Hedgehog signaling plays a critical role in directing correct primordial follicle formation in the mouse ovary

Honors Thesis
Presented to the College of Agriculture and Life Sciences,
Department of Animal Science
of Cornell University
in Partial Fulfillment of the Requirements for the
Research Honors Program

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May 2007

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Abstract:

The Hedgehog (HH) signaling pathway has been identified to play an essential role in cell differentiation, cell fate determination and cell proliferation in both *Drosophila* and mammalian models. While much is known regarding HH actions in the *Drosophila* ovary, its role in the developing mammalian ovary remains a mystery. In the days following birth, the mouse ovary transitions from an organ filled with ovigerous cords to one filled with primordial follicles, which are oocytes surrounded by a layer of flattened pre-granulosa epithelial cells, enclosed by a basement membrane, and associated with mesenchymal cells. The purpose of this study was to determine the role HH plays in primordial follicle formation in the mouse ovary. HH signaling was blocked by injecting cyclopamine, a plant alkaloid known to inhibit HH signaling, on days 0, 1, 2 and 3 after birth. Immunohistochemistry using an antibody for laminin, a common basement membrane protein, revealed a significantly higher number of discontinuous basement membranes around primordial follicles in ovaries extracted from cyclopamine-treated mice. H&E staining revealed a significantly lower ratio of mesenchymal cells to oocytes in ovaries from cyclopamine-treated mice, as compared to controls. The reduced HH signaling in this study is thought to have disrupted proper ovigerous cord breakdown and interfered with normal cell communication among oocytes, epithelial cells and mesenchymal cells, suggesting that the HH signaling pathway does play a critical role in directing correct primordial follicle development. This study provides valuable insight into the role HH plays in the developing mouse ovary.

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Introduction:

In mice, the formation of ovarian follicles occurs during the first few days of life. At birth, the mouse ovary contains structures referred to as ovigerous cords which are membrane-bound sacs of oocytes and epithelial cells surrounded by mesenchymal cells. Functional and structural organization of the ovary occurs during the first 4 to 8 days postpartum. During this time, ovigerous cords breakdown and somatic cells associate with oocytes to form primordial follicles [1]. Primordial follicles are composed of a quiescent oocyte surrounded by a flattened layer of pre-granulosa cells, enclosed by a basement membrane. Mesenchymal cells in the region surrounding primordial follicles are eventually incorporated into an outer theca layer as the follicle grows and develops [1]. Primordial follicle formation is directed by cell to cell signaling by locally produced autocrine and paracrine growth factors [2]. Inappropriate expression and action of these factors can lead to incomplete cord break down and altered ovarian physiology [2]. Primordial follicles represent the total follicular reserve. This is the total number of follicles available for development and ovulation during a female's lifespan, and as such is a critical indicator of fertility.

The HH signaling pathway was first identified in *Drosophila*, and was shown to play an important role in cell differentiation, cell fate determination and cell proliferation. It was also shown to be essential for proper ovarian function. In addition, the HH pathway was found to play an important role in numerous aspects of development in mammals. Its function in the mammalian ovary is just beginning to be studied [3, 4, 5].

In mammals, the HH signaling pathway utilizes three protein ligands: sonic (SHH), desert (DHH) and Indian (IHH) [6]. These ligands bind to the transmembrane receptor

patched (PTCH). In the absence of the HH ligand, PTCH blocks the activity of the transmembrane signal transducer protein smoothened (SMO) [7]. The binding of HH to PTCH removes the inhibition of SMO, resulting in activation of downstream transcription factors that then alter gene transcription [7].

Results of several studies have demonstrated that a functional HH pathway is present in the theca and granulosa cells of the mouse ovary [3, 4]. Research to date has focused on the role of HH in developing follicles in the immature and adult ovary [3, 4, 5]. Yet, components of the HH signaling pathway are also expressed in the newborn ovary [3], suggesting a potential role for HH in the formation of primordial follicles. Preliminary data from Dr. Quirk's lab suggest that early follicle development in the neonatal ovary is altered when HH signaling is reduced experimentally. The goal of the research reported in this thesis was to determine the role of the HH signaling pathway in the development of the mouse ovary. The strategy used was to examine the effects of reducing HH signaling in the neonatal mouse ovary on the assembly of primordial follicles. The hypothesis tested is that HH signaling is required to direct correct primordial follicle formation.

Literature Review:

The mouse ovary is incompletely developed at the time of birth. During the first 4 to 8 days postpartum the ovary undergoes substantial functional and structural organization as it completes development. This process involves the rearrangement of associations among somatic cells and germ cells to form primordial follicles. This is a dynamic process that is just beginning to be fully understood, but is believed to be directed by a variety of cell

signaling pathways. The focus of the research reported in this thesis is to determine whether the hedgehog (HH) signaling pathway is involved in this early ovarian development.

Hedgehog Signaling

The HH signaling pathway was first found in *Drosophila* while screening for mutations that disrupt the larval body plan [6]. Since its discovery, HH has been studied extensively in *Drosophila* and shown to be important for development of wings, legs, eyes, abdomen, gut, trachea and gonads [6]. In the gonad, it is involved in germ cell migration, cell proliferation and polarization [6].

The HH pathway also plays an important role in mammals. It is essential for proper embryonic development and adult tissue maintenance. In the embryo, HH is involved in the development of multiple organs by regulating cell proliferation and differentiation, as well as directing cell fate [6]. In adult tissue, the HH pathway can be reactivated by tissue damage and stimulates tissue repair [8].

HH signals are received, transmitted and interpreted using pathways that have been highly conserved during the divergent evolution of insects and mammals [7]. While only one HH ligand has been found in *Drosophila*, three HH ligands exist in the mammal: sonic (SHH), Indian (IHH), and desert (DHH), with the DHH ligand being most closely related to the *Drosophila* ligand [6]. All three HH ligands are secreted proteins that bind to the transmembrane receptor patched (PTCH).   In the absence of the HH ligand, PTCH blocks the activity of the transmembrane signal transducer protein smoothened (SMO) [7]. The binding of HH to PTCH removes the inhibition of SMO. SMO is then able to undergo a

conformational change that allows it to activate a downstream group of transcription factors which act to alter target gene expression [7].

