A Novel Molecular Pathway Involving GPR10, REST, and PRICKLE1 in the Pathogenesis of Uterine Leiomyoma

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Abstract

Uterine Leiomyoma (UL), also known as Uterine Fibroids, are benign, hormone-sensitive tumors arising in the smooth muscle tissue layer of the uterus, the myometrium. UL are the most common female reproductive tract tumors, with lifetime occurrence in up to 77% of all women. A third of women with UL require treatment for clinically significant symptoms of menorrhagia, severe pain, anemia and fertility complications, resulting in over 200,000 hysterectomies and up to $34.4 billion in medical costs in the United States each year. Despite the prevalence of UL, currently no treatment for UL is long-term, cost effective and leaves fertility intact. There is a pressing need to develop better pharmacotherapies for the treatment of UL.

Remarkably, very little is known about the molecular pathogenesis of UL. Environmental estrogen exposure is one of the most recognized risk factors associated with the development of UL tumors. At the cellular level, extensive evidence has linked the cell proliferation, survival and growth of UL tumors to the over-activation of the PI3K/AKT-mTOR pathway. This work establishes, for the first time, a comprehensive molecular pathway starting from known environmental estrogen risk factors leading to activation of the most crucial cell proliferation pathway in UL.

We identified the most overexpressed G-protein coupled receptor in UL as the neuron-specific GPR10 (PRLHR), which activates the PI3K/AKT-mTOR pathway in UL cells upon stimulation by its ligand, PrRP (Prolactin-Releasing Peptide). Epigenetic silencing of GPR10 in non-neuronal cells is accomplished by the tumor-suppressor REST (Repressor Element Silencing Transcription factor), which we found to be drastically down-regulated at the protein level in UL tissue. In addition to GPR10, many of the most dysregulated genes in UL tissue are direct targets
of REST, implicating the central role of the loss of REST in the pathogenesis of UL. In our investigations on the degradation of REST in UL, we found significant under-expression of PRICKLE1, the protein required for REST localization to the nucleus. We have found that PRICKLE1 expression in the uterine myometrium is regulated by estrogen through Estrogen Receptor-α and that the loss of PRICKLE1 leads to the destabilization and degradation of REST protein in UL. We found overexpression of the polycomb repressor complex protein EZH2 (Enhancer of Zeste Homolog 2) participates in repression of PRICKLE1 in UL. Furthermore, we provide two important preclinical mouse models, which are among the first in the UL field that recapitulate genes dysregulated in human UL. We show that mice expressing hGPR10 in the uterus, and a conditional knockout of REST in the reproductive tract, show UL phenotype. This work establishes a novel molecular pathway in UL pathogenesis linking upstream estrogen signaling to downstream PI3K/AKT-mTOR pathway activation and provides potential drug targets and preclinical mouse models for improved treatment of UL.
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Chapter 1

Introduction
Uterine fibroids, also known as uterine leiomyoma (UL), are hormone-responsive, monoclonal tumors of the smooth muscle tissue layer (myometrium) of the uterus (Bulun, 2013). Although UL are considered benign as they do not metastasize, uterine fibroids are the source of major quality of life issues for approximately 25% of all women, who suffer from the clinically significant symptoms of UL (Cramer & Patel, 1990; Parker, 2007; Stewart, 2001; Walker & Stewart, 2005). Symptoms include pelvic discomfort, dysmenorrhea, menorrhagia, anemia, urinary incontinence, recurrent pregnancy loss, preterm labor and in some cases infertility (Bulun, 2013; Catherino, Parrott, & Segars, 2011; H. Cook, Ezzati, Segars, & McCarthy, 2010; Parker, 2007; Pritts, Parker, & Olive, 2009). It is estimated that 3 out of every 4 women will develop UL tumors in their lifetime, with incidence starting as early as menarche and peaking in perimenopause (Cramer & Patel, 1990; Parker, 2007; Walker & Stewart, 2005). Risk factors for the development of UL include obesity, age at menarche and parity, which are related to overall lifetime exposure to estrogen (Vilos et al., 2015; Walker & Stewart, 2005). Women of African-American descent are more at risk for developing UL, often have more severe symptoms, larger tumors and are diagnosed at an earlier age than Caucasian women, although the reason for this discrepancy is not yet known (Kjerulff, Langenberg, Seidman, Stolley, & Guzinski, 1996). As the most common female reproductive tumor, UL accounted for $5.9-34.4 billion in medical costs in 2010 in the United States (Cardozo et al., 2012). Taken together, UL are widespread, debilitating, and treatment costly tumors.

Despite the urgent need, there is currently no cure or treatment option for UL that is long-term, cost effective and preserves fertility. The standard treatment for UL is hysterectomy, removal of the entire uterus, or more rarely, myomectomy, removal of only the UL (Al-Hendy & Salama, 2006; Gambadauro, 2012; Martin, Bhanot, & Athreya, 2013; Parker, 2007). These
surgical treatments are invasive, costly and terminate fertility, creating a major burden for women in a society in which the average age for childbearing is steadily increasing (Balasch & Gratacos, 2011; Gambadauro, Gudmundsson, & Torrejon, 2012; Martin et al., 2013; Pritts et al., 2009). Other treatment options include hormonal therapy, such as GnRH antagonists (Bifulco et al., 2004), selective estrogen receptor modulators (SERMs)(Walker, 2002) and synthetic progestins (Bulun, 2013). However, these drug therapies are typically only used for short term treatment of symptoms, can only treat a subset of UL tumors, and do not preserve fertility during treatment (Gambadauro, 2012; Gambadauro et al., 2012; Moravek et al., 2015). To identify and develop improved treatment options for UL, the pathogenesis of UL must be better elucidated.

It is not yet known how steroid hormones, estrogen and progesterone, differentially affect UL. Furthermore, there exists a gap in our understanding of UL etiology regarding the molecular link between estrogen sensitivity and downstream cell proliferation pathways. Extensive evidence indicates the activation of the PI3K/AKT-mTOR pathway as one of the most overactive molecular pathways in UL leading to decreased apoptosis, increased cell growth and proliferation, and ultimately the development of UL tumors (Karra et al., 2010; Makker, Goel, Das, & Agarwal, 2012). Evidence for involvement of the PI3K/AKT-mTOR pathway in UL includes activation of PI3K/AKT pathway proteins and targets, including elevated p-AKT, p-GSK3 and CD2 proteins as well as dysregulated PTEN levels, in UL compared to myometrial tissue (Karra et al., 2010; Kovacs et al., 2003). Some studies also suggest overexpression of other signaling pathways, including the IGF-I pathway leading to MAPK activation of mTOR in UL (Yu et al., 2008). However, IGF-I pathway proteins are only shown to be overexpressed in 20-40% of human patients, which would not account for the molecular pathogenesis of the vast majority of UL, but could be a contributing factor (Peng et al., 2009; J. Wei, Chiriboga,
Mizuguchi, Yee, & Mittal, 2005). In contrast, PI3K/AKT-mTOR proteins are consistently over activated in UL compared to myometrial tissue, indicating the central role of the PI3K/AKT-mTOR pathway in UL pathogenesis (Kovacs et al., 2003; Kovacs et al., 2007).

It is also well established that UL are highly estrogen sensitive. UL have increased expression of steroid hormone receptors and are dependent on stimulation by their ligands, including estrogen, for growth and development (Burns & Korach, 2012; D'Aloisio, Baird, DeRoo, & Sandler, 2012; Hermon et al., 2008; Walker, 2002). Exposure to estrogenic compounds in our environment, such as genistein and diethylstilbestrol (DES), are major risk factors for the development of UL (D'Aloisio et al., 2012; Greathouse et al., 2008).

Environmental estrogens are compounds which have structural similarities to endogenous hormones and can interfere with normal endocrine function, such as hormone synthesis, metabolism and signaling (Betancourt, Eltoum, Desmond, Russo, & Lamartiniere, 2010; Biro et al., 2010; Okasha, McCarron, Gunnell, & Smith, 2003; Roy, Chakraborty, & Chakraborty, 2009; Walker, 2011). Environmental estrogens, also referred to as endocrine disruptors, are found commonly in everyday use, including polycarbonate plastics used for food and beverage storage, in pesticides and in flame retardants (Roy et al., 2009). The effect of increasing exposure to environmental estrogens, especially before puberty during the critical window of susceptibility to environmental factors, is multifaceted in terms of UL risk. Directly, prepubertal exposure to environmental estrogens has been shown to result in altered developmental programming of reproductive organs, including increased risk of UL in the uterus (Moller et al., 2012; Walker, 2002, 2011). More indirectly, increasing environmental estrogen exposure has been linked with a steadily declining age of menarche, which increases risk of UL due to increased overall lifetime
exposure to estrogen (McGuinn, Ghazarian, Joseph Su, & Ellison, 2015; Nagao, Saito, Usumi, Kuwagata, & Imai, 1999; Roy et al., 2009; Walker & Stewart, 2005).

Both early estrogenic signaling and PI3K/AKT-mTOR pathway activation are important contributors to UL pathogenesis. Moreover, these two causative events are linked by evidence showing that PI3K and mTOR are necessary for estrogen-dependent cell growth in UL and myometrial cells (Yin, Wang, & Khan-Dawood, 2007). However the molecular link between estrogen exposure and downstream activation of the PI3K/AKT-mTOR pathway leading to leiomyoma growth is still unknown.

To discover possible drug targets upstream of the cell proliferation pathways in the treatment of leiomyoma, we identified the most aberrantly expressed G-protein coupled receptor in UL, GPR10 (Varghese et al., 2013). Normal expression of GPR10 is limited to the reticular thalamic nucleus, the hypothalamus, and the adrenal medulla (Roland et al., 1999; Samson et al., 2003). Although GPR10 is activated by the peptide ligand PrRP (prolactin-releasing peptide) its role in prolactin release has been invalidated; instead, GPR10 functions in the central nervous system in the stress response, food consumption and the opioid system (Laurent et al., 2005; Lin, 2008). Our preliminary data indicates GPR10, normally expressed only in specific neurons (Roland et al., 1999), is highly expressed in UL tissue, where it activates the PI3K/AKT-mTOR pathway (Varghese et al., 2013). Our data identifies GPR10 as the previously unknown activator of cell proliferation pathway PI3K/AKT-mTOR in UL, and reveals a promising drug target for treatment of UL.

The aberrant expression of the neuron-specific protein, GPR10, outside the central nervous system in UL led us to explore the mechanism of GPR10 misexpression in the uterus. It has been proposed that the repression of GPR10 in non-neuronal tissues is accomplished by the
epigenetic silencer REST/NRSF (RE-1 Silencing Transcription factor/ Neuron Restrictive Silencing Factor)(Ballas & Mandel, 2005; Kemp, Lin, Ubeda, & Habener, 2002). REST binds to a 21bp repressor element (RE-1) sequence, predicted to occur in the promoters of about 2000 genes including GPR10, and recruits cofactors, including coREST and histone modifiers, to silence neuronal gene transcription in peripheral tissues (Andres et al., 1999; Kemp et al., 2002; Roopra et al., 2000). We have found the promoter of GPR10 is associated with REST in myometrial, but not matched UL cells, where REST expression is significantly reduced (Varghese et al., 2013). The expression of GPR10 outside of the central nervous system in UL tissue, and the subsequent activation of the PI3K/AKT pathway in leiomyoma cells likely contributes significantly to the etiology of the disease.

The work presented in this dissertation suggests that the loss of REST is a key molecular event in the development of UL. REST has been classified as a tumor-suppressor in epithelial tissues, where the loss of REST results in the de-repression of neuronal genes (Westbrook et al., 2005). The loss of REST plays a prominent role in the development of several tumor types, including colon cancer (Z. Huang & Bao, 2012). In breast cancer, 20% of which are REST-dependent, the loss of REST has been proposed to induce hyperphosphorylation of PI3K and AKT, leading to increased cell proliferation (Westbrook et al., 2005). In small cell lung carcinoma (SCLC), decreased REST expression has been shown to increase activation of AKT, leading to mTOR signaling and cell growth and proliferation (Kreisler et al., 2010). Crucially, we have found that REST protein, but not mRNA, is absent in UL tissue, and many of the most significantly up-regulated genes in UL are direct targets of REST (Varghese et al., 2013). This suggests an important role for the loss of REST at the protein level in UL.
The function of REST as a neuronal gene silencer requires that REST be present in the nucleus of all non-neuronal cells. Nevertheless, our data have shown REST, in reduced levels, localized outside the nucleus in primary UL cells and tissue (Varghese et al., 2013). To explain this discrepancy, we focused on the protein implicated in bringing REST into the nucleus, PRICKLE-1 (Shimojo, 2011; Shimojo & Hersh, 2003, 2006). Remarkably, we have found that PRICKLE-1 is the essential link between estrogen exposure and the loss of REST in UL. PRICKLE-1, also known as REST-interacting LIM Domain protein (RILP), directly binds REST and is required to bring REST to the nucleus (Shimojo, 2008; Shimojo & Hersh, 2006). It is crucial for REST to associate with its binding partner PRICKLE-1 in order to perform epigenetic functions in the nucleus (Shimojo & Hersh, 2003, 2006). PRICKLE-1 has a well-established role during development in specifying planar cell polarity in the non-canonical WNT signaling pathway (Carreira-Barbosa et al., 2003; D. W. Chan, Chan, Yam, Ching, & Ng, 2006; Mlodzik, 2009; Veeman, Slusarski, Kaykas, Louie, & Moon, 2003; Yang et al., 2014), however recently studies have explored additional functions for PRICKLE-1. PRICKLE-1 putatively functions as a tumor suppressor in liver cells, where the loss of PRICKE-1 can result in human hepatocellular carcinoma (D. W. Chan et al., 2006). Related to our findings in UL, in cardiac development, the transient suppression of PRICKLE-1 decreases the amount of REST in the nucleus to regulate proper REST target gene expression (Shimojo, 2011). Our data indicates PRICKLE-1 expression is markedly decreased in human UL and that PRICKLE-1 directly regulates REST expression in primary cultured myometrial and UL cells. These data strongly suggest a role for PRICKLE-1 leading to the loss of REST and downstream epigenetic changes in UL.

Finally, to complete this novel molecular cascade in UL, our data also show that PRICKLE-1 expression is regulated by estrogen in the uterus, which positions PRICKLE-1 as
the previously unknown molecular link between downstream proliferation pathways and upstream estrogen signaling. Prepubertal exposure to environmental estrogens is one of the major risk factors for UL incidence (D’Aloisio et al., 2012; Walker, 2011). We have found that environmental estrogens, including DES and genistein, as well as prepubertal estrogen treatment, negatively regulate PRICKLE-1 expression \textit{in vivo}. Our data indicating that estrogen regulates PRICKLE-1 is a relationship that has not been previously demonstrated. Although estrogen typically functions as an activator of target genes, there is emerging evidence of estrogen acting in an inhibitory role in gene regulation, including inhibition of IL-6 (Burns & Korach, 2012). We have discovered a novel inhibitory role for estrogen in the regulation of PRICKLE-1 in UL using \textit{in vivo} models to strongly support our findings.

Furthermore, we provide the first preclinical genetic mouse models expressing a gene overexpressed in UL, GPR10, as well as a conditional knock out model for a tumor suppressor protein under expressed in UL, REST. These animal models provide novel genetic tools to study human UL. Currently, the predominantly used UL animal model, known as the Eker rat, has mutations in the $TSC1$ and $TSC2$ (\textit{tuberous sclerosis}) genes, which disrupt regulation of mTOR signaling, leading to leiomyoma growth (Walker, Hunter, & Everitt, 2003). However, Eker rats develop additional severe complications, including renal cell carcinoma, which limit the potential for the Eker rat in providing long term studies in UL-specific drug development (J. D. Cook & Walker, 2004). Mouse models with myometrial specific $TSC2$ mutations also quickly develop fatal complications, including lymphangioleiomyomatosis (LAM), which is smooth muscle cell growth in the lungs (Prizant et al., 2013). Moreover, although the mTOR pathway is consistently dysregulated in UL, the only genetic mutations identified to promote UL are the very rare fumarate hydratase mutations, and the MED12 somatic mutation (Ding et al., 2008;
Makinen et al., 2011; Mittal et al., 2015; Tomlinson et al., 2002; H. Wang, Shen, Ye, & Ye, 2013); the TSC2 gene is not mutated in human UL. Therefore, TSC mutations in animal models provide an incomplete representation of human UL in vivo. For progress in understanding the complications of drug development for UL in human health, new animal models, such as the transgenic hGPR10 and REST cKO mice generated in our laboratory, are needed to better phenocopy the human condition. Here, we present two novel animal models which express genes dysregulated in human UL, GPR10 and REST that show distinctive UL phenotype. These animal models are novel both for the basic understanding of the etiology of UL, and in new approaches to drug development for treating the disease.

By combining in vitro approaches and mouse geneic models, we provide a comprehensive molecular pathway from estrogen to the PI3K/AKT-mTOR pathway resulting in the development of UL. We address in depth the role of PRICKLE-1, REST and GPR10 in UL pathogenesis, including the de-repression of GPR10 in activating cell proliferation and survival pathways (Chapter 3), the impact of the loss of REST on epigenetic reprogramming in UL (Chapter 4) and the regulation of PRICKLE-1 by estrogen and its significance in the pathogenesis of UL (Chapter 5). We provide in depth supporting data, as well as preclinical genetic models for genes aberrantly expressed in UL, including a transgenic mouse expressing human GPR10 and a conditional knockout for REST in the uterus. Finally, as a G-protein coupled receptor, normally expressed only in the brain, GPR10 makes a practical and exciting new target for small molecule peptide drugs that do not pass the blood brain barrier.

This work demonstrates for the first time, a complete molecular pathway starting from known environmental estrogen sources leading to activation of the most dysregulated cell proliferation pathways in UL. Our work provides new knowledge in understanding the molecular
cascades initiating UL pathogenesis and novel therapeutic targets for the development of improved treatment options for UL. The meticulous construction of this novel pathway in UL increases our understanding of the disease etiology, provides targets for new pharmacotherapy treatments of the disease, and offers a complete model linking risk factors to dysregulated cellular pathways.
Figure 1. 2: Schematic representation of novel molecular pathway in uterine leiomyoma pathogenesis.
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Chapter 2

Literature Review
Uterine Leiomyoma

A.1. Uterine leiomyoma characteristics.

Uterine leiomyoma (UL), or uterine fibroids, are steroid hormone-responsive, benign monoclonal tumors of the smooth muscle tissue layer (myometrium) of the uterus (Walker & Stewart, 2005). As the most common reproductive tumor in women, UL present a major quality of life problem for a large fraction of the population. It is estimated that up to 77% of all women will develop UL in their lifetime and 15-30% of these women suffer from substantial symptoms, including pelvic discomfort, dysmenorrhea, menorrhagia, anemia, urinary incontinence, recurrent pregnancy loss, preterm labor and in some cases infertility (Bulun, 2013; Catherino et al., 2011). In 10-40% of pregnancies with UL present, complications occur and miscarriage is up to 2-fold higher in women with symptomatic UL (H. Cook et al., 2010). UL can range in size from 10mm to greater than 20cm and are often found in multiple nodules within one uterus (Bulun, 2013).

UL are characterized by increased proliferation of disordered smooth muscle cells, altered extracellular matrix deposition and enhanced responsiveness to sex steroid hormones (Bulun, 2013). A major defining characteristic of UL is the overproduction and disorganized nature of fibrous extracellular matrix (ECM), as compared to myometrial tissue (Walker & Stewart, 2005). The ECM is composed of a network of structural proteins, growth factors, cytokines, proteases and other molecules, which are secreted by surrounding cells to create a unique environment of physical and biochemical support for cells (A. Patel, Malik, Britten, Cox, & Catherino, 2016). The ECM plays an important role in tissue composition and cellular and organ function. Extracellular matrix profiles in UL include increased collagens, fibronectin and proteoglycans (Barker et al., 2015; Fujisawa & Castellot, 2014). Several studies have found
increased levels of genes involved in ECM, including Transforming Growth Factor-β3 (TGF-β3), CD24, Insulin-like Growth Factor I (IGF-1), collagens type I and III, fibronectin and glycosaminoglycans (Arici & Sozen, 2000; W. Catherino et al., 2004; Stewart, Friedman, Peck, & Nowak, 1994; Wolanska, Sobolewski, Drozdzewicz, & Bankowski, 1998). Excessive collagen fibrils formed in UL are abnormal in formation and disorganized compared to myometrial tissue (Leppert et al., 2004; Parker, 2007). Interestingly, the ECM proteoglycan dermatopontin (DPT), an important organizer of collagens, is decreased in UL as well as in keloids, a fibrous lesion with disordered ECM similar to UL tissue (W. H. Catherino et al., 2004). In addition to altering the organization of ECM collagens, the low levels of DPT in UL may also contribute to altered TGF-β3 signaling in UL (Okamoto, Fujiwara, Abe, & Sato, 1999). Normal myometrial smooth muscle cells in culture grow uniformly and in parallel with the cells’ major axis, whereas UL cells form ball-like aggregates or nodules in culture, contributing to the disorganized nature of UL cells (Kobayashi et al., 1996). The excessive ECM in UL tissue may contribute to the disorganized cellular structure of UL cells.

The location of a particular UL within the uterus contributes to the symptoms presented. UL are classified as subserosal, intramural, submucosal or pedunculated depending on their location (Bulun, 2013). Submucosal UL are most likely to cause menorrhagia, interfere with fertility, and can cause preterm birth or miscarriage (Gambadauro, 2012). Large submucosal ULs can lead to pressure on adjacent organs resulting in pain, urinary symptoms and constipation (Stewart, 2001). Submucosal UL also lead to lower pregnancy, implantation and delivery rates in women undergoing in-vitro fertilization (H. Cook et al., 2010; Pritts et al., 2009). There is evidence of cross talk from UL to adjacent endometrial cells that can lead to decreased endometrial receptivity and poor blastocyst implantation (Makker & Goel, 2013). In addition, it
has been suggested that submucosal UL may disrupt normal uterine peristaltic movements and contractility, impeding sperm arrival at the oviducts, embryo movement into the uterus, or causing increased contractions leading to preterm labor (H. Cook et al., 2010).

A.2. Etiology of uterine leiomyoma.

a. Risk factors.

Despite the problematic symptoms and prevalence of UL, very little is known about the etiology of these tumors. UL are most often diagnosed in the perimenopausal years, but can become symptomatic much earlier in some women and incidence declines after menopause (Walker & Stewart, 2005). UL incidence increases with age, peaking in the early 40s (Marshall et al., 1997; Ross et al., 1986). However, this could be a result of previously asymptomatic UL becoming more noticeable after years of growth and exposure to endogenous steroid hormone, or greater likelihood of older women to seek fertility-ending UL treatment (Flake, Andersen, & Dixon, 2003). After menopause, UL are present in comparable numbers to premenopausal women; however, these tumors tend to be smaller, fewer and often asymptomatic (Cramer & Patel, 1990).

Age at menarche has also been suggested to be a risk factor for UL, with an earlier age indicating higher risk of developing UL (Marshall, Spiegelman, Goldman, et al., 1998). Parity is also a UL risk factor, with a decrease in UL incidence relative to the number of live births (Flake et al., 2003; Lumbiganon et al., 1996; Marshall, Spiegelman, Goldman, et al., 1998). Both age at menarche and parity as risk factors could be attributed to levels of lifetime exposure to sex steroid hormones, although the exact reason is still unclear.

Lifestyle choices can have a significant impact on risk of UL development. Obesity, diet, lack of exercise, and smoking have been correlated with UL incidence. Several studies have shown high BMI (body mass index) correlates with increased UL incidence (Lumbiganon et al.,
1996; Marshall, Spiegelman, Manson, et al., 1998; Ross et al., 1986; Sato, Nishi, Kudo, & Miyake, 1998). Notably, a study by Ross et al. showed an over 20% increase in UL risk for every 10 kilogram increase in body weight (Ross et al., 1986), and Lumbiganon et al. found a 6% increase of UL for each BMI unit increase (Lumbiganon et al., 1996). Diet and exercise have also been linked to UL incidence, although it is not well established whether these contribute to body weight or constitute a risk factor alone. A study by Chiaffarino et al. showed a relationship between higher UL risk in women with diets high in red meat and lower incidence in those with a diet high in green vegetables (Chiaffarino et al., 1999).

b. Race.

UL disproportionately appear in higher percentage in women of African American descent. Even after controlling for high BMI, parity, socioeconomic status, and other risk factors, African American women have higher incidence, larger tumors at diagnosis, more severe symptoms and earlier age at diagnosis than white, Hispanic or Asian American women (Kjerulff et al., 1996; Marshall et al., 1997). UL incidence by age 50 is over 80% among African American women, compared to 70% in Caucasian women (Parker, 2007). Women of African-American descent seek treatment for fibroids more often than other ethnic group in the United States. African American women are 2.4 times more likely to have a hysterectomy for treatment of fibroids and have a 6.8 fold higher rate of myomectomy treatment for removal of fibroids compared to Caucasian women (Wechter, Stewart, Myers, Kho, & Wu, 2011). A study by Huyck et al. looked at racial differences in UL severity in a sister study which included African American and Caucasian women who have a sister previously diagnosed with UL. Black study participants exhibited higher likelihood for some known UL risk factors, including earlier age at menarche, higher likelihood of being obese. However, black participants also scored lower on other UL risk
factors including less history of smoking and less consumption of red meat. Nevertheless, when other risk factors were controlled for, this study showed African American women had a significantly younger age at diagnosis of UL, more severe pain associated with UL and a higher UL rate compared to Caucasian women (Huyck et al., 2008). African American women are also more likely to report that UL interfere with relationships between significant others, family and friends and report a higher negative impact on self-esteem and emotional issues (Stewart, Nicholson, Bradley, & Borah, 2013). In a self-reporting study, African American women in the United States were 77% more likely to miss work due to severe UL symptoms than Caucasian women (Stewart et al., 2013). Despite the disproportionate severity and incidence of UL in women of African American descent, the underlying cause for the discrepancy is not well understood. More studies on the etiology of UL are needed to discover the reason for racial risk factors in UL.

c. Steroid hormones.

It is well established that UL are sensitive to sex steroid hormones. UL in vivo and UL cells in culture are dependent upon stimulation from hormones, especially estrogen, for growth and development (Walker, 2002). UL can become symptomatic starting after puberty, when endogenous estrogen levels rise, and UL regress after menopause (D'Aloisio et al., 2012). Local uterine tissue concentrations of hormones and hormone receptors differ between UL and healthy myometrial tissue. UL have higher concentrations of estradiol, aromatase, progesterone receptor (PR) and estrogen receptor alpha (ERα) (Parker, 2007). Increased expression of ERα and PR is independent of tumor size, can be heterogeneous within tumors of one patient, and is consistent throughout all the menstrual cycle phases (Stewart, 2001). As such, UL seem to have a hypersensitivity to sex steroid hormones, distinct from the normal myometrial response to
estrogen and progesterone. While the normal myometrium has a limited response to estrogen, and becomes quiescent in the luteal phase, UL tissue shows an increase in estrogen-regulated genes in the luteal phase (Maruo, Ohara, Wang, & Matsuo, 2004). In addition to this loss of temporal/cyclical regulation by estrogen, UL also grow in response to progesterone, which typically has a suppressive effect on the myometrium (Moravek et al., 2015). It is not yet understood when this change in sensitivity to sex steroid hormones occurs in UL. Blood serum levels of estrogen and progesterone are similar in women with and without UL (Parker, 2007). However, African American women have 18% higher estradiol levels compared to Caucasians and no difference in progesterone levels (Stewart, 2001). Despite extensive evidence showing UL to be responsive to estrogen both in vitro and in vivo, the link between estrogen exposure and activation of the downstream PI3K/AKT-mTOR pathway leading to UL growth is still poorly understood.

Although the growth of UL has been shown to be highly dependent on stimulation by steroid hormones, the exact role of progesterone in UL pathogenesis is still poorly understood. Whereas estrogen has been shown to be essential for UL cell growth (Barbarisi et al., 2001), progesterone treatment in vitro has been shown to be both supporting of growth (Barbarisi et al., 2001; Hoekstra et al., 2009) and also inhibitory (Maruo et al., 2004). In addition, estrogen stimulation increases fibroid growth in the most commonly used UL animal model, the Eker rat, while progesterone does not stimulate growth in these rodents (Burroughs, Fuchs-Young, Davis, & Walker, 2000). However, studies in a xenograft mouse model with human UL inserted under the kidney capsule, demonstrated progesterone is necessary for UL growth (Ishikawa et al., 2010). Despite conflicting results in cell culture and animal studies of progesterone action in UL, a strong support for progesterone involvement in UL growth comes from anti-progestin
therapies. The anti-progestin drugs, RU-486, proellex (CDB4124), ulipristal acetate (CDB2914) and mifepristone cause regression of UL tumor size and symptoms as well as a decrease in ECM formation in UL (Eisinger, Meldrum, Fiscella, le Roux, & Guzick, 2003; Kim, Kurita, & Bulun, 2013; Murphy, Morales, Kettel, & Yen, 1995; A. Patel et al., 2016). The selective progesterone receptor modulator, asoprisnil, is also used as a short-term effective treatment for UL tumor symptom and size (Chwalisz et al., 2007; Kim et al., 2013; Williams et al., 2007). Low circulating 25-hydroxyvitamin D among African American women has been associated with higher incidence of UL and supplementation of vitamin D3 has been suggested as a potential long-term therapeutic option for UL prevention and treatment (Baird, Hill, Schectman, & Hollis, 2013; Paffoni et al., 2013; Wu & Segars, 2015).

i. Environmental estrogens and uterine leiomyoma.

One of the major risk factors for the development of UL is pre-pubertal exposure to environmental estrogens, likely resulting in developmental reprogramming of the uterus (D’Aloisio et al., 2012). Environmental estrogens mimic estrogen signaling in the cell by binding to estrogen receptors and altering hormonal activity (Yu et al., 2012). Also referred to as estrogen-like endocrine disrupting chemicals (EEDC), these environmental estrogens can also alter the function of the endocrine system through altered hormone synthesis or metabolism (Roy et al., 2009). Chemical environmental estrogens are referred to as xenoestrogens, including diethylstilbestrol (DES) and Bisphenol A (BPA). Pytoestrogens, such as genistein, come from plant sources (Jefferson, Patisaul, & Williams, 2012; Shen et al., 2013). EEDCs can be found frequently in plastics and cans containing food products, pesticides, and flame retardants, and have been shown to be readily absorbed by humans, especially at high temperatures (Roy et al., 2009). The structural similarity of many EEDCs, including dichlorodiphenyltrichloroethane
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(DTT), BPA, DES, Genistein, dioxin, and polychlorinated biohenyls (PCBs) can cause symptoms of developmental reprogramming, such as early age of menarche in girls (Roy et al., 2009). Developmental reprogramming refers to the altered response to normal physiological stimuli in adulthood due to exposure to an adverse stimulus early in life (Walker, 2011). Recently, awareness that early life adverse exposures and events have strong association with disease later in life has gained attention in areas of reproductive health and breast cancer (Okasha et al., 2003). Vulnerability to developmental reprogramming by synthetic estrogen exposure on the reproductive tract is the highest before puberty in humans, when the protective influence of progesterone is not yet in place (Jefferson et al., 2012). This window of sensitivity corresponds to postnatal days 1-5 in rodent models (Jefferson et al., 2009). The effects of exposure to environmental estrogens have been shown in both animal model and human studies to be most pronounced at this critical stage before and during puberty. During this time, development of reproductive organs, including the uterus, as well as development of the brain during puberty, can be permanently affected by environmental factors. One prominent concern for both increased UL incidence, as well as higher incidence of hormone-responsive breast cancer, is the effect of environmental estrogen exposure on early onset of puberty in young girls. The average age of menarche has been declining in recent years (Biro et al., 2010; Herman-Giddens, 2006). This early onset of puberty increases the overall lifetime exposure to estrogen, which increases risk of UL development and can also lead to adverse health problems later in life, such as depression and obesity (Black & Klein, 2012; Jefferson et al., 2012).

