermix and primers. Each sample was setup in triplicate and the reults were averaged. Gene expressions were normalized to 18S expression, which is an internal reference gene.

Primers

Primer	**Melting Point ºC**
HBP1	
CCN5	
Axin II	
MED 12	57.5
ESR 1	

Table 2. Primers that were used with melting points.

RNA Sequence and Ingenuity Pathway Analysis (IPA)

RNA from two patients, 16 and 17, with a myometrium and two fibroid samples, were submitted for RNA sequence at Tufts University. The data from the RNA Sequence was analyzed to fine genes that had a P value that was less than or equal to .05 and submitted to IPA.

Results

Loss of CCN5 mRNA Expression in Leiomyomas

As discussed in the introduction, decreased expression of CCN5, an inhibitor of smooth muscle cell proliferation, is a common feature of fibroid tumors. Therefore, we sought to test that the fibroids we obtained from our patient set did show this common molecular marker. The mRNA expression of CCN5 in the fibroids was measured by qRT-PCR using 18S RNA as an invariant internal control. In all four patients, there was a decrease in the CCN5 mRNA levels, with a range of mRNA levels from 50% of normal myometrium expression, Patient 17 TB, to almost no expression, Patient 16 TB (Fig. 6 and 7). These results confirm the previous findings of the Castellot lab (REFS) and confirm that our patient samples were consistent with being fibroid tumors.

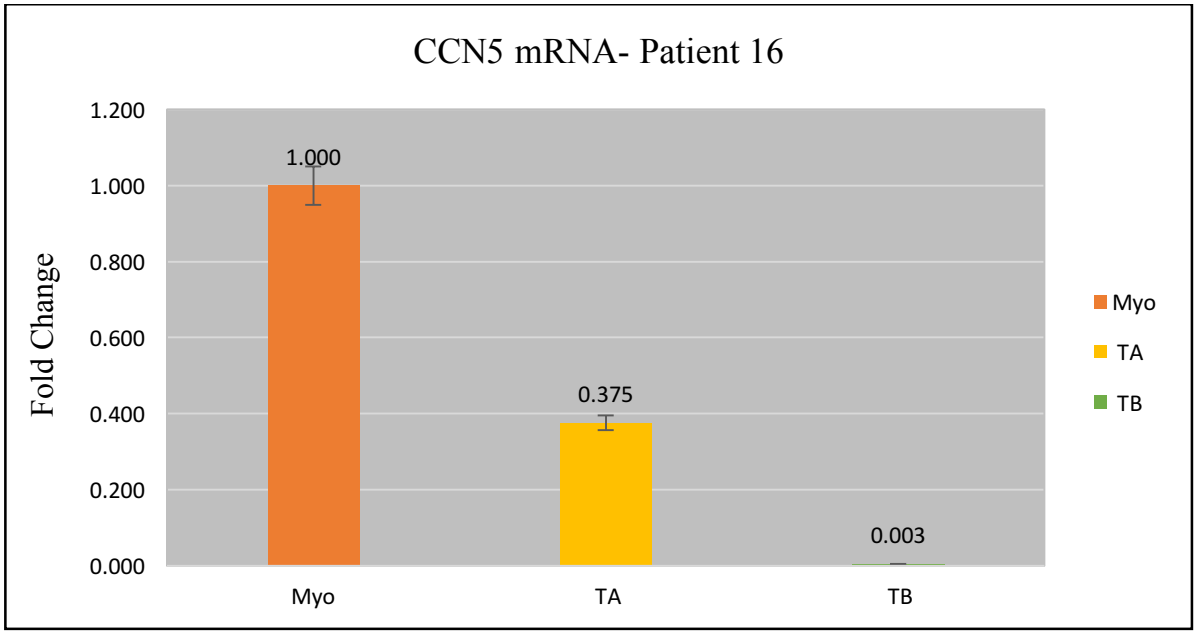

Figure 6: CCN5 mRNA expression levels in Patient 16. *Both tumor A (TA) and tumor B (TB) have a decrease in CCN5 mRNA expression level.*

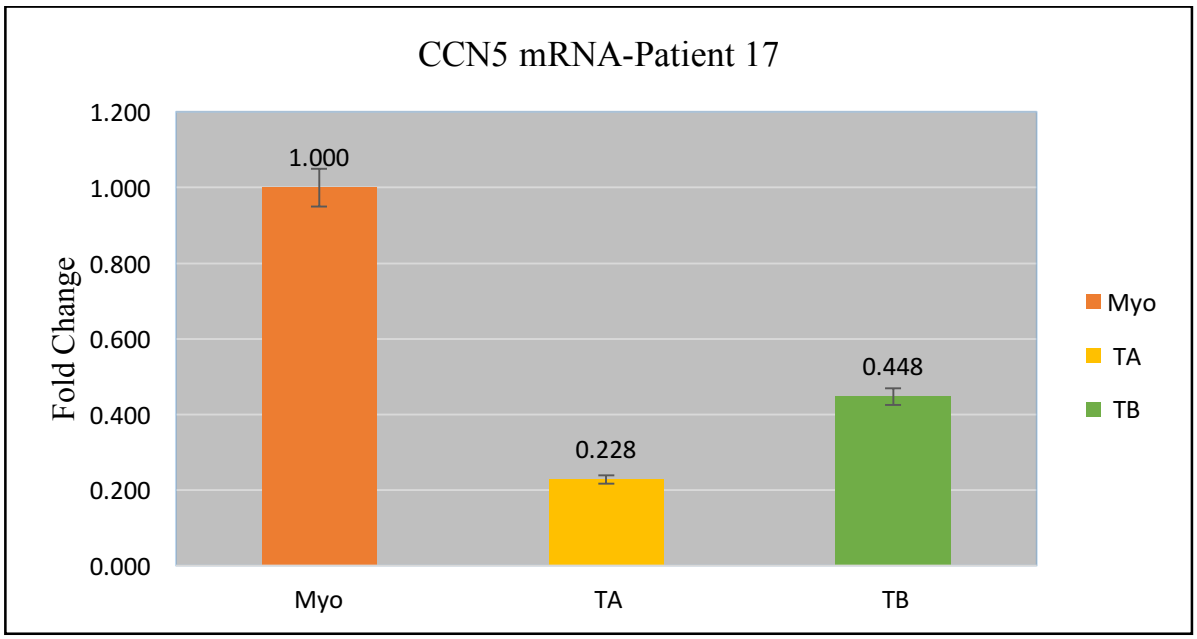

Figure 7: CCN5 mRNA expression levels in Patient 17. *Both tumor A (TA) and tumor B (TB) have a decrease in CCN5 mRNA expression level.*

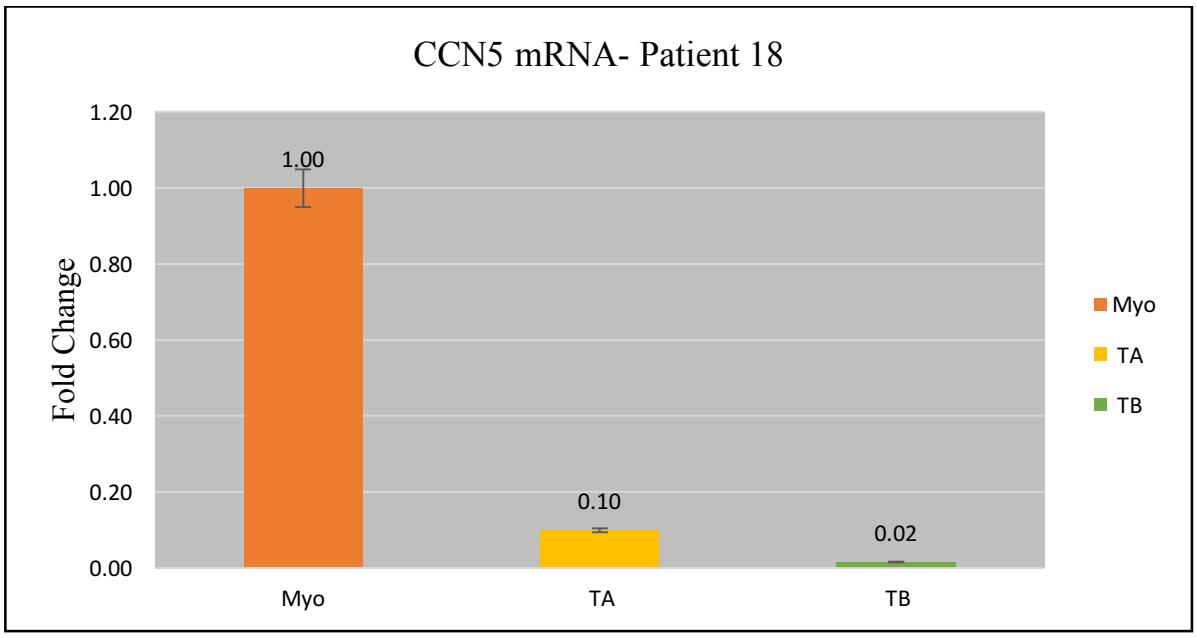

Figure 8: CCN5 mRNA expression levels in Patient 18. *Both tumor A (TA) and tumor B (TB) have a decrease in CCN5 mRNA expression level.*

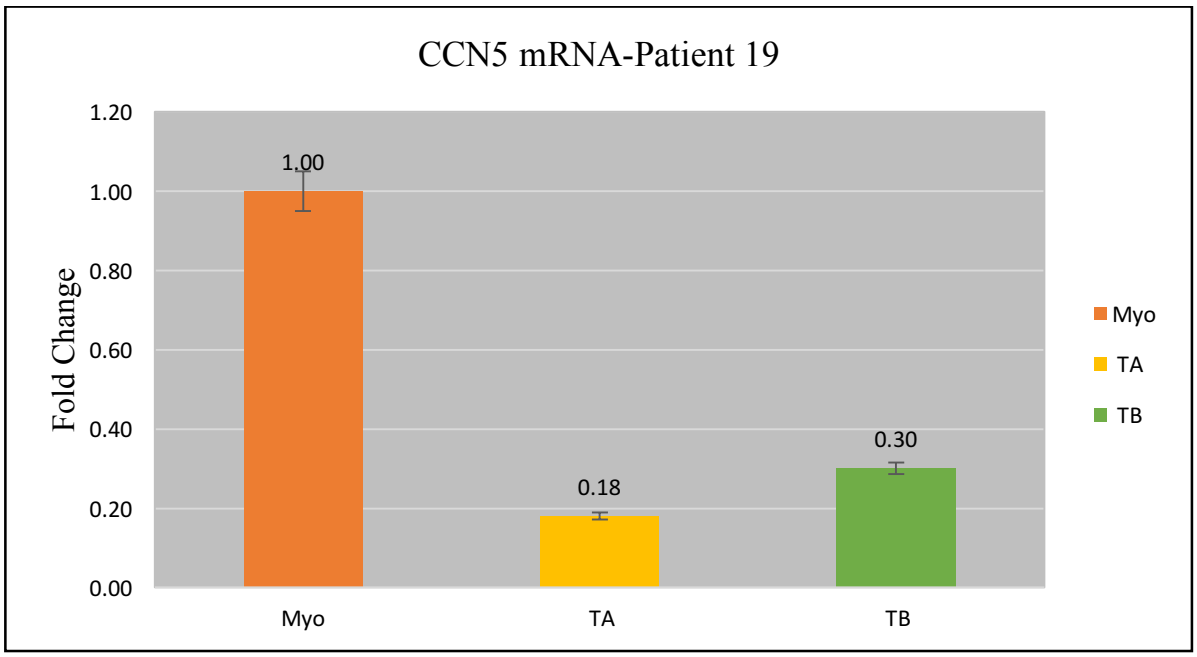

Figure 9: CCN5 mRNA expression levels in Patient 19. *Both tumor A (TA) and tumor B (TB) have a decrease in CCN5 mRNA expression level.*

HBP1 Expression in Leiomyoma

As discussed, the least common deletion in the 7q22-31 region encompassed 10 genes, of which HBP1 was one (Fig. 4). Given the role of HBP1 as a potential tumor suppressor and inhibitor of Wnt signaling, we hypothesized that decreased HBP1 expression, whether through genetics (i.e. 7q22.3 deletion) or another mechanism, contributed to human fibroid pathophysiology. Therefore, we performed experiments to examine if HBP1 expression was decreased in uterine leiomyomas as compared to the normal myometrium. We prepared mRNA from myometrium and fibroid tumors from four patient samples. This was a technological challenge, as the tumors are extremely fibrous, and extracting the RNA proved to require highly disruptive techniques to open the tissue and remove sufficient RNA for analysis. We determined the expression of HBP1 using qRT-PCR analysis, with 18S Ribosomal RNA as an internal

control. HBP1 mRNA levels varied between the fibroids within each patient, for the same patient. As seen in Figs. 10-13, 5 of 8 individual fibroid tumors did indeed have low HBP1 expression, while 3 of 8 had normal to even higher expression relative to myometrium from the same patients. The total percent of tumors with a low HBP1 gene expression was 62.5 %.

