DOMINANT ACTIVATION OF HEDGEHOG SIGNALING ALTERS DEVELOPMENT OF THE FEMALE REPRODUCTIVE TRACT

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ABSTRACT

The hedgehog (HH) signaling pathway regulates the development of multiple organs in the embryo as well as remodeling in adult tissues. Signaling occurs through binding of one of three secreted HH ligands (Indian, desert and sonic) to the membrane receptor patched (PTCH); this relieves inhibition of the signal transducer smoothened (SMO), and downstream signaling occurs through GLI transcription factors. The objective of this study was to determine the role of HH signaling in development of the female reproductive tract. Mice were created in which expression of a dominant active allele of *Smo*, known as *SmoM2*, and a fusion protein, yellow fluorescent protein (YFP), are induced in the Müllerian duct by CRE-mediated recombination using the *Amhr2*$^{cre/+}$ allele. The *Amhr2*$^{cre/+}$ allele is known to be expressed during embryonic development of the Müllerian duct and the ovary. In *Amhr2*$^{cre/+}$*SmoM2* mutant mice, YFP was detected in the reproductive tract on the day of birth, confirming that expression of the *SmoM2/Yfp* fusion gene had been induced. At 24 days of age, levels of mRNA for genes that are known transcriptional targets of HH signaling, *Gli1*, *Ptch1* and *Hhip*, were increased in uteri and oviducts of *Amhr2*$^{cre/+}$*SmoM2* mutant mice compared to genotype-matched *Amhr2*$^{+/+}$*SmoM2* controls. Thus, over-activation of HH signaling continued in prepubertal mutant mice. The reproductive tract of mutant mice failed to develop normally. Unlike the uterus in control mice, in which the luminal epithelium consists of a single layer of columnar epithelial cells, the luminal epithelium in mutant mice had stratified squamous cells, resembling those of a normal cervix. In addition, the uterus of mutant mice lacked glands, had a disorganized myometrium and the oviduct lacked coiling. These characteristics suggested partial homeotic transformation of the uterus in mutant mice

to resemble the more posterior cervix and failure of the oviduct to develop normally. The anterior-posterior axis of the reproductive tract is known to be regulated by differential expression of genes in the *Hox* family. *Wnt7a* and *Wnt5a* null mice have defects in patterning of the reproductive tract. Furthermore, neonatal treatment with DES was reported to alter development of the reproductive tract and the expression of *Hox* and *Wnt* genes. Therefore, steady state levels of mRNA for *Hoxa9, Hoxa10, Hoxa11, Hoxa13, Wnt5a* and *Wnt7a* were determined in oviduct, uterus and vagina of 24-day old *Amhr2*$^{cre/+}$*SmoM2* mutant and control mice. In control mice, expression of *Hoxa9* was most prevalent in the oviduct, *Hoxa10* and *Hoxa11* in the uterus and *Hoxa13* in the vagina. mRNA levels of *Hoxa9, Hoxa10* and *Hoxa11* in the oviduct and uterus of mutant mice were similar to that in control mice. In contrast, mRNA of *Hoxa13* in uterus of mutants was 12-fold higher than in controls. Steady state levels *Wnt7a* mRNA were was similar in the reproductive tract of mutants and controls. In contrast, *Wnt5a* expression was increased 1.7-fold in the oviducts and 1.8-fold in the uterus of mutant mice compared to control mice, but it did not differ in the vagina. Mutant mice were infertile, in large part due to an ovarian defect that prevented ovulation. In addition, mating with either fertile or vasectomized wild-type males induced a severe inflammatory response in the reproductive tract by a mechanism that remains to be determined. In summary, over-activation of HH signaling results in a defect in patterning of the reproductive tract that is associated with an altered pattern of expression of *Hoxa13* in the uterus and elevated expression of *Wnt5a* in the oviduct and uterus. These results show that development of the reproductive tract can be influenced by HH signaling.

BIOGRAPHICAL SKETCH

Fernando Franco Migone Rhor was born December 23, 1985 in Lima, Peru. In 2004 he attended Universidad Nacional Agraria La Molina, where he studied in the Department of Animal Science for three and a half years. During this time he discovered his passion for reproductive physiology through the guidance of his mentor Enrique Alvarado and he was involved in research on dairy cattle with Amalia Gallegos. In 2007, he transferred to Southern Illinois University where he earned his bachelor degree in Animal Science, graduating with honors. During his time in SIU, Fernando was mentored by Dr. Karen Jones and he became committed to research and teaching under the guidance of Drs. Amer Abughazaleh, Todd Winters and Nancy Henry. In 2009, he started the journey of graduate school, focusing on reproductive physiology in the Department of Animal Science at Cornell University and mentored by Dr. Susan Quirk. Fernando has enjoyed every second at Cornell, from doing research in the laboratory, attending lectures with great professors and attending high quality seminars and conferences. He looks forward to continue learning in order to achieve his dream of conducting research in his own lab, and teaching and transmitting the knowledge obtained to people in Peru.

Dedicado a mi abuelo Eduardo y a mi abuela Rosa

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CHAPTER 1

INTRODUCTION TO THE THESIS

Development of the female reproductive tract

The female reproductive tract (FRT) is essential for continuity of life because indispensable processes such as fertilization, implantation and embryo development occur there. In humans, congenital abnormalities in the FRT occur in 0.1 to 3% of births and result in later infertility or embryonic loss (Christopoulos et al., 2009). The development of the FRT occurs in two stages: prenatal organogenesis and postnatal morphogenesis. The FRT is composed of the oviduct, uterus, cervix and vagina, all of which derive from the Müllerian duct. By contrast, in the male reproductive tract, the epididymis, vas deferens, and seminal vesicles originate from the Wolffian duct.

Prenatal organogenesis begins with development of Müllerian and Wolffian ducts. Females and males develop both ducts during embryogenesis independent of the chromosomal sex. In mice, differentiation of the intermediate mesoderm results in initiation of Wolffian duct formation at embryonic day 9.5, and by day 10.5 the duct has extended in length and has reached the cloaca. The Müllerian duct originates from an invagination of the mesonephros in the urogenital ridge at embryonic day 11.5 and the duct extends and reaches the cloaca at day 13.5 (Spencer et al., 2005). In males, the *Sry* gene, which is located on the Y chromosome, leads to development of testes which will produce testosterone, anti Müllerian hormone (AMH) and insulin-like growth factor 3. AMH promotes regression of the Müllerian duct. In contrast, in females, absence of male hormones leads to regression of the Wolffian duct (Kobayashi and Behringer, 2003; Spencer et al., 2005).

Using gene modification in mice, several genes were identified to play a role in development of the Müllerian duct during embryogenesis. Deletion of wingless-related MMTV integration site 4 (*Wnt4*) inhibits development of the Müllerian duct. Furthermore, in *Wnt4* null females, the Wolffian duct does not regress due to secretion of androgens from ectopic Leydig cells in the ovaries (Vainio et al., 1999). Mice with deletion of paired box gene 2 (*Pax2*) undergo normal formation of the Müllerian duct at embryonic day 11.5; however, the duct begins to regress by embryonic day 12.5 (Torres et al., 1995). Empty spiracles homeobox 2 (*Emx2*) deletion inhibits formation of kidney, gonads and Müllerian duct, and these mice die *in utero* (Miyamoto et al., 1997). Finally, deletion of Lim homeodomain transcription factor 1 (*Lim1*) eliminates Müllerian duct formation (Kobayashi et al., 2004).

Differentiation of the Müllerian duct into the structures of the FRT requires anterior-posterior and radial patterning. Anterior-posterior patterning determines organization of tissue in a cranial to caudal fashion. In the case of the FRT this patterning identifies molecular cues that will determine which part of the Müllerian duct becomes oviduct, uterus or vagina. By contrast, radial patterning regulates the histoarchitecture of a given portion of the tract.

In mice, anterior-posterior patterning of the Müllerian duct begins close to birth and some genes of the homeobox (*Hox*) family are essential for the process. *Hoxa9, Hoxa10, Hoxa11* and *Hoxa13* are expressed uniformly along the Müllerian duct up to embryonic day 15.5. At embryonic day 18.5 expression domains of these genes become restricted to the following pattern: *Hoxa9* is expressed in the oviduct, *Hoxa10* in the uterus, *Hoxa11* in the uterus and cervix, and *Hoxa13* in the cervix and vagina. The anterior-posterior pattern of expression of these genes continues through adulthood and is conserved among mammals (Benson et al., 1996; Daftary and Taylor, 2006; Du and Taylor, 2004; Taylor et al., 1997; Zhao and Potter, 2001). Targeted

gene deletion of members of the *Hoxa* family involved in FRT development alters anterior-posterior patterning of the tract. Deletion of *Hoxa10* produces homeotic transformation of the anterior uterus into oviduct (Benson et al., 1996), deletion of *Hoxa11* generates homeotic transformation of the uterus into oviduct (Branford et al., 2000; Gendron et al., 1997), while replacement of *Hoxa11* with *Hoxa13* results in a uterus with characteristics of the cervix and vagina (Zhao and Potter, 2001). In addition to *Hox* genes, two members of the *Wnt* family play a role in establishing anterior to posterior and radial patterning of the FRT. Deletion of *Wnt5a* impairs the development of the posterior structures of the Müllerian duct (i.e. cervix and vagina) (Mericskay et al., 2004). Finally, deletion of *Wnt7a* alters normal development of the oviduct and uterus (Miller and Sassoon, 1998).

