REASSESSMENT OF INTRA- AND EXTRA-OVARIAN EXPRESSION OF GROWTH DIFFERENTIATION FACTOR-9

A Thesis by

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Bachelor of Science, Wichita State University, 2003

Submitted to the College of Liberal Arts and Sciences
and the faculty of the Graduate School of
Wichita State University in partial fulfillment of
the requirements for the degree of
Master of Science

May 2006

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I have examined the final copy of this Thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Biological Sciences

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ACKNOWLEGMENTS

I especially want to thank my advisor Dr. Jeffery May. Without his professional support, advice, and guidance throughout my educational endeavor here at Wichita State this would have not been possible; his wisdom and passion for science has been inspiring. My appreciation extends to my committee members Dr. William Hendry and Dr. Michael VanStipdonk. Thank you for your guidance, assistance, and insightful comments. A special thank you goes to Dr. Aaron Hsueh and Dr. David McDonald for their technical contributions to this project, and to Fawn Beckman and Kathy Dandurand for their exceptional maintenance of the animal care facility.

I would like to thank Isabel Hendry, Maria Russell, Dulce Maroni, Rebecca Lackey, and numerous individuals, too many to name, whom have given me helpful advice either scientifically or personally over the course of my studies.

Finally, to my family I extend a sincere appreciation for instilling in me to never settle for mediocrity no matter what my endeavor(s) may be.

ABSTRACT

Mammalian ovarian organogenesis is characterized temporally by oogonial mitosis, oogonial apoptosis, rescue of germ cells via interaction with somatic cells to form primordial follicles, and entrance into meiosis. Recent studies suggest that germ cell's activity participate in this process via the production of local regulatory factors. Growth differentiation factor-9 (GDF-9), a novel transforming growth factor-β family member, is expressed in ovaries of various species as a crucial factor in ovarian follicular development. Likewise, the expressional pattern of GDF-9 in nonovarian tissues has remained elusive, as current data continues to support the exclusive expression of GDF-9 within the mammalian ovary. Recently, our lab became interested in the pattern of expression associated with GDF-9 as it pertains to ovarian organogenesis and follicle formation in the neonatal hamster ovary due to the ability to obtain ovaries at specific stages of follicular organization. Further interests include investigating the possible expression of GDF-9 in nonovarian tissues. Consensus oligonucleotide primer pairs spanning at least one intron for GDF-9 were determined by analysis of the gene sequences for human, mouse, rat, bovine, and ovine for use in the hamster. mRNA for GDF-9 in ovarian and non-ovarian tissues was detected by RT/PCR using total RNA. PCR products were sequenced to determine the degree of homology for the gene relative to that of other species (mouse 91%, rat 90%, and human 84%). mRNA for GDF-9 was detected in ovarian samples for all days examined (Days 1, 3, 5, and 6-9 post delivery [Day 0]. This time frame reflects periods of active oogonial mitosis (Days 1 and 3),

oogonial atresia (Day 5), and primordial follicle formation (Days 6-9). Translation of GDF-9 was observed prior to primordial follicle development (Day 5).

For non-ovarian analysis mRNA, expression of GDF-9 was detected in several tissues (liver, kidney, spleen, and testis) at various stages of growth and development. Furthermore, white blood cells retrieved from adult hamsters revealed mRNA expression of this novel growth factor. Kidney perfusion experiments supported the idea that mRNA expression of GDF-9 in non-ovarian tissues could be the resultant of circulating white blood cells as a 35% reduction was seen in perfused samples. Immunological analyses of hamster ovarian sections detected expression of GDF-9 within the highly vascularized regions of the corpus luteum. In fact, re-investigations of early stage immunological analyses for GDF-9 suggest that mast cells are potential sites of GDF-9 protein expression.

Reports suggest that GDF-9 is expressed exclusively in the ovary, specifically within the oocytes, and that expression of GDF-9 is not seen prior to primordial follicle formation. Our results conclude several revolutionary findings pertaining to ovarian and non-ovarian expression of GDF-9. Importantly we report that GDF-9 mRNA is expressed in the ovary prior to and during primordial follicle formation, and after ovarian senescence. And that GDF-9 is non-ovarian specific as previously indicated by scientific literature as we have localized non-ovarian transcription and translation of GDF-9 to white blood cells. Currently, only one type of immunologic cell, mast cells, has been identification to express GDF-9. Further individual white blood cell assays will be required to identify additional specific white blood cell expression of GDF-9.

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LIST OF ABBREVIATIONS/NOMENCLATURE

AAALAC	Association for Assessment and Accreditation of Laboratory Care
AEC	Aminoethyl Carbazole
bFGF	Basic Fibroblast Growth Factor
Bax	bcl-2 associated-x gene
bcl-2	B-cell leukemia/lymphoma-2
BMP-4	Bone Morphogenetic Protein-4
BMP-7	Bone Morphogenetic Protein-7
BMP-8	Bone Morphogenetic Protein-8
BMP-15	Bone Morphogenetic Protein-15
bp	Nucleotide Base Pair
C	Celsius
cc	Cubic Centimeters
CO_2	Carbon Dioxide
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
DDT	1,1,1-Trichloro-2,2-bis(p-chlorophenyl) Ethane
DEPC	Diethylprocarbonate
DES	Diethylstilbestrol
DNA	Deoxyribonucleic Acid
ECF-A	Eosinophil Chemotactic Factor of Anaphylaxis
EDTA	Ethylenediaminetetraacetic Acid
EEC	Extra-embryonic Ectoderm

LIST OF ABBREVIATIONS/NOMENCLATURE (continued)

ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
EtOH	Ethanol
Figα	Factor in the Germline α
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF-3	Growth Differentiation Factor-3
GDF-9	Growth Differentiation Factor-9
HSRL	Histo-Scientific Research Laboratories Inc.
IGF-1	Insulin-like Growth Factor 1
IACUC	Institutional Animal Care and Use Committee
kDA	Kilodalton
kg	Kilogram
KL	Kit Ligand
LAP	Latency Associated Peptide
LH	Lutenizing Hormone
m	Molar
MIS	Mullerian Inhibiting Substance
ml	Milliliter
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
ng	Nanogram

LIST OF ABBREVIATIONS/NOMENCLATURE (continued)

nm	Nanometer
OB/GYN	Obstetrician/Gynecologist
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PGC	Primordial Germ Cells
pmol	Picomole
POF	Polyovular Follicles
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDS	Sodium Dodecylsulfate
SF-1	Steroidogenic Factor 1
TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor β
ug	Microgram
ul	Microliter
UV	Ultraviolet
v/v	Volume to Volume

LIST OF ABBREVIATIONS/NOMENCLATURE (continued)

WT1	Wilms Tumor Suppressor 1
w/v	Weight to Volume
ZP	Zona Pellucida Proteins

INTRODUCTION

Embryonic Indifferent Gonad

During the initial phases of mammalian embryonic development, male and female embryos are morphologically indistinguishable. Gender-specific genetic information, received by the embryo at fertilization, remains inactive; resulting in an undifferentiated, indifferent embryologic stage. Early formation of the bipotential gonad begins shortly after gastrulation as mesodermal populations (intermediate mesoderm) arise in the trunk of the embryo, forming the early precursor of the urogenital system [1]. Structurally, the urogenital system develops into three individual lineages: the pronephros, mesonephros, and the metanephros. The pronephros serves a role in the temporary excretory function in the embryo but is absent from the adult where the metanephros ultimately differentiates into the adult excretory kidney. The mesonephros serves little function in the execratory system of the embryo and eventually differentiates into the indifferent gonad [2].

Thickening of the coelomic epithelium on the ventromedial surface of the mesonephros gives rise to the bipotential, indifferent gonad. Proliferation of the mesothelial cells of the mesonephros forms a subsurface longitudinal ridge, structurally known as the gonadal ridge [1]. Differentiation of the ridge is dependent upon the expression of sufficient levels of Wilms Tumor Suppressor 1 (WT1) and Steroidogenic Factor 1 (SF-1). Gene knockout of transcription factor WT1 in rodents prevents development of kidneys or gonads [3]. Furthermore, deletion of the orphan nuclear

receptor SF-1 results in destabilization of intermediate mesoderm in the undifferentiated gonad [4].

The gonadal primordia are initially formed exclusively by somatic, mesoderm-derived cells: mesenchymal cells of mesonephric origin and coelomic epithelial cells that cover the surface of the gonadal ridge [5]. In the indifferent gonad, these cell lineages assume a non-sex-specific identity. Through genetic signals, external regulating factors, and endocrine cues, these cells eventually form the internal framework of either the testis or the ovary [6]. Each cell type in the gonad must differentiate accordingly. In the gender-determined gonad, the supporting cell lineage gives rise to cells that immediately surround and support the developing germ cell; notably referred to as granulosa cells in the female gonad, and Sertoil cells in the male. The steroidogenic lineage differentiates into theca cells of the female gonad and Leydig cells in the male embryo while the stromal cell line patterns the structural spaces of the ovary and the testis [1] .

Colonization of the Genital Ridges by Primordial Germ Cells

While the developing indifferent gonad differentiates from the mesonephric mesenchyme, a small group of alkaline-phosphatase positive cells of the epiblast begin to migrate, at a very early stage of embryonic life, through the primitive streak into the extra-embryonic region at the base of the allantois [7]. These migrating pluripotent cells, known as primordial germ cells (PGCs), remain shielded from the major morphological rearrangements occurring in the embryo proper; including the differentiating influences of active growth factors and morphogens [1]. Transplantation experiments suggest that factors produced by the EEC are required for PGC formation. Epiblast cells that were

placed in close proximity to the EEC developed into PGCs, as opposed to those that were dispersed beyond the EEC region. Two EEC derived factors, bone morphogenetic protein 4 (BMP4) and bone morphogenetic protein 8 (BMP8), are responsible for the generation and development of PGCs from the extraembryonic ectoderm [8-10]. Inactivation of both growth factors results in the failure of PGC development in the embryo [10].

The movement of PGCs from the base of the allantois through the hindgut into the mesoderm of the mesonephros and hence into the indifferent gonad is influenced by morphogenetic rearrangements and chemoattractive signals [11]. The targeting of the PGCs to the gonadal ridge is partially dependent upon integrins [12] and the c-Kit/Steel signaling pathway [13]. Migration of PGCs also depends on interactions with the extracellular matrix proteins fibronectin and laminin [12, 14]. During relocation of these PCGs to the genital ridge, mitosis occurs within these cells so that several thousand arrive in the indifferent gonad [15].

Ovarian Organogenesis and Primordial Follicle Development

With the migration of PGCs into the indifferent gonad during midgestation, gender-specific genetic signals and endocrine regulators transform the undifferentiated bipotential gonads into either the testis or ovary. Loose cordlike structures, known as germinal cords, develop from the invagination of coelomic epithelial of the gonads. In the ovary these germinal cords encase the PGCs, and keep them closely associated with the outer edge of the cortical zone of the ovary [16, 17]. Cytoplasmic bridges are seen microscopically between the germ cells, developing structurally as cysts. Gradually, fine cytoplasmic extensions from nearby pre-granulosa cells begin to segregate between the

germ cell cysts, (oogonial nests) [16]. These somatic cell inversions eventually disperse around each individual germ cell (oocyte), forming a single layer of squamous, epithelialised cells [18]. This single layer of granulosa cells that surrounds the oocyte becomes encapsulated by the basal lamina that forms on the periphery of the follicle and separates the newly formed primordial follicle from the stromal cells of the ovary [18]. The breakdown of these large cysts are crucial to the formation of primordial follicles [19].

Close apposition of oogonia to the somatic granulosa cells results in direct signaling and autocrine/paracrine communication between the two and ultimately leads to primordial follicle formation [20]. Even with the coordinal expression of local organizers, not all oogonia will proceed onward to organize into a primordial follicle. Germ cells that fail to associate with nearby pre-granulosa cells undergo cell death (atresia). Proteins encoded by the B-cell leukemia/lymphoma-2 (bcl-2) family are responsible for the regulation of atresia in primordial germ cells. Two factors, bcl-2 and bcl-2 associated-x gene (Bax), serve opposing roles in the regulation of cell death [21]. Up-regulation of the bcl-2 gene protects germ cells against atresia whereas increased expression of Bax results in germ cell degradation. Theoretically, only oogonia are able to down regulate Bax at the end of meiotic prophase can escape atresia [22].

Primordial germ cells that survive the process of oogonial atresia become encapsulated by a single layer of squamous epithelium followed by arrestment in prophase of meiosis I. Structurally, this defines the first cohort of primordial follicle development in the mammalian ovary [23, 24]. An oocyte specific factor, factor in the germline α (Fig α), plays an obligatory role in the formation of primordial follicles. In

mice null for Fig α, gonadogenesis proceeds normally but primordial follicles fail to develop properly [25]. Fig α is also a critical transcription factor for genes encoding zona pellucida (ZP) proteins. Combined expression of all ZP proteins may provide an extracellular matrix that allows for the attachment of granulosa cells to the membrane of the germ cell [26]. Gap junction proteins, connexin 43 and connexin 37, also play an important regulatory role in the formation of primordial follicles. Studies dealing with mice null for connexin 43 show a lack in the normal development and progression of primordial follicles [27]. Similar experiments dealing with connection 37-null rodents resulted in the complete failure of granulosa cells to organize around individual oogonia and in the degradation of germ cells [28].

Primordial follicles are a quiescent stage of follicle development that lacks physical growth of the germ cell and its granulosa cell compartments. A basic doctrine of reproductive biology is that most mammalian females fail to possess the capability of post-natal germ-cell renewal. Thus, a fixed number of primordial follicles are endowed prior to birth, where most are maintained in a resting state until menarche [29]. However, recent studies challenge this age-old theory suggesting that juvenile and adult mouse ovaries possess mitotically active germ cells that are needed to continuously replenish the follicle pool [30]. Nevertheless, growth of some of these dormant follicles occurs prior to reproductive maturity of the female; resulting in the recruitment and entrance of the primordial follicles into a follicular growth phase (folliculogenesis). Follicles will progress through higher stages of follicle organization until follicle development reaches a stage of growth that requires gonadotropin support. With the absence of follicle stimulating hormone (FSH) and lutenizing hormone (LH) in the system, development

ceases and follicles undergo atresia [29, 31, 32]. Upon pubertal maturation recruited follicles are rescued from these early stages of follicular atresia. With the needed gonadotropin support, follicles proceed to higher organizational levels and ultimately lead to the ovulation of the oocyte or follicular atresia [29, 31, 32]. Through continual recruitment, depletion of the primordial follicle pool will occur and eventually result in ovarian follicle exhaustion and senescence [33].

Folliculogenesis

As primordial follicles are recruited from the resting pool they developmentally attain successively higher levels of follicular organization through cell proliferation and cytodifferentiation, a process known as folliculogenesis. Folliculogenesis occurs in a continuous manner consisting of several stages: primordial follicle recruitment, preantral follicle development, selection and growth of a Graafian follicle, and follicle atresia [34]. Primordial follicles are recruited by unknown factors that stimulate the follicles to progress into primary follicles. The transition of a primordial follicle into a primary follicle involves transformation of the surrounding squamous pre-granulosa cells into a single cuboidal layer covering the growing oocyte [35, 36]. Soon afterwards, the structure of the primary follicle begins to change into a secondary follicle. With acquisition of mitotic potential in the granulosa cells of the follicle, several layers of granulosa cells encapsulate the enlarging oocyte. Secondary follicles continue to accumulate increasing numbers of granulosa cells through proliferation and eventually the follicle is considered a tertiary follicle [37]. Early stages of tertiary follicle development consist of a fully grown oocyte surrounded by multiple layers of granulosa

cells [37]. These follicles are also characterized by the formation of thecal cell lineages on the outer surface of the follicle. Two layers of theca appear, an inner theca interna and an outer theca externa [38]. Formation of numerous capillaries within the theca tissue accompanies the development of the tertiary follicle [37]. The coordinal progression of a primary follicle into a secondary and then an early tertiary follicle defines the preantral development of folliculogenesis.

Selection and growth of a Graafian follicle begins in the late stages of tertiary follicle formation. The late tertiary follicle, also known as a Graafian follicle or antral follicle, is marked by the formation of a fluid-filled cavity adjacent to the oocyte called the antrum [37]. Granulosa cells of the Graafian follicle begin to differentiate into several subtypes: corona radiata that surrounds the zona pellucida, membrana that is interior to the basal lamina, periantral that is adjacent to the antrum, and cumulus oophorous that connects the membrana and corona radiata granulosa cells [37].

In response to FSH stimulation, a dominant follicle is selected from the cohort of small developing Graafian follicles [39]. Granulosa cells and theca cells of the dominant follicle continue to divide rapidly due to the abundant uptake of circulating gonadotropins. However, because of reduced gonadotropin support caused from the formation of a dominate follicle, proliferation of the steroidogenic and supporting lineages of the remaining Graafian follicles decline; resulting in follicular atresia [39, 40]. A mid-cycle surge of LH stimulates prostoglandins to interact with the surface epithelium of the lone surviving Graafian follicle. Proteolytic enzymes are released and begin to degrade the underlying tissues; causing the surface epithelial cells to detach from the basement membrane. A stigma develops on the exterior surface of the follicle and

creates a pathway for the release of the oocyte [41]. After ovulation, the remaining granulosa and thecal compartments collapse inward, filling the follicular cavity. Processes of hyperplasia and hypertrophy transform the granulosa and theca cells into luteinized cells, forming the corpus luteum. The corpus luteum is one of the most vascularized tissues in the body, establishing an extensive vascular network that is essential for corpus luteum development [42, 43]. With the formation of the corpus luteum, progesterone is secreted by the lutein granulosa cells which will support pregnancy upon fertilization of the oocyte. If fertilization does not occur the corpus luteum degenerates and is replaced by connective tissue, forming a corpus albicans [41].

Local Organizers and External Impacting Factors

Folliculogenesis is dependent on sequential changes in local organizing factors, cell-cell interactions, and external gonadotropin support [26]. A number of factors influence the development, regulation, and support of the oocyte, steroidogenic cells, and supporting cell lineages of the mammalian follicle. Knockout experiments showed that follicular growth is arrested in mice lacking the FSH receptor present on granulosa cells [44]. FSH stimulation is critically important in the developmental progression of preovulatory Graafian follicles [26]. Follicle growth is also dependent on insulin-like growth factor I (IGF-1) and its receptor [45, 46]. IGF-1 alone cannot drive the development of follicles, but it enhances the effects of FSH within the system [26]. The presence of both estrogen receptors (ERα and ERβ) [47, 48] on granulosa cells of growing follicles are important in their normal development. Double knockout studies

involving both estrogen receptors leads to the impairment of follicle growth and a reversal of the female phenotype [49].

The exact mechanisms that drive the transition of primordial follicles into the process of folliculogenesis are not well defined. Studies showing that rodent primordial follicles are fully capable of undergoing follicle assembly to form primary follicles in serum-free culture [50] support the non-dependency of gonadotropin involvement in early follicle development. The transition of a primordial follicle to a primary follicle is then dependent on local organizing factors expressed by the oocyte and the supporting granulosa cells. Possible factors regulating the transitional phase of the primordial follicles includes kit ligand (KL) [51], basic fibroblast growth factor (bFGF) [52], bone morphogenetic protein 7 (BMP-7) [53], bone morphogenetic protein 15 (BMP-15) [54], and growth differentiation factor 9 (GDF-9) [55].

Both KL and bFGF appear to act in synergistic manner to promote the formation of primordial follicles to primary follicles [56]. Granulosa cells of organizing follicles (primordial follicles) express KL [57] with its receptor present on the surrounding granulosa cells [58]. *In vitro* studies suggest that both KL and bFGF must be active in order to maximally promote changes of a primordial follicle to a primary follicle, utilizing cell to cell support to increase expression and induce change [56].

Other factors, specifically growth factors, act locally within the ovary to regulate the primordial to primary follicle transition and follicle growth in early stages of folliculogenesis. BMP-7, an abundantly expressed factor involved in embryonic myocardium development [59], is also involved in transition of primordial follicles to primary follicles by increasing granulosa cell proliferation around the changing follicle

[53]. Two structurally similar oocyte-derived growth factors, BMP-15 and GDF-9, influence the development and growth of the oocyte, granulosa cells, and theca compartments of follicles undergoing increased organizational phases throughout folliculogenesis. BMP-15, an oocyte expressed factor, stimulates proliferation of pre-antral granulosa cells and inhibits FSH-stimulated progesterone production by late-stage granulosa cells [54]. Likewise, GDF-9, an oocyte specific factor [60], forms a communication network between the surrounding lineages; signaling associated granulosa cells to proliferate, theca lineages to organize, and oocytes to expand [61-63]. In GDF-9 deficient mice, primordial follicles and primary follicles form but follicular development is blocked completely beyond this one-layer follicle stage thus resulting in infertility [61]. Unlike GDF-9, BMP-15 appears to be of minimal importance for normal progression of folliculogenesis as female mice lacking BMP-15 are subfertile and exhibit little ovarian histopathological defects [64].

Transforming Growth Factor-β Superfamily

Several factors involved in ovarian organogenesis and folliculogenesis are members of the transforming growth factor-β (TGF-β) superfamily. This superfamily is a large group of extracellular growth factors that are important in cell activation, adhesion, proliferation, migration, differentiation, and maturation throughout various stages of mammalian development [65, 66]. TGF-β related proteins include TGF-βs, activins, inhibins, Mullerian inhibiting substance (MIS), BMPs, GDFs, Nodal, and leftys [66]. Ligands of the TGF-β family are translated as prepropeptide precursors with an N-terminal signal peptide followed by the prodomain and the mature domain. Six to nine

conserved cysteine residues in the mature region are characteristic of the TGF-β family; forming intramolecular and intermolecular disulfide bonds that create covalent dimerization of the ligands [66, 67]. Noncovalently associated subunits, growth differentiation 3 (GDF-3), GDF-9, and BMP-15 lack one or several conserved cysteines. Instead, the cysteines are substituted with serines which ultimately form more labile dimers [66, 68].

Molecular activity of the mature domain of the ligand family is masked by a propeptide region known as, latency associated peptide (LAP). Furin-like endoproteinases can cleave such LAP complexes, from the mature domain during their secretion but the LAP remains linked to the protein through non-covalent associations [69]. Thus, TGF-β ligands are secreted as biologically latent forms [70, 71] and complete dissociation of the LAP results in ultimate activation of the protein [66].

Several TGF-β family members are functionally diversified among a variety of developmental and regulatory events. For instance, BMP-7 [53] and BMP-8 [9, 10] are involved with the regulation of the reproductive system yet also responsible for the development of non-reproductive structures; as BMP-7 directs myocardium formation [59] and BMP-8 regulates skeletal muscle mass [72]. In contrast, the TGF-β family member GDF-9, is solely responsible for reproductive activities such as regulation of follicle development and oocyte growth; with its expression remaining exclusively restricted to the mammalian ovary [68].

Growth Differentiation Factor 9

In 1992 McPherron et al. [68] identified a new TGF-β superfamily member, GDF-9, through the use of degenerate oligonucleotides. The COOH-terminal of the two-intron/one-exon [73] GDF-9 gene was homologous with other TGF-β family members but the remainder of the sequence was significantly divergent [68]. Of the seven conserved cysteine residues of the COOH-terminal, standard to TGF-β family members, GDF-9 structurally lacks the fourth cysteine of the seven, which is replaced by a serine in the sequence. Substitution of the cysteine, believed to participate in intermolecular disulfide bond formation during dimmerization, results in a noncovalent association of the subunits [68].