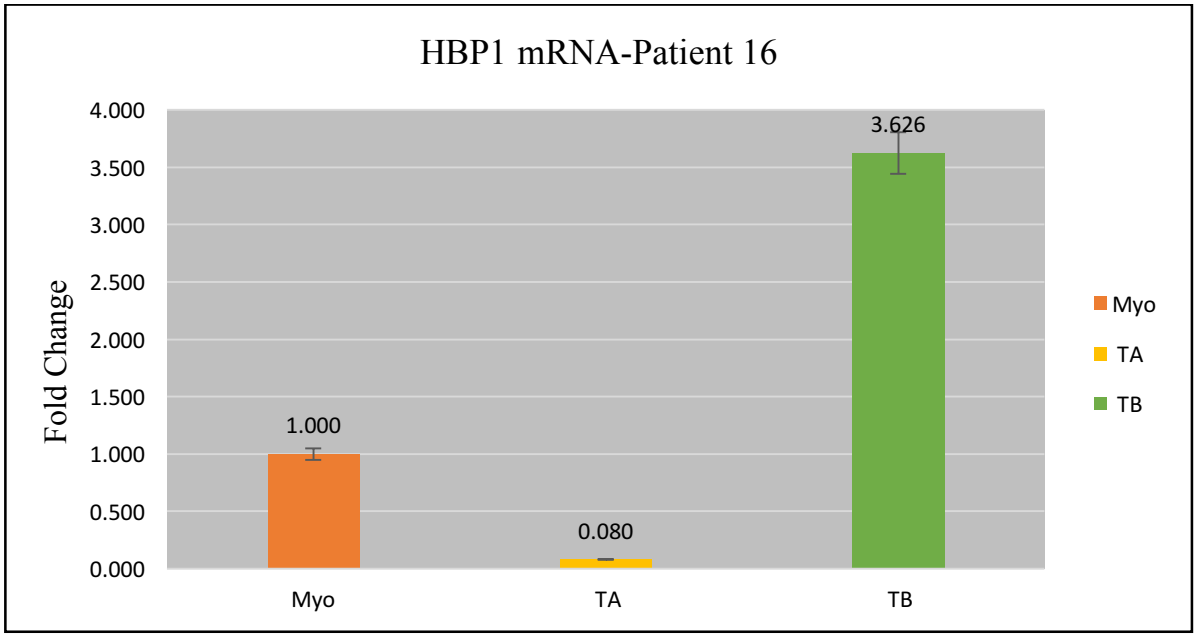

Figure 10: HBP1 mRNA expression levels in Patient 16. *In this patient sample, tumor A (TA) has decrease expression of HBP1 mRNA expression, as compared to tumor B (TB), which has an increase.*

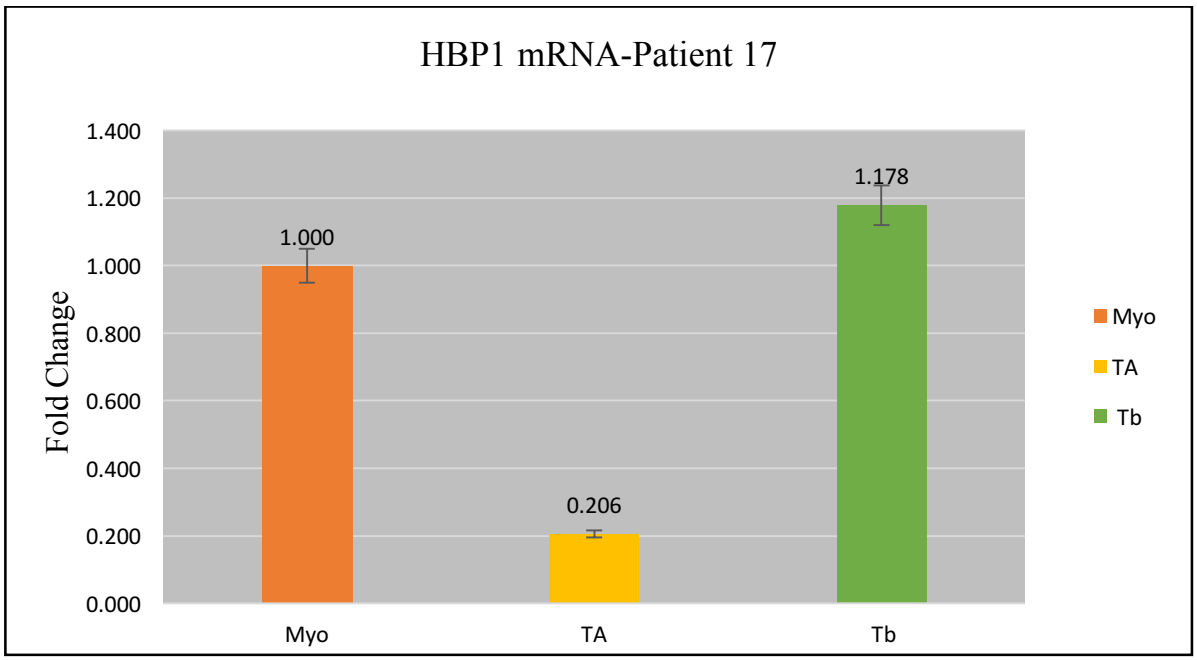

Figure 11: HBP1 mRNA expression levels in Patient 17. *In this patient sample, tumor A (TA) has low HBP1 expression and tumor B (TB) has an increase level of HBP1 expression.*

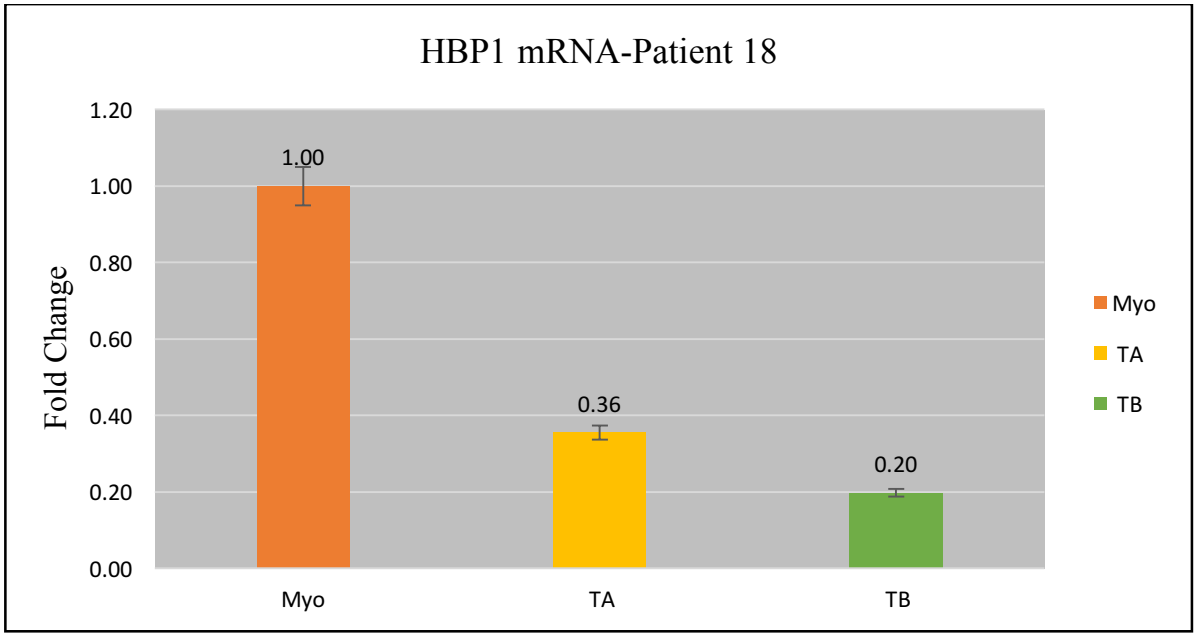

Figure 12: HBP1 mRNA expression levels in Patient 18. *In this patient sample, tumor A (TA) and tumor B (TB) has low HBP1 expression.*

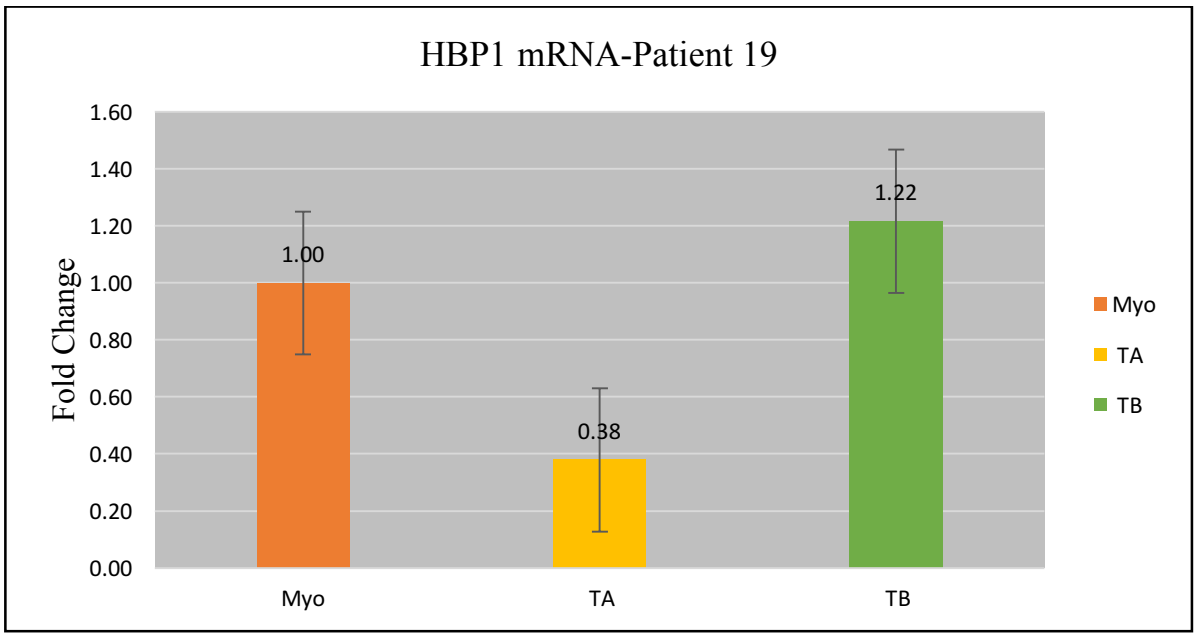

Figure 13: HBP1 mRNA expression levels in Patient 19. *In this patient sample, tumor A (TA) has low HBP1 expression and tumor B (TB) has an increase level of HBP1 expression.*

Wnt Pathway Activity in Leiomyoma and Myometrium

Decreased expression of HBP1 in many fibroid tumors suggested the possibility that these tumors had increased Wnt signaling. β-catenin antibody was used to probe if there is an increase in Wnt signaling in the HBP1-low expressing leiomyoma as compared to the myometrium. Unexpectedly, there was a large increase in β-catenin in almost all the tumors relative to control myometrium from the same patients, suggesting that activation of Wnt signaling was universal for fibroid tumors. To provide further evidence of Wnt signaling activation, phosphorylation of GSK3b at serine 9 was analyzed by western blot. Phosphorylation of serine 9 is a measure of GSK3b inactivation induced by Wnt ligand (see introduction), and thus the increase in b-catenin. The quantification showed lack of phosphorylated GSK3b in the myometrium and induction in the leiomyoma and was completely consistent with the β-catenin

data. This data further confirmed that Wnt signaling is activated in fibroid tumors regardless of HBP1 expression.