In *Drosophila*, the target transcription factor is the segment-polarity gene: *cubits interruptus* (*Ci*) [7]. In resting cells, *Ci* is phosphorylated, which serves to repress transcription of target genes. In the presence of HH signaling, however, *Ci* is not phosphorylated and is able to activate transcription of HH target genes [7].   In mammals transcription factors include three Ci-related proteins from the GLI-Kruppel family, GLI1, GLI2, and GLI3, which act as either repressors or activators depending on posttranslational processing [6]. GLI1 serves as an activator of HH target gene transcription, while GLI3 acts as a repressor of both HH target gene transcription and *Shh* expression [6]. HH target genes include *Ptch* and *Gli1* [6], suggesting that the HH signaling pathway works to modulate its own activity.

Disruption of HH signaling has been shown to have devastating effects upon an individual.   In mice, the absence of HH signaling prenatally results in embryo lethality [9]. Mutation and deregulation of the HH pathway plays a role in tumorigenesis, with over expression of the HH pathway being linked to basal cell carcinomas and other cancers [8]. Furthermore, in male mice in which the *Dhh* gene has been deleted, abnormal testes development and sterility are observed [10, 11].

In *Drosophila*, HH signaling stimulates proliferation of ovarian somatic stem cells, promotes polarization of follicle cells, and migration of the primordial germ cells to the gonads in the embryo [6, 12, 13]. Since HH is essential for proper ovarian function in *Drosophila*, it has been postulated that it is also essential in the mammalian ovary.

Primordial Follicle Formation in the Mouse Ovary

At birth, the mouse ovary consists of structures called ovigerous cords, which are basement-membrane-enclosed sacs of oocytes and epithelial-type somatic cells surrounded by mesenchymal-type cells [1]. The basement membrane surrounding the cords is not thought to be a permanent continuous structure, and as such, intermingling between cells in different regions may occur [1]. The oocytes within the cords are interconnected via intracellular bridges, creating a cyst-like structure [13]. Research suggests this cyst-like structure is the result of synchronous division of a progenitor germ cell during the embryonic time period [13]. Cyst breakdown occurs around the time of birth; however before it occurs mitochondria migrate via intracellular bridges to adjacent oocytes [14]. It has been postulated that this ensures that oocytes destined for future primordial follicle formation acquire functional mitochondria [14].

During the first few days of life, the ovigerous cords become fragmented and tissue remodeling leads to the formation of the first follicles, primordial follicles. Epithelial-type pre-granulosa cells envelop individual oocytes to form primordial follicles. A primordial follicle is composed of a quiescent oocyte surrounded by a flattened layer of epithelial pre-granulosa cells and enclosed by a newly synthesized basement membrane [1]. Mesenchymal cells in the region surrounding primordial follicles eventually become incorporated into an outer theca cell layer of the follicle as it grows and develops, in a process that is poorly understood [1]. Recent research suggests that HH signals from granulosa cells may act on the mesenchymal cells to promote development of the theca [4].

Synthesis of the basement membrane around follicles is a poorly understood process. It has been suggested that the basement membrane components (type I collagen, type IV

collagen, laminin and fibronectin) may all be primarily synthesized by the theca compartment surrounding the outside of the basement membrane of the follicle [1, 15]. In the testis, it has been suggested that epithelial and mesenchymal cells interact to assemble the basement membrane [16]; however the appearance of a similar mechanism in the ovary has yet to be investigated. Laminin appears to be the only protein present in basement membrane surrounding primordial follicles in mice [17]. In primordial follicles, laminin forms a ring around each granulosa cell compartment, which functions to define the basement membrane that separates the primordial follicle from the surrounding area. Each laminin molecule is composed of one α, one β, and one γ chain, yet multiple varieties of each chain occur. The composition of the laminin chain affects the fate of the associated cells, and thus laminin chain composition varies in different tissues [18]. Previous studies suggest that the α1, β1, and γ1 laminin chains are present in the basement membrane surrounding primordial follicles in cows, pigs and rabbits [18, 19].

Primordial follicle formation does not occur in a uniform pattern throughout the ovary. At birth, ovigerous cords are more densely packed in the medulla of the ovary [20]. These cords contain a high number of oocytes with few epithelial cells. As the cords break down, primordial follicles first develop in the medulla [21]. These first primordial follicles are characterized by round, cubodial pre-granulosa cells. Primordial follicles in the cortex appear a little later and differ in the fact that the pre-granulosa cells are flattened, squamous cells [21]. In the first few days postpartum, it is common to see areas of fully enclosed follicles neighboring areas still exhibiting cord-like patterns [20].

Primordial follicle formation is under the control of cell to cell signaling; however which cells are signaling is still debatable. An old idea suggests that signaling between pre-

granulosa cells and the oocyte directs primordial follicle formation [22]. A second idea suggests that signals from mesenchymal cells direct primordial follicle formation since their presence is thought to be required for the organization of the basement membrane that encloses the primordial follicle [22]. In general, a basement membrane is thought to be organized when epithelial cell and mesenchymal cell interactions occur, suggesting the possibility that epithelial pre-granulosa cells and mesenchymal cells interact to form the basement membrane that surrounds primordial follicles [22].

Recent research suggests both of these ideas may be correct, with cell signaling occurring between the pre-granulosa cells and oocyte, as well as the pre-granulosa cells and the mesenchymal cells. Primordial follicle formation is coordinated by locally produced paracrine and autocrine growth factors [2, 9]. Interactions between the pre-granulosa cells and the oocyte as well as interactions between the pre-granulosa cells and mesenchymal cells may be needed for proper primordial follicle assembly, such that inappropriate expression and actions of these factors may result in abnormal ovarian physiology [2].

Primordial follicles represent the follicular reserve, the total number of follicles available for development during a female's lifespan, and are thus critical for fertility. Primordial follicles become quiescent once formed. Throughout an individual's lifetime, primordial follicles are continuously recruited from this follicle reserve to undergo further growth and development. Previous studies suggest that the overall number of quiescent follicles in the ovary affects the rate at which follicles are recruited into the growing pool [23]. There is an inverse correlation between the number of primordial follicles and the rate at which they are stimulated to grow [23]. A reduction in the size of the primordial stockpile will result in an increase in the proportion of follicles beginning to grow. If too many

follicles are recruited at once it could have devastating effects on fertility, as the animal may run out of follicles prematurely. A complex network of cell to cell signaling, including the actions of locally produced growth factors, is required for the transition from primordial to a growing primary follicle [2]. If disruption of these signals occur, potentially too many primordial follicles could be recruited into the growing pool leading to depletion of the follicle reserve.

