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THE ROLE OF OOCYTE- AND EMBRYO-SECRETED FACTORS IN CUMULUS CELL DIFFERENTIATION AND THEIR RELATIONSHIP TO EMBRYO QUALITY AND DEVELOPMENTAL COMPETENCE

© Anil Dhawan

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THESIS OUTLINE

FOREWORD

All is one and one is all.

Himself as in all beings,
And all beings in himself,
Sees he whose self is disciplined in discipline,
Who sees the same in all things.
Who sees Me in all,
And sees all in Me,
For him I am not lost,
And he is not lost for Me.
Whoso reveres Me as abiding in all things,
adopting the belief in oneness,
though abiding in any possible condition,
that disciplined man abides in Me.

Bhagavad Gita [vi. 29-31]

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That my destiny led me to spend my graduate studies under the supervision of Dr.Barbara Vanderhyden, I am very, very grateful. It is no overstatement to say that I truly acquired *education* under Dr. Vanderhyden's tutelage.

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I hope that all of the mice that I sacrificed were done so dutifully and I hope that their lives will benefit us all.

A final and sincere thanks to those kind souls who I have not mentioned...please accept my humble gratitude and may you all achieve Divine Success.

Pranaam

God Bless

ABSTRACT

Reproductive technologies could be markedly improved by the development of methods to evaluate oocyte and/or embryo quality in a non-invasive, quantitative manner. Previous work from this lab has shown that human embryos secrete factors that inhibit progesterone and estradiol production by granulosa cells. To determine if the presence of the progesterone-inhibitory factor (PIF) and estradiol-regulatory factor in human embryo-conditioned (HEC) media correlates with the health and developmental capacity of the embryos, an interspecies bioassay was established. Oocyte-cumulus complexes were isolated from superovulated mouse ovaries, the oocytes were microsurgically removed and the oocytectomized complexes were cultured in HEC media for 48 hours in the presence of FSH and testosterone. Steroid accumulation in the media was determined by radioimmunoassay. Despite the potential limitations of very small volumes of HEC media to evaluate, and the need to freeze this media at the source, the bioassay was able to detect PIF activity in HEC media, but no success was achieved measuring estradiol-regulatory factor. Most embryos produced PIF activity, but the degree of inhibition did not correlate with the ability of the oocytes to be fertilized nor with embryo morphology or their ability to cleave and develop after transfer. The role of the oocyte-specific protein, growth differentiation factor-9 (GDF-9), in cumulus expansion and steroidogenesis was also investigated to determine its potential as a marker for oocyte and embryo quality. Results with HEC media demonstrated that the secretion of PIF by human embryos could be measured by this bioassay and that human PIF could inhibit murine granulosa cell steroidogenesis; however, PIF activity did not correlate with human embryo quality or developmental competence. GDF-9 was shown to enable cumulus expansion and inhibit progesterone and stimulate estradiol production by murine cumulus granulosa cells.

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LIST OF ABBREVIATIONS AND CHEMICAL FORMULAE

8-bromo-cAMP	8-bromo-adenosine-3':5'-monophospate, cyclic
bp	base pair
ANOVA	analysis of variance
ART	assisted reproduction technology
BSA	bovine serum albumin
°C	degrees Celcius
cAMP	cyclic adenosine monophosphate
CEEF	cumulus expansion-enabling factor
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
DO	denuded oocytes
dbcAMP	dibutyryl-adenosine-3':5'-monophosphat, cyclic
DNA	deoxyribonucleic acid
ERF	estradiol-regulatory factor
FCS	fetal calf serum
FSH	follicle stimulating hormone
E_2	estradiol
EGF	epidermal growth factor
GDF-9	growth differentiation factor-9
GIFT	gamete intrafallopian transfer
h	hour(s)
HAS-2	hyaluronan synthase-2

HEC	human embryo-conditioned
HTF	human tubal fluid
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
kb	kilobase pair
kD	kilodalton
KL	kit ligand
l	litre
LH	luteinizing hormone
MC	meiotically competent
MI	meiotically incompetent
min	minutes
ml	millilitre(s)
mm	millimetre
mRNA	messenger ribonucleic acid
O_2	oxygen
OCC	oocyte cumulus complex
OOX	oocytectomized cumulus complex
N_2	nitrogen
NaCl	sodium chloride
ng	nanogram
P_4	progesterone
PIF	progesterone inhibitory factor