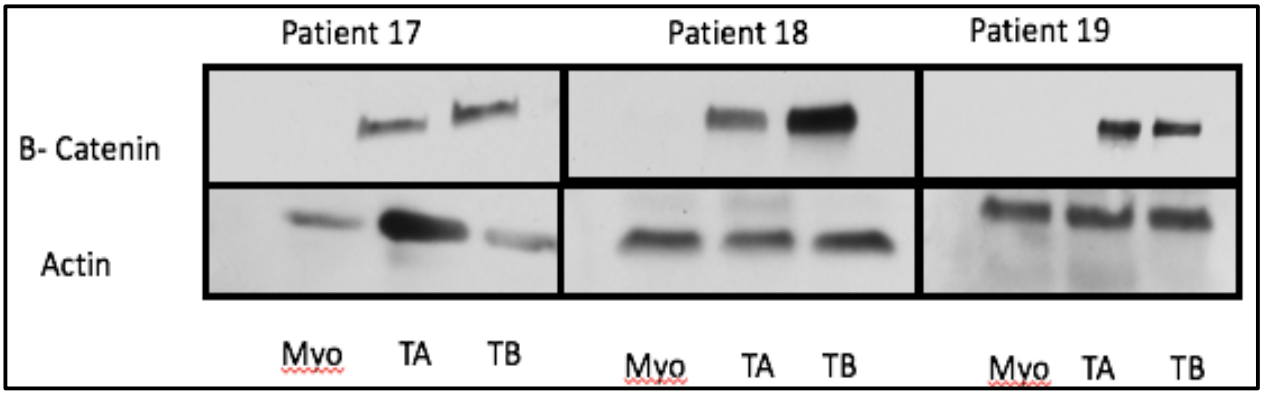

Figure 14: Western blot analysis of B- catenin expression in patient matched myometrium and leiomyoma. *All the myometrium patient samples have no expression of / β- catenin protein, while tumor A (TA) and tumor B (TB) have an increase in the expression. This explains the increase in Wnt/ β- catenin activation in the leiomyoma.*

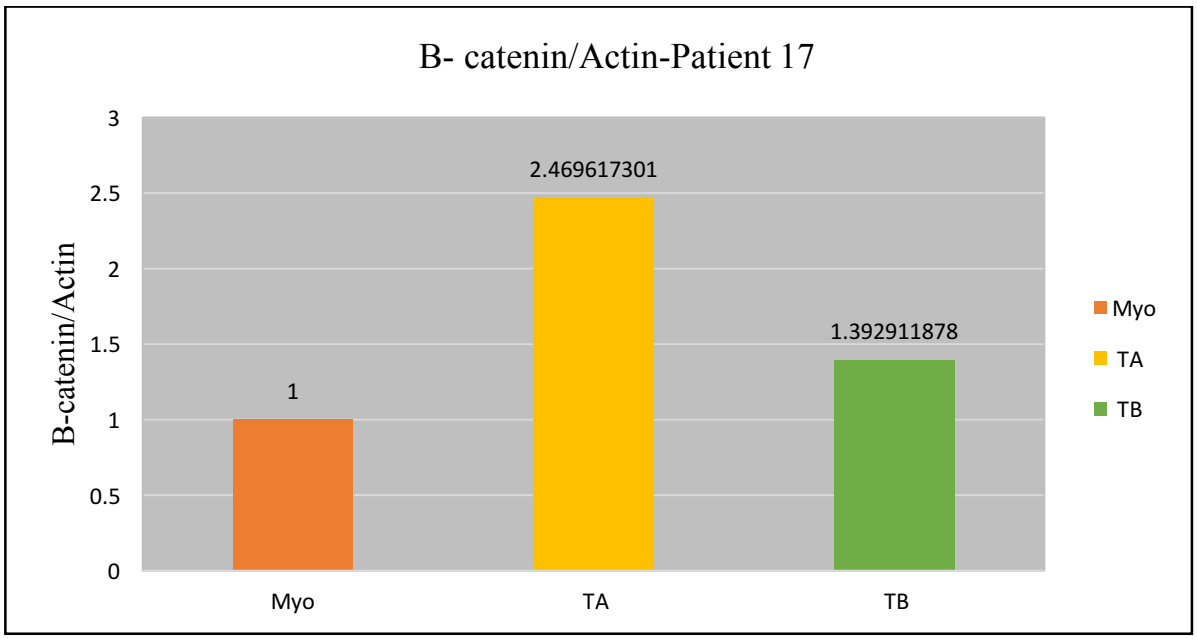

Figure 15: Densitometry quantification of Western Blot data of Patient 17. *Image J was used to quantify Western blot data.* The analysis shows the increase in Wnt/ *β- catenin pathway in the leiomyoma*.

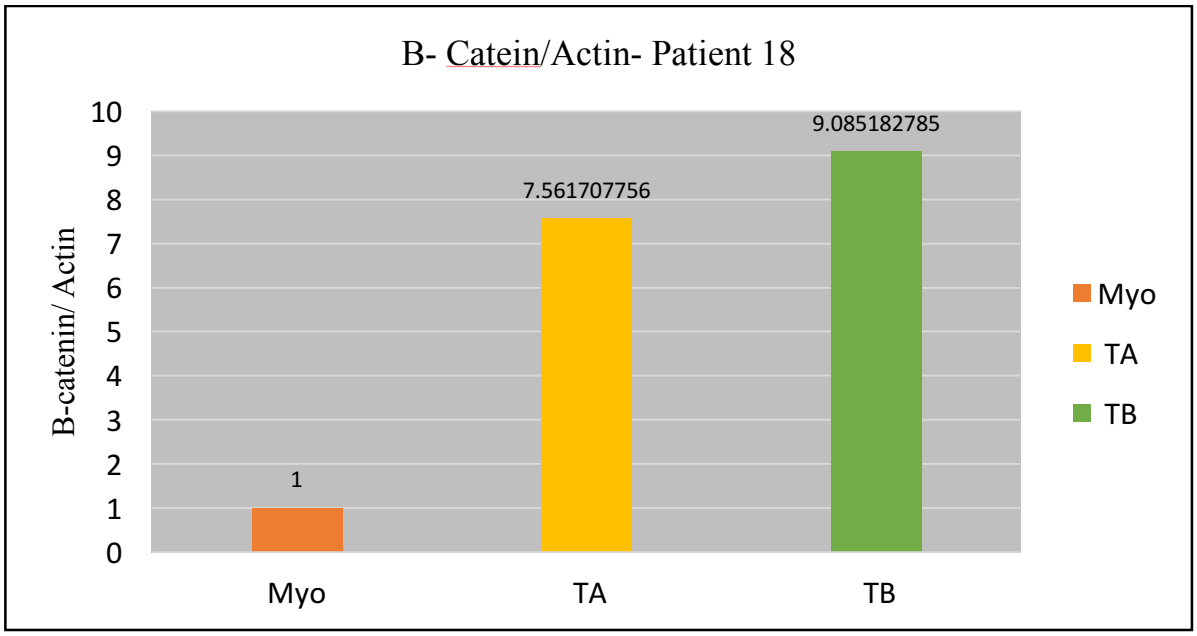

Figure 16: Densitometry quantification of Western Blot data of Patient 18. *Image J was used to quantify Western blot data.* The analysis shows the increase in Wnt/ *β- catenin pathway in the leiomyoma*.

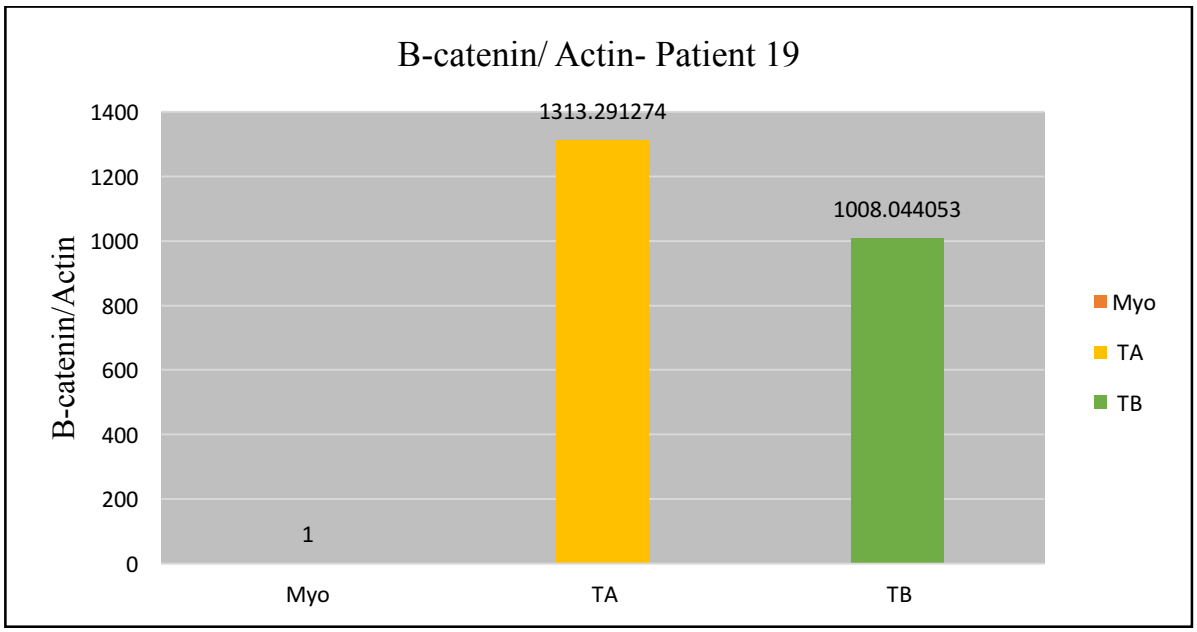

Figure 17: Densitometry quantification of Western Blot data of Patient 19. *Image J was used to quantify Western blot data. The analysis shows the increase in Wnt/ β- catenin pathway in the leiomyoma.*

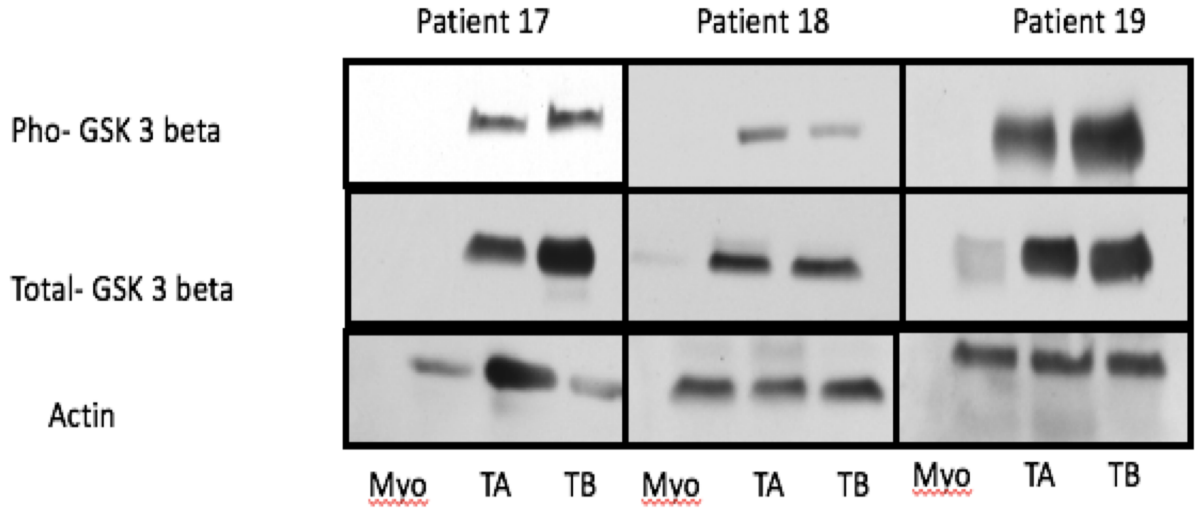

Figure 18: Western blot analysis of Phosphorylated GSK 3 Beta and Total GSK 3 Beta expression in patient matched myometrium and leiomyoma. *All the myometrium patient samples have no expression of Pho- GSK 3 beta and Total- GSK 3 beta. While tumor A (TA) and tumor B (TB) have an increase in the expression. This explains the increase in Wnt/ β- catenin activation in the leiomyoma.*

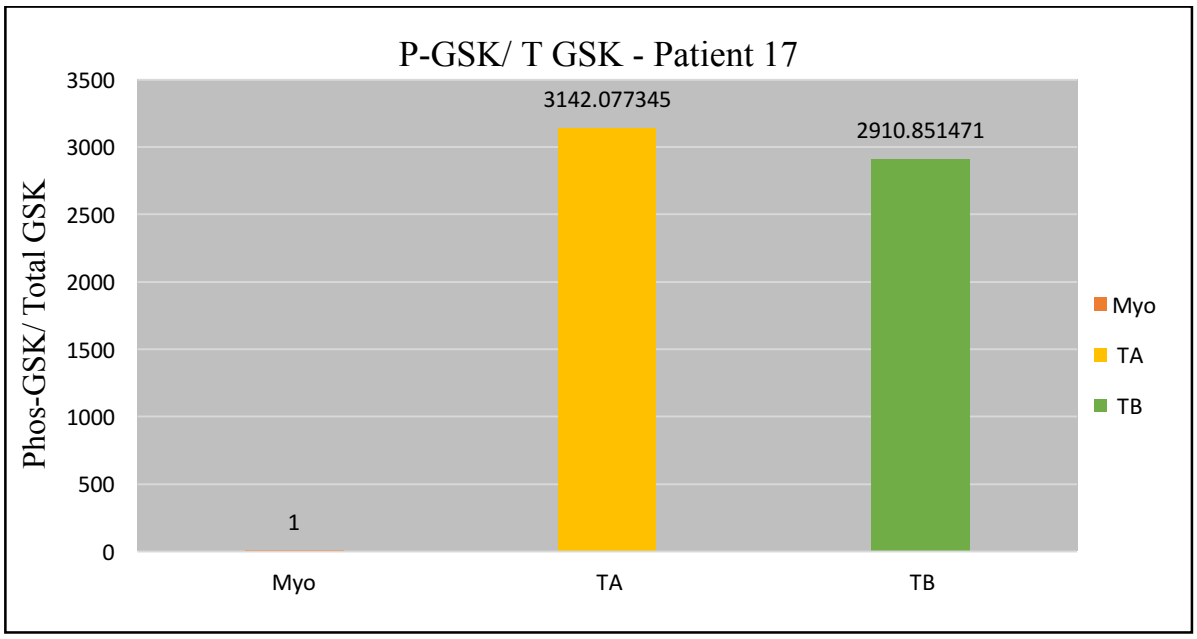

Figure 19: Densitometry quantification of Western Blot data of Patient 17. *Image J was used to quantify Western blot data. The analysis shows the increase in GSK 3 beta in the leiomyoma.*