The expression pattern of GDF-9 was initially screened by Northern analysis in a variety of adult rodent tissues. Poly(A)-selected RNA isolated from the tissues resulted in a 1.7 kilobase mRNA band specific to the ovary [68]. *In situ* hybridization of post-natal mouse ovaries, representing stages of early and progressed follicular growth, detected GDF-9 mRNA in oocytes of all follicle stages, except primordial follicles, whereas ovaries removed from pre-natal mice failed to express GDF-9 transcripts [60]. Interestingly, ovulated oocytes retained mRNA activity until fertilization [60]. Northern hybridization of neonatal rat ovaries detected GDF-9 RNA transcripts at low levels, whereas expression dramatically increased as ovaries retained higher organized follicles [74]. *In situ* analyses of human and rat ovarian sections localized GDF-9 mRNA exclusively in oocytes of primary follicles and in follicles of higher organizational levels [74-76]. Oocyte-specific expression of GDF-9 mRNA in non-rodent species, sheep, ovine, and bovine, conflict with findings seen in rodents and humans; according to *in situ*

hybridization studies [77, 78]. In domestic ruminants, GDF-9 gene expression is detected in oocytes of primordial follicles and developed follicles of folliculogenesis [77, 78]. In contrast to rodent models, GDF-9 mRNA was undetectable in oocyte-free follicles, nodules, and granulosa cells [60, 68, 74, 75, 77, 78].

In 1998, Fitzpatrick et al. [76] published a paper challenging the ovarian specific expression of GDF-9. Northern analyses of poly(A)-selected RNA from rodents generated a 1.6 kilobase GDF-9 mRNA transcript identified in hypothalamic, testis, and ovarian tissues. Amplification of the mRNA products by RT-PCR followed by sequencing, confirmed that the products were indeed GDF-9 [76]. In similar regards, northern analyses detected GDF-9 mRNA in reproductive and non-reproductive human tissues including the bone marrow. RT-PCR and sequence analyses of human pituitary and testis samples established GDF-9 mRNA expression to be non-ovarian specific [76]. With results that conflicted with previous expression studies, Southern blots were preformed using mouse and human genomic DNA; verifying that GDF-9 alone was responsible for the non-ovarian expression observed [76]. Therefore, the ovarian specific nature of GDF-9 is questionable and so the non-ovarian expression and function of GDF-9 deserves investigation.

The focal point of GDF-9 since its discovery is the gene's ability to regulate the development and growth of non-gonadotropin dependent follicles [61, 79-81]. The exact growth factor(s) responsible for directing follicular expansion of the oocyte, granulosa cells, and theca layers during early mammalian folliculogenesis are not well defined. It is known that growth factors synthesized by ovarian somatic cells directly affect oocyte growth and function [61, 82, 83], but was questionable whether oocyte-secreted factors

play a similar role in modulating somatic cell functions [61]. Targeted deletions of exon 2 that encodes the mature region of GDF-9 in mice causes follicular arrest beyond the primary follicle stage [61, 62]. Molecular defects affecting follicle organization occur within the follicle in the absence of GDF-9, as gene deficient follicles lacked normal ovarian function [63]. Follicles are incapable of transmitting signals relied upon for proper recruitment of theca cell precursors. Granulosa cells up-regulate KL and inhibin-α which may act in a paracrine fashion to support oocyte survival in the absence of GDF-9 [63].

Through exploitation of an *in vitro* bioassay containing denuded oocytes and cocultured ovarian granulosa cells, it became clear that bidirectional communication of oocytes with surrounding somatic cells is crucial for the normal progression of folliculogenesis [84, 85]. *In vitro* studies showed that GDF-9 stimulates the growth of preantral follicles and thecal cell differentiation in rodents [80, 81] and the development of primary and early secondary follicles in human ovarian cultures [86]. Treatment of rodent granulosa cells with recombinant GDF-9 stimulated proliferation and differentiation processes in preantral follicles, antagonized the FSH induction of steroidogenesis [87], enabled cumulus expansion, and reduced LH receptor expression in cultured granulosa cells [88].

GDF-9, an oocyte-secreted factor, regulates folliculogenesis by controlling a broad range of activities associated with the growth and development of follicles. Identification of the expressional pattern of the GDF-9 protein throughout the various organizational phases of folliculogenesis has been minimal among non-rodent species. Immunohistochemical analyses of mice, rat, and human ovarian sections reveal GDF-9

protein to be expressed in oocytes throughout folliculogenesis, initially beginning at the primary follicle stage [74, 80]. Immunoflorescence detection of GDF-9 from in vivo and *in vitro* studies of ovaries from Syrian Golden hamsters illustrated that protein expression occurs prior to primordial follicle formation [89], an observation that significantly diverges from similar analyses in other rodent models [74, 80]. Immunoblots analysis of the GDF-9 protein using prepared antibodies directed against the pro-region of the GDF-9 gene detected the pro-form of the GDF-9 protein at an approximate molecular weight of 37,000 kDA [60]. Immunoblot analyses of recombinant GDF-9 protein under reducing conditions detected the mature form at 15,000 kDA - 19,000 kDA, and the apparent full-length GDF-9 protein at about 60,000 kDA - 80,000 kDA [80]. Expression of GDF-9 protein in ovine and bovine species has yet to be reported.

Endocrine Disruptors

Because of GDF-9's profound involvement in regulating the development and growth of mammalian ovarian follicles, it was purposed by our lab that GDF-9 may be a target for endocrine disruption (specific aims). An endocrine disruptor is commonly characterized as an exogenous chemical or environmental agent that interferes with the normal function(s) of the endocrine system [90]. Endocrine disruptors are natural products (phytoestrogens) or synthetic chemicals (xenoestrogens) that mimic or antagonize the actions of naturally occurring hormones thereby leading to the disruption of several biological processes [91]. Phytoestrogens occur naturally in plants. Specific examples of phytoestrogens include coumestrol, formononetin, daidzein, biochanin A, and genistein [90]. Dietary-mediated phytoestrogen exposure in grazing animals causes

significant reproductive chaos and irreversible infertility [92]. Xenoestrogens such as alkylphenols, bisphenol A, diethylstilbestrol, polychlorinated biphenyls, etc., can induce a variety of anatomic malformations and functional abnormalities of sensitive organs [91, 93]. Extreme differences among species in their susceptibility to these chemicals may depend upon several diverse factors such as chemical sensitivity, individual species response, age at exposure, and dose received [91, 93]. Exposure to disruptors during fetal development is of particular concern because many feedback mechanisms functioning in the adult are absent and adverse effects may be occur lower doses than those observed in the adult [91, 93].

Generally within all developing systems, there lies a particularly sensitive time-point at which they are most vulnerable to disruptive stimuli. Reproductive distress caused by exposure to an endocrine disruptor affects the survival and viability of species and the proceeding generations of both males and females [94, 95]. For example, administration of the xenoestrogen methoxychlor to newborn rats at a dose level of 0.5 ug/day caused accelerated puberty and loss of fertility [93]. Similarly, newborn female rats injected with 1 mg/day of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) had an early transition into menarche followed by an accelerated loss in fertility [93].

Diethylstilbestrol

Diethylstilbestrol (DES) is a potent, non-steroidal, synthetic estrogen that was widely administered to pregnant women during the 1940s through the 1960s to prevent miscarriages [90]. Ultimately, DES was recognized as an endocrine disruptor because severe malformations occurred in the reproductive organs of daughters and sons of

mothers who had received DES while pregnant. Women exposed to DES during embryonic development suffered reproductive organ dysfunctions, reduced fertility and/or infertility, immunological problems, and vaginal adenosis. In prenatally exposed males, sperm numbers and mobility declined, genitals were malformed and reduced in size, and incidents of prostate cancer increased [90, 91, 93].

Intercellular communications between epithelial and stromal cell tissues in reproductive structures are essential in the development and maintenance of the system and so are particularly sensitive to endocrine disruptors [95]. DES-induced uterine disruption in neonatal hamsters displayed hypertrophic and hyperplastic effects among the columnar epithelium of the endometrium [95]. In neonatal DES-treated rats, hypertrophic effects of the uteri were seen immediately after exposure, yet resulted in a hypotrophic/hypoplastic physiology in adults [95, 96]. Mice exposed to DES *in utero* developed a hypertropic/hyperplastic response, but had the adverse effects with neonatal exposure (reviewed by Hendry et al.) [95, 97]. Thus, differences in exposure outcomes may be mediated by species specificity, dose response, hormonal receptor abundance, and age at exposure [91, 93, 95].

Subsequent studies showed that the ovary is also a target of DES-mediated disruption. In mice, prenatal DES exposure (100 ug/kg) induced oophoritis, altered interstitial physiology, and increased steroidal production [98]. Ovaries of various species exposed perinatally to DES developed follicles that contained multiple oocytes per follicle, i.e., polyovular follicles (POF) [99, 100]. This abnormality, thought to be caused by incomplete oocyte division during primordial follicle formation, is a rare occurrence within a normally functioning ovary however, significant in perinatally DES-

treated ovaries [101, 102]. Studies, conducted by May et al. [102] in 1999 confirmed and expanded the spectrum of morphological abnormalities imposed upon the ovary following neonatal DES exposure: cystic unovulated follicles, abnormal theca, hyperplasia of the stroma, hypertrophy of the theca, and absence of corpus luteum [102]. Dose-dependent exposure of DES (0.1, 1, and 100 ug), in neonatal hamsters, induced substantial development of POFs, even at the lowest dose. Thus, neonatal administration of a single, low dose of DES is sufficient to disrupt the long-term development and morphologic arrangement of follicles [102].

Hamster Ovarian Morphology

Relative to most species, the hamster represents a convenient model to study early ovarian organogenesis and initial primordial follicle formation. At birth, the hamster ovary is developmentally immature; containing massive amounts of mitotically dividing oogonia (germ cells) [102, 103]. Mesenchymal cells migrate from the meduallary region of the ovary, encapsulate neighboring oogonial nest and eventually form the granulosa layer of the follicle. By day 5, the hamster ovary is actively undergoing oogonial atresia. The initial germ cell population of the ovary quickly declines as oogonia fail to associate with nearby somatic/granulosa cells [102, 103]. The hamster ovary will not acquire follicle development until the sixth post-natal day [89] which is equivalent to the organization seen in late stage pre-natal rodents [55]. At post-partum day 9 folliculogenesis is well underway within the ovary. Primordial follicles reside in the outer cortex of the ovary as primary and secondary follicles develop towards the medulla [102, 103].

Moreover, with the proposal to examine the direct effects of endocrine disruptors during ovarian organogenesis and ovarian senescence, DES can be administered to the hamster pups; without directly exposing the mother. Arguably, exposure of the experimental model through the mother represents a more "physiological" and practical means of observing ovarian dysfunction. Adversely, it is possible that direct exposure of neonates to DES will better define the actual levels needed to induce an ovarian effect [102].

GDF-9 Oligonucleotide Development

To analyze GDF-9 mRNA expression in the hamster ovary whether in terms of ovarian specificity, presence during follicular formation and/or development, or as a target for endocrine disruption, two individual sets of oligonucleotide primers pairs were generated for RT/PCR analysis: GDF-9 184–515, and GDF-9 Sp up–dw. Unfortunately, unlike highly utilized experimental models, the hamsters' genome is unsequenced. Therefore primers were generated from conserved regions of known species. The GDF-9 184-515 primer set was developed by compared sequence data from rodent models. GDF-9 184 (sense: 5' TGT AGA TGG GAC TGA CAG GTC 3') was generated to reside within exon 2. The reverse primer of the set, GDF-9 515 (anti-sense: 5' TCA GCG TGT ATA GCA AGA CCG 3'), was designed to coincide with a sequence spanning the intron between exons 1 and 2 of the gene.

GDF-9 Sp up–dw was generated for RT/PCR expressional studies within multiple species, including the hamster. Sequences form a diverse range of species were analyzed for amino acid and nucleotide commonalities Conserved nucleotide bases between

species were carried over into the formation of either the sense or anti-sense strands (figure 1). Differences among species' oligonucleotides were accommodated into the primer design by selecting bases that were common to the majority of species. Thus, GDF-9 Sp up (sense: 5' AAG CTC TAT AAG ACA TAT GCT A 3') was generated to a sequence contained within exon 2 of GDF-9; while GDF-9 Sp dw (anti-sense: 5' AGA CTG ATT TGA GTC AGT GTT C 3') was developed to span the intron between exon 1 and 2 of the gene.

SPECIFIC AIMS

(1) Characterize whether GDF-9, an oocyte specific factor, is expressed exclusively in developing, and maturing oocytes.

A) Observe qualitatively the expression of GDF-9 mRNA at time periods that reflect ovarian organogenesis, in terms of oogonial mitosis, oogonia atresia, primordial follicle formation, folliculogenesis, and ovarian senescence.

B) Determine which oocytes at differing stages of folliculogenesis express the GDF-9 protein.

(2) Confirm that GDF-9 is an ovary specific factor involved in ovarian organogenesis; expressed exclusively by oogonia, oocytes of primordial follicles, and oocytes at all levels folliculogenesis.

A) Identify the expression of GDF-9 mRNA in ovarian and non-ovarian tissues.

(3) Determine whether neonatal DES exposure modifies GDF-9 expression and leads to alterations in the normal course of primordial follicle formation.

A) Observe, through semi-qualitative analyses, the effect neonatal DES exposure has upon the mRNA expression of GDF-9 during ovarian organogenesis, oogonial mitosis, oogonia atresia, primordial follicle development, and folliculogenesis.

SPECIFIC AIMS (continued)

B) Determine the effects of neonatal DES exposure on the expression of GDF-9 mRNA in the aging ovary.

C) Quantify the appearance of POFs in neonatal and adult ovaries.

MATERIALS AND METHODS

Experimental Animals

Syrian Golden hamsters and C57BL/6Nsd black mice were purchased from Harlan Spague Dawley (Indianapolis, IN) and maintained accordingly to the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Care (AAALAC).

Ovarian Collection for Neonatal DES Impact upon GDF-9 Expression

Timed pregnant Syrian Golden Hamsters were obtained from Harlan Spague Dawley. Within six hours following birth (Day 0), litters were adjusted to optimize the number of females pups per mother, with totals being limited to eight. Pups from six litters were injected with 50ul of corn oil (control), while the remaining six litters were injected with 50ul of corn oil containing 100ug DES. At pre-determined time points reflecting ovarian development and early follicular growth (days 1, 3, 5, 6, 7, 8, and 9), neonates were randomly selected from litters and terminated by CO_2 asphyxiation and decapitation. Ovaries were isolated from the reproductive tract microscopically (Nikon Dissecting Microscope) (Figure 1). Those destined for utilization in analysis of GDF-9 mRNA were snap-frozen in a 1.5ml DEPC-treated microfuge tube and then stored at -80C. The remaining ovaries were then placed in 4% paraformaldehyde, followed by 70% EtOH. Fixed tissues were sent off to Histo-Scientific Research Laboratories Inc. (HSRL, Woodstock, VA) for histomorphological sectioning and staining.

Ovarian Collection for Neonatal GDF-9 Expression Analyses

Timed pregnant Syrian Golden Hamsters were obtained from Harlan Spague Dawley. Within six hours following birth (Day 0), litters were adjusted to optimize the number of females pups per mother with totals being limited to eight. At pre-determined time points reflecting ovarian development and early follicular growth (days 1, 3, 5, 6, 7, 8, and 9), neonates were randomly selected from litters and terminated by CO_2 asphyxiation and decapitation. Ovaries were isolated from the reproductive tract microscopically (Nikon Dissecting Microscope) (Figure 1). Those destined for utilization in analysis of GDF-9 mRNA were snap-frozen in a 1.5ml DEPC-treated microfuge tube and then stored at -80C. The remaining ovaries were then placed in 4% paraformaldehyde, followed by 70% EtOH. Fixed tissues were sent off to Histo-Scientific Research Laboratories Inc. (HSRL, Woodstock, VA) for sectioning and embedding in paraffin for eventual analysis by immunohistochemistry. Several DES exposed neonates were selected out and allowed to mature for use in aging GDF-9 mRNA analyses.

Aging Ovary Expression of GDF-9

Retired birth mothers of hamster litters were allowed to progress to time points representative of reproductive ovarian senescence (> 40 weeks; 280 days). At various ages before, during, and after follicular depletion hamsters were terminated by CO_2 asphyxiation and by cervical dislocation. Ovaries were removed and placed in a 1.5ml DEPC-treated microfuge tube; snap-frozen and then stored at -80C for GDF-9 mRNA analysis.

Non-Ovarian Tissue Retrieval

At varying ages animals were randomly selected; anesthetized and terminated using CO_2 and cervical dislocation. Tissue samples were collected individually by segregated instruments pre-soaked in 70% EtOH. The removals of the tissues were coordinated in a logical manner; moving in the direction of increasing vascularity. Isolated tissues were immediately snap-frozen in 1.5ml DEPC-treated microfuge tubes for mRNA analysis.

GDF-9 Oligonucleotides

Since the hamster gene sequence for GDF-9 is unknown, two sets of oligonucleotide primers pairs were generated (as previously discussed) with conserved regions of (mouse and rat; or mouse, rat, human, cow, and pig) GDF-9 based upon sequence comparisons from the NCBI website (www.ncbi.nih.gov) (Table 1). For semi-quantitative analysis, primers were also developed for a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH); detailed in Table 1.

RNA Isolation and RT/PCR Analysis

For semi-quantitative RT/PCR analysis, total RNA was extracted from various tissues using Ultraspec RNA isolation reagent (Biotecx Inc.). Tissues were homogenized in one milliliter of Ultraspec with individual polytrons pre-soaked in a 10% solution of SDS/DEPC-treated H_2O followed by a 95% EtOH wash. After a 5 minute period 0.2ml of chloroform was added to all tubes followed by a 15 second period of vigorous inversions. Samples were incubated on ice for another 5 minutes. Tubes were then centrifuged for 15 minutes @ 12,000 RPM (4C). The supernatants were carefully

removed and placed in new DEPC treated microfuge tubes. An equal volume of chilled isopropanol was added to all samples and inverted. Tubes were stored at -20C overnight to facilitate the precipitation of the RNA. Precipitated RNA was concentrated by centrifugation at 12,000 RPM for 10 minutes (4C). RNA pellets were washed twice with 1.0ml of 75% EtOH in DEPC-treated H_2O. RNA was centrifuged for 5 minutes at 7,500 RPM (4C) following each wash. The RNA pellet was dried by centrifugation for 10 minutes using a Savant Vacuum Dryer. For resuspension of the RNA, 20ul of nuclease-free water was added to each tube and incubated at 55C for 15 minutes. Total RNA was quantitated via UV spectroscopy at 260/280nm using an Eppendorf Biophotometer.

Aliquots of total RNA (2.0ug, 5.0ug, or 10ug) were reversed transcribed using a Promega Reverse Transcription System, random hexamer and oligo-dT primers. Samples were incubated at room temp for 10 minutes followed by a 30 minute incubation at 42C and a 3 minute incubation at 94C. Ten ug of each RT reaction was used in each 25ul PCR reaction (Promega PCR Core Kit 1) primed with 2.5ul of GDF-9 specific oligonucleotides, preformed under the following conditions: 35 cycles of denaturation at 94C for 1 minute, annealing at 55C for 1 minute, and extension at 72C for 2 minutes. Amplification of the GDF-9 184-495 set yields a product of 331 bp. Amplification of GDF-9 sp up-dw set yields a product of 202 bp. PCR products were separated on a 2% agarose gel containing ethidium bromide and resulting bands were visualized by UV light and photographed using a Kodak digital camera coupled with a Kodak EDAS photodocumentation system.

DNA Gene Sequencing

PCR products were extracted from agarose gels and purified using a QIAEX II Gel Extraction Kit (Qiagen, Inc., Valecia, CA). DNA content was analyzed via UV spectroscopy at 260/280 nm. 100ng of purified PCR product and 25ul of the diluted corresponding primer set (10 pmol/ul) were sent to the University of Kansas Medical School Biotechnology Facility in Kansas City, KS., for sequencing.

Immunoblot Detection

Ovaries from both hamsters and mice were homogenized in 1 ml of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, in phosphate-buffered saline, pH 7.4) containing protease inhibitors (Sigma P-8340). Lysates were incubated on ice for 30 minutes then centrifuged at 10,000 RPM for 10 minutes at 4C. The supernatant, containing the total cell lysate, was removed and stored at -20C. Total protein content of the lysate was determined using a BRC Protein Assay Kit (BioRad 500-0113, 500-0114, 500-0115). Lysates were dilluted in a 4 to 5 ratio with 5x Laemmli sample buffer containing reducing agent β-mercaptoethanol (Sigma). Samples were heated at 94C for five minutes. Various volumes, of equal protein content, were loaded into a 12% acrylamide/Tris-glycine pre-cast gel (In Vitrogen 6010633). Pre-stained molecular weight markers (In-Vitrogen) and secondary antibody reactive molecular weight markers (Santa Cruz 10905) flanked the samples. Under constant voltage, at room temperature, samples were separated and then electrotransferred to PVP hydrophobic membranes overnight at 4C. Membranes were blocked for 1 hour with 100 ml of Blotto A (5% w/v non-fat dry milk, 0.05% v/v Tween 20). Membranes were

exposed to either a polyclonal goat anti-mouse GDF antibody (Santa Cruz 7407) or goat IgG (Santa Cruz 2028) in 15ml of Blotto A for 2 hours; room temperature. Afterwards, membranes were washed 3 times in 5 minute incurables with TBS/Tween 20. Membranes were then incubated with a horseradish peroxidase linked donkey anti-goat secondary antibody (Santa Cruz 20330), at room temperature, for 1 hour. Following the incubation with the secondary antibody, membranes were washed twice for five minutes in TBS/Tween 20 and once in TBS. Membranes were then covered with 3ml of Luminol reagent (Santa Cruz 2048) for 1 minute. Bands were detected using a chemiluminescent BioRad Versadoc digital photoimaging system.

Immunohistochemistry

Ovaries were collected from 5, 9, 75, and 88-day-old Sprague Dawley Syrian golden hamsters and fixed in 4% paraformaldehyde for 24 hours. After fixation samples were placed in 70% EtOH and sent off to Histo-Scientific Research Laboratories Inc. (Woodstock, VA) for embedding and sectioning. Ovarian sections were deparaffinized at 37C then rehydrated in a graded series of EtOH solutions. Slides were incubated at 37C for 4 hours in 10% normal goat serum in PBS, pH 7.5 containing 0.05% Tween 20 followed by an overnight incubation at 4C with either anti-rat GDF-9 antiserum (1:200) [courtesy of Dr. Aaron Hsueh, Department of OB/GYN, Stanford University, CA] or mouse IgG (1:200). Sections were washed repeatedly in PBS/Tween pH 7.5 then incubated at 37C for 1 hour in PBS/Tween containing goat anti-mouse conjugated with horseradish peroxidase (1:400) (Santa Cruz # sc-2005). After three five minute consecutive washes, tissues were incubated at room temp for 10 minutes with peroxidase

substrate NovaRed (Vector Laboratories # sk-4800) followed by several 5 minute washes of distilled water to halt the reaction.