Early exposure to the environmental estrogen diethylstilbestrol (DES) can result in hyper-responsiveness to normal levels of estrogen hormone as an adult, and can result in higher UL risk (Mahalingaiah et al., 2014; S. A. Patel & Sunde, 2014). DES was first produced in the 1930s as a
synthetic estrogen prescribed to pregnant women through the 1970s to prevent miscarriage and ease morning sickness. This resulted in an estimated 5-10 million pregnant women and their children, termed DES-daughters and DES-sons, exposed to DES during the window of sensitivity to environmental estrogens. DES daughters, exposed in utero to DES, present later in life with clear cell adenocarcinomas, uterine structural difference, higher chance of preterm labor or ectopic pregnancy, infertility and increased incidence of UL. DES sons display higher risk of infertility, epididymal cysts and reproductive tract cancers. Studies in rodent models show perinatal exposure to DES can cause developmental reprogramming of the female rat reproductive tract. Symptoms in the adult animal include persistent estrus, early vaginal opening, infertility, early reproductive senescence, multi-oocyte follicles and uterine adenocarcinomas (Iguchi, Kamiya, Uesugi, Sayama, & Takasugi, 1991). Eker mice treated with DES have an increased incidence of UL. DES signals through ERα, as evidenced by Esr1 null mice which are resistance to DES-induced uterine “reprogramming” (J. D. Cook & Walker, 2004; Walker et al., 2003).

An increased risk of UL development has been linked to early-life exposure to genistein, a common source of dietary estrogen (Hoey, Rowland, Lloyd, Clarke, & Wiseman, 2004; Jefferson et al., 2009). Genistein is a phytoestrogen found naturally in plant sources such as soybeans and fava beans (Adlercreutz, Yamada, Wahala, & Watanabe, 1999). Genistein is best known as a tyrosine kinase inhibitor of EGFR and is used as a treatment for cancers due to its vascular inflammation modulation and angiogenesis inhibition properties (Cojocneanu Petric et al., 2015). However, genistein can also activate estrogen receptors to influence estrogen-responsive genes (Moller et al., 2012). Genistein has a higher affinity for ERβ but has been shown to activate ERα-responsive genes in the uterus (Moller et al., 2012). As a common
Isoflavone, genistein can be found often in the diet, including soy-based products and beer (Adlercreutz et al., 1999; Lapcik, Hill, Hampl, Wahala, & Adlercreutz, 1998). Infants fed with soy-based infant formula have a 6-11 fold higher exposure, based on body weight, to genistein than the levels known to cause hormonal effects in adults (Hoey et al., 2004; Setchell, Zimmer-Nechemias, Cai, & Heubi, 1997). This high concentration of phytoestrogens in infant formula has an estrogenic affect in the female reproductive tract during the critical window of sensitivity to environmental estrogens, including re-estrogenization of vaginal cells at 6 months of age (Bernbaum et al., 2008). Mice treated neonatally with genistein at 50mg/kg/day, a level corresponding to serum concentrations in infants fed soy-based formula (6.8µM), have disrupted cyclicity and ovulation and are infertile due to uterine and oviduct abnormalities (Jefferson et al., 2009). Rats treated prepubertally with genistein show myometrial gene expression differences understood to prime the uterus for adverse consequences as adults (Moller et al., 2012).

In addition to DES and genistein, there is evidence that phenolic environmental estrogens, including BPA, octylphenol (OP), and nonylphenol (NP) may be involved in UL pathogenesis. BPA is a synthetic estrogen used during manufacturing of plastic products and resins commonly found in packaging of foods (McGuinn et al., 2015). Differential levels of xenoestrogens in blood serum levels and urine concentrations of patients with and without UL show correlation between environmental estrogen exposures and UL incidence (Shen et al., 2013). In addition, UL cells in culture increase proliferation and show increased expression of genes involved in UL pathogenesis when treated with BPA (Shen et al., 2014). Studies on rodent models exposed to high levels of BPA have shown disrupted reproductive organ development, including delayed vaginal opening and delayed testicular descent (Ashby & Tinwell, 1998; Nagao et al., 1999), as well as hypothalamis-pituitary-gonadal axis disruption (Rasier, Toppari,
Parent, & Bourguignon, 2006) and increased cancer risk (Betancourt et al., 2010). However, some studies show no effect of BPA on UL incidence at concentrations commonly found in adults (Pollack et al., 2015). In summary, prepubertal exposure to environmental estrogenic compounds increase risk of UL and result in reprogramming of reproductive organs.

d. Cellular etiology.

i. Cytogenic abnormalities in uterine leiomyoma.

Karyotypic abnormalities occur in 40-50% of UL, and tumors from the same uterus often show different chromosomal changes (Bulun, 2013). The most common abnormalities are translocations on chromosome 12, deletion of chromosomes 3q and 7q, trisomy 12 and rearrangements on chromosomes 6, 10 and 13 (Bulun, 2013). These chromosomal abnormalities may contribute to disruption of genes aberrantly expressed in UL, including HGMA2, ESR2, and RAD5 (Walker & Stewart, 2005). Recently, research on a somatic mutation (c.131G>A) in the mediator complex subunit 12 (MED12) has gained attention as this is an important contributor to UL etiology. The mutation in exon 2 of MED12 is present in approximately 70% of UL and is not found in surrounding myometrial tissue (Makinen et al., 2011). MED12 is a highly conserved 250kDa protein that is involved in the transcriptional regulation of the RNA polymerase II complex. MED12 is part of the CDK8 module, which, upon activation by cyclin C, phosphorylates the C-terminal domain of the large subunit of RNA polymerase II and inhibits formation of the transcriptional initiation complex (H. Wang et al., 2013). Mice expressing the c.131G>A MED12 mutation in the uterus show uterine hyperplasia, UL-like tumor formation, and genomic instability, demonstrating the importance of this mutation in UL etiology (Mittal et al., 2015). However, the mechanism resulting in the MED12 mutation in the otherwise categorically quiescent myometrial tissue is still unknown. In addition to the MED12 mutation, a
small percentage of UL are due to familial genetic mutations in the Fumarate Hydrase (HLRCC) genes; however, these do not account for the vast majority of UL. HRLCC mutations are present only in approximately 100 families worldwide (Walker & Stewart, 2005).

**ii. Dysregulation of microRNAs in uterine leiomyoma.**

Several miRNA have been shown to have differential expression between normal myometrium and UL tissue. A study by Wang *et. al* in 2007, revealed 45 miRNAs including the *let-7* family, *miR-21, miR-23b, miR-29b,* and *miR-197* were significantly dysregulated in UL compared to healthy myometrial tissue (T. Wang *et al.*, 2007). In addition, a significant racial difference in miRNA expression between UL from Caucasian and African American women, specifically *miR-21, miR-23b,* and *miR-197* was found (T. Wang *et al.*, 2007). A study by Marsh *et. al* also identified 46 miRNAs differentially expressed between UL and the myometrium, many of which were also dysregulated in other tumors (Marsh *et al.*, 2008). Since UL are steroid hormone-sensitive tumors, of particular interest were miRNAs associated with sex-steroid hormones in breast and prostate cancers that were also found to be increased in UL, including *miR-21, miR-34a, miR-125b* and *miR-150.* A study by Luo & Chegini (Luo & Chegini, 2008) identified 91 miRNAs differentially expressed in UL and found that 27 of these were similarly dysregulated in at least one of two studies by Marsh or Wang described above. Crucial *in vivo* evidence for the tumor suppressor function of miR29b was provided recently by Qiang *et al.* using a kidney capsule transplant model of UL (Qiang *et al.*, 2014).

**iii. Cell signaling pathways in uterine leiomyoma**

**PI3K/AKT-mTOR pathway**

Extensive evidence indicates the activation of the PI3K/AKT-mTOR pathway as one of the most overactive molecular pathways in UL, leading to decreased apoptosis, increased cell growth and
proliferation, and ultimately the development of UL (Karra et al., 2010; Makker et al., 2012). Phosphatidylinositol 3’-kinase (PI3K) regulates fundamental cellular functions including cell proliferation, growth, survival, transcription and translation through activation of protein kinase B (AKT) (Osaki, Oshimura, & Ito, 2004). PI3K is activated by receptor tyrosine kinases and by G-protein coupled receptors (GPCRs)(Osaki et al., 2004). PI3K forms phosphatidylinositol-(3,4,5)-triphosphate (PIP3), which activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), resulting in activation of AKT.

AKT signaling acts to increase cell survival by blocking the function of the pro-apoptotic protein, BAD. AKT also blocks FOXO-mediated transcription of pro-apoptotic target genes. AKT signals cell proliferation through inhibition of GSK3β, which degrades β-catenin and activates pro-apoptotic p53 (Manning & Cantley, 2007). AKT increases cell growth by inhibiting TSC1 and TSC2, which are negative regulators of mTOR (Carrera, 2004). mTOR is a serine-threonine kinase involved in promoting biosynthesis of proteins, lipids and organelles and limiting catabolic processes such as autophagy. mTOR acts in association with mLST8 and either Raptor (mTOR complex 1) or Rictor (mTOR complex 2) (Zaytseva, Valentino, Gulhati, & Evers, 2012). Disruption of the PI3K-AKT-mTOR pathway is associated with poor balance between cell proliferation and cell survival. Over-activation of the PI3K/AKT-mTOR pathway is associated with many tumors, including cancers, diabetes mellitus, and autoimmune diseases, and is being targeted by signal transduction therapies (Karra et al., 2010; Osaki et al., 2004).

The PI3K/AKT-mTOR pathway has been identified as one of the most up-regulated signaling pathways in UL, based on evidence from protein and transcriptional profiling of human UL, as well as in the Eker rat animal model (Crabtree et al., 2009). At the protein level, increased activation of PI3K/AKT pathway proteins and targets, including PTEN, p-AKT, p-GSK3 and
CD2 proteins, in UL compared to myometrium shows involvement of PI3K/AKT signaling in UL pathogenesis (Karra et al., 2010; Kovacs et al., 2003). In addition, there is evidence that PI3K and mTOR are necessary for estrogen-dependent cell growth in UL and myometrial cell cultures (Yin et al., 2007). Up-regulation of PI3K/AKT-mTOR proteins is evident in uterine cells of the Eker rat, which is a common UL animal model (Crabtree et al., 2009).

Targeting of the PI3K/AKT-mTOR pathway as a therapeutic option for UL is currently being explored by some laboratories (Borahay, Al-Hendy, Kilic, & Boehning, 2015; Lara et al., 2015). The AKT inhibitor, MK-2206, shows promise in the lab in limiting UL growth and increasing cell death (Borahay et al., 2015). However, side effects of rash, diarrhea, fatigue and mucositis in patients treated with MK-2206 are common due to the pervasive extent of AKT signaling in normal physiology (Lara et al., 2015). These side effects may limit the use of AKT inhibitor therapies for UL treatment.

**Ras/Raf/MEK/ERK pathway**

The Ras/Raf/MEK (mitogen-activated protein kinase)/ ERK (extracellular-signal-regulated kinase) pathway is involved in regulation of cell proliferation, differentiation and cell survival (Kolch, 2000). This pathway is activated by growth factors secreted by cells into the extracellular space. Growth factors bind receptor tyrosine kinases (RTKs) on the target cell surface to activate the Ras/Raf/MEK/ERK pathway. Upon activation, the RTK binds son of sevenless protein (SOS) and recruits Grb2. These proteins act to exchange GDP for GTP on the G-protein, Ras. Ras then activates Raf kinases, which phosphorylates MEK. Activated MEK phosphorylates and activates ERK. ERK can then activate the Activating protein-1 (AP-1) family of transcription factors to regulate expression of target genes (Borahay et al., 2015). Interestingly, two members of the AP-1 family transcription factors, c-Fos and c-Jun show lower levels of mRNA in UL compared to
healthy myometrium (Gustavsson et al., 2000; Lessl et al., 1997). However, a role for increased Ras/Ref/MEK/ERK signaling in UL is likely based on overexpression in UL of several proteins involved in the pathway. Shc, Grb2, and ERK and 15 distinct RTKs are more highly expressed in UL compared to healthy myometrium (Yu et al., 2008). In addition, a study by Nierth-Simpson et al showed increased activation of ERK following estrogen treatment occurs in UL, but not in myometrial cells, again suggesting a possible role for the Ras/Ref/MEK/ERK signaling pathway in UL pathogenesis (Nierth-Simpson et al., 2009).

**Wnt signaling pathway**

There is some evidence of wingless-type (Wnt) signaling in UL tumor etiology. The canonical Wnt signaling pathway begins with the binding of Wnt family protein ligands to the cell surface receptor Frizzled, which activates the cytoplasmic protein Dishvelled (Dvl). In canonical Wnt signaling, β-catenin accumulates in the nucleus and leads to activation of specific transcription factors (Ono et al., 2014). Wnt signaling genes, including Wnt5b, are overexpressed in UL (Mangioni et al., 2005). Wnt11 and Wnt16 expression is also increased specifically in UL cells after estrogen treatment (Borahay et al., 2015). Studies by Ono et al (Ono et al., 2014) show inhibitors of WNT and β-catenin may block UL growth and proliferation, suggesting canonical Wnt signaling may play a role in UL. Finally, studies by Tanwar et al have shown constitutively active beta-catenin drives UL-like tumors in the mouse uterus and may contribute to activation of TGF-β and mTOR signaling in these tumors (Tanwar et al., 2009).

**Growth factors**

Several growth factors also appear to play a role in cell proliferation and UL growth. Major growth factors overexpressed in UL include transforming growth factor-β (TGF-β),
epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) (Parker, 2007).

Over-activation of TGF-β signaling in UL has been identified as a major contributor to UL etiology, especially in regards to ECM regulation and smooth muscle cell proliferation (Arici & Sozen, 2000; Arslan et al., 2005; W. Catherino et al., 2004; Ciarmela et al., 2011). TGF-β signaling begins with binding to cell surface serine/threonine kinase receptors called Type I or Type II receptors. These receptors phosphorylate intracellular proteins called Smads, which complex with Co-Smads and accumulate in the nucleus to influence gene transcription (Savage-Dunn, 2005). In UL, substantial evidence indicates that TGF-β increases smooth muscle cell proliferation and extracellular matrix deposition and can be activated by PI3K/AKT pathway proteins (Borahay et al., 2015; Ciarmela et al., 2011). Three distinct isoforms of TGF-β (TGF-β1, -2, and -3) occur in the myometrium. TGF-β-1 & -2 are expressed at similar levels between the myometrium and UL tissue, whereas TGF-β-3 is highly overexpressed in UL (Ciarmela et al., 2011). A study by Arici & Sozen found a 3.5-5-fold higher expression of TGF-β-3 in UL compared to myometrial cells, and treatment of cells with TGF-β led to increased fibronectin and cell proliferation in UL cells (Arici & Sozen, 2000). Furthermore, treatment of the Eker rat, a UL animal model, with TGF-β inhibitors reduces leiomyoma severity and incidence in these animals (Laping et al., 2007). Increased levels of phosphorylated SMAD 2/3 proteins in UL also suggest possible involvement of TGF-β in UL etiology (Chegini, Luo, Ding, & Ripley, 2003). Interestingly, TGF-β expression in the uterus is sensitive to sex steroid hormones and acts downstream of environmental estrogen signaling, a prominent risk factor for UL development (Di et al., 2012; Shen, Wu, Lu, Zhang, & Ren, 2015). TGF-β-3 expression levels fluctuate during each phase of the menstrual cycle, peaking at the secretory phase, suggesting regulation by
progesterone and estrogen (Ciarmela et al., 2011). TGF-β and downstream Smad proteins show altered expression at high levels of environmental estrogen exposure, including genistein, bisphenol A (BPA), and nonylphenol (Di et al., 2012; Shen et al., 2015). In addition, all three isoforms of TGF-β are reduced in women undergoing treatment with GnRH agonists, which diminish steroid hormones in the uterus (Ciarmela et al., 2011).

EGF, PDGF and VEGF-A levels have been found at significantly higher levels in UL tissue compared to myometrial tissue, although the receptor for EGF, EGFR, is expressed at similar levels between the tissues (Borahay et al., 2015; Ren et al., 2011). Overexpression of PDGF in UL has been linked to increased collagen α1, and proliferating cell nuclear antigen (PCNA), both of which are also increased in UL tissue (Borahay et al., 2015). EGF stimulates DNA synthesis and increases mitogenesis through MAPK signaling pathways exclusively in UL tissue (Parker, 2007). Importantly, EGF expression seems to be highly regulated by steroid hormones, especially progesterone and levels of EGF are drastically reduced upon GnRH-agonist treatment (Flake et al., 2003).

Analysis of mRNA and protein of UL tissue shows approximately one third of UL patient samples have increased levels of IGF-1, which has been shown to stimulate mitogenesis by activating the MAPK signaling (Borahay et al., 2015; Parker, 2007). Peng et. al found a correlation between increased IGF-1 and increased activation of pAKT in UL, levels of which also correlated with increased UL size (Peng et al., 2009). Studies in the Eker rat, also indicate possible involvement of IGF-1 in UL pathogenesis. A study by Burroughs et. al showed a 7-fold increase in IGF-1 levels in the Eker rat leiomyoma, as well as a significant increase of a protein down-stream of IGF-1, insulin receptor substrate-1 (IRS-1) in UL (Burroughs et al., 2002). Finally, IGF-1 has also been shown to increase in leiomyoma cultured cells after estrogen
treatment (Swartz, Afshari, Yu, Hall, & Dixon, 2005). Thus, altered IGF-1 signaling may play a role in a considerable portion of UL.

**A.3. Treatment of uterine leiomyoma.**

*Hysterectomy and myomectomy.*

The majority of UL are asymptomatic and require no treatment. However in about 25% of cases severe symptoms necessitate treatment, of which the most common is hysterectomy, removal of the entire uterus, or in some cases myomectomy, removal of only the UL (Vilos et al., 2015). Hysterectomy is considered the definitive treatment for UL, resulting in over 200,000 hysterectomies per year in the United States (Bulun, 2013). However, hysterectomy as a treatment option is costly, risky, and poses problems for women wishing to preserve fertility. In the United States in 2010 over $34 billion was spent on medical costs due to UL, including time spent off work for long post-surgical recovery (Cardozo et al., 2012). Following surgery for UL there is a 15-38% risk for major complications, including post-operative hemorrhage, fever, and injury to adjacent organs (Al-Hendy & Salama, 2006). Despite the drawbacks, hysterectomy remains the standard treatment for UL, especially in women past child-bearing age, because it results in immediate and complete resolution of UL tumors and symptoms (Bulun, 2013). Many of the long-term risks associated with hysterectomy are related to oophorectomy, or removal of ovaries, that accompanies hysterectomy in approximately 73% of cases (Buie, Owings, DeFrances, & Golosinskiy, 2010). Oophorectomy has been shown to lead to increased risk of cardiovascular disease, dementia, osteoporosis and mood disorders (Stewart, Shuster, & Rocca, 2012). However, even in cases of hysterectomy alone, without removal of the ovaries, there are long-term complications, including early menopause. Early menopause often leads to further adverse events such as increased risk of cardiovascular problems and shorter lifespan (Stewart et
al., 2012). Despite the widespread use of hysterectomy for UL treatment, the long-term adverse effects of organ removal for a benign condition may outweigh the benefits in many cases.

**b. Minimally invasive surgical therapies.**

An option for a less invasive surgical treatment for UL includes uterine artery embolization (UAE). UAE involves blocking uterine arteries with small particles to diminish blood supply to the uterus and cause ischemic necrosis of UL (Gambadauro, 2012). A study by Pron et al. reported improvement of UL-associated bleeding in 77% of women following UAE and showed an average reduction of 33% in UL tumor size (Pron et al., 2005). However, UAE is only recommended for women not seeking to preserve fertility due to high risk of reduced placental blood flow, preterm labor and intrauterine growth restriction following UAE. Other complications of UAE for UL treatment include damage to the ovaries due to decreased blood supply and infection damaging the fallopian tubes, both of which impair or destroy fertility. It is estimated that 80% of women seek re-intervention treatment in the 5 years following UAE (van der Kooij, Bipat, Hehenkamp, Ankum, & Reekers, 2011). Despite its limitations, UAE does result in shorter hospitalization time as compared to hysterectomy and leads to less post-surgical complications in the short term (Gupta, Sinha, Lumsden, & Hickey, 2006).

Similar to UAE, other treatments for UL directed to reduce blood supply to the uterus and cause UL ischemia are vaginal occlusion of uterine arteries, laparoscopic occlusion and transvaginal clamping. These techniques show alleviation of UL symptoms in the majority of cases, but are not available to women who wish to preserve future fertility, women who use an intrauterine device or have a history of anemia or deep vein thrombosis (Zupi et al., 2015).

Another surgical alternative treatment option for UL is Magnetic Resonance Imaging-guided High-frequency Ultrasound (MRgFUS). This technique uses MRI technology to visualize
the UL tumors and uses focused ultrasonic energy to target UL tumors without damaging adjacent healthy myometrium. Relief of UL symptoms in the majority of patients is seen following MRgFUS; however, it is only a viable treatment option for UL located immediately adjacent to the anterior abdominal wall (Gambadauro, 2012; Pron et al., 2005; Zupi et al., 2015). A retrospective study by Behera et. al found 47% of patients were considered ineligible for MRgFUS treatment of UL, due mainly to large UL tumor size and high cost of the procedure (Behera, Leong, Johnson, & Brown, 2010).


i. GnRH agonists.

Gonadotropin-releasing hormone (GnRH) agonists are one of only two drugs currently approved by the United States Food and Drug Administration (FDA) for treatment of UL. GnRH agonists are approved only for the 3 to 6 months preceding surgery and must be taken with iron supplementation for reduction of anemia symptoms (Doherty, Mutlu, Sinclair, & Taylor, 2014). GnRH agonists function by shutting down the hypothalamic-pituitary-gonadal axis. GnRH is a hormone released by the hypothalamus which functions by binding to GnRH receptors on gonadotropins in the pituitary, stimulating release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which in turn stimulate sex steroid hormone production in the ovary. GnRH agonists, including leuprolide acetate and nafarelin, function by binding to GnRH receptors, causing an initial flare-up in LH and FSH, followed by down regulation of GnRH receptors in the pituitary and inhibition of ovarian sex steroid hormone production by the hypothalamic-pituitary-gonadal axis (American College of & Gynecologists, 2008; Doherty et al., 2014). In addition, GnRH agonists decrease aromatase production in UL cells, which further decreases estrogen in the uterus (Shozu et al., 2001). Short-term treatment with GnRH agonists
results in a 35%-65% decrease in tumors size and reduces bleeding associated with UL (Minaguchi, Wong, & Snubes, 2000). The reduction in UL size is beneficial because it reduces blood loss during surgery and allows less invasive surgery options (Doherty et al., 2014). A study by Vercillini et al. of 60 women undergoing hysterectomy surgery for UL showed that treatment with GnRH agonists for 12 weeks prior to surgery allowed a less invasive vaginal surgery, rather than abdominal hysterectomy in 47% of women (Vercellini et al., 1998). Drawbacks of GnRH agonists for treatment of UL are related to the hypoestrogenic state induced by long-term use of the drug. Side-effects include hot flashes, vaginal dryness, bone loss and headaches. Bone resorption becomes irreversible after 6 months of treatment, limiting the duration of safe use of GnRH agonists alone (Doherty et al., 2014; Zupi et al., 2015). Another drawback to GnRH agonist treatment for UL is that tumors return to pre-treatment size and symptom severity once treatment is stopped (Zupi et al., 2015). Recently, add-back therapy in conjunction with GnRH agonists have gained attention as a longer-term drug treatment solution for UL. This involves supplementing GnRH agonists with estrogen, progesterone or both to offset the hypoestrogenic side-effects of GnRH agonists alone. However, the sensitivity of UL to hormone stimulation creates a barrier to add-back therapy, as tumors have been shown to return to pre-treatment size when add-back therapy is used (Doherty et al., 2014; Friedman et al., 1994).

ii. Synthetic estrogen and progestins.

Synthetic estrogens and progestins have been used for treatment of abnormal bleeding associated with UL. Often estrogen and progesterone are given in combination in the form of oral contraceptive pills. Although this course of treatment can help control symptoms of excessive bleeding through action in the endometrium, there is no reduction in tumor size, and in some cases an increase in UL tumor size is seen (Owen & Armstrong, 2015). More recently, oral
contraceptive pills have been used in combination with GnRH agonists to help offset the hypoestrogenic side effects of GnRH agonists, but have shown limited improvement in reducing UL tumor size. Synthetic progestin-only pills, including Mirena (levonorgestrel-releasing intrauterine device), are FDA approved to treat menorrhagia associated with UL, but these do not reduce tumor size (Vilos et al., 2015).

iii. Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) are small molecule drugs which bind estrogen receptors and can act as either antagonists or agonists to estrogen activity depending on the co-activators and co-repressors present in specific target tissues (Vilos et al., 2015). Two SERMs that have been used as treatments for UL are Tamoxifen and Raloxifene. Tamoxifen is best known as a treatment for hormone-responsive breast cancers, as it is antagonistic in breast tissue and is an agonist in bone, cardiovascular tissues, and the endometrium (Doherty et al., 2014). Women with UL treated for 6 months with Tamoxifen showed a 40-50% reduction in blood loss and a lessened severity of pain associated with UL; however, UL tumor size was not changed and patients suffered from substantial side effects of dizziness and hot flashes (Sadan et al., 2001). In addition, the agonist activity of Tamoxifen on the endometrium raises concerns about uterine hyperplasia and cancer. Therefore, the use of Tamoxifen for treatment of UL is discouraged (Doherty et al., 2014; Sadan et al., 2001). Raloxifene is a SERM with agonistic activity in bone and circulating lipoproteins, but has antagonistic activity in the endometrium, eliminating the major concern of uterine hyperplasia in long-term use (Baker et al., 1998). Raloxifene has shown to be effective in reducing UL tumor size in postmenopausal women (Palomba et al., 2001). However, generally UL symptoms and incidence are drastically decreased after menopause, rendering postmenopausal treatment unnecessary (Walker &
Stewart, 2005). In premenopausal women, initial studies have found no significant difference in UL tumor size or bleeding following treatment with Raloxifene (Palomba, Orio, et al., 2002). However, when used as an add-back therapy for GnRH agonists, UL tumor size does regress with fewer side effects than GnRH agonists alone (Palomba, Russo, et al., 2002). Future development of SERMs which are brain-sparing and ovary-neutral may be an important advancement for use of SERMs for UL treatment without severe side-effect limitations.

iv. Anti-progestins and progesterone receptor modulators.

The newly defined role of progesterone and progesterone receptor (PR) in the proliferation of UL cells has spurred advancements in progesterone antagonistic drugs for UL treatment (Talaulikar & Manyonda, 2012). Although progesterone classically has an anti-proliferative effect on the uterus, several studies have shown contradictory results in UL tissue. Progesterone can constrain UL cell growth by down-regulation of IGF-1 (Talaulikar & Manyonda, 2012). However, progesterone has also been shown to increase UL cell growth by influencing gene expression of epidermal growth factor (EGF), Bcl-2 and tumor necrosis factor-α (TNF-α) (Maruo et al., 2000). Evidence for a role of progesterone in UL tumor progression has led to off-label treatment of UL with progesterone receptor modulators (PRMs), including mifepristone (RU-486), asoprisnil (J867) and ulipristal acetate (CDB-2914) (Spitz, 2009; Talaulikar & Manyonda, 2012). Studies on these PRMs, have shown promising results for short term use. Women using PRMs for 3-4 months report less pain associated with UL, decreased bleeding and show smaller UL size. However, long-term use of PRMs, beyond 4 months, is contraindicated due to side effects of endometrial thickening (Spitz, 2009).

v. Aromatase inhibitors.
Aromatase, an enzyme involved in the conversion of androgens to estrogen, is shown to be overexpressed in UL tissue compared to healthy myometrial tissue, and is believed to increase the local concentration of estrogen in UL tumors (Doherty et al., 2014). Therefore, inhibition of aromatase has been suggested as an experimental option in treatment of UL. Aromatase inhibitors, including letrozole, anastrozole and fadrozole, create an immediate hypoestrogenic state, followed by high levels of gonadotropins, due to loss of negative inhibition by estrogen on the pituitary (Doherty et al., 2014). Studies in post-menopausal women with UL, who have no ovarian production of estrogen, demonstrate significant reduction in UL tumor size when treated with aromatase inhibitors (Doherty et al., 2014; Kaunitz, 2007). In premenopausal women treated with aromatase inhibitors studies have shown decreased bleeding and a slightly reduced tumor size, without the bone loss seen in GnRH-agonist therapy (Gurates et al., 2008). However, the increased gonadotropins caused by long-term use of aromatase inhibitors leads to formation of ovarian cysts (Doherty et al., 2014). A study by Gurates et al of women with UL treated for 3 months with a high dose (5mg/day) of letrozole resulted in over half (56%) of the participants developing follicular ovarian cysts (Gurates et al., 2008). To reduce this side effect, aromatase inhibitors are currently used in combination with GnRH agonists. However, this consequentially induces bone mineral density loss in many patients, and is therefore only used short-term (3-4 months) preceding surgery (Doherty et al., 2014).

vi. Anti-fibrotics.

One of the hallmark characteristics of UL is the overabundance of extracellular matrix; hence, anti-fibrotic drugs present an attractive option for UL treatment. The anti-fibrotic pirfenidone decreases TGF-β, collagen types I and III and reduces proliferation of UL cells in culture (Doherty et al., 2014). However, patients report severe side effects including nausea, rash and
fatigue when taking pirfenidone, which limits its long-term use for UL (Raghu, Johnson, Lockhart, & Mageto, 1999). Similarly, other treatments targeting extracellular matrix and growth factors proposed for UL treatment, including SB-525334, interferon-α therapy, and thiazolidinediones produce severe side-effects which preclude use for UL treatment (Doherty et al., 2014). However, novel anti-fibrotic, such as halofuginone, show reduction in ECM proteins in UL cells in vitro and may be used in the future for UL treatment (Nowak, 2000). Halofuginone has also shown promise in inhibiting cell proliferation and increasing tumor cell apoptosis in a mouse xenograft model (Koohestani et al., 2016).