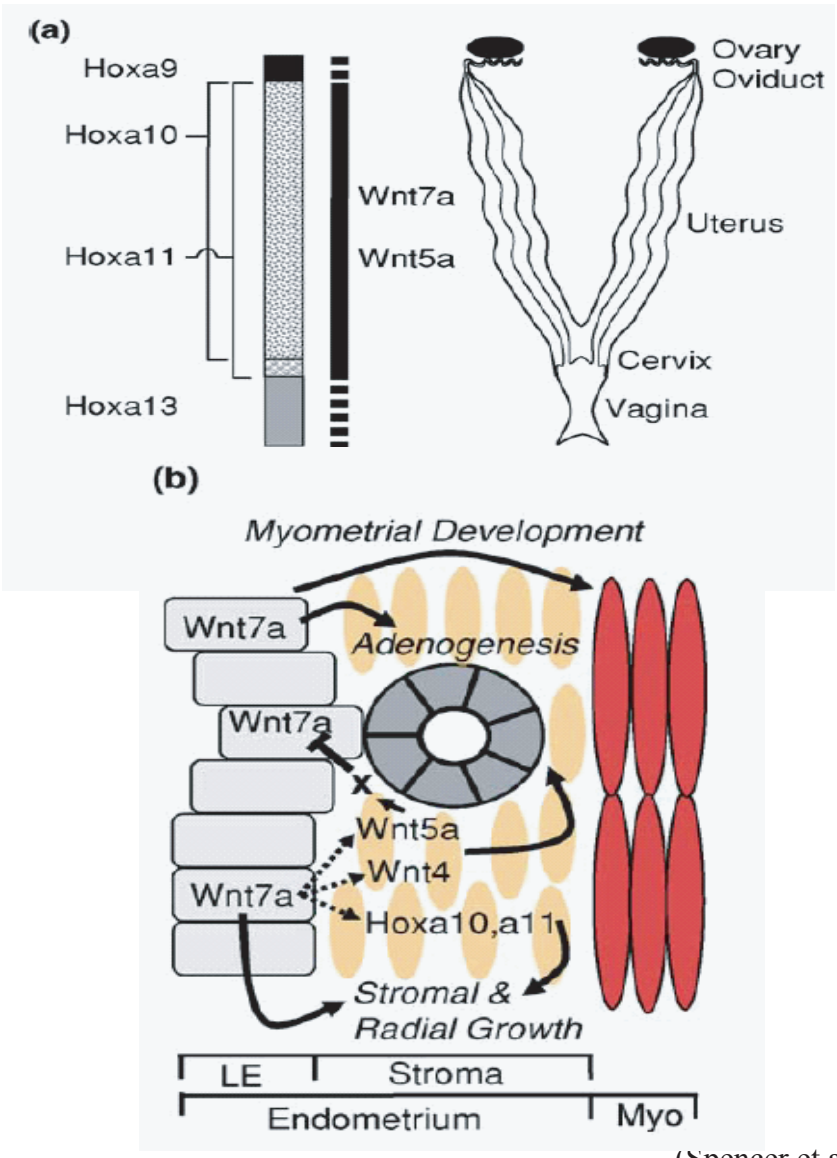
At birth, the reproductive tract consists of a simple luminal epithelium and an undifferentiated mesenchyme. Tissues within the FRT require establishment of radial patterning to provide typical characteristics of the luminal epithelium, stroma and muscle layer of the oviducts, uterus, cervix and vagina. Radial patterning begins postnatally, and in mice, by two weeks of age the tract is completely differentiated and resembles the adult tract. In the oviduct, the luminal epithelium differentiates into two populations of cells: secretory cells and ciliated cells. These cells are expressed in different ratios within the four compartments of the oviduct (infundibulum, ampulla, isthmus and uterotubal junction). The epithelium is surrounded by stroma which has no glands and by circular and longitudinal muscle layers (Yamanouchi et al., 2010). In addition, coiling of the oviduct begins as early as embryonic day 16 and is completed by two weeks of age (Newbold et al., 1983).

In the uterus, the luminal epithelium is simple columnar and is surrounded by a glandular stroma and myometrium (circular and longitudinal muscle layers) (Spencer et al., 2005). At birth, it is not possible to identify the area of the mesenchyme that

will differentiate into stroma or muscle layers. However, by day 5, the area that will form the myometrium can be identified by smooth muscle actin staining and by day 10 circular and longitudinal muscle layers can be identified either by histology or with smooth muscle actin staining (Brody and Cunha, 1989). In addition, the stroma forms uterine glands which produce and secrete substances essential for implantation (Gray et al., 2001). At birth no glands are present in the stroma, but by day 5 the first invaginations of the luminal epithelium are observed, and the first glands are present by day 7. By day 15, uterine gland formation is complete (Gray et al., 2001; Spencer et al., 2005). There is evidence to suggest that interactions among *Wnt* and *Hox* genes regulate uterine differentiation (Fig. 1.1) (Spencer et al., 2005).

In mice, the vagina originates from the Müllerian duct and the urogenital sinus. At birth, the area of the vagina that originates from the Müllerian duct consists of a lumen lined with columnar epithelium and surrounded by mesenchyme. On the other hand, the area that originates from the urogential sinus consists of a solid epithelial cord. Within the first weeks after birth, both epithelia stratify and Müllerian or sinus origin is no longer identifiable (Boutin and Cunha, 1996). The epithelium is surrounded by an aglandular stroma and muscle layers, similar to the layers present in other regions of the FRT.

Based on studies to understand how radial pattering is regulated, it is widely believed that mesenchymal-epithelial interactions are essential. Elegant studies using tissue recombination demonstrated that the stroma is a key mediator in the differentiation of the luminal epithelium in the different regions of the FRT. In these studies, epithelial and mesenchymal fragments of the FRT were recombined and grafted under the renal capsule of adult female mice. In the oviduct, recombination of stromal and epithelial tissue of the ampulla, isthmus and infundibulum was performed. This experiment demonstrated that the luminal epithelium of the grafted tissue



(Spencer et al., 2005)

Figure 1.1. Members of the *Hox* and *Wnt* families are involved in estabilshing anterior to posterior and radial patterning during the development of the FRT (a). Radial differentiation of the developing uterus requires interactions of *Hox* and *Wnt* genes in order to develop glands and differentiate luminal epithelium and myometrium (b).

acquired ciliated and secretory cells in a ratio typical of the region of the tract from which the stroma was obtained (Yamanouchi et al., 2010). In a similar experiment, vaginal and uterine stroma were recombined with vaginal and uterine luminal epithelium. Here, independent of the origin, luminal epithelia differentiated into luminal epithelium typical of the region from which the stroma was derived (Kurita et al., 2001). In these two experiments, stroma-induced differentiation of the luminal epithelium was only successful if tissue was obtained during the first weeks of life and not in adulthood, suggesting an early period of plasticity of the tract.

Mesenchymal-epithelial interactions are also involved in mediating responses to stimuli. Experiments showed that ablation of the luminal epithelium *in vivo* inhibited the ability of stromal cells to undergo decidualization in response to progesterone and estrogen (Lejeune et al., 1981). Furthermore, tissue recombination combined with targeted deletion of genes demonstrated that estrogen receptor alpha (*Esr1*) in the stroma, but not in the epithelium, is essential for proliferation of the luminal epithelium in response to estrogen. Similarly, stromal aryl hydrocarbon receptor (*Ahr*) in the stroma is necessary to regulate proliferation in the luminal epithelium while *Ahr* in the luminal epithelium is not required for this process (Cunha et al., 2004). Furthermore, progesterone receptor (PGR) in the stroma and not in the epithelium of the uterus was shown to increase expression of *Ihh* in the luminal epithelium after administration of progesterone (Simon et al., 2009). These studies revealed the importance of mesenchymal-epithelial interactions in mediating responses to hormones in the FRT. This concept was recently challenged, however, when a study using conditional deletion of *Pgr* in the uterine luminal epithelium demonstrated that signals within the uterine luminal epithelium rather than within the stroma were necessary to induce expression of progesterone-dependant genes such as *Ihh* in the epithelium (Franco et al., 2010). These studies suggest that changes in gene

expression and function within the epithelium may be mediated by signals directly to the epithelium rather than indirectly by signals from the stroma.

The Hedgehog signaling pathway

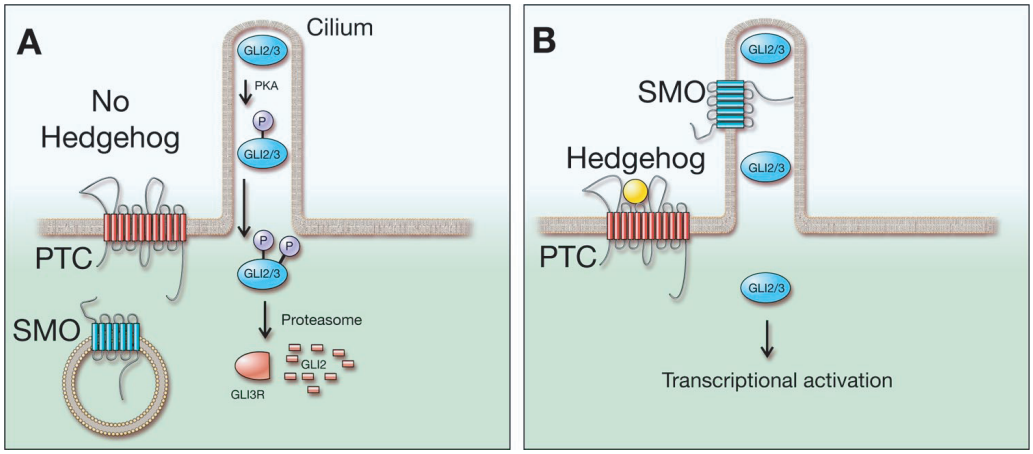
The hedgehog (*HH*) gene was first discovered in a genetic screen looking for mutations in cuticle formation in *Drosophila* embryos. Mutation of *HH* generated a disorganized and spiky pattern of the thoracic and abdominal segments of the embryo resembling a hedgehog (Nusslein-Volhard and Wieschaus, 1980). In mammals, three paralogous genes to the *Drosophila HH* have been identified, Sonic HH (*Shh*), Indian HH (*Ihh*) and Desert HH (*Dhh*) (King et al., 2008). *HH* genes are involved in cell proliferation, cell differentiation and tissue patterning. Using the mouse, chick and frog models, HH signaling has been identified to play a role in development and pattering of the brain, bone, gut, heart, lung and hair follicle among other tissues (Bellusci et al., 1997; Echelard et al., 1993; Sato et al., 1999; St-Jacques et al., 1999; Sukegawa et al., 2000). The HH signaling pathway is also involved in the anterior-posterior polarity of the limb-bud (Riddle et al., 1993) and modulation of lateral symmetry of the embryo (Meyers and Martin, 1999). Additionally, HH signaling has a role in proliferation and differentiation of hematopoietic stem cells and myeloid progenitors (Merchant et al., 2010), smooth muscle cells in the urinary bladder, (Tasian et al., 2010), progenitor cells of the adrenal capsule (Huang et al., 2010) and intestinal stem cells (Kosinski et al., 2010).

In order for HH proteins to be released from producing cells, they need to undergo a maturation process. Precursors of HH proteins are synthesized as ~45 kD proteins (Lee et al., 1994). These precursor proteins are autocleaved to form an N-terminal fragment of ~ 19 kD which forms the mature HH protein, while no role is known for the ~21 kD segment (Hall et al., 1995). Cholesterol is covalently bound to

the C-terminal glycine of the HH polypeptide and plays a role in anchoring this polypeptide to the membrane (Mann and Beachy, 2004; Porter et al., 1995). Finally, palmitoylation of the cysteine closest to the N-terminus completes maturation of HH protein (Buglino and Resh, 2008; Pepinsky et al., 1998). Addition of these lipids has been suggested to make HH proteins highly hydrophobic, giving them the ability to form micelle-like structures when secreted (Chen et al., 2004) and the property to establish gradients in tissue after diffusion (Ingham and McMahon, 2001).