Isolating White Blood Cells

Adult Syrian Golden hamsters of varying ages were anesthetized and terminated using CO_2 and cervical dislocation. A vertical incision was made at the base of the vaginal opening; continuing diagonally through the peritoneal cavity. A lateral cut through the diaphragm and the sternum was made to expose the heart. Due to a brief period of electrical misfiring of the heart after termination, ventricular pulsation occurs momentarily allowing for blood flow to continue through the circulatory system. An 18 gauge needle; 3cc syringe filled with 200ul of 0.5m EDTA (Sigma) was inserted into the right ventricle of the heart. Total blood volume obtained per animal was 3cc. The blood collected from each animal was transferred into individual 14 ml polystyrene tubes and centrifuged at 7,000 RPM for 15 minutes. The upper white buffy coat was removed and placed into a 1.5ml DEPC treated microfuge tubes followed by centrifugation at 7,500 RPM for 5 minutes (4C). The supernatant was discarded and the remaining pellet washed with 1 milliliter of PBS. Samples underwent a final centrifugation at 7,500 for 5 minutes. Supernatants were discarded from the tubes and the remaining pellet stored at -80C for eventual RNA analysis.

Kidney Perfusion

Adult golden hamsters were anesthetized and terminated as previously described. Incisions were made on the ventral surface of the animal to expose the interior organs. A

horizontal cut extending through the diaphragm was made to relieve excess tension placed upon the ribs and the lobes of the liver due to the dismemberment of the sternum. One of the two kidneys was randomly clapped off at the renal artery junction. An 18 gauge; 3cc syringe containing 1x saline was inserted into the descending aorta at a point that was just inferior to the right ventricle, yet superior to the liver. In a simultaneous manner the inferior vena cava was cut several centimeters below the midline of the renal vessels while gentle pressure was placed on the syringe to initiate the flushing. Positive results were noticeable by a distinct color change in the flushed kidney. Both kidneys were removed and snap froze in 1.5ml DEPC treated microfuge tubes for RNA analysis.

Microscopic Analysis

Immunohistochemistry and hematoxylin/eosin-stained sections were analyzed and digitally photographed by using a Nikon Eclipse E800 microscope equipped with a MagnaFire digital camera (Optronics, Goleta, CA) and AnalySIS® digital imaging software (Soft Imaging System Corp., Lakewood, CO).

RESULTS

Aim (1) Characterize whether GDF-9, an oocyte specific factor, is expressed exclusively in developing, and maturing oocytes.

A) Observe qualitatively the expression of GDF-9 mRNA at time periods that reflect ovarian organogenesis, in terms of oogonial mitosis, oogonia atresia, primordial follicle formation, folliculogenesis, and ovarian senescence.

B) Determine which oocytes at differing stages of folliculogenesis express the GDF-9 protein.

Neonatal Ovarian Morphology and GDF-9 mRNA Analyses

At birth, the hamster ovary is developmentally immature, relative to most mammalian species, and is undergoing extensive organogenesis. Morphologically, mitotic oogonia are subdivided into "oogonial nests" by cortical mesothelium arising from the medullary hilus (Figure 3, A). Oogonial mitosis continues throughout post-natal Day 3. GDF-9 mRNA was expressed at Day 1 (Figure 3, B) and Day 3 post-partum. (Figure 3, C). Two oligonucleotide primer pair sets; GDF-9 184-515 and GDF-9 Sp up-dw, were utilized to study GDF-9 mRNA expression. Both post-natal Day 1 and Day 3 produced products that correlated with the predicted size: GDF-9 184-515 (331 bp) and GDF-9 Sp up-dw (202 bp). Importantly, GDF-9 mRNA is expressed at a developmental period that lacks follicle organization and/or formation.

Immense changes occur in the ovary by post-partum Day 5. Oogonia become individually segregated by somatic pre-granulosa cells, as "oogonial nests" disassociate.

During this re-organizing period, germ cells begin to associate with adjacent pre-granulosa cells in order to escape atretic processes. Ovarian morphology during this period exhibits numerous apoptotic oogonia (Figure 4, A). Extensive cell shrinkage and nuclear degradation is characteristic of this irreversible process. Ovarian GDF-9 mRNA analysis at post-natal Day 5 (Figure 4, B) revealed transcriptional expression of this growth factor. GDF-9 184-515 primers produced an expected 331 bp band, while GDF-9 Sp up-dw produced an expected 202 bp product.

Complete encapsulation of the oocyte by squamous pre-granulosa cells occurs within post-partum Day 6 (Figure 5, A). These structures, known as primordial follicles, arrest developmentally within prophase I of meiosis and contribute to the "resting germ cell pool". Atretic oogonia, dispersed throughout the ovarian cortex, continue to the depletion of germ cells. Post-natal Day 6 mRNA expression was visually evident (Figure 5, B). GDF-9 184-515 and GDF-9 Sp up-dw expressional bands were relative to expected products: 331 bp and 202 bp.

Populations of quiescent primordial follicles transform into primary follicles throughout post-partum Day 7 and Day 8. Indication of primordial to primary transformation is evident by the appearance of the granulosa cell layer: single and cuboidal (Figure 6, A). Development of primary follicles signifies the beginning of an irreversible ovarian process known as folliculogenesis. Transcription of GDF-9 was clearly evident at post-natal Day 7 and Day 8 (Figure 6, B and C). GDF-9 expression was denoted by RT/PCR alignment of GDF-9 184-515 at 331 bp and GDF-9 Sp up-dw at 202 bp.

Morphology of a post-partum Day 9 ovary exhibited characteristics that were markedly different than earlier developmental periods previously described. Notably, oogonial atresia, which occurred extensively in post-natal days prior to Day 9, was non-apparent among the structures (Figure 7, A). Development of secondary follicles was visually prevalent within the central cortical region of the ovary. These follicles adapted a similar conformation to those of primary follicles; differing in the magnitude of surrounding cuboidal granulosa layers. Sizeable populations of primary and primordial follicles were localized in a gradient fashion as primordial follicles were rarely identified within the meduallary region of the ovary. Post-natal Day 9 mRNA expression of GDF-9 was notably distinct (Figure 7, B). RT/PCR analysis of ovarian tissues with GDF-9 184-515 produced an expected 331 bp product, while analyses with GDF-9 Sp up-dw exhibited an expected 202 bp product.

Adult Ovarian Morphology and GDF-9 mRNA Analyses

Morphological characteristics of a Day 75 ovary reveal every aspect of folliculogenesis: primordial recruitment, preantral follicle development, selection and growth of a Graafian follicle, and atresia (Figure 8). Contrary to the physiology of neonatal ovaries, reproductively active ovaries include highly differentiated theca compartments (Figure 8, B and C), preovulatory follicles (Figure 8, A), corpora lutea (Figure 8, C), and remnants of luteinized follicles. GDF-9 mRNA expression was clearly evident at Days 75, 138, 166, and 229. Transcription of the message was indicated by 331 bp RT/PCR products; an expected size of GDF-9 184-515 (Figure 9, A). Identical

expressional trends were observed at Days 75, 138, 166, and 229 by GDF-9 Sp up-dw: 202 bp products (data not shown).

As the aging ovary reached stages consistent with a dwindling and/or exhausted follicle pool (> 280 Days), GDF-9 mRNA retained expressional similarities equal to what was observed at periods reflecting follicle organization and development. At Days 209 and 258, GDF-9 expression was prevalent, as marked by the appearance of 202 bp products indicative of GDF-9 Sp up-dw (Figure 9, B). At extended time points, Days 440 and 531, which reflected ovarian senescence GDF-9 mRNA was detected as illustrated by 202 bp products (Figure 9, B). GDF-9 184-515 analyses at Days 209, 258, 440, and 531 produced equivalent results (data not shown).

GDF-9 mRNA expression was unaffected by the varying developmental processes that occur throughout ovarian organogenesis, folliculogenesis, and reproductive senescence (Table 3). Expression of GDF-9 was clearly evident during periods of follicle formation (prenatal Day 6) and active folliculogenesis (> post-pubertal Day 21). GDF-9, a known oocyte specific factor, was expressed at time periods (Days 1, 3, 5, and Days 440, and 531) that lacked follicle appearance. Analyses of all time periods the with primer sets GDF-9 184-515 and GDF-9 Sp up-dw retained identical expressional results.

Sequence Homologies

Isolation of cDNA products from ovarian PCR analyses was utilized to confirm GDF-9 expression. Analysis of sequenced ovarian products was compared against GDF-9 gene sequence data of known species. Oligonucleotide homology of the hamster ovarian product produced by the use of the GDF-9 184-515 primer set was 91%

homologous to sequences of both the rat and mouse (Table 2). GDF-9 184-515 product homology against the human GDF-9 sequence was 80% compatible. Products sequenced from the GDF-9 Sp up-dw set produced similar results. Comparisons of the sequence against rat and mouse exhibited 91% homology, and 84% homology aligned to human sequences.

Ovarian GDF-9 Protein Expression

For protein analysis of GDF-9, two commercially obtained antibodies (R&D and Santa Cruz) were utilized among various protein assays. These two commercially purchased antibodies are polyclonal antibodies of mouse origin produced in goats. The selection of these antibodies was based upon the following criteria: 1) current literature produced evidence of protein expression in an identical experimental model; 2) availability of commercially obtainable GDF-9 antibodies were limited to only the two polyclonals selected here; and 3) Arguably, polyclonal antibodies are capable of recognizing multiple epitopes of an antigen.

R&D and Santa Cruz Goat Polyclonal GDF-9 Antibodies

Reproductively active (Day 60) hamster and mice ovaries were analyzed for GDF-9 protein expression by Immunoblot assays under reducing conditions. The polyclonal R&D antibody was analyzed at 1:1000, 1:500, 1:200, and 1:100 dilutions; equally replicated (Table 4). Negative results were identified for each replicate and dilution. Total protein content was increased from 50ug per well to 125ug per well and

re-evaluated at 1:1000, 1:500, 1:200, and 1:100 dilutions. However, results remained negative.

Immunonblots utilizing the polyclonal Santa Cruz antibody were preformed as mentioned above for the R&D antibody. Replicates of 50ug protein contents analyzed at 1:1000, 1:500, 1:200, and 1:100 produced negative results (Table 4). Increased protein content, 125ug per well at 1:500 and 1:200 dilutions, produced products at 132 kDa and 43 kDa (Figure 10, A). However, neither bands correlated to published molecular weights for GDF-9. Under reducing conditions, GDF-9 is expected to express the mature form at 15,000 kDA - 19,000 kDA, and the apparent full-length GDF-9 protein at about 60,000 kDA - 80,000 kDA [80]. To determine the relevancy of the 132 kDa and 43 kDa products, since neither were expressed by the non-immune control, additional replicates were preformed at 1:500 and 1:200 dilutions using a 2:1 ratio of a GDF-9 peptide blocker and the GDF-9 antibody. Peptide block analyses confirmed that bands of 132 kDa and 43 kDa were non-specific products (Figure 10, B).

Immunohistochemical assay of Day 75 hamster ovarian sections, were probed by the polyclonal R&D antibody at 1:500, 1:200, and 1:100 dilutions (Table 5). Numerous replicates were preformed under conditions of antigen and non-antigen retrieval along with extended periods of blocking, primary and secondary incubations, and substrate exposure. All compiled attempts produced negative results.

Analyses with the polyclonal Santa Cruz antibody initially produced identical results (Table 5). At 1:500, 1:200, and 1:100 dilution strengths, extreme non-specific signals were identified in non-immune controls. Protocol parameters were optimized: extended periods of blocking, increased primary and secondary exposures, non-antigen

retrieval, and addition of hydrogen peroxides with substrate. A single positive result was identified (Day 75) at a 1:200 dilution, after a four hour block, overnight primary antibody incubation, one hour secondary incubation, and a five minute substrate exposure.

Hsueh Monoclonal GDF-9 Antibody

With insufficient results occurring with the use of polyclonal antibodies R&D and Santa Cruz, an individual lab developed rat monoclonal GDF-9 antibody was obtained: courtesy of Dr. Aaron Hsueh, Department of OB/GYN, Stanford University, CA. This monoclonal antibody was developed against the COOH-terminal region of the mature protein. Published results showed this antibody to be effective in recognizing ovarian-expressed GDF-9 through immunohistochemical and recombinant immunoblot assays [80]. Due to the acquired volume of the antibody being minimal, only two immunoblot analyses were performed each with 125ug of total protein per well (Table 4). Dilution strengths of 1:8000 and 1:500 were used in the two trials. Both dilutions, analyzed in Day 60 hamster and mouse ovaries, resulted in negative outcomes.

However, immunohistochemical analyses utilizing the Hsueh monoclonal GDF-9 antibody produced visually evident results as positive immune signals for the GDF-9 protein was expressed in Day 75 ovarian sections (Figure 11). Expression of GDF-9 was localized specifically to the oocyte. Several highly organized follicles produced an intense (red) signal for the GDF-9 protein. Day 88 ovarian sections produced similar results (Figure 12). GDF-9 was expressed exclusively within the oocyte of highly developed tertiary follicles and secondary follicles. The protein was also detected in

primary follicles however; the signal was noticeably less intense than the higher organized follicles.

Expression of the GDF-9 protein prior to highly organized follicle development was analyzed in Day 5 and Day 9 neonatal hamster ovarian sections. At Day 9, expression of GDF-9 was detected in primordial and primary follicles (Figure 13). Expression was evident within dispersed primary follicles and was localized to the interior medullary region of the ovary and in primordial follicles gathered near the ovarian cortex. In Day 5 ovarian sections indicative of extensive oogonial atresia phases GDF-9 was expressed in non-follicle organized oogonia.

Aim (2) Confirm that GDF-9 is an ovary specific factor involved in ovarian organogenesis; expressed exclusively by oogonia, oocytes of primordial follicles, and oocytes at all levels folliculogenesis.

A) Identify the expression of GDF-9 mRNA in ovarian and non-ovarian tissues.

GDF-9 mRNA Expression in Non-Ovarian Tissues

Through Northern Blot analyses, GDF-9 was identified as an ovary-specific factor localized exclusively to the oocyte [68]. To confirm or disprove this observation, highly sensitive RT/PCR was used to analyze non-ovarian tissues for GDF-9 mRNA expression. Non-ovarian transcription of GDF-9 was observed in Day 235 hamster tissues (Figure 15, A). Various lineages of the reproductive and gastrointestinal system notably indicated extensive non-ovarian expression: uterus, testis, liver, kidney, and spleen. GDF-9 184-515 produced products at the expected 331 bp position. Analysis with GDF-9 Sp up-dw at Day 235 produced an identical pattern of tissue expression (data not shown). At Day 19, similar RT/PCR results produced mRNA signals form non-ovarian tissues: liver and testis (Figure 15, B). Products were visualized at the expected 202 bp position of GDF-9 Sp up-dw. Non-ovarian expression at Day 19 was also confirmed by GDF-9 184-515 aligned products (data not shown).

Non-ovarian expression of GDF-9 was analyzed at numerous developmental stages including neonatal, pre-pubertal, reproductively active, and geriatric (Table 6). In neonatal periods (Day 2 and Day 9) GDF-9 mRNA expression was evident in the liver and kidney. At Day 2, organ identification was difficult due to identical coloration of tissues, thus isolation of only the liver and kidney were performed and analyzed for GDF-

9 mRNA expression. Expanded expressional studies at Day 9 included analyses of other non-ovarian tissues such as the spleen, muscle, heart, uterus, and testis. GDF-9 expression was indicated by the respective GDF-9 184-515 and GDF-9 Sp up-dw base pair products. Pre-pubertal Day 19 non-ovarian samples from liver, kidney, spleen, muscle, heart, uterus, and testis resulted in similar expressional products. Identical results were seen in non-ovarian tissues of reproductively active and geriatric adults. Expression analyses of GDF-9 in male reproductive tissues were conducted at Day 9, 19 and Day 60. No expressional information was generated for muscle extracts at Days 42 and 166. Difficulty in the manual homogenization of the muscle tissue, during RNA isolation, resulted in the acquirement of insufficient RNA for RT/PCR analyses.

Negative controls were employed in various non-ovarian expression assays. Negative products consisted of: 1) RNA + PCR master mix + GDF-9 primers; designated as a Non-RT sample; and 2) RT master mix + PCR master mix + GDF-9 primers; elected as a Non-RNA sample. Figure 16 illustrates an ovarian and non-ovarian PCR at Day 19 including the two negative controls. GDF-9 mRNA expression was evident in the ovary and liver (products produced at 331 bp) but was absent in both ovarian and non-ovarian negative controls. Negative trials were consistently added to an array of non-ovarian tissues analyses as a means to detect and/or disprove possible DNA contaminants. Through these negative analyses, we confirmed that non-ovarian expression of GDF-9 was not due to DNA contamination.

Rodent Non-Ovarian GDF-9 mRNA Expression

Since information regarding the exclusive ovarian expression of GDF-9 was advocated primarily from rodent models, non-ovarian expression of GDF-9 was analyzed in Day 39 and Day 60 mice. At Day 39 transcription of GDF-9 occurred in ovarian and in non-ovarian tissues including the liver, kidney, and spleen (Figure 17). A 331 bp product, indicative of GDF-9 184-515, identified the expression of GDF-9 in rodent tissues. Replicates were performed utilizing both the GDF-9 184-515 and GDF-9 Sp up-dw primer sets and identical expressional results were observed (data not shown).

GDF-9 mRNA Expression in Isolated White Blood Cells

The surprising finding of GDF-9 expression in non-ovarian tissues prompted us to evaluate the possible commonality that exists between these non-ovarian tissues. One obvious similarity between the liver, kidney, spleen, muscle, heart, etc. is their acquisition of vascularity i.e. blood supply. In order to isolate total RNA from blood nucleus rendering white blood cells had to be separated from the remaining blood inhabitants. Acquirement of blood samples were obtained through a right ventricular puncture of terminated adult hamsters and immediately centrifuged to isolate white blood cell residents. Density gradient separations of specific white blood cell populations were not performed. Instead, all white blood cell populations were extracted from centrifuged products and utilized for expressional analyses.

mRNA analysis of GDF-9 expression was clearly defined by the 331 bp product of GDF-9 184-515 on Day 60 (Figure 18, A) and Day 341 (Figure 18, B). Mirror results were generated at Day 60 and Day 341, with analyses utilizing GDF-9 Sp up-dw primers

(Figure 18, C and D). To verify that non-ovarian expression of GDF-9 was not an artifact of DNA contaminants, negative controls were analyzed in conjunction with processed white blood cell products. At Day 60, GDF-9 expression was clearly visible, producing the expected 331 bp product of GDF-9 184-515 (Figure 19). Negative controls exhibited no expression of GDF-9 mRNA.

Human GDF-9 mRNA Expression

A dilemma of scientific research when performed in rodent and non-rodent species is whether acquired results are comparably similar to humans. A preliminary study involving human blood was analyzed for similar expression of GDF-9 mRNA. A single 3cc blood sample was obtained from the radial vein. Isolation of the white blood cells from erythrocytes was similar to the centrification process described above. Analyses with GDF-9 184-515 and GDF-9 Sp up-dw produced expected 331 bp and 202 bp products (Figure 20). Negative controls utilized in the assay lacked GDF-9 expression.

Reduced Expression of GDF-9 mRNA by Kidney Perfusions

The presence of GDF-9 in white blood cells raised the possibility that non-ovarian expression of GDF-9 in various tissues could be due to circulating blood within the structure and/or white blood cell inhabitants. To resolve this predicament hamster kidney perfusions were performed. The kidney was decided upon because; 1) they are highly vascularized as the entire blood content of the system passes through the kidney regularly for filtration, and 2) they are paired which allows for a non-perfused control.

Unilateral kidney perfusions were performed in Day 258 and Day 431 hamsters. Isolated kidneys were identified as flushed (kidney that underwent blood removal by 1x saline) and non-flushed (kidney that was clamped off prior to blood removal). Results indicated that GDF-9 expression in the flushed kidney of Day 258 hamsters was reduced by 33% compared with the non-flushed kidney (Figure 21, A). Day 431 samples produced similar results as evaluations by densitometry indicated that GDF-9 mRNA was reduced by 35% in the flushed kidney (Figure 21, B). Generally, we did not expect a 100% reduction in GDF-9 expression in the flushed kidney since some white blood cells migrate out of the vasculature into the tissue compartments of the structure.

Non-Ovarian Sequence Homologies

Isolation of cDNA products from non-ovarian PCR analyses (liver and blood) was utilized to authenticate the non-ovarian expression of GDF-9. Results of sequenced products were compared against GDF-9 gene sequence data of known species. Oligonucleotide homology of the hamster liver product generated with the GDF-9 184-515 primer set was 91% homologous to sequences of both the rat and mouse (Table 7). GDF-9 184-515 product homology against the human GDF-9 sequence was 79%. Blood products sequenced from GDF-9 184-515 produced similar results. Comparisons of the sequence against rat and mouse exhibited 91% homology, and 80% homology aligned to human sequences.

Immunohistochemical Expression of GDF-9 in the Corpus Luteum

The corpus luteum is one of the most vascularized tissues in the body [42, 43]. Re-evaluation of immunohistochemical analyses in Day 75 and Day 88 hamster ovarian sections revealed GDF-9 protein expression to be identified within this highly vascular structure. At Day 75, the protein was localized to the outer cortex of the corpus luteum; noted by the reddish appearance of the signaling substrate (Figure 22). The strength of the signal in the corpus luteum was visually equivalent to the intensity expressed by the nearby tertiary follicle. Similar results were observed in the corpora lutea of Day 88 ovarian sections (Figure 23).

Potential Mast Cell Expression of GDF-9

Localization of mast cells within the system's structures is widespread; with relative abundances occurring in the dermis, digestive, and reproductive tracts. Morphologically, mast cells are circular composites of connective tissues that are easily identified by their internal basophilic granules [104]. Expression of GDF-9, by apparent mast cells, was detected by immunological analyses of Day 9 hamster ovarian sections (Figure 24). Non-immune control sections were negative for staining however, sections exposed to the GDF-9 antibody exhibited characteristics of GDF-9 expression in non-oocyte like structures. Higher magnification of sections identified mast cells as the source of non-oocyte expressed GDF-9.

Aim (3) Determine whether neonatal DES exposure modifies GDF-9 expression and leads to alterations in the normal course of primordial follicle formation.

A) Observe, through semi-qualitative analyses, the effect neonatal DES exposure has upon the mRNA expression of GDF-9 during ovarian organogenesis, oogonial mitosis, oogonia atresia, primordial follicle development, and folliculogenesis.

B) Determine the effects of neonatal DES exposure on the expression of GDF-9 mRNA in the aging ovary.

C) Quantify the appearance of POFs in neonatal and adult ovaries.

Modification of GDF-9 Expression due to Neonatal DES Exposure

Because of GDF-9's profound involvement in regulating the development and growth of mammalian ovarian follicles, we proposed that GDF-9 may be a target for endocrine disruption. Day 0 neonatal hamsters were exposed to 100ug of DES, a non-steroidal disruptor, and then euthanized at various developmental periods reflecting oogonial mitosis, oogonia atresia, primordial follicle development, and early folliculogenesis. Semi-quantitative mRNA analyses of post-partum Day 1 and Day 3 ovarian samples, periods reflective of extensive oogonial mitosis, displayed similar expression of GDF-9 between the control and DES groups (Figure 25). At post-partum Day 5, indicative of oogonial mitosis, GDF-9 mRNA expression appeared to be non-affected by DES.