PMSG	pregnant mares' serum gonadotropin
RIA	radioimmunoassay
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
SSPE	NaCl, $NaH_2PO_4 \cdot H_20$, EDTA
SEM	standard error of mean
StAR	steroidogenic acute regulator protein
TE	Tris•Cl, EDTA
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
μg	microgram
μl	microlitre
WAY	waymouth media
ZIFT	zygote intrafallopian transfer

INTRODUCTION

1. Assisted Reproductive Technology

1.1 Ovarian Physiology at a Glance

The ovaries' primary function as human female reproductive organs is to produce haploid ova from its germ cells for fertilization by spermatozoa. The production of gametes in the female is governed by the processes of mitotic cell proliferation and the shuffling and rearrangement of genetic material by meiosis. Unlike the testis, the ovary does not rely heavily on mitosis, as only one egg is released during each cycle. The length of an entire human ovarian menstrual cycle is approximately 24-30 days. The ovary's approach to gametogenesis is such that, once having entered the gonad, primordial germ cells continue their mitotic proliferation well after the establishment of ovarian morphology. The mitotic activity of the oogonia, termed as such at this stage of development, terminates finally shortly before birth.

Shortly following the initiation of meiosis, at between 16-24 weeks of gestation, the primordial germ cells become surrounded by ovarian mesonephric (granulosa) cells to form the primordial follicle. Once the oocytes reach the diplotene stage of the first meiotic prophase, their development is arrested. This 'dictyate stage' is characterized by the enclosure of the chromosomes within the nucleus, also known as the germinal vesicle. Surprisingly, the arrested oocytes may stay at this stage for up to 50 years in women, waiting for the signal to resume growth. The reason for storing the oocytes in this remarkable 'frozen' meiotic state is unknown (Johnson and Everitt, 1980).

The dictyate pool of primordial follicles consists of somatic and germ cells which grow in synchrony, via elaborate communication pathways, throughout follicular development. The fully-grown follicle, termed a Graafian follicle, is characterized by an enlarged oocyte with prominent germinal vesicle enclosed within an extracellular coat, the zona pellucida. The germinal vesicle soon disappears, under the command of an, as of yet, unbeknownst factor(s), signaling the oocyte's ability to resume meiosis (meiotic competence). The somatic component of the Graafian follicle is comprised of several cell layers: inner layers of columnar cells (corona radiata or cumulus granulosa cells), several layers of mural granulosa cells with a fluid-filled cavity or antrum, and several outer layers of thecal cells in communication with blood vessels (Pollard, 1994). The granulosa and theca cell compartments are separated by a basement membrane. Meiosis resumes with the preovulatory surge of luteinizing hormone (LH), taking the oocyte from the dictyate stage of the first meiotic division to metaphase of the second meiotic division. Other metabolic changes occur within the oocyte that allow for its activation at fertilization. The growth and differentiation of follicles either culminates in ovulation or, more likely, atresia, the spontaneous degeneration of the follicle (estimate > 99%) (Johnson and Everitt, 1980). Follicular atresia can occur at any stage during development (Figure 1).

This remarkable feat of follicular development is orchestrated by sophisticated and meticulous endocrine activity. Unlike the testis, the ovary releases its oocytes in an episodic manner, termed ovulation, which is governed by systematic fluctuations in steroid levels. The release of the oocyte is characterized by estrogen dominance before ovulation, and progesterone dominance following ovulation: the former is often called the

follicular phase, and the latter, post-ovulatory period, the *luteal* phase. The cyclic release of steroids effects the entire body, in addition to the ovary, acting to support two distinct functions of the genital tract: (1) transport of oocytes to the site of fertilization and (2) preparing the site for implantation of the fertilized egg. The coordination of events is such that the estrogen period prepares the Fallopian tube for the receipt of spermatozoa and fertilization of the oocyte, whereas the combination of estrogen and progesterone after ovulation prepares the uterus to receive and nurture the embryo, if fertilization happens to occur. Following the rupture and subsequent ovulation of the cumulus complex, granulosa cells begin to differentiate or luteinize, and theca cells and blood capillaries intermingle to give rise to the corpus luteum (Johnson and Everitt, 1980).