The secreted mature HH proteins bind to the receptor patched1 (PTCH1) or patched2 (PTCH2). There are also known agonists and antagonists of this binding that can play a regulatory role in HH signaling. Hedgehog interacting protein (HHIP) is a negative regulator that competes with PTCH for binding to HH proteins. On the other hand, immunoglobulin/fibronectin-repeat-containing proteins, CDO and BOC, and glycosidyl-phosphatidylinositol-anchored membrane-bound protein, GAS1, can enhance binding of HH ligands to the receptor (Heretsch et al., 2010). Binding of HH ligand to PTCH, relieves the inhibition by PTCH on the seven- pass transmembrane signal transducer smoothened (SMO). SMO is present in the cell in three different states: an internalized inactive form (SMOA), a cilium-bound inactive form (SMOB) and an active form (SMOC) (Fig. 1.2) (van den Brink, 2007).

Release of SMO from inhibition by PTCH, promotes the activation of glioma-associated oncogene homolog (GLI) transcription factors (GLI1, GLI2 and GLI3), which regulate gene expression (King et al., 2008; Rohatgi and Scott, 2007). Within the set of genes that are transcribed in response to binding of HH are feedback regulatory genes that can enhance the pathway, such as *Gli1* (Regl et al., 2002; Ruiz i Altaba, 1999), or suppress it, such as *Ptch* and *Hhip* (Yang and Lin, 2010). In the absence of HH ligands, GLI2 and GLI3 proteins bind with suppressor of fussed (SUFU) protein (Pearse et al., 1999) and the complex is phosphorylated by protein



(van den Brink, 2007)

Figure 1.2. The HH signaling pathway: In the absence of HH ligand, receptor PTCH inhibits signal transducer SMO which is internalized in intracellular vesicles. GLI2 and GLI3 transcription factors are phosphorylated, GLI2 is completely degraded while GLI3 is processed into a repressor form (A). When HH binds to PTCH, it relives the inhibition on SMO by PTCH. SMO is translocated to the primary cilium were it promotes transcriptional activation (B).

kinase A (PKA), glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) (Pan et al., 2006). Phosphorylated GLI2 and GLI3 bind to beta-transducin repeat-containing homologue protein (βTrCP) which mediates ubiquitination of GLI2 and GLI3 proteins (Wang and Li, 2006). Ubiquitination results in partial degradation of GLI3 to generate its repressor form (GLI3-R) and total degradation of GLI2 (Murdoch and Copp, 2010).

The HH signaling pathway in mammals requires the primary cilium to mediate signal transduction (Wilson and Chuang, 2010). The primary cilium is composed of an arrangement of microtubules that protrude from the plasma membrane and is present on almost all non-dividing cells. Formation and maintenance of the primary cilium depends on intraflagellar transport (ITF) and kinesin II and dynein motors (Rosenbaum and Witman, 2002). Deletion of genes involved in ITF such as *Kif3a* and *Ift88* deregulates repressor and activator roles of GLI transcription factors (Huangfu and Anderson, 2006) and the phenotype in mice with these deletions mimics the phenotypes observed in deletion or ectopic activation of components of HH signaling (Goetz and Anderson, 2010; Goetz et al., 2009). Furthermore, the components of the HH signaling pathway –*Gli1, Gli2, Gli3, Ptch1, Smo* and *SuFu*– are localized in the primary cilium (Chen et al., 2009; Corbit et al., 2005; Haycraft et al., 2005; Rohatgi and Scott, 2007).

Most of what we know about HH signaling in mammals has been investigated using the mouse model. In mice, deletion of *Shh* results in embryonic death characterized by reduction in size of the brain and spinal cord and severe growth retardation (Chiang et al., 1996). Targeted deletion of *Ihh* generates mice that die at embryonic stages due to circulatory problems or at birth due to respiratory failure (St-Jacques et al., 1999). In contrast, mice with deletion of *Dhh* are viable; however, males are infertile (Bitgood et al., 1996; Clark et al., 2000). Furthermore, deletion of

the signal transducer *Smo* produces a phenotype similar to that observed in *Shh* and *Ihh* null mice (Zhang et al., 2001). Deletion of the receptor *Ptch* results in embryonic death due to an enlargement of the neural tubes (Goodrich et al., 1997). Finally, deletion of *Gli2* produces embryonic lethality while mice with deletion of *Gli1* and *Gli3* are viable (Park et al., 2000). Due to the lethality observed when components of the HH signaling pathway are deleted, development of mice with conditional alteration of genes has been used to obtain insight into the role of HH in particular tissues.

Hedgehog signaling pathway and the FRT

The HH signaling pathway plays a role in multiple tissues; however, little is known about the role of HH signaling in the development of the FRT. As previously discussed, mesenchymal-epithelial interactions are essential for several processes during the development of the FRT. In addition, components of the HH signaling pathway are expressed at mesenchymal-epithelial boundaries during development of organs such as lung, gut, hair follicle and tooth (Walterhouse et al., 2003). These findings suggest that the HH pathway may have a role in the female tract as well.

During postnatal glandular development in the porcine uterus, *Ihh* mRNA and protein are expressed in the uterine luminal and glandular epithelium. In addition, the receptor PTCH1 localizes in the stroma and luminal epithelium (Bartol et al., 2006). Furthermore, *Ihh Dhh* and the HH target genes, *Ptch1*, *Gli1* and chicken ovalbumin upstream promoter transcription factor II (*Coup-TfII*) are expressed in the uterus of immature female rats at 20-days of age (Katayama et al., 2006).

In mice, expression of *Ihh* is increased by signaling through the progesterone receptor (PGR). Expression of *Ihh* is increased 6 hours after administration of progesterone to ovariectomized mice (Takamoto et al., 2002). Expression of *Ihh*

peaks in the glandular and luminal epithelium of the uterus between day 3 and 4 of pregnancy in mice, while expression of transcriptional target genes, *Ptch1, Hhip1* and *Coup-TfII,* increase in the uterine epithelium and stroma between day 4 and 6 (Matsumoto et al., 2002; Takamoto et al., 2002; Wakitani et al., 2008). In addition, similar to the mouse, expression of components of the HH signaling pathway increases around the time of implantation in rats (Kubota et al., 2008; Kubota et al., 2010) and hamsters (Khatua et al., 2006). In another experiment, pieces of uterus in which the luminal epithelium was enzymatically removed were cultured *in vitro*. Removal of the HH source impaired expression of responsive genes in the stroma and decreased stromal proliferation, indicating the importance of mesenchymal-epithelial interactions in the female tract (Matsumoto et al., 2002).

To assess whether *Ihh* is a key regulator of implantation, researchers created mice in which *Ihh* was conditionally deleted in the female tract. Making a conditional deletion was necessary due to embryonic lethality produced when components of the hedgehog signaling pathway were ablated. Mice which had *Cre* inserted into the *Pgr* locus ($Pgr^{cre/+}$ mice) were crossed with mice harboring the floxed *Ihh* gene. The offspring produced, $Pgr^{tm1(cre)Lyd/+}Ihh^{tm1Blan}$ mice, developed phenotypically normal reproductive tracts, but were infertile due to failure of blastocysts to implant. Additionally, these mice had reduced proliferation and vascularization in the uterus, and failed to undergo a decidual response (Lee et al., 2006). $Pgr^{tm1(cre)Lyd/+}Ihh^{tm1Blan}$ mice had altered estrogen signaling in the uterus, shown by increased expression of estrogen receptor 1 (*Esr1*) and increased expression of Mucin 1 (*Muc1),* which depends on estrogen signaling in the uterus (Franco et al., 2010a). Furthermore, we conditionally deleted floxed alleles of *Smo* in the reproductive tract using CRE-mediated recombination driven by *Amhr2* which begins to be expressed as early as embryonic day 12.5. The phenotypic characteristics of the FRT were apparently not

altered; however, females had reduced liter size due to a delay in the time of implantation [manuscript submitted (Harman et al., 2010)]. These experiments suggest that the components of the HH signaling pathway, *Ihh* and *Smo,* are not essential for development of the FRT; however they play a role at implantation.

This year, Franco et al. (2010b) reported a conditional mouse model generated to study implantation under conditions of over-activation of HH signaling in the FRT. $Pgr^{cre/+}$ mice were crossed with mice expressing a dominant active allele of the signal transducer *Smo* known as *SmoM2*. $Pgr^{cre/+}SmoM2$ mice were infertile due to an implantation defect. In addition, these mice had developmental abnormalities in the reproductive tract such as reduced number of uterine glands, altered extracellular matrix composition in the uterus (shown by increased collagen, mucopolysaccharides and glycosaminoglycans) and atypical multilayered uterine luminal epithelium (Franco et al., 2010b). This mouse model indicated that over-activation of HH signaling in the FRT postnatally altered development and led to inhibition of implantation in adults. However, recombination using $Pgr^{Cre/+}$ mice does not provide information about what would happen if over-activation of HH signaling begins at embryonic stages. During the last several years, we created a mouse model that has conditional over-activation of HH signaling in the developing Müllerian duct as early as embryonic day 12.5. These mice, known as $Amhr2^{cre/+}SmoM2$, were generated by crossing *SmoM2* mice with $Amhr2^{cre/+}$ mice. Here we report results of research on $Amhr2^{cre/+}SmoM2$ mice and describe the importance of HH signaling regulation for proper development of the FRT at prenatal and postnatal stages.

Inflammation and reproduction

$Amhr2^{cre/+}SmoM2$ mice had altered development of the FRT and were infertile. In addition when bred, they underwent a severe inflammatory reaction in the FRT

(discussed later). An inflammatory response is a process that occurs in response to exposure to microorganisms, tissue injury, or other kinds of trauma. After stimuli, cytokines, adhesion molecules, and growth factors are secreted and guide leukocytes, lymphocytes and other inflammatory cells to repair the tissue in question. During this process, changes in extracellular matrix, microcirculation and removal of apoptotic and necrotic cells and microbes occur (Coussens and Werb, 2002; Jabbour et al., 2009; Schmid-Schonbein, 2009). Processes such as ovulation, menstruation, mating, implantation and parturition involve inflammation (Goswami et al., 2008).