Semi-quantitative observations at post-partum Day 6, a period of initial primordial follicle development, indicated slight reductions in GDF-9 expression within

the DES exposed group (Figure 26). Continual analyses at Day 7 and Day 8 post-partum detected similar declines of GDF-9 expression in the DES groups. In neonatal Day 9 GDF-9 mRNA expression was also reduced in the DES-exposed group.

Morphologically, neonatal DES exposure was evident by isolation of the reproductive tract. DES exposed and non-exposed reproductive tracts were isolated from Day1 and Day 5 neonates. DES tracts at Day 1, exhibited a slight hypertrophic response in comparison to the non-exposed tract (Figure 27, A). At Day 5, morphology of the DES exposed reproductive tract indicated abrupt disruption; clearly marked by the distention of the oviduct and the abnormal expansion of the uteri (Figure 27, B).

DES Effects on GDF-9 Expression in the Aging Ovary

In situ analyses of human and rat ovarian sections localized GDF-9 mRNA exclusively to oocytes of primary follicles and follicles of higher organizational levels [74-76]. By correlating with oocyte supply, GDF-9 initially represented an opportunity to observe the effects of an endocrine disruption upon ovarian folliculogenesis; moreover, whether premature decline and/or termination of reproductive function would result. A selected amount of DES exposed neonates were allowed to mature and develop to reproductively active ages and beyond. Sequential order of ovarian isolation was purposed to cover areas that marked active follicle function, follicle decline, and follicle depletion. Unsuspected problems arose in isolating ovaries (Table 8): 1) DES exposed ovaries were difficult to locate because of extreme hypertrophic effects occurring within the reproductive tracts (Figure 28); 2) expansion of the oviduct by liquid puris and dead neutrophils effected concise identification of the ovary; 3) hamsters began to suffer and

ultimately die off from other non-reproductive traumas: liver and spleen cysts, and renal failure. For humane purpose, the experiment was discontinued and remaining DES animals terminated. The small amounts of data collected were inconclusive and non-replicatable.

Neonatal and Adult Quantification of Ovarian Polyovular Follicles

Ovaries of various species, exposed to DES, developed follicles that accommodate multiple oocytes per follicle, i.e., polyovular follicles (POF) [99, 100]. Quantification of POFs in DES exposed and non-exposed neonatal and adult ovarian sections was initially purposed to evaluate the amount of disruption occurring within each developmental stage of the ovary. Neonatal sections of Day 6 and Day 9 were viewed for relative appearance of POFs. At Day 6, indicative of primordial follicle formation, comparisons between non-exposed and DES exposed ovarian sections revealed no apparent disruption of ovarian and follicle development, and no POF formation (Figure 29, A and B). Visualizing Day 9 ovaries, which are in the beginning stages of folliculogenesis, indicated that DES ovarian sections contained approximately equivalent numbers of POFs seen in non-exposed ovarian sections (Figure 29, C and D). Further quantitative analyses of POFs, in response to the aging ovary were never achieved as endocrine disruptive studies were discontinued for reasons mentioned above.

DISCUSSION

Neonatal GDF-9 Ovarian Expression

The exact growth factors responsible for directing follicular growth and expansion of oocytes, granulosa cells, and theca layers during early mammalian folliculogenesis are not well defined. However, it is known that oocyte secreted factors directly affect follicular growth and development [61, 82, 83]. GDF-9, an oocyte expressed growth factor, has shown to regulate the growth and development of non-gonadotropin dependent follicles: i.e. primordial, primary, and secondary follicle stages [61, 79-81].

Analyzing GDF-9 expression in neonatal hamsters was ideal for observing specific stage emergence of GDF-9 transcription. Relative to most mammalian species, the hamster ovary at birth is developmentally immature. Oogonial mitosis is extensively occurring through post-natal Day 1 up to Day 3. In rodent models oogonial mitosis is prevalent during fetal periods where at birth the ovary has already organized oocytes into primordial and primary follicles [7]. Observation of GDF-9 expression prior to primordial follicle development in rodents would have required the deviant task of pre-natal removal. Ultimately, in the neonatal hamster we were able to track the expression of GDF-9 prior to primordial follicle organization and/or development.

mRNA expression of GDF-9 was evident prior to primordial follicle formation. In fact, GDF-9 was expressed during periods of oogonial mitosis (Day 1 and Day 3) and oogonial atresia (Day 5). Occurrence of primordial follicle formation was observed by post-natal Day 6 along with GDF-9 mRNA expression. *In situ* hybridization of pre-natal rodent ovaries failed to demonstrate GDF-9 expression prior to follicular formation, yet

post-natal analyses detected GDF-9 mRNA in oocytes of all follicle stages except primordial [60]. *In situ* hybridization in ovaries of non-rodent species, ovine and bovine, revealed GDF-9 expression beginning at the primordial follicle stage [77, 78]. Consistent with non-rodent expression of GDF-9 we indicated that transcription of GDF-9 occurs with primordial follicle development and continues with higher pre-antral developed follicles, as seen at Day 9. Importantly, we identified GDF-9 mRNA expression in ovaries lacking any type of follicle organization, whether primordial or primary. Thus, we can initially conclude that GDF-9 could possibly be an important factor that regulates oogonial survival during ovarian organogenesis and ultimately a factor responsible for the progressional transition of primordial follicles to primary.

In vitro studies show that rodent primordial follicles are fully capable of undergoing follicle assembly, forming primary follicles in serum-free culture [50]; supporting the non-dependency of gonadotropin involvement in early follicle development. However, the idea that GDF-9 is the delegated factor that promotes primordial to primary transitions generally may be divergent among species as *in situ* analyses of human and rat ovarian sections have localized GDF-9 mRNA exclusively to oocytes of primary follicles and follicles of higher organizational levels [74-76]. Results suggest the possibility of GDF-9 being species specific in the role of follicle organization and development. Reproductive specific factors and regulators are not uncommon to differ between species. For instance, insulin-like growth factor-I (IGF-I) and IGF-II increase mitogenesis and synergistically augment the stimulatory effects of gonadotropins on steroidogenesis. IGF-I and/or -II stimulus to the ovary depends on the specie, as human follicles contain IGF-II mRNA and not IGF-I mRNA; whereas

granulosa cells of rats contain IGF-I and not IGF-II mRNA, and bovine granulosa cells contain both [105]. Translational expression of GDF-9 during early ovarian organization phases and initial follicle development will signify whether GDF-9 is actively participating in these processes and/or if GDF-9 is being transcriptionally regulated; as is common among TGF-β family members.

Because of the novel expression of GDF-9 mRNA prior to follicular development, an un-sequenced genome, and the development of primers from compared consensus sequences of rodent and non-rodent species PCR products were isolated and sequenced for evaluation of homology. Results indicated percentages that ranged well into the 90's, among rodent and human gene sequences. Therefore, mRNA products observed through RT/PCR analyses were truly indicative of GDF-9 expression.

R&D and Santa Cruz Polyclonal Antibodies; and Hsueh Monoclonal Antibody

At a transcriptional level, we had discovered that GDF-9 was expressed prior to primordial follicle formation, a finding that was unrelated to the expression seen in rodents and humans. To identify whether translation of GDF-9 is also occurring at these early organizational and developmental stages two commercially developed GDF-9 polyclonal antibodies (R&D and Santa Cruz) were initially acquired for immunoblotting and immunohistochemical purposes. The selection of these antibodies was based upon: 1) current literature produced evidence of protein expression in an identical experimental model [89]; 2) availability of commercially obtainable GDF-9 antibodies were limited to only the two selected here; and 3) Arguably, polyclonal antibodies are capable of recognizing multiple epitopes of an antigen.

Review of scientific literature involving immunoblot analyses for GDF-9 indicated only two available references for result comparisons [60, 80]. Unfortunately, identical replications of protocols utilized by the two previously published reports were inapplicable due to; 1) inactive pro-form of GDF-9 was specifically analyzed, 2) individually derived specie specific monoclonal antibodies were used, and 3) mature region results were generated through recombinant GDF-9 analyses.

Immunoblotting analyses were initially performed with reproductively active hamster ovarian samples in order to optimize protein concentrations and antibody dilution efficiencies needed for recognition of neonatal expressed GDF-9. Tribulations were prevalent with the R&D and Santa Cruz polyclonal antibodies as both failed to produce products of expected molecular weights characteristic of GDF-9. It became apparent that these commercially obtained antibodies could possibly be; 1) non-compatible with epitopes of cross-species; or 2) non-sufficient for immunoblot protocols.

R&D and Santa Cruz polyclonal antibodies were manufactured against the COOH terminus of the mature region of mice, thus immunoblots were analyzed by the addition of rodent ovarian tissues to test whether the commercially obtained antibodies were insufficient for species other than the derived specie origin. Analyses produced unidentified products non-reflective of GDF-9 as confirmed by blocking peptide assays. Antibodies of polyclonal origin were assumed to be optimal for GDF-9 protein analyses due to their ability to recognize multiple epitopes of the defined antigen and their general ability to retain functionality after assay processing and/or exposure to stringent conditions.

Even with specie specific conditions antibodies failed to produce positive results. The fact that only two immunoblots have ever been successfully achieved, for GDF-9, raises concern in whether the protein is capable of antigen/antibody reactivity after being structurally disrupted. Typically, immunoblots contain a denaturing agent, sodium dodecylsulate (SDS), which functions to break apart non-covalent bonds holding proteins in their native conformation; and a sulfhydryl reducing agent, β-mercaptoethanol, that further denatures the protein by disrupting disulfide bonds holding peptide chains together.

Structurally, GDF-9 lacks the fourth of the seven conserved cysteine residues of the COOH-terminal, standard to TGF-β family members. Substitution of the cysteine believed to participate in intermolecular disulfide bond formation during dimmerization results in a noncovalent association of GDF-9 peptide subunits [68]. Generally, optimal immunobloting results are established through linearization of the protein by SDS where further disruption of the globular conformation of the protein and its peptide chains can be achieved by addition of β–mercaptoethanol. The noncovalent relationship of GDF-9 creates an inevitable circumstance for immunoblot analyses. Structurally, GDF-9 appears to be susceptible, even in the presence of SDS, to protein degradation moreover, resulting in the demolishment of the antigen. Arguably, to achieve a favorable immunoblot assay for GDF-9, antibodies must be developed against the structural conformation of the protein after exposure to denaturing agents and castment through gel matrixes thus optimizing the efficiency of antigen/antibody recognition.

The actual scientific sufficiencies of our two commercially obtained polyclonal antibodies were re-evaluated within available publications. GDF-9 protein analyses were

generally obtained through immunohistochemical protocols that utilized individually lab developed monoclonal species specific antibodies [74, 80]. Immunohistochemical analyses conducted with the Santa Cruz polyclonal were scarcely established, as experimentally results were inconsistent within various identical trials [74]. However, immunoflorescence detection of GDF-9 from, in vivo and *in vitro*, ovaries of Syrian Golden hamsters, was obtained through assays using the polyclonal Santa Cruz antibody [89].

Consequently, immunochemical assays are considerably reduced in stringency compared to immunoblotting protocols. The natural tertiary conformation of the protein is never severally disrupted thus exposed antigens retain immunological abilities to recognize presented antibodies. With evidence to support immunohistochemical identification of GDF-9, and identical specie recognition by commercial antibodies immunohistochemistry protocols were optimized for recognition of GDF-9.

Various immunochemical replications of reproductively active hamster ovarian sections were analyzed by the commercially obtained antibodies. Results were never consistent between trials. The Santa Cruz polyclonal antibody produced immune signals that appeared to be representative of GDF-9 however; intensity of the response was minimal and inconsistent.

Due to insufficient results occurring with the use of polyclonal antibodies R&D and Santa Cruz extensive adjustments were established for continual attempts to detect the translation of GDF-9. Extreme protocol alterations were marked by the discontinued use of commercially obtained polyclonal antibodies. Instead an individual lab developed

rat monoclonal GDF-9 antibody, developed against the mature region COOH-terminus, was obtained through an acquaintance: Dr. Aaron Hsueh, for immunohistochemistry use. We anticipated positive results as published studies have revealed the antibody to be effective in recognizing ovarian expressed GDF-9 through immunohistochemical and recombinant immunoblot assays [80].

Other immunohistochemical modifications, dealing with identification substrates, were adapted. An intensive illuminating substrate, aminoethyl carbazole (AEC), was attained for use because of its ability to produce a distinctive red signal with attachment to systematically present horseradish peroxidase secondary antibodies. Pervious immunochemical assays were analyzed by a 3,3'-diaminobenzidine (DAB) substrate, which visually appeared brown and inevitably was identical in appearance to the normal stromal compartments of the ovary causing identification errors. Counter staining of the ovarian sections was also discontinued due to apparent expression of GDF-9 in locations other than the oocyte.

Initial trial runs with the adapted modifications produced immediate results. Reproductively active hamster ovarian sections revealed GDF-9 to be distinctly present in oocytes of highly organized follicles. Ultimately, the efficiency of the rat monoclonal antibody to recognize GDF-9 in hamster ovarian sections presented information that supports a structure commonality between species. Initially, polyclonal antibodies were used due to their commercial attainability and their ability to recognize several epitopes of the antigen. Production of these polyclonal antibodies specifically were developed from COOH terminal regions of rodent species thus we contemplated possible dissimilarities among the structure when compared across species, which though

polyclonal use, would allow for GDF-9 expression in hamster ovarian sections. However, scientific results were inconsistent and unreplicatable with the use of the polyclonal antibodies. The only source of usable data was generated through analyses utilizing the Hsueh monoclonal antibody. Monoclonal antibodies are known to be specific for one epitope of the desired antigen. Because of this specificity the antibody is highly effective however; the antigen must have the specific epitope present and available when utilizing immunochemical assays. The difficulty of acquiring or developing an antibody for GDF-9 may explain why translational data for non-rodent species is presently non-existent.

Neonatal Ovarian Translation of GDF-9

Identification of the expressional pattern of the GDF-9 protein throughout the various organizational phases of folliculogenesis has been negligible among non-rodent species. Immunohistochemical analyses of mice, rat, and human ovarian sections reveal GDF-9 protein to be expressed in oocytes throughout folliculogenesis; initially beginning at the primary follicle stage [74, 80]. Previously, through transcription analyses, we observed expression of GDF-9 mRNA during periods of oogonial mitosis, oogonial atresia, primordial follicle development, and folliculogenesis. Pre-mature appearance of expressed GDF-9 during extensive ovarian organogenesis suggests the involvement of this growth factor in the regulation of these phases. However, TGF-β family members are known for their ability to be translational regulated thus assurance of GDF-9's involvement during ovarian organogenesis is dependent upon the expression of the protein.

Literature sighted observations of immunoflorescence detection of GDF-9 from *in vivo* and *in vitro* ovaries of Syrian Golden hamsters' illustrated protein expression to occur at initial periods of primordial follicle organization [89]. Likewise, through neonatal immunohistochemical analyses we have identified GDF-9 to be expressed at developmental periods indicative of extensive oogonial atresia and dispersal. Actual translation of GDF-9 prior to primordial follicle formation suggests an involvement in the regulation of oogonial survival and/or development prior to and during follicle formation. Translational expression of GDF-9, in hamster ovarian sections, was well defined in follicles encompassing initial follicular development, i.e. primordial follicles and organized folliculogenesis, preantral follicles, Graafian follicles, and preovulatory follicles.

GDF-9 Expression in Oocyte Depleted Follicles

In situ analyses of human and rat ovarian sections have localized GDF-9 mRNA exclusively to oocytes of primary follicles and follicles of higher organizational levels [74-76]. Thus, oocyte specific GDF-9 should reflect a declining expression of GDF-9 as the ovary reaches reproductive senescence. GDF-9 mRNA expression was visualized throughout time periods that reflected active reproductive function, a result that was expected because of GDF-9's regulatory actions during folliculogenesis [61, 79-81]. As the hamster reached the ages of follicular decline (> Day 28) we expected to see a reduction of expressed GDF-9 however, GDF-9 mRNA expression was well reflected after follicle decline and reproductive senescence. Current controversial studies dealing with germ cell renewal of aging ovaries would imply existence of germ cells up to and

partially beyond reproductive capacities [30]. However, the statistical counts of the supposed renewal germ cells are not sufficient to maintain identical numbers seen during reproductively active stages. Thus, if continual renewal is occurring after reproductive decline the levels of GDF-9 expression would still be hypothesized to be decreased.

Non-Ovarian Expression of GDF-9

In an unanticipated manner, we deliberated why GDF-9 would be expressed in ovarian tissues that reflected reproductive senescence when scientifically published *in situ* analyses report GDF-9 mRNA to be localized exclusively to oocytes of mammalian follicles [74-76]. Surprisingly, through ovarian and non-ovarian tissues analyses we revealed mRNA expression of GDF-9 in a vast array of non-reproductive tissues.

GDF-9 was initially identified as an ovary specific factor by poly(A)-selected Northern analyses [68], later *in situ* hybridization assays lead to the direct localization of GDF-9 within the oocyte [60, 74-78]. With infinite amounts of scientific results revolved around GDF-9's ability to regulate follicular growth and development the possibility of GDF-9 being non-ovarian specific factor has been argumentative and notably disregarded.

Through RT/PCR analyses we have indicated mRNA expression of GDF-9 in reproductively associated tissues (uteri and testis) other than exclusively the ovary. Our findings correlate to those by Fitzpatrick et al. [76] who identified non-ovarian expression of GDF-9, through poly(A)-selected Northern analyses in the hypothalamus and testis [76]. Further investigations into ectopic expression of GDF-9 revealed transcription of the gene to be expressed in non-reproductive tissues. In fact, we have

identified GDF-9 to be expressed in the liver, kidney, spleen, heart and muscle. To assure products were GDF-9; 1) negative controls were instigated into RT/PCR protocols, and 2) sequence analyses were performed on non-ovarian PCR products; results confirming homology to published GDF-9 sequences.

It is not unusual for a factor to be initially identified as "exclusively specific". For instance BMP-7, a highly expressed factor involved in embryonic myocardium development [59], was identified to be specific in neonatal heart development. However, recent studies have shown the factor to be expressed and involved in the stimulation of granulosa cell proliferation [53]. Thus, non-ovarian expression of GDF-9, an initially identified ovarian specific factor, could have been expected.

Scientific difference seen in recognizing non-ovarian expression of GDF-9, relates to the sensitivity of the assays. Northern analyses were the initial assays utilized for mRNA detection. However, advancements in scientific protocols lead to the development of RT/PCR. The sensitivity of RT/PCR is increased several fold compared to Northern analyses. Expression of GDF-9 in non-ovarian tissues may be retained at lower levels than to what is produced in the ovary, thus expressional analyses utilizing Northern blots may be insufficient to detect transcription. Therefore, PCR analyses optimize these low transcribed levels and visually make them prevalent for analyzing purposes.

GDF-9 Expression in White Blood Cells

Expression of GDF-9 mRNA in a diverse range of non-reproductive tissues was a revolutionary finding. However, we questioned whether a commonality existed between

non-ovarian expressed GDF-9 and the tissues identified to transcribe the message. After evaluating the situation, we proposed that the vascularity of the organs and tissue could possible link the non-ovarian expression of GDF-9. In analyzing white blood cell extractions, we observed GDF-9 to be expressed in the blood regardless of reproductive stature: active or senescent; and importantly, GDF-9 expression is observed across species: hamster and human.

With the appearance of GDF-9 transcription in white blood isolates, we contemplated what might happen to the expression of GDF-9 if the blood was removed from the tissue. Since GDF-9 expression was identified in the kidney, a major blood detoxification structure, and is replicated into two identical structures we utilized this structure for perfusion purposes. Densitometry results indicated that GDF-9 mRNA expression was significantly reduced in kidneys that were pefused. A complete reduction of GDF-9 expression was not expected, as tissues retain numerous white blood cells dispersed within the tissue matrix of the organ. Only circulating white blood cells would be susceptible to the perfusion.

We concluded that white blood cell expression of GDF-9 was partially and/or fully responsible for the non-ovarian expression seen in various tissues. However, we cannot define the functionality of GDF-9 in the blood. Under certain conditions TGFs-β will demonstrate anti-proliferative effects on endothelial cells, macrophages, and T and B-lymphocytes with effects including the secretion of immunoglobulins and the suppression of hematopoiesis, myogenesis, adipogenesis, and adrenal steroidogenesis [65-67]. Therefore, GDF-9, a member of the TGF-β superfamily, may be involved in

regulating processes other than mammalian folliculogenesis; possibly angiogenesis and/or hematopoiesis.

Translation of GDF-9 in White Blood Cells

Structurally, the corpus luteum is a highly vascularized ovarian structure that results from collapsing granulosa and theca cell compartments of ovulated follicles. The established extensive vascular network, formed with the development of the corpus luteum, is essential to the functionality of the structure [42, 43]. Identification of translated GDF-9 has been correlated to ovarian oocytes [74, 80]. With the general idea of GDF-9 being oocyte specific GDF-9 expression within corpora lutea would have been nonexistent as the oocyte has long been released from the follicle mass. However, we have consistently observed non-ovarian expression of GDF-9 in a vast array of tissues, and have ultimately found GDF-9 to be expressed within white blood cells. Re-evaluation of immunohistochemical analyses of reproductively active ovarian sections revealed the translation of GDF-9 within corpus luteums. Identification of GDF-9 in the corpus luteum supports the non-ovarian association of this growth factor to white blood cell populations. In fact, our results suggest that GDF-9 is expressed by mast cells; an immunologic white blood cell.

Localization of mast cells, within the system is widespread with relative abundances occurring in the dermis, digestive, and reproductive tracts [104]. Mast cells are filled with basophilic granules that act as a storage refuge for chemical mediators of inflammatory responses. These granules contain histamine, neutral proteases, and importantly eosinophil chemotactic factor of anaphylaxis (ECF-A). This tetrapeptide

binds to receptors on the surface of eosinophils and activates tissue repair processes [106]. In correlation to the ovary mast cells are widely dispersed through the interstitial cortical stroma and are highly prevalent in corpora lutea structures. After the corpus luteum has performed its systematic reproductive function the structure degrades forming a tissue scared corpus albicans. Released basophilic granules from localized mast cells contribute to the repair of the damaged tissue. Thus, GDF-9 expression, within mast cells, may signify an identical functional role for the factor. Further experimental assays involving isolation of specific types of white blood cells will reveal the exact location(s) of GDF-9 expression.

Our ability to identify the expression of GDF-9 in mast cells was optimized by substation of substrate identifiers and elimination of background counterstaining. With previous immunohistochemical trials utilizing GDF-9 antibodies we noticed expression of the factor outside of the oocyte. GDF-9 is known to be a secreted factor but the amount viewed appeared extensive to what would be secreted thus, we eliminated counterstaining of the sections. However, the DAB substrate used to identify GDF-9 expression was identical in contrast to that of the stromal tissue so it was unclear whether staining was specific or merely background tissue appearance. Thus, a red intensifying substrate (AEC, Vector Nova Red) was used. We were able to identify that the expression of GDF-9 in the corpus luteum and stromal dispersed mast cells were immune specific.