At birth, the human ovary contains approximately two million germ cells; however, by puberty, the female retains 400,000 germs cells and eventually ovulates only 400-500 of these germs cells in her lifetime, due to the high rate of follicular atresia (Johnson and Everitt, 1980).

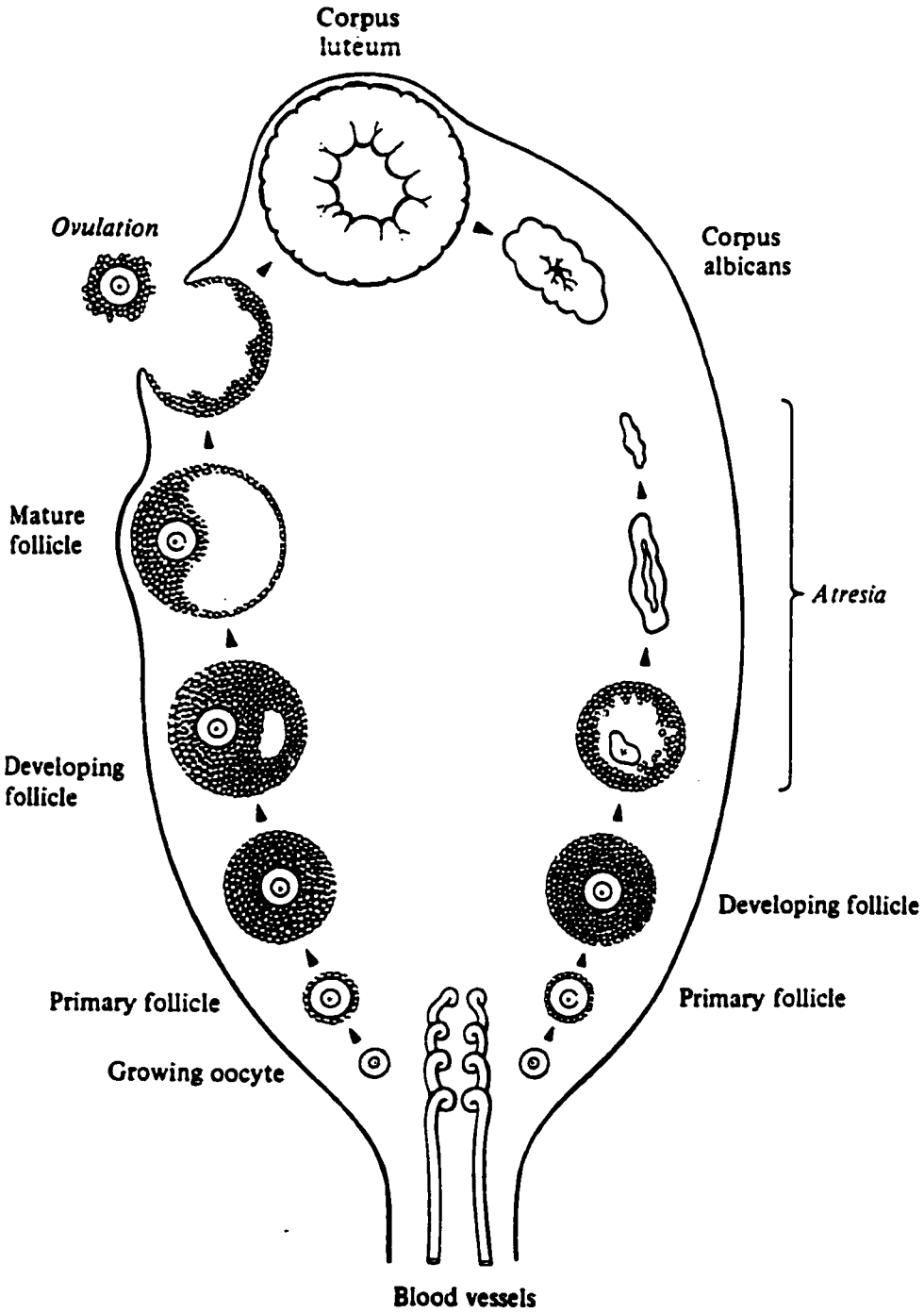

Corpus luteum
Ovulation
Corpus albicans
Mature follicle
Atresia
Developing follicle
Developing follicle
Primary follicle
Primary follicle
Growing oocyte
Blood vessels