Two inflammatory responses in female reproductive biology are relevant to this thesis: the responses after mating and at implantation. After mating, there is an inflammatory response in the uterus of mice, humans, rats and rabbits. This inflammatory response develops due to the presence of high molecular weight seminal vesicle proteins (Robertson et al., 1996). The sequence of events in this inflammatory response has been extensively studied in mice. First, proteins of the seminal plasma, especially transforming growth factor beta (TGFβ), interact with the superficial layers of the cervix and uterus (Tremellen et al., 1998). This interaction stimulates the uterine epithelial cells to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) as early as 12 hours after mating (Robertson et al., 1996). This secreted protein recruits macrophages, granulocytes and dendritic cells into the stroma, epithelium and lumen of the uterus (Robertson et al., 2003; Robertson et al., 2000). Immune cells remain at high levels on day 1 and 2 of pregnancy and then decline (Robertson and Sharkey, 2001). For the duration of the inflammatory response, mRNA and protein of IL-1 and TNF-α, which are mediators of inflammation, are elevated (McMaster et al., 1992). The inflammatory response after mating has been hypothesized to recognize antigens present in sperm that will later

provide uterine-immunotolerance to the embryo, increasing the success of embryo implantation and placental development (Robertson and Sharkey, 2001).

The second inflammatory response occurs at the time of implantation in mice, at day 4 after mating. Here, the embryo produces cytokines that induce inflammatory pathways in the endometrium (Sherwin et al., 2007). A similar response to that generated by the embryo can be stimulated by scratching the uterus, or injecting oil into the tract of females primed by treatment with progesterone and estrogen (Dekel et al., 2010). The signal generated by the embryo induces infiltration of leukocytes, modifications of the extracellular matrix and increased vascular permeability in the endometrium; however, once the embryo is attached the inflammatory response regresses (Hess et al., 2007; Jabbour et al., 2009; Wood et al., 1999). The role of this inflammatory response is to degrade molecules that surround the luminal epithelium such as Mucin1, allowing the blastocyst to implant at the appropriate place (Dekel et al., 2010; Meseguer et al., 2001).

The two inflammations described above are normal processes in female reproductive biology. However, targeted modification of certain genes produced an abnormal increase in the intensity of the inflammatory response, suggesting that these genes could be involved in controlling the strength of the inflammatory response. One example is mice in which both endogenous isoforms of follistatin had been deleted and in which the human follistatin isoform, FST315, had been inserted. At day 4 postcoitum, these mice had excessive infiltration of leukocytes into the lumen and degeneration of the luminal epithelium. Furthermore, when females remained with males, the inflammatory response in the uterus failed to regress (Lin et al., 2008). Similar abnormal inflammation in the FRT was observed in our studies with *Amhr2*$^{cre/+}$*SmoM2* mice (discussed later).

In summary, the role of the HH signaling pathway has been studied in many tissues; however, little is known about the functions of the pathway during development of the FRT. It has been documented that signaling by IHH is required for implantation (Lee et al., 2006) . In our studies with mice in which conditional deletion of *Smo* was induced in the FRT during embryonic development, implantation was compromised but the phenotypic characteristics of the FRT were apparently normal. In the present study, we over-activated the HH signaling pathway in the developing Müllerian duct to obtain insight into whether appropriate HH signaling is required for the development of the FRT.

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CHAPTER 2

DOMINANT ACTIVATION OF HEDGEHOG SIGNALING ALTERS DEVELOPMENT OF THE FEMALE REPRODUCTIVE TRACT

Introduction

In humans, congenital abnormalities in the female reproductive tract occur in 0.1 to 3% of births and result in later infertility or embryonic loss (Kobayashi and Behringer, 2003). Therefore, determination of the genes and their interactions involved in the development and maintenance of the reproductive tract is essential for better understanding of human pathology.

The HH signaling pathway regulates the development of multiple organs in the embryo as well as remodeling of adult tissues (King et al., 2008; McMahon et al., 2003). However, the potential role of HH signaling in the development of the FRT is not known. In mammals there are three secreted HH ligands, Indian (IHH), desert (DHH) and sonic (SHH) HH. There are two homologous transmembrane receptors, patched 1 (PTCH1) and patched 2 (PTCH2), which are thought to act similarly, and a signal transducer protein, smoothened (SMO). When PTCH is not bound to HH ligand, it represses the activity of SMO. Binding of HH ligand to PTCH relieves inhibition of SMO, and downstream signaling occurs through glioma-associated oncogene homolog transcription factors (GLI1, GLI2, GLI3) (Huangfu and Anderson, 2006).

Components of the HH signaling pathway are expressed in the uterus of immature rats (Katayama et al., 2006) and gilts (Bartol et al., 2006). In addition, expression of *Ihh* in the luminal and glandular epithelium of the uterus is increased in response to signaling though PGR (Khatua et al., 2006; Lee et al., 2006b; Matsumoto

et al., 2002; Simon et al., 2009; Takamoto et al., 2002; Wakitani et al., 2008) and expression of *Ihh* in the epithelium increases before implantation (Kubota et al., 2008; Kubota et al., 2010; Lee et al., 2006a; Wakitani et al., 2008). Conditional deletion of *Ihh* in the mouse uterus blocked implantation (Franco et al., 2010; Lee et al., 2006a).

Much of what we understand about the early development of the reproductive tract has been obtained using the mouse model. Formation of the FRT begins with the development of the Müllerian duct at embryonic day 11.5 and from these ducts the oviduct, uterus, cervix and upper vagina are formed (Spencer et al., 2005). At birth the tract consists of a single layer of luminal epithelium surrounded by undifferentiated mesenchyme (Spencer et al., 2005; Yin and Ma, 2005). During the first 15 days of life, mesenchyme of the uterus differentiates to form the stroma and inner circular and outer longitudinal muscle layers. In addition, glands form in the stroma which are the source of proteins required for implantation (Gray et al., 2001; Spencer et al., 2005; Yin and Ma, 2005).

Genes within the *Hox* family regulate the anterior-posterior patterning of the reproductive tract. Expression of *Hoxa9, Hoxa10, Hoxa11* and *Hoxa13* is evenly distributed throughout the length of the Müllerian duct at embryonic day 15.5. Close to birth and thereafter, expression of these genes becomes regionally restricted in an anterior to posterior pattern with expression of *Hoxa9* primarily in the oviducts, expression of *Hoxa10* in the uterus, *Hoxa11* in the uterus and cervix and *Hoxa13* in the cervix and vagina (Daftary and Taylor, 2006; Du and Taylor, 2004; Taylor et al., 1997). *Hoxa10* null mice are infertile and the anterior portion of the uterus has oviductal-like coiling (Benson et al., 1996). *Hoxa11* null mice are infertile and have a reduced number of uterine glands (Branford et al., 2000). *Hoxa13* null mice rarely survive to birth due to a dramatic hypoplasia of the cervix and vaginal canal (Zhao and Potter, 2001).

Members of the *Wnt* family are also involved in patterning of the reproductive tract. *Wnt4* is expressed throughout the Müllerian duct starting at embryonic day 12.5, and expression is absent in the regressing Wolffian duct. After birth, *Wnt4* continues to be expressed in the oviduct, uterus and vagina. Deletion of *Wnt4* in mice, inhibits formation of the Müllerian duct and results in failure of the Wolffian duct to regress (Heikkilä et al., 2001; Vainio et al., 1999). At embryonic day 17, *Wnt5a* is expressed in the mesenchyme of the Müllerian duct, and after birth it is expressed in the stroma of the uterus (Miller et al., 1998b). Deletion of *Wnt5a* results in small uterine horns, failure to form the cervix and the vagina and early postnatal death. To circumvent lethality and to obtain insight into the role of *Wnt5a* in postnatal development of the tract, pieces of uterus were grafted under the kidney capsule. Grafted uterine tissue from *Wnt5a* null mice had circular and longitudinal layers of muscle of reduced thickness and a reduced number of uterine glands (Mericskay et al., 2004). W*nt7*a is expressed within the epithelium of the Müllerian duct at embryonic day 17, and its expression is restricted to epithelium of the oviduct and uterus in adulthood (Miller et al., 1998a). In *Wnt7a* null mice, the uterus is shortened and the luminal epithelium is stratified squamous similar to that normally found in cervix and vagina. In addition, oviducts lack coiling and there is no clear division between the posterior end of the oviduct and the anterior portion of the uterus (Miller and Sassoon, 1998).

The objective of this study was to gain insight into the role of HH signaling in development of the FRT by studying transgenic mice in which a dominant active allele of *Smo* is expressed in the Müllerian duct.

Materials and Methods

Generation of mice

Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All studies were approved by the Cornell University Institutional Animal Care and Use Committee. *Gt(ROSA)26Sor*$^{tm1(smo/YFP)Amc}$*/J* mice (The Jackson Laboratory, Bar Harbor, ME) (Jeong et al., 2004) were bred to mice in which *Cre* recombinase is expressed through the anti-Müllerian hormone receptor 2 gene (*Amhr2*$^{cre/+}$) (provided by Dr. Richard Behringer) (Jamin et al., 2002). The pups produced were genotyped from tail DNA using protocols provided by The Jackson Laboratory, and identified as controls (*Amhr2*$^{+/+}$*SmoM2*) or mutants (*Amhr2*$^{cre/+}$*SmoM2*). In studies to test fertility, control and mutant females were caged with fertile CD-1 males. In other experiments, females were bred to vasectomized CD-1 males. Reproductive tracts were fixed for histological analysis or immediately frozen and stored at -80 ˚C until preparation of RNA.

Flow cytometry

Enriched fractions of epithelial and stromal cells were prepared from uteri of 24-day old *Amhr2*$^{+/+}$*SmoM2* control and *Amhr2*$^{cre/+}$*SmoM2* mutant mice. Reproductive tracts were removed and trimmed in Dulbecco's Modified Eagle Medium-Ham's F-12 Nutrient Mixture (DMEM-F12). Uterine horns were sliced open longitudinally, exposing luminal epithelial cells. Tissues from individual animals were incubated in 750 µl DMEM-F12 containing 0.075% trypsin, 0.3 mM EDTA and 10 µg/ml deoxyribonuclease I at 37 ˚C. Luminal epithelial cells were dislodged from underlying tissue by gentle triteration with a large bore pipette every 30 minutes. After 90 minutes, media containing luminal epithelial cells was transferred to a 1.5 ml tube, and 100 µl fetal bovine serum (FBS) plus 500 µl media was added to stop

enzymatic activity. Cells were pelleted by centrifugation, rinsed with DMEM-F12 and suspended in 80% ethanol. Remaining uterine tissue was placed in 750 µl fresh enzyme solution and digested at 37 ˚C for 2 hours with gentle triteration every 30 minutes. At the end of 2 hours, undigested myometrium was discarded. Media containing stromal cells and glandular epithelial cells was transferred to a 1.5 ml tube, enzyme activity was terminated as above, and cells were rinsed, pelleted and resuspended in 80% ethanol as above. Purity of the cell preparations was assessed by culture of representative cell fractions and examination of morphology and by immunohistochemical detection of cytokeratin (data not shown). These tests indicated that the luminal epithelial cell preparations were essentially free of stromal cells and the stromal cell preparations contained approximately 10% epithelial cells, likely due to glandular epithelial cells. Within 24 h of storage at 4 ˚C, cells were centrifuged and reconstituted in PBS, and YFP fluorescence was determined using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Cells were excited at 488 nm and emission detected at 530±30 nm. Data were analyzed using WinMDI software (The Scripps Research Institute, San Diego, CA). Single cells were selected from a plot of side scatter *vs.* forward scatter and YFP fluorescence was plotted *vs.* side scatter. Non-specific background fluorescence was determined by analyzing cells from control mice and using the data to construct a gated area in which 95% of the cells resided within the gated area. This gate was applied to plots of fluorescence for all samples, and the percent of cells with specific fluorescence above the gated area was thereby determined.