DES effects on GDF-9 Expression

Initially the principal aim of this project regarded examining the effects of neonatal exposure to an endocrine disruptor, DES, upon the ovarian follicular reserve and

the expression of the oocyte-specific growth factor GDF-9. Ultimately, exposure to an endocrine disruptor during critical developmental stages induces adverse effects upon the system, thus we purposed DES exposure would negatively impact follicle organization during ovarian organogenesis. Preliminary mRNA analyses indicated a decline in neonatal GDF-9 expression however, the reduction was observed toward the later stages of development (Day 6-9). We had anticipated that the effects of DES would be instantaneous with the initial expression of GDF-9 however; from our observations we concluded these effects were attributed to an indirect response. Therefore, GDF-9 is not an ideal gene for analyzing direct effects of ovarian endocrine disruption.

Concurrently, we had attempted to analyze the effects of DES on GDF-9 expression as the ovary entered reproductive decline. Unsuspected problems arose in isolating ovaries: 1) DES exposed ovaries were difficult to locate because of extreme hypertrophic effects occurring within the reproductive tracts, 2) extreme expansion of the oviduct by liquid puris and dead neutrophils effected concise identification of the ovary; 3) hamsters began to suffer and ultimately die off from other non-reproductive traumas: liver and spleen cysts, and renal failure. The small amounts of data collected were inconclusive and non-replicatable.

Simultaneously occurring mRNA analyses of non-endocrine exposed ovarian tissues revealed GDF-9 expression to be unassociated with follicular decline. Subsequent expressional studies in non-ovarian tissues revealed GDF-9 to non-exclusive to the ovary, as previously reported. Thus, further analyses of this experiment were discontinued and the remaining DES animals terminated.

CONCLUSIONS

Folliculogenesis is dependent on sequential changes in local organizing factors, cell-cell interactions, and external gonadotropin support. A number of factors are responsible for the development, regulation, and support of the oocyte, steroidogenic cells, and supporting cell lineages of the mammalian follicle. Because of GDF-9's profound involvement in regulating the development and growth of oocytes, its reproductive functions have been widely examined. In rodents GDF-9 is shown to be expressed in follicles beyond the primary follicle stage whereas, non-rodent species display GDF-9 expression prior to primordial follicle formation. In hamsters we have identified that transcription of GDF-9 occurs throughout ovarian organogenesis, primordial follicle formation, folliculogenesis, and ovarian senescence. Importantly, we observe expression of the protein in neonatal ovarian sections lacking any type of follicle organization, whether primordial or primary. Contradicting to expressional findings seen in rodents, we conclude that GDF-9 is an essential factor that regulates oogonial survival, primordial follicle formation, and primordial to primary follicle transition during ovarian organogenesis and folliculogenesis.

Because of GDF-9 being previously identified as "oocyte specific" we had expected the expression of this growth factor to decline as the germ cell supply of the ovary diminished. However, we observed GDF-9 to be expressed significantly after ovarian senescence. To clarify whether GDF-9 was indeed exclusively localized to the ovary we analyzed several non-ovarian tissues for GDF-9 expression. Our results indicated that GDF-9 was not ovary specific. In fact, we identified GDF-9 mRNA to be

expressed in white blood cell extractions. Reduced expression of GDF-9 mRNA through kidney perfusion analyses supported the idea that white blood cell populations residing in the system's structures may be partially and/or fully responsible for the non-ovarian expression of GDF-9 seen in analyzed tissue samples. Through re-evaluations of immunohistochemical analyses we observe GDF-9 to be translated within the highly vascular corpora lutea of the ovary, thus supporting our revolutionary finding of GDF-9 expression in the blood. Moreover, we have localized GDF-9 protein expression to mast cells. Therefore, GDF-9, a member of the TGF-β superfamily, may be involved in regulating processes other than mammalian folliculogenesis; possibly angiogenesis and/or hematopoiesis. Further individual white blood cell assays will be required to identify additional specific white blood cell expression of GDF-9.

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APPENDICES

Table 1: Oligonucleotide primers. GDF-9 primers, 184-515 and Sp up-dw were generated from conserved sequences of various species.

Gene	Oligonucleotide primer pairs	Compared Species	BP
GDF-9 (184 – 515)	Sense 5' TGT AGA TGG GAC TGA CAG GTC 3' Anti-sense 5' TCA GCG TGT ATA GCA AGA CCG 3'	Mouse Rat	331
GDF-9 (up – dw)	Sense 5' AAG CTC TAT AAG ACA TAT GCT A 3' Anti-sense 5' AGA CTG ATT TGA GTC AGT GTT C 3'	Mouse Rat Human Cow Pig	202
GAPDH	Sense 5' CAT CAC CAT CTT CCA GGA GCG 3' Anti-sense 5' ACT TGG GAG GTT TCT CCA GGC 3'	Mouse Rat Chinese Hamster	545

Table 2: Sequence homology. PCR products of hamster ovarian tissues were isolated and sequenced to verify the visualization of expressed GDF-9 mRNA. Homology of the generated sequence was compared to GDF-9 sequences of known species.

HAMSTER OVARY: VERSES	GDF-9 184 – 515 (331 bp)	GDF-9 Sp up - dw (202 bp)
MOUSE	91%	91%
RAT	91%	91%
HUMAN	80%	84%

Table 3: Summary of GDF-9 mRNA analyses in hamster ovarian samples. Transcriptional expression of GDF-9 was seen during periods reflecting oogonial mitosis oogonial atresia, primordial follicle formation, preantral, folliculogenesis, declining reproductive function, and reproductive senescence.

Time Periods (Days)	Ovarian Morphology	GDF-9 184-515	Replicates	GDF-9 Sp up-dw	Replicates
1	Oogonial Mitosis	++++	4	+++	3
3		++++	4	+++	3
5	Oogonial Atresia	++++	4	+++	3
6	Initial Primordial Follicle Formation	+++	3	+++	3
7	Primary Follicle Formation	+++	3	+++	3
8		+++	3	+++	3
9	Preantral Follicle Formation	++++	3	+++	3
15		++	2	++	2
21	Graafian Follicles	++	2	++	2
42	Folliculogenesis	++	2	+	1

+ Positive Results - Negative Results

Table 3: (continued)

Time Periods (Days)	Ovarian Morphology	GDF-9 184-515	Replicates	GDF-9 Sp up-dw	Replicates
60	Folliculogenesis	+++	3	++	2
75		++	2	+	1
88		++	2	+	1
110		+	1	++	2
138		++	2	++	2
166		++	2	++	2
209	Decline Folliculogenesis	+++	3	+++	3
229		++	2	++	2
258		++	2	+	1
440	Ovarian Senescence	++	2	+	1
531		++	2	+	1

+ Positive Results - Negative Results

Table 4: Summary of immunoblot analyses of GDF-9 protein expression. Immunonblots utilizing the R&D and Santa Cruz polyclonal antibodies; and the Hsueh monoclonal antibody were analyzed for GDF-9 expression in Day 60 hamster and mouse ovarian tissues.

Immunoblots	50ug per well		
Antibody	Dilution	Replicates	Results
GDF-9 R&D Polyclonal	1:1000	2	Negative
	1:500	2	Negative
	1:200	2	Negative
	1:100	2	Negative
GDF-9 Santa Cruz Polyclonal	1:1000	3	Negative
	1:500	3	Negative
	1:200	3	Negative
	1:100	3	Negative

Immunoblots	125ug per well		
Antibody	Dilution	Replicates	Results
GDF-9 R&D Polyclonal	1:1000	2	Negative
	1:500	2	Negative
	1:200	2	Negative
	1:100	2	Negative
GDF-9 Santa Cruz Polyclonal	1:1000	3	Negative
	1:500	3	Positive ?
	1:200	3	Positive ?
	1:100	3	Negative
GDF-9 Hsueh Monoclonal	1:8000	1	Negative
	1:500	1	Negative

Final Results	
Peptide Block	Negative
Peptide Block	Negative

Table 5: Summary of immunohistochemistry analyses. Consistent positive results for, GDF-9 protein expression, were identified in Day 75 and Day 88 hamster ovarian sections by the Hsueh monoclonal antibody. Negative results were generally viewed in both R&D and Santa Cruz polyclonal antibodies.

DAY 75

Immunohistochemistry				
Antibody	Dilution	Antigen Ret.	Replicates	Results
GDF-9 R&D Polyclonal	1:500	+	2	Negative
		-	2	Negative
	1:200	+	2	Negative
		-	2	Negative
	1:100	+	1	Negative
		-	1	Negative
GDF-9 Santa Cruz Polyclonal	1:500	+	2	Negative
		-	2	Negative
	1:200	+	5	Negative
		-	25	Positive *
	1:100	+	1	Negative
		-	3	Negative
GDF-9 Hsueh Monoclonal	NA	NA	NA	NA
	1:200	-	7	Positive
	NA	NA	NA	NA

Negative *

* Results inconsistent between positive and negative

DAY 88

Immunohistochemistry				
Antibody	Dilution	Antigen Ret.	Replicates	Results
GDF-9 R&D Polyclonal	NA	NA	NA	NA
GDF-9 Santa Cruz Polyclonal	1:500	+	2	Negative
		-	2	Negative
	1:200	+	1	Negative
		-	17	Negative
	NA	NA	NA	NA
GDF-9 Hsueh Monoclonal	NA	NA	NA	NA
	1:200	-	5	Positive
	NA	NA	NA	NA

* Results inconsistent between positive and negative

Table 6: Summary of GDF-9 mRNA analyses in non-ovarian hamster tissues. Time points studied represent a variety of developmental phase: neonatal, pre-pubertal, reproductively active adult, and geriatric.

	Non-Ovarian Tissue Samples						
Time Points (Days)	Liver	Kidney	Spleen	Heart	Muscle	Uterus	Testis
2	x xx	x xx					
9	x xx	x xx	x xx	x xx	x xx	x xx	x xx
19	x xx	x xx	x xx	x xx	x xx	x xx	
42	x xx	x xx	x xx	x xx	------	x xx	
60	x xx	x xx	x xx	x xx	x xx	x xx	x xx
166	x xx	x xx	x xx	x xx	------	x xx	
209	x xx	x xx	x xx	x xx	x xx	x xx	
235	x xx	x xx	x xx	x xx	x xx	x xx	
440	x xx	x xx	x xx	x xx	x xx	x xx	

x GDF-9 184-515 xx GDF-9 Sp up-dw

Table 7: Sequence homology. GDF-9 184-515 products of hamster non-ovarian tissues were isolated and sequenced to verify expressed GDF-9 mRNA. Homology of the generated sequence was compared to GDF-9 sequences of known species.

HAMSTER OVARY: VERSES	GDF-9 184-515 LIVER	GDF-9 184-515 BLOOD
MOUSE	91%	91%
RAT	91%	91%
HUMAN	79%	80%

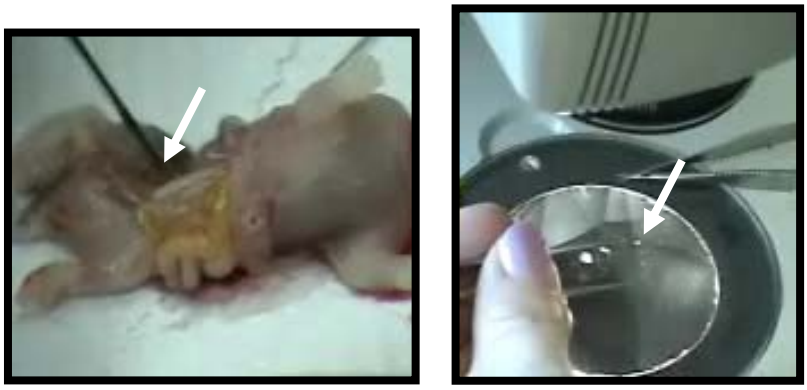
Table 8: DES reproductive abnormalities. Unsuspected problems arose in isolating ovaries for GDF-9 analyses in aging DES exposed hamsters. Exposed ovaries were difficult to locate because of extreme hypertrophic effects occurring within the reproductive tract

		CONTROL		DES	
		Ovaries Retrieved	Reproductive Morphology	Ovaries Retrieved	Reproductive Morphology
Time Points (Days)	42	4*	Visible CL Appears Normal	2*	Extreme Hypertrophy, Major Adhesions on Peritoneal Cavity, Cyst on Spleen
	45	2*	Visible CL Appears Normal	2*	
	127	2*	Visible CL Appears Normal	1*	Extreme Distention of Oviduct (puss)
	134	2*	Visible CL Appears Normal	0	Unable to Locate Ovaries; Severe Hypertrophy of Uterine Tract and Extreme Distention of the Oviduct. Multiple Cyst Are Present on the Liver, Spleen. Cavity Filled with Fluid and Kidneys Reduced in Size.
	196	2*	Visible CL Appears Normal	0	
	209	4*	Normal Appearance	0	
	233	2*	Reduced CL Appears Normal	0	

* Ovaries collected underwent RNA Isolation and RT/PCR Analyses

AA		Lys	Les	Tyr	Lys	Thr	Tyr	Ala	Thr	Lys	Glu	
Human	5'	AAG	CTC	TAT	AAG	ACA	TAT	GCT	ACC	AAG	GAA	3'
Cow	5'	AGG	CTC	TAT	AAA	GCA	TAT	GCT	ACC	AAG	GAA	3'
Mouse	5'	AAG	CTC	TAT	AAG	ACG	TAT	GCT	ACC	AAG	GAA	3'
Rat	5'	AAG	CTC	TAT	AAA	ACA	TAT	GCT	ACC	AAG	GAA	3'
Pig	5'	AGG	CTG	TAT	AAG	ACA	TAT	GCT	ACC	AAG	GAA	3'
Consensus		AAG	CTC	TAT	AAG	ACA	TAT	GCT	A			

Figure 1: Oligonucleotide and amino acid species comparisons. To generate primers for use in GDF-9 mRNA expressional studies, consensus sequences form various species were analyzed for similarities. Conserved nucleotide bases between species were carried over into the formation of either the sense or anti-sense strands. The above figure (GDF-9 Sp up-dw; sense strand) represents the alignment of nucleotide bases, for a portion of the GDF-9 gene, in several species. Differences in bases (squares) were accommodated by selecting the bases that were an overall commonality between species.



A **B**

Figure 2: Microscopic removal of the neonatal reproductive tracts. Image A represents a one-day-old Syrian Golden hamster pup. Arrows (image A and B) indicate the location and size of the reproductive tracts. Upon isolation of the tracts, the ovary had to be microscopically removed from the mass.

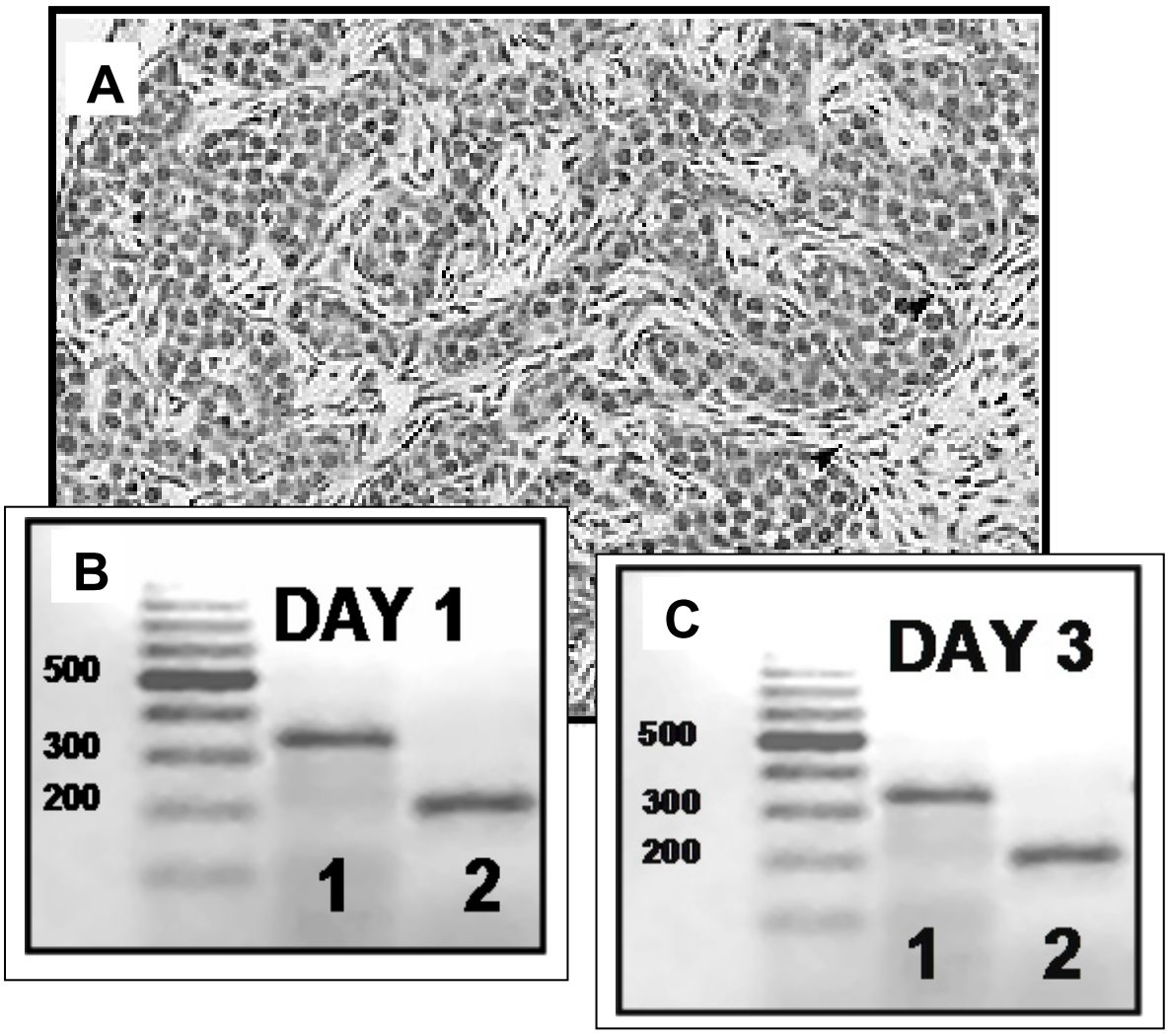


Figure 3: Neonatal ovarian morphology and RT/PCR analyses. (A) Morphological representation of ovarian development in a one to three-day old (post-partum) hamster ovary. Ovarian organization begins as mitotic oogonia compact amongst one another, forming oogonial nest. Mesothelium, originating form the medullary hilus, subdivides the various nests; representing the early supporting cell linage of pre-granulosa cells. Magnification: 662 X. (B) RT/PCR analysis of hamsters ovaries at one-day post-partum and (C) three-days post-partum. Lane 1 represents primer set GDF-9 185-515 and Lane 2 indicates the GDF-9 Sp up-dw primer set. GDF-9 mRNA expression is clearly evident at both time points.

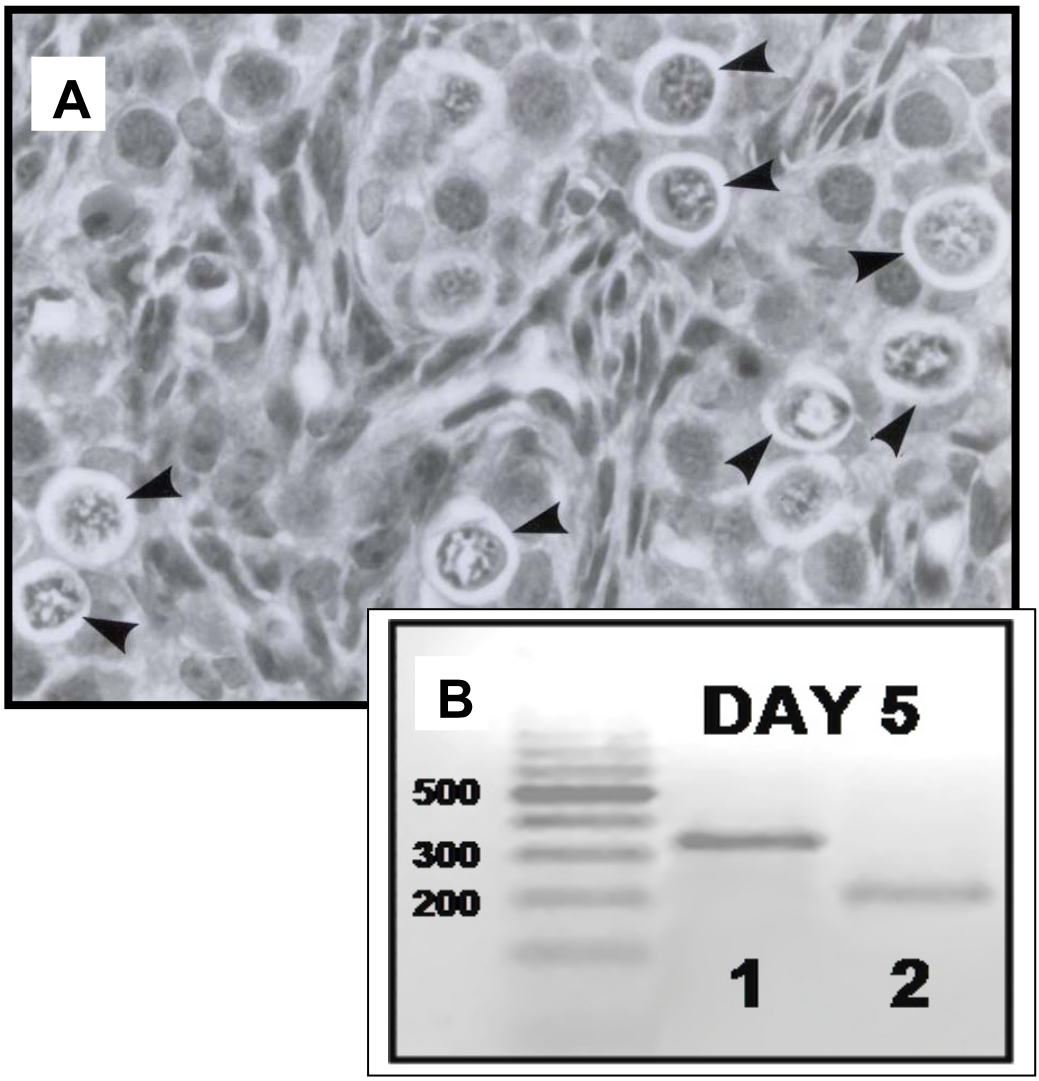


Figure 4: Neonatal ovarian morphology and RT/PCR analyses. (A) Morphology of a five-day-old (post-partum) hamster ovary. Oogonial atresia is actively occurring throughout the ovary as indicated by the arrows. Atretic cells exhibit extensive nuclear degradation and cell shrinkage (arrows). Magnification: 2,646 X. (B) RT/PCR analysis of five-day old hamster ovaries. Lane 1 represents GDF-9 expression amplified with GDF-9 184-515 primers, where lane 2 represents GDF-9 primers Sp up-dw. GDF-9 mRNA is visually present during this highly atretic period of the ovary.