Hedgehog's Role in the Mouse Ovary

The HH pathway has previously been found to be expressed in the adult mouse ovary. Granulosa cells express all three HH ligands, while granulosa and theca cells express PTCH and SMO [3]. The oocyte is thought to be unable to respond to HH because it doesn't express *Smo* [4]. Granulosa cells of primary follicles have been found to secrete HH, which can act upon the surrounding pre-theca compartment [4]. Granulosa cells have also been identified as a target for HH signaling, with an increase in HH signaling causing a small increase in granulosa cell proliferation and increased expression of the transcriptional target gene *Gli1* [3]. Components of the HH signaling pathway are also expressed in the newborn ovary, with *Shh*, *Ihh* and *Dhh* all being expressed in low levels on day 0 [3]. This suggests a potential role for HH in the formation of primordial follicles.

Materials and Methods:

Inhibition of HH signaling

Cyclopamine is a plant alkaloid and a known pharmacological inhibitor of HH signaling. Cyclopamine (BioMol, Plymouth Meeting, PA) was dissolved in 2-hydroxypropyl-

β-cyclodextrin solution (Sigma-Aldrich Inc., St. Louis, MO). CD-1 female pups were injected i.p. with two different doses of cyclopamine (2mg/kg or 5mg/kg) or vehicle (2-hydroxypropyl-β-cyclodextrin solution) on days 0 (day of birth), 1, 2 and 3 of age. Ovaries were then isolated on day 4 for analysis. Ovaries were also isolated from uninjected control pups on day 0. In addition, some mice that were treated with either vehicle or high dose cyclopamine from days 0-3 were allowed to reach puberty and were then caged with an adult, fertile CD-1 male. At day 30, ovaries from these mice were collected for analysis.

Immunohistochemistry:

Day 4 ovaries from vehicle-injected and cyclopamine-injected mice (2mg/kg and 5mg/kg) were fixed in 2% paraformaldehyde. Fixed ovaries were embedded in paraffin wax and 5μM sections were cut by the Histology Core Facility, Cornell University, NYS College of Veterinary Medicine. Sections were deparaffinized in xylene, rehydrated by stepwise incubation in 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol and then rinsed twice in PBS. Nonspecific binding was blocked at room temperature for 30 minutes in 2% normal goat serum in PBS. Sections were incubated with rabbit polyclonal antiserum (8 μg/mL) raised against laminin-1 isolated from Englebreth Holm-Swarm (EHS) tumors (Sigma-Aldrich Inc., St. Louis, MO) in PBS + 2% BSA in a humidified chamber for 2 hrs at room temperature. Sections were rinsed with PBS four times and then incubated with AlexaFluor555 goat anti-rabbit IgG (0.25 μg/mL; Molecular Probes, Eugene, OR) in PBS + 2% BSA for 1 hr at room temperature. Slides were rinsed 4 times in PBS and then counterstained with Hoechst (Molecular Probes, Eugene, OR). Staining was performed on

ovary sections from day 4 control and cyclopamine-injected mice, as well as day 0 uninjected control ovaries (n =3 per treatment).

Stained sections were evaluated using a fluorescence microscope. Pictures of 4 unique fields were taken from 2 to 4 ovary sections per mouse. The completeness of the laminin rings around primordial follicles was then evaluated in each field, such that each mouse had four fields evaluated. The number of complete and incomplete laminin rings found in the four fields was summed and a percent of incomplete and complete laminin rings was calculated for each mouse.

Laminin encapsulation of primordial follicles was considered to be complete if there were no gaps or fragmentation of the laminin ring around the primordial follicle. Laminin encapsulation was scored as incomplete if there were gaps or fragmentation present in the laminin ring. The degree to which the laminin ring was incomplete was then graded on a rubric scale to assign the following scores: 0, presence of a complete laminin ring; 1, presence of a laminin ring around an oocyte that is greater than 50% complete; 2, presence of a laminin ring around an oocyte that is half complete; 3, presence of a laminin ring around an oocyte that is less than 50% complete.

Hematoxylin & Eosin staining:

Morphology of the developing follicles was evaluated in sections of day 4 ovaries fixed in Bouin's solution and stained with hematoxylin and eosin. Ovaries from three control and three high dose cyclopamine-treated (5mg/kg) mice were evaluated. The number of oocytes, pre-granulosa (epithelial) cells and mesenchymal cells were counted in 4 unique fields per mouse to yield average epithelial: oocyte and mesenchymal: oocyte ratios for each

mouse. Epithelial cells were identified by their dark, central nucleus and the fact that staining by hematoxylin was lighter than that for mesenchymal cells. Mesenchymal cells were identified by their more flattened shape and darker staining with hematoxylin. Day 30 ovaries from control and high-dose cyclopamine-treated mice (n =3 per treatment) were also stained. The morphology of these ovaries was then observed.

RT-PCR for Gli1 expression:

In order to determine whether HH signaling had been inhibited through this model, *Gli1* expression in the ovary was evaluated. RNA was prepared from whole ovaries from day 4 control and high dose cyclopamine-treated mice (5mg/kg) using a RNeasy MiniKit. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real time PCR for *Gli1* was performed on an ABI Prism 7000 using kits purchased from Applied Biosystems, Foster City, CA (assay number Mm00494645_m1). The assay used spans exons 2 and 3. cDNA from RNA will be amplified with this assay, but genomic DNA will not be amplified because of the large introns present in genomic DNA between exons 2 and 3. A standard curve was constructed from cDNA prepared from RNA of pooled whole ovaries. 18s rRNA concentration was also measured (assay number 4319413E). Results were standardized by dividing by the 18s rRNA concentration and multiplying by 100.

Fertility Evaluation:

Some mice that were treated with low and high doses of cyclopamine from days 0-3 were allowed to reach puberty and were then caged with an adult, fertile CD-1 male.

Litter sizes were recorded for all females that became pregnant.

Statistical Analysis:

A T-test was used to test the difference in the total number of incomplete laminin rings in control versus cyclopamine-treated mice. A t-test was also used to test differences in the ratios of epithelial cells: oocytes and mesenchymal cells: oocytes in control and cyclopamine-treated mice. The differences in ovarian *Gli1* expression in cyclopamine-treated and control mice were tested by a t-test.