**A.4. Animal models for uterine leiomyoma.**

The most commonly used and best characterized preclinical model for UL is the Eker rat. Eker rats have mutations in the tuberous sclerosis complex 2 (Tsc2) gene, involved in regulation of mTOR Complex 1. These mice develop frequent spontaneous UL tumors in up to 65% of females by 15 months of age (J. D. Cook & Walker, 2004). Similar to human UL, Eker rat leiomyomas are highly estrogen sensitive and share biochemical and phenotypic profiles, including sex steroid hormone receptor expression, dysregulation of HMGA2, IGF-1, and the mTOR pathway and histological features (Crabtree et al., 2009; Walker & Stewart, 2005). In addition, tumor uterine cells in culture isolated from the Eker rat, known as ELT3 cells, have been shown to retain their UL phenotype and have been used extensively to explore possible therapeutic options for UL, including SERMs, anti-fibrotics and anti-progestins (Arslan et al., 2005). However, Eker rats also present with renal and liver cancers, which are often fatal and limit long-term studies (Everitt, Wolf, Howe, Goldsworthy, & Walker, 1995). Similar to the Eker rat, a mouse model with mutations in the Tsc2 gene has been developed recently. This mouse model presents with uterine leiomyoma, as well as myometrial tumors in the lungs (Prizant et al.,
2013; Walker & Stewart, 2005). Despite their usefulness in studying UL, the Eker rat and Tsc2 null mouse models are not ideal UL animal models, as the Tsc2 gene mutated in these animals is not affected in human UL.

Recently, a mouse model with a UL-specific Med12 mutation has been developed (Mittal et al., 2015). This mouse model expresses UL-specific c.131G>A missense mutation in the reproductive tract under Med12 conditional null background, driven by Amhr2^{+/Cre} (Makinen et al., 2011; Mittal et al., 2015). These mice develop myometrial tumors, which mimic UL at the histological level, including development of nodules with increased ECM. In addition, evidence of chromosomal instability is seen in this mouse model (Mittal et al., 2015). Further characterization of this mouse model will be important for understanding the emerging role of the common MED12 mutations in UL pathogenesis.

Other UL models include xenografts of human UL tumors in immune-compromised mice (Suo, Sadarangani, Lamarca, Cowan, & Wang, 2009). This approach is useful for studying tumor formation in vivo but lacks myometrial controls, since the matched human myometrial cells do not form xenografts easily in this system (Suo et al., 2009). The guinea pig has also been used as an alternative mammalian model for UL, which forms frequent leiomyoma after ovary removal and estrogen treatment (Walker & Stewart, 2005). However, these leiomyoma show little similarity to human UL based on histological studies (Everitt et al., 1995).

**Molecular Players**

**B.1. GPR-10.**

GPR10, also known as prolactin releasing hormone receptor (PRLHR), is a neuron-specific G-protein coupled receptor (GPCR) expressed in the reticular nucleus of the thalamus, periventricular nucleus (PVN) of the hypothalamus, adrenal glands and medulla oblongata (Dodd
& Luckman, 2013). The gene for GPR10 is located on human chromosome 10 and was first cloned in hypothalamic tissue as an orphan GPCR. In cultured rat pituitary somatotrope tumor cell lines (GH3 cells), GPR10 activates the PI3K/AKT signaling cascade through G\textsubscript{i}/G\textsubscript{0} (Hayakawa et al., 2002). In support of the role of GPR10 in PI3K/AKT activation, studies have shown calcium release upon activation of GPR10 in neuronal cell culture (Xia & Arai, 2011). There is also evidence of GPR10 activation of extracellular signal-related kinase (ERK) and c-Jun N-terminal protein kinase (JNK), although the principal signaling pathway of GPR10 appears to be the PI3K/AKT cascade (Hayakawa et al., 2002; Kimura et al., 2000).

The peptide ligand Prolactin-releasing peptide (PrRP) activates GPR10. This is a 21 amino acid RF-amide peptide, meaning the carboxyl terminus has an amidated phenylalanine motif (Osugi, Ukena, Sower, Kawauchi, & Tsutsui, 2006). Although the name PrRP suggests a role in prolactin release, studies showing the involvement of PrRP in release of prolactin were performed in vitro in anterior pituitary tumor cell lines (Hinuma et al., 1998) and subsequent in vivo studies did not substantiate the evidence (Seal et al., 2002). Instead, PrRP and GPR10 have been shown to be involved in broad functions in the brain, including satiety, stress, nociception, and sleep (Lawrence, Celsi, Brennand, & Luckman, 2000). Furthermore, GPR10 and PrRP have well-established roles in feeding behavior, the stress response, and in pain sensitivity. In addition to binding to GPR10, PrRP can act as a ligand for other receptors, including neuropeptide FF (NPFF-R2). Therefore, its expression is more ubiquitous than GPR10 (Engstrom, Brandt, Wurster, Savola, & Panula, 2003; Gouarderes, Puget, & Zajac, 2004). PrRP is found in the brain as well as periphery, including adrenal glands, pancreas, placenta and testis (Dodd & Luckman, 2013). In contrast, the only ligand known to activate GPR10 is PrRP, and expression of GPR10 outside of the central nervous system has not been shown.
During digestion, GPR10 activation by PrRP mediates the central nervous system response to cholecystokinin (CCK) release from enteroendocrine cells, signaling satiety, and the CNS response to leptin (Lawrence et al., 2000). GPR10 and PrRP signal sensations of fullness after food consumption through nucleus solitaries neurons leading to the hypothalamus (Xia & Arai, 2011). Gpr10-null and PrRP-null mice are obese and hyperphagic, and central administration of PrRP decreases feeding and bodyweight in rats and mice, supporting the role of GPR10 in signaling satiety (Dodd & Luckman, 2013; Lawrence et al., 2000). Treatment of mice with leptin and PrRP results in decreased eating and weight loss, however the hypophagic effect of leptin is impaired in PrRP-null and Gpr10-null mice (Gu, Geddes, Zhang, Foley, & Stricker-Krongrad, 2004; Takayanagi et al., 2008).

GPR10 plays a role in the hypothalamic-pituitary-adrenal axis (HPA-axis) secondary stress response. GPR10 expressing neurons support release of corticotropin-releasing hormone (CRH), ultimately resulting in sympathetic nervous stimulation including elevated blood pressure, increased heart rate and increased vasoconstriction (Dodd & Luckman, 2013). Central administration of PrRP results in release of ACTH, oxytocin and cortisol into systemic circulation (Samson, Resch, & Murphy, 2000).

Evidence also shows GPR10 activation by PrRP has a role in offsetting the opioid system, and possibly sleep regulation. GPR10 null mice have higher pain threshold and reduced tolerance for morphine. Treatment of wild-type mice with PrRP reverses the pain relieving effect of morphine. GPR10 is also highly expressed in neurons found in the preoptic nuclei, the histaminergic tuberomammillary nucleus and the locus coeruleus, which are involved in sleep regulation, suggesting a role for GPR10 and PrRP in sleep behavior (Xia & Arai, 2011).
B.2. REST/NRSF.

a. REST function.

RE1-Silencing Transcription factor (REST), also known as Neuron-Restrictive Silencer Factor (NRSF), is an epigenetic silencer which broadly functions to restrict neuronal gene expression to the central nervous system by silencing target gene expression in the periphery (Ballas & Mandel, 2005). The protein structure of REST consists of eight zinc-finger domains at the N-terminus, which are required to bind to the repressor element-1 (RE-1) sequence, a highly basic region, followed by a series of proline repeats and a zinc finger at the C-terminus. REST is located on chromosome 4 (Shimojo, 2006).

REST silences target genes through binding to a 21 base-pair canonical RE-1 consensus sequence (TTCAGCACCACGGACAG), found in the regulatory regions of the target genes (Bruce et al., 2004). Although over 2000 genes contain the highly conserved RE-1 sequence, REST only binds some of these genes in certain cell types, and REST binding is lost or gained depending on the state of differentiation of the cell (Bruce et al., 2004; Negrini, Prada, D'Alessandro, & Meldolesi, 2013). REST targets include neuron-specific genes such as GPR10, GRIN2A (the glutamate NMDA receptor channel, involved in long term potentiation), NEFH (the subunit of neurofilaments), GRIA2 (the glutamate receptor AMPA subunit), Doublecortin (involved in neural migration), and ADAM12 (involved in EGF signaling)(Bruce et al., 2004; Gopalakrishnan, 2009; Kemp et al., 2002).

As a transcriptional repressor, REST acts as a molecular scaffold, recruiting co-repressors and chromatin remodelers to epigenetically silence target gene transcription (Ballas & Mandel, 2005). Depending on the cell type, REST has been shown to complex with several different co-repressors and epigenetic modifiers. These unique complexes determine the affinity of REST
binding and the degree of gene repression (Negrini et al., 2013). REST is known to bind the co-repressor mSin3, which forms a complex with histone deacetylases HDAC1 and HDAC 2 and nuclear hormone receptor co-repressor (N-Co-R)(Roopra et al., 2000). Co-REST is an important binding partner of REST at the C-terminus which bridges REST and methyl CpG binding protein (MeCP-2), lysine specific demethylase (LSD-1), chromodomain Y-like protein (CDYL), and histone H3-lysine 9 methyltransferase G9a (Andres et al., 1999; Gopalakrishnan, 2009). Interestingly, REST has been shown to complex with MED12, which is mutated in up to 77% of UL (Ding et al., 2008). The interaction between REST and MED12 with regard to the common MED12 mutation seen in UL is still not fully understood. However, MED12 mutations identified in X-linked mental retardation and Lujan’s syndrome interrupted G9a mediated binding of REST to the mediator complex in cell culture (Ding et al., 2008; Gopalakrishnan, 2009). It is possible that a similar disturbance exists between the REST and mutated MED12 in UL.

Expression of REST in the periphery is well established, as REST is considered universally expressed in non-neuronal tissues (Negrini et al., 2013). REST expression is also well established as critical during brain development as evidenced by Rest null mice showing embryonic lethality due to brain malformation on day E9.5 (Chen, Paquette, & Anderson, 1998). However, in the central nervous system, recent studies have illuminated a more complex expression pattern for REST than previously thought. Instead of insignificant expression in all neurons, REST is now thought to be up-regulated in neural precursor cells and post mitotic neurons, where it functions to maintain cells in an undifferentiated state through epigenetic suppression of neuron-specific genes (Ballas, Grunseich, Lu, Speh, & Mandel, 2005). Maintenance of REST is associated with an undifferentiated phenotype in neural cells, whereas
many mature cells in the central nervous system show significant expression of REST (Ballas & Mandel, 2005).

In neural cells, REST is targeted for degradation by the E3 ubiquitin ligase, β-TRCP, at the C-terminal DEGXXS sequence. Lambda-phosphatase treatment prevents degradation of REST, showing phosphorylation of REST is needed for interaction with β-TRCP. Increased expression of herpes virus-associated ubiquitin-specific proteasomes (HAUSP) results in deubiquitination of REST, even in the presence of β-TRCP, keeping expression of REST high (Negrini et al., 2013). The balance between β-TRCP ubiquitination and HAUSP deubiquitination is thought to maintain the appropriate level of REST expression in neuronal cells (Z. Huang & Bao, 2012). In support of REST degradation by proteasomal targeting in other cell types, it has been shown in He-la cells that REST degradation can be prevented through proteasomal inhibition by MG132 treatment (Z. Huang & Bao, 2012).

Compelling evidence shows a role for REST upstream of the tumorigenic signaling pathway PI3K-AKT, which is significantly up-regulated in UL. Silencing of REST increases PI3K-AKT signaling and PI3K activity is required for REST-induced cellular transformation in human mammary epithelial cells (Westbrook et al., 2005). Loss of REST function in lung cancers and breast cancer cells enhances cell proliferation and survival by AKT pathway activation. However, the mechanism by which REST activates the PI3K-AKT pathway is unclear, and may likely activate the pathway through several molecular networks (Westbrook et al., 2005).

**b. REST dysfunction.**

REST has opposite roles in epithelial cells and in neurons. REST is considered a tumor suppressor in epithelial cells and an oncogene in neuronal cells. As such, an abnormally high
level of REST in neuroblastoma cells stimulates cell proliferation, whereas low REST expression contributes to cell proliferation in epithelial cancer cells (Negrini et al., 2013). Dysregulation of REST in both the central nervous system and in epithelial cells has been linked to several types of cancers. Overexpression of REST in the central nervous system has been implicated in the etiology of neuroblastomas, medullablastomas and glioblastomas. Conversely, down-regulation of REST has been shown in epithelial cell cancers such as breast cancer, small cell lung carcinoma (SCLC) and colorectal cancer (Lv et al., 2010; Wagoner et al., 2010; Westbrook et al., 2005). In addition, low REST expression in muscle cells has been observed in association with overexpression of neuronal genes in myotilinopathy, a disease of muscle tissue (Gopalakrishnan, 2009).

Causes of REST dysfunction can take place by several distinctive mechanisms, depending on the disease and cell type. These include genetic mutation, alternative splicing, proteasomal degradation, mislocalization, and RE-1 sequence mutation.

Genetic deletion causes a frame-shift mutation in the REST gene in colorectal cancer. A single-nucleotide deletion in exon 4 of REST results in a frame shift mutation, causing expression of a truncated REST protein, only 85kDa in size, in colon cancer cell lines (Westbrook et al., 2005).

Alternative splicing is responsible for REST dysfunction in both pheochromocytoma and small cell lung carcinoma cells. In adrenal medulla cancer cells alternative splicing results in expression of a dominant negative form of REST called REST4, leading to repression of REST epigenetic function and tumor formation (Shimojo, Paquette, Anderson, & Hersh, 1999). In small cell lung carcinoma (SCLC), an SCLC specific form of REST is expressed which
decreases efficiency of REST, leading to activation of tumorigenic pathway activation (Westbrook et al., 2008).

REST function is compromised by inappropriate protein degradation in neuroblastomas, medullablastomas, and glioblastomas, due to imbalance in the activity of β-TRCP, the E3 ubiquitin ligase responsible for targeting REST for degradation, and the expression of HAUSP, responsible for de-ubiquitination of REST (Z. Huang & Bao, 2012; Negrini et al., 2013). Similarly, β-TRCP overexpression in human mammary epithelial cells causes oncogenic transformation due to increased REST degradation (Westbrook et al., 2008). In addition, recent evidence has shown REST expression is increased in neurons as a protective measure in response to cortical and hippocampal injury, such as ischemic stroke, aging and stress (Calderone et al., 2003). However, in Alzheimer’s disease the protective effect of REST expression is compromised by degradation of REST, found in puncta within autophagosomes, instead of the nucleus (Lu et al., 2014).

Mislocalization of REST is the cause of disrupted REST function in cardiomyocyte differentiation, progressive myoclonus epilepsy and Huntington’s disease. In cardiomyocyte differentiation, during the G2 phase of cell growth, REST is held outside of the nucleus to allow expression of its target gene, atrial natriuretic peptide (Shimojo, 2011). In progressive myoclonus epilepsy, a mutation in the REST binding partner, PRICKLE-1, blocks interaction with REST, resulting in mislocalization and therefore disrupted activity of REST (Bassuk et al., 2008). In Huntington’s Disease, the protein huntingtin is mutated so that it cannot bind the REST/PRICKLE-1 complex through dynactin p150 to keep REST in the cytoplasm, resulting in REST localization to the nucleus and suppression of critical genes such as Brain-Derived Neurotrophic Factor (BDNF)(Shimojo, 2008).
In addition to the previously described REST dysregulation in disease states, other possible routes of control of REST expression have been postulated, but not yet seen in vivo, including regulation of REST transcription and miRNA regulation. It has been shown that the canonical WNT pathway can regulate REST expression during neuronal development, and feasibly, aberrant WNT signaling could lead to altered REST expression and downstream epigenetic changes (Negrini et al., 2013; Nishihara, Tsuda, & Ogura, 2003). Reciprocal repression of REST and miR-124a, or other miRNAs could have an effect on REST regulation (Negrini et al., 2013).

**B.3. PRICKLE1.**

Prickle homolog 1 (PRICKLE1), also known as Rest-interacting LIM-domain Protein (RILP) is a perinuclear protein involved in the nuclear localization of REST, planar cell polarity (PCP), and neuronal migration. The human PRICKLE1 gene is located on chromosome 12 and was first characterized in drosophila, where PRICKLE1 mutations created flies with prickle-like cuticular hairs, sensory bristles and eye units (Gallagher, 2009). Eight exons encode the protein, which has 3 LIM domains, three nuclear localization sequences (NLS), and a PET domain. Mutation of any of the NLS results in PRICKLE1 mislocalization (Shimojo & Hersh, 2006). The PET domain has been demonstrated to be essential for the binding of PRICKLE1 with REST, and mutations of the PET domain, particularly R104Q, results in the prevention of PRICKLE1 co-immunoprecipitation with REST (Bassuk et al., 2008). PRICKLE1 has three mRNA transcripts that differ only in the 5’ untranslated region (Shimojo & Hersh, 2003). Depending on the study, PRICKLE1 has been described as both a nuclear receptor and also as a cytoplasmic protein.
PRICKLE1 is best known for its critical role in non-canonical WNT/Planar Cell Polarity (PCP) signaling (Veeman et al., 2003). PCP is important in orienting sheets of cells in a polarized plane and especially important during development, including gastrulation and neuronal migration. PCP involves asymmetric distribution of specific PCP core proteins across the cell and is termed “non-canonical WNT” due to the absence of β-catenin involvement. During the development of Drosophila eyes and wings, PCP proteins FRIZZLED, DISHEVELLED (DVL) and DIEGO localize at the distal end of the cell while the proximal end of the cell membrane recruits STRABISMUS/VANGOGH and PRICKLE1. The latter interacts with DVL and prevents an association between DVL and FRIZZLED, facilitating a differential distribution of proteins at each end of the cell (Veeman et al., 2003). PRICKLE1 is kept at the proximal side of the cell by Par6 activation of Smurf 1/2 E3 ubiquitin ligases at the distal side, which target PRICKLE1 for proteasomal degradation. PRICKLE1 location also depends on phosphorylation by MINK1, and PRICKLE1 is cytosolic when MINK1 is silenced (Daulat et al., 2012).

PRICKLE1 is also known as Rest-interacting LIM domain protein (RILP) due to its major role in the nuclear localization of REST (Shimojo & Hersh, 2006). PRICKLE1 is a binding partner of REST, required to bring REST into the nucleus to perform epigenetic repression. Silencing of PRICKLE1, or mutation of PRICKLE1 NLS, results in mislocalization of REST and aberrant expression of genes with the RE-1 sequence. In vitro studies in myocardiocytes also show a major role for PRICKLE1 in REST localization to the nucleus, resulting in REST target gene regulation (Shimojo, 2011). In cortical neurons, PRICKLE1 is part of a complex shown to be necessary for proper REST localization, which is disrupted in Huntington’s disease (Shimojo, 2008). In addition to bringing REST to the nucleus, PRICKLE1
is also necessary to bring REST4, a dominant negative regulator of REST, to the nucleus (Shimojo & Hersh, 2006; Shimojo et al., 1999).

Several post-translational modifications of PRICKLE1 have been observed. PRICKLE1 is ubiquitinated and targeted for proteasomal degradation by SMURF1/2 (Mlodzik, 2009). PRICKLE1 can be phosphorylated by MINK1, which regulates its Rab5-dependent endosomal trafficking (Daulat et al., 2012). PRICKLE1 can also be phosphorylated by PKA; however, the function of this phosphorylation is unknown since PRICKLE1 still functions in PKA-deficient cell lines (Shimojo & Hersh, 2006). At the C-terminus of PRICKLE1, a farnesylation motif, as well as three NLS allows entry into the nucleus. Treatment with a farnesyllation inhibitor results in PRICKLE1 mislocalization (Shimojo & Hersh, 2006). It has been suggested that depending on its phosphorylation state, PRICKLE1 could either keep REST in the cytoplasm or the nucleus. Indeed, in different cell types PRICKLE1 can keep REST cytoplasmic, as seen in cortical neurons (Bassuk et al., 2008; Shimojo, 2008) or transfers REST to the nucleus as seen in epithelial cells (Shimojo & Hersh, 2003, 2006).

Disruption of PRICKLE1 expression or function has been linked to several disease conditions. In autosomal recessive ataxia epilepsy, a common missense mutation (R104Q) in the PRICKLE1 gene blocks the interaction between PRICKLE1 and REST leading to mislocalization of REST and aberrant expression of target genes, ultimately inducing seizures (Bassuk et al., 2008). In Huntington’s disease, disruption of the interaction between REST and PRICKLE1 leads to REST localization in the nucleus and disruption of the normal REST target gene expression in these neurons (Shimojo, 2008). In human hepatocellular carcinoma (HCC), PRICKLE1 under-expression contributes to disease etiology by disrupting Wnt signaling in HCC cells (D. W. Chan et al., 2006). Disrupted Wnt signaling is also seen in patients with cleft palate,
in which a high frequency mutation in *PRICKLE1* (C251X), results in disrupted Wnt signaling and improper palate development (Yang et al., 2014). Similarly, disruption of Wnt signaling due to *PRICKLE1* mutation stunts limb growth in mice (Gallagher, 2009). Interestingly, *PRICKLE1* dysfunction has also been linked to MED12, a gene mutated in the majority of UL. A study by Rocha *et. al.*, showed a mutation in *Med12* in mouse embryos caused disruption of Prickle1 positioning within the cell and disrupted the Wnt/PCP pathway, ultimately leading to problems with neural tube closure in these mice (Rocha, Bleiss, & Schrewe, 2010). The relationship between *PRICKLE1* and other proteins known to be involved in UL pathogenesis will be of interest for future study.

**B.4. EZH2.**

Enhancer of Zeste Homolog 2 (EZH2) is a histone methyltransferase polycomb group (PcG) protein. PcG proteins make up the protein complexes called Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). These complexes act as a memory systems for chromatin structure and gene expression through stable epigenetic inheritance during cell division (Cao et al., 2002). EZH2 is the catalytic subunit of the PRC2 complex and functions to trimethylate histone H3 lysine 27, a repressive chromatin mark (Volkel, Dupret, Le Bourhis, & Angrand, 2015). However, EZH2 requires two other proteins, including SUZ12 and EED for its enzymatic activity, EZH2 alone has no histone methyltransferase activity (Volkel et al., 2015). As such, EZH2 is regulated by its binding partners, which change during differentiation to regulate genes essential to development and cell differentiation (Cao et al., 2002; Czermin et al., 2002) The importance of EZH2 in gene regulation is evidenced by embryonic lethality of EZH2-null mice (Kuzmichev et al., 2005).
Dysregulation of EZH2 is often observed in several different cancers, and the degree of malignancy correlates with EZH2 dysregulation (Volkel et al., 2015). Depending on the disease, EZH2 acts as an oncogene or tumor suppressor. For example, in prostate, breast, bladder, lung, renal pancreatic and brain cancers, EZH2 is overexpressed. However, in myeloproliferative neoplasms, pediatric cancers and pediatric gliomas EZH2 activity is decreased (Volkel et al., 2015).

EZH2 overexpression in hormone sensitive cancers, including breast cancer, prostate cancer, and lymphoma mediates tumor differentiation (Hwang et al., 2008; Kleer et al., 2003; Varambally et al., 2002; Visser et al., 2001). For example, EZH2 silencing in prostate cancer cell lines reduces proliferation and decreases cell growth, whereas overexpression leads to a more invasive phenotype (Croonquist & Van Ness, 2005; Varambally et al., 2002). In estrogen-sensitive cancers, EZH2 is recruited to estrogen response element (ERE) containing promoters by its binding partner, Repressor of Estrogen receptor Activity (REA) to repress estrogen dependent transcription (Hwang et al., 2008).

Although EZH2 is best known as a gene silencer, there is also evidence of EZH2 function independent of its methyltransferase activity. In breast cancer MCF-7 cells, EZH2 acts at target gene promoters to link ERα to WNT signaling proteins β-catenin and TCF, which activates transcription of genes, such as MYC, independent of the methyltransferase activity of EZH2 (K. M. Chan et al., 2013; Shi et al., 2007; Volkel et al., 2015).

Long non-coding RNAs can increase PRC2 recruitment at chromatin in cancers. One IncRNA which interacts with EZH2 is HOTAIR. HOTAIR overexpression has been shown to increase invasiveness of epithelial cancers by bringing the PRC2 complex to target genes and therefore changing the epigenetic pattern expected in these epithelial cell types (Volkel et al.,
2015). Silencing of HOTAIR in cancer cells with high EZH2 expression can also decrease invasiveness (Rinn et al., 2007). Interestingly, HOTAIR also acts as a scaffold to link the PRC2 complex with REST and its binding partner, CoREST, which also control many epigenetic programs (Tsai et al., 2010). This interaction between EZH2 and REST could coordinate histone modification and gene silencing.

In ELT3 cells derived from the EKER rat, there is some evidence that the suppression of EZH2 is responsible for epigenetic changes in UL. Treatment of the rat uterus with genistein reduces activity of EZH2, while BPA treatment increases the activity (Greathouse et al., 2012).
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Chapter 3

The Role of GPR10 in the Pathogenesis of Uterine Leiomyoma
Abstract

Uterine leiomyoma (UL) are tumors arising in the smooth muscle tissue of the myometrium. UL are present in up to 77% of women, often causing severe pain, bleeding and discomfort (Cardozo et al., 2012). Despite their prevalence, there is no long-term pharmaceutical treatment for UL; due largely to the lack of understanding about the molecular pathogenesis of UL. It is well established that there is greater activation of PI3K/AKT-mTOR pathway in UL compared to the healthy myometrium. However, molecular mechanisms leading to the activation of PI3K/AKT-mTOR pathway in UL are unknown. In an attempt to identify potential drug targets upstream of PI3K/AKT-mTOR, we show here that the neuron-specific G-protein coupled receptor, GPR10, is highly expressed in UL tissue, whereas it is not normally present in the healthy myometrium. We show GPR10 activates the PI3K/AKT-mTOR pathway and increases mitogenic activity in primary UL cells. Furthermore, we show the consequence of GPR10 overexpression in vivo through generation and characterization of transgenic mice expressing hGPR10 in the myometrium. We provide compelling evidence that these transgenic mice display fibroid phenotype, including UL specific gene expression, increased extracellular matrix deposition, and UL tumor formation. Our transgenic mice results confirm the potential of GPR10 as a therapeutic target for UL and highlight the importance of the first biologically relevant animal model for a widely dysregulated gene in UL. Taken together, our data elucidate the pathogenic role of aberrant expression of GPR10 in UL and provide a preclinical mouse model for therapeutic purposes.
Introduction

Uterine leiomyoma (UL) are monoclonal smooth muscle cell tumors originating in the myometrial layer of the uterus (Walker & Stewart, 2005). UL are the most common tumor of the female reproductive tract, accounting for over 200,000 hysterectomies and billions of dollars in medical costs in the United States each year (Cardozo et al., 2012). Up to 77% of women are estimated to have UL and about a third of these women have clinically significant symptoms, including severe pain and bleeding, requiring treatment (Bulun, 2013).

Despite the prevalence of UL, there is currently no treatment for UL that is long-term, cost effective and that leaves fertility intact. Hysterectomy, surgical removal of the uterus, or myometectomy, removal of only the UL, are the standard of care for these tumors (Vilos et al., 2015). Unfortunately, high risk of major complications following surgery, are estimated to be 15-38% among patients (Al-Hendy & Salama, 2006). These complications are in addition to major side effects of hysterectomy surgery, and commonly accompanied oophorectomy, including early menopause, increased bone fracture risk, pelvic floor dysfunction and cardiovascular problems (Stewart et al., 2012). In addition, surgical treatment of UL often decreases or eliminates fertility; a growing concern for a population that is steadily increasing the age of childbearing (Balasch & Gratacos, 2011; Catherino et al., 2011). Other treatment options for UL include uterine artery embolization (UAE) and magnetic resonance-guided ultrasound. However, these treatments can result in organ damage and are considered viable options for a fraction of women presenting with UL (Gambadauro, 2012). Alternatively, a limited number of drug options are available for the treatment of UL, such as gonadotropin releasing hormone (GnRH) agonists and synthetic progestins. These hormonal treatments are typically used only short-term to treat symptoms before surgical removal of UL due to hypo-estrogenic side-effects such as
decreased bone mineral density, hot flashes, mood disorders and long-term cardiovascular problems (Bulun, 2013). There is a pressing unmet need to develop pharmacotherapies for the treatment of UL. To do so, more about the molecular pathogenesis of UL must be discovered.

Currently, the molecular etiology of UL is poorly understood. Risk factors for the development of UL include obesity, parity, race and increased lifetime exposure to estrogenic compounds (Walker & Stewart, 2005). However, the link between these global risk factors and the initiation of tumorigenic molecular cascades in UL cells is as yet undetermined. At the cellular level, several genetic alterations have been described in UL. Rare hereditary mutations in the Fumarate hydrase (FH) gene lead to the development of UL, but are only seen in 100 families worldwide (Walker & Stewart, 2005). In contrast, approximately 40% of UL tumors show chromosomal mutations, including chromosomal translocations, rearrangements and deletions (Bulun, 2013). In addition, up to 70% of UL tumors display a functional missense mutation in the mediator complex subunit 12 (MED12), which contributes to UL formation (Makinen et al., 2011; Mittal et al., 2015). Despite these common cytogenic abnormalities in UL, it is still unknown how the categorically quiescent myometrial tissue gives rise to frequent chromosomal instabilities.

Several recent studies have highlighted the central role of altered cell signaling in the molecular etiology of UL. The PI3K/AKT-mTOR signaling pathway has been identified as one of the most up-regulated signaling pathways in UL (Karra et al., 2010; Makker & Goel, 2013). Increased activation of proteins within this tumorigenic pathway, including p-AKT, have been shown in human UL compared to healthy myometrial tissue. Furthermore, PI3K and mTOR are essential for estrogen-dependent cell growth of UL cells (Kovacs et al., 2003; Yin et al., 2007).

Despite the prominence of altered PI3K/AKT-mTOR signaling in UL, the molecular
cascade resulting in the activation of this pathway is still poorly understood. In an effort to identify a novel drug target upstream of the activated PI3K/AKT-mTOR pathway, we focused on G-Protein coupled receptor (GPCR) proteins. We found that the neuron-specific GPR10 is the most highly up-regulated GPCR in human UL. GPR10 is activated by its peptide ligand, Prolactin-Releasing Peptide (PrRP), which was originally thought to promote prolactin release from the pituitary, but this role has since been disproven (Seal et al., 2002). Instead, GPR10 and PrRP play important roles upstream of the PI3K/AKT-mTOR pathway in the central nervous system response to stress, in satiety signaling and in nociception (Dodd & Luckman, 2013). Expression and function of GPR10 outside of the central nervous system has not been described previously. However, here we show the near-ubiquitous overexpression of GPR10 in UL is unparalleled among dysregulated genes in UL. We demonstrate that the overexpression of GPR10 in UL functionally promotes tumor cell proliferation and contributes to the pathogenesis of UL. In addition, we provide an important pre-clinical mouse model expressing hGPR10 in the myometrium. This work contributes significantly to the understanding of UL etiology and the development of a drug treatment option for UL.
Materials and Methods

Chemicals and reagents.