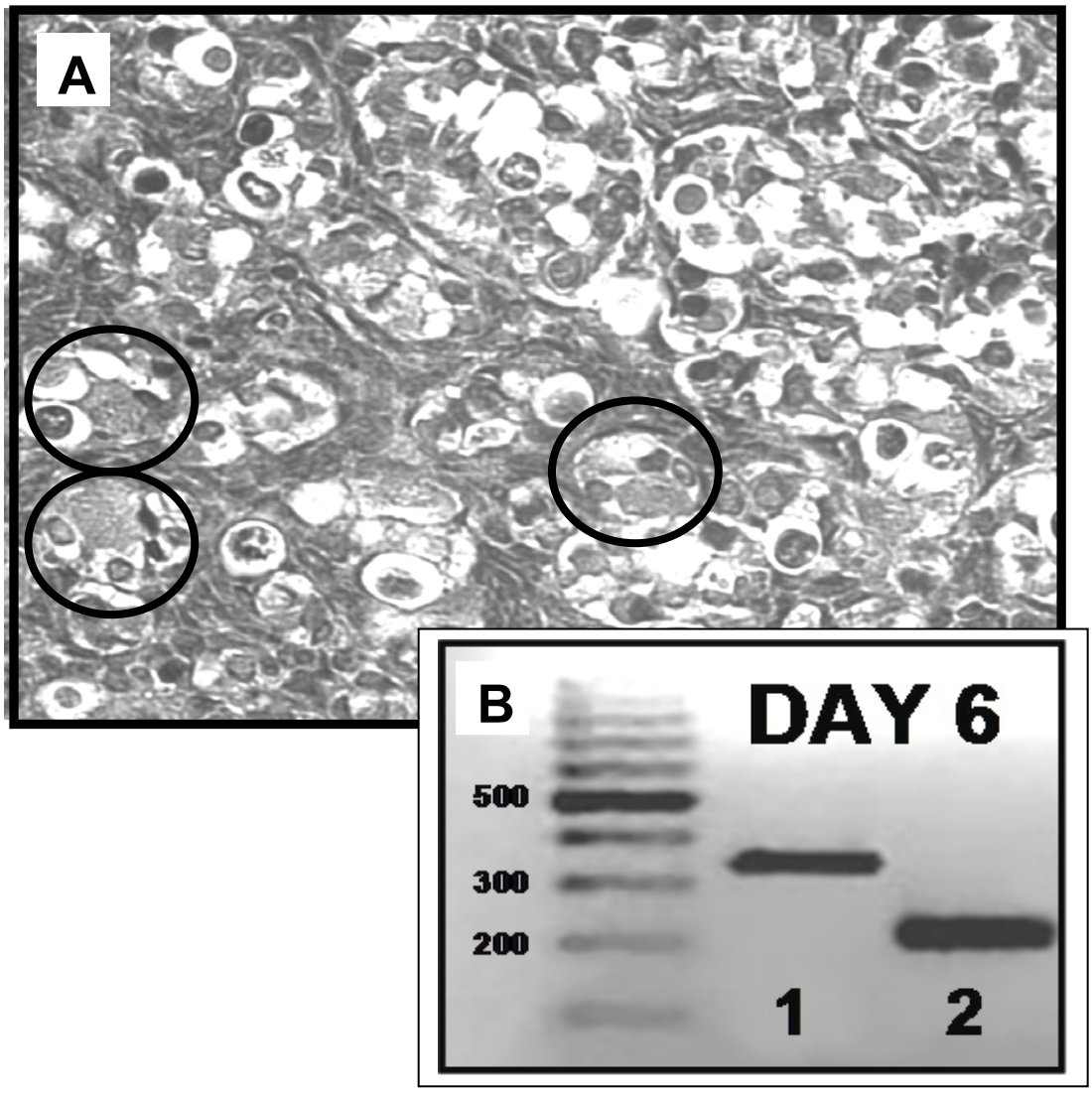


Figure 5: Neonatal ovarian morphology and RT/PCR analyses. (A) Morphology of a six-day old (post-partum) hamster ovary. Day 6 of the ovary represents the initial period of organized follicle formation, as the first population of primordial follicles become visible (circles) within the ovarian structure. Magnification: 200X. (B) RT/PCR analysis of GDF-9 expression is clearly evident at this stage. Lane 1 represents primer set GDF-9 185-515 and Lane 2 indicates the GDF-9 Sp up-dw primer set.

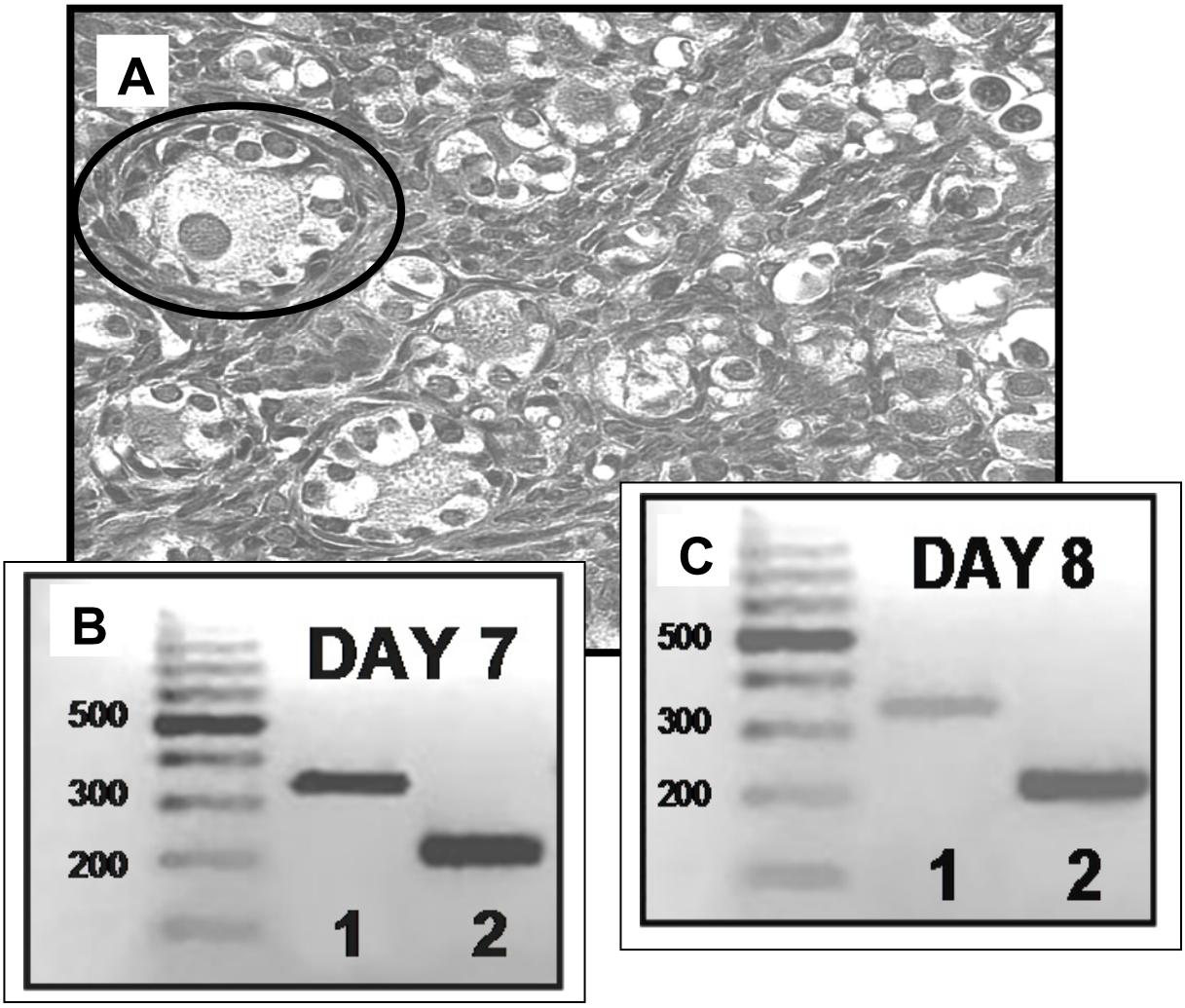


Figure 6: Neonatal ovarian morphology and RT/PCR analyses. (A) Morphological representation of ovarian development in a seven to eight-day old (post-partum) hamster ovary. Developmentally, the ovary is beginning to enter the first initial stage of folliculogenesis; i.e. primary follicle formation (circle). During these time-points certain primordial follicles are recruited to become primary follicles. Magnification: 200 X. (B) and (C) RT/PCR analyses of hamster ovarian tissues during postnatal days seven and eight. Lane 1 represents primer set GDF-9 185-515 and Lane 2 indicates the GDF-9 Sp up-dw primer set. GDF-9 mRNA expression is clearly evident at both time points.

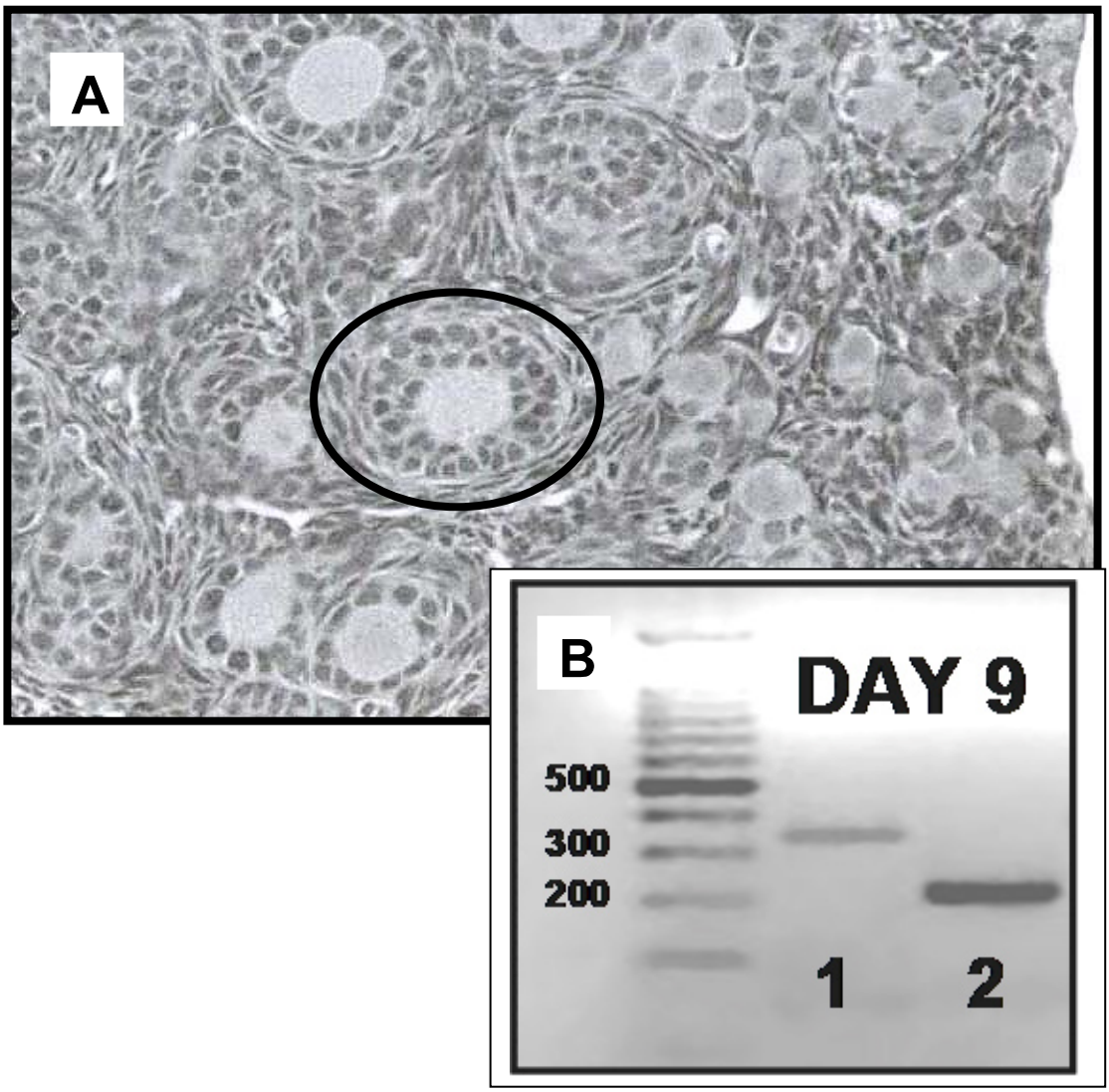


Figure 7: Neonatal ovarian morphology and RT/PCR analyses. (A) Morphology of a nine-day old (post-partum) hamster ovary is characterized by the explosion of organized follicle development. The process of folliculogenesis is actively occurring at this developmental stage. Primordial follicles can be seen within the cortex of the ovary, as primary and secondary follicles (circle) develop towards the interior region of the organ. Magnification: 662 X. (B) RT/PCR analysis of day nine hamster ovaries. GDF-9 mRNA is clearly expressed. Lane 1 represents GDF-9 184-515 primers and lane 2 represents GDF-9 Sp up-dw.

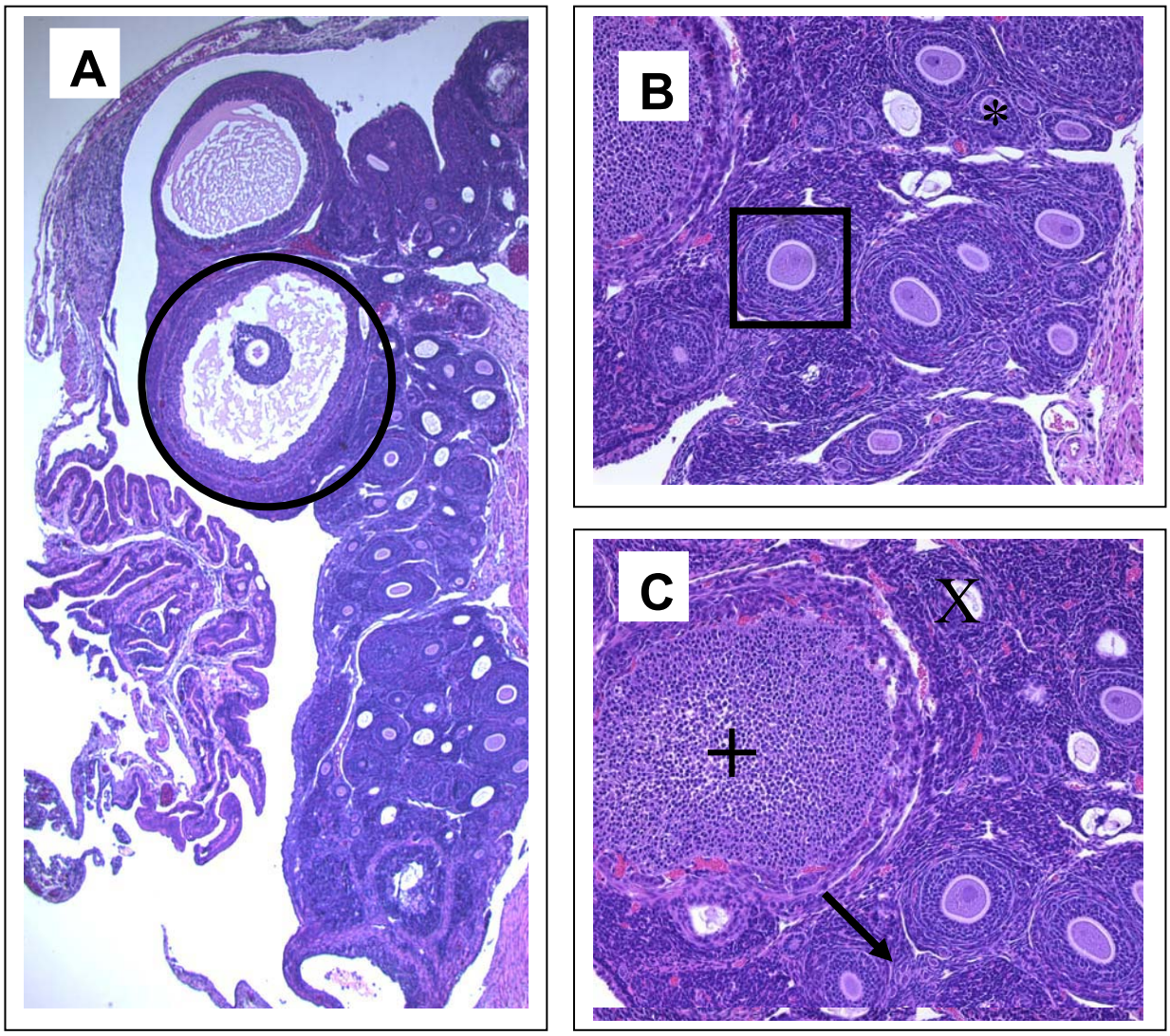


Figure 8: Histomorphology of hematoxylin and eosin-stained Day 75 ovarian sections. (A) Morphology of a reproductively active ovary. All stages of folliculogenesis can be seen occurring simultaneously. Figures A, B and C visually indicate formation of primordial (arrow), primary (*), secondary (square), Graafian (circle), atretic follicles (X), and corpus luteums (+). Magnification of panel A: 20 X, panels B and C: 100 X

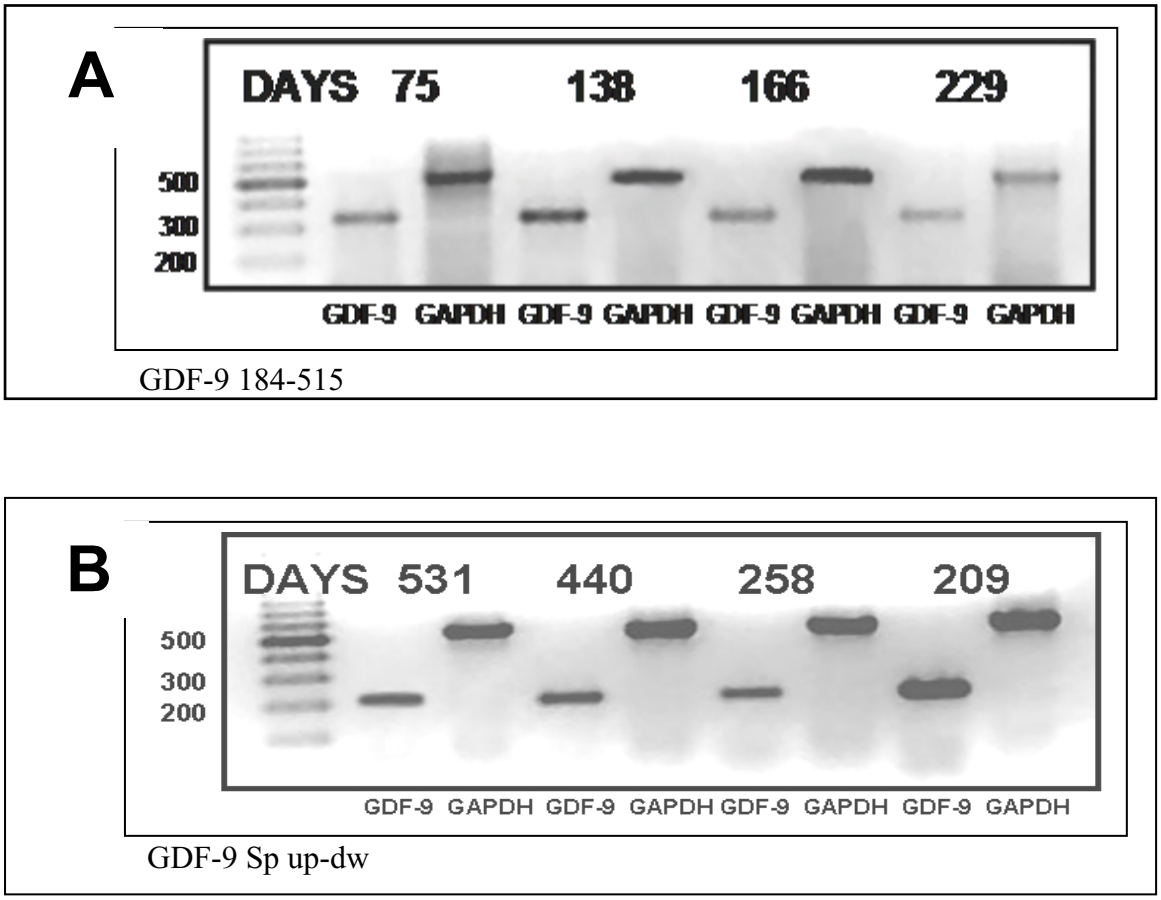


Figure 9: GDF-9 mRNA expression in reproductively active and senescent ovarian tissues. (A) Expression of GDF-9 was seen at Days 75, 138, 166 and 229; of which marks periods of active follicle recruitment, folliculogenesis, and ovulation. GDF-9 184-515 produced an expected product at 331 bp. Similar expression was seen with GDF-9 Sp up-dw (data not shown). (B) GDF-9 mRNA expression was visible at Days 209, and 258; time periods that mark the decline reproductive ability of the ovary. By Days 440 and 531 reproductive senesces in the ovary has occurred, however GDF-9 expression is visually evident. Expression was marked by the 202 bp product produced by GDF-9 Sp up-dw. Similar results were seen with GDF-9 184-515 (data not shown).