Results:

Quantitative real time RT-PCR was used to evaluate *Gli1* expression levels in vehicle-injected and high dose cyclopamine-injected ovaries. This was done to determine if HH signaling had been inhibited by the cyclopamine injections. 18s ribosomal RNA, a "housekeeping" gene, was used to standardize the values. High dose cyclopamine-treated ovaries had a significantly lower expression level of *Gli1* (p= 0.05) (Fig 1). This shows that cyclopamine injections reduced expression of the HH transcriptional target gene *Gli1*, suggesting that cyclopamine treatment did reduce HH signals.
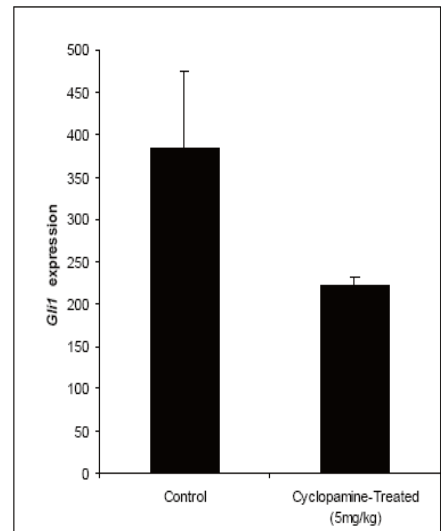

Figure 1: Expression level of downstream transcription factor, *Gli1*, in control and cyclopamine-treated animals (n=3). *Gli1* expression was determined using quantitative real time RT-PCR. Values were standardized through comparison to 18s RNA results. Mean ± SE is shown. *Gli1* expression is significantly lower in cyclopamine-treated mice (p = 0.05).

Immunohistochemistry to detect laminin in ovaries of uninjected mice on day 0 revealed the presence of ovigerous cords, as expected. Oocytes were arranged mainly in long

chains that were surrounded by somatic cells and enclosed by a laminin-containing basement membrane (Fig 2A). The ovigerous cords varied in size, with sections through some containing only two oocytes and others containing as many as 10 to 15.

Ovaries from vehicle-injected mice on day 4 showed expected cord breakdown and primordial follicle development. Single oocytes were surrounded by a flattened ring of somatic cells and a laminin-containing basement membrane (Fig 2B). On average, 69.8% of
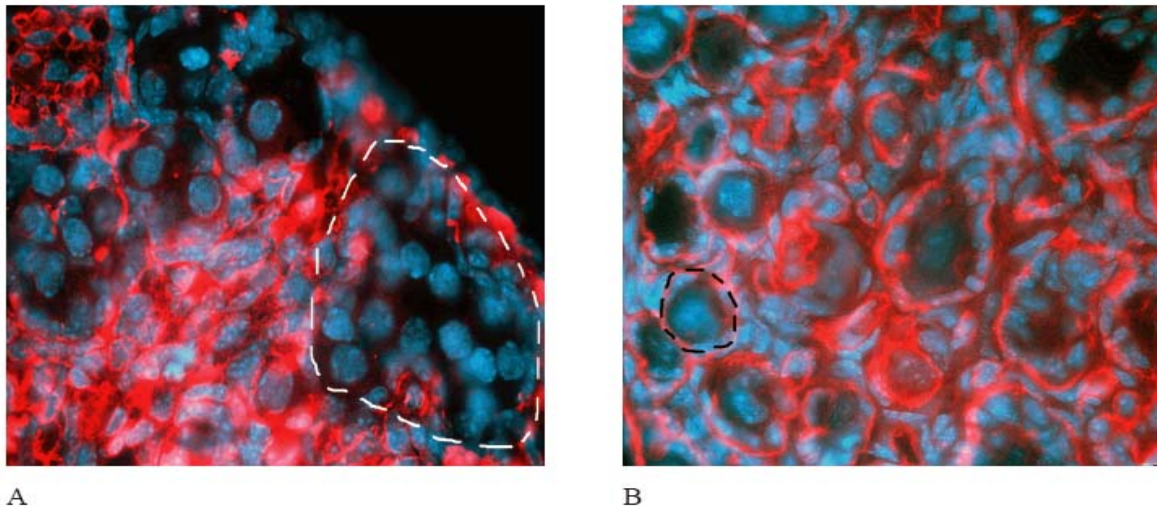

A   B

Figure 2: Laminin staining for day 0 (A) and day 4 (B) control ovaries from CD-1 mice at 100x magnification. Red is laminin stain. Blue is nuclear stain. The day 0 ovary shows ovigerous cords (example indicated within the white dashed line) with oocytes and somatic cells enclosed in a basement membrane. The day 4 ovary shows primordial follicles (example indicated within the black dashed line) enclosed by complete basement membranes.

Table 1: Percentage of primordial follicles surrounded by a basement membrane at each laminin ring score

Treatment Group	Laminin Ring Score			
	0*	1*	2*	3*
Control	69.8	14.1	12.3	3.7
Low-dose cyclopamine (2 mg/kg)	60.8	13.8	15.7	10.2
High-dose cyclopamine (5mg/kg)	26.1	21.8	27.2	24.9

*0, 1, 2 and 3 are the laminin ring scores, which is the degree to which the laminin ring around the follicle was incomplete with: 0 being a completely continuous laminin ring, 1 being a mostly complete laminin ring with a few breaks, 2 being a half-enclosed laminin ring and 3 being a very discontinuous laminin ring with less that 50% of the follicle enclosed by laminin.

primordial follicles were enclosed by a complete basement membrane, with a score of 0

(Table 1). There were few areas of the ovaries in which basement membranes surrounding

primordial follicles were incomplete. Only 3.7% of follicles had basement membranes that

were very discontinuous, with a score of 3. Primary follicles were present in day 4 ovaries

and were concentrated in the center, medullary region of the ovary.

In contrast to control mice, mice treated with low and high doses of cyclopamine had

an increased frequency of primordial follicles surrounded by basement membranes that were

incomplete. This effect was most pronounced in mice treated with the higher dose of

cyclopamine in which 24.9% of follicles had discontinuous membranes with a score of 3 and

only 26.1% of follicles had complete basement membranes with a score of 0 (Table 1). As

shown in Figure 3C, ovaries consisted mainly of primordial follicles enclosed by incomplete

basement membranes with large gaps. In addition, a portion of oocytes seemed to have

associations with only a few somatic cells and had minimal amounts of laminin surrounding

them. The effect was less pronounced in mice treated with a low dose of cyclopamine

(2mg/kg) in which only 10.2% of follicles had discontinuous basement membranes with a

score of 3 and 60.8% of follicles had complete basement membranes with a score of 0. As

shown in Fig 3B, areas of primordial follicles with complete basement membranes and areas

with incomplete basement membranes were both apparent.