FIGURE 1: ***Post-Natal Development of the Ovary***

Once a germ cell emerges from the ovarian pool of non-growing follicles, it grows and begins to form an intricate relationship with the surrounding granulosa cells (e.g., formation of gap junctions). The one layer of undifferentiated cells that surrounds the oocyte of the primordial follicle begins to proliferate, forming a pre-antral follicle (oocyte at prophase I: meiotic arrest), and eventually an antral follicle characterized by the formation of a fluid-filled antrum (oocyte acquires meiotic competence). Granulosa cells differentiate into two distinct populations: mural (or membrana granulosa) and cumulus granulosa cells which immediately surround the oocyte. Approximately 36 hours after the LH surge (in humans), the cumulus complex is ovulated from the preovulatory follicle (Graafian follicle) and the remaining follicular cells form the corpus luteum. Fertilization and embryo development see the maintenance of the corpus luteum, whereas lack of fertilization results in degeneration of the corpus luteum. (From R. Meadows. *Pocket Atlas of Human Histology*. Oxford University Press (1980).)

1.2 Infertility: A New Epidemic?

A couple is considered to be infertile when no conception has occurred after having unprotected coitus of average frequency for approximately one year. About 60-70% of couples who try to conceive will be successful within 6 months. Another 20% will conceive within a year. The remaining 20% will have a medical condition that underlies and interferes with conception. It has been reported that approximately 15% of couples in developed nations are involuntarily infertile, while infertility in developing nations is considerably higher (e.g., 30-40% of women in parts of tropical Africa) (Pollard, 1994). The primary causes of infertility in developing nations are sexually transmitted diseases (e.g., gonorrhoea and chlamydia) and poor hygiene at the time of childbirth, miscarriage, or abortion, which can lead to subsequent infertility.

Of the 80 million cases of infertility around the world, the World Health Organization attributes 30-40% to an exclusively male factor, 40% to a predominantly female factor, 25% to both male and female factors, and in the remaining circumstances a diagnosis cannot be made (Pollard, 1994). Human female fertility decreases with age in both natural and assisted reproduction systems (ART) with the rate of infertility increasing from 30 years of age to menopause.

The recent rise in infertility (Pollard, 1994) is likely attributable to the postponing of pregnancy by women until they are in their thirties, along with the exposure of both women and men to low concentrations of toxins at the workplace: thousands of chemicals are continuously released in the environment, the majority of which have never been tested for their effects on the reproductive system. The increased presence of women in the workplace and their exposure to occupational hazards of heat, noise, vibrations, toxic

chemicals, radiation, etc. has likely compromised the female reproductive tract. The same hazards are likely to compromise male reproduction, affecting semen quality, fertility and libido. Between 50-70% of all male infertility is asymptomatic and without any demonstrable cause (Pollard, 1994). Infertility in women usually arises from a variety of impairments: failure to ovulate, hormonal imbalances, abnormalities of the uterus, and production of sperm antibodies are among a few.

In order for pregnancy to occur, the woman must produce a healthy egg, or ovum, and the man must produce quality sperm in an abundant quantity. Upon entry of the sperm in the vagina, they must travel up to the uterus, eventually meeting the ovum within the Fallopian tubes. Although the sperm may live for a few days in the female reproductive tract, the ovum can only be fertilized within a 12- to 24- hour period after ovulation. Upon fertilization, the egg must travel down the Fallopian tubes for successful implantation within the endometrium of the uterus.