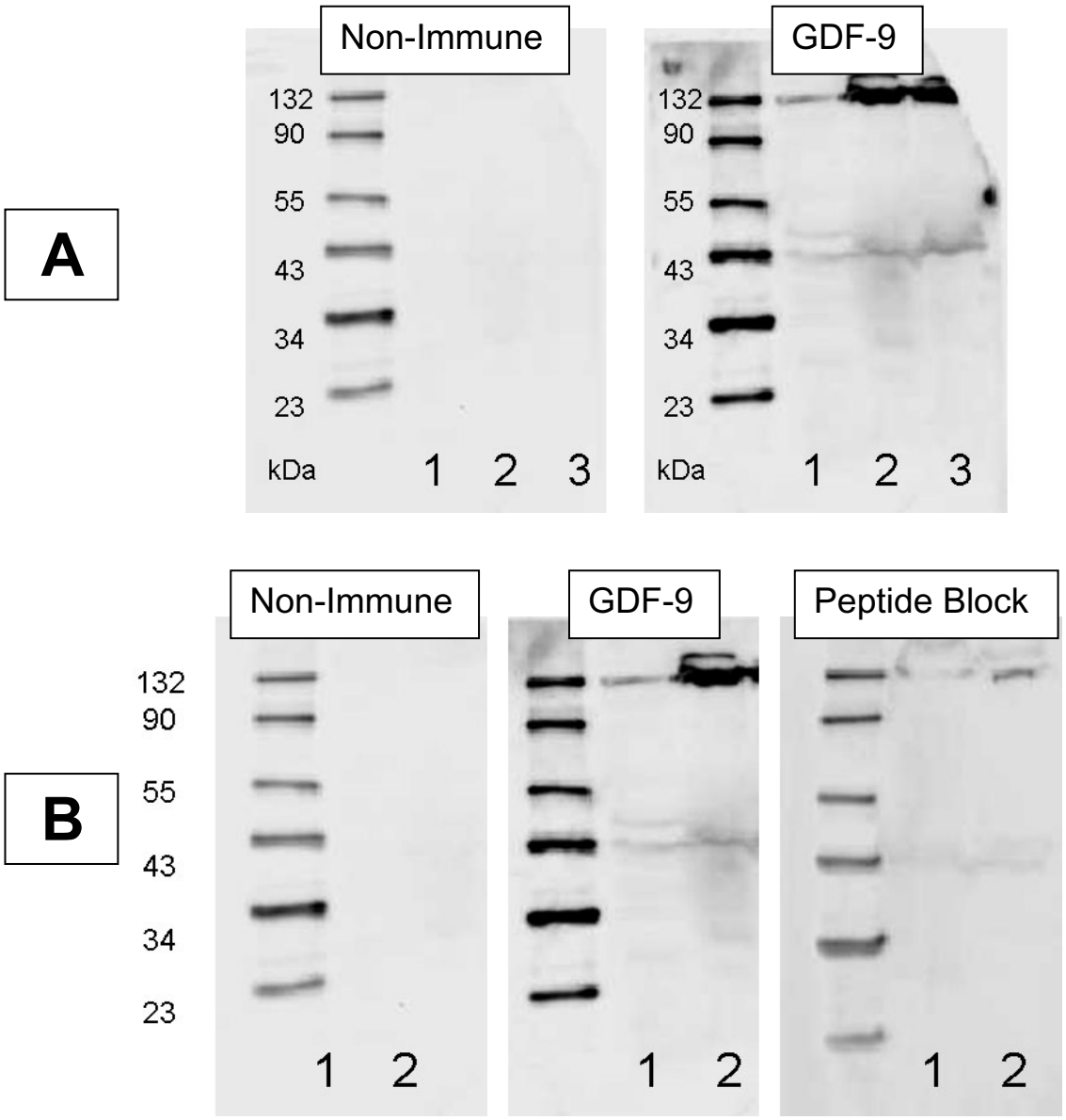


Figure 10: Immunoblot analysis of protein expression in reproductive tissues. (A) Immunoblot analysis of Day 60 male and female reproductive tissues using a commercially obtained goat polyclonal GDF-9 antibody. Non-immune samples were negative for GDF-9. (B) Peptide block of the GDF-9 antibody confirmed that bands seen in immune samples were due to non-specific products. Lanes: (1) Day 60 mouse ovary, (2) Day 60 hamster ovary, and (3) Day 60 hamster testis

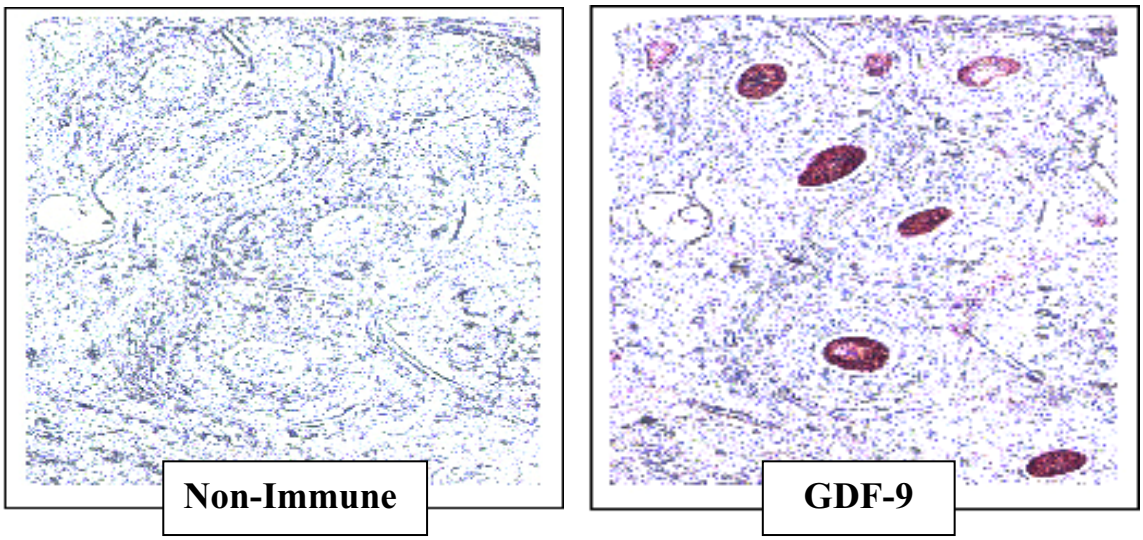


Figure 11: Immunohistochemistry results for GDF-9 in reproductively active ovarian sections (Day 75). Positive expression of the protein was clearly evident in oocytes of highly organized follicles. No signal was identified in the non-immune control. Vector Nova Red substrate was used to identify the protein; positive signal appears dark red. Magnification: 100 X.

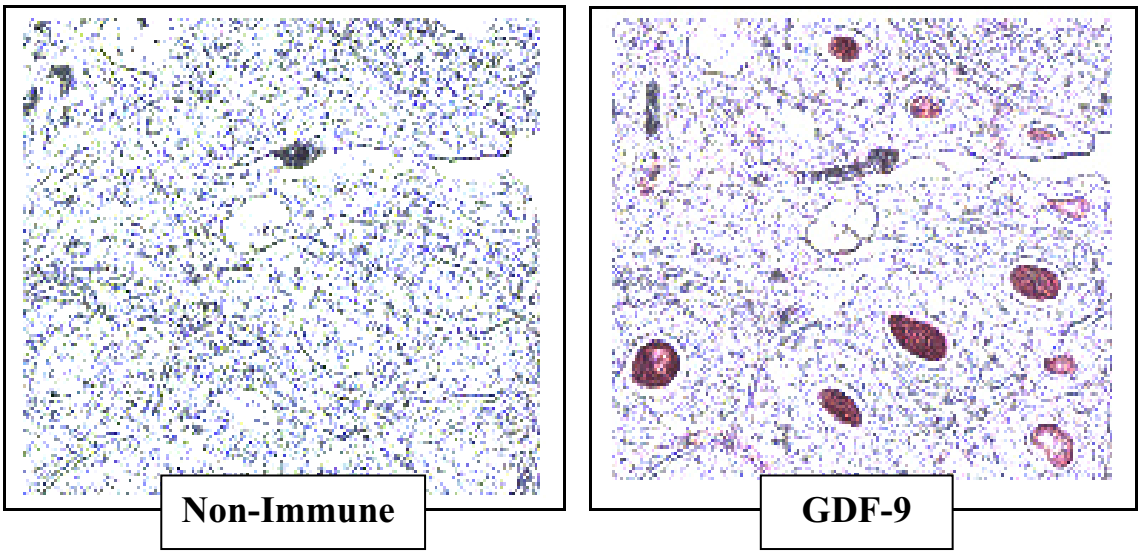


Figure 12: Immunohistochemistry expression of GDF-9 in Day 88 hamster ovarian sections. The expression of the GDF-9 protein is clearly visible (RED) within the oocyte of organized follicles: primary, secondary, and tertiary. Vector Nova Red substrate was used to identify the protein; positive signal appears dark red. Magnification: 100 X.

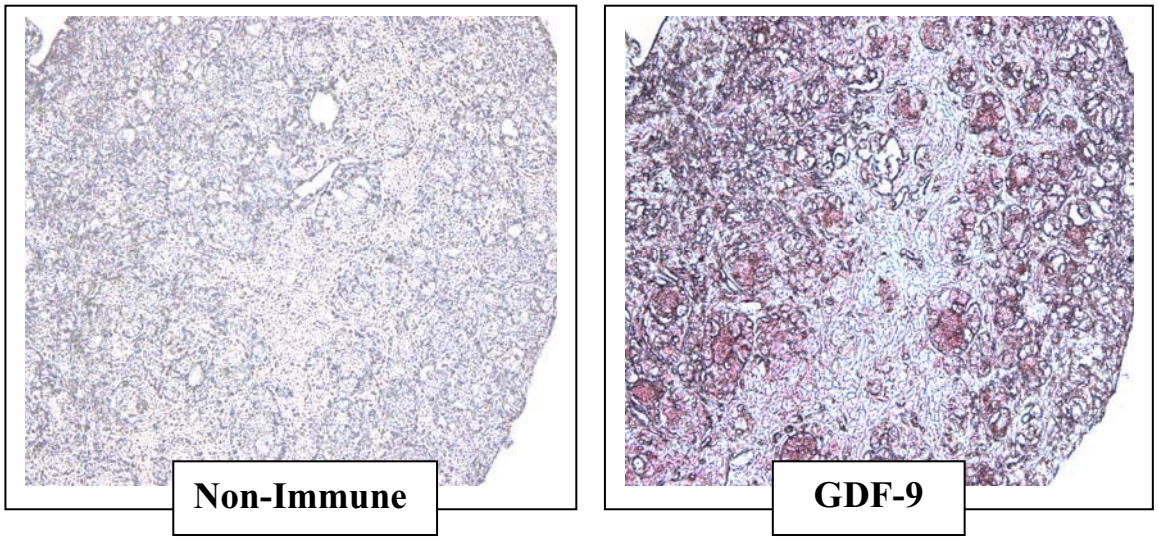


Figure 13: Immunohistochemistry analyses of Day 9 neonatal ovarian sections. At post-natal Day9 ovaries are consumed by populations of primordial follicles, primary follicles and a few secondary follicles, i.e. preantral follicle formation. GDF-9 is seen expressed within primordial follicles (cortex) and preantral follicles (medullary). Magnification 400 X.

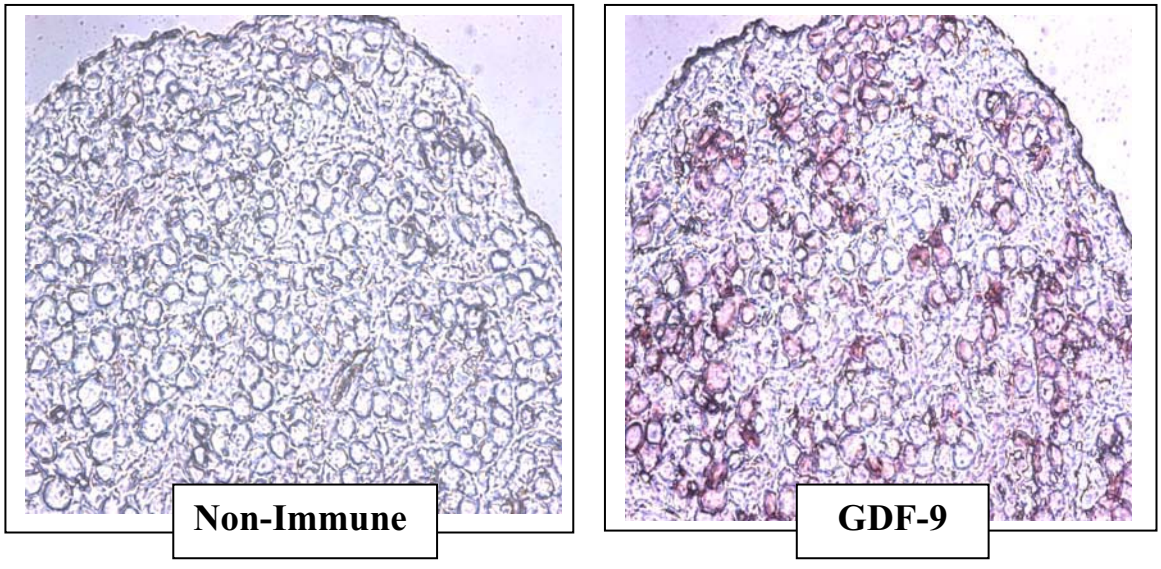


Figure 14: Immunohistochemistry analyses of Day 5 neonatal ovarian sections. Oogonial atresia is highly present at this developmental stage, Translation of GDF-9 is visually evident within the oocyte populations. Magnification 400 X

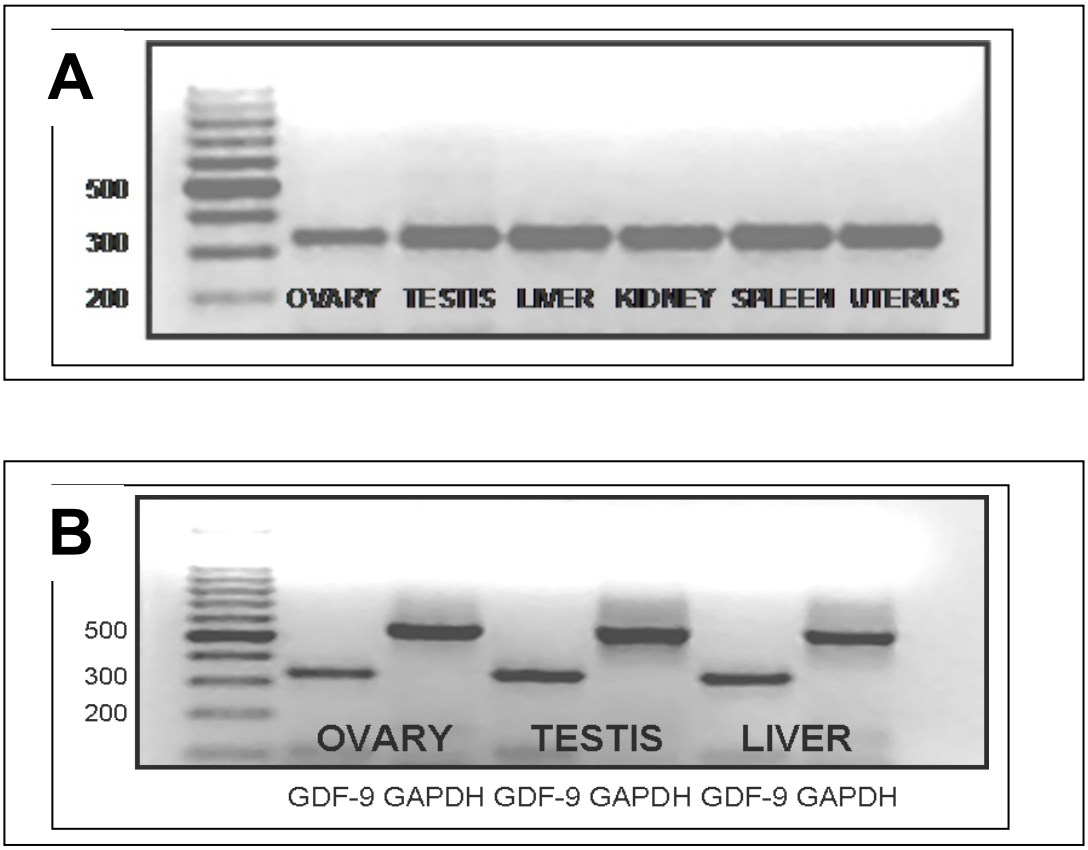


Figure 15: Ovarian and non-ovarian GDF-9 mRNA expression. Various lineages of reproductive and gastrointestinal tissues were analyzed for GDF-9 mRNA expression. (A) Day 235 and (B) Day 19, GDF-9 184-515 produced visually evident products that aligned at the expected 331 bp position.

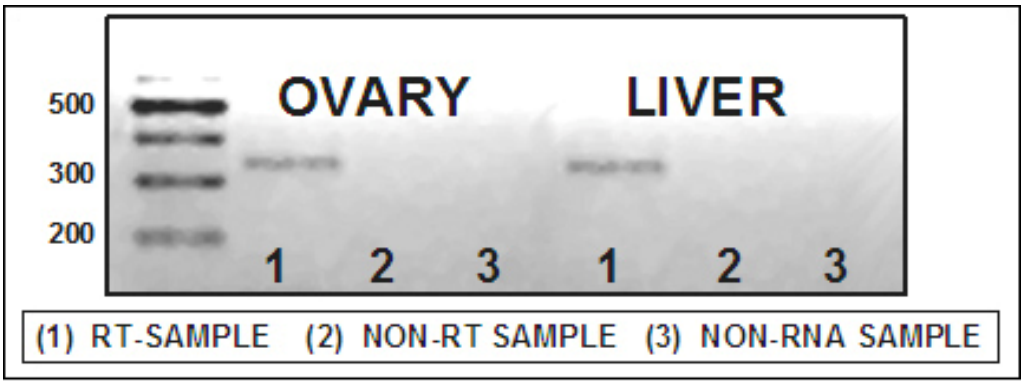


Figure 16: Ovarian and non-ovarian expression of GDF-9 utilizing negative controls. GDF-9 mRNA expression was evident in the ovary and liver, products produced at 331 bp (GDF-9 184-515), but was absent in both ovarian and non-ovarian negative controls. Negative controls: Non-RT; RNA + PCR mix + GDF-9 184-515 primers, Non-RNA; RT mix + PCR mix + GDF-9 184-515 primers.

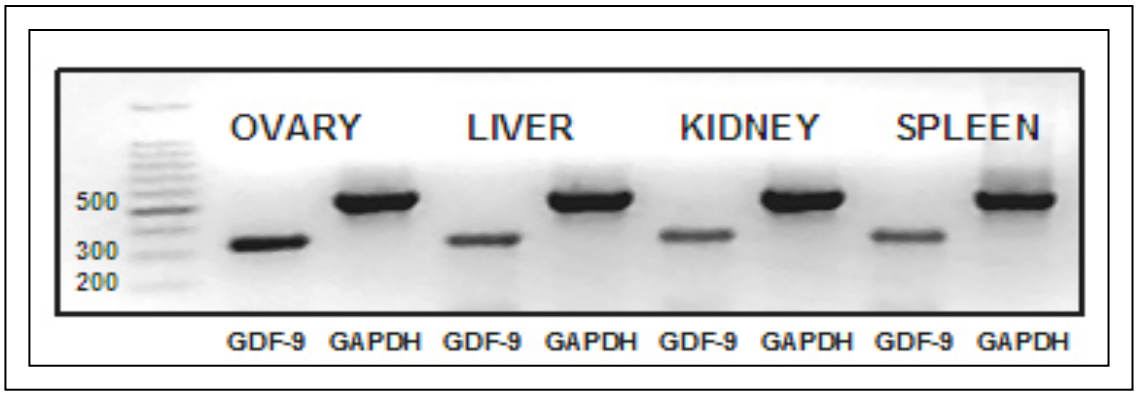


Figure 17: Ovarian and non-ovarian GDF-9 mRNA expression. In Day 39 mice, transcription of GDF-9 was found to occur in ovarian and non-ovarian tissues: liver, kidney and spleen. PCR analysis was utilized by GDF-9 184-515 primers.

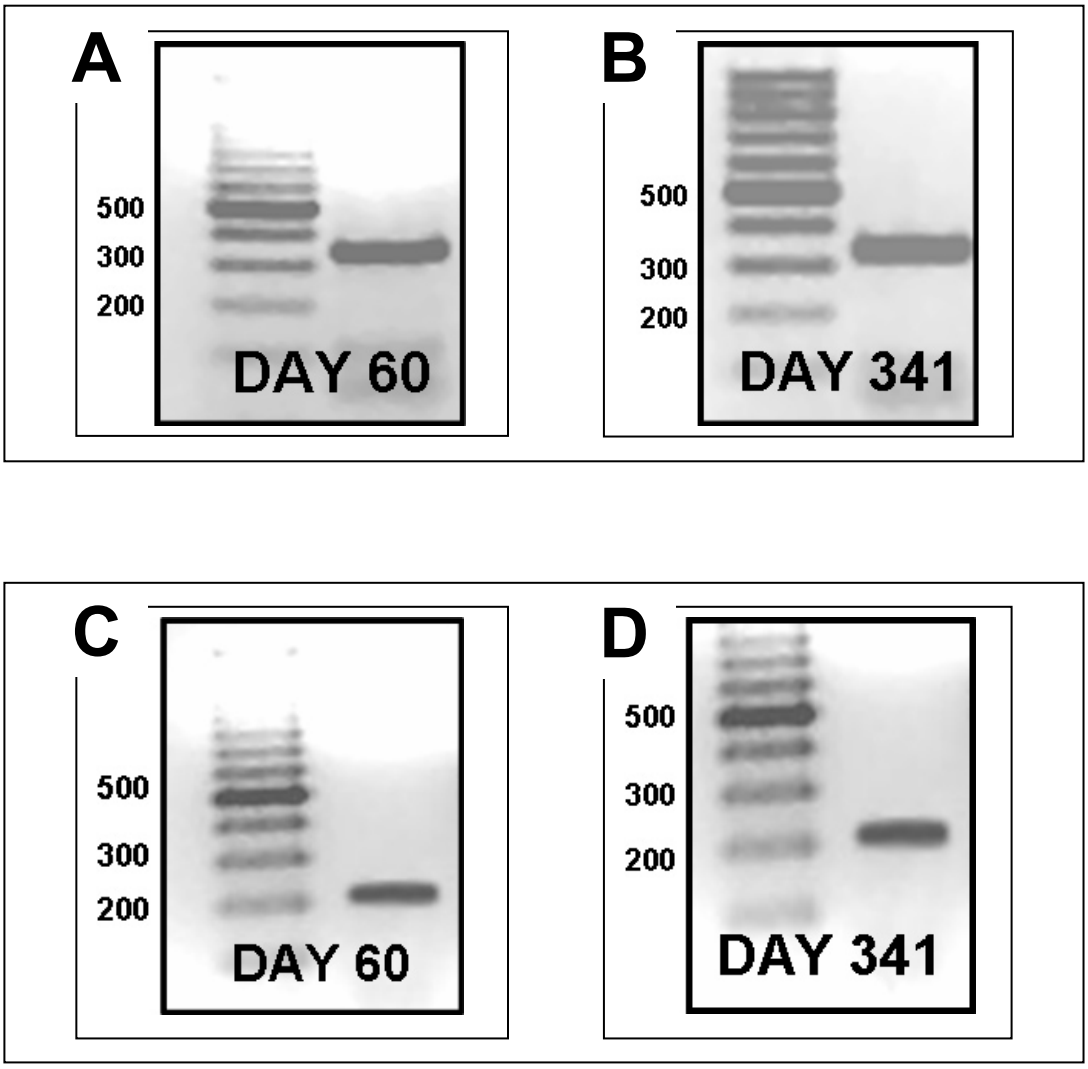


Figure 18: White blood cell GDF-9 mRNA analyses. mRNA expression, Day 60 and Day 341, was clearly defined by the 331 bp product of GDF-9 184-515 (Figure 18, A and B). Identical transcription of GDF-9 mRNA was observed in GDF-9 Sp up-dw primers; i.e. 202 bp products, at Day 60 and Day 341 (Figure 18, C and D).

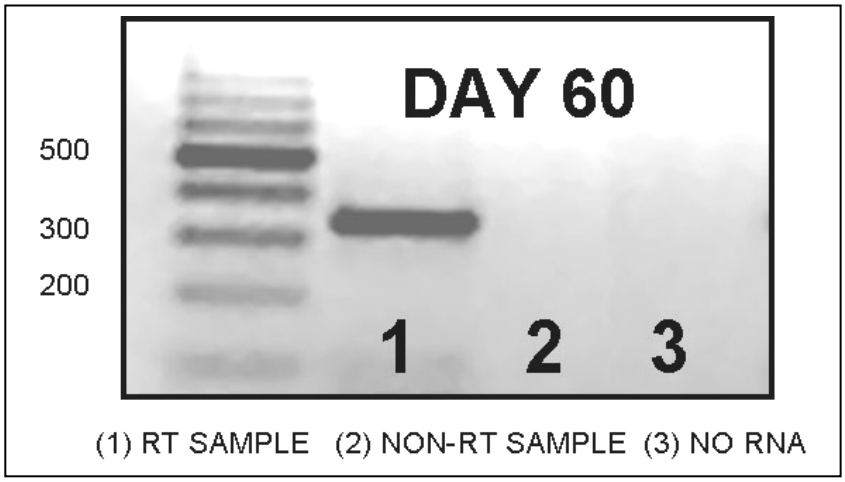


Figure 19: Ovarian and non-ovarian expression of GDF-9 utilizing negative controls. At Day 60, GDF-9 expression was clearly visible; producing the expected 331 bp product of GDF-9 184-515 in blood cell samples. Negative controls exhibited no expression of GDF-9 mRNA.