The phenotype observed in mice treated with the high dose of cyclopamine was

variable. Ovaries from two of the mice had the phenotype described above. The ovary from

a third mouse had areas of relatively normal primordial follicle development in which

oocytes were surrounded by a ring of flattened somatic cells and enclosed by a basement

membrane that was often incomplete. However, the same ovary had areas of densely packed
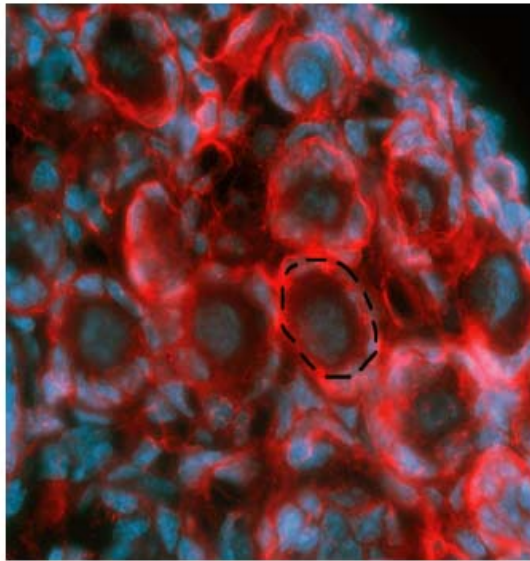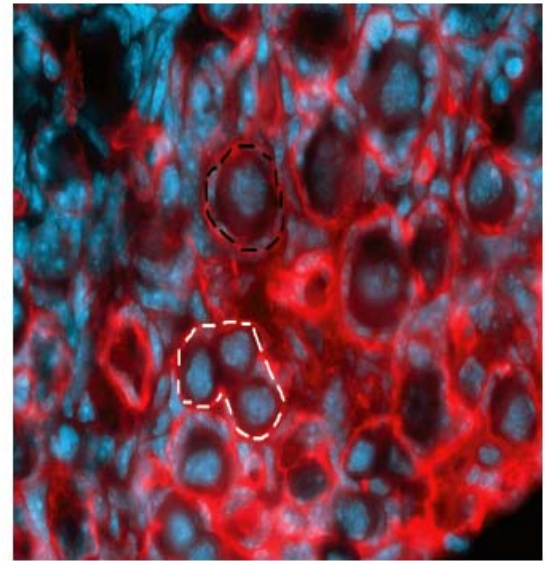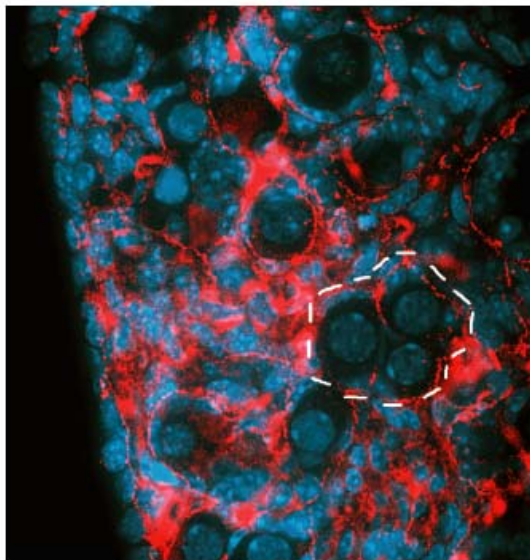
Figure 3: Laminin staining for day 4 control and cyclopamine-treated mice. Red is laminin stain. Blue is nuclear stain. Control ovary section (A), low-dose cyclopamine (2 mg/kg) ovary section (B) and high-dose cyclopamine (5 mg/kg) ovary section (C) are shown at 100x magnification. Incomplete laminin rings (example indicated within the white dashed line) are seen most frequently the high-dose cyclopamine animals, while complete laminin rings (example indicated within the black dashed line) are seen most frequently in controls. Low-dose cyclopamine has an intermediate phenotype.
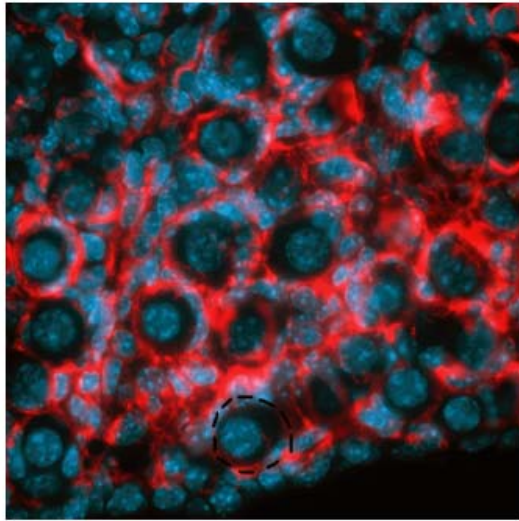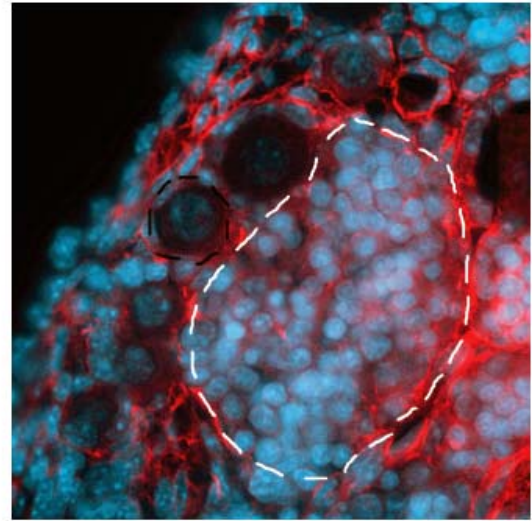
A   B

Figure 4: Laminin staining for day 4 high-dose cyclopamine-treated (5mg/kg) mice at 100x magnification. Red is laminin stain. Blue is nuclear stain. There was a large diversity in the expressed phenotype in high-dose mice. Some mice showed the expected phenotype (A) with discontinuous basement membranes and multiple oocyte follicles. One mouse (B) showed a variant phenotype. This mouse had sections with primordial follicles surrounded by incomplete laminin rings (example within the black dashed line), but also sections of densely-packed cells enclosed by a laminin ring (example within the white dashed line). Oocytes do not seem apparent in these regions of densely-packed cells.

cells containing mostly somatic cells and a few scattered oocytes, surrounded by a basement membrane (Fig 4B).