Dulbecco's Modified Eagle's medium (DMEM; D5671), Accustain Trichrome Stain Kit (HT15), BCIP/NBT solution (B6404), Saline-Sodium Citrate buffer (S6639), Tween-20 (P1379), Triton-X 100 (93443), Trizma base (T6066) normal goat serum (D9663) and protease and phosphatase inhibitor cocktails (P0044) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin (17-602), and L-glutamine (17-605) were purchased from Biowhittaker Lonza (Walkersville, MD). Dulbecco’s PBS/Modified (SH30028), fetal bovine serum (SH30071) and bovine calf serum (SH30073) were purchased from Hyclone (Logan, UT). Ethidium homodimer (E3599), Alexa Fluor conjugated secondary antibodies (ab150073), high-capacity cDNA reverse transcription kit (4368814) and Collagenase (14072) were obtained from Life Technologies (Grand Island, NY). Anti-GPR10 antibody (NBP1-00854, rabbit polyclonal) was obtained from Novus Biologicals (Littleton, CO). FuGene 6 transfection reagent (E2691) and colorimetric BrdU cell proliferation ELISA kit (11296736001) were from Roche (Indianapolis, IN). TaqMan primer-probe sets for PRLHR/ GPR10 (Hs00244685_s1, ABI) was obtained from Applied Biosystems (Foster City, CA). Taqman assays for Col1A1 (Mm.PT.47.6999992), Col3A1 (Mm.PT.47.9778198), Acta2 (Mm.PT.47.7024949), Tgfb3 (Mm.PT.47.10648587), and Dpt (Mm.PT.47.17098032) were purchased from IDT (Coralville, IA). Locked nucleic acid probes (300514-15) were obtained from Exiqon Inc. (Woburn, MA). PrRP peptide was obtained from Phoenix Pharmaceuticals (Burlingame, CA). Antigen unmasking solution (H-3300) was purchased from Vector (Burlingame, CA). NE-PER nuclear extraction reagent (78833), SuperSignal West Pico Chemiluminescent Substrate (34080),
Dharmafect (T2002), Proteinase K (25530-015), Restore PLUS Western Blot Stripping buffer (46430), xylene (C8H10), RNAlater solution (AM7021) and charcoal-stripped FBS (12676011) were purchased from Thermo Fisher Scientific (Rockford, IL). RNeasy Mini Kit (74104) and RNAlater (76104) were obtained from Qiagen (Valencia, CA; 74104). Primary antibodies against AKT (9272), p-AKT (40605), p-4EBP1 (9459S), p-70S6K (4206S), p-mTOR (2971) and mTOR (2983) were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin (SC1616) and anti-goat IgG HRP conjugated antibody (SC2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRIS/Glycine/SDS Buffer (161-0732), Blotting-grade blocker (170-6404) and gradient 4-15% mini-PROTEAN TGX gels (456-1086) were purchased from Bio-Rad (Hercules, CA). Paraformaldehyde solution (15710) was obtained from Electron Microscopy Sciences (Hatfield, PA).

Tissue collection and cell culture.

Human UL and healthy myometrial tissue samples were obtained from hysterectomies of pre-menopausal women at Carle Foundation Hospital (Urbana, IL) and University of Kansas Hospital (Kansas City, KS). Patient criteria included women who had hysterectomy with UL as the primary indication, without complicating reproductive tract diseases such as cancer or adenomyosis, and have not had hormone therapy in the three months preceding surgery. Tissue samples were processed for protein, RNA and primary cell culture.

Smooth muscle cells were isolated from specimen by mincing the tissue into 2-3mm cubes and digesting in DMEM containing 1.5mg/ml collagenase for 4-6 hours at 37 degrees. After digestion, cells were cultured in DMEM medium containing 10% serum (FBS and FCS), 1% L-glutamine, and 1% penicillin-streptomycin in an incubator maintaining 95% humidity and 5% CO2 at 37 degrees. Cells were not passaged past p6 to maintain phenotype as close to in vivo
as possible. Cells were serum starved for 24h in the presence of 0.1% charcoal stripped FBS prior to treatment with PrRP (0.1 – 2 μM) ligand. Serum starved cells were treated for 24h with BrdU labeling reagent in the presence of PrRP and were analyzed using the BrdU labeling detection ELISA kit (Roche).

**RNA isolation and qRT-PCR analyses.**

Total RNA was isolated from tissue samples stored in RNAlater or cultured cells using RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol. After quantification using Nanodrop spectrophotometer, aliquots of RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Applied Biosystems). TaqMan assays for *PRLHR/GPR10* (Hs00244685_s1, ABI), *Col1A1* (Mm.PT.47.6999992, IDT), *Col3A1* (Mm.PT.47.9778198, IDT), *Acta2* (Mm.PT.47.7024949, IDT), Tgfb3 (Mm.PT.47.10648587, IDT), and *Dpt* (Mm.PT.47.17098032, IDT) were used to quantify gene expression utilizing the delta delta C(T) method relative to 18s rRNA.

**Transgenic construct and generation of CaBP9K-hGPR10 mice.**

A full-length cDNA encoding human *GPR10* (NM_004248) was obtained from Open Biosystems (Huntsville, AL 35806 USA). The rat calbindin D9K short (-117 to+365bp) promoter (reference sequence Genbank: X16635.1) was PCR amplified with primers containing HindIII site at the 5’ end and a reverse primer containing overlapping GPR10 sequence up to a unique Apal site close to the start ATG. The following primers were used for gene amplification/cloning: Forward (*Cabp9K*) primer: GAG TCT TAA GCT TGG TCT CAG, reverse hybrid primer: 5’ CTG GGG CCC CGA GTG GTC GAT GAG GCC ATT TTT CTG TGC TGT AAT TTG G (overlapping *GPR10* sequence including the Apa1 site underlined). The PCR product was cloned into a vector upstream of the Apa1 site of GPR10 as shown in Figure 3.
1. The construct was used to generate the D9K-hGPR10 transgenic mouse mice. We obtained three (2 male, 1 female) positive founders (Figure 3. 2). These transgenic founders were bred to WT C57BL6 mice to obtain F1 progeny. Transgenic mice were genotyped by PCR using a rat Cabp9K forward primer (5’ CCA CTA ATG CTG TTC CGA CCT GTC) and a GPR10 reverse primer (5’ CAC CAG CTG CAG GCT CTG GAA G).
Figure 3.1: CaBP9K-hGPR10 transgenic construct and sequence. (A). Schematic showing CaBP9K-hGPR10 construct. (B). DNA sequence of transgenic construct. Restriction sites are shown in black, the myometrial-specific truncated rat CaBP9K promoter sequence is shown in red, and the GPR10 open reading frame is shown in blue.
Figure 3. 2: CaBP9K-hGPR10 founder mice genotyping and qPCR. (A) PCR genotyping of transgenic founder mice showing the presence of three transgenic mice (lanes 2, 4, and 5) (B) TaqMan RT-PCR analysis of uterine RNA of F₀ female founder (TG1, lane 4 in B, 6 months old) and F₁ females (3 months old) derived from the two male founder lines (TG2, TG3, lanes 2 and 5 in B) showing hGPR10 expression. Values are average ± SD for three mice in each group except for the F₀ female founder (TG1) where the average ± SD of three independent RT-PCR estimates is plotted. Error bars indicate ± SD, Paired T-test was performed, *P<0.05.
siRNA knockdown of GPR10.

Cultured primary leiomyoma SMCs were transfected with On-TARGETplus SMARTpool siRNAs (J-005524-05, J-005524-06, J-005524-07, and J-005524-08, Dharmacon, ThermoFisher) to GPR10 using DharmaFECT 2 transfection reagent according to manufacturer’s protocol. Control experiments included ON-TARGETplus non-targeting scrambled siRNA#2 (D-001810-02-05). After 24 hours, transfected cells were serum starved in 0.1% FBS overnight followed by GPR10 ligand treatment (PrRP 1uM in PBS) for 1h. Protein extracts from the cells were then analyzed for western blotting.

Histological staining.

Uterine tissues were fixed in paraformaldehyde (4% w/v, in PBS) and processed for paraffin embedding. Tissue sections (5 µm thickness) were deparaffinized in xylene, rehydrated through a series of ethanol solutions and stained with Hematoxylin and Eosin (H&E). For Masson’s trichrome staining, to visualize collagen, the deparaffinized tissue sections were made mordant in Bouin’s fixative, stained with Weigert’s iron hematoxyline, Biebrich scarlet acid-fucshin and aniline blue solutions according to the Accustain® Trichrome protocol (Sigma-Aldrich). For immunofluorescence, rehydrated tissue sections were subjected to antigen retrieval by heating in citrate buffer. Slides were next washed with PBS (5 minutes, 3 times), blocked for 30 minutes in blocking agent (5% normal goat serum) followed by primary antibody incubation in blocking buffer overnight at 4°C. Following washes in PBS (5min, 3 times), slides were reincubated with AlexaFluor® 488 or AlexaFluor® 555 labeled secondary antibodies (Invitrogen, Life Technologies) for 1 hour at 37°C. Nuclei was stained with EthD-1, 5uM in PBS for 5 min at room temperature and washed in PBS prior to being mounted in 50% glycerol in
PBS. An Olympus 1X71 inverted microscope and TE2000U 3 laser inverted confocal microscope were used for imaging.

**In-situ hybridization.**

Deparaffinized sections (5µm) were digested with proteinase-K (25µg/mL in PBS) at 37°C for 15 min followed by 3 washes in DEPC water. Sections were next treated with digoxigenin labeled LNA probes at 60°C for 5 min and hybridized to the probes overnight at 37°C in a humid chamber. The next day, slides were washed with 0.2% BSA in 0.2x Saline sodium citrate buffer, incubated with alkaline phosphatase conjugated anti-digoxigenin antibodies (37°C, 1 hour) followed by NBT/BCIP reaction to visualize target mRNA. Custom LNA antisense probe for hGPR10 (probe sequence: 5DigN/ACG TAA GAC AGG AGG ATG ACCA/3Dig_N) or custom LNA control sense probe (probe sequence: 5DigN/TGG TCA TCC TCC TGT CTT ACGT/3Dig) were used for the hybridization. An additional LNA mRNA detection control probe (Product Number 300514-15, 5DigN/GTG TAA CAC GTC TAT ACG CCCA/3Dig_N, Exiqon, Woburn, MA) was used to confirm assay specificity.

**Protein extraction and western blotting.**

Frozen tissue samples were homogenized in a hypotonic buffer (40mM NaCl, 10mM KCl, 20mM Tris-HCl pH 7.4, 0.1% Triton-X100, 0.1% Tween 20) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO). Homogenates were centrifuged at 10,000 rpm at 4°C. Post-nuclear supernatants were combined with extracts from the nuclear pellets and solubilized using the above buffer supplemented with 300mM NaCl or NE-PER nuclear extraction buffer (Thermo Scientific, Rockford, IL). Western blots were performed with lysates in 6x Laemmli buffer loaded on 10% polyacrylamide SDS gels and
subjected to electrophoresis at 70 volts at room temperature. Transfer to PVDF membrane was at 4 degrees for 1.5 hours at 100 volts. Membranes were blocked in 5% skim milk for 1 hour. Overnight incubation at 4°C was with anti-GPR10 (1:2000), anti-AKT (1:2000), anti-p-AKT (Ser473) (1:1000), anti-p-4EBP1 (Thr37/48) (1:1000), anti-p-70S6K (Thr389) (1:1000), anti-p-mTOR (Ser2448) (1:2000) or anti-actin (7.5:10000). Secondary antibody incubation was with IgG HRP conjugated antibodies (1: 5000) at room temp for 1 hour. Signal was developed with SuperSignal West Pico Chemiluminescent Substrate purchased from Thermo Scientific (Rockford, IL).

**Cell proliferation assay.**

Low passage serum-starved leiomyoma smooth muscle cells (SMCs) were treated with increasing concentrations of PrRP-31 ligand from 0.1 to 2µM overnight. Treatment controls included PrRP treatment of patient matched myometrial cells, which do not express GPR10, and myometrial SMCs and leiomyoma SMCs receiving PBS in place of PrRP. A 10mM concentration of BrdU labeling reagent was applied to cells, followed by fixation and staining with a 1:100 dilution of anti-BrdU antibody and substrate for detection by absorbance. Assay controls included cells that do not receive BrdU reagent and absorbance blanks.

**RNA-sequencing.**

RNA isolation from whole uteri of CaBP9K-hGPR10 and littermate wild-type mice aged 4 months was performed using phenol:chloroform. RNA samples were prepared by the KUMC Genomic Sequencing Facility using the TruSeq Stranded Total RNA LT Sample Preparation Kit with Ribo-Zero Mouse. Briefly, RNA samples were fragmented, the first two strands of cDNA were synthesized in thermal cycler, followed by adenylation of 3’ ends, adapter ligation, and enrichment of DNA fragments. Libraries were adjusted to 2nM concentration and pooled for
multiplex sequencing. Libraries were validated using the Agilent 2100 Bioanalyzer using the Agilent DNA 1000 Chip. Stranded Total RNA-Sequencing was performed at a 2 x 100 bp paired end resolution in an illumina HiSeq 2500 sequencing machine (Illumina, San Diego, CA). The Wt (Wild-type) and Tg (GPR10-transgenic) samples were analyzed in biological triplicates giving six samples in total. Each sample generated between 108.4 and 126.8 million fragments (reads). Reads were mapped to the mouse genome (GRCm38) using the STAR software, version 2.3.1z (Dobin et al., 2013). Between 82.6% and 88.2% of the sequenced reads mapped to the mouse genome (See Table XX). Differential gene expression analysis was performed using Cuffdiff, version 2.1.1 (Trapnell et al., 2012). The resulting p-values were adjusted for multiple hypothesis testing using the Benjamini and Hochberg method (Benjamini & Hochberg, 1995).

Table XX

<table>
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<th>Sample</th>
<th>Total reads</th>
<th>Mapped reads</th>
<th>Percent mapped</th>
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<td>104,001,160</td>
<td>88.21%</td>
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<tr>
<td>Sample - WT2</td>
<td>121,506,764</td>
<td>100,385,782</td>
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<td>89,648,652</td>
<td>82.68%</td>
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</table>

Data mining and Ingenuity Pathway Analysis.

GEO dataset GSE13319 was analyzed for the expression of GPR10 in myometrial and leiomyoma samples. The dataset was preprocessed for analysis using the Robust Multichip Averages Procedure (RMA). Statistical analysis was performed on biological triplicates. Biological functional and pathway analysis were performed using Ingenuity Systems Pathway Analysis software [IPA, version 7.6; Ingenuity Systems (www.ingenuity.com)] on the significantly (fold change ≥ 1.5, p-value ≤ 0.05) differentially expressed genes between myometrium and leiomyoma, or between wild-type and transgenic mice.
**Calcium imaging.**

Myometrial and leiomyoma SMCs grown on chamber slides were serum starved as described above and incubated with Fura2-AM. Fluorometric imaging techniques were used to monitor the intracellular calcium concentration ([Ca2+]). PrRP (1 mM) or vehicle (PBS) was added during the live confocal imaging of cells.

**Statistical Analysis.**

Leiomyoma samples were compared to myometrial controls from the same patient. For animal studies, transgenic mice uterine samples were compared to wild-type littermates at the same estrus cycle. Quantitative experiments, including qRT-PCR and densitometry of western blots, were repeated with at least three independent biological replicates. Statistical significance was determined using paired sample T-test to determine change from the control sample. For paired tissue samples, the study was powered to measure changes in gene expression between the tumor and normal tissues. Using paired t-test, 11 pairs gave us 80% power to detect a difference of 0.81 (fold change of 1.75) standard deviation units or larger in gene expression with one-sided level of significance of 0.05. Significance was set at $P < 0.05$ for all comparisons. For RNA-sequencing results, genes used in Ingenuity Pathway Analysis were filtered for $p < 0.05$, $q < 0.01$, and fold change of 1.5 or higher.
Results

**Aberrant expression of GPR10 in uterine leiomyoma.**

In an effort to identify drug targetable, signaling molecules upstream of the mTOR pathway that might be aberrantly regulated in UL, we analyzed gene expression profiling data available from the Geo database. Analysis of human UL and normal myometrial samples from dataset GSE13319 revealed that GPR10, a GPCR with known functions in PI3K/AKT pathway activation, was the most highly dysregulated GPCR in UL (Figure 3. 3). TaqMan RT-PCR was used to confirm the expression of GPR10 mRNA in UL (Figure 3. 4). Expression of GPR10 was significantly upregulated in 13 out of 14 patient samples. The aberrant expression of GPR10 was further confirmed by western blotting. Expression of the receptor protein was evident in all the UL tissue samples tested whereas expression was either absent or negligible in matched normal myometrial tissues (Figure 3. 5).

Since UL tumor growth is known to be associated with activation of the PI3K/AKT pathway leading to the activation of mTOR, we compared the expression of GPR10 to the status of AKT phosphorylation. The AKT phosphorylation levels in individual patient samples reflected the level of expression of GPR10 in those samples (Figure 3. 6), indicating that the aberrant expression of GPR10 and PI3K/AKT pathway activation may be related.
Figure 3. 3: Microarray analysis of GPR10 gene expression in patient samples. Analysis of GPR10 expression in uterine leiomyoma samples (red) compared with that in matched normal myometrial samples (blue) from the gene expression dataset GSE13319.
Figure 3. 4: TaqMan qRT-PCR analysis of GPR10 in paired patient samples. GPR10 expression in 14 pairs of matched normal myometrial and leiomyoma samples. Shown on a logarithmic scale.
Figure 3.5: **Western blot analysis of GPR10 in paired patient samples.** Protein extracts from patient (P1-P8) samples comparing GPR10 expression in normal myometrium to matched leiomyoma samples. Lower panel shows β-actin expression used as protein loading control.
Figure 3.6: AKT activation in correlation with GPR10 expression. Western blot analysis of phosph-AKT (Ser 473) and GPR10 expression in three patient (PA, PB, and PC)-matched myometrial and leiomyoma samples. Total AKT and β-actin were used as loading controls.
**Activation of GPR10 leads to PI3K/AKT – mTOR pathway activation and leiomyoma cell proliferation.**

Next we tested whether the activation of GPR10 leads to regulation of the PI3K/AKT – mTOR pathways in primary myometrial and leiomyoma smooth muscle cells (SMCs) cultured in vitro. Abundant expression of GPR10 was maintained in cultured leiomyoma SMC (Figure 3. 7A; LC1, LC2, LC3) whereas in normal myometrial SMCs GPR10 expression was negligible (Figure 3. 7A; MC1, MC2, MC3). Persistence of differential GPR10 expression in cultured primary cells enabled us to study the effect of its activation by PrRP. We treated low passage serum-starved leiomyoma SMCs with increasing concentrations of PrRP-31 ligand from 0.1 to 2µM. Cells were incubated for 0, 30 or 60 minutes to determine changes in protein phosphorylation over time. Treatment controls included PrRP treatment of patient matched myometrial SMCs, which do not express GPR10, and myometrial SMCs and leiomyoma SMCs receiving PBS in place of PrRP. Treatment of leiomyoma SMCs with PrRP-31 peptide resulted in robust phosphorylation of AKT (Ser473) within 1h (Figure 3. 7B) whereas phosphorylation of AKT was not affected by PrRP treatment in myometrial SMCs. Knockdown of GPR10 in leiomyoma SMCs using siRNA resulted in the loss of AKT phosphorylation upon treatment with PrRP peptide (Figure 3. 8), indicating that the effect of PrRP is transduced by the activation of GPR10. Relatedly, functional coupling of GPR10 to Gq and to intracellular calcium is known to occur(Cardozo et al., 2012), and familial mutations to the GPR10 gene resulting in altered calcium homeostasis have been reported (Langmead et al., 2000). Using a calcium fluorometric assay, we found that treatment of cultured leiomyoma cells, which express GPR10, with 1µM PrRP resulted in the mobilization of intracellular Ca2+ (Figure 3. 9), whereas leiomyoma cells
treated with vehicle did not show this increase in intracellular Ca2+. These results demonstrate the functional role of PrRP activation of GPR10 in leiomyoma cellular activity.

We hypothesized that the activation of GPR10 and ensuing AKT phosphorylation in leiomyoma SMCs may trigger the mTOR pathway that is usually activated in uterine fibroids. Leiomyoma SMCs treated with the ligand PrRP showed increased mTOR phosphorylation at Ser2448, activation of p70S6K (Thr389) and 4EBP1 (Thr37/48) (Figure 3. 10). Phosphorylation of mTOR, p70S6K and 4EBP1 were unchanged in normal myometrial SMCs treated with PrRP. These results indicate that GPR10 activation may trigger the mTOR pathway specifically in UL cells.

Activation of GPR10 in leiomyoma SMCs had significant mitogenic effects compared to that in normal myometrial SMCs in culture as indicated by BrdU incorporation (Figure 3. 11). Under serum starvation, vehicle treated control myometrial and leiomyoma SMCs showed equivalent BrdU incorporation. Leiomyoma SMCs treated with 2 µM PrRP showed greater than 2.5 fold increase in BrdU incorporation in 24 hours compared to vehicle treated cells (Figure 3. 11). The mitogenic effect of PrRP on myometrial cells was significantly lower and reflected the lower level of GPR10 expression (Figure 3. 11 inset).
Figure 3. 7: Protein expression of GPR10 with PrRP treatment in cultured primary leiomyoma cells. (A) Western blot analysis of GPR10 expression in primary myometrial (MC1-3) and autologous leiomyoma smooth muscle cells (LC1-3). β-actin was used as a protein loading control. (B) Anti-phospho-AKT (s473) Western blot showing that the activation of GPR10 using PrRP (1μM) leads to the phosphorylation of AKT in leiomyoma smooth muscle cells. Total AKT was used as a protein loading control.
Figure 3. 8: Silencing of GPR10 and inhibition of AKT phosphorylation in leiomyoma cells.

(A) TaqMan qPCR analysis of GPR10 mRNA after siRNA knockdown. Replicates of samples treated with control vehicle, scrambled siRNA, and siGPR10 for 24 hours were serum starved for an additional 24 hours. Error bars indicate ±SD.  (B) Representative anti-pAKT (s473) Western blot and densiometric analysis of Western blots from three independent experiments, showing that siRNA knockdown of GPR10 leads to a loss of PrRP response on AKT phosphorylation.
Figure 3. 9: **Calcium fluorometric assay of PrRP treated leiomyoma cells.** Fluorometric analysis of intracellular calcium release in leiomyoma smooth muscle cells upon treatment with 1μM PrRP-31 peptide.
Figure 3. 10: **Western blot analysis of mTOR pathway activation by PrRP.** Myometrial and leiomyoma smooth muscle cells with or without PrRP treatment (1μM) probed for p-mTOR (Ser2448), p-p70S6K (Thr389), and p-4EBP1 (Thr37/48). B-actin was used as a loading control.
Figure 3.11: **Cell proliferation analysis of primary cultured cells treated with PrRP.**

Mitogenic effect of PrRP on myometrial and leiomyoma cells after serum starvation (0.1% FBS for 24 hours followed by 1μM pRRP treatment for 24 hours). Levels of BrDU incorporation in relation to vehicle-treated controls were plotted. *(Inset)* Western Blot analysis of GPR10 in the same cells used in proliferation assay. Paired T-tests were used to demonstrate significant increase in cell proliferation compared to control. Error bars indicate ±SD, *P* < 0.05.
Transgenic overexpression of hGPR10 in the mouse myometrium leads to leiomyoma phenotype.

Activation of aberrantly expressed GPR10 triggering PI3K-AKT-mTOR pathways and UL cell proliferation in vitro convinced us to test whether transgenic overexpression of hGPR10 in the myometrium would result in a fibroid-like phenotype in vivo. To target GPR10 to the myometrium, we used a DNA sequence from the 5’ flanking region of the rat calbindin D9K gene, -117 to +365bp. This truncated sequence of the calbindin D9K promoter has been used previously to target the expression of SV40 large T-antigen specifically to the myometrium in the mouse, and is activated endogenously by estrogen (Romagnolo et al., 1996). For our transgenic construct, we cloned the full open reading frame of the human GPR10 gene (exon 2) downstream of this truncated calbindin D9K promoter (Figure 3.1A). The construct was cloned into a vector including the SV-40 poly adenylation sequence. This construct was used by the KU Medical Center Transgenic and Gene Targeting Institutional Facility to generate CaBP9K-hGPR10 transgenic mice. Three founder mice were bred to wild-type C57BL6 mice to obtain hemizygous transgenic CaBP9K-hGPR10 progeny.

We obtained 3 founder mice (1 female, 2 male) following pronuclear injection of the transgenic construct as determined by PCR (Figure 3.2). All three of the mouse lines expressed hGPR10 in the uteri (Figure 3.2B). Expression of the transgene mRNA was specific to the myometrial compartment of the uterus as indicated by in situ hybridization using a LNA probe (Figure 3.12). Additionally, immunofluorescence microscopy using anti-GPR10 antibodies demonstrated myometrial expression of GPR10 protein in the transgenic CaBP9K-hGPR10 mice (Figure 3.13). Interestingly, histomorphometric analysis of uterine cross sections revealed that
the transgenic *CaBP9K-hGPR10* mice had a 2-fold increase in myometrial cross sectional area compared to littermate WT controls (Figure 3. 14A). Increase in overall uterine thickness was noticeable in all the adult transgenic *CaBP9K-hGPR10* mice compared to estrus cycle stage matched control littermates (representative cross sections at equal magnification are shown in Figure 3. 12).

Since UL are known to be associated with increased TGFβ signaling and with altered expression of a number of genes that encode proteins of smooth muscle cells and the extracellular matrix, we tested the expression of *Col1A1* and *Col3A1*, *Tgfb3*, dermatopontin (*Dpt*), and alpha smooth muscle actin (*Acta2*) in uteri from 2 to 4 month-old mice. Our results indicated that the transgenic *CaBP9K-hGPR10* mice expressed significantly higher levels of *Col1A1*, *Col3A1*, *Tgfb3* and *Acta2* and lower level of *Dpt* compared to WT mice (Figure 3. 14 B-F). Dysregulation of these genes in the transgenic *CaBP9K-hGPR10* mice represent a phenotype similar to UL. We stained sections of uteri from littermates of 6 months old mice using Masson’s trichrome to visualize collagen in the extracellular matrix. Collagen deposition in the transgenic *CaBP9K-hGPR10* uterus was dramatically increased compared to that in the uterus of wild type littermates (Figure 3. 15). Myometrial tissue sections from transgenic *CaBP9K-hGPR10* mice stained with H&E were morphologically identical to those of human UL samples (Figure 3. 16). In addition, uteri from 6-9 month-old transgenic *CaBP9K-hGPR10* mice contained SMC tumors with excessive collagen deposition (Figure 3. 17).

Next, we performed sequencing of RNA of the uteri of 4 month old *CaBP9K-hGPR10* mice and littermate wild-type controls, with three mice in each group. To confirm *hGPR10* expression in the uteri of *CaBP9K-hGPR10* mice, the reads from RNA-sequencing were mapped to the human genome. In Figure 3.18, we show human *GPR10* expression has a 115.87 higher
fold change in the \textit{CaBP9K-hGPR10} mouse compared to littermate wild-type controls. Our RNA-sequencing results revealed many genes are dysregulated in the \textit{CaBP9k-hGPR10} mouse as a result of \textit{hGPR10} expression in the uterus (Figure 3.19). In addition, Ingenuity Pathway Analysis of genes significantly over or under expressed in the \textit{CaBP9k-hGPR10} mouse show similarities to gene expression profiles of diseases such as cancer, reproductive system disease, dermatological and renal conditions (Figure 3.20).
Figure 3.12: **mRNA localization of GPR10 transcript in CaBP9K-hGPR10 mouse.**

Representative *in-situ* hybridization using an antisense LNA probe to *hGPR10* showing myometrial expression of the transgene in wild-type and transgenic mouse uteri (N=3). LNA oligo corresponding to the sense strand sequence was used as control.
Figure 3.13: **Histological analysis of GPR10 protein localization in CaBP9K-hGPR10 myometrium.** Immunofluorescence staining of representative uterine cross-sections using anti-GPR10 antibodies showing myometrial expression of GPR10 (green) in the CaBP9K-hGPR10 mice (N=3). Control experiments used no primary antibodies. Sections were co-stained with EthD-1 (red) for visualization of nuclei.
Figure 3. 14: **Gene expression profiles of CaBP9K-hGPR10 transgenic mice.** (A) Transverse cross-sections of uteri from 4 month old CaBP9K-hGPR10 transgenic mice and WT littermates (N = 4) were stained with H&E. Using histomorphometry, total myometrial area of 20 cross-sections in each genotype were measured and represented as relative values to that of WT. (B-F) TaqMan RT-PCR analysis of gene expression in the uteri from four CaBP9K-hGPR10 and WT mice (3-4 months old) (N = 4), measuring Col1A1, Acta2, DPT, Col3A1 and Tgfb3, respectively. All samples were analyzed in triplicates. Error bars indicate ±SD, *P<0.05.
Figure 3.15: **Histological analysis of collagen deposition in Tg CaBP9K-hGPR10 uteri.**

Masson’s trichrome staining of uterine myometrial sections showing excessive collagen deposition (blue) in the transgenic tissue. Adjacent sections were stained with H&E for comparison.
Figure 3.16: **Histological comparison of myometrial tissue in CaBP9K-hGPR10 transgenic mice and human leiomyoma.** Tissue sections from human myometrial and leiomyoma (A and B), WT and transgenic mouse myometrium (C and D) were stained with hematoxylin & eosin for morphological comparison.
Figure 3.17: **CaBP9K-hGPR10 Tg mouse fibroid-like tumor.** (A) H&E and (B) Masson’s trichrome stained cross-sections showing a submucosal smooth muscle tumor protruding into the cervix of a CaBP9K-hGPR10 mouse (6 months old).
Figure 3. 18: **Human PRLHR (GPR-10) gene expression in CaBP9K-hGPR10 Tg mouse uteri.** Expression of the human GPR10 gene in wild-type versus CaBP9K-hGPR10 Tg mouse from RNA-sequencing of the entire uterus, including endometrium (N = 3). Gene expression has been normalized to Phix and displayed on a logarithmic scale. * $P < 0.05$. 
Figure 3.19: **RNA-sequencing analysis of the most dysregulated genes in CaBP9K-hGPR10 mouse.** Logarithmic expression of the genes most significantly dysregulated (fold-change of 2 or higher; all have $P < 0.05$) between wild-type (blue) and CaBP9K-hGPR10 (red) whole mouse uteri, from RNA-sequencing analysis of 4 month old mice in diestrus (N = 3).
Figure 3. 20: **Ingenuity Pathway Analysis of CaBP9K-hGPR10 mouse activated genes.**

Genes which show a significant \( P < 0.05 \) differential expression based on RNA-sequencing results of whole uteri of 4 month old WT and CaBP9K-hGPR10 mice \( (N = 3) \) were used for pathway prediction by Ingenuity Pathway Analysis software.
Discussion

Extensive evidence has linked the cell proliferation, survival and growth of UL tumors to the over-activation of the PI3K/AKT-mTOR pathway (Karra et al., 2010; Makker et al., 2012). However, molecular cascades initiating the downstream tumorigenic signaling pathway, PI3K/AKT-mTOR, in UL is unknown. In addition, there is a lack of animal models specific for the UL phenotype. Here we present work which significantly contributes to the fundamental understanding of the molecular etiology of UL, and also provides a potential drug target and preclinical mouse model essential for the development of pharmacotherapy options for UL. We show that aberrant expression of the neuron-specific receptor protein, GPR10, and its activation by PrRP, lead to PI3K/AKT-mTOR pathway activation in leiomyoma SMCs. Crucially, we have developed the first transgenic mouse model expressing a gene dysregulated in human UL. We show that transgenic mice expressing the human GPR10 gene in the myometrium develop a UL phenotype, validating the role of this signaling molecule in the pathogenesis of UL in vivo.