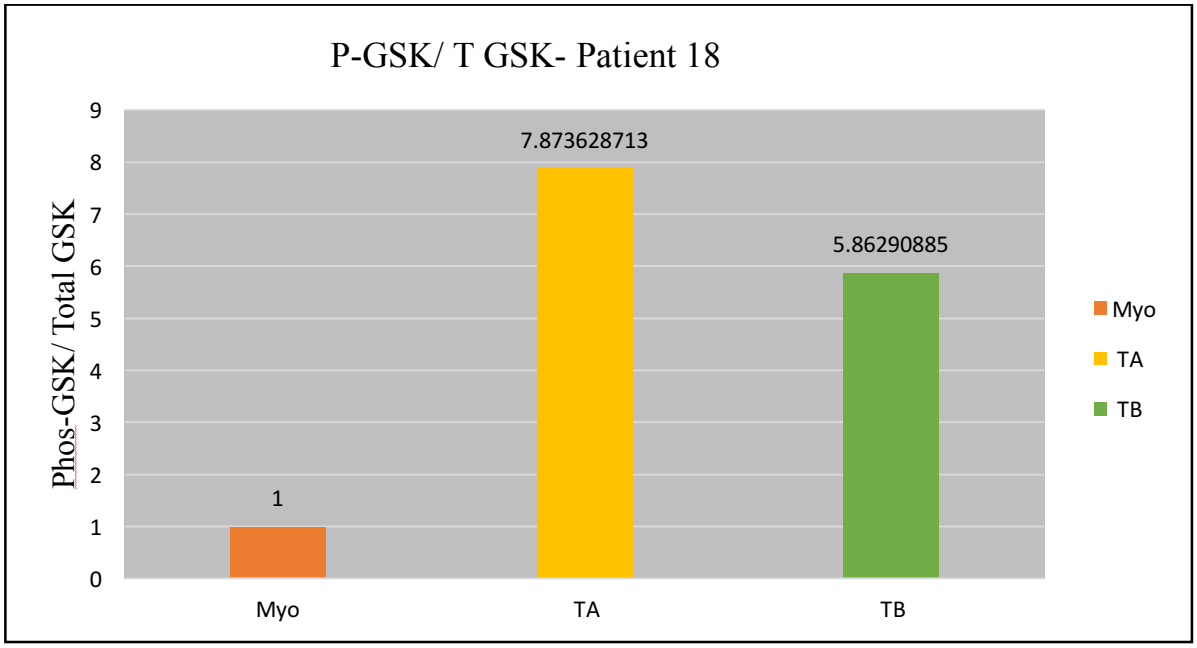

Figure 20: Densitometry quantification of Western Blot data of Patient 18*. Image J was used to quantify Western blot data. The analysis shows the increase in Pho- GSK 3 beta and Total-GSK 3 beta in the leiomyoma.*

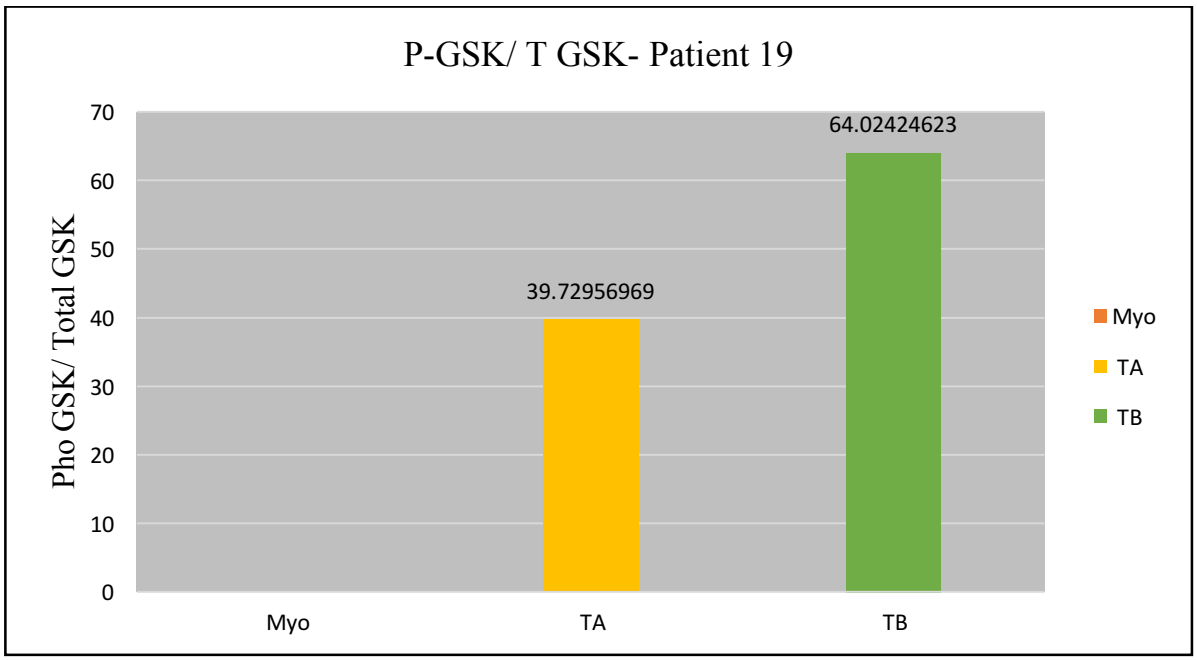

Figure 21: ***Densitometry quantification of Western Blot data of Patient 19****. Image J was used to quantify Western blot data. The analysis shows the increase in Pho- GSK 3 beta and Total-GSK 3 beta in the leiomyoma.*

In the data presented above, the was a significant increase in β- catenin and phosphorylated GSK3b, thus suggesting an increase in Wnt pathway in fibroids, compared to the myometrium. As previously stated, the proposed mechanism of action for the increase in β-catenin was a loss of HBP1 gene expression. However, the HBP1 data did not explain the variation in Wnt signaling between the patient samples and myometrium. As another test of downstream Wnt signaling activity, we measured expression of a Wnt target gene, Axin II. Interestingly, Axin II, is also part of the b-catenin destruction complex and so is also a Wnt effector. We determined the expression of Axin II using qRT-PCR analysis, with 18S Ribosomal RNA as an internal control. A broad analysis of Axin II was not explored. As shown in Figs. 21-24, there was a decrease in many but not all the fibroids. One exception is Patient 17 TB, which had an HBP1 low profile and high β-catenin, which may have been sufficient to

maintain Axin II expression. However, the overall decrease in Axin II expression was a surprise. Nevertheless, we did not undertake a broad analysis of Wnt target gene expression, which may have provided a better picture of downstream Wnt activity. Many genes are regulated by multiple signaling pathways, and the Axin II results may simply be an outlier. As we will show in the next section, that conclusion is likely correct as additional evidence suggests that there is significant Wnt target activation.

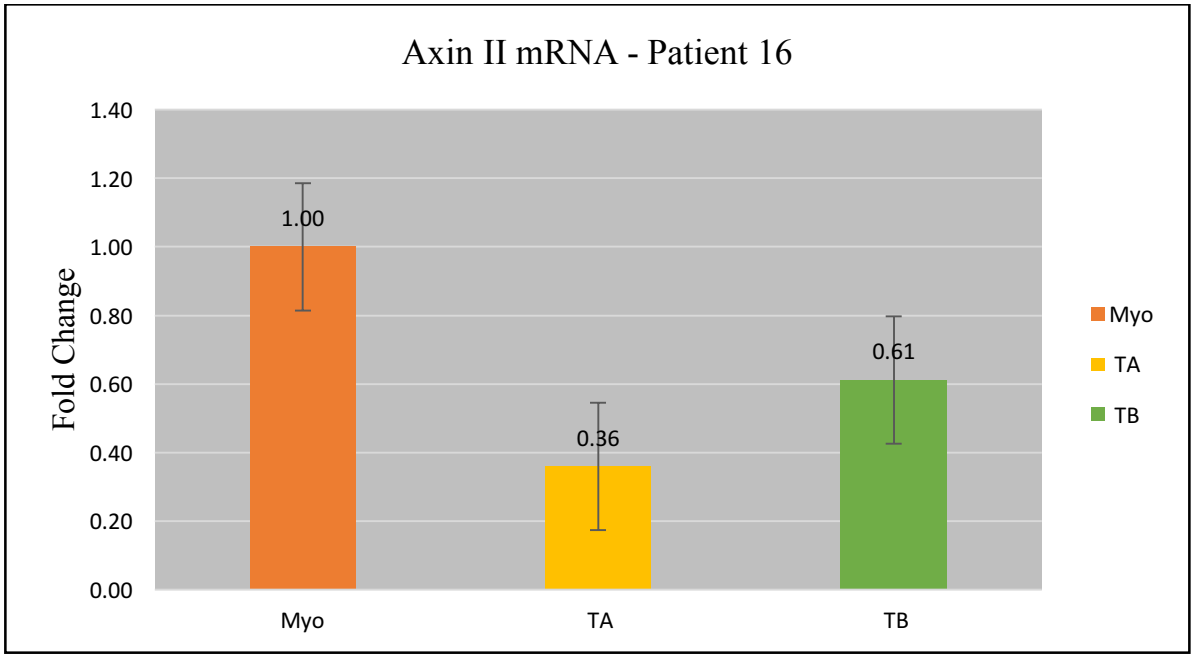

Figure 22: Axin II mRNA expression levels in Patient 16. *Both tumor A(TA) and tumor B (TB) have a decrease expression of Axin II, compared to the myometrium.*

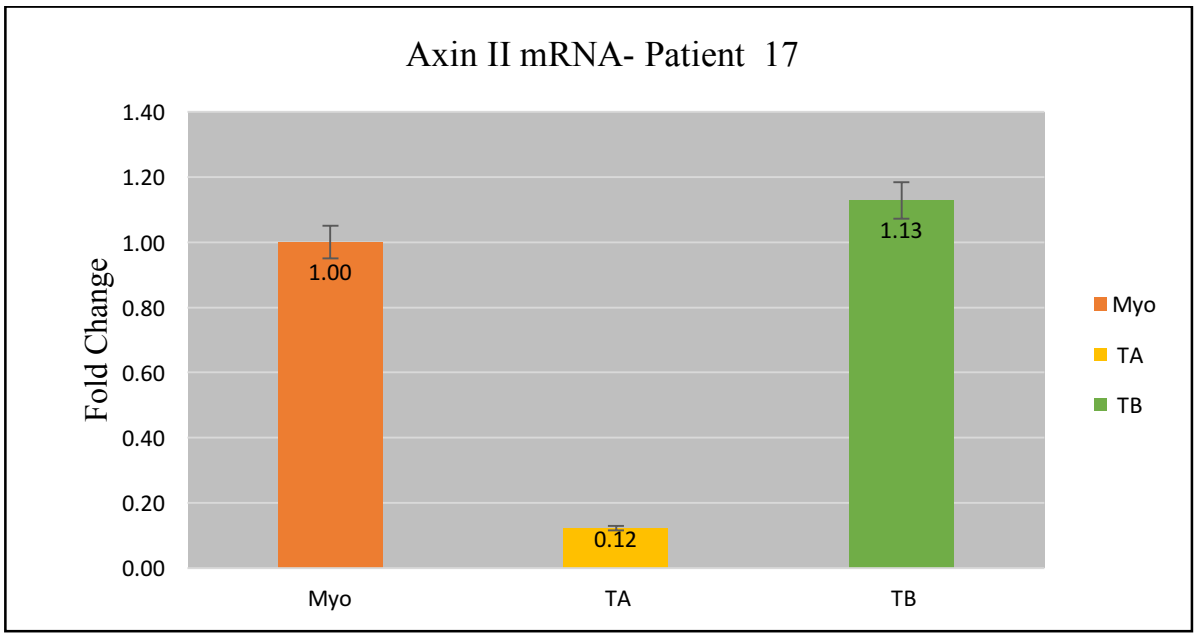

Figure 23: Axin II mRNA expression levels in Patient 17. *Tumor A (TA) has a decrease expression and tumor B (TB) have an increase expression of Axin II.*