1.3 In Vitro Fertilization, ICSI, and Emerging Assisted Reproductive Technologies (ART)

The increase in infertility treatment in the last few decades has been quite dramatic, perhaps due to the encouraging results generated by the rapid evolution of reproductive technologies. One of the more frequently utilized assisted reproductive treatments for infertility is *in vitro* fertilization, popularly known as IVF. IVF, the first assisted reproduction technology procedure, was developed on rabbits in the late 1960s (Brackett and Williams, 1968), and was used later to treat human infertility caused by the blockage or damage of Fallopian tubes. The first baby to be born from this technique was Louise

Brown, in 1978 (Steptoe and Edwards, 1978). IVF technology involves retrieving sperm and eggs from the bodies of male and female partners, and placing them together in a petri dish to allow for '*in vitro*' fertilization. After approximately 48-72 hours, fertilized eggs are replaced back into the female partner's uterus for implantation and eventual development.

The success rate for IVF in the U.S. is approximately 20% (Society for Assisted Reproductive Technology 1995). Some ART centres report IVF success rates as high as 30% per retrieval (Society for Assisted Reproductive Technology 1995). The success rates are higher for women under 40 than over, indicative of the declining quality of eggs in women as they age. Considering that 1 in 4 couples who hope to have a child actually achieve conception, the success rates for IVF offer an optimistic treatment option for some infertile couples.

IVF is applied to only 3% of all infertile couples who enter treatment. In fact, many other effective forms of treatment are available for men and women. For some women who have problems with regular ovulation, brief treatment with hormones that induce ovulation may allow for successful conception and pregnancy. For men, analysis of sperm may detect an infection or an antibody problem that can be treated with the appropriate medication and a few visits to the physician. If sperm analysis detects low sperm count or low motility the couples may be introduced to the treatment of intracytoplasmic sperm injection (ICSI). ICSI is the newest of the micromanipulation techniques and is a promising new treatment for male infertility. The procedure involves the injection of a single sperm into the egg, bypassing both the requirements for the sperm to swim up the reproductive tract to fertilize the egg and the need to have the

sperm penetrate the egg. As in IVF, the female is prepared for egg recovery by ovarian stimulation. ICSI is probably the most effective of the new ART techniques, with a success rate of approximately 26% (pregnancies per cycle) (Speroff et al., 1994), and some centres reporting success rates as high as 35% (Skakkebaek et al., 1994).

Another commonly used ART procedure is gamete intrafallopian transfer (GIFT). As a variation of standard IVF, GIFT was developed in 1984 and has been used successfully as an alternative for couples with unexplained infertility or infertility caused by cervical or immunological factors, mild endometriosis or some cases of male infertility. The primary distinction between IVF and GIFT is that the former technique occurs 'outside' of the woman's body, whereas GIFT involves placing a mixture of eggs and sperm directly into the woman's Fallopian tubes where fertilization should occur. Thus, the fertilization occurs in vivo, where it naturally would in a normally fertile woman. After placement of the fertilized egg within the Fallopian tube, normal embryo development should occur, with its eventual migration into the uterus for implantation.

An additional form of IVF is zygote intrafallopian transfer (ZIFT), also known as tubal embryo transfer. For ZIFT, fertilization of the egg occurs outside of the woman's body, via the same procedure of ovarian stimulation, monitoring, and egg retrieval that is followed for IVF and GIFT. In the case of ZIFT, those eggs that have been fertilized *in vitro* are transferred directly into the Fallopian tubes, via mini-laparotomy or laparoscopy, rather than into the uterus. The benefit of this technique over GIFT is that it is possible to determine whether fertilization has taken place.

2. Markers for Reproductive Success

2.1 Oocyte and Embryo Quality

Human IVF is a sophisticated process involving a number of stages, which include ovarian stimulation, aspiration of mature ova, semen collection and preparation, IVF, embryo culture and transfer. Of primary interest to both research scientists and clinicians alike is the accurate assessment of oocyte and embryo quality prior to use in IVF, as the major cause for the low success rate in IVF is the tremendous variation in oocyte/embryo quality. It is important to select the most viable oocytes/embryos, i.e. those most likely to develop into healthy fetuses and reach term, for transfer and to keep