GPR10, also known as prolactin releasing hormone receptor (PRLHR), is a neuron-specific G-protein coupled receptor (GPCR) expressed in the reticular nucleus of the thalamus, periventricular nucleus (PVN), hypothalamus, adrenal glands and medulla oblongata (Dodd & Luckman, 2013). The peptide ligand which activates GPR10, PrRP, is a 21 amino acid RF-amide peptide which is present both in the central nervous system, consistent with its role as the ligand for GPR10, and also in the periphery where it is proposed to activate other receptors, including neuropeptide FF (NPFF-R2)(Engstrom et al., 2003). Evidence for PrRP’s role in the periphery is rather tenuous, considering the administration of this peptide ligand peripherally had no effect in mice, whereas central administration of PrRP (i.c.v.) elicited expected effects in experimental animals (Dodd & Luckman, 2013; Roland et al., 1999). GPR10 and PrRP have well established
roles in the central nervous system in integrating peripheral cues of satiety, nociception and stress response. Cues from afferent neurons and enteroendocrine cells activate GPR10, upstream of the PI3K/AKT-mTOR pathway in the hypothalamus and medulla oblongata (Dodd & Luckman, 2013). For the first time, we show GPR10 expression, and its functional activation by PrRP, outside of the central nervous system in UL. We found GPR10 expression in over 90% of UL samples. In sharp contrast, other growth factor receptor pathways, such as insulin-like growth factor 2 (IGF2), are up-regulated in up to one third of UL (Peng et al., 2009; J. Wei et al., 2005). We show the presence of GPR10 in the uterus in UL has functional significance in the activation of the tumorigenic PI3K/AKT-mTOR pathway. Like GPR10, many of the most overexpressed genes in UL are neuron specific, which suggests possible epigenetic changes allowing derepression of neuronal genes, including GPR10 in UL.

Although the exact etiology of UL is still poorly understood, it is well established that aberrant cell proliferation signaling pathways play a critical role in UL tumor formation. Extensive evidence from transcriptional profiling of human UL indicates the PI3K/KT-mTOR pathway is one of the most significantly activated tumorigenic pathways in UL (Crabtree et al., 2009). UL show increased activation of PI3K/AKT pathway proteins and targets, including PTEN, p-AKT, p-GSK3 and CD2 proteins, compared to normal myometrium (Karra et al., 2010; Kovacs et al., 2003). In addition, there is evidence that PI3K and mTOR are necessary for estrogen-dependent cell growth in UL and myometrial cell cultures (Yin et al., 2007). Up-regulation of PI3K/AKT-mTOR proteins is also evident in uterine cells of the Eker rat, which is a common UL animal model (Crabtree et al., 2009). Some evidence exists in literature showing involvement of other signaling factors, including Transforming Growth Factor β (TGF-β) and Insulin-like Growth Factor (IGF) in UL etiology (Parker, 2007). TGF-β can be activated by the
PI3K/AKT pathway proteins, and increases smooth muscle cell proliferation and extracellular matrix deposition in UL (Borahay et al., 2015). IGF activation of mTOR is seen in only 20-40% of human patients and would not account for the vast majority of UL.

Although it is well recognized that the PI3K/AKT-mTOR pathway is crucial to UL development, the initiation of this pathway in UL cells remains a mystery. Since GPR10 activates the PI3K/AKT-mTOR pathway in hypothalamic neurons and cancer cell lines (Dodd & Luckman, 2013), we hypothesized that aberrantly expressed GPR10 may act upstream of the mTOR pathway in UL. In the central nervous system GPR10 activates the PI3K/AKT pathway through Gi/Go and has been shown to increase intracellular calcium (Hayakawa et al., 2002; Xia & Arai, 2011). We present compelling evidence that PrRP activation of GPR10 in UL directly activates PI3K/AKT-mTOR proteins. In UL tissue samples, we found that GPR10 expression reflected the extent of phospho-AKT levels (Figure 3.6), suggesting that the underlying mechanisms of GPR10 expression and PI3K/AKT-mTOR pathway activation in UL may be interrelated. Using primary cultured leiomyoma and myometrial SMCs, we confirmed the important functional role of overexpressed GPR10 upstream of the tumorigenic PI3K/AKT-mTOR pathway in UL. Activating PI3K/AKT-mTOR pathway proteins through phosphorylation, including AKT, 70S6K, 4EBP1, and mTOR, are known to promote cell growth and proliferation (Makker et al., 2012). Here, we revealed PrRP activation of GPR10 results in phosphorylation of these PI3K/AKT-mTOR proteins, mobilization of intracellular calcium and mitogenic responses in UL cells. Our data advances an important novel and functional role for GPR10 overexpression in UL cell signaling and proliferation. In support of our findings, a recent study by Mehine et. al (Mehine et al., 2016), analyzed gene transcripts from 94 patients with UL and categorized leiomyomas into subtypes based on the mutations present, including High
Mobility Group AT-hook -(HMGA1), HGMA2, Mediator complex subunit 12 (MED12), Fumarate Hydrase (FH), and Collagen type IV (COL4A) mutations. Remarkably, UL harboring mutations in HGMA1, HGMA2, MED12 and COL4A5-6, which collectively account for the vast majority of UL, all result in GPR10 expression and downstream pathway activation in UL (Mehine et al., 2016). This study reinforces the importance of the near-ubiquitous functional expression of GPR10 in UL presented here and highlights the practicality of GPR10 as a potential all-encompassing drug target for UL with a variety of common gene mutations.

Currently, the UL field lacks biologically relevant preclinical animal models for fibroids. The best characterized rodent model for UL is the Eker rat, which has mutations in the Tsc2 gene, leading to aberrant mTOR signaling and a leiomyoma-like phenotype (Walker et al., 2003). Despite its common use to study UL, the Eker rat is an incomplete representation of human UL in vivo, as the TSC2 gene is not mutated in the human condition. Importantly, we present the first animal model for UL expressing a gene which is overexpressed in human UL. Using an estrogen dependent CaBP9K promoter, we targeted the human GPR10 gene specifically to the mouse myometrium (Figure 3.12 & Figure 3.13). Our results indicate that the transgenic CaBP9K-hGPR10 mice display myometrial-specific robust GPR10 expression (Figure 3.18). Although normal GPR10 expression is limited to the wild-type mouse brain, the peptide ligand for GPR10, PrRP, has been shown to be present in the peripheral circulation due to his role in activation of other receptor proteins, such as Neuropeptide FF receptor 2 (NPFF-R2) (Dodd & Luckman, 2013). As such, we show functional activation of GPR10 in vivo with increased activation of signaling molecules downstream of GPR10 (Figure 3.14). In addition, the transgenic CaBP9K-hGPR10 mice show increased myometrial thickness, altered uterine smooth muscle gene expression, enhanced extracellular matrix deposition, and UL-like tumor
formation. RNA-sequencing results further confirm the usefulness of the CaBP9k-hGPR10 mouse as a model for UL study. Several of the most significantly dysregulated genes in the CaBP9k-hGPR10 mouse (Figure 3.19) encode histone proteins, including Hist1h3h, Hist1h4f, Hist1h2bl, Hist1h2bk, Hist1h4b, Hist1h2bg, and Hist1h3i. Interestingly, a study by Zhu et. al found evidence of increased histone proteins as a biomarker for estrogen receptor-mediated cell proliferation (Zhu, Edwards, & Boobis, 2009), which could imply that our CaBP9K-hGPR10 mouse model demonstrates estrogen-responsive gene expression even in diestrus. This increase in estrogen sensitivity is similar to the responsiveness of human UL seen in the luteal phase. Our RNA-sequencing results also show a similarity in gene expression profiles of our CaBP9k-hGPR10 mouse to human UL-related conditions. Ingenuity Pathway Analysis pathway prediction shows strong similarity of CaBP9k-hGPR10 dysregulated genes with cancer, organismal injury, reproductive system disease, renal disease and dermatological disease (Figure 3.20). Relatedly, UL are known to share commonly disrupted ECM proteins, including DPT and collagens type I and III, with dermatological conditions such as keloids (W. H. Catherino et al., 2004; Sun, Wang, & Lee, 2014). One theory to the origin of UL, based on the similarity of UL to dermatological disorders, is that UL are a result of abnormal wound healing in response to injury of the uterus, perhaps during menstruation (Leppert, Catherino, & Segars, 2006). The similar gene expression profile of the CaBP9k-hGPR10 mouse to renal disease is also relevant because genes commonly disrupted in UL, including TSC2 and mTOR, are also implicated in renal cell carcinoma (Commandeur, Styer, & Teixeira, 2015). Overall the similarity of gene expression profiles of our CaBP9k-hGPR10 mouse to UL-related conditions further promotes this mouse model as a valuable tool for the study of UL and as a preclinical mouse model for the development of better UL treatment options.
Available drug therapy options for the treatment of UL target the symptoms of UL tumors, but do not address the underlying etiology of the disease. FDA approved drug treatments for UL include GnRH agonists and synthetic progestins. GnRH agonists provide short-term therapy for reducing tumor size and bleeding, but are only used short-term due to potential for bone resorption and hypo-estrogenic side effects. Similarly, progestins reduce menorrhagia associated with UL but do not reduce tumor size and eliminate fertility during treatment. In addition, after hormonal therapy is stopped, UL return to pre-treatment size and symptoms reappear (Vilos et al., 2015; Walker & Stewart, 2005). Targeting of the PI3K/AKT-mTOR pathway as a therapeutic option for UL is currently being explored, where it shows promise in limiting UL growth and increasing cell death, but may have broad side-effects on off-target proliferative tissues (Borahay et al., 2015). Here we provide a promising potential drug target for treatment of UL in the cell receptor protein, GPR10. Development of a brain-sparing peptidomimetic or small molecule antagonist to GPR10 shows promise due to the functional role of GPR10 we have demonstrated in vivo. Functional activation of GPR10 in our transgenic CaBP9K-hGPR10 mice leads to UL-like tumor development in myometrium (Figure 3.16 & Figure 3.17). Hence, our transgenic CaBP9K-hGPR10 mice may be important pre-clinical animal models for the development of drug treatments for UL. Development of a compound which blocks PrRP binding or inhibits downstream signaling capabilities of GPR10 and does not pass the blood-brain barrier, would yield negligible side effects due to endogenous expression of GPR10 restricted only to central nervous system neurons (Dodd & Luckman, 2013). In addition, as a cell surface G-protein coupled receptor (GPCR), GPR10 makes an excellent drug target.

In conclusion, our research provides an important role for GPR10 as a novel molecular activator of tumorigenic signaling pathways involved in UL growth and development. The
abundant expression of GPR10, outside of its normal expression pattern, could be a result of modified epigenetic programming and could provide additional clues to the consequences of genetic and epigenetic abnormalities in UL.
Bibliography


Chapter 4

The Role of REST in the Pathogenesis of Uterine Leiomyoma
Abstract

Every year in the United States, an estimated 200,000 women undergo invasive surgical removal of the entire uterus (hysterectomy), as treatment for uterine leiomyoma (UL) tumors. UL, also known as fibroids, are the most common tumors of the female reproductive tract. UL develop in the smooth muscle compartment (myometrium) of the uterus and are benign, yet cause devastating symptoms of pain, bleeding, anemia and fertility complications in up to 25% of women. Despite the prevalence and severity of UL, there is a scarcity of available drug treatment options for UL, due largely to the lack of understanding about the etiology of these tumors. Extensive evidence shows cytogenetic abnormalities and altered cell signaling pathways, especially the PI3K/AKT-mTOR pathway, contribute to the pathogenesis of UL. However, the molecular mechanisms that trigger these cellular defects are currently unknown. Previously, we found that aberrant overexpression of the neuronal protein, GPR10, activates the PI3K/AKT-mTOR pathway in UL cells. Here, we further show the dramatic reduction in protein expression of the epigenetic silencer, REST, yields de-repression of GPR10 in UL and activation of downstream PI3K/AKT-mTOR pathway proteins. We show the GPR10 promoter is associated with REST only in myometrial, not UL, cells and the silencing of REST in primary uterine cells results in overexpression of REST-target genes, including GPR10. Critically, we have developed an important UL preclinical mouse model with conditional deletion of Rest in the uterus resulting in UL phenotype. Taken together, we demonstrate the critical role of REST in myometrial cell gene regulation, including GPR10 expression, and confirm the function of REST in vivo with a novel mouse model.
Introduction

Uterine leiomyoma (UL), also known as fibroids, are benign smooth muscle cell tumors of the myometrium (Walker & Stewart, 2005). UL represent the most frequent clinical indication for hysterectomy that prematurely ends a woman's reproductive capability (Bulun, 2013). Despite this, there are currently no approved drugs that can provide effective, long-term treatment for these tumors. In the year 2010, the estimated annual cost of UL tumors in the United States was $5.9–34.4 billion (Cardozo et al., 2012). Although UL are the most common tumors of the female reproductive tract and a major quality of life issue for a significant percentage of women, the mechanisms that initiate UL growth and pathogenesis are still not well understood.

Extensive evidence shows chromosomal abnormalities and instabilities; as well as rare genetic risk factors, contribute to the etiology of UL (Parker, 2007; Stewart, 2001; Vilos et al., 2015; Walker & Stewart, 2005). It is estimated that 40-50% of UL have karyotypic abnormalities (Bulun, 2013). The most common chromosomal rearrangements in UL include 17% of UL with a deletion of chromosome 7q, 20% with the 12q15 rearrangement and 5% of UL with the 6p21 rearrangement (Mehine et al., 2013). These chromosomal abnormalities may contribute to disruption of genes aberrantly expressed in UL, including HGMA2, ESR2, and RAD5 (Walker & Stewart, 2005). In addition to cytogenic abnormalities, a somatic gain-of-function mutation in the mediator complex subunit 12 (MED12) has recently been reported in up to 70% of UL, and is not found in the surrounding myometrium (Makinen et al., 2011; Mittal et al., 2015). Although the contribution of the MED12 mutation appears to be relevant to the etiology of UL, its developmental origins in the quiescent myometrial tissue is unknown. Finally, rare genetic mutations in the Fumarate hydrase (FH) gene lead to familial occurrence of UL, but such rare
mutations, occurring in less than 100 families worldwide, do not account for the vast majority of UL (Gross et al., 2004; Harrison et al., 2015). Alternative factors involved in UL pathogenesis including epigenetic variations, microRNA-mediated posttranscriptional gene regulation, and altered cell signaling have gained traction as putative disease initiating events (Luo & Chegini, 2008; Navarro et al., 2012; Pan, Luo, & Chegini, 2008, 2010; Peng et al., 2009; J. J. Wei et al., 2006).

Several recent studies have demonstrated a central role for a dysregulated phosphoinositide 3-kinase–protein kinase B/AKT (PI3K/AKT) pathway leading to the activation of mammalian target of rapamycin (mTOR) in the pathogenesis of UL (J. D. Cook & Walker, 2004; Crabtree et al., 2009; Karra et al., 2010). Critically, we have found that the neuron specific G-protein coupled receptor, GPR10, functionally activates the tumorigenic PI3K/AKT-mTOR pathway in UL cells (Chapter 3)(Varghese et al., 2013). We showed GPR10, previously only found in areas of the hypothalamus, thalamus and medulla oblongata, is highly expressed in UL, where its activation by the peptide ligand, Prolactin-releasing peptide (PrRP) leads to tumor cell signaling activation (Dodd & Luckman, 2013; Varghese et al., 2013). This led us to explore the mechanism allowing expression of GPR10 outside of the central nervous system in UL. Studies in cell lines have shown transcriptional repression of GPR10 in the periphery is conferred by the epigenetic silencer RE1 silencing transcription factor/ neuron-restrictive silencing factor (REST)(Kemp et al., 2002). REST binds a canonical 21bp repressor element (RE-1), which is present in the GPR10 promoter, as well as in up to 2000 gene promoters, rendering REST a fundamental regulator in epigenetic repression of genes, including GPR10 (Gopalakrishnan, 2009; Kemp et al., 2002). Importantly, the activation of GPR10 as well as the loss of
REST/NRSF, is shown to trigger PI3K/AKT signaling and proliferation in tumor cell lines (Hayakawa et al., 2002; Samson et al., 2003; Westbrook et al., 2008; Westbrook et al., 2005).

REST is an important tumor suppressor protein that broadly functions to restrict target gene expression to the central nervous system through epigenetic regulation (Ballas et al., 2005; Ballas & Mandel, 2005). REST acts as a molecular scaffold to recruit co-factors including co-REST, mSin3A, histone deacetylases, histone methyltransferases and DNA methyltransferases to epigenetically silence target gene transcription in the periphery (Y. Huang, Myers, & Dingledine, 1999; Mulligan et al., 2008). Several studies have indicated the loss of REST plays a critical role in tumorigenesis of colon cancer, breast cancer and small cell lung carcinomas (Z. Huang & Bao, 2012; Kreisler et al., 2010; Westbrook et al., 2005). The loss of REST is also known to result in genomic instability in tumor cells, similar to frequent chromosomal translocations seen in UL (Gross et al., 2003; Guardavaccaro et al., 2008). Importantly, proteasomal degradation of REST, or expression of dominant negative forms of REST, have been shown to lead to over-activation of proteins in the PI3K/AKT-mTOR pathway in epithelial tumor cells (Negrini et al., 2013).

Interestingly, REST is also known to interact with MED12 (Ding et al., 2008). The potential impact of the loss of REST on the occurrence of gain-of-function of MED12 missense mutations, often seen in UL is currently unknown.

Here, we reveal REST protein, but not mRNA, is significantly reduced in UL tissue and that many of the most aberrantly expressed genes in UL are direct REST targets, including GPR10, which activates the PI3K/AKT mTOR pathway (Varghese et al., 2013). The loss of the crucial epigenetic regulator, REST, in UL tissue is a promising novel mechanism for the pathogenesis of UL. We propose the degradation of REST leads to long term epigenetic changes in UL cells, resulting in de-repression of key tumorigenic genes, including GPR10. The
following experiments demonstrate the role of REST in myometrial smooth muscle cell gene
regulation, including regulation of GPR10 expression, and confirm the function of REST in vivo
using a conditional REST knockout mouse model generated in our laboratory.
Materials and Methods

Chemicals and reagents.

Dulbecco's Modified Eagle's medium (DMEM; D5671), Accustain Trichrome Stain Kit (HT15), BCIP/NBT solution (B6404), Saline-Sodium Citrate buffer(S6639), Tween-20 (P1379), Triton-X 100 (93443), Trizma base (T6066) normal goat serum (D9663) and protease and phosphatase inhibitor cocktails (P0044) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin (17-602), and L-glutamine (17-605) were purchased from Biowhittaker Lonza (Walkersville, MD). Dulbecco’s PBS/Modified (SH30028), fetal bovine serum (SH30071) and bovine calf serum (SH30073) were purchased from Hyclone (Logan, UT). Ethidium homodimer (E3599), Alexa Fluor conjugated secondary antibodies (ab150073), Protein A agarose beads (15918-014), high-capacity cDNA reverse transcription kit (4368814) and Collagenase (14072) were obtained from Life Technologies (Grand Island, NY). Anti-GPR10 antibody (NBP1-00854, rabbit polyclonal) was obtained from Novus Biologicals (Littleton, CO). FuGene 6 transfection reagent (E2691) and colorimetric BrdU cell proliferation ELISA kit (11296736001) were from Roche (Indianapolis, IN). TaqMan primer-probe sets for PRLHR/ GPR10 (Hs00244685_s1, ABI) was obtained from Applied Biosystems (Foster City, CA). Taqman assays for Col1A1 (Mm.PT.47.6999992), Col3A1 (Mm.PT.47.9778198), Acta2 (Mm.PT.47.7024949), Tgfβ3 (Mm.PT.47.10648587), and Dpt (Mm.PT.47.17098032) were purchased from IDT (Coralville, IA). Locked nucleic acid probes (300514-15) were obtained from Exiqon Inc. (Woburn, MA). PrRP peptide was obtained from Phoenix Pharmaceuticals (Burlingame, CA). Antigen unmasking solution (H-3300) was purchased from Vector (Burlingame, CA). NE-PER nuclear extraction reagent (78833), SuperSignal West Pico Chemiluminescent Substrate (34080), Dharmafect (T2002), Proteinase K (25530-015), Restore
PLUS Western Blot Stripping buffer (46430), xylene (C8H10), RNAlater solution (AM7021), ON-TARGETplus SMARTpool siRNA to REST (L-006466-00-0005), ON-TARGETplus non-targeting scrambled siRNA#2 (D-001810-02-05) and charcoal-stripped FBS (12676011) were purchased from Thermo Fisher Scientific (Rockford, IL). RNeasy Mini Kit (74104) and RNAlater (76104) were obtained from Qiagen (Valencia, CA; 74104). Primary antibodies against AKT (9272), p-AKT (40605), p-4EBP1(9459S), p-70S6K (4206S), p-mTOR (2971), β-TRCP (4394), Co-REST (14567) and mTOR (2983) were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin (SC1616) and anti-goat IgG HRP conjugated antibody (SC2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRIS/Glycine/SDS Buffer (161-0732), Blotting-grade blocker (170-6404) and gradient 4-15% mini-PROTEAN TGX gels (456-1086) were purchased from Bio-Rad (Hercules, CA). Paraformaldehyde solution (15710) was obtained from Electron Microscopy Sciences (Hatfield, PA). Anti-REST antibodies (Catalog numbers: 09-019, 05-1477, and 17-641 for western blotting, immunofluorescence and ChIP assays respectively) were obtained from EMD Millipore Corporation (Billerica, MA). REST silencer Select siRNA (s11934) was obtained from Ambion. Cell culture chamber slides were purchased from Tissue Tek (Hatfield, PA).

**Tissue collection and cell culture.**

Leiomyoma samples were obtained from pre-menopausal women undergoing hysterectomy at Carle Foundation Hospital (Urbana, IL) and the University of Kansas Hospital (Kansas City, KS). Criteria for patients in this study exclude women undergoing hysterectomy for a primary condition other than uterine leiomyoma and those taking hormone therapy in the three months preceding surgery. Smooth muscle tissue cells (SMCs) were prepared from the samples by mincing the tissue and digesting in DMEM containing 1.5mg/ml collagenase for 4-6
hours at 37 degrees. After digestion, cells were cultured in DMEM medium containing 10% serum (FBS and FCS), 1% L-glutamine, and 1% penicillin-streptomycin in an incubator maintaining 95% humidity and 5% CO2 at 37 degrees. Cells were not passaged past p6. For paired tissue samples, the study was powered to measure changes in gene expression between the tumor and normal tissues. Using paired t-test, 11 pairs gave us 80% power to detect a difference of 0.81 (fold change of 1.75) standard deviation units or larger in gene expression with one-sided level of significance of 0.05.

**RNA isolation and qRT-PCR analyses.**

Total RNA was isolated from tissue samples or cultured cells stored in RNAlater (Qiagen, Valencia, CA) using RNeasy Mini Kit (Qiagen) according to manufacturers protocol. After quantification using Nanodrop spectrophotometer, aliquots of RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Applied Biosystems). TaqMan assays for PRLHR/ GPR10 (Hs00244685_s1, ABI), Col1A1 (Mm.PT.47.6999992, IDT), Col3A1 (Mm.PT.47.9778198, IDT), Acta2 (Mm.PT.47.7024949, IDT), Tgfb3 (Mm.PT.47.10648587, IDT), and Dpt (Mm.PT.47.17098032, IDT) were used to quantify gene expression utilizing delta delta C(T) method in comparison with 18s rRNA.

**Generation of REST conditional knockout mice.**

Floxed Rest embryonic stem cell clones were acquired from the European Conditional Mouse Mutagenesis Program ([http://www.eucomm.org](http://www.eucomm.org)) (Figure 4. 16). The embryonic stem cells (ES cells) were used to generate chimeric founder mice, which were mated to C57BL6 WT mice to create heterozygous Rest fl/+ mice. These mice were crossed with FLPo expressing mice (B6.129S4-Gt(ROSA) 26Sor<sup>tm1(FLP1)Dym/RainJ</sup>) to remove FRT flanked βgal-neo sequences. These were bred together to obtain homozygous Rest fl/fl mice, which were crossed with
Amhr2+/cre recombinase mice, to produce Rest cKO mice, specific to the female reproductive tract (Figure 4. 3). The mice were genotyped by PCR using forward Rest primer (5’TGTAAGTTTCCAAACTGTGAAGTTCG) and two reverse Rest primers (5’TGAAGTTTCCAAACTGTGAAGTTCG) (5’GCTACCAAATGCGTAAGTTCAAGG), as well as primers for Amhr2 (5’ GGA CAT GTT CAG GGA TCG CCA GGC) (5’ CGA CGA TGA AGC ATG TTT AGC TG) (Figure 4. 4)
Figure 4.3: Breeding scheme for generation of Rest cKO mice.
Figure 4.4: **Rest cKO targeted locus and genetic construct sequence.** (A) Wild-type sequence of Rest targeted in knockout mouse model. Showing genotyping primers (blue, green, red) and sequence removed in genetic construct (underlined). (B) Genetic construct sequence at exon 2 of Rest locus used in knockout mouse model. Showing genotyping primers (blue, red) and synthetic sequence added (orange).
Histology.

Uterine tissues were fixed in 4% paraformaldehyde and processed for paraffin embedding. Tissue sections (5 um thickness) were deparaffinized in xylene, rehydrated through a series of ethanol and stained with Hematoxylin and Eosin (H&E). For immunofluorescence, rehydrated tissue sections were subjected to antigen retrieval by heating in citrate buffer (Vector Laboratories Inc., Burlingame, CA). SMCs were cultured on chamber slides overnight, followed by fixation with 4% paraformaldehyde and permeabilization with 0.3% triton x-100 prior to immunofluorescence experiments. The slides were washed with PBS (5min, 3 times), blocked for 30 min in blocking agent (5% normal goat serum) followed by primary antibody in blocking buffer overnight at 4°C. The slides were washed with PBS (5min, 3 times) and incubated with AlexaFluor® 488 or AlexaFluor® 555 labeled secondary antibodies (Invitrogen, Life Technologies) for 1 hour at 37°C. The slides were treated with nuclear dye (Ethidium Homodimer, 5uM in PBS) for 5 min at room temperature and washed in PBS (5min, 3 times). The slides were mounted using 50% glycerol in PBS and visualized using an Olympus 1X71 inverted microscope and TE2000U 3 laser inverted confocal microscope.

Transfections.

SiRNA knockdown of REST in primary myometrial SMCs was performed using Silencer Select siRNA (Ambion) with Lipofectamine 2000 transfection reagent (ThermoFisher Scientific). SMCs were transfected 12 hours after plating and RNA and protein expression were analyzed 24h and 48h after transfection. The results were further confirmed by using ON-TARGETplus SMARTpool (L-006466-00-0005, Dharmacon, ThermoFisher) siRNAs to REST. Control experiments included ON-TARGETplus non-targeting scrambled siRNA#2 (D-001810-02-05). Protein extracts from the cells were then analyzed for western blotting. For GFP-REST
experiments, primary cultured cells were cultured overnight followed by transfection with 2.5µg GFP CAM V6-NRSF vector and 10µl FUGENE transfection reagent for 24 hours. pEGFP C1 vector was used as a control. Cells were fixed and analyzed using an Olympus 1X71 inverted microscope and TE2000U 3 laser inverted confocal microscope for imaging. For protein stability experiments, cells were transfected with GFP CAM V6-NRSF vector (as described above) overnight, followed by treatment with 50µg/ml cyclohexamide or MG132, and protein was extracted at 0, 3, 6, 12 or 24 hours and analyzed by western blotting.

**Protein extraction and western blotting.**

Frozen tissue samples were homogenized in a hypotonic buffer (40mM NaCl, 10mM KCl, 20mM Tris-HCl pH 7.4, 0.1% Triton-X100, 0.1% Tween 20) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO). The homogenates were centrifuged at 10,000 rpm at 4°C. The post-nuclear supernatants were combined with extracts from the nuclear pellets, solubilized using the above buffer supplemented with 300mM NaCl or NE-PER nuclear extraction buffer (Thermo Scientific, Rockford, IL). Western blots were performed with protein loaded with 6x Laemmli buffer on 10% polyacrylamide SDS gels, run by electrophoresis at 70 volts at room temperature. Transfer to PVDF membrane was at 4 degrees for 1.5 hours. Membranes were blocked in 5% skim milk for 1 hour. Overnight incubation at 4 degrees was with anti-REST (1:2000), anti-GPR10 (1:2000) or anti-actin (7.5:10000). Secondary antibody incubation was with IgG HRP conjugated antibodies (1: 5000) at room temp for 1 hour. Signal was developed with SuperSignal West Pico Chemiluminescent Substrate purchased from Thermo Scientific (Rockford, IL).

**Chromatin immunoprecipitation and PCR.**
Primary leiomyoma SMC and patient matched myometrial SMCs were cross-linked using 1% formaldehyde and chromatin was fragmented to nucleosomes using sonication, followed by immunoprecipitation (IP) for REST associated DNA fragments. 10µg of Anti-REST ChIPAb+ antibody (Millipore, 01821) or Acetylated histone H3 antibody (used as a marker for gene activation) were added to chromatin with 50µl of Protein A Agarose beads and incubated overnight at 4°C with rotation. Samples were centrifuged for 2 minutes at 1500 rpm and the supernatant removed. Beads were washed five times with 500µl cold lysis buffer and DNA was isolated. This procedure was performed in at least three different patient pairs of cultured cells, and performed in triplicates. Positive controls for IP include input samples consisting of lysate before IP, and negative controls include IP across IgG beads to detect non-specific binding to columns. DNA was analyzed by PCR amplification of the GPR10 promoter containing the RE-1 sequence to which REST binds. PCR primers spanning the conserved RE1 element in the promoter of GPR10 were designed based on the genomic sequence (GenBank: AL356865.19, bp 80348 – bp 80466) to amplify an 119bp PCR product. The primer sequences were: 5’ CTT GGC TGC AGC GCG CTC A and 5’ CTG CTC CTC CCC ACA TCA TC.

RNA-sequencing.

RNA isolation from whole uteri of Rest cKO and control mice aged 4 months was performed using phenol:chloroform. RNA samples were prepared by the KUMC Genomic Sequencing Facility using the TruSeq Stranded Total RNA LT Sample Preparation Kit with Ribo-Zero Mouse. Briefly, RNA samples were fragmented, the first two strands of cDNA were synthesized in thermal cycler, followed by adenylation of 3’ ends, adapter ligation, and enrichment of DNA fragments. Libraries were adjusted to 2nM concentration and pooled for multiplex sequencing. Libraries were validated using the Agilent 2100 Bioanalyzer using the
Agilent DNA 1000 Chip. Sequencing was performed on the Illumina HiSeq2500 Sequencing System.