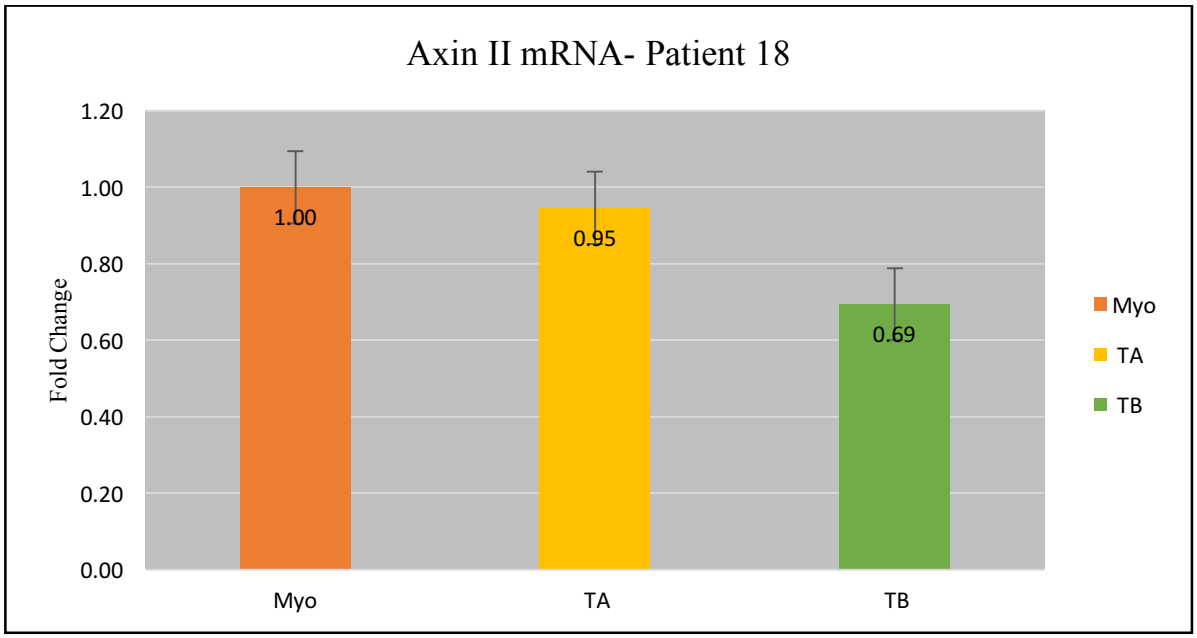

Figure 24: Axin II mRNA expression levels in Patient 18. *Both tumor A (TA) and tumor B (TB) have a decrease expression of Axin II, compared to the myometrium.*

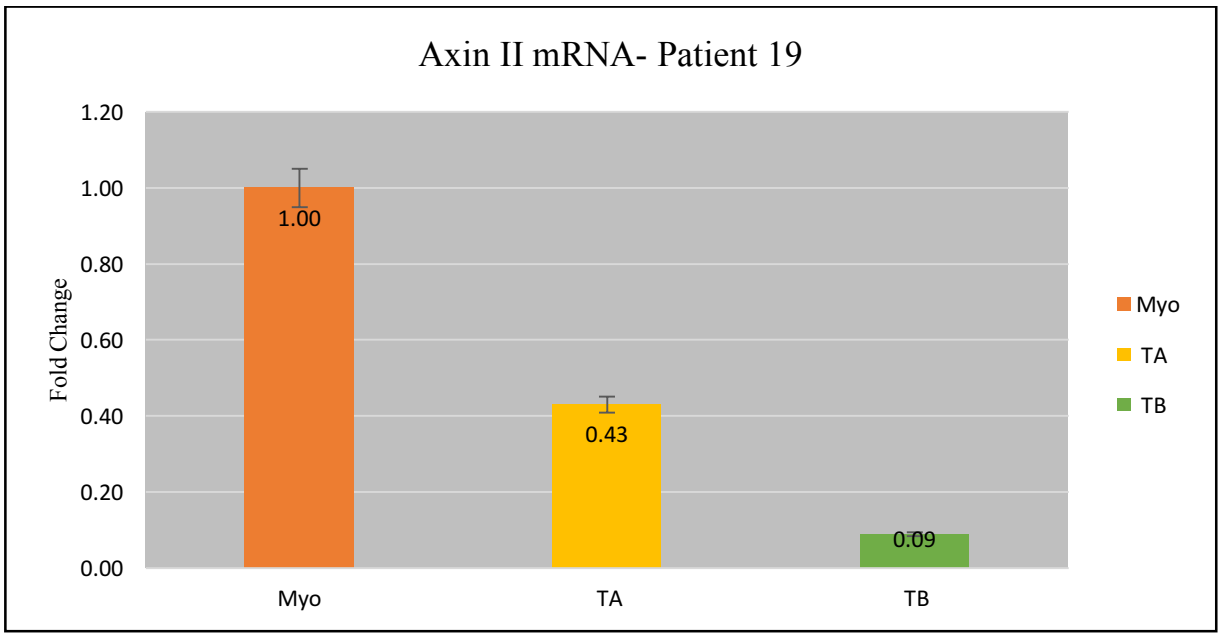

Figure 25: Axin II mRNA expression levels in Patient 19. *Both tumor A (TA) and tumor B (TB) have a decrease expression of Axin II, compared to the myometrium.*

Bioinformatic Analysis of Fibroid Tumors

Our initial observations, consistent with the published fibroid genetics in the 7q22-31 region encompassing the HBP1 gene, suggested that there was a set of fibroid tumors with low HBP1 expression, as well as tumors with normal HBP1 expression. However, all tumors analyzed suggested that elevated Wnt signaling was a common feature of most fibroid tumors. To identify common and unique aspects of fibroid tumor biology, we decided to perform a transcriptomic analysis of control myometrium and tumors from patients 16, 17 and 19, followed by an unbiased bioinformatic analysis. RNA from patients 16, 17 and 19 was sequenced using RNAseq in the Tufts Core facility, with moderately deep sequence analysis of 25-28 million reads. After normalization (Tufts Core Facility) individual gene transcript levels for each tumor

were obtained and individual pairwise analyses performed to determine mRNA changes between samples.

As an initial bioinformatic test, we examined whether decreased HBP1 expression had an effect on tumor function. The RNA levels of patient 16 tumor B and patient 17 tumor A, which had low HBP1 relative to their control myometrium, where compared to each other, producing a list of negative and positive regulated genes. The list was culled to use only genes with a p value of less than or equal to .05 and 216 up-regulated and 46 down-regulated genes were common between the samples. An unbiased analysis of the gene expression data was done using Ingenuity Pathway Analysis (IPA). IPA is a bibliographic knowledge base that provides multiple platforms to organize data based on criteria such as signaling pathway organization and activation, as well as disease or biological function prediction. Our main goals in this initial analysis were to elucidate there were common upstream biological causes and downstream effects on cellular changes in fibroids with a decrease in HBP1 gene expression.

As shown in Figure 25, one of the top biological pathways in the HBP1 low fibroids is Wnt/ β- catenin pathway. However, IPA stated that there is a negative z- score. But a closer look at the Wnt/ β- catenin pathway analysis shows that the only downregulated gene in the signaling pathway is Oct- 4, which is required for the correct maintenance of pluripotent cells (Figure 26). β- catenin is highly upregulated in the pathway, thus correlating with the western blots previously shown in the results. Interestingly, there are about 30 genes that are directly upregulated through β- catenin and 2 genes that are downregulated. The biological active functions in the HBP1 low genes specifically includes cellular growth and proliferation, cellular development, and tissue development. This correlates to the idea that fibroids are benign tumors that have a change in the cell morphology compared to the myometrium (Figure 28).

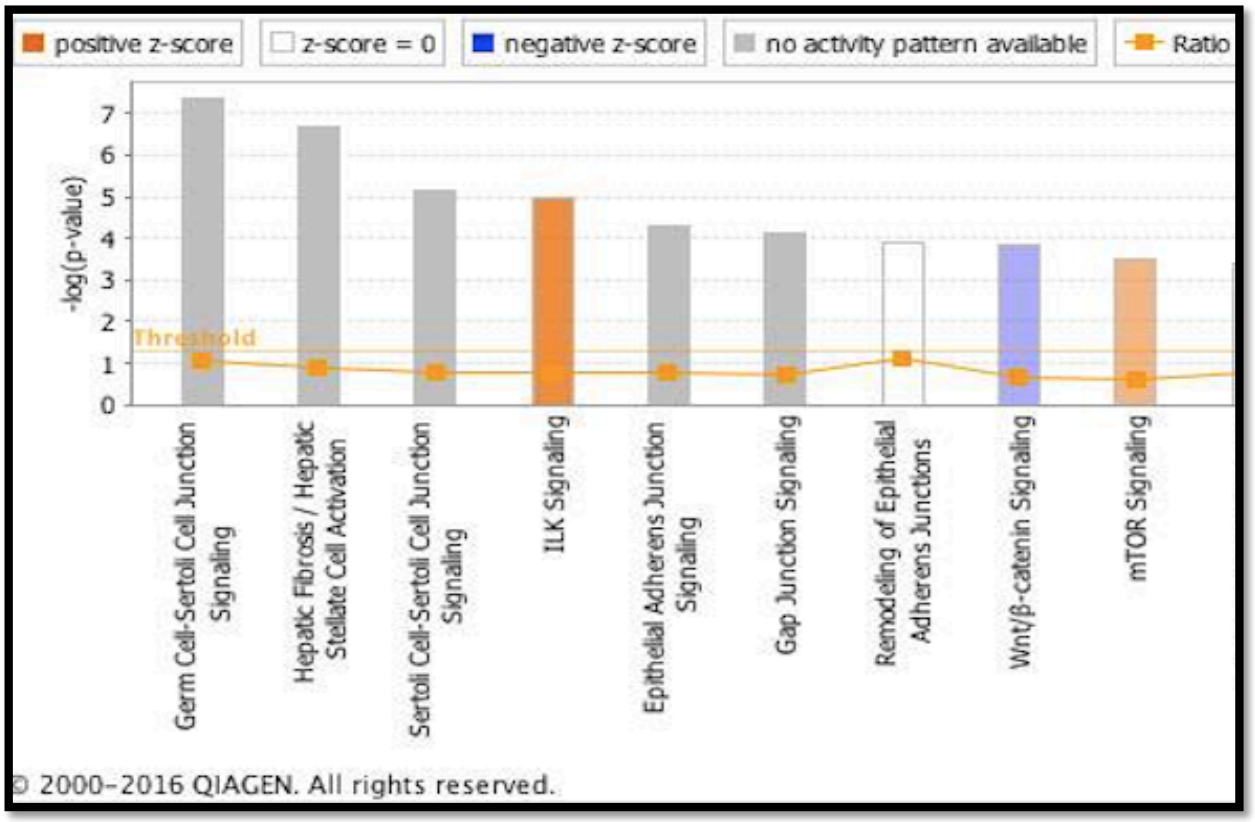

Figure 26: Ingenuity Pathway Assist (IPA) analysis of significant signaling pathways involved in fibroids. *Canonical pathway analysis identified the pathways from the IPA library that were most significant to the data set. Pathway names are indicated on the x axis and the statistical significance of gene-set over-representation is indicated on the y axis (–log base 10 scale). The numbers at the top of each bar is a ratio of the number of genes in each pathway present in the obese downregulated dataset to the total number of genes representing that pathway in the IPA knowledge base. The pathways that were altered by fibroid formation including Wnt/ β-catenin signaling and mTOR signaling pathway.*