Histology

Tissues were fixed overnight in Bouins, dehydrated in ethanol, embedded in paraffin and 5µm sections prepared. Slides were stained with hematoxylin and eosin

or using Masson's trichrome protocol. Sectioning and staining were performed by the Histology Laboratory, New York State College of Veterinary Medicine.

Analysis of gene expression

Total RNA was extracted from oviduct, uterus and vagina of 24-day old mutant and control mice using an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) using mouse-specific Taqman Gene Expression Assays (Table 2.1) according to the manufacturer's instructions. A sample of pooled RNA from uteri of control and mutant mice was used as a standard. Values were normalized to the concentration of 18S rRNA. For each gene, results were obtained in a single assay using one plate.

Statistical analysis

Gene expression data were log transformed and analyzed by one-way ANOVA using JMP8 software (SAS, Cary, NC). Student-Newman-Keuls procedure was used to compare individual means. Flow cytometry analysis of YFP expression was analyzed by two-way ANOVA and significance determined by Student-Newman-Keuls procedure.

Results

Generation of mice with over-activation of the HH signaling pathway in the reproductive tract

Homozygous mice expressing a dominant active allele of *Smo*, known as *SmoM2*, and yellow fluorescent protein (YFP) were mated with heterozygous mice

Table 2.1. Quantitative real-time RT-PCR assays

Symbol	Gene name	Assay Identification[a]	Exons[b]
Hhip	Hedgehog-interacting protein	Mm00469580_m1	12-13
Gli1	GLI-Kruppel family member GLI1	Mm00494645_m1	2-3
Ptch1	patched homolog 1	Mm00436026_m1	17-18
Hoxa9	Homeo box A9	Mm00439364_m1	1-2
Hoxa10	Homeo box A10	Mm00433966_m1	1-2
Hoxa11	Homeo box A11	Mm00439360_m1	1-2
Hoxa13	Homeo box A13	Mm00433967_m1	1-2
Wnt5a	Wingless-related MMTV integration site 5a	Mm00437355_m1	2-3
Wnt7a	Wingless-related MMTV integration site 7a	Mm00437347_m1	4-5
18s rRNA		4319413E	

[a] Taqman® Gene Expression Assays (Applied Biosystems)
[b] Exons in which forward and reverse primers anneal.

expressing the *Cre* recombinase sequence inserted into the *Amhr2* gene. CRE-mediated recombination driven by the *Amhr2*^{cre} allele begins between embryonic days 12.5 and 13.5 (Arango et al., 2005; Jamin et al., 2002). In our previous study using *Amhr2*$^{cre/+}$*SmoM2* mutant mice, YFP was detected in the reproductive tract and ovaries on the day of birth, confirming that expression of the *SmoM2/Yfp* fusion gene had been induced (Ren et al., 2009).

To determine the efficiency of CRE-mediated recombination in the reproductive tracts of juvenile mice, the percentage of cells that expressed the YFP fusion protein was determined by flow cytometry of dispersed luminal epithelial and stromal cells. In *Amhr2*$^{cre/+}$*SmoM2* mutant mice, fluorescence was detected in 31% of stromal cells and only background fluorescence was observed in luminal epithelial cells. This data confirmed that recombination occurred in the stroma and not in the epithelium of mutant mice. In contrast, epithelial and stromal cell suspensions of genotype-matched *Amhr2*$^{+/+}$*SmoM2* control mice had background levels of fluorescence (Fig. 2.1).

To determine whether expression of dominant active *SmoM2* in the reproductive tract of mutant mice caused over-activation of HH signaling, expression of mRNA for known transcriptional targets of HH signaling –*Gli1*, *Hhip* and *Ptch1*– were examined in 24-day old mice. Level of mRNA of *Hhip* and *Ptch1* were elevated in the oviduct, uterus and vagina of mutant mice compared to controls, while mRNA for *Gli1* was elevated in the oviduct but not in the uterus or vagina (Fig. 2.2).

Over-activation of HH signaling alters development of the reproductive tract

To determine if over-activation of HH signaling alters development of the reproductive tract, gross morphology and histology were analyzed in *Amhr2*$^{+/+}$*SmoM2* control and *Amhr2*$^{cre/+}$*SmoM2* mutant mice. The normal coiling of the oviduct

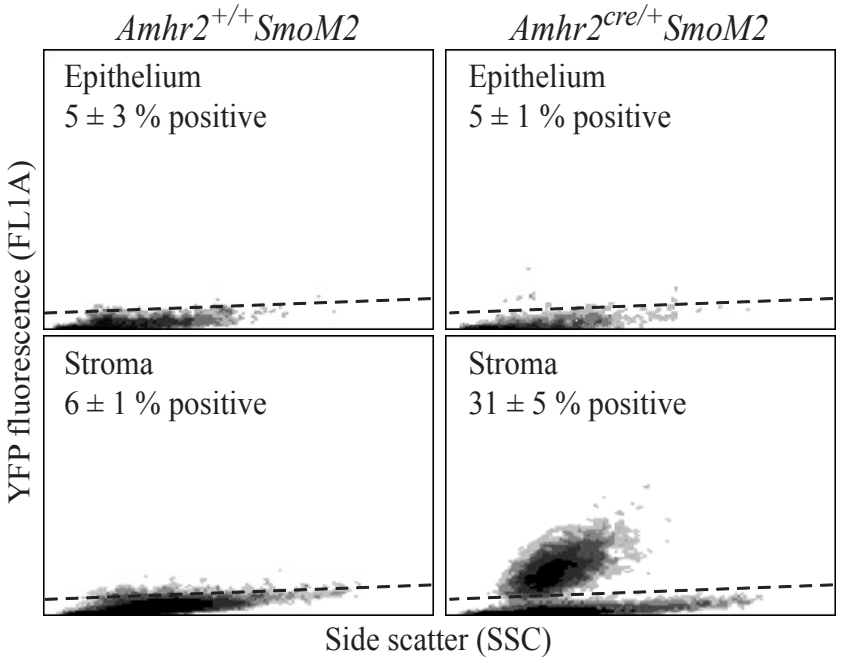

Figure 2.1. Assessment of the efficiency of CRE-mediated recombination in uterus of 24-day old *Amhr2*$^{cre/+}$*SmoM2* mice. The percentage of cells positive for YFP detected by flow cytometry in luminal epithelium and stroma. Each panel shows the mean percent YFP positive cells ± SEM (3 replicates) and a representative plot of fluorescence vs. side scatter.

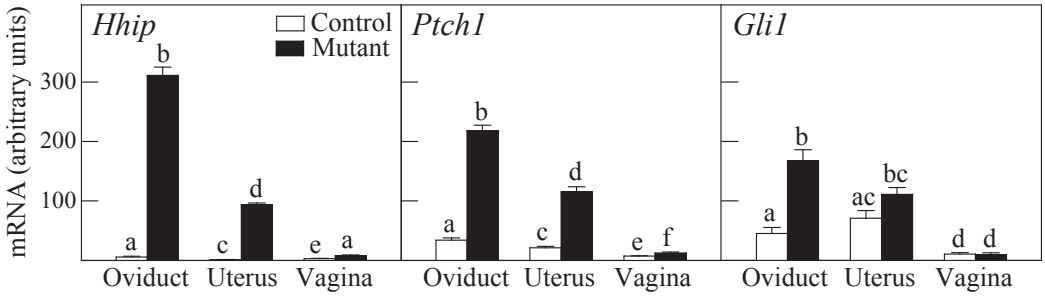

Figure 2.2. Steady-state levels of mRNA for transcriptional targets of HH signaling in the reproductive tract of 24-day old *Amhr2*$^{+/+}$*SmoM2* control and *Amhr2*$^{cre/+}$*SmoM2* mutant mice. Data are mean ± SEM, n=3. Bars with different superscripts are significantly different ($p < 0.05$).

observed in control mice was absent in mutant mice (Fig. 2.3A,B). Oviducts of mutants had a larger diameter and the junction between the posterior end of the oviduct and the anterior end of the uterus was ill-defined. Histological analysis confirmed that oviducts of mutant mice were uncoiled and revealed that the stromal area was increased and the luminal space was reduced compared to that in control mice (Fig. 2.3C,D). In addition, the infolding of the epithelium was absent in oviducts of mutant mice.

Uterine horns of mutant mice had a somewhat flattened, ribbon-like appearance (Fig. 2.4B) that was not observed in control mice (Fig. 2.4A). In uteri of control mice, the number of glands increased between 22 days of age (not shown) and 60 days of age (Fig. 2.4C). In contrast, uteri of mutant mice were essentially devoid of glands at all ages examined (60 days in Fig. 2.4D; 8, 22, 36 and 120 days, not shown). The luminal epithelium of the uterus in control mice consisted of a single layer of columnar cells (Fig. 2.4E), while in mutant mice large areas of the luminal epithelium had a stratified squamous morphology typical of that normally found in cervix and vagina (Fig. 2.4F). In control mice, the typical longitudinal and circular layers of the myometrium were easily visualized in sections stained with Mason's trichrome (Fig. 2.4G). While these muscle layers were also present in uteri of mutant mice, they had a less organized appearance (Fig. 2.4H).