**Data mining and Ingenuity Pathway analysis.**

GEO dataset GSE13319 was analyzed for the expression of REST associated/regulated genes in myometrial and leiomyoma samples. The dataset was preprocessed for analysis using the Robust Multichip Averages Procedure (RMA). Statistical analysis was performed on biological triplicates. Biological functional and pathway analysis were performed using Ingenuity Systems Pathway Analysis software [IPA, version 7.6; Ingenuity Systems (www.ingenuity.com)] on the significantly (fold change ≥ 1.5, p-value ≤ 0.05) differentially expressed genes between myometrium and leiomyoma, or between wild-type mouse and genetically modified mouse.

**Statistical Analysis.**

Leiomyoma samples were compared to myometrial controls from the same patient. For animal studies, Rest cKO mice uterine samples were compared to age-matched control mice at the same stage of estrus cycle. Quantitative experiments, including qRT-PCR and densitometry of western blots, were repeated with at least three independent biological replicates. Statistical significance was determined using paired sample T-test to determine change from the control sample. For paired tissue samples, the study was powered to measure changes in gene expression between the tumor and normal tissues. Using paired t-test, 11 pairs gave us 80% power to detect a difference of 0.81 (fold change of 1.75) standard deviation units or larger in gene expression with one-sided level of significance of 0.05. Significance was set at $P < 0.05$ for all comparisons. For RNA-sequencing results, genes used in Ingenuity Pathway Analysis were filtered for $p < 0.05$, $q < 0.01$, and fold change of 1.5 or higher. Comparison between RNA-sequencing results of
Rest cKO mice versus human microarray data (shown in Figure 4.21) were analyzed by hypergeometric statistical analysis, where the hypergeometric $p-value = \sum_{t=(F\cap P)}^{\min(F,P)} \frac{\binom{t}{i}\binom{N-F}{p-i}}{\binom{N}{p}}$.
Results

REST expression is decreased in uterine leiomyoma.

We have found GPR10, a neuronal gene normally silenced in the periphery, is expressed in vast majority of UL (Varghese et al., 2013). The activation of cell proliferation and growth pathways in UL resulting from exposure to the GPR10 ligand, PrRP, (Chapter 3) led us to explore the molecular mechanism allowing GPR10 expression in UL tissue. Because the repression of GPR10 in non-neuronal tissues is accomplished by the epigenetic silencer REST (Kemp et al., 2002), we tested the expression levels of REST in UL tissue. REST protein levels, determined by Western blotting in 7 paired patient samples, indicated that compared with normal myometrial tissues, patient-matched UL samples expressed markedly reduced levels of the protein (Figure 4. 5A & Figure 4. 6) This result was further confirmed by immunofluorescence staining of REST in tissue sections from normal myometrium and UL (Figure 4. 5B). Normal myometrial samples showed intense REST staining, whereas the UL samples showed lower levels of REST. Further, in UL samples the residual level of REST protein present was predominantly cytoplasmic (Figure 4. 5B panel 5 &6, Figure 4. 7), suggesting that REST-mediated repression of a multitude of genes could be compromised in fibroids. Interestingly, mRNA levels of REST are unchanged between the myometrium and UL (Figure 2.7), suggesting that the loss of REST in UL may occur post-translation.
Figure 4.5: Decreased REST protein expression in uterine leiomyoma. (A) Western blotting for REST in matched myometrial and leiomyoma tissues showing down-regulation of REST protein in leiomyomas (N = 7). β-actin was used as a protein loading control. (B) Immunofluorescence staining of representative myometrial and leiomyoma tissues (panels 1–4; N = 3) and SMCs (panels 5 and 6) with anti-REST (green) antibody. Nuclei are stained in panels 1–4 with EthD-1 (red).
Figure 4. 6: **Western blot analysis of REST expression in paired patient samples.** (A)

Western blot showing lower REST expression in leiomyoma (L) samples compared to matched myometrium (M) from additional patients (P4-P7; N = 7). (B) Densitometric analysis of western blots showing significantly lower relative REST levels in leiomyomas. Error bars indicate ± SD.
Figure 4. 7: **Histological analysis of REST protein localization in uterine leiomyoma.** (A & B) Immunofluorescence analysis of REST (green) in myometrium and leiomyoma tissue samples. Nuclei were stained with EthD-2 (red). (C &D). Immunofluorescence analysis of GFP-REST(green) transfected myometrial smooth muscle cells and leiomyoma smooth muscle cells.
The loss of REST permits GPR10 expression.

Our data show down-regulation of REST at the protein level in UL tissue, and the retention of low REST levels in cultured primary UL cells (Figure 4. 5 & Figure 4. 6), making it feasible to study the role of REST in cell culture. To perform its repressive epigenetic function, REST binds a 21bp RE-1 sequence in the promoter of target genes(Ballas & Mandel, 2005). To demonstrate the direct regulation of GPR10 by REST, we performed chromatin immunoprecipitation (ChIP) assays in primary cultured UL and myometrial cells, using ChIPAb + REST antibodies. Our results indicate that REST is associated with the GPR10 promoter in normal myometrial cells (Figure 4. 8). Conversely, in UL cells, the RE1 element in the GPR10 promoter was not associated with REST but was specifically associated with acetylated histone H3, indicating that the chromatin is permissive to transcription (Figure 4. 8). Additionally, siRNA knockdown of REST in normal myometrial cells led to expression of GPR10 mRNA (Figure 4. 9A) and protein expression (Figure 4. 9B), indicating that the loss of REST may mediate the expression of GPR10 in myometrial SMCs as illustrated (Figure 4. 10).
Figure 4.8: Chromatin immunoprecipitation analysis of the GPR10 RE-1 site. Chromatin immunoprecipitation using REST and acetylated histone H3 antibodies at the GPR10 promoter RE-1 site in matched myometrial and leiomyoma SMCs (N = 3).
Figure 4. 9: Silencing of REST in myometrial smooth muscle cells. (A) Quantitative RT-PCR on REST and GPR10 mRNA in myometrial SMCs silenced with siREST and scrambled siRNA (N = 3) Error bars indicate ± SD. (B) Expression of REST and GPR10 in myometrial SMCs silenced with siREST and scrambled siRNA for 24 and 48 h.
Figure 4. 10: **Schematic representation of REST loss and functional GPR10 expression in uterine leiomyoma.** Working model depicting the link between loss of REST and the overexpression of GPR10 in myometrium.
REST target genes are overexpressed in uterine leiomyoma.

Because the loss of REST could potentially lead to de-repression of a large number of its targets in the periphery, we used data mining to identify additional REST target genes expressed in UL. In fact, several of the most aberrantly expressed genes in UL, including glutamate receptor, ionotopic, AMPA 2 (GRIA2); stathmin-like 2 (STMN2); glutamate receptor, ionotopic, N-methyl D-aspartate 2A (GRIN2A); neurofilament heavy polypeptide (NEFH); sal-like protein 1 (SALL1); secretogranin II (SCG2); and cerebellin 1 (CBLN1) are known REST-repressed targets (Figure 4.11; dataset GSE13319). Pathway analysis (using IPA; Ingenuity Systems, www.ingenuity.com) of the gene expression dataset also revealed that two additional genes down-regulated in UL samples, PRICKLE1 and HBEGF were REST related but not direct transcriptional targets of REST (Figure 4.11). PRICKLE1 (also known as RILP for REST/NRSF-interacting LIM-domain protein) has been shown to influence nuclear localization of REST (Shimojo & Hersh, 2006). The aberrant expression of a number of REST target genes in addition to GPR10 supports a role for the loss of this tumor suppressor in fibroid pathogenesis. Because down-modulation of REST in cultured primary myometrial cells leads to GPR10 expression, we further queried whether additional REST target genes with potential functions in UL show concomitant changes in expression. Gene expression profiling using Affymetrix microarrays (U133 Plus 2.0) indicated that several REST target genes with functions in connective tissues were also up-regulated in myometrial cells after knockdown of REST (Figure 4.12, GEO dataset GSE41386).
Figure 4. 11: **Pathway analysis of REST target genes differentially expressed in uterine leiomyoma.** (A) Expression of known REST-related genes in uterine fibroids from dataset GSE13319, pathway analysis (using IPA; Ingenuity Systems). Note that REST mRNA expression is unchanged.
Figure 4.12: **Gene expression analysis of REST knockdown in myometrial smooth muscle cells.** Gene expression analysis using Affymetrix microarray (U133 Plus 2.0, GEO dataset GSE41386) showing siRNA knockdown of REST in myometrial smooth muscle cells leads to overexpression of REST-repressed targets.
**REST regulation is at the protein level in uterine leiomyoma.**

Our data showed protein levels of REST were drastically reduced in UL (Figure 4. 5). Interestingly, *REST* mRNA expression was unchanged in UL compared with that in the normal myometrium. The results also corroborated the expression data for *REST* from GEO dataset GSE13319 (Figure 4. 11) and suggests the regulation of REST at the protein level in UL. In addition, GFP-REST protein expressed from a transfected construct was less stable in UL cells compared with that in myometrial cells (Figure 4. 13). Ubiquitinylation and proteasomal degradation of REST mediated by beta-transducin repeat containing protein (β-TRCP), contributing to oncogenic transformation in cell lines, has been reported (Guardavaccaro et al., 2008; Z. Huang & Bao, 2012; Westbrook et al., 2008). This led us to test whether β-TRCP or REST corepressor 1 (CoREST), known regulators of REST expression and function, were dysregulated in UL. Our data indicated that the levels of expression of Co-REST and β-TRCP were not significantly altered in UL (Figure 4. 14). Altered protein–protein interactions of REST with Co-REST or with β-TRCP may also influence its repressor function or stability. Our results suggest that the mechanism of loss of REST in UL may be unique.
Figure 4.13: Altered stability of REST in leiomyoma smooth muscle cells. Western blot (A) and densitometric analysis (B) showing the accelerated degradation of GFP-REST in leiomyoma SMCs. MSMCs and LSMCs (N=3) were transfected with 2.5µg GFP CAM V6-NRSF vector for 24 hours, followed by treatment with 50µg/mL cyclohexamide or MG132 for control. Protein was extracted at 0,3,6 or 12 hours and analyzed by western blot.
Figure 4.14: Expression of REST co-regulators in uterine smooth muscle cells. (A) Western blots and densitometric analysis (B) showing that Co-REST and β-TRCP are not significantly altered in leiomyomas and myometrial primary cultured cells. β-Actin was used as a protein loading control.
Conditional knock-out of REST in the mouse myometrium leads to leiomyoma phenotype.

Our *in vitro* data strongly supported a role for the loss of REST in the de-repression of downstream genes, including *GPR10*. REST is highly expressed in the mouse myometrium (Figure 4. 15), which substantiates the significance of a knockout mouse model for REST. Conventional *Rest* KO results in embryonic lethality (Chen et al., 1998), therefore, to determine the role of the degradation of REST in UL *in vivo* we have developed a conditional knock out of *REST* in the mouse uterus.

Floxed REST embryonic stem cell clones were acquired from the European Conditional Mouse Mutagenesis Program (http://www.eucomm.org). (Figure 4. 16A) The ES cells were used to generate chimeric founder mice, which were mated to C57BL6 WT mice to create heterozygous *RESTfl/+* mice. These mice were crossed with FLPe expressing mice (B6.129S4-Gt(ROSA) 26Ser<sup>tm1(FLP1)Dym</sup>/RainJ) to remove FRT flanked βgal-neo sequences. The resulting offspring were bred together to obtain homozygous *RESTfl/fl* mice (Figure 4. 16B) which were crossed with Amhr2<sup>+/cre</sup> recombinase mice (Hernandez Gifford, Hunzicker-Dunn, & Nilson, 2009), to produce conditional *Rest* KO mice, specific to the reproductive tract, including the uterus and ovaries (Figure 4. 3).

Our results showed increased myometrial thickness in the *Rest cKO* compared to control mice (Figure 4. 17). Western blot analysis showed decreased Rest expression in the uterus led to overexpression of Gpr10, which is not normally present in the mouse myometrium (Figure 4. 18). In addition, we show increased activating phosphorylation of proteins involved in the PI3K/AKT-mTOR pathway, including p-4EBP1, pAKT, and p-p70S6K (Figure 4. 18).
Previously, we showed these PI3K/AKT-mTOR proteins are activated in UL cells as a result of GPR10 activation (Chapter 3).

We performed RNA-sequencing of the entire uterus, including the endometrium, of 4 month old Rest cKO mice in diestrus. Control mice were Rest^fl/fl mice which did not express Amhr2^+/cre. Sequencing results were analyzed using Ingenuity Pathway Analysis (IPA) for predicted pathways. Gene expression profiles of the Rest cKO mice matched genes involved in pathways including hepatic fibrosis, a condition related to UL especially in terms of extra fibrous ECM, as well as retinoic acid signaling which has been implicated in human UL pathogenesis (Figure 4.17). Upstream regulators identified by IPA include REST (Figure 4.17), and network analysis reveals many direct (Figure 4.19) and indirect (Figure 4.20) REST-associated genes are significantly dysregulated in the Rest cKO, which provides substantial evidence of REST deletion in the myometrium of the Rest cKO mice. Disease expression profiles that are similar to gene expression profiles of the Rest cKO mice include cancers, dermatological diseases and neurological disease, as expected for a UL-specific mouse model with deletion of a major epigenetic repressor of neuronal genes (Figure 4.17).

Although the normal myometrium shows a limited response to estrogen in the luteal phase, UL show a hypersensitivity to estrogen and increased expression of sex steroid-responsive genes even in the luteal phase (Maruo et al., 2004). Similarly, we note increased uterine size and morphology in the Rest cKO mice even during diestrus, when estrogen levels are low (Figure 4.15). β-estradiol was identified by IPA analysis as a highly significant upstream regulator of genes expressed in the Rest cKO uterus (Figure 4.17). Therefore, we analyzed Esr1 associated genes which are significantly (p<0.05) dysregulated in the Rest cKO RNA-sequencing results
and found many ERα targets, including known REST target genes which are increased in human UL, such as GRIA2 and STMN3 (Figure 4.18).

Next, we compared RNA-sequencing results of the Rest cKO to microarray data from 23 paired patient samples of human UL. 15,094 genes were found in both experiments, with 888 genes significantly dysregulated in human UL, and 703 genes significantly dysregulated in the Rest cKO mouse whole uteri (Figure 4.21). Significance was set a \( q < 0.01 \) for human samples, due to larger sample size of \( N = 23 \), and \( p < 0.05 \) for Rest cKO (\( N = 3 \)). Genes included in the analysis had a 1.5 fold change or higher. Importantly, 94 genes were identified as significantly dysregulated in both human UL and Rest cKO and expressed in the same direction in both. This overlap was determined to have a significance of 3.2E-14, which is highly statistically significant (Figure 4.21). This genetic overlap is considerable, even given that the Rest cKO sample consisted of the whole uterus, including the endometrium, despite Rest deletion only targeted to the myometrium. Further examination of the 94 genes found in common between human UL and Rest cKO revealed several REST-target genes with known neuron-specific function, as indicated by www.genecards.org (Figure 4.22). IPA analysis of the 94 mutually dysregulated genes showed similarity in gene expression profiles to UL-specific conditions such as benign neoplasia, leiomyomatosis, smooth muscle tumor, benign neoplasm of female genital organ, and leiomyoma itself (Figure 4.23). In addition, gene profiles show similarity to dermatological conditions, including skin lesion, skin tumor and skin cancer (Figure 4.23), which is relevant due to similarities between dermatological cancers and UL (Leppert et al., 2006; Sun et al., 2014).

Overall, we show that conditional deletion of Rest in the mouse uterus results in epigenetic changes leading to aberrant expression of genes involved in uterine leiomyoma
pathogenesis. This animal model provides valuable information on the functional outcome of REST down-regulation in the pathogenesis of UL.
Figure 4.15: **Histological expression of Rest in the wild-type mouse myometrium.**

Immunofluorescence staining of a wild-type mouse uterus (N = 1) with anti-REST (green) antibody. Nuclei are stained with EthD-1 (red).
Figure 4. 16: Genetic construct and genotyping for generation of a Rest conditional knockout in the mouse myometrium. (A) Rest cKO targeting construct. Floxed Rest embryonic stem cell clones were acquired from the European Conditional Mouse Mutagenesis Program. (B) PCR genotyping of Rest $^{fl/fl}$ mice (lanes 4 & 5), Rest heterozygous mice (lanes 2,3, & 7), and control mice (lane 6).
Figure 4.17: **Morphology of Rest cKO mouse uteri.** (A) Representative photograph and (B) H&E stain showing increased size and morphology of Rest cKO compared to control mouse uteri in diestrus. Magnification bars represent 50µm.
Figure 4. 18: **Protein expression analysis of Rest cKO mice uteri.** Western blot analysis of GPR10 expression and activation of downstream tumorigenic signaling proteins in total uterine tissue of Rest cKO and control mice (N = 1). GAPDH was used as a protein loading control.
Figure 4. 19: **Ingenuity Pathway Analysis of gene expression profiles of Rest cKO.** Genes which show a significant ($P < 0.05$) differential expression based on RNA-sequencing of whole uteri of 4 month old Rest cKO and control mice (N = 3) were used for pathway prediction by Ingenuity Pathway Analysis software.
Figure 4. 20: **Analysis of Esr1 associated genes in Rest cKO mouse.** Gene network analysis of Esr1 associated genes using Ingenuity Pathway Analysis software. Genes which were significantly ($p < 0.05$) up-regulated (red) or down-regulated (green) in RNA-sequencing results of 4-month old Rest cKO mouse whole uteri in diestrus (N = 3).
Figure 4. 21: Gene network analysis of direct REST targets in Rest cKO. Genes which are known direct targets of REST and are significantly ($p < 0.05$) down-regulated (fold-change of 1.5 or higher) in Rest cKO uteri. Analysis performed with IPA software on RNA-sequencing study of whole uteri from 4 month old Rest cKO and control mice (N = 3).
Figure 4.22: Gene network analysis including indirect REST associated genes in Rest cKO.

Ingenuity Pathway Analysis of genes associated with REST directly and indirectly. Genes included in the pathway are significantly ($p < 0.05$) up-regulated (red) or down-regulated (green) in the whole uteri of 4 month old Rest cKO mice in diestrus compared to control mice (N = 3).
Figure 4. 23: **Comparison of human uterine leiomyoma to Rest cKO global gene expression.**

Analysis of GEO dataset GSE 13319 (green; N = 23) in comparison to RNA-sequencing of whole uteri of Rest cKO and control mice (blue; N = 3). Genes significantly dysregulated (human 888 genes with fold-change > 1.5; q value < 0.01) (mouse 703 genes with fold-change > 1.5; p-value < 0.05) in both sets (yellow) total 94 genes, and is highly statistically significant (3.2E-14; hypergeometric p-value).
<table>
<thead>
<tr>
<th>GENE</th>
<th>FULL NAME</th>
<th>FUNCTION (<a href="http://www.Genecards.org">www.Genecards.org</a>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEX1</td>
<td>Brain Expressed X-Linked 1</td>
<td>Signaling adapter molecule involved in p75NTR/NGFR signaling. Plays a role in cell cycle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>progression and neuronal differentiation.</td>
</tr>
<tr>
<td>COL9A2</td>
<td>Collagen Type IX Alpha 2</td>
<td>This gene encodes one of the three alpha chains of type IX collagen, the major collagen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>component of hyaline cartilage.</td>
</tr>
<tr>
<td>CRMP1</td>
<td>Collapsin Response Mediator</td>
<td>Encodes a member of a family of cytosolic phosphoproteins expressed exclusively in the</td>
</tr>
<tr>
<td>Protein 1</td>
<td></td>
<td>nervous system.</td>
</tr>
<tr>
<td>GRIA2</td>
<td>Glutamate Ionotropic Receptor</td>
<td>Glutamate receptors that are sensitive to AMPA, and function as ligand-activated cation</td>
</tr>
<tr>
<td>AMPA Type Subunit 2</td>
<td></td>
<td>channels.</td>
</tr>
<tr>
<td>LRRTM1</td>
<td>Leucine Rich Repeat Transmembrane</td>
<td>Exhibits strong synaptogenic activity, restricted to excitatory presynaptic differentiation,</td>
</tr>
<tr>
<td>Neuronal 1</td>
<td></td>
<td>acting at both pre- and postsynaptic level.</td>
</tr>
<tr>
<td>NPTX2</td>
<td>Neuronal Pentraxin 2</td>
<td>Involved in excitatory synapse formation.</td>
</tr>
<tr>
<td>NRXN2</td>
<td>Neurexin 2</td>
<td>The products of this gene function as cell adhesion molecules and receptors in the</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrate nervous system.</td>
</tr>
<tr>
<td>QPRT</td>
<td>Quinolinate Phosphoribosyltransferase</td>
<td>Quinolinate acts as a most potent endogenous excitotoxin to neurons. Elevation of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>quinolinate levels in the brain has been linked to the pathogenesis of neurodegenerative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>disorders such as epilepsy, Alzheimer's disease, and Huntington's disease.</td>
</tr>
<tr>
<td>RIMS2</td>
<td>Regulating Synaptic Membrane</td>
<td>Rab effector involved in exocytosis. May act as scaffold protein. Plays a role in dendrite</td>
</tr>
<tr>
<td>Exocytosis 2</td>
<td></td>
<td>formation by melanocytes.</td>
</tr>
<tr>
<td>SCG2</td>
<td>Secretogranin II</td>
<td>Involved in the packaging or sorting of peptide hormones and neuropeptides into secretory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vesicles.</td>
</tr>
<tr>
<td>SCG5</td>
<td>Secretogranin V</td>
<td>Acts as a molecular chaperone for PCSK2/PC7, preventing its premature activation in the</td>
</tr>
<tr>
<td></td>
<td></td>
<td>regulated secretory pathway.</td>
</tr>
<tr>
<td>SRPX2</td>
<td>Sushi Repeat Containing Protein, X-Linked 2</td>
<td>May play a role in the development of speech and language centers in the brain.</td>
</tr>
<tr>
<td>STMN2</td>
<td>Stathmin 2</td>
<td>Function in microtubule dynamics and signal transduction. The encoded protein plays a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>regulatory role in neuronal growth and is also thought to be involved in osteogenesis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reductions in the expression of this gene have been associated with Down's syndrome and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alzheimer's disease.</td>
</tr>
</tbody>
</table>

Figure 4. 24: **Functions of select genes dysregulated in both UL and Rest cKO mice.** Gene function descriptions obtained from www.genecards.org.
Figure 4. 25: **Pathway analysis of genes dysregulated in both human UL and Rest cKO mouse.** Genes which were found to be significantly dysregulated in both human UL (GEO dataset GSE 13319; N = 23) and RNA-sequencing results of 4 month-old Rest cKO in diestrus (N = 3) were analysed using pathway prediction in IPA software.
Discussion

The molecular mechanisms that initiate UL growth and pathogenesis are still not completely understood. Here we provide evidence that the loss of the tumor suppressor REST and the ensuing de-repression of GPR10 play a role in the pathogenesis of UL. We propose that the loss of REST acts as a trigger for the proliferation of quiescent smooth muscle cells in the myometrium. Crucially, we show that mice with a conditional deletion of Rest in the myometrium develop a UL phenotype, validating the role of this epigenetic silencer in the pathogenesis of UL in vivo.

In chapter 3, we established that the near ubiquitous expression of GPR10 in UL activates the PI3K/AKT-mTOR pathway, providing a previously unknown function for GPR10 in UL (Varghese et al., 2013). We confirmed the important role of GPR10 overexpression in UL with a transgenic mouse model expressing the human GPR10 gene in the myometrium. Most importantly, near universal expression of GPR10 (PRLHR) was also observed in UL carrying various known mutations and chromosomal translocations in a recent genetic association study (Mehine et al., 2016). These results implicate that a master regulatory switch may exist further upstream of GPR10 and the genetic mutations observed in a majority of fibroids. The presence of an RE1 element in the promoter of GPR10 has been suggested to preclude the expression of this GPCR in non-neuronal cells (Kemp et al., 2002). Thus, its widespread expression in UL is very fascinating and suggested to us that REST may be dysregulated in UL. In addition to GPR10, a number of other known targets of REST including GRIA2, GRIN2A, DCX, STMN2, SCG2, SALL1, and CBLN1 were among the most significantly up-regulated genes in UL (Figure 4. 11), strongly suggesting that the function of the tumor suppressor, REST, is severely compromised in UL. Our results indicated that, whereas REST mRNA levels were comparable in
normal myometrium and UL, REST protein levels were markedly reduced in UL (Figure 4. 5, Figure 4. 6 & Figure 4. 11). The loss of REST in UL has not been previously shown, possibly due to the similar mRNA levels of REST in myometrial and leiomyoma tissues. Furthermore, results from chromatin immunoprecipitation and gene knockdown experiments (Figure 4. 8 & Figure 4. 9) confirmed that REST regulates GPR10 expression in the myometrium and that loss of REST leads to aberrant expression of GPR10 in UL cells. The loss of this master regulator of epigenetic long-term gene silencing provides a compelling mechanism for the pathogenesis of UL and links the activation of PI3K/AKT pathway (Westbrook et al., 2005) and the proposed regulation of REST target genes by estrogen (Bronson, Hillenmeyer, Park, & Brodsky, 2010) to UL.

As a master epigenetic silencer of neuronal genes, REST is ubiquitously expressed in non-neuronal cells where it binds a 21-23-bp repressor element (RE1) sequence present in an estimated 2000 gene promoters within the human genome (Abrajano et al., 2010; Liu, Liu, Niu, Cheng, & Fei, 2009). In addition, REST can also bind non-canonical RE1 sequences, making REST an important epigenetic regulator of a multitude of genes (Bruce et al., 2004). REST accomplishes epigenetic silencing of target genes by acting as a molecular scaffold to recruit numerous co-repressors and gene regulatory proteins to promoter sites (Coulson, 2005). REST is considered a tumor suppressor in epithelial cells as down-regulation of REST has been shown in epithelial cell cancers such as breast cancer, small cell lung carcinoma (SCLC) and colorectal cancer (Lv et al., 2010; Wagoner et al., 2010; Westbrook et al., 2005). Overexpression of alternatively spliced forms of REST that function as dominant negatives has also been shown to promote tumorigenesis (Coulson, 2005; Palm, Metsis, & Timmusk, 1999). Importantly, down-regulation of REST has been shown to enable gene expression that promotes vascular SMC
proliferation and atherosclerosis (Cheong et al., 2005). Here, we show the loss of REST in uterine smooth muscle cells leads to gene expression consistent with tumorigenesis cell signaling in UL (Figure 4. 12). We found the loss of REST leads to the de-repression of GPR10 (Figure 4. 9), which activates the PI3K/AKT-mTOR signaling pathway (Figure 2.6). In addition, we show the important role of REST in UL in vivo with a conditional knockout of REST in the mouse uterus. The role of REST in myometrial smooth muscle cells or its loss in the pathogenesis of UL has not been reported previously. We found that β-TRCP, the ubiquitin ligase known to regulate REST stability in tumor cells (Westbrook et al., 2005), and Co-REST, an important regulator of REST activity (Lunyak et al., 2002), were expressed at comparable levels in UL and myometrium (Figure 4. 14). Elucidation of the exact mechanism leading to the accelerated REST degradation in UL (Figure 4. 13) is not yet known. It is conceivable that the loss of REST protein triggers myometrial cell proliferation and transformation of the cells to a UL phenotype.

An important factor contributing to the pathogenesis of UL is the common cytogenic abnormalities present in 40-50% of UL (Bulun, 2013). The most common abnormalities are translocations on chromosome 12, deletion of chromosomes 3q and 7q, trisomy 12 and rearrangements on chromosomes 6, 10 and 13 (Bulun, 2013). Interestingly, loss of REST is known to result in mitotic arrest deficient-like 1 (MAD2)-mediated genomic instability in tumor-derived cell lines (Guardavaccaro et al., 2008). These REST dependent genomic instabilities could likely cause recurring chromosomal translocations and gene mutations shown to exist in UL. Because REST is known to interact with MED12 (Ding et al., 2008), a gene frequently mutated in UL (Makinen et al., 2011; McGuire et al., 2012), we investigated whether such somatic mutations resulted in altered protein–protein interactions that may contribute to improper tumor suppressor function of REST. We were unable to detect altered REST–MED12
interactions or aberrant subcellular localization of MED12 in UL cells. Somatic missense mutations occurring in MED12 at high frequencies indicate upstream mechanisms that trigger initial steps of myometrial cell proliferation, considering that the adult normal myometrium is essentially quiescent. Based on our results showing the overexpression of REST target genes in UL, it will be important to test whether MED12 mutation occurs as a “second hit” that leads to the activation of these genes. Further studies are needed to determine if the loss of REST leads to MED12 mutations or if missense mutations in MED12 may influence REST function. It may also be important to understand whether accumulation of mutations in MED12 (Makinen et al., 2011; McGuire et al., 2012), TSC2 (Crabtree et al., 2009), HMGA2 (Hunter et al., 2002; Velagaleti et al., 2010), or REST pathway genes occur after the loss of REST and resultant chromosomal instability.