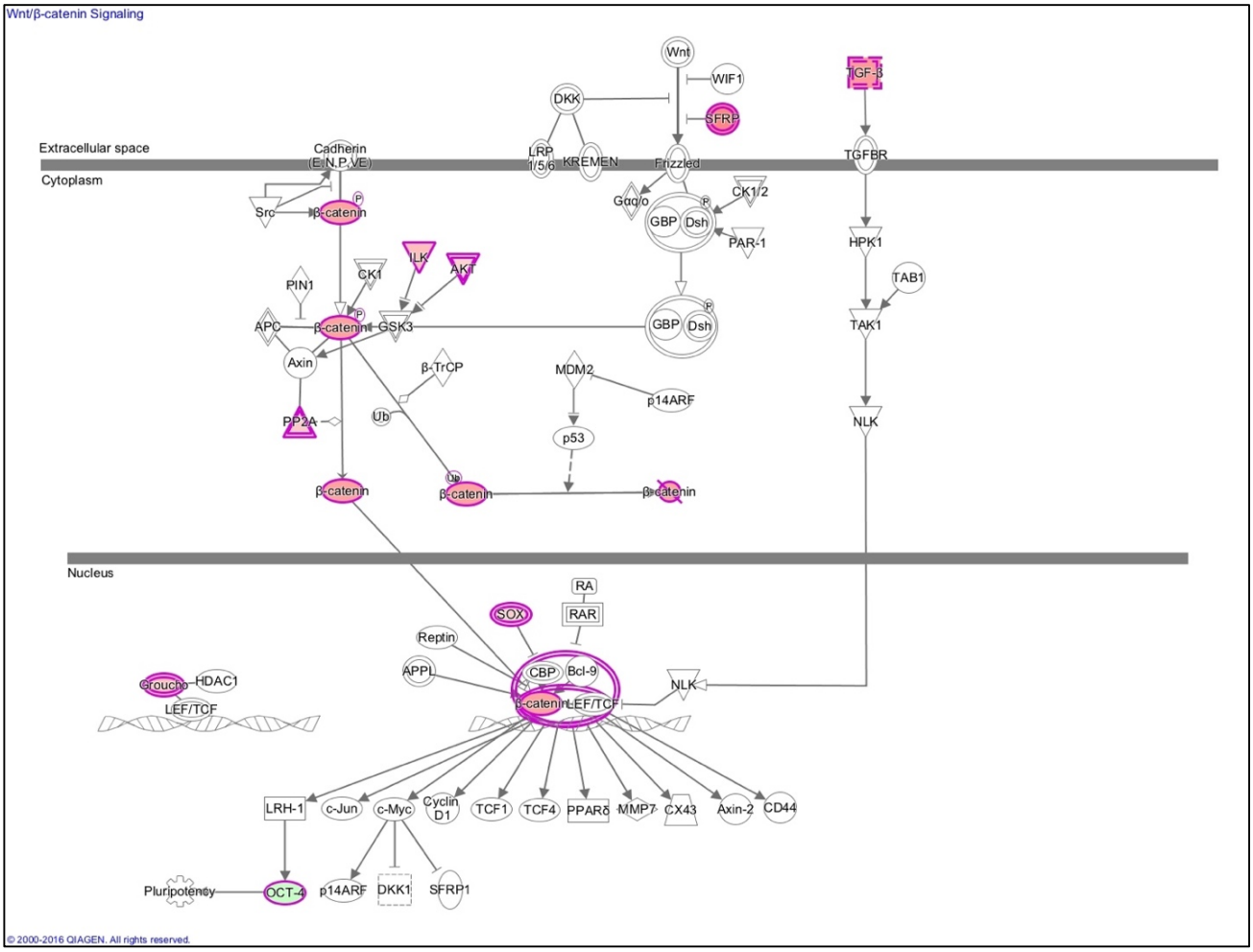

Figure 27: IPA enrichment pathway of Wnt/ B- catenin signaling. *The map shows the genes that can be affected through the Wnt/ B- catenin pathway. All the genes that are in pink are activated.*

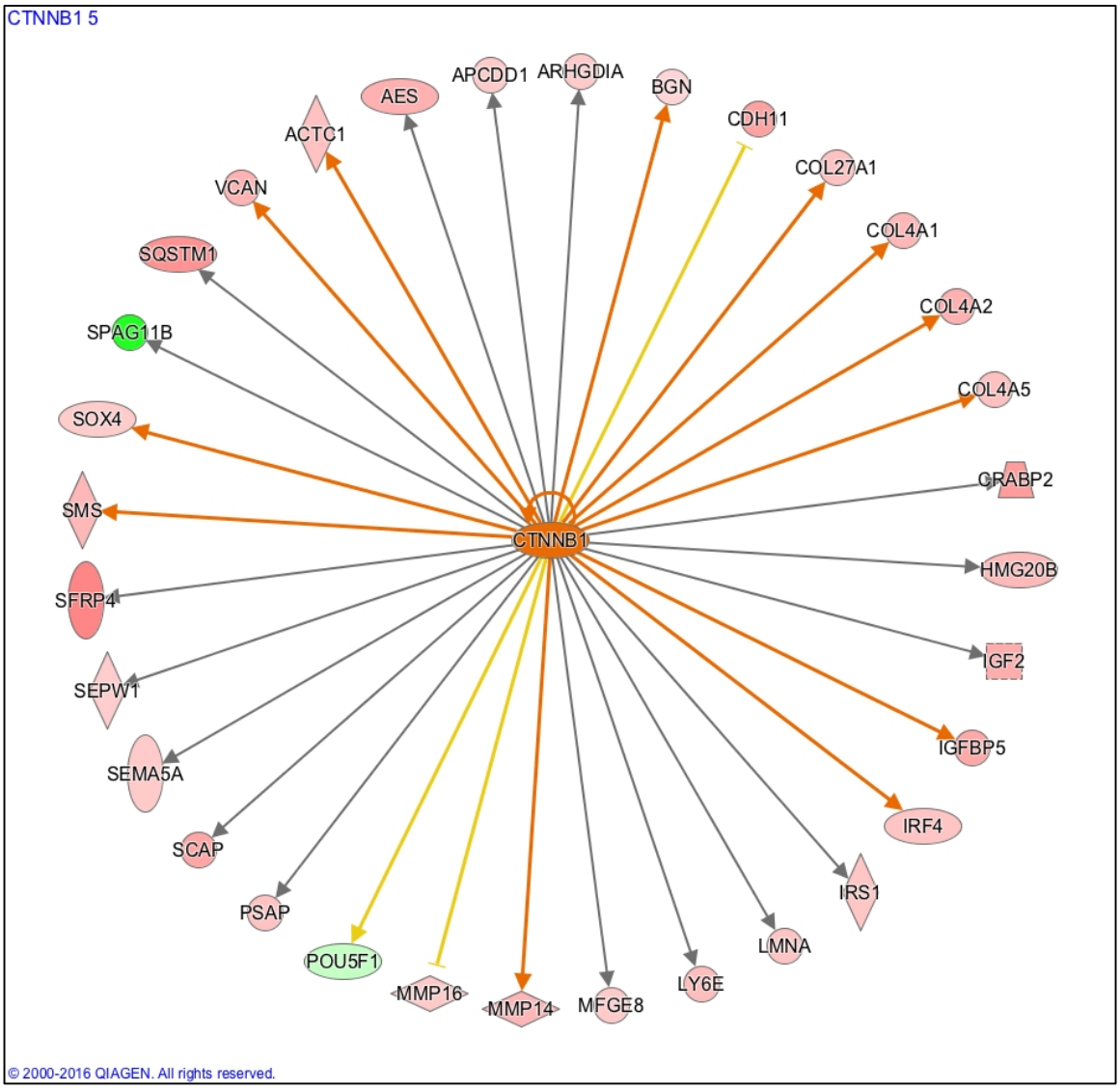

Figure 28: B-catenin upregulated and downregulated genes. *Overactive Wnt signaling leads to constitutively active b-catenin, which via LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors leads to inappropriate activation of Wnt target genes. The pink shades represent a prediction of upregulation and the green shade represents a prediction of downregulation. The orange line predicts a relationship that is leading to activation, the yellow like predicts a relationship that is leading to downstream molecule activation, and the grey lines are predicting no effect.*

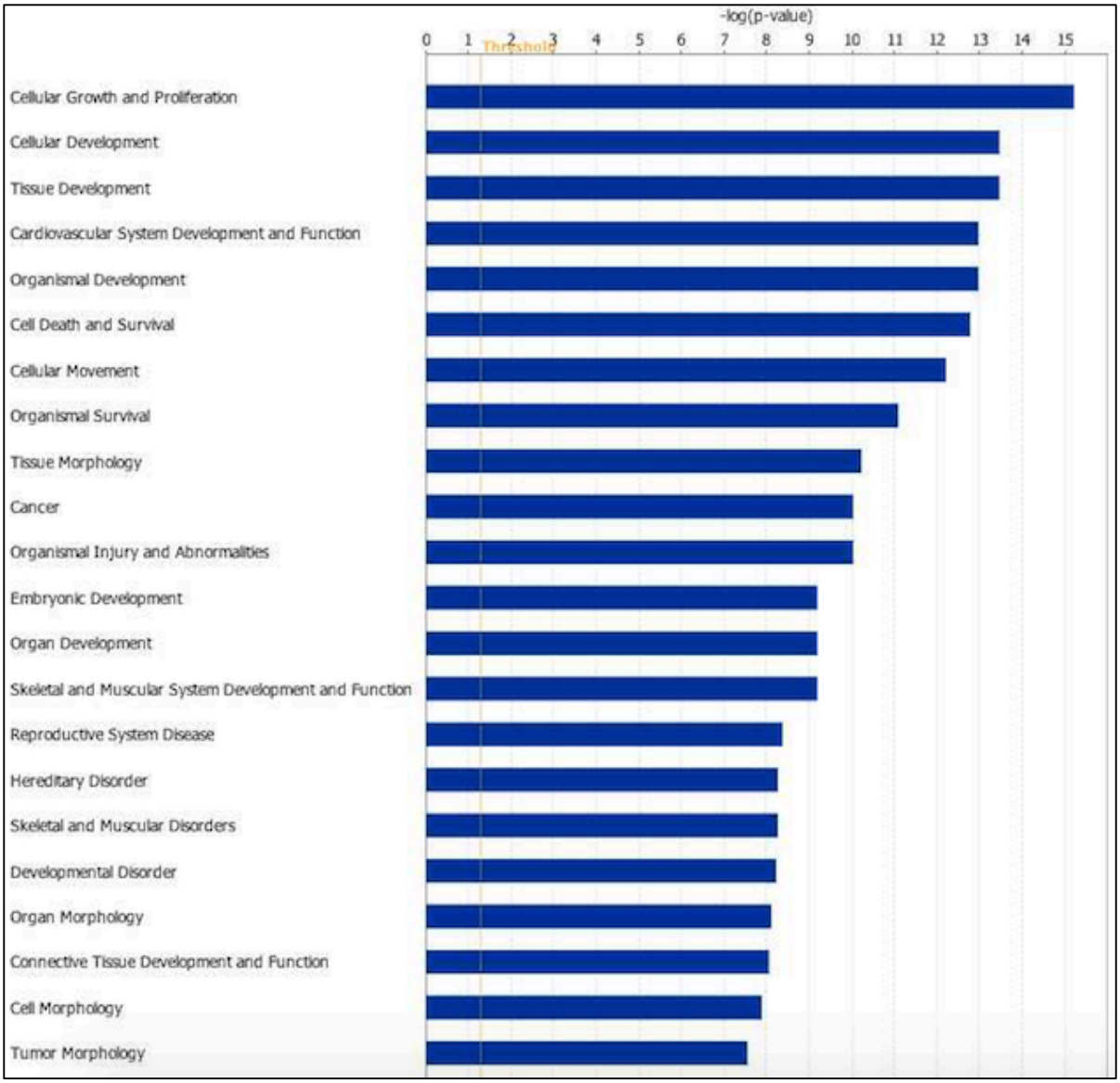

Figure 29: Ingenuity Systems Pathway Analysis (IPA) software was used to determine the highest biologically active functions in the HBP1 low fibroids. *The top pathways include cellular growth and cellular development, which support the hypothesis that tumor suppressor gene for cell proliferation and development are inhibited. Thus, allowing for the abnormal growth of the uterine tissue.*

MED 12 Mutation in Leiomyomas

The mediator complex contains the following subunits, MED12, MED13, CDK8, CycC. MED 12 is part of the mediator complex and is a transcriptional activator of Wnt- signaling, by binding directly to β- catenin. First, the TCF/LEF family, of DNA-binding proteins, positions β-catenin to activate transcription from the promoters of Wnt target genes. The interaction between β-catenin and the MED12 interface in, the Mediator Complex, appears to be functionally important for transduction of Wnt signals through β-catenin to RNA polymerase II (Kim et. al., .2006). MED 12 is on chromosome X and through previous published data, is shown to be mutated in fibroids. Random inactivation of the X chromosome, will lead to random expression of either the paternal or maternal MED 12 locus in uterine myometrial cells. However, there can be a mutation in the MED 12 locus that is randomly being expressed. Since cytogenetic mutation are common in fibroid, our initial hypothesis is that some, but not all, patient fibroid samples will have a mutation in MED 12 gene. We determined the expression of MED 12 using qRT-PCR analysis, with 18S Ribosomal RNA as an internal control. As shown in the Figs. 30-34, 5 of 8 individual fibroid tumors did indeed have low MED 12 expression, while 3 of 8 had normal to even higher expression relative to myometrium from the same patients. Specially in Patient 18, both fibroid samples have an increase in the MED 12 expression, compared to the myometrium. The increase in expression could contribute to the increase of Wnt gene targets that are becoming activated through the Westen blots as previously shown. Patient 16 and 17 have fibroid samples that have a decrease in MED 12 expression, therefore there is a limited interaction with β-catenin and the Mediator Complex. Therefore, downstream Wnt target genes could potentially not become activated.