Over-activation of HH signaling alters expression of genes from the Hox and Wnt families

To gain insight into genes that could be altered by expression of dominant active *SmoM2,* expression of genes within the *Hox* and *Wnt* families that are known to regulate reproductive tract development were examined. In control mice, *Hoxa9* was expressed at highest levels in the oviduct and uterus and at low levels in the vagina

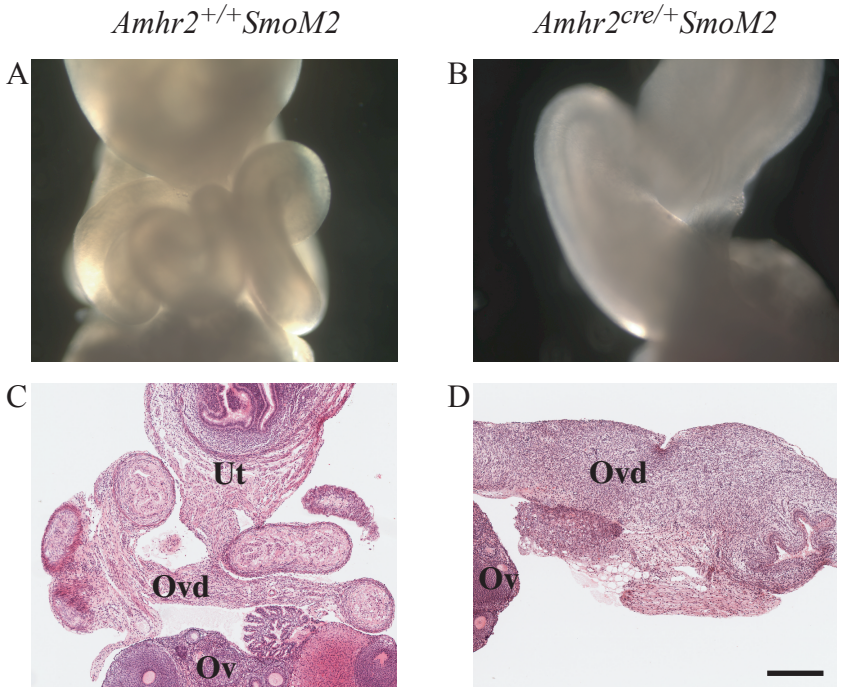

Figure 2.3. Morphological characteristics of the oviduct of *Amhr2*$^{+/+}$*SmoM2* control and *Amhr2*$^{cre/+}$*SmoM2* mutant mice. Gross morphology of oviducts of 8-day old mice (A,B). H&E stained sections through oviducts of 60-day old mice (C,D). Ovary (Ov), oviduct (Ovd), uterus (Ut). The size reference bar shown in panel D represents the following sizes in different panels: 400 µm (A,B), 450 µm (C,D).

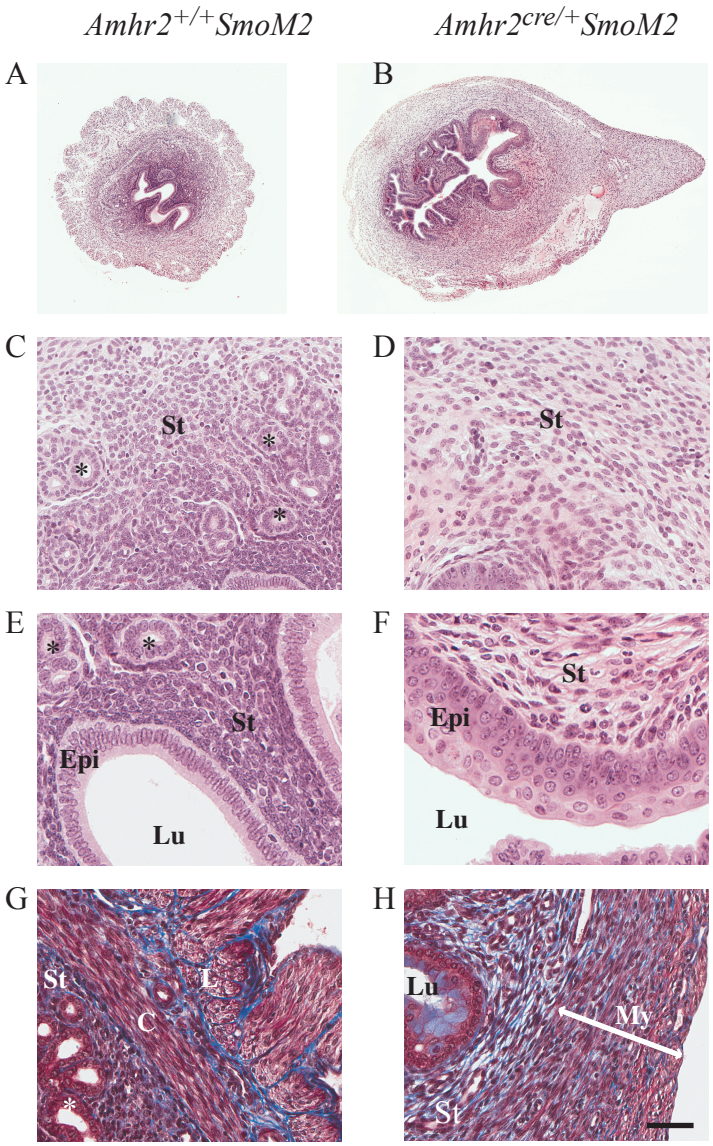

Figure 2.4. Morphological characteristics of the uterus of *Amhr2*$^{+/+}$*SmoM2* control and *Amhr2*$^{cre/+}$*SmoM2* mutant mice. H&E stained sections through uteri (A-F) and Masson's trichrome stained sections of uteri (G,H) of 60-day old mice. Myometrium (My), longitudinal muscle layer (L), circular muscle layer (C), stroma (St), luminal epithelium (Epi) and lumen (Lu). Asterisks mark examples of uterine glands. The size reference bar shown in panel H represents the following sizes in different panels: 300 µm (A,B), 80 µm (C,D), 50 µm (E,F) and 70 µm (G,H).

(Fig. 2.5). *Hoxa10* and *Hoxa11* were both expressed primarily in the uterus while *Hoxa13* was expressed primarily in the vagina (Fig. 2.5). While the pattern of expression of most of the *Hox* genes did not differ in the reproductive tract of mutant mice compared to controls, levels of *Hoxa13* were 12-fold higher in the uterus of mutant mice. Expression of *Wnt5a* and *Wnt7a* were highest in the oviduct and uterus of both control and mutant mice with relatively low expression in the vagina (Fig. 2.5). While expression of *Wnt7a* did not differ between mutants and controls, expression of *Wnt5a* was increased 1.7-fold in the oviduct and 1.8-fold in the uterus of mutant mice compared to controls (Fig. 2.5).

Inflammation of the reproductive tract after mating in Amhr2$^{cre/+}$SmoM2 mice

In order to asses fertility, *Amhr2$^{+/+}$SmoM2* control and *Amhr2$^{cre/+}$SmoM2* mutant mice at 55 to 68 days of age were caged with CD-1 males of proven fertility. While control females generated litters normally, mutant females failed to produce offspring. Five out of six mutant mice appeared to be in distress, and a bloody discharge from the vagina was sometimes observed. Mutant mice were therefore euthanized after 48±3 days of being caged with males. At necropsy, the uterus of one mutant mouse was apparently normal while the uteri of five mice were swollen and inflamed (Fig. 2.6A,B). Histology revealed extensive infiltration of leukocytes into the uterine lumen and the presence of necrotic cells in the lumen that had exfoliated from the luminal epithelium and stroma (Fig. 2.6C). In addition, large areas of the muscle layers appeared necrotic (Fig. 2.6D). The stroma appeared hyperplasic with evidence of cell proliferation (shown later).

To determine whether the inflammatory response may have been triggered by the presence of sperm in the tract, two control and four mutant females from 39 to 41 days of age were caged with vasectomized CD-1 males for 21 days and then

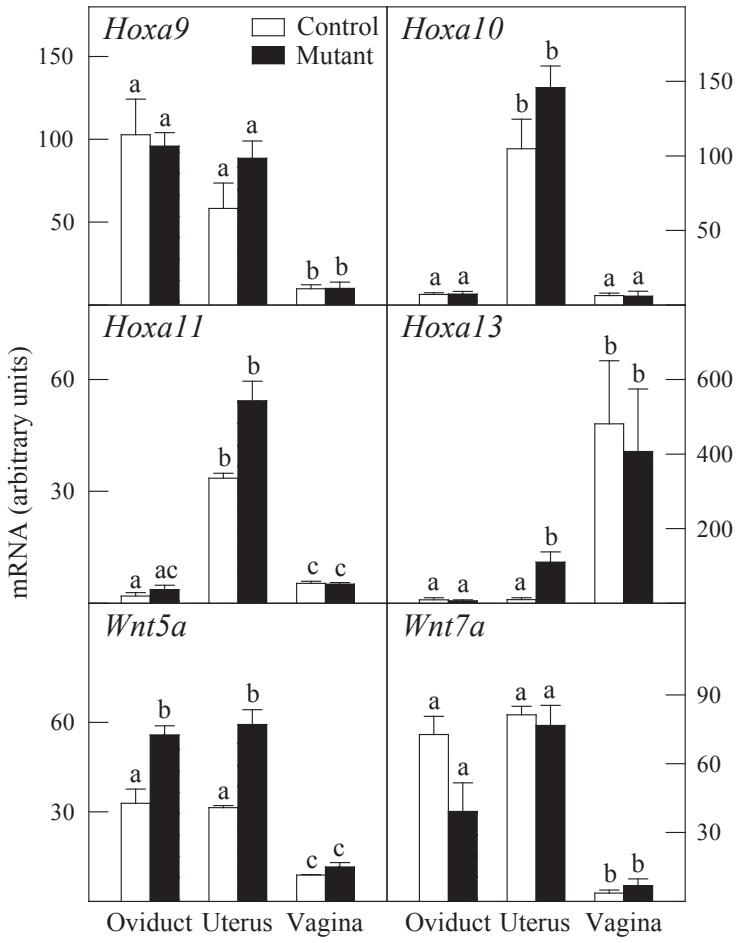

Figure 2.5. Steady-state levels of mRNA for members of the Hox and Wnt families in the reproductive tract of 24-day old *Amhr2*$^{+/+}$*SmoM2* control and *Amhr2*$^{cre/+}$*SmoM2* mutant mice. Data are mean ± SEM, n=3. Bars with different superscripts are significantly different ($p < 0.05$).