Despite some variation in the phenotype of mice treated with the high dose of cyclopamine, the percentage of follicles with basement membranes that were incomplete was significantly higher than in control mice ($p < 0.05$) (Fig 5). A similar statistical analysis was not possible for mice treated with the low dose of cyclopamine because the sample size was too small (n=2).
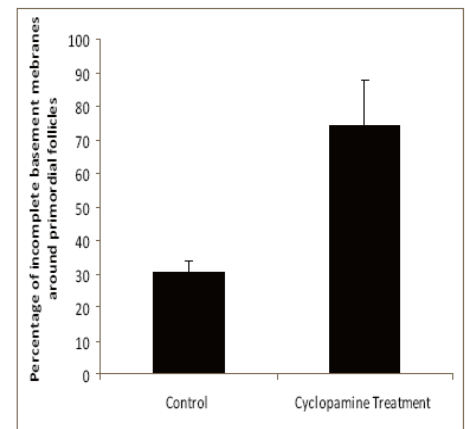

Figure 5: The percentage of incomplete laminin rings around primordial follicles in day 4 ovaries from vehicle-injected control and high-dose cyclopamine-injected (5mg/kg) mice (n=3). An incomplete laminin ring is a discontinuous basement membrane surrounding the primordial follicle. To calculate the percentage incomplete, the percentages of primordial follicles with laminin ring scores of 1, 2 and 3 were added. Mean ± SE is shown. The percent incomplete is significantly higher in cyclopamine-treated animals, compared to controls ($p < 0.05$).
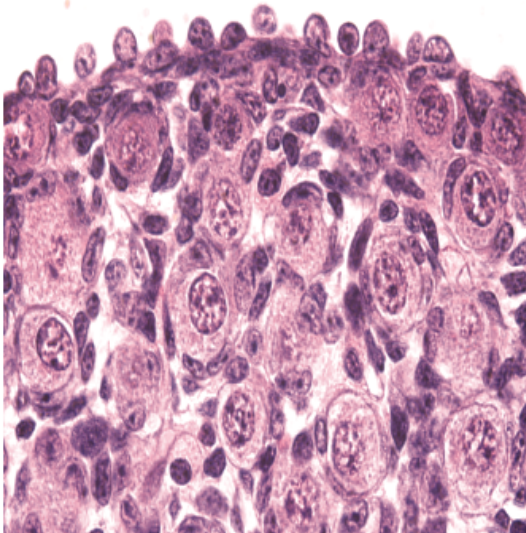
20

Control

Cyclopamine (5mg/kg)
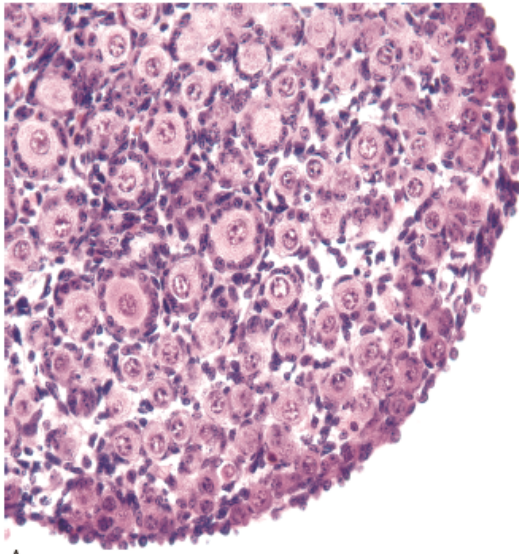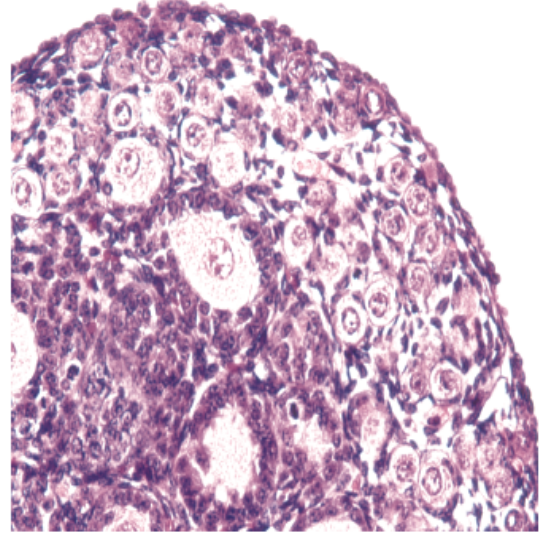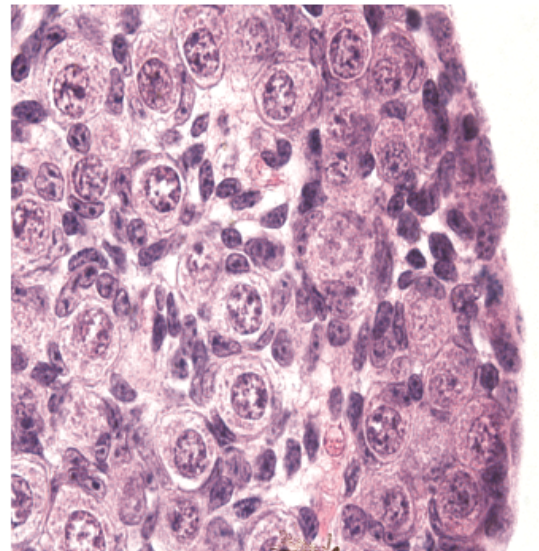
Figure 6: H&E staining of day 4 control and high-dose cyclopamine-treated (5mg/kg) ovaries. Control ovary sections at 40x (A) and 100x (C) magnification, and high-dose cyclopamine (5 mg/kg) at 40x (B) and 100x (D) are shown. Epithelial cells are identified by their dark, central nucleus and lighter staining with hematoxylin. Mesenchymal cells are identified by their flattened shape and darker staining with hematoxylin. In control animals, mesenchymal cells have become associated with the primordial follicles. In cyclopamine-treated animals, mesenchymal cells are visible but occur in streaks or patches rather than being evenly distributed among primordial follicles.