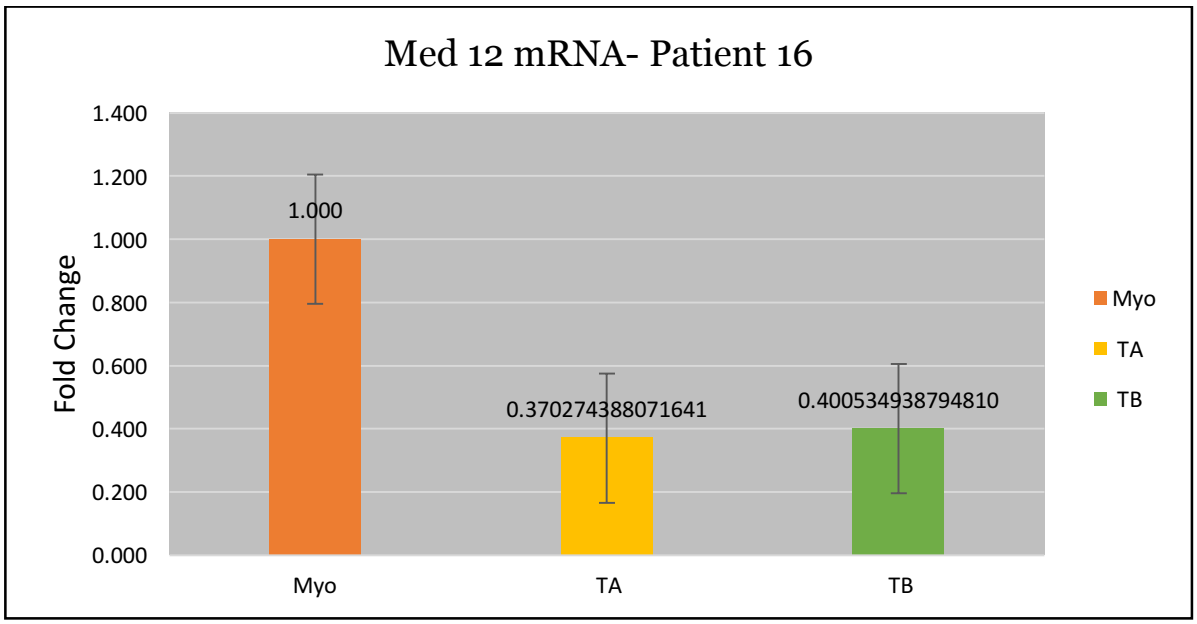

Figure 30: Med 12 mRNA expression levels Patient 16. *Both tumor A (TA) and tumor B (TB) have a decrease expression of Med 12 mRNA.*

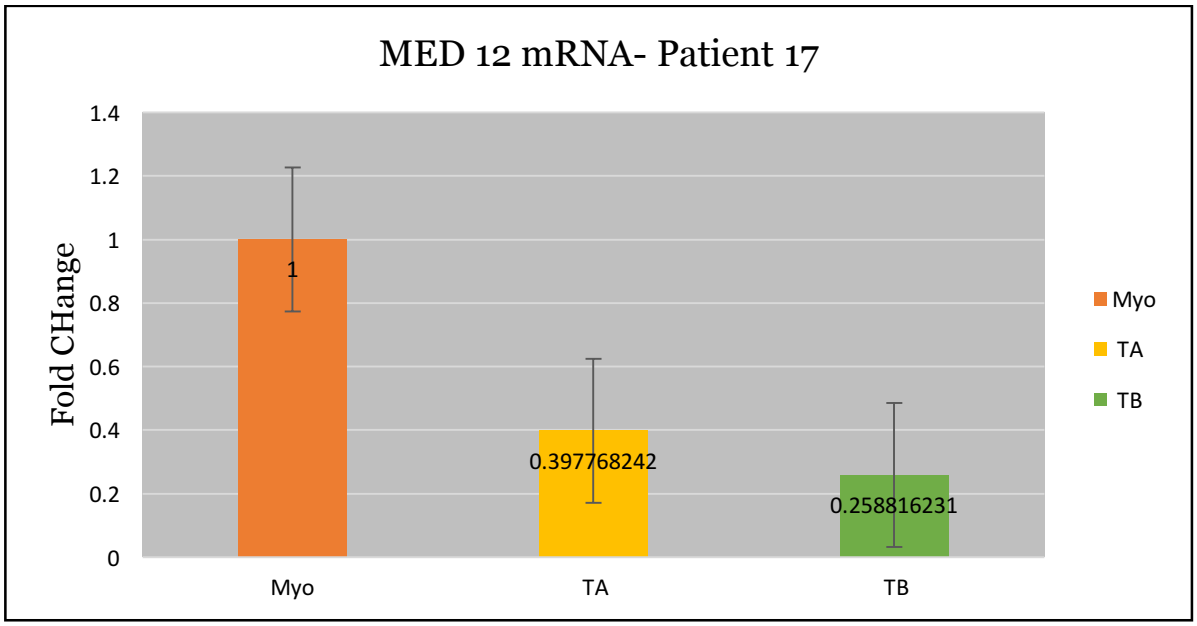

Figure 31: Med 12 mRNA expression levels in Patient 17. *Both tumor A (TA) and tumor B (TB) have a decrease expression of Med 12 mRNA.*

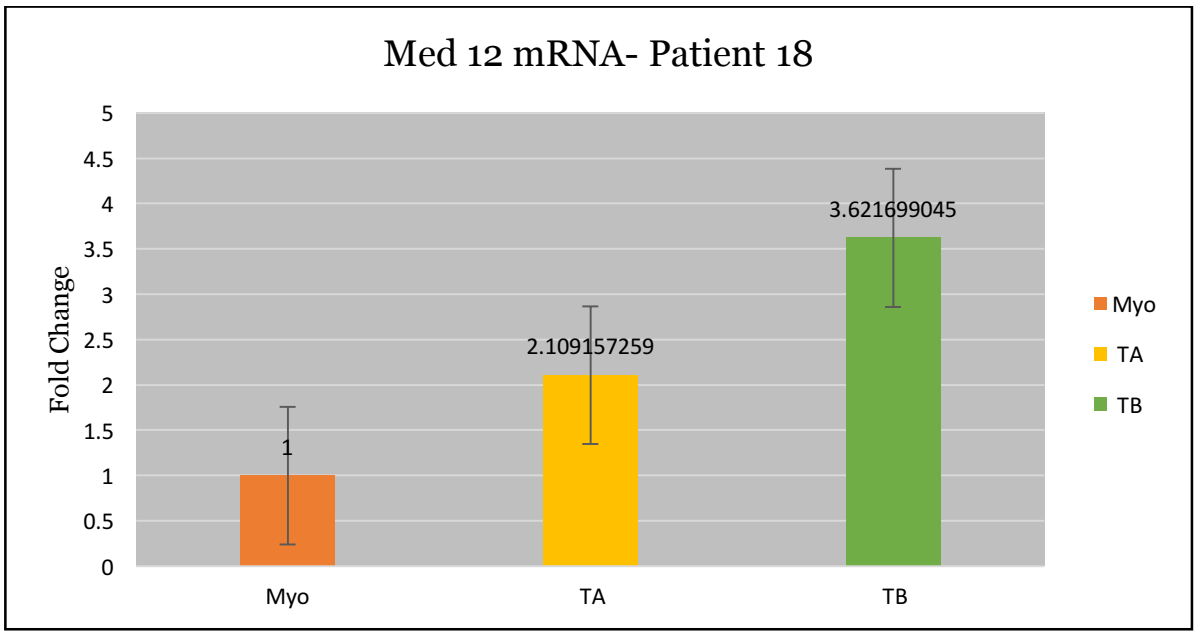

Figure 32: Med 12 mRNA expression levels in Patient 18. *Both tumor A (TA) and tumor B (TB) have an increase expression of Med 12 mRNA.*

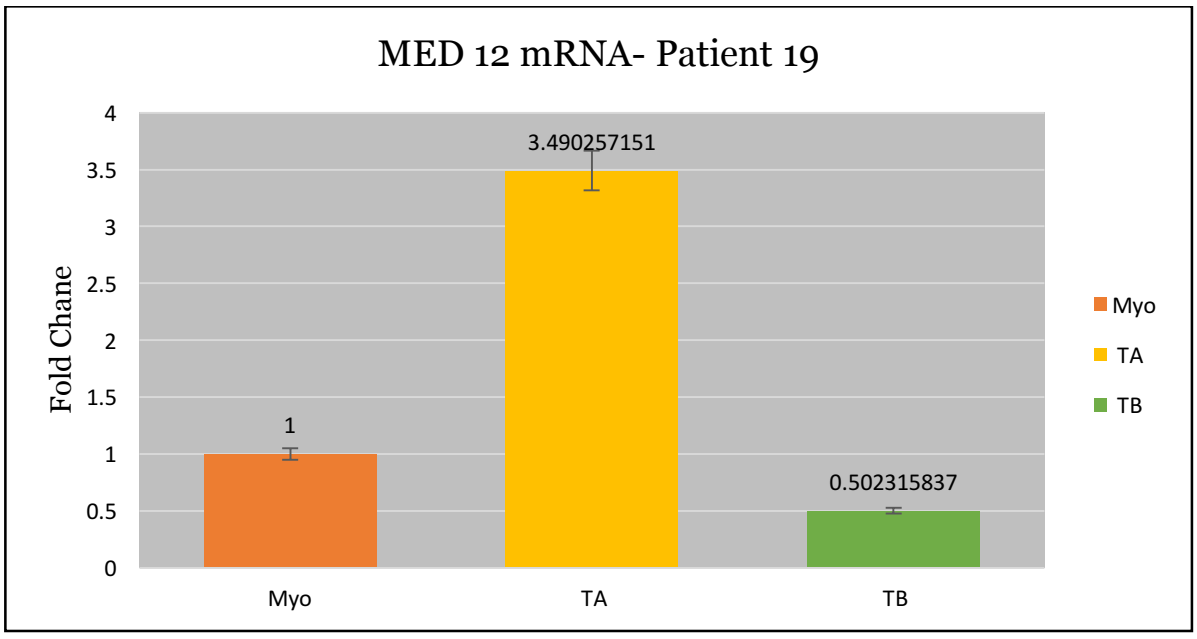

Figure 33: Med 12 mRNA expression levels in Patient 19. *Tumor A (TA) has a increase and tumor B (TB) have a decrease expression of Med 12 mRNA.*

Estrogen Receptor expression in Leiomyomas

The observation that leiomyomas occur primarily in women of reproductive age and regress during menopause supports the widely held view that ovarian steroids, like estrogen, stimulate leiomyoma growth. Moreover, disruption of ovarian estrogen or progesterone production during treatment results in size reduction of leiomyomas, an effect that is reversed once treatment is stopped. Estrogens exert their effects on target cells through the activation of estrogen receptors. As transcriptional modulation, endogenous estrogen binds to estrogen receptors (ER), the estrogen-ER complex regulates transcriptional activities. Estrogen, up-regulates the expression of several genes thought to play a role in leiomyoma pathogenesis, including multiple growth factors, collagens, and the estrogen and progesterone receptors.

Some studies have reported increased ER expression in leiomyoma compared with the surrounding myometrium, whereas other studies report no such difference. In the IPA, estrogen receptor was upregulated in the HBP1 low fibroid tumor. We determined the expression of ESR1 using qRT-PCR analysis, with 18S Ribosomal RNA as an internal control. As shown in the Figs. 35-38, the expression of ESR1 varied among patient samples. Patient 16 tumor TA and Patient 17 tumor TB, both have a HBP1 low profile, had an increase in ESR 1 expression. Interetsly, it could be stated that a Similar to Patient 18, where both fibroids had a HBP1 low expression and an increase in ESR1 mRNA. However, the Patient 19, had an increase in ESR1 expression and low HBP1 expression. The variation in expression of ESR1 between patient samples could support the difference in Wnt targets. However, another factor to consider about the variation in estrogen receptors would be the time of menstruation for each patient. Since this information is not provided about the patient, it could be a factor as to how the estrogen receptor expression is different.