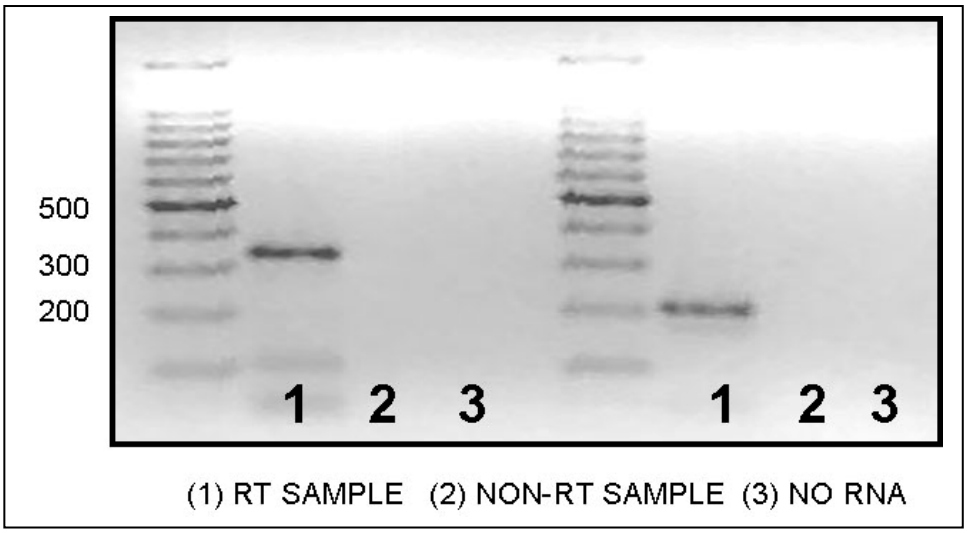


Figure 20: Human white blood cell GDF-9 mRNA expression. Preliminary study involving isolation of human blood for transcriptional expression of GDF-9. Analyses with GDF-9 184-515 and GDF-9 Sp up-dw produced expected 331 bp and 202 bp products. Negative controls utilized in the assay lacked GDF-9 expression.

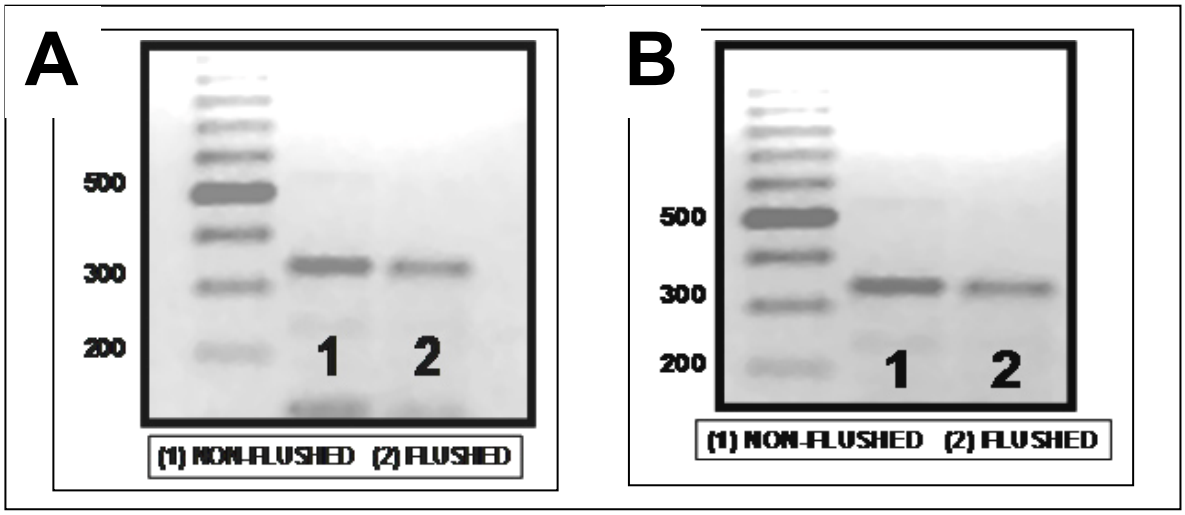


Figure 21: Kidney perfusion. (A) Expressional results, seen in Day 258, indicated that GDF-9 expression in the flushed kidney was reduced by 33% in comparison with the non-flushed kidney. (B) In Day 431, similar reduced results were indicated. Evaluation of declined expression, by densitometry indicated that GDF-9 mRNA was reduced by 35% in the flushed kidney.

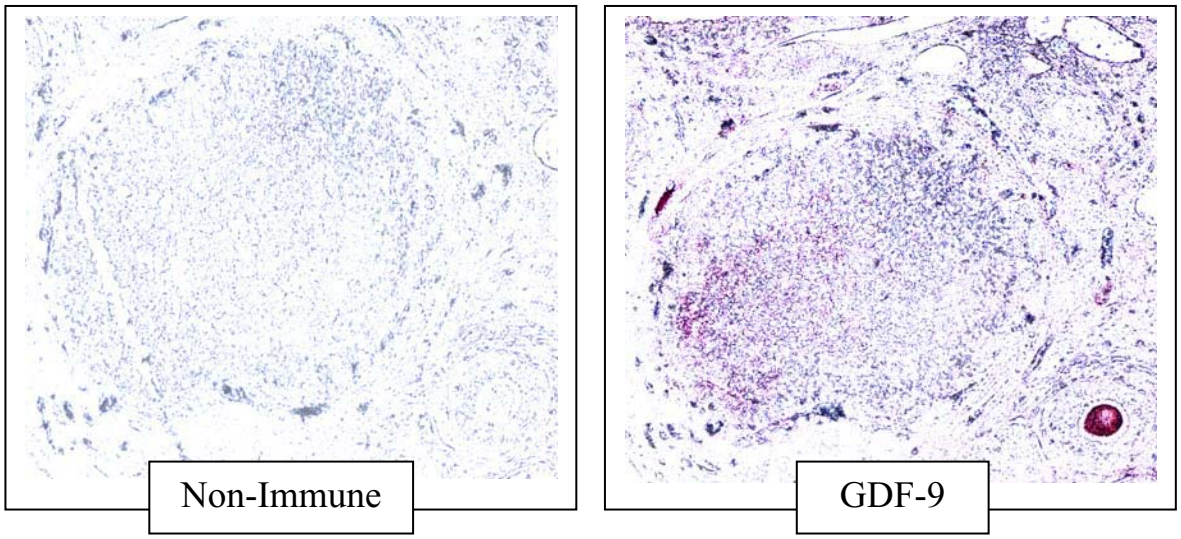


Figure 22: Immunohistochemistry. Re-evaluations of GDF-9 protein expression in reproductively active ovarian sections, Day 75, indicated expression of GDF-9 within highly vascular corpus luteums. Magnification 200 X.

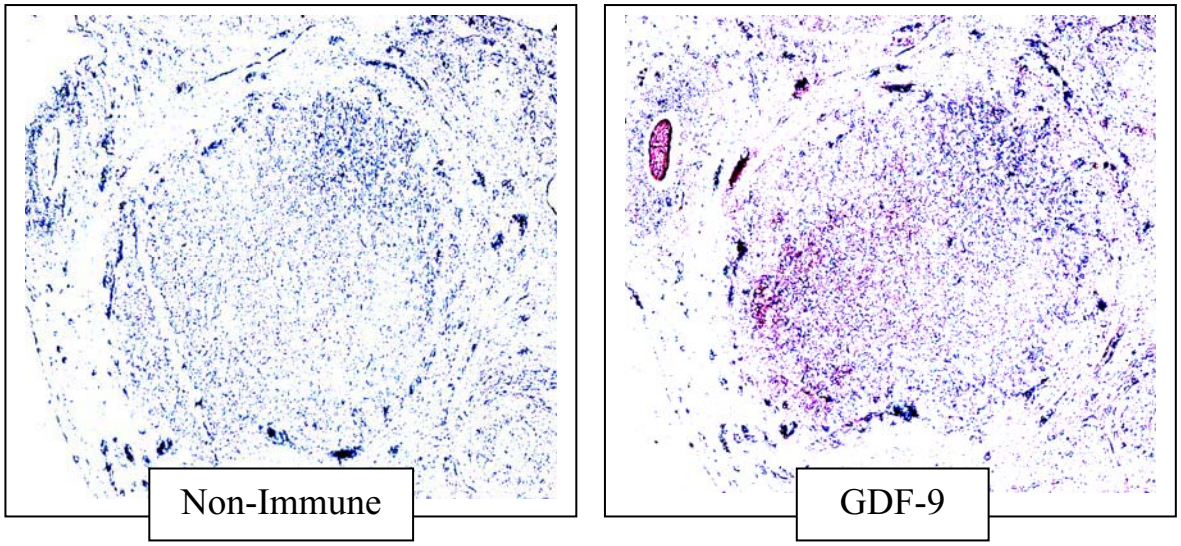


Figure 23: Immunohistochemistry. At Day 88, expression of the GDF-9 protein can be seen localized to the outer cortex of the corpus luteum; noted by the red appearance. Magnification: 200 X

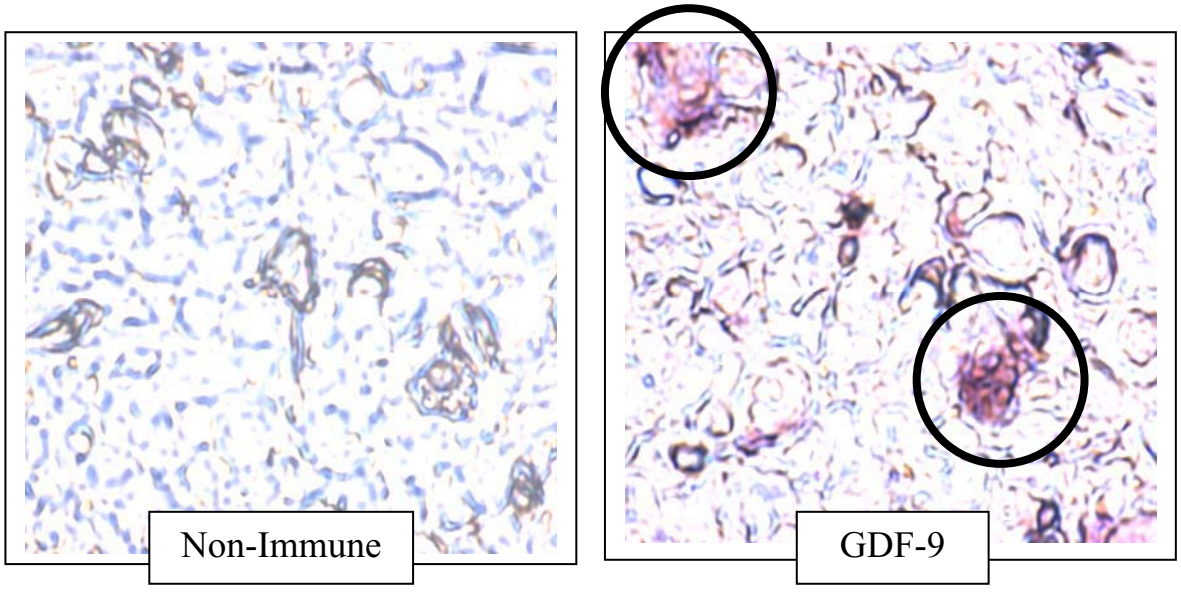


Figure 24: Immunohistochemistry expression of GDF-9 in mast cells. Expression of GDF-9, by apparent mast cells (black circles), was identified in immunological analyses of Day 9 hamster ovarian sections (Figure 24). Non-immune sections were negative for immune staining. Magnification: 1000 X.

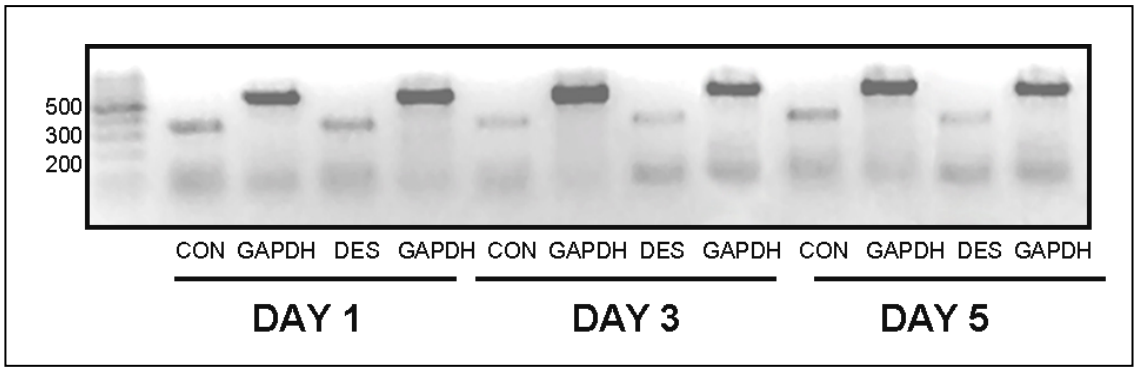


Figure 25: Neonatal effects of DES on GDF-9 expression. Semi-quantitative results using GDF-9 184-515 primer set. GDF-9 mRNA expression was non-effected by neonatal DES exposure. Time points representing oogonial mitosis, Day 1 and Day 5, and oogonia atresia, Day 5, visually appeared to remain similar among control and DES groups.

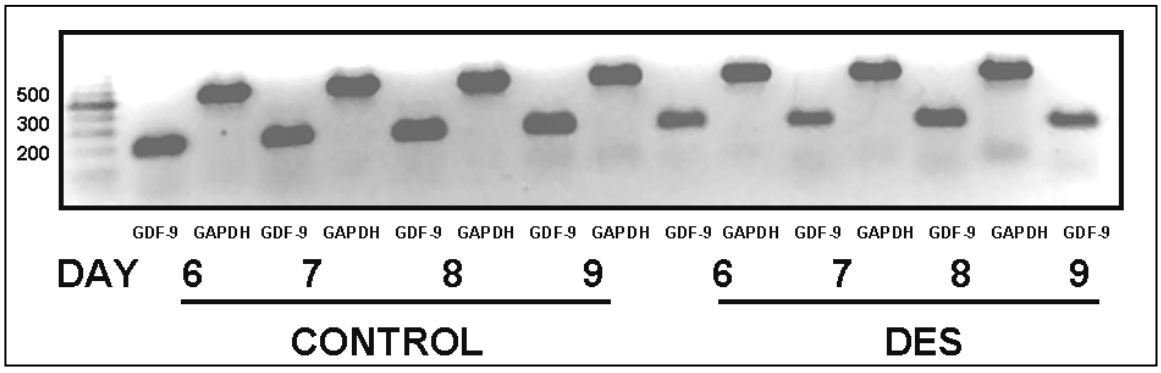


Figure 26: Neonatal effects of DES on GDF-9 expression. Semi-quantitative results using GDF-9 Sp up-dw primer set. GDF-9 mRNA expression appeared to decline in DES exposed ovarian samples. Time points reflect initial primordial follicle formation, primary follicle development, and folliculogenesis.

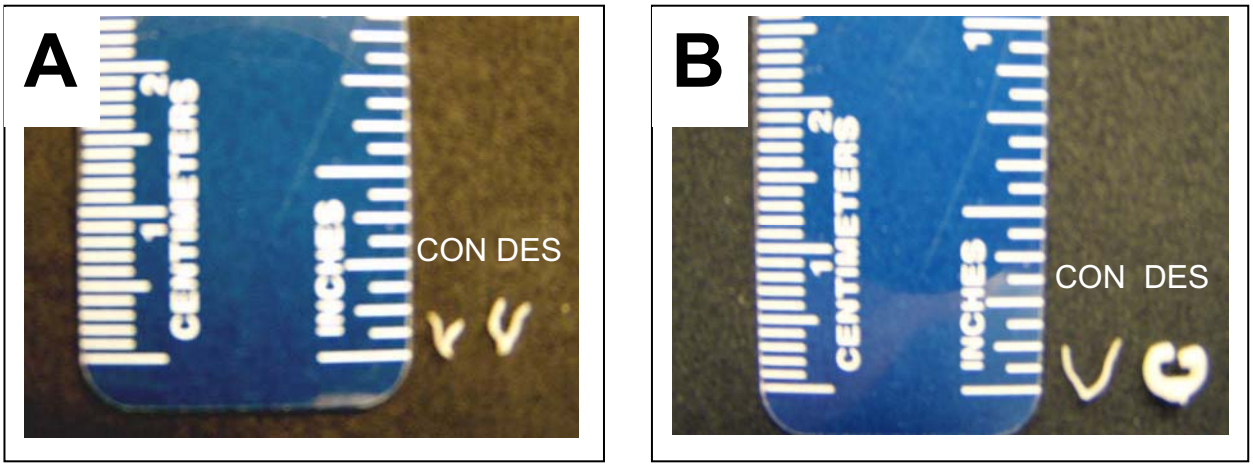


Figure 27: Morphology of neonatal reproductive tracts. DES exposed and non-exposed reproductive tracts were isolated from Day1 and Day 5 neonates. (A) DES tracts at Day 1, exhibited a slight hypertrophic response in comparison to the non-exposed tract. (B) At Day 5, DES exposure is noticeably evident; marked by the inflamatic engorgement of the structure.

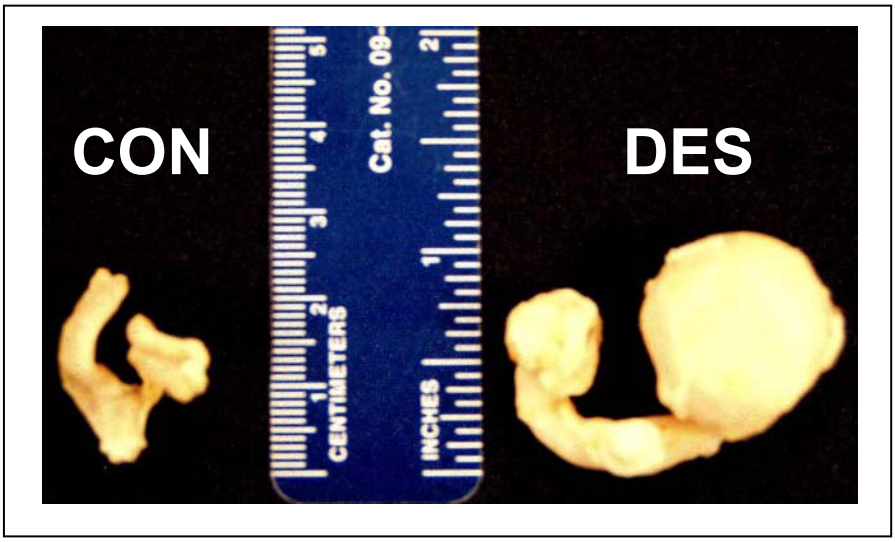


Figure 28: Morphology of DES exposed reproductive tract. Neonatal hamsters exposed to DES had a range of reproductive tract abnormalities as the animals progressed in age. Uterine horns exhibited hypertrophic effects as the oviducts became severally distended. DES aging studies were discontinued.

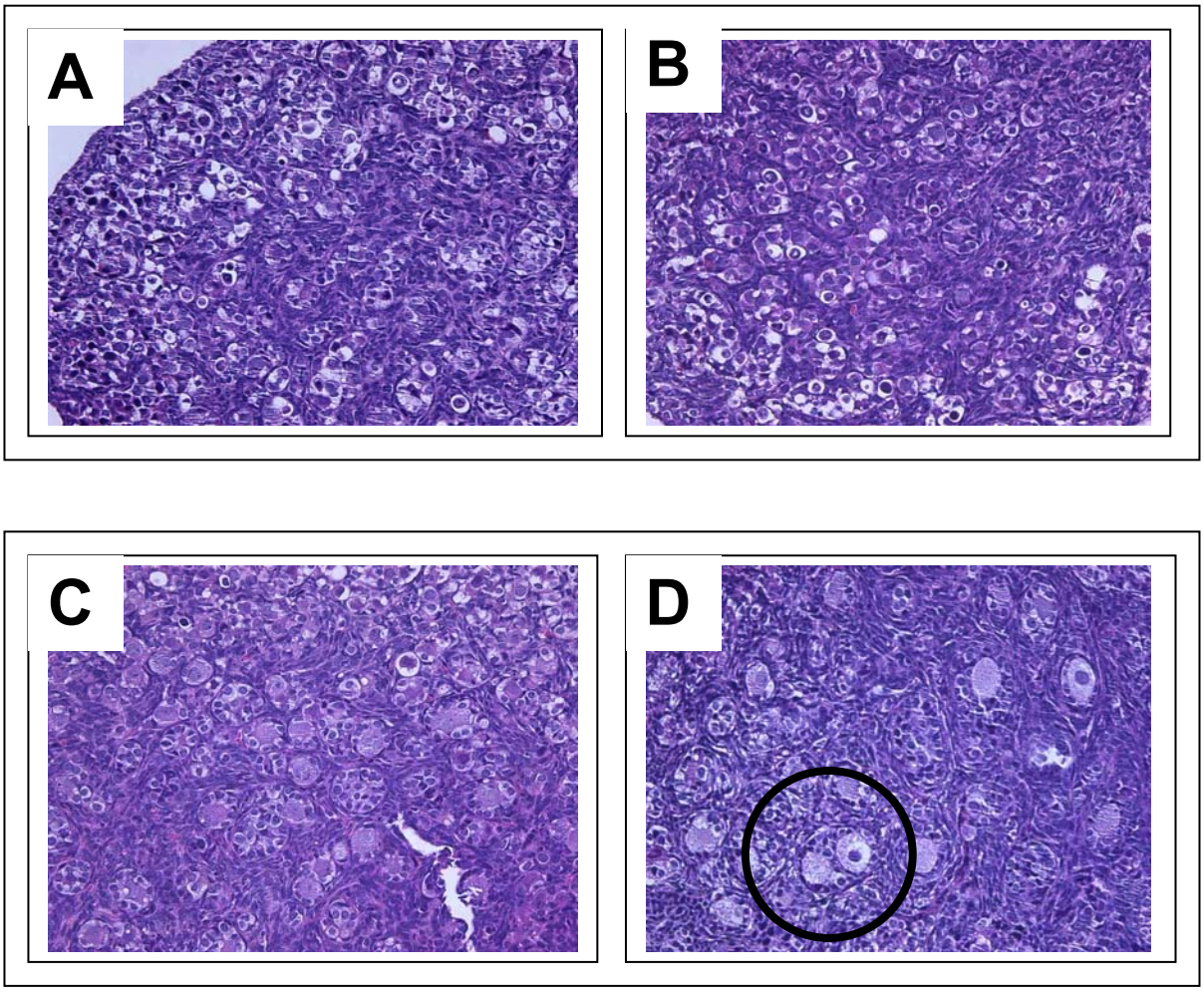


Figure 29: Histomorphology of hematoxylin and eosin-stained neonatal, Day 6 and Day 9, ovarian sections. At Day 6, indicative of primordial follicle formation, comparisons between (A) non-exposed and (B) DES exposed ovarian sections revealed no apparent disruption of ovarian and follicle development, and no POF formation. (Figure 29, A and B). Visualizing Day 9 ovaries, which are in the beginning stages of folliculogenesis, indicated that DES ovarian sections contained approximately equivalent numbers of POFs (black circle) seen in non-exposed ovarian sections. Magnification: 200 X.