There is currently a scarcity of appropriate preclinical animal models available for the study of UL in vivo. The most widely used model is the Eker rat, which has mutations in the Tsc2 gene resulting in UL-like tumors as well as renal cell carcinoma, which limits its use in long-term studies of UL (J. D. Cook & Walker, 2004; Walker et al., 2003). In addition, the Eker rat model is an incomplete representation of human UL since the TSC2 gene is not affected in the human condition. Recently, a mouse model with a UL-specific Med-12 mutation has been shown to develop UL-like tumors with increased ECM and chromosomal instabilities (Makinen et al., 2011; Mittal et al., 2015). Further development of this Med-12 mouse model will be important in understanding UL development. Here, we present an animal model for UL with myometrial specific deletion of a gene, Rest, which is drastically reduced in human UL. We found our conditional knockout mouse model of Rest in the uterus has increased myometrial size and mirrors human UL at the protein activation level (Figure 4.17 & Figure 4.18). We found
myometrial expression of Gpr10 in the *Rest* cKO mouse uterus, whereas Gpr10 is not normally found in the mouse myometrium, showing de-repression of this REST target gene (Figure 4.18). Further, we found activation of PI3K/AKT-mTOR pathway proteins downstream of Gpr10 in our *Rest* cKO mouse model. RNA-sequencing of the entire mouse uterus, including endometrium, of *Rest* cKO and control mice in diestrus show additional evidence of *Rest* conditional deletion in the myometrium. *Rest* mRNA levels in RNA-sequencing results were not found to be significantly reduced in *Rest* cKO mice compared to controls (data not shown). This is most likely due to robust expression of Rest in endometrial glandular and luminal epithelial cells, which were included in tissue samples sequenced. However, IPA analysis of RNA-sequencing results identify REST as a major upstream regulator of significantly dysregulated genes (Figure 4.17), and further gene network analysis reveals a large number of REST targets and indirect REST-associated genes are significantly dysregulated in the *Rest* cKO mouse, even considering the inclusion of endometrial tissue (Figure 4.19 & 4.20). This is further evidence that *Rest* is ablated in our knock-out mouse. Another interesting upstream regulator identified in IPA analysis of *Rest* cKO mice is *Esr1*, considering the mice used in this study were in diestrus. UL display an increased sensitivity to estrogen and show estrogen-responsive gene expression in the luteal phase, when the normal myometrium becomes quiescent (Maruo et al., 2004). Further analysis of *Esr1* associated genes by IPA network analysis reveal up-regulation of several *Esr1* responsive genes in *Rest* cKO mice in diestrus, including known REST-target neuronal genes such as GRIA2 and STMN3 (Figure 4.18). In addition, the *Rest* cKO mice show a substantial increase in uterine size and thickness in diestrus compared to matched control mice uteri (Figure 4.15).
RNA-sequencing of the Rest cKO whole uterus analyzed by Ingenuity Pathway Analysis provides compelling evidence that the Rest cKO mouse model is an excellent approximation of human UL. Pathway analysis results show significant similarities in gene expression profiles to pathways involved in hepatic fibrosis and retinoic acid signaling as well as diseases including cancer, reproductive tract disease, dermatological conditions and neurological disease (Figure 4.17). UL share similarities with hepatic fibrosis gene expression, especially in terms of fibrous extracellular matrix components (Ebrahimi, Naderian, & Sohrabpour, 2016). The similarity of gene expression profiles of Rest cKO mice to retinoid X receptors (RXR) inhibition is of interest considering that UL are known to express differential levels of RXR compared to the myometrium, and retinoic acid signaling has been shown to have an effect on UL pathogenesis (Borahay et al., 2015). An evidence that the Rest cKO mice are a good approximation of human UL comes from the similarity of gene expression profiles of Rest cKO mice to dermatological conditions, as UL are known to share commonly disrupted ECM proteins, including DPT and collagens type I and III, with dermatological conditions such as keloids (W. H. Catherino et al., 2004; Sun et al., 2014). In fact, an emerging theory to the pathogenesis of UL, based on the similarity of UL to dermatological disorders, is that UL result from abnormal wound healing in response to injury of the uterus (Leppert et al., 2006). Moreover, IPA analysis of the Rest cKO mice transcriptome also show strong similarity to gene expression profiles of reproductive system disease and cancer (Figure 4.17). Further evidence supporting the similarity of the Rest cKO mice to human UL was provided by comparison of the Rest cKO mice transcriptome to human microarray data (Figure 4.21). A highly significant number of genes were expressed in the same direction in the Rest cKO mouse uteri and human UL, even considering the Rest cKO mice uteri samples included the endometrial epithelium, in which Rest would not be removed by
Amhr2<sup>+/cre</sup> (Figure 4.21). This strong indication of the Rest cKO mouse as an excellent UL model is supported by investigation of the genes dysregulated in common between Rest cKO mice and human UL. Many of the genes are neuron-specific, yet they are being expressed in the uteri in Rest cKO mice and human UL, which support the prominent role that the loss of the major neuronal epigenetic silencer, REST, plays in UL pathogenesis (Figure 4.22). In addition, IPA pathway analysis of the 94 genes dysregulated in common between Rest cKO and human UL reveal significant similarity to expression profiles of smooth muscle tumors, leiomyomatosis, benign neoplasm of female genitalia and leiomyoma, again indicating the Rest cKO mouse is approximating the human condition (Figure 4.23). In conclusion, the phenotype of the Rest cKO mice represent a good approximation of the human condition, providing invaluable knowledge about the etiology of UL.

Our work provides a novel and critical role for the epigenetic regulator, REST, in UL. We have found that REST protein levels are severely decreased in UL tissue and that the silencing of REST in myometrial cells leads to increased expression of GPR10 and other UL specific genes. We show that the loss of the tumor suppressor REST leads to the de-repression of epigenetically silenced genes including GPR10, leading to the pathogenesis of UL. In addition, we present a unique myometrial-specific loss of function mouse genetic model (Rest<sup>f/f</sup> Amhr2<sup>Cre<sup>+</sup></sup>) to confirm the role of REST in the pathogenesis of UL.
Bibliography


encoding neuronal genes. *Science, 298*(5599), 1747-1752. doi:10.1126/science.1076469


Chapter 5

Estrogenic Regulation of PRICKLE1 in the Pathogenesis of Uterine Leiomyoma
Abstract

Uterine leiomyoma (UL), benign tumors of the myometrial smooth muscle layer, are present in over 75% of women, often causing severe pain, menorrhagia and reproductive dysfunction. The molecular pathogenesis of UL is poorly understood. We recently showed that the loss of REST (RE-1 Silencing Transcription factor), a tumor suppressor, in UL leads to aberrant activation of PI3K/AKT-mTOR pathway. We report here a critical link between estrogen receptor alpha (ERα) and the loss of REST, via PRICKLE1 (REST interacting LIM-domain protein - RILP). We found that PRICKLE1 is severely suppressed in UL, and that the suppression of PRICKLE1 significantly down regulates REST protein levels. Conversely, overexpression of PRICKLE1 resulted in the restoration of REST in leiomyoma smooth muscle cells. Crucially, mice exposed neonatally to environmental estrogens, proven risk factors for UL, expressed strikingly low levels of PRICKLE1 and REST in the myometrium. Using mice that lack endogenous estrogen (Lhb⁻/⁻ mice), or ERα (Esr1⁻/⁻ mice), we demonstrate that Prickle1 expression in the myometrium is suppressed by estrogen through ERα. Enhancer of zeste homolog 2 (EZH2) participates in the repression of specific ERα target genes. UL express increased levels of EZH2 that inversely correlate with the expression of PRICKLE1. Further, the down modulation of EZH2 expression, but not its histone methyltransferase activity, leads to restoration of PRICKLE1 in UL cells. Collectively, our results identify a novel link between estrogen exposure and PRICKLE1/REST-regulated tumorigenic pathways in UL.
Introduction

Uterine fibroids, also known as uterine leiomyomas (UL), are the most common tumors of the female reproductive tract. UL result from aberrant clonal expansion of smooth muscle cells in the myometrium and are a major health concern among women. It is estimated that the cumulative incidence of UL tumors by age 50 was greater than 80% for African American women and nearly 70% for Caucasian women. Over 25% of all women have clinical symptoms of pain, pressure, excessive bleeding, anemia and in acute cases, infertility (Baird, Dunson, Hill, Cousins, & Schectman, 2003; Cramer & Patel, 1990; Walker & Stewart, 2005). Currently, the leading treatment for UL is hysterectomy, which is not only invasive and risky, but also presents a substantial financial burden. UL accounted for up to $34.4 billion in medical costs in the United States in 2010 and continue to be the leading cause for hysterectomies (Cardozo et al., 2011). Despite the widespread need for a more efficacious treatment for UL, there is currently no approved pharmacotherapy treatment option for fibroids that is long term, cost effective and that leaves fertility intact. Remarkably, the molecular mechanisms that trigger the pathogenesis of UL are poorly understood.

Recent evidence has indicated the central role of the PI3K/AKT/mTOR signal transduction pathway leading to cell growth, proliferation and cell survival in UL, as well as in malignant tumors (J. D. Cook & Walker, 2004; Crabtree et al., 2009; Martini, De Santis, Braccini, Gulluni, & Hirsch, 2014). In Chapter 3, we reported that the activation of GPR10, an aberrantly expressed G-protein coupled receptor in UL by its ligand, PrRP, leads to activation of the PI3K/AKT-mTOR pathway (Varghese et al., 2013). Normally, GPR10 is repressed in non-neuronal tissues, including the myometrium, by its transcriptional silencer REST (Repressor Element Silencing Transcription factor/Neuron-Restrictive Silencing Factor) (Kemp et al., 2002;
Varghese et al., 2013). REST is a critical tumor suppressor that prevents neuronal gene expression in non-neuronal tissue by epigenetic regulation of target genes (Westbrook et al., 2005). We reported in Chapter 4 that the loss of REST protein in UL allows aberrant expression of GPR10, leading to cell growth and survival via the PI3K/AKT-mTOR signal transduction pathway. In addition to GPR10, we reported that many of the most aberrantly expressed genes in UL are direct targets of REST, indicating that the loss of REST has an important role in downstream epigenetic changes leading to UL development and growth (Chapter 4). Despite its emerging critical role in UL pathogenesis, the molecular mechanisms that promote the loss of REST in UL are unknown.

Pre-pubertal exposure to environmental estrogens including DES, BPA and genistein has been implicated as a primary risk factor for the development of UL later in the reproductive life of women (Baird & Newbold, 2005; J. D. Cook, Davis, Goewey, Berry, & Walker, 2007; Di et al., 2008; Jeong et al., 2013; Newbold, Jefferson, & Padilla-Banks, 2007; K. H. Wang, Kao, Chang, Lin, & Kuo, 2013). Studies in rodent models have indicated that long-lasting epigenetic modifications that accompany early exposure to estrogenic compounds may promote UL formation (J. D. Cook et al., 2007; Greathouse et al., 2008). Because REST is a major epigenetic regulator of long-term gene repression in the periphery, we hypothesized that environmental estrogens promote development of UL by causing a decrease in REST protein levels and thereby impairing its function in the uterus.

Altered expression or function of REST has been reported in disease states, such as colon cancer and small cell lung cancer, where the REST gene is mutated or deleted (Z. Huang & Bao, 2012; Wagoner et al., 2010). In differentiating neuronal progenitor cells, the degradation of REST is mediated by the E3 ubiquitin ligase, β-TRCP, which targets it for proteasomal
degradation (Chen et al., 1998; Schoenherr & Anderson, 1995; Weissman, 2008). In addition, the loss of REST by the ubiquitin-proteasome pathway has been reported in various cancers (Westbrook et al., 2008; Westbrook et al., 2005). Recently, the loss of REST expression by an unidentified contributing mechanism was shown to promote the pathogenesis of Alzheimer’s disease (Lu et al., 2014). Interestingly, in UL the REST mRNA level is unchanged in comparison to healthy myometrium, indicating that REST expression is regulated post-transcriptionally (Chapter 4, Figure 4.11). REST is also degraded at a faster rate in cultured UL cells compared to myometrial cells despite normal β-TRCP levels in both (Varghese et al., 2013)(Chapter 4, Figure 4.13 & Figure 4.14). Given the widespread loss of REST and the relatively normal levels of β-TRCP expression in UL, we hypothesized that alternative mechanisms exist for the functional loss of REST in UL cells.

In an effort to identify the mechanism of loss of REST in UL, we focused on PRICKLE1, a Wnt/PCP protein that associates with REST. Here we show that UL expressed significantly lower levels of PRICKLE1, and its expression mirrors that of REST. PRICKLE1, also known as RILP (REST-interacting LIM domain protein) regulates nuclear localization of REST (Shimojo & Hersh, 2003, 2006). Interestingly, the Prickle1 promoter contains estrogen response elements (ERE) that are directly bound by estrogen receptor alpha (ERα) in the mouse uterus (Hewitt et al., 2012), presenting a potential link to the well recognized role of environmental estrogens in the pathogenesis of UL. Using a series of in vitro and in vivo methods, we demonstrate here that PRICKLE1 is downregulated by environmental estrogens in the myometrium and that the loss of PRICKLE1 leads to the loss of REST in UL. Additionally, we identify a direct role for ERα and the polycomb repressive complex protein EZH2 (enhancer of zeste homolog 2) in the repression of PRICKLE1 in leiomyoma smooth muscle cells. Taken together, we identify a novel pathway
that connects environmental estrogen exposure, suppression of PRICKLE1, loss of REST
dependent epigenetic control, and aberrant gene expression in UL.

Materials and Methods

Chemicals and reagents.

Dulbecco’s Modified Eagle’s medium (DMEM; D5671), penicillin-streptomycin (17-602), and L-glutamine (17-605) were purchased from Biowhittaker (Walkersville, MD). Dulbecco’s PBS (SH30028), fetal bovine serum (FBS; SH30071) and bovine calf serum (BCS; SH30073) were purchased from Hyclone (Logan, UT). Anti-PRICKLE-1 (506-520) anti-rabbit polyclonal antibody (R3782), protease and phosphatase inhibitor cocktails (P0044) and anti-Flag M2 antibody (F1804) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-REST rabbit polyclonal (07-579) and anti-trimethyl-Histone H3 (Lys27) (07-449) antibodies were purchased from Millipore (Temecula, CA). Anti-actin (sc-47778) and donkey anti-goat IgG HRP conjugated antibody (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ethidium homodimer (E3599), Alexa Fluor conjugated secondary antibodies (ab150073), lipofectamine 2000 reagent (11668027), collagenase type II (17101-015), and high-capacity cDNA reverse transcription kit (4368814) were obtained from Life Technologies (Grand Island, NY). Anti-rabbit IgG HRP conjugate antibody (W401B) was purchased from Promega (Madison, WI). pGateway 3XFlag Prickle1 vector (24644) was purchased from Addgene (Cambridge, MA). TaqMan gene expression master mix (4304437) was purchased from Applied Biosystems (Foster City, CA). NE-PER nuclear extraction reagent (78833) and SuperSignal West Pico Chemiluminescent Substrate (34080) were purchased from Thermo Scientific (Rockford, IL). DRAQ5 (4084L), EZH2 (D2C9) XP Rabbit mAb, GAPDH pAb, were purchased from Cell Signaling (Danvers, MA) Taqman primer and probe sets, and PRICKLE-1 siRNAs
(HSC.RNAI.N001144881.12.2, HSC.RNAI.N001144881.12.4, and HSC.RNAI.N001144881.12.8) and EZH2 siRNAs (HSC.RMAI.N152988.12.2_2nm, HSC.RMAI.N152988.12.7_2nm, HSC.RMAI.N152988.12.8_2nm) were purchased from IDT (Coralville, IA). EPZ-6438 (A-1623) was purchased from Active Biochemicals (Wan Chai, Hong Kong). Gradient 4-15% mini-PROTEAN TGX gels (456-1086) were purchased from Bio-Rad (Hercules, CA). RNeasy Mini Kit (74104) was obtained from Qiagen (Valencia, CA). Antigen unmasking solution (H-3300) was purchased from Vector (Burlingame, CA).

**Tissue collection and cell culture.**

Leiomyoma tissue samples were obtained from hysterectomy specimen of pre-menopausal women at The University of Kansas Medical Center (Kansas City, KS). Patient criteria included women who had UL as the primary indication, without complicating reproductive tract diseases, and have not had hormone therapy in the three months before surgery. For paired tissue samples, the study was powered to measure changes in gene expression between the tumor and normal tissues. Using paired t-test, 11 pairs gave us 80% power to detect a difference of 0.81 (fold change of 1.75) standard deviation units or larger in gene expression with one-sided level of significance of 0.05. Smooth muscle cells (SMCs) were isolated from tissue by dicing the tissue into 2-3mm sections and digesting in DMEM containing 1.5mg/ml collagenase for 4-6 hours at 37 degrees. After digestion, cells were cultured in DMEM medium with 10% FBS and FCS, 1% L-glutamine, and 1% penicillin-streptomycin in an incubator at 95% humidity and 5% CO2. Cells were not passaged past p6 to maintain phenotype as close to *in vivo* as possible.

**Histology.**
Human and mice uterine tissue sections were fixed in 4% paraformaldehyde and paraffin embedded. Deparaffinization was with xylene and rehydration with graded alcohol. Samples were then boiled in 1% antigen unmasking buffer. Tissue sections were blocked in 5% normal goat serum and 2% BSA in PBS for 1 hour, then incubated with anti-PRICKLE-1 polyclonal antibody (1:100) or anti-REST polyclonal antibody (1:100) overnight at 4°C. Fluorescence was developed using Alexa Flour 488 conjugated secondary antibody (1:300). Nuclei were stained with Ethidium Homodimer (1:300) or DRAQ5 (1:500). An Olympus 1X71 inverted microscope and TE2000U 3 laser inverted confocal microscope were used for imaging.

**Protein isolation and western blotting.**

Leiomyoma and myometrial tissues were homogenized, and primary cells were lysed, in 1x cell lysis buffer containing 1% protease and phosphotase inhibitor cocktails. Supernatant was collected and the pellet was used for nuclear extraction using NE-PER nuclear extraction buffer. Cytoplasmic and nuclear extracts were combined and loaded with 6x Laemmli buffer on 4-15% gradient polyacrylamide SDS gels, run by electrophoresis at 70 volts at room temperature. Transfer to PVDF membrane was at 4°C for 1.5 hours. Membranes were blocked in 5% skim milk for 1 hour followed by an overnight incubation at 4°C with anti-prickle-1 (1:6000), anti-REST (1:2000), anti-FLAG (1:1000), anti-EZH2 (1:1000), anti-H3K27me3 (1:1000), anti-GAPDH (1:1000) or anti-actin (7.5:10000) antibodies. Secondary antibody incubation was with IgG HRP conjugated antibodies (1: 5000) at room temp for 1 hour. Signal was developed with SuperSignal West Pico Chemiluminescent Substrate. ImageJ software from the National Institutes of Health was used for densitometric analysis.

**Cell transfections.**
Primary leiomyoma or myometrial cells were cultured to 70% confluence in 6-well plates and incubated overnight in antibiotic-free DMEM medium before transfection with 25nM *PRICKLE-1* siRNAs (HSC.RNAI.N001144881.12.2, HSC.RNAI.N001144881.12.4, and HSC.RNAI.N001144881.12.8; IDT) or *EZH2* siRNA (HSC.RMAI.N152988.12.2_2nm, HSC.RMAI.N152988.12.7_2nm, HSC.RMAI.N152988.12.8_2nm) or 4ug of pGateway 3XFlag *PRICKLE-1* vector using Lipofectamine 2000. siRNA or vector containing medium was removed 24 hours after transfection and cells were harvested 24 hours later.

**RNA isolation and real time qPCR.**

RNA was extracted using Qiagen RNeasy kit. cDNA was prepared with the High Capacity cDNA Reverse Transcription kit using 1 µg RNA. Probes for total *PRICKLE-1* (Hs.PT.49a.20111671), *REST* (Hs.PT.47.3525906: IDT), and *EZH2* (Hs.PT.58.1924301) were used in combination with Taqman Gene Expression Master Mix. Four experimental replicates were performed for each sample. An ABI 7900HT sequence detection system was used for real time PCR amplification. The comparative CT method ((ΔΔCt) was applied for data analysis. Relative fold differences in gene expressions were normalized to 18S (Hs.PT.49a.3175696.g; IDT) expression as internal control.

**EZH2 inhibitor treatment.**

Primary myometrial cells were cultured to 70% confluence in 6-well plates in DMEM medium with serum, followed by incubation with EZH2 inhibitor, EPZ-6438, in DMSO at 2µM final concentration or DMSO control for 72 hours. Following incubation, cells were washed in PBS and protein was isolated for western blot analysis.

**Global Run-On Sequencing.**
Global Run-On Sequencing Assays were performed in collaboration with the lab of Lee Kraus of the University of Texas Southwestern. Day 22 immature mice (6-7 mice per biological replicate) were treated with either vehicle (ethanol/corn oil mixed at 1:9 ratio) or estradiol (20μg/ml in the vehicle, 5μl/g mouse weight) for 40 min. Mice were euthanized, uteri collected on ice and GRO-Seq from isolated nuclei was performed as reported earlier (Hah et al., 2011).

Briefly, nuclei were mixed for 5 minutes at 30°C with equal volume reaction buffer containing 10mM Tris-Cl, 5mM MgCl2, 1mM DTT, 300mM KCl, 1% sarkosyl, 500μM ATP, GTP and Br-UTP, 2μM CTP and α-32P-CTP. Next 23μl of DNAsel buffer and 10μl DNase were added. Next, protein digestion was performed using a buffer containing Tris-Cl, SDS, EDTA and Proteinase K for 1 hour. RNA extraction was with phenol:chloroform, followed by base hydrolysis, immuno-purificatoin of Br-U RNA, end repair and adapter ligation protocols. Next, the RNA was reverse transcribed and amplified, followed by PAGE purification of NRO-RNA libraries. Samples were sequenced on Illumina 1G Genome Analyzer for alignment of GRO-seq reads to the human genome. The Kraus lab of UT Southwestern provided the GRO-seq browser tracks using their unpublished data.

Animals.

Animals were handled according to National Institutes of Health National Institute of Environmental Health Sciences guidelines under approved animal care and use protocols.

Female CD-1 mice were injected subcutaneously on PND1–PND5 with corn oil (control) or Genistein (50 mg/kg/day; Sigma, St. Louis, MO), followed by isolation of uteri and fixation in 4% paraformaldehyde. (Jefferson et al., 2009). Sections of uteri were mounted to slides and processed as described in histology section.
Esr1-/- mice (B6.129P2-Esr1tm1Ksk/J) were purchased from The Jackson Laboratory. Uteri of 3-4 month old female Esr1-/- mice were isolated and processed for histology.

Lhb-/- mice were generated and genotyped as described previously (Ma, Dong, Matzuk, & Kumar, 2004). Adult female Lhb-/- or littermate control mice were treated with 5µg/kg estradiol or placebo time release pellets for 7 days before sacking and uteri collection.

Results

PRICKLE1 is aberrantly expressed in uterine leiomyoma in association with REST and REST target genes.

In a candidate gene approach to understand the molecular basis for the loss of REST in UL, we investigated the status of REST-associated genes in UL using gene expression profiling data available from the GEO dataset (GSE13319). Ingenuity™ Pathway Analysis (IPA) showed that many of the most significantly overexpressed genes in UL, such as GRIA2, GRIN2A, DCX, STMN2, NEFH, GPR10, SCG2 and SALL1 are direct targets of REST mediated long-term repression (Figure 5.1). Conversely, PRICKLE1 and HB-EGF, two of the REST linked genes with putative roles in the regulation of REST, were down regulated in UL (Figure 5.1). Gene expression analysis in replicates of matched myometrial and leiomyoma samples from patients confirmed the low expression of PRICKLE1 in ULs compared to myometrial tissue (Figure 5.2 & Figure 5.5). In addition, PRICKLE1 protein levels were also significantly reduced in ULs as indicated by immunoblot (Figure 5.3) and immunofluorescence (Figure 5.4) analyses. Interestingly, lower levels of PRICKLE1 also mirror the low REST levels in UL compared to myometrial tissue (Figure 5.3). Because PRICKLE1 associates directly with REST and is essential for REST function and nuclear localization, we explored the role of PRICKLE1 as a candidate for the regulation of REST and for the pathogenesis of UL. Importantly, while normal
myometrial smooth muscle tissue sections show robust expression and nuclear localization of REST, the traces of REST remaining in UL samples have a predominantly cytoplasmic localization (Chapter 4, Figure 4. 7), suggesting that PRICKLE1-mediated nuclear localization of REST may be dysfunctional in UL. Furthermore, ectopically expressed REST-GFP fusion protein was improperly localized to the cytoplasm in leiomyoma SMCs (Chapter 4, Figure 4. 7), suggesting that the lower PRICKLE1 expression occurring in tumor cells may play a role in REST localization and function. The coupled loss of both PRICKLE1 and REST and the improper localization of REST in UL cells suggest a direct role for PRICKLE1 in the loss of REST in UL.
Figure 5. 1: Gene expression of PRICKLE1, in association with REST, and REST-target genes in uterine leiomyoma. Analysis of REST associated genes in uterine leiomyoma tissues using gene expression profiling dataset GSE13319.
Figure 5. 2: *PRICKLE1* gene expression analysis. PRICKLE1 gene expression in uterine leiomyoma and myometrial patient samples.
Figure 5.3: **Immunoblot analysis of PRICKLE1 and REST expression in uterine leiomyoma and myometrial tissues.** Protein expression of PRICKLE1 and REST in paired patient samples (N = 4). β-Actin was used as loading control. Statistical analysis by paired T-test; Error bars represent SD; * $P < 0.05$. 

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Relative Expression

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* $P < 0.05$. 
Figure 5.4: **Immunofluorescence analysis of PRICKLE1 expression in myometrial and leiomyoma tissue samples.** Representative histological sections of paired patient leiomyoma tumor and adjacent myometrium (N = 4) probed for PRICKLE1 (green). Nuclei were stained with EthD-2 (red). Magnification bars represent 50µm.
Figure 5.5: Real Time qRT-PCR analysis of PRICKLE-1 mRNA expression in uterine leiomyomas. Gene expression analysis of *PRICKLE1* in paired patient myometrium and leiomyoma tissues (N = 8). Error bars represent SD; statistical significance by paired T-test *$P < 0.05$*.
PRIKLE-1 regulates REST expression in uterine leiomyoma at the protein level.

To elucidate the role of PRIKLE1 in REST stability and to establish a molecular mechanism for their concomitant loss in UL, we tested whether varying PRIKLE1 expression levels altered REST protein levels. We silenced PRIKLE1 expression in cultured primary myometrial smooth muscle cells (MSMC) using siRNA transfection and probed for REST expression (Figure 5. 6). PRIKLE1 knockdown caused a corresponding decrease in REST protein levels. Furthermore, PRIKLE1 overexpression in cultured primary leiomyoma smooth muscle cells (LSMC) caused an increase in REST protein levels (Figure 5. 6). To confirm that REST regulation by PRIKLE1 occurs at the post-transcriptional level, we further analyzed PRIKLE1 vector transfected LSMCs by quantitative PCR (Figure 5. 7). As expected, PRIKLE1 mRNA expression was significantly increased in transfected cells, but there was no change in REST mRNA, indicating that REST protein stability rather than mRNA expression was affected. These data suggest that PRIKLE1 functions to stabilize REST in the myometrium, and that loss of PRIKLE1 leads to the destabilization and degradation of REST in UL.
Figure 5. 6: **Western blot analysis of PRICKLE1 regulation of REST expression in uterine leiomyomas.** (A) Immunoblot analysis of REST expression upon *PRICKLE1* silencing in myometrial smooth muscle cells (N = 3). β-Actin was used as loading control. (B) Analysis of REST expression in leiomyoma smooth muscle cells overexpressing FLAG-PRICKLE1 (N = 3). GAPDH was used as loading control.
Figure 5.7: *PRICKLE1* and *REST* gene expression in cells transfected with FLAG-*PRICKLE1* vector. Real time qRTPCR analysis of *PRICKLE-1* and *REST* mRNA in leiomyoma smooth muscle cells overexpressing FLAG-PRICKLE-1 (N = 5). Error bars represent SD. Statistical analysis by paired T-test. *P < 0.05
**Estrogen down regulates PRICKLE1 expression in vivo.**

Pre-pubertal exposure to estrogenic chemicals has been proposed as a major risk factor for UL (Baird & Newbold, 2005; Di et al., 2008; Moore et al., 2007; Newbold et al., 2007). In an effort to determine the sensitivity of the PRICKLE1-REST pathway to estrogen exposure in vivo, we examined mice treated on postnatal days (PND) 1 - 5 with genistein, a common source of dietary phytoestrogen. Immunofluorescent staining for REST in PND5 uteri from genistein-treated and vehicle treated control mice showed that mice exposed neonatally to genistein had significantly reduced levels of REST expression (Figure 5.8). Further, immunostaining for PRICKLE1 in these tissues showed markedly lower expression of PRICKLE1 in the myometrium (Figure 5.8), suggesting that early exposure to estrogenic chemicals may have significant effects on PRICKLE1 – REST mediated epigenetic control in the uterus.

To test the putative direct role of endogenous estradiol in the regulation of PRICKLE1 expression in the mouse uterus we used luteinizing hormone beta-null (Lhb/-) mice that have extremely low estrogen levels (Nagaraja, Agno, Kumar, & Matzuk, 2008). PRICKLE1 expression in the myometrium of uteri from Lhb/- mice was remarkably higher than in WT controls (Figure 5.9). Importantly, Lhb/- mice treated with estradiol revert PRICKLE1 levels to the significantly lower wild-type level (Figure 5.9), confirming a substantial down-regulation of PRICKLE1 expression by estrogen.
Figure 5.8: **Histological staining of PRICKLE1 protein in mouse uteri treated with environmental estrogen.** Immunofluorescence analysis of PRICKLE1 (green) and REST (green) expression in vehicle (A & C) and genistein-treated (B & D) mouse myometrium. Nuclei were stained with EthD-2 (red) or DRAQ5 (blue). Magnification bars represent 50µm. M: myometrium.
Figure 5.9: **Histological analysis of PRICKLE1 expression in \( Lhb \) null mouse uteri.**

Immunofluorescence analysis of PRICKLE1 (green) in \( Lhb \) null mouse myometrium treated with or without estradiol. Nuclei were stained with DRAQ5 (blue).
**Estrogen receptor α suppresses PRICKLE1 expression in the myometrium.**

A recent ChIP-sequencing study showed that in the adult mouse uterus, the Prickle1 promoter is among the sequences associated with ERα, making PRICKLE1 a potential direct link between estrogens and downstream tumorigenic pathways in the myometrium (Hewitt et al., 2012). To distinguish between the possible mechanisms of estrogen regulation of PRICKLE-1, we examined Esr1-null mouse myometrium using fluorescent immunochemistry. In the absence of Esr1, PRICKLE1 expression was significantly increased (Figure 5. 10) despite very high circulating estrogen levels in these mice (Couse et al., 1995), indicating that ERα mediates estrogen-induced repression of PRICKLE1.

In order to confirm that estrogen down regulates PRICKLE1 transcription directly through ERα, we used ChIP-sequencing data from mouse uteri (Hewitt et al., 2012) in combination with genomic run-on-sequencing (GRO-Seq) of estrogen regulated gene transcription in the mouse uteri (Gro-seq browser tracks provided by the Kraus lab of the University of Texas Southwestern). It is evident from the ChIP-sequencing data that ERα binds to upstream target sequences within Prickle1 promoter in the ovariectomized mouse uteri within 60 min after estrogen treatment (peaks marked by arrowheads in browser track B, Figure 5. 11). Most importantly, the rate of Prickle1 transcription in the mouse uteri, measured by the frequency of nascent transcript sequences detected in isolated nuclei by GRO-Seq, was significantly and rapidly reduced upon estrogen treatment of immature mice (Figure 5. 11). Thus, estrogen treatment results in the recruitment of ERα to Prickle1 promoter and direct suppression of Prickle1 transcription.
Figure 5. 10: **Immunofluorescence analysis of PRICKLE1 expression in Esr1 null mouse uteri.** Expression of PRICKLE1 in representative WT and Esr1 null mouse myometrium (N = 4). Nuclei were stained with EthD-2 (red). Magnification bars represent 50µm. M: myometrium.
Figure 5.11: **Gro-seq at the PRICKLE1 promoter.** ChIP-sequecing data depicting ERα binding sites in ovariectomized mouse uteri treated with vehicle (A) or estrogen (B). (C). Gro-seq data indicating rate of transcription of *PRICKLE-1* in vehicle (C) or estrogen (D) treated mice after 1 hour.
**Enhancer of zeste homolog 2 (EZH2) participates in the repression of PRICKLE1.**

EZH2, the histone methyltransferase subunit of the polycomb repressor complex, binds to PRICKLE1 promoter and suppresses its expression (Cheng et al., 2011). It is also known that EZH2 assists in the repression of ER target genes through association with REA (repressor of estrogen receptor activity; Phb2) an estrogen receptor co-repressor (Hwang et al., 2008). We analyzed the expression status and a potential role that EZH2 may have in the repression of PRICKLE1 in UL. Analysis of GEO dataset GSE: 13319 showed that EZH2 mRNA levels were significantly increased in UL samples compared to normal myometrium, showing an inverse correlation with PRICKLE1 expression (Figure 5.12). TaqMan qRT-PCR analysis of matched human myometrium and UL samples confirmed the increased expression of EZH2 (Figure 5.13). Further, western blotting analysis of patient samples showed that EZH2 protein expression was higher in leiomyoma samples compared to patient matched normal myometrium with a consistent inverse relation to PRICKLE1 expression (Figure 5.14 & Figure 5.15). Furthermore, siRNA knockdown of EZH2 in cultured primary leiomyoma SMCs resulted in the increase of PRICKLE1 expression (Figure 5.16), showing that EZH2 overexpressed in UL, may play a role in the repression of PRICKLE1.