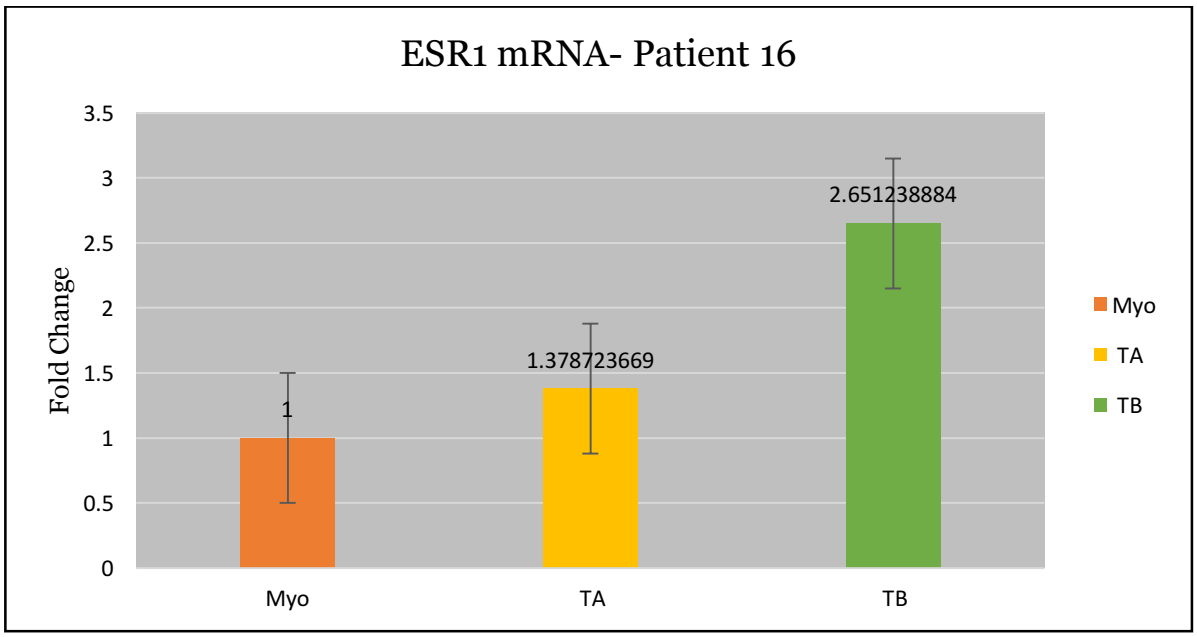

Figure 34: ESR1 mRNA expression levels in Patient 16. *Both tumor A (TA) and tumor B (TB) have an increase in estrogen receptors compared to the myometrium.*

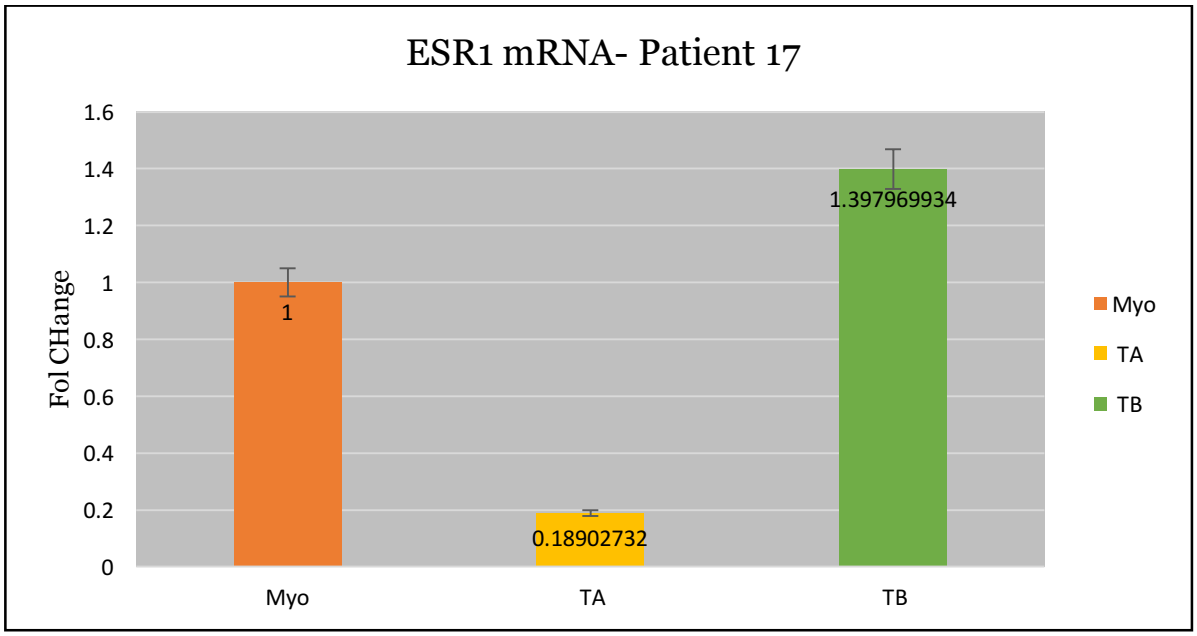

Figure 35: ESR1 mRNA expression levels in Patient 17. *Tumor A (TA) has a decrease in estrogen receptor and tumor B (TB) has an increase in estrogen receptor mRNA expression.*

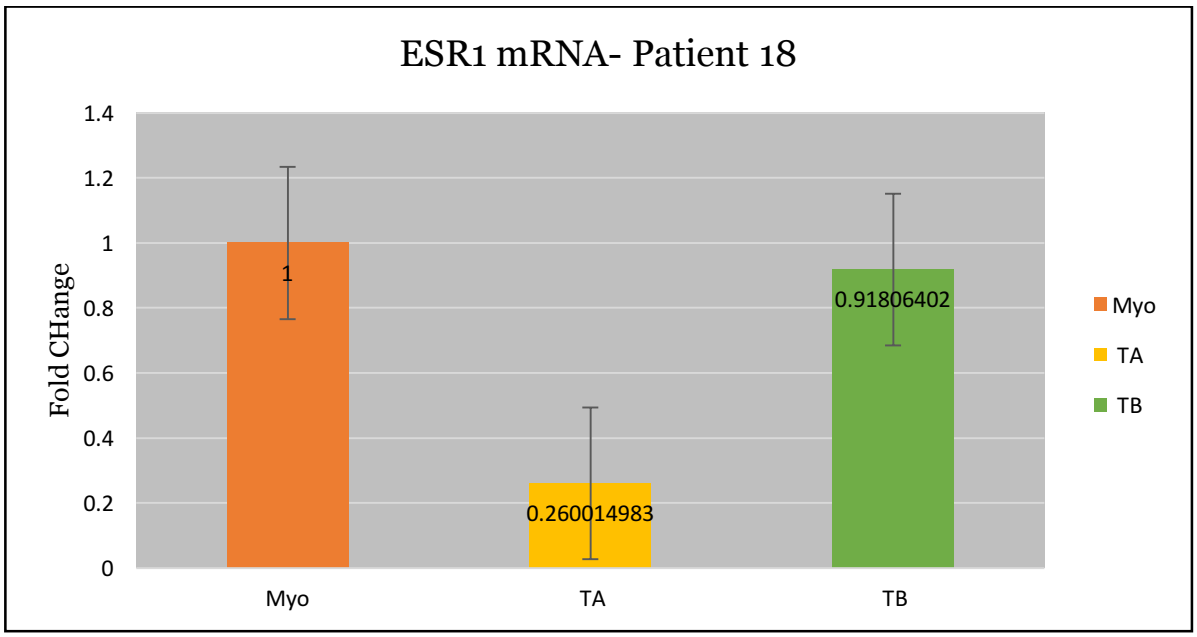

Figure 36: ESR1 mRNA expression levels in Patient 18. *Tumor A (TA) has a decrease in estrogen receptor and tumor B (TB) has an increase in estrogen receptor mRNA expression.*

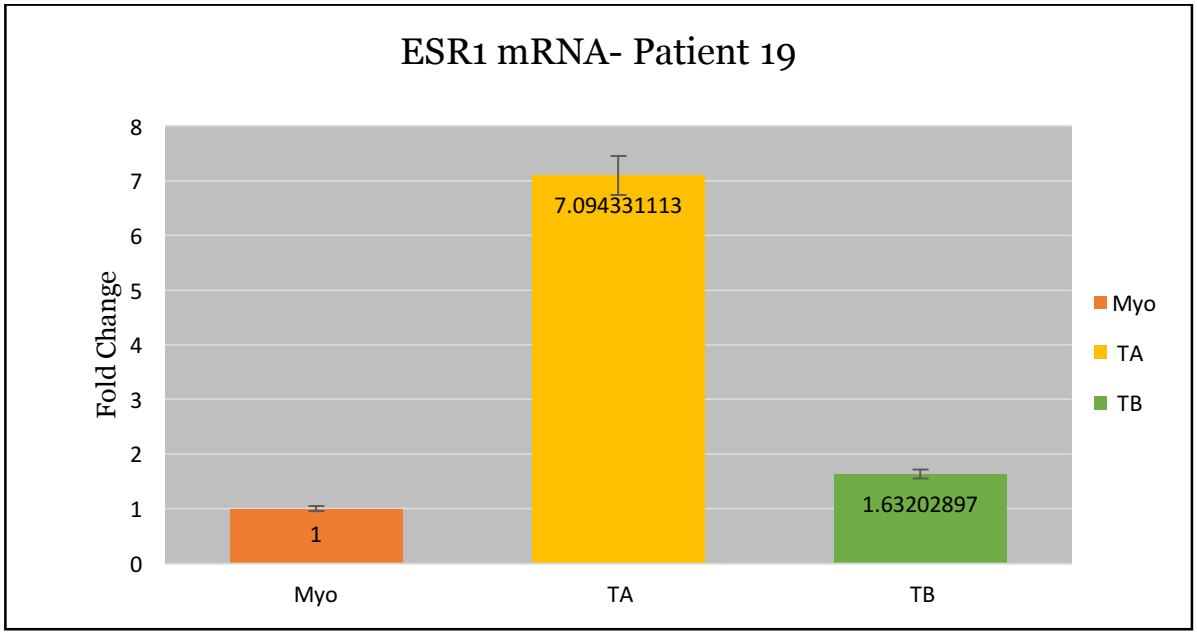

Figure 37: ESR1 mRNA expression levels in Patient 19. *Tumor A (TA) has an increase in estrogen receptor and tumor B (TB) has a decrease in estrogen receptor mRNA expression.*

Discussion

Potential Therapeutic Wnt/ β- catenin Inhibitors

The hallmarks of uterine leiomyoma development and growth are proliferation of leiomyoma cells along with a deposition of excessive disordered extra cellular matrix. The processes of development and growth are synchronized through a complex network of interconnected signaling pathways. Each patient sample fibroid is monoclonal and will give independent results, as compared to the myometrium.

Through previous published work, the activation of the Wnt/ Beta catenin signaling pathway has been shown to be a potential maker for the characterization of fibroids. β catenin, a transcriptional protein, was greatly increased in the monoclonal fibroids for each patient. Because many β-catenin target genes, such as Axin II and WISP 2, are involved in cell proliferation, β-catenin signaling plays an important role in development and neoplasia. These observations led us to hypothesize that WNT/β-catenin could be a factor in somatic stem cell function in the myometrium and in uterine fibroids tissue.

Based on the results that have been presented, a potential therapeutic target for the treatment of fibroids could be Wnt inhibitors, that are currently in clinical trials. Therapeutic agents that specifically target the WNT pathway have only recently entered clinical trials, although a few FDA-approved drugs do affect WNT signaling. There are a number of issues that must be considered in the development of therapeutic strategies to modulate the WNT pathway. Since, the WNT pathways is a crucial for embryo development, it becomes to difficult target because there could be many side effects problems. Given the important role of WNT signaling in homeostasis in multiple organ systems like the intestine, haematopoietic system, skin and hair similar side effects, could be bone loss and breakage. Also, women who are pregnant or plan on

becoming pregnant should not take WNT inhibitors because of the potential side effect on fetal development.

There are nonspecific Wnt pathway modulators that are already FDA approved drug and can be used for targeting different parts of the WNT cascade. Non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and COX2 inhibitor Celecoxib (Celebrex), inhibit the activity of COX. Various experimental, an increased COX-generated prostaglandin E_2 (PGE_2) suppresses β-catenin degradation, and so inhibition of the WNT–β-catenin signaling pathway is one of the potential mechanisms of action of NSAIDs (Maier et. al., 2005). Recently, it has also been suggested that both vitamin A and vitamin D might produce inhibitory proteins of the WNT–β-catenin signaling pathway. Vitamin A could induce Disabled homolog 2 (DAB2) and vitamin D may induce and DKK1, which is an inhibitor of WNT pathway. (Pendás et. al., 2008). These already approved FDA could be a potential therapy for fibroids because their safety profile is already approved. Also, it could be use to prescribe to patients for an off- label use.

Tankyrase 1 and 2 are part of a large family of enzymes call the the poly(ADP)-ribosylating enzymes (PARP). Tankyrases have been shown to interact with a domain of Axin II and promote its ubiquitylation and degradation (Huang et. al., 2009), which would lead to a destabilization of the β-catenin destruction complex and activating the Wnt cascade. A potential drug class are tankyrase inhibitors that successfully restrain WNT-mediated cellular responses, by stabilizing Axin II and a potentially viable strategy for inhibiting the WNT–β-catenin signaling cascade. The small molecular inhibitor, XAV939, prolongs the half-life of Axin II and promotes β-catenin degradation by inhibiting tankyrase. XAV939 induces the stabilization of Axin II by inhibiting the poly(ADP)-ribosylating enzymes (PARP) tankyrase 1 and tankyrase 2

(Huang et. al., 2009). A potential drug for the treatment of fibroids could be XAV939, however there are various WNT pathways inhibitors.

Another inhibitor of the Wnt pathway could be, Porcupine (PORCN), a membrane bound O-acyltransferase that is required for Wnt palmitoylation, secretion, and biologic activity. All human Wnt pathway activation requires PORCN for proper activity, suggesting that inhibition of PORCN could be an effective treatment for cancers dependent on excess Wnt activity. Palmitoylation is essential for WNT to interact with Frizzled receptors outside the cell (Kurayoshi et. al., 2007). The small molecular inhibitor, C59 is a porcupine inhibitor, and currently in clinical trials. A Novartis PORCN inhibitor, LGK974, is in early-phase clinical trials (NCT01351103) although no peer-reviewed published information is available regarding its activity or efficacy.

The data reported in this thesis have shed light on an important and previously under-studied area of fibroid pathophysiology, namely, the role of Wnt/b-catenin signaling. In particular, my results implicate both CCN5 and HBP1 as potentially important negative regulators of leiomyoma growth. Furthermore, CCN5 and HBP1 may be useful therapeutic targets in the treatment of fibroids, although much additional work will be required to substantiate this possibility.

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