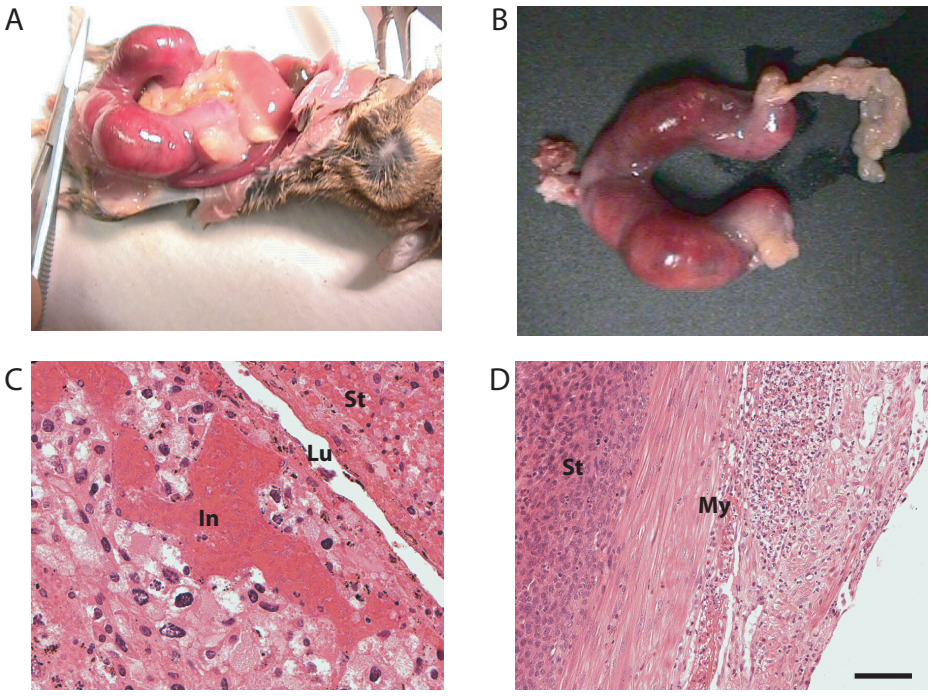

Figure 2.6. Inflamed tracts of *Amhr2*$^{cre/+}$*SmoM2* mutant females caged with intact males for approximately 48 days. Myometrium (My), stroma (St), lumen (Lu) and infiltrating cells (In) and cellular debris from exfoliated dead cells. The size reference bar shown in panel D represents the following sizes in different panels: 1 cm (B), 45µm (B) and 80 µm (C).

sacrificed. Two of the mutant females had uteri that were swollen and inflamed (Fig. 2.7B), while the gross appearance of tracts in the other two mutant mice was apparently normal. None of the control females displayed signs of inflammation (Fig. 2.7A). The histological appearance of the tracts of affected mice was similar to that described above for mutant mice caged continuously with intact males. However, the infiltration of inflammatory cells into the lumen was less pronounced and this made it easier to observe hyperplasia of the stroma (Fig. 2.7C), lack of infolding of the uterine luminal epithelium (Fig. 2.7D; compared to virgin control and mutant mice of similar age shown in Fig. 2.4) and the exfoliation of the luminal epithelium (Fig. 2.7E). Histology of the tracts that appeared grossly normal in two mutant mice revealed some infiltration of leukocytes and less dramatic signs of stromal hyperplasia and exfoliation of cells into the lumen (data not shown). To examine the early stages of inflammation, control and mutant females from 60 to 100 days of age were caged with vasectomized CD-1 males and sacrificed on the afternoon of day 3 of pseudopregnancy. At this time point, early signs of stromal hyperplasia and leukocyte infiltration were observed in mutant mice (Fig. 2.8B), but not in control females (Fig. 2.8A).

Discussion

The HH signaling pathway plays an essential role in implantation, acting downstream of the PGR (Lee et al., 2006b; Wakitani et al., 2008). Administration of progesterone increases expression of components of the HH signaling pathway –*Ihh*, *Ptch1* and *Gli* transcription factors – in mice (Lee et al., 2006a; Matsumoto et al., 2002; Takamoto et al., 2002), hamsters (Khatua et al., 2006), rats (Katayama et al., 2006; Kubota et al., 2008; Kubota et al., 2010), and humans (Wei et al., 2010).

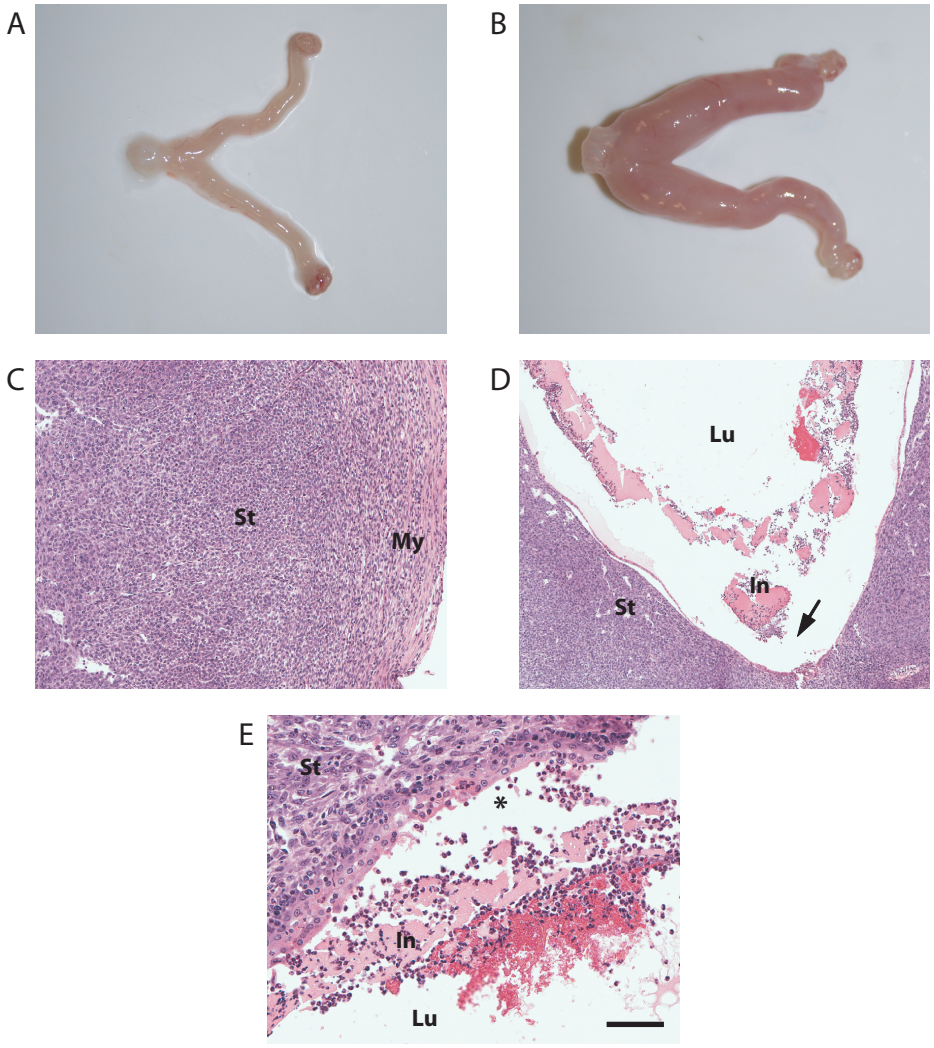

Figure 2.7. Inflamed tracts of *Amhr2*$^{+/+}$*SmoM2* control (A) and *Amhr2*$^{cre/+}$*SmoM2* mutant (B-E) females caged with with vasectomized males for 21 days (D-F). Myometrium (My), stroma (St), lumen (Lu) and infiltrating cells (In) and cellular debris from exfoliated dead cells. Arrow (D) points to the absence of luminal epithelium due to exfoliation, while the asterisk (E) shows exfoliation of the luminal epithelium into the lumen. The size reference bar shown in panel E represents the following sizes in different panels: 0.6 cm (A,B), 150 µm (C), 250 µm (D) and 60 µm (E).

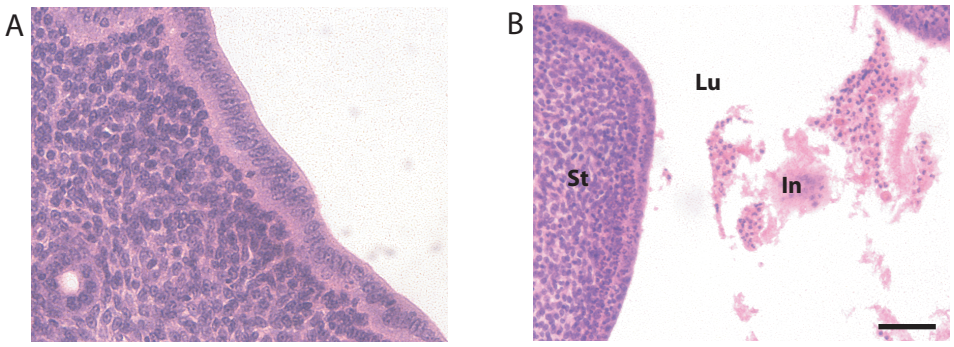

Figure 2.8. Uterus of *Amhr2*$^{+/+}$*SmoM2* control (A) and *Amhr2*$^{cre/+}$*SmoM2* mutant females (B) caged with vasectomized males and euthanized 3 days after copulatory plug was seem. Stroma (St), lumen (Lu) and infiltrating cells (In) and cellular debris from exfoliated dead cells. Scale bar represents 70 µm.

A potential role for the HH signaling pathway in the development of the reproductive tract has not been determined. Over-activation of the HH signaling pathway in the Müllerian duct in *Amhr2*$^{cre/+}$*SmoM2* mice altered the development of the reproductive tract. The major characteristics observed were lack of oviductal coiling, reduced number of uterine glands, and atypical stratified squamous luminal epithelium in the uterus. During the course of my studies using the *Amhr2*$^{cre/+}$*SmoM2* mouse model, a report was published on mice generated by crossing *SmoM2* mice with mice in which *Cre* sequence was inserted into the *Pgr* locus (Franco et al., 2010b). *Pgr*$^{cre/+}$*SmoM2* mice had uteri with atypical stratified squamous luminal epithelium and a reduced number of glands. In addition, the mice were infertile due to a defect in implantation and failed to undergo a decidual response. Because CRE-mediated recombination driven by PGR occurs in the luminal and glandular epithelium and stroma between 10 days to 28 days of age and in the myometrium at 8 weeks of age (Franco et al., 2008; Franco et al., 2010b) the phenotype observed is likely generated postnatally. In contrast, CRE-mediated recombination driven by *Amhr2*^{cre} occurs in the mesenchyme of the Müllerian duct as early as embryonic day 12.5 and, therefore, the phenotype observed in *Amhr2*$^{cre/+}$*SmoM2* mice may result from changes induced pre- and post-natally. *Pgr*$^{cre/+}$*SmoM2* mice and *Amhr2*$^{cre/+}$*SmoM2* mice had phenotypic similarities in processes that begin after birth, such as atypical stratified uterine luminal epithelium and reduced number of uterine glands. However, events that begin during embryonic life such as oviductal coiling, were abnormal in *Amhr2*$^{cre/+}$*SmoM2* mice but not in *Pgr*$^{cre/+}$*SmoM2* mice. Based on the phenotypes of *Amhr2*$^{cre/+}$*SmoM2* and *Pgr*$^{cre/+}$*SmoM2* mice we can conclude that processes such as development of uterine glands and differentiation of uterine luminal epithelium are affected by over-activation of HH signaling postnatally rather than prenatally. Oviductal coiling is impaired only when HH signaling is over-activated prenatally.