Intriguingly, the inhibition of histone methyltransferase activity, indicated by the decrease in trimethylated lysine 27 of histone H3, by treatment with EPZ-6438, did not change the level of PRICKLE1 expression in cultured leiomyoma SMCs (Figure 5.17). Thus, an unknown mechanism may be present in UL that result in the suppression of PRICKLE1 by EZH2.
Figure 5. 12: Gene network analysis of *PRICKLE1* associated genes in uterine leiomyoma.

Analysis of GEO dataset GSE 13319.
Figure 5.13: Gene expression analysis of **EZH2 in paired patient samples**. Real time qRT-PCR analysis of **EZH2** mRNA in leiomyoma and myometrial tissues (N = 14). Error bars represent SE. * $P < 0.05$
Figure 5. 14: **Immunoblot analysis of EZH2 and PRICKLE1 in matched human leiomyoma and myometrial tissues.** (A) Western Blot analysis of EZH2 and PRICKLE1 protein in paired patient samples (N = 4). GAPDH was used as a loading control. (B) Densitometry analysis of western blot. Error bars represent SE, paired T-test gives significance of * $P < 0.05$
Figure 5.15: **EZH2 protein expression in additional paired patient samples.** Immunoblot analysis of EZH2 in paired patient myometrium and leiomyoma tissues (N = 6). GAPDH was used as loading control.
Figure 5.16: **Immunoblot analysis of EZH2 and PRICKLE1 knockdown in myometrial smooth muscle cells.** Representative western blot analysis of PRICKLE1 and EZH2 expression in EZH2 and PRICKLE1-silenced ULSMCs respectively (N = 3). GAPDH was used as a loading control.
Figure 5. 17: Western blot analysis of leiomyoma cells treated with EZH2 inhibitor.

Immunoblot analysis of PRICKLE1 and H3K27me3 in leiomyoma smooth muscle cells upon inhibition of methyltransferase activity of EZH2 by EPZ-6438 (N = 3). GAPDH was used as loading control.
Discussion

The pathogenesis of UL is poorly understood despite the widespread occurrence (Cramer & Patel, 1990), significant morbidity (Walker & Stewart, 2005), and the ever expanding medical cost (Cardozo et al., 2011) associated with the disease. While near ubiquitous existence of specific somatic missense DNA mutations (Makinen et al., 2011; McGuire et al., 2012) or chromosomal translocations (van Rijk et al., 2009) have been reported in UL, the mechanisms that initiate those events in the relatively quiescent myometrial smooth muscle tissue have not yet been identified. We recently reported that the loss of tumor suppressor REST in uterine myometrial smooth muscle cells leads to aberrant gene expression and promotes the pathogenesis of UL (Varghese et al., 2013). REST, via direct binding to the RE-1 sequence elements in upstream regulatory regions is known to control the long-term silencing of about 2000 genes by epigenetic mechanisms (Abrajano et al., 2010; Mortazavi, Leeper Thompson, Garcia, Myers, & Wold, 2006). The loss of REST by b-TRCP mediated ubiquitination and proteasomal degradation is suggested to result in PI3K/AKT dependent tumor cell proliferation and cell survival (Westbrook et al., 2008; Westbrook et al., 2005). Intriguingly, the loss of REST in UL was not associated with enhanced b-TRCP mediated ubiquitin-proteasomal degradation of REST (Varghese et al., 2013), suggesting a novel mechanism may exist for its loss in UL.

In a candidate gene approach to identify the mechanism for the loss of REST in UL, we examined the regulation of REST related genes in UL. Interestingly, while many of the REST target genes encode the most upregulated mRNAs in UL, PRICKLE1, an interacting partner of REST involved in its nuclear localization (Shimojo & Hersh, 2003), was significantly down regulated in UL (Figure 5. 2 & Figure 5. 3). Further, the remarkable decrease of PRICKLE1
expression in patient samples mirrored the status of REST expression in leiomyomas (Figure 5. 3), suggesting that the loss of these protein partners may be linked. Importantly, we had reported earlier that, in leiomyoma SMCs transfected with a GFP-REST construct, the GFP-tagged REST protein was unstable and had failed to localize to the nucleus, indicating that the nuclear localization of REST was specifically affected in UL (Varghese et al., 2013). An inherited homozygous mutation on PRICKLE1 that disrupts its interaction with REST was reported to cause progressive myoclonus epilepsy (PME) with symptoms of neurological decline, including ataxia and dementia (Bassuk et al., 2008). PRICKLE1, also known as the REST interacting LIM domain protein (RILP) is a key regulator of the noncanonical Wnt/PCP pathway (Yang, Bassuk, & Fritsch, 2013). Prickle1 plays a crucial role during mouse embryo development by regulating the expression of Vangl2, BMP4, Fgf8 and Wnt5a (Yang et al., 2013). Function blocking mutation to Prickle1 (C251X/C251X) in mice results in major phenotypic changes in tissues of mesenchymal lineage (Yang et al., 2013). Most relevant to the well-recognized link between environmental estrogen exposure and the pathogenesis of UL, a recent ChIP-sequencing study revealed that estrogen receptor alpha (Esr1) was associated with Prickle1 promoter in the mouse uterus (Hewitt et al., 2012).

We hypothesized that the loss of PRICKLE1 expression in leiomyoma SMCs is mechanistically linked to the loss of REST and smooth muscle tumor development in the uterus. We observed that upon siRNA-mediated knockdown of PRICKLE1 in cultured primary myometrial SMCs, REST expression was correspondingly reduced (Figure 5. 6A), indicating that the low PRICKLE1 levels in UL may indeed trigger the decline in REST stability and function. Conversely, overexpression of Flag-tagged PRICKLE1 in cultured primary leiomyoma SMCs led to a matching restoration of REST expression (Figure 5. 6B) without altering REST
mRNA expression, confirming that the loss of PRICKLE1 expression in UL is linked to the destabilization of REST. Given the function of PRICKLE1 in the nuclear localization of REST, these results further support our observation that GFP-REST failed to localize to the nuclei of primary leiomyoma SMCs upon transfection (Chapter 4). The loss of normal REST function in various pathological states has thus far been linked to genetic mutations or to β-TRCP mediated degradation (Coulson, 2005; Z. Huang & Bao, 2012; Qureshi & Mehler, 2009). Also, while the loss of REST function has been linked recently to the development of Alzheimer’s disease, a consistent mechanism for the loss of REST has not been identified (Lu et al., 2014; Orta-Salazar et al., 2014). The widespread destabilization and loss of REST in the absence of PRICKLE1 expression seen here in UL is entirely novel, and may provide future therapeutic approaches for restoring REST function.

Somatic missense mutations to the mediator complex protein MED12 have been reported in ULs (Makinen et al., 2011). MED12 is known to interact with REST through G9a, a methyl transferase responsible for repressive H3K9 mono- and di-methylation (Ding et al., 2008). Additionally, a Med12 hypomorphic mutation in mouse embryos results in asymmetric distribution of Prickle1 and disruption of the Wnt/PCP pathway (Rocha et al., 2010). How the missense mutations to MED12 reported in ULs affect PRICKLE1 and the Wnt/PCP pathway, in addition to REST localization and function are currently unknown.

Pre-pubertal exposure to environmental or dietary phyto-estrogens such as DES, BPA, and genistein has been suggested to predispose women to the development of UL (Baird & Newbold, 2005; Di et al., 2008; Moore et al., 2007; Shen et al., 2014; K. H. Wang et al., 2013). Also, the long-term effects of such endocrine disruptors in the uterus suggest the involvement of epigenetic gene regulatory mechanisms. Since REST is a major epigenetic regulator of gene
expression, we investigated the impact of neonatal estrogen exposure on PRICKLE1-REST pathway. Our results indicated that the expression of PRICKLE1 and REST in the mouse uterus is negatively regulated by genistein (Figure 5.8). Further, in the absence of endogenous estrogen in Lhb-/- mice, Prickle1 expression was significantly higher in the myometrium compared to that in the wild type or in estrogen treated mice (Figure 5.9). Additionally, in Esr1 knockout mouse model, the expression of Prickle1 was significantly higher than in the WT uterus (Figure 5.10). The most compelling evidence that ERα binds to the promoter and suppresses Prickle1 expression rapidly in response to estradiol treatment comes from our analysis of available ERα ChIP-sequencing data ((Hewitt et al., 2012)) in combination with the GRO-Sequencing study for the mouse uterus (Figure 5.11). The GRO-sequencing data indicate that the rate of transcription from the Prickle1 locus decreases significantly within 40 minutes of estradiol treatment of immature mice, coinciding with the recruitment of ERα, obtained from the ChIP-sequencing study.

Enhancer of Zeste Homolog 2 (EZH2), a polycomb group histone methyltransferase, is known to regulate the expression of estrogen responsive genes in breast and prostate cancer cells via its association with the repressor of estrogen activity (REA), an estrogen receptor co-repressor (Hwang et al., 2008). Additionally, EZH2 has been shown to directly bind to the promoter and to negatively regulate the expression of PRICKLE1 in hepatocellular carcinoma cells (Cheng et al., 2011), evoking the possibility for both EZH2 and estrogen receptor alpha together playing a role in the suppression of PRICKLE1 in leiomyoma. Intriguingly, based on studies using ELT3 cells, it has also been suggested that the down regulation of EZH2 by environmental estrogens via non-genomic pathways promotes the pathogenesis of UL through aberrant epigenetic changes in the myometrium (Walker, 2011), though no patient data to
indicate the suppression of EZH2 in UL was presented. In contrast to this hypothesis, our results indicated that EZH2 expression is significantly upregulated in UL samples both at mRNA and protein levels and this upregulation was inversely correlated with the expression of PRICKLE1 and REST (Figure 5. 14 & Figure 5. 15). Most importantly, the knockdown of EZH2 using siRNA, but not the inhibition of its histone methyl transferase activity, resulted in the restoration of PRICKLE1 in leiomyoma cells (Figure 5. 16 & Figure 5. 17). Our results indicate that ERα - EZH2 mediated regulation of PRICKLE1 suppression may not involve changes in H3K27 methylation in its promoter. Our results support a novel ERα/ EZH2 – PRICKLE1 – REST mediated target-specific epigenetic disruption model rather than an estrogen - mediated suppression of EZH2 and the ensuing global epigenetic disarray in UL.

Estrogen receptor alpha is known to positively regulate a vast majority of its target genes in the uterus, contributing to the uterotrophic effect of estrogen. Several selective estrogen receptor modulators (SERMs) have been investigated as potential treatment for UL with mixed to moderate success (Dhingra, 1999). The SERMs inhibit the effect of estrogen in the uterus by promoting the recruitment of co-repressors to the estrogen receptor bound transcriptional targets. Since PRICKLE1 seems to be negatively regulated by estradiol and environmental estrogens, it may be crucial to test in the future how PRICKLE1, REST, and the epigenetic status of downstream REST – target genes is affected by novel SERMs. Our findings on the role of PRICKLE1 and environmental factors on the stability of REST may have wider implications in cancer and other conditions such as Alzheimer’s disease where loss of REST has been reported. Taken together, our results provide a novel mechanism for the loss of REST and identify potential targets for the development of treatment for UL in the future.
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Chapter 6

Summary and Future Directions
The overall goal of my dissertation was to understand the molecular connection between known environmental estrogenic risk factors and the activation of one of the most dysregulated tumorigenic pathway, PI3K/AKT-mTOR, in the pathogenesis of uterine leiomyoma (UL). I hypothesized that: a) GPR10 overexpression in UL activates the PI3K/AKT-mTOR pathway, b) loss of REST in UL leads to epigenetic reprogramming in the uterus, including de-repression of GPR10, c) REST degradation is a result of PRICKLE1 loss in UL, d) PRICKLE-1 expression is sensitive to environmental estrogen signaling through ERα and EZH2 in the uterus and e) mice expressing hGPR10 in the myometrium, and a conditional knock-out of REST in the reproductive tract will show UL phenotype and serve as important preclinical animal models for the pathogenesis of UL.

Using *in vitro* model systems of primary cultured UL and myometrial smooth muscle cells, as well as *in vivo* mouse models, I was able to confirm all of the above hypotheses. In summary, the main findings of my research studies are:

1. The G-protein coupled receptor protein, GPR10, is aberrantly overexpressed in UL. Normal expression of GPR10 is limited to neurons of the central nervous system. Expression of GPR10 in the periphery has not been reported previously. In Chapter 3, we show significant expression of GPR10 protein and RNA in UL in the majority of patient samples.

2. Activation of GPR10 by its peptide ligand, PrRP, leads to an increase in PI3K/AKT-mTOR signaling, increased mobilization of intracellular calcium, and increased cell proliferation specifically in primary cultured leiomyoma smooth muscle cells.

3. Transgenic overexpression of GPR10 in the mouse myometrium leads to a leiomyoma phenotype. Gene expression profiles of the transgenic *CaBP9K-hGPR10* mice mirror
human UL gene expression, specifically with significantly higher levels of *Col1A1, Col3A1, Tgfb3 and Acta2* and lower level of *Dpt* compared to WT mice. Histological studies demonstrate morphological similarity to human UL tissue, and evidence of increased collagen deposition, a hallmark of UL.

4. Upstream of GPR10, protein levels of the epigenetic silencer REST are drastically decreased in UL, while its mRNA levels were unchanged. This loss of REST is a key event in UL pathogenesis, as many of the most aberrantly expressed genes in UL, including GPR10, are REST targets.

5. In cultured primary uterine smooth muscle cells, REST knockdown leads to increased expression of GPR10 RNA transcripts and GPR10 protein, as well as functional activation of PI3K/AKT-mTOR pathway proteins. In addition, gene expression profiling revealed several REST target genes with important roles in connective tissue are also increased after REST knockdown.

6. Conditional knock-out of REST in the mouse uterus leads to UL phenotype, including increased myometrial thickness, increased GPR10 expression, and increased activation of PI3K/AKT-mTOR pathway proteins.

7. PRICKLE1, the protein required for REST nuclear translocation, is significantly reduced in UL and its expression mirrors low expression of REST in UL.

8. PRICKLE1 regulates the protein expression of REST in UL, as evidenced by knockdown of PRICKLE1 in primary cultured myometrial cells resulting in dramatic reduction of REST at the protein, but not mRNA level. Moreover, overexpression of PRICKLE1 in cultured leiomyoma smooth muscle cells resulted in increased REST protein levels.
9. Early exposure to estrogenic compounds has significant effects on PRICKLE1 and REST regulation in the uterus. We found mice treated neonatally with the phytoestrogen, genistein, had significantly reduced levels of Prickle1 and Rest. Furthermore, Prickle1 expression in the myometrium of Lhb-/- and Esr1-/- mice was considerably higher than in WT controls and subsequent treatment with estradiol rescued Prickle1 expression to WT levels in the Lhb-/- mice, confirming down-regulation of Prickle1 by estrogen.

10. Estrogen treatment results in the recruitment of ERα to the Prickle1 promoter and direct suppression of Prickle1 transcription. Using ChIP-sequencing data from mouse uteri, in combination with genomic run-on sequencing (GRO-Seq) in ovariectomized mice treated with estrogen for 60 minutes we found Prickle1 transcription was rapidly reduced upon ERα binding of the Prickle1 promoter.

11. Enhancer of Zeste homolog 2 (EZH2) participates in the suppression of PRICKLE1 in UL. We found expression of EZH2 is considerably increased in UL tissue, and knockdown of EZH2 in cultured primary UL cells resulted in the increase of PRICKE1 expression. However, the histone methyltransferase activity of EZH2 is not required for regulation of PRICKLE1, as evidenced by unchanged PRICKLE1 expression levels after treatment of cultured UL cells with an inhibitor of EZH2 enzymatic activity.
Figure 6.1: Schematic showing the regulation of *PRICKLE1* by ERα and regulation of REST by PRICKLE-1 in myometrium and leiomyoma.
This work demonstrates for the first time a molecular pathway linking environmental estrogenic exposure to the activation of downstream tumorigenic pathways in UL pathogenesis. In addition, this novel pathway provides therapeutic targets for development of drug treatment options for UL, as well as preclinical mouse models for genes aberrantly expressed in UL. This work increases our understanding of the etiology of UL and reveals several areas of exciting future study on the pathogenesis of UL. The following are potential questions and topics related to this research that will be of future interest to the UL field:

**GPR10 as a novel drug target for improved pharmacotherapy for UL.**

Currently, there is no available drug therapy option for treatment of UL that is long-term, cost-effective and leaves fertility intact. Most drug therapies are used either short-term to reduce tumor size and bleeding preceding surgical myomectomy or hysterectomy, or are prescribed to treat the symptoms of UL, but not the underlying etiology of the disease. Current drug treatment options include Gonadotropin-releasing hormone (Gn-RH) agonists, synthetic progestin, selective estrogen receptor modulators, aromatase inhibitors and antifibrotics (Vercellini et al., 1998; Walker, 2002). These drug treatments have mixed results on tumor-size and bleeding, but cannot be used long-term due to potential for bone-resorption, loss of fertility and other menopause like side effects due to the patient entering a hypo-estrogenic state. In addition, UL tumors tend to return to pre-treatment size after therapy is stopped.

Our results demonstrate the important role of GPR10 in the pathogenesis of UL (Chapter 3). The natural peptide activator of GPR10 is a 21 amino acid peptide called PrRP. Development of a peptidomimetic or a small molecule antagonist which would bind GPR10 and inhibit binding of PrRP or disrupt downstream molecular signaling
pathways of GPR10 would be an interesting avenue for treatment of UL. GPR10 as a potential drug target is especially exciting considering that GPR10 is a G-protein coupled receptor, which serve as targets for a significant number of drugs on the market today. In addition, the natural expression of GPR10 is limited to areas of the brain. Therefore, development of a peptidomimetic / small molecule which displays proper polar surface area to prevent passage across the blood-brain-barrier would preclude potential side effects of the drug.

The early drug discovery process includes small molecule hit identification from HTS, followed by specificity assays in cell lines, and efficiency testing in animal models. Although not a part of the thesis, an HTS Ready (Millipore) cell based assay using intracellular calcium as the readout for GPR10 activation was optimized and used in the laboratory. This dissertation work not only identifies and validates GPR10 as a promising drug target, but also provides two pre-clinical mouse models for testing of a potential drug. Our CaBP9K-hGPR10 transgenic mouse and conditional REST knockout mouse show UL phenotype, which will make them useful in development of an effective novel drug for UL.

**Further characterization of CaBP9K-hGPR10 transgenic mice.**

Data presented in this dissertation indicate the CaBP9K-hGPR10 transgenic mouse phenotype mirrors human UL. Further characterization of this novel animal model will explore the complete functional result of overexpression of hGPR10 in the uterus. Based on our *in vitro* data and preliminary results from the CaBP9K-hGPR10 transgenic mouse model, we expect many of the aberrantly expressed genes of human UL to also be dysregulated in our mouse model, although species specific differences may be expected in global gene expression profiles. It will be of interest to explore the effect of exogenous PrRP injection on PI3K/AKT-mTOR pathway activation in CaBP9K-hGPR10 mice. Although PrRP is present in the bloodstream as a
circulating peptide ligand (Lin, 2008), acute injection of exogenous PrRP may lead to hyper-activation of GPR10, resulting in robust activation of the PI3K/AKT-mTOR pathway.

Finally, UL have been indicated as a major factor in up to 10% of infertility cases, and have been suggested to hinder endometrial receptivity for implantation during pregnancy (Makker & Goel, 2013). Although UL typically do not become symptomatic during early adulthood, infertility due to UL is becoming more prominent as women delay child-bearing to later in life (Balasch & Gratacos, 2011; Gambadauro, 2012). Considering the myometrial-specific expression of GPR10 presents a UL phenotype in vivo, reduced fertility in these mice may an important factor to consider. Furthermore, the CaBPD9K promoter is activated by endogenous estrogen during puberty in the GPR10 mice (Romagnolo et al., 1996), therefore, a possible protective effect of parity on UL incidence for mice mated young at 2 months of age could also be investigated.

**Further characterization of Rest cKO mice**

Work presented here has shown the phenotype of the Rest cKO mice reflect the human condition, providing valuable knowledge about the etiology of UL and contributing significant advancement to the field of UL study. Complete characterization of the Rest cKO mice will be necessary to confirm the UL phenotype. Histological evaluation should include H&E and Masson’s Trichrome staining to identify Rest cKO tissue organization and ECM deposition. Considering the prominent role of estrogen in the PRICKLE1-REST pathway we have elucidated in our novel molecular pathway, as well as the high risk of UL development following prepubertal environmental estrogen exposure (D’Aloisio et al., 2012), it will be important to explore the acute effect of estrogen treatment of the Rest cKO mice. Ovariectomy of the Rest cKO mice, followed by estradiol, progesterone, and combinations of steroids at various time
points should be performed in the future to determine the role of steroid hormones in this mouse model. Similarly, it would be valuable to cross the Rest cKO mice with Esr1-/- or Lhb-/- mice to study the effect of low E2 or ERα function on the myometrium.

**Myometrial specific cre recombinase for Rest cKO mice.**

To produce our Rest cKO mice, homozygous Rest floxed mice were crossed with Amhr2+/-cre mice to create a loss of Rest expression specific to the female reproductive tract. In addition to the myometrium, conditional gene deletion by Amhr2cre mice has been reported in the endometrium, granulosa cells, as well as in unanticipated organs such as brain, pituitary, heart liver and tail (Hernandez Gifford et al., 2009). Rest has been confirmed as a vital developmental gene as shown by embryonic lethality in conventional Rest knockout studies (Chen et al., 1998). Despite the possibility of complications arising from the expression of Amhr2+/-cre recombinase outside the myometrium, we have shown UL phenotype in our Rest cKO mice. However, it will be extremely advantageous to confirm the phenotype seen in our Rest cKO is based on myometrial-specific deletion of Rest. We have already generated a myometrial-specific cre recombinase mouse in our lab, under the CaBPD9K promoter, which could be mated with Rest floxed mice to generate a myometrial-specific Rest cKO. Characterization of these mice will be helpful in confirming the in vivo role of the loss of REST in the uterus.

**Prickle cKO mouse model for uterine leiomyoma.**

In Chapter 5, we show deletion of PRICKLE1 leads to the destabilization and degradation of REST in UL. However, this relationship has not yet been demonstrated in vivo. Development of a conditional knock-out of Prickle1 in the mouse myometrium would be exceptionally beneficial in further studies of the role of PRICKLE1 in UL. These mice could be generated using floxed Prickle1 embryonic stem cells and cre-expressing mice as described in
Chapter 4 for the *Rest cKO*.

**Exploration of additional cell proliferation pathways activated in uterine leiomyoma.**

Our *in vitro* data clearly shows activation of the PI3K/AKT-mTOR pathway in UL smooth muscle cells. However, other pathways may also be regulated by GPR10 signaling in UL. Transforming growth factor (TGFβ), especially the isoform TGFβ3, as well as its receptors and downstream signaling mediators, Smads 2/3, are up-regulated in UL and have been suggested to be involved in UL pathogenesis (Salama, Diaz-Arrastia, Kilic, & Kamel, 2012). Importantly, in our CaBP9K-hGPR10 mouse model, our qRT-PCR data show a significant increase in TGFβ3, mirroring the human phenotype (Chapter 3). To further elucidate the process of UL tumorigenesis, an alternative approach could include *in vitro* experiments examining the activation of TGFβ3 and downstream Smads in response to PrRP treatment in UL cells and in our CaBP9K-hGPR10 transgenic mouse model. Additionally, GPR10 is known to activate MAPK/ERK pathway in pituitary tumor cells (Dodd & Luckman, 2013). Experiments may also be designed to examine the status of MAPK/ERK pathway in our CaBP9K-hGPR10 transgenic mice.

**Studies in human cells on the PRICKLE1-estrogen link.**

The results presented in Chapter 5, showed the regulation of PRICKLE1 expression by ERα *in vivo* using several mouse models. It will be essential to confirm this regulation of PRICKLE1 in human primary cells as well. The presence of EREs in the human *PRICKLE-1* promoter is a promising indication of PRICKLE-1 regulation by estrogen in human uterine cells. To confirm this regulation, myometrial smooth muscle cells, which appropriately express PRICKLE-1, must be treated with estrogen *in vitro*. However, the rapid loss of steroid hormone receptors upon culturing of uterine smooth muscle cells may present a challenge to testing
PRICKLE-1 sensitivity to E2 in cell in culture. As an alternative, 3D cell culture shows improved hormone receptor expression in 3D culture, indicating the feasibility of confirming PRICKLE1 regulation by E2 in 3D cell culture. Alternatively, it would be feasible to clone the PRICKLE-1 promoter, with and without the ERE sites, upstream of a luciferase reporter construct. Myometrial smooth muscle cells could be transfected with the construct, followed by treatment of E2 and detection of luciferase activity. We have also obtained adenovirus that overexpress ESR1 to supplement ERalpha in the primary cells.

**REST-MED12 interaction in uterine leiomyoma.**

An important aspect of REST function is the interaction between REST and MED12, a subunit of the transcriptional mediator complex. A missense mutation in the *MED12* gene has been shown in up to 60% of human UL (Makinen et al., 2011). Importantly, REST is known to recruit MED12 as part of a gene regulatory complex (Gopalakrishnan, 2009). It is conceivable that REST - MED12 protein complexes with mutations have aberrant functions in UL. Our lab has already developed vectors containing the mutant and wild-type *MED12* genes for transfection in stable cell lines. Future studies will include in-depth analysis of the REST and MED12 interactions. It will be interesting to determine if the frequent MED12 mutation in the essentially quiescent myometrium occurs as a “second hit” following the loss of REST in UL, and how the function of REST is impacted by the MED12 mutation.

**Additional REST targets in uterine leiomyoma pathogenesis.**

Results presented in Chapter 4 have shown REST regulation of GPR10 in UL. Moreover, REST potentially targets up to 2000 additional genes containing RE1 consensus sequences in their regulatory regions (Gopalakrishnan, 2009), and many of these are among the most dysregulated genes in UL. It is likely the expression of other genes, in addition to GPR10,
contribute to the full pathogenesis of UL. Chromatin-immunoprecipitation studies in uterine smooth muscle cells could lead to exciting candidate genes which could be further explored for a role in UL development in the future. Our Rest cKO mouse model will also be useful in determining additional REST target genes contributing to UL pathogenesis.

**PRICKLE1 in tissue patterning of uterine leiomyoma.**

In addition to the role of PRICKLE1 as a REST binding partner, PRICKLE1 also has a well-established role during development of planar cell polarity in organization of tissue patterning. Relatedly, UL display a characteristic disorganization of smooth muscle cells and extracellular matrix proteins. It would be interesting to determine if the loss of PRICKLE1 in UL contributes to the disorganization of smooth muscle tissue in UL. Generation of a conditional knockout mouse model for Prickle1 in the myometrium, described above, would be an excellent model for elucidation of the role of PRICKLE1 in UL tissue patterning.

**Mechanism of EZH2 regulation of Prickle1.**

Increased levels of EZH2 in UL regulate PRICKLE1 expression in primary cultured UL cells (Chapter 5). However, the methyltransferase activity of EZH2 is not required for the suppression of PRICKLE1 expression. It will be of interest to determine how EZH2 contributes to the suppression of PRIKCLE1 in UL. Chromatin immunoprecipitation and chromatin conformation capture experiments to determine the nature of the suppressed PRICKLE1 promoter in UL would be helpful in determining the epigenetic control of the gene.

**Role of REST in uterine leiomyoma hypersensitivity to steroid hormones.**

UL tissue displays an altered response to steroid hormones in comparison to healthy myometrial tissue. During the luteal phase myometrial tissue becomes quiescent, whereas UL
shows a loss of negative regulation by steroid hormones and instead present an increase in estrogen-related genes and growth. Furthermore, UL grow in response to progesterone, which normally has a suppressive effect in the myometrium. The reason for this loss of inhibition by steroid hormones in the luteal phase is not well understood. Interestingly, our Rest cKO mouse model presents with significantly increased uterine size throughout the mouse estrus cycle, which would suggest a role for the loss of REST in steroid hormone sensitivity in UL. Studies of cultured cell lines derived from the Rest cKO mouse model with subsequent steroid hormone treatment would be interesting to further elucidate the role of REST in steroid hormone sensitivity.

**Characterization of the protein complex of REST in uterine leiomyoma.**

As an epigenetic silencer, REST functions as a molecular scaffold, recruiting co-factors such as Co-REST, G9A, MED12, and SIN3a to target gene promoters (Z. Huang & Bao, 2012; Shimojo & Hersh, 2003; Westbrook et al., 2008). REST also interacts with proteins thought to be involved in its stability and proteasomal degradation, including SCFβ-TRCP, an E3 ubiquitin ligase and PRICKLE-1 (Shimojo & Hersh, 2003; Westbrook et al., 2008). The specific proteins that form a complex with REST differ depending on the cell type and can change during development (Shimojo et al., 1999). The protein complex of REST in UL or in healthy myometrial tissue has not yet been determined. It is likely that altered protein-protein interactions occur as a result of the degradation of REST at the protein level and contribute to UL pathogenesis. Proteomic analysis to characterize the protein complex of REST and to possibly identify novel REST-associated proteins in UL will be an avenue of future interest. We expect results to show altered protein complexes and possible clues to the consequence of REST downregulation in UL. Of particular interest is the long non-coding RNA HOTAIR. HOTAIR
has been shown to link the epigenetic Polycomb repressor complex 2 (PRC2 complex) containing EZH2 with REST. It will be of interest to further elucidate this dynamic interaction between EZH2 and REST, via HOTAIR, in epigenetic control of target genes in UL and may further confirm the importance of the proteins involved in the novel molecular pathway presented in this dissertation.

**PRICKLE1 estrogen sensitivity and REST regulation in Alzheimer’s disease and cancers.**

The loss of REST occurs in steroid-sensitive cancer, including breast cancer (Z. Huang & Bao, 2012; Westbrook et al., 2005). In addition, REST degradation has recently been found to be a possible causative factor in Alzheimer’s disease (Lu et al., 2014). Interestingly, several studies have confirmed a definite link between estrogen levels and development of Alzheimer’s disease (Engler-Chiurazzi, Singh, & Simpkins, 2015). Results in chapter 5 are the first to identify the sensitivity of PRICKLE1 expression to estrogen. These results on estrogen regulation of PRICKLE1 and subsequent destabilization and degradation of REST could have wider implications in other steroid-sensitive cancers with loss of REST, as well as Alzheimer’s disease. Furthermore, the role of estrogen in PRICKLE1 regulation could be studied in other tissues, such as hair cells of the auditory system, where PRICKLE1 expression has been shown to important for appropriate function.
In conclusion, the findings of my dissertation have contributed significantly to the understanding the pathogenesis of UL. This work shows meticulous construction of a novel molecular pathway starting from known endocrine risk factors and ending with tumorigenic pathway activation resulting in UL tumor formation. The novel pathway has identified several important proteins and mechanisms involved in UL etiology, reveals novel drug targets for improved treatment of UL, and has generated two animal models unique to the UL field.
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