H&E sections of day 4 ovaries from control and high dose cyclopamine-treated

mice (n=3) were evaluated for differences in the ratio of epithelial and mesenchymal cells

to oocytes present. In control and cyclopamine-treated mice, primordial follicles were

surrounded by a layer of flattened pre-granulosa (epithelial) cells (Fig 6). In controls, a

layer of mesenchymal cells is often found surrounding the epithelial layer associated with

an individual oocyte (Fig 6A and C). Mesenchymal cells appeared to be interspersed

between individual follicles. Contrastingly, in cyclopamine-treated animals

mesenchymal cells were most often in long chains or clumps, rather than interspersed

between and associating with individual follicles (Fig 6B and D).

The epithelial cell to oocyte
ratio was not different between
controls and cyclopamine-treated mice
($p>0.05$) (Fig 7). The mesenchymal
cell to oocyte ratio was significantly
less in cyclopamine-treated mice
compared to controls ($p<0.05$) (Fig 7).

Ovaries from day 30 vehicle-
injected and high dose cyclopamine
injected mice (n =3), stained with
H&E, were obersevered to identify any
striking morphology abnormalities due
to cyclopamine-treatment. Ovaries
from both vehicle-injected and high
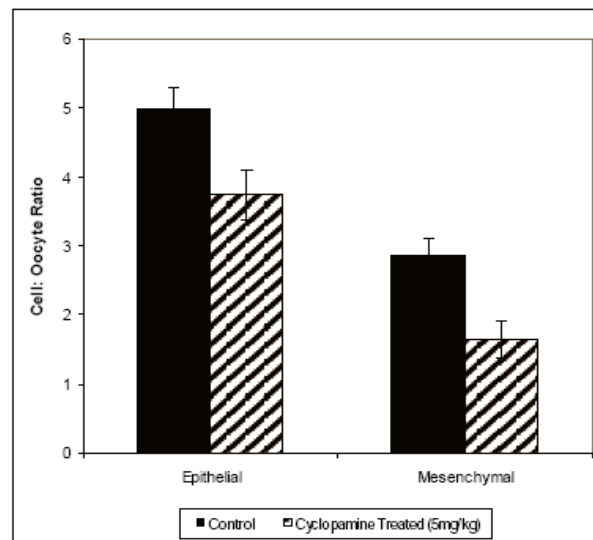
Figure 7: Epithelial: oocyte and mesenchymal: oocyte cell count ratios for day 4 control and cyclopamine-treated (5mg/kg) animals (n=3). Cell counts were conducted on H&E stained sections of day 4 ovaries. Solid bars represent control animals. Dashed bars represent cyclopamine-treated animals. Mean ± SE is shown. The epithelial: oocyte ratio is not different between the two groups ($p>0.05$). The mesenchymal: oocyte ratio is significantly different between the two groups ($p<0.05$).

dose cyclopamine injected mice had follicles at all stages of development. Some ovaries had corpus luteua present suggesting the mouse had ovulated previously. Neither the number of follicles at each stage of development nor the total number of primordial follicles present was counted.

The fertility study conducted found that low dose (n= 6) and high dose (n =1) cyclopamine-treated mice had litter sizes similar to those of vehicle-injected mice (n = 2). Average litter size ranged between 13 and 15 pups for controls, 12 to 15 pups for low dose cyclopamine-treatment and 9 pups for high dose cyclopamine-treatment. These are within the normal range for CD-1 mice suggesting all cyclopamine-treated mice were still fertile and able to become pregnant.

Discussion:

Primordial follicle formation is a complex process involving multiple cell types and cell signals. The findings of this study suggest that the HH signaling pathway plays an important role in directing correct primordial follicle formation in the mouse. Inhibiting HH signaling through cyclopamine injections resulted in improper primordial follicle formation, as characterized by incomplete basement membranes and altered association of mesenchymal cells with primordial follicles.

As expected, control mice showed a normal phenotype, with normal cord breakdown and primordial follicle formation occurring between day 0 and day 4. Day 4 ovaries exhibited proper assembly of primordial follicles. The disappearance of cord structures and the presence of primordial follicles as well as growing primary follicles in

these mice suggest that the period of primordial follicle formation is almost complete by day 4.

An altered phenotype, however, was seen in cyclopamine-treated mice, including poor basement membrane formation around primordial follicles. High dose cyclopamine-treatment resulted in the most severe phenotype, while low-dose cyclopamine treatment resulted in more moderate changes. There was variation in the phenotype in both groups, with the most variation among the high dose cyclopamine-treated mice. The variation in the phenotype in the mice treated with the high dose of cyclopamine suggests that cyclopamine may have affected some animals more drastically than others. The areas of densely packed somatic cells surrounded by basement membranes observed in one mouse may have once been cords in which all the oocytes underwent apoptosis. Further research is needed to determine if cyclopamine treatment causes oocyte apoptosis. This could be accomplished by increasing the number of replicate mice and by studying the time course of the effects after cyclopamine treatment to determine if loss of oocytes occurs. Interestingly, in day 4 ovaries stained for laminin from transgenic mice (Amhr2$^{Cre/+}$smoM2) that have a continually activated HH pathway, similar areas of densely-packed somatic cells were observed. In those mice, there are areas containing densely-packed somatic cells with no apparent oocytes enclosed by a basement membrane, as well as areas of relatively normal primordial follicle development. This suggests that altering HH signaling by either depressing it or up-regulating it can have similar effects on follicle development and suggest that precisely regulated HH signaling is required for proper primordial follicle development.