To obtain insight into effects of reducing HH signaling, two mouse models were created. One model consisted of ablation of floxed alleles of *Ihh* by Pgr^{cre} ($Pgr^{cre/+}Ihh^{f/f}$). These mice are infertile due to a defect in implantation of blastocysts. In addition, uterine expression of Mucin1 (*Muc1*) was elevated, and vascularization and proliferation of the stroma were reduced at the time of implantation (Lee et al., 2006). Furthermore, estrogen-induced signaling was increased and epidermal growth factor-mediated signaling was altered (Franco et al., 2010a); however the development of the FRT was normal. Another mouse model was created when mice with a null allele and a floxed allele of *Smo* were mated to $Amhr2^{cre/+}$ mice [manuscript submitted, (Harman et al., 2010)]. $Amhr2^{cre/+}Smo^{null/flox}$ mice had reduced litter size due to a delay in timing of implantation, but no obvious differences in the gross structures or histology of the tract were observed. These experiments suggest that conditional deletion of *Ihh* or *Smo* does not compromise development of the FRT; however HH signaling is necessary for successful implantation.

Components of the HH signaling pathway were expressed at detectable levels in the uterus of prepuberal rats (Katayama et al., 2006) and gilts (Bartol et al., 2006). Furthermore, microarray analysis showed that components of the HH signaling pathway were detected in uteri of 3-month old mice (Franco et al., 2010b). In real time RT-PCR assays in the current study, critical thresholds ranged between 21 and 29 for *Ptch1* and *Gli1* in the reproductive tract of 24-day old prepubertal control mice. These data suggest that HH signaling occurs at basal levels in prepubertal rats, gilts and mice. In $Amhr2^{cre/+}SmoM2$ mice, HH signaling was over-activated as demonstrated by elevated expression of transcriptional targets of the HH signaling pathway, *Gli1, Ptch1* and *Hhip* compared to control mice.

The process of oviductal coiling extends over the prenatal and postnatal periods. At embryonic day 17, the cranial section of the Müllerian duct has reduced

diameter compared with the rest of the duct. After birth, the oviduct begins to fold and coiling is complete by day 15 (Newbold et al., 1983; Price et al., 1969). Deletion of *Wnt7a* (Miller and Sassoon, 1998) or β-catenin (Deutscher and Hung-Chang Yao, 2007) or prenatal exposure to diethylstilbestrol (DES) (Newbold et al., 1983) inhibited coiling of the oviducts in mice. In *Amhr2*$^{cre/+}$*SmoM2* mutant mice, the level of *Wnt7a* in the oviduct was not significantly different from that in *Amhr2*$^{+/+}$*SmoM2* control mice; however, expression of *Wnt7a* was slightly reduced. In mice treated prenatally with DES, expression of *Wnt7*a in the reproductive tract was reduced at birth but not in older mice and the phenotype of the tract resembled that in *Wnt7a* null mice (Miller et al., 1998). It is possible, therefore, that expression of *Wnt7a* may have been altered at an earlier age in *Amhr2*$^{cre/+}$*SmoM2* mutant mice. *Amhr2*$^{cre/+}$*SmoM2* mutant mice had higher levels of *Wnt5a* in the oviduct and uterus compared to control mice. In *Wnt5a* null mice, development of the cervix and vagina were impaired while development of the oviduct occurred normally (Mericskay et al., 2004). It is possible that a particular level of expression of *Wnt5a* is required for normal development of the oviduct and that in *Amhr2*$^{cre/+}$*SmoM2* mice the elevated expression of *Wnt5a* in the oviducts may have contributed to the lack of oviductal coiling.

In mice, uterine tissue undergoes changes postnatally including formation of glands, differentiation of the myometrium and acquisition of the typical columnar morphology of the luminal epithelial cells. This process, known as radial patterning, is compromised in *Amhr2*$^{cre/+}$*SmoM2* mutant mice; uterine glands fail to form, the luminal epithelium aquires a stratified squamous organization typical of that of cervix and vagina and organization of the myometrium is somewhat abnormal. In addition, expression of *Hoxa13* and *Wnt5a* were elevated compared to control mice. *Hoxa13* is normally expressed in the cervix and vagina of mice (Warot et al., 1997). In an experiment in which *Hoxa11*, which is expressed in the uterus, was replaced by

Hoxa13, mice failed to develop uterine glands and luminal epithelium had a stratified squamous organization (Zhao and Potter, 2001). Furthermore, deletion of *Wnt5a* compromised gland development and muscle organization in uterine tissues (Mericskay et al., 2004). It is possible that increasing the expression of *Wnt5a* in the tract may also cause abnormal development. It is likely that ectopic expression of *Hoxa13* and increased expression of *Wnt5a* in the uterus of *Amhr2*$^{cre/+}$*SmoM2* mutant mice altered radial patterning of the tract.

Mesenchymal-epithelial interactions play an important role in patterning the FRT. In experiments in which vaginal and uterine stroma were recombined with vaginal and uterine luminal epithelium and then transplanted under the kidney capsule, luminal epithelia differentiated in a manner consistent with the stroma of the transplanted tissue (Kurita et al., 2001). In addition, estrogen receptor α (*Esr1*) in the stroma and not in the epithelium mediates the action of estrogen in the epithelium (Kurita et al., 2000). These data provides insight into the role of the mesenchyme in regulating development and hormonal control of the epithelium of the reproductive tract. *Wnt5a*, which is expressed in the uterine stroma, also plays a role in mesenchymal-epithelial interactions in the uterus by down regulating expression of *Wnt7a* in the luminal epithelium in response to estrogen (Mericskay et al., 2004). In *Amhr2*$^{cre/+}$*SmoM2* mutant mice, CRE-mediated recombination occurs in the mesenchyme and yet characteristics of both the stroma and epithelium of the tract are altered. It is hypothesized that mesenchymal-epithelial interactions in the FRT may be altered, and that altered expression of *Wnt5a* may be the causative factor.

In a number of tissues, HH has been shown to regulate muscle development. In *Xenopus*, inhibition of the HH signaling pathway by cyclopamine, prevented formation of the muscle layer of the lymph heart (Peyrot et al., 2010). In mice with deletion of *Shh* or *Ihh*, the thickness of the circular muscle layer of the intestine was

reduced at embryonic day 17.5 (Ramalho-Santos et al., 2000). In *Amhr2*$^{cre/+}$*SmoM2* mutant mice, the muscle layers of the uterus appeared disorganized compared to that in control mice

A severe inflammatory response of the reproductive tract occurred after mating in mutant mice. There are two normal inflammatory processes that occur in the uterus after mating and that are believed to play a role in pregnancy. The first occurs after mating and is caused by a class of high molecular weight proteins from the seminal vesicle (Robertson et al., 1996). This inflammatory response is characterized by the presence of a large number of leukocytes, lymphocytes and dendritic cells on day 1 of pregnancy and regression of inflammation by day 2 (Piazzon et al., 1985; Robertson et al., 2000; Tremellen et al., 1998). The inflammatory response after mating is thought to facilitate recognition of male antigens and maternal tolerance to the embryo. The second inflammatory response occurs at implantation and plays a role in selectively degrading molecules like MUC1 that are abundant in the uterus and that regulate implantation of the blastocyst at the appropriate site (Dekel et al., 2010). During this period, the uterus is characterized by expression of IL-6, IL-8 and TNF-α (Koga and Mor, 2008), and by the presence of natural killer cells, macrophages and dendritic cells (Dekel et al., 2010).

When *Amhr2*$^{cre/+}$*SmoM2* mutant females were bred, they did not produce any litters and they underwent a severe inflammatory response that was characterized by loss of luminal epithelium, and infiltration of immune cells into the lumen of the uterus. The inflammation occurred in response to mating with intact males or with vasectomized males, ruling out the possibility that sperm triggered the response. On day 3 of pseudopregnancy, mutant mice had immune cells in the lumen of the uterus, indicating symptoms of inflammation at a time point when the normal inflammatory response after mating should have regressed and the inflammatory response of

implantation should not have occurred. This suggests that over-activation of HH signaling in the reproductive tract or changes in the phenotypic characteristics of the tract lead to the increased intensity of the normal inflammatory response after mating. Similar inflammation in the uterus occurred after mating in mice in which the endogenous follistatin gene was deleted and a gene encoding an isoform of human follistatin (hFst315) was inserted. In this model, a large number of neutrophils were found in the lumen of the uterus after mating and the luminal epithelium degenerated (Lin et al., 2008). The mice had shorter uterine horns but the other aspects of reproductive tract development appeared normal. In *Pgr*$^{cre/+}$*SmoM2* mice, an obvious inflammatory reaction after mating was not reported (Franco et al., 2010b). This is interesting in light of the fact that a number of characteristics of the uterus in *Pgr*$^{cre/+}$*SmoM2* mice resemble that in *Amhr2*$^{cre/+}$*SmoM2* mice. Although definitive conclusions about the cause of inflammation cannot be drawn from my studies, phenotypic changes in the tract may not be primarily responsible.

Conclusion

Proper regulation of HH signaling is required for development of the FRT. Expression of dominant active *SmoM2* induced over-activation of the HH signaling pathway. Ectopic expression of *Hoxa13* in the uterus of *Amhr2*$^{cre/+}$*SmoM2* mutant mice could mediate the absence of uterine glands and the formation of atypical stratified squamous luminal epithelium. Because *Wnt5a* is expressed in the stroma of the tract and is known to mediate mesenchymal-epithelial interactions, increased expression of *Wnt5a* in the oviduct and uterus of mutant mice may interfere with normal signaling essential for tract development. It is also possible that increased expression of *Hoxa13* and *Wnt5a* in the reproductive tract of mutant mice represents an effect rather than a cause of abnormal patterning. Over-activation of HH signaling

in mutant mice may increase the intensity of the normal inflammatory response that occurs at mating.

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