Since *Gli1* expression analysis shows that HH was inhibited in the cyclopamine-treatment model, it can be suggested that reduced HH signaling caused incomplete basement membranes to be formed. While there is no previous study regarding the effects of HH signaling on basement membrane formation in the ovary, prior studies support the idea that *Dhh* modulates the production of the basal lamina around seminiferous tubules [10]. When HH signaling was inhibited in the testes of *Dhh* knockout mice the basal lamina around seminiferous tubules contained numerous areas with small gaps, giving the appearance of a discontinuous and incomplete basal lamina [10]. Prior studies have shown all three HH ligands to be present in granulosa cells [3]. Furthermore granulosa and theca cells have been shown to exhibit PTCH and SMO [3], suggesting that HH acts on both granulosa and theca cells. It is thought that theca cells may synthesize basement membrane components [1], when they interact with epithelial cells via cell signaling pathways [22]. The finding that granulosa cells of primary follicles secrete HH, which can then act upon the surrounding mesenchymal-derived theca cells [4], suggests that perhaps HH acts on mesenchymal cells surrounding cords to initiate basement membrane formation around primordial follicles. Reduction of HH signaling in cyclopamine-treated mice may have prevented a signal from being expressed by the pre-granulosa cells, resulting in disrupted communication between the mesenchymal and epithelial cells. The mesenchymal cells may not have been able to synthesize basement membrane components, including laminin, in appropriate quantities due to this blocked cell interaction. Thus a lack of cell communication between epithelial and mesenchymal cells may have caused the high incidence of discontinuous basement membranes around primordial follicles observed in the cyclopamine-treated mice.

The decreased ratio of mesenchymal cells to oocytes in cyclopamine-treated mice suggests that HH also plays a role in this part of primordial follicle formation. HH has been identified as a key player in regulating cell proliferation in multiple organs, including the ovary [5]. This suggests a potential role for HH in regulating mesenchymal cell proliferation in the ovary. In the developing palate, epithelial-expressed SHH was found to regulate palatal mesenchyme cell proliferation through the maintenance of cyclin D1 and D2 expression [24]. HH also regulates mesenchyme cell proliferation in the gut [25] and in the developing lungs [26] through epithelial cell and mesenchymal cell interactions. In these studies, the absence of HH led to decreased mesenchymal cell proliferation. Since HH is an important regulator of cell proliferation in other organs and a decreased ratio of mesenchymal cells to oocytes was observed in this study, it is possible that reduced HH signaling in the ovary inhibited mesenchymal cell proliferation. Mesenchymal cells proliferate extensively in both the embryonic and neonatal ovary [20]. In the present study, cyclopamine was used to inhibit HH signaling after birth and thus may have prevented mesenchymal cells from proliferating during that period. The ratio of epithelial cells to oocytes was similar between controls and cyclopamine-treated mice. Epithelial cells only appear to proliferate in the embryonic ovary, not after birth [20]. Since HH was blocked by cyclopamine treatment after birth, and epithelial cells are not proliferating at this time, it is not surprising that the epithelial cell to oocyte ratio was not reduced.

The reduced mesenchymal cell to oocyte ratio in cyclopamine-treated mice could have also been caused by failure of the mesenchymal cells to localize appropriately around primordial follicles. In the day 0 ovary, mesenchymal cells are arranged in long

chains surrounding the ovigerous cords, but by day 4 these cells become localized around primordial follicles. The fact that these cells were still arranged in chains in ovaries of cyclopamine-treated mice on day 4 rather than localized around individual primordial follicles suggests that HH signaling is necessary to control the localization of mesenchymal cells around primordial follicles.

Preliminary studies were performed in which mice treated with high dose cyclopamine on days 0 -3 were examined at 30 days of age. Cyclopamine-treated mice caged with fertile adult males had litter sizes similar to controls, indicating that the cyclopamine-treated mice were fertile. Histology of day 30 ovaries from cyclopamine-treated mice showed that follicles at all stages of development were present. This is as expected, since all cyclopamine-treated mice still had a certain percentage of correctly formed primordial follicles. These primordial follicles would be able to enter the growing pool, develop normally and ovulate. The mice had normal sized litters, suggesting that the number of follicles that ovulated was not affected. This result may be due to a compensatory mechanism within the ovary. The ovary generates the species-specific number of ovulatory follicles, even when the size of the primordial follicle pool is reduced. There is an inverse correlation between the number of primordial follicles and the rate at which they are stimulated to grow, such that a reduction in the size of the primordial stockpile will result in an increase in the proportion of follicles beginning to grow [23]. This occurs to ensure that the correct number of follicles ovulate.

A number of cell signaling pathways have been shown to be involved in primordial follicle development [9]. For example, the Notch pathway has been shown to be expressed in oocytes and pre-granulosa cells prior to the assembly of primordial

follicles [27]. Furthermore, when Notch signaling was suppressed in cultured newborn mouse ovaries, there was a marked reduction in primordial follicles and an increase in the number of germ cells that remained in cords [27]. Thus, multiple pathways may direct primordial follicle formation and the inhibition of one may result in decreased efficiency of primordial follicles formation rather than complete inhibition. Even if HH is effectively blocked, some primordial follicles may form as the other required pathways remain intact.

This study is the first to examine the effects of HH signaling on primordial follicle formation in the mouse ovary. While this study offers evidence for an important role of HH signaling in primordial follicle formation, additional research is necessary to support this. Cyclopamine should be administered for 8 days after birth to fully understand HH's role in primordial follicle development. While a majority of primordial follicle formation occurs during the first four days of life, primordial follicle formation is not absolutely complete at this time. This leaves open the possibility that additional follicle formation could occur after termination of cyclopamine treatment on day 3. Inhibition of the HH signaling pathway through day 8 might be expected to generate a more severe reduction in primordial follicle formation.

Whether the treatment of newborn mice with cyclopamine resulted in a reduced number of primordial follicles at day 30 was not examined, but ovaries have been collected and fixed for future study. In the future, the number of primordial follicles and follicles at various other stages of development will be assessed to confirm an effect on reduced follicle formation and to determine if inhibition of HH signaling early in life affects the dynamics of follicle development in the mature ovary. Levels of follicle

atresia in day 30 cyclopamine-treated mice should also be evaluated. Preliminary studies with Amhr$^{cre/+}$Smo$^{flox/null}$ mice, a transgenic mouse model in which the *Smo* gene has been conditionally deleted in the ovary and reproductive tract, show an abnormally high level of atresia in primary follicles. Therefore, it would be interesting to determine whether this effect also occurs in adult mice that had been treated with cyclopamine neonatally.

The results of this study are consistent with the hypothesis that HH signaling plays a role in primordial follicle formation in the mouse ovary. It appears to do so by affecting the mesenchymal cell to oocyte ratio as well as basement membrane formation. When HH signaling is reduced, it appears that mesenchymal cells may not proliferate as extensively or that they may not localize normally around developing primordial follicles. This may result in decreased signaling between epithelial and mesenchymal cells, such that primordial follicle formation and basement membrane deposition is disrupted. Future research is necessary to support these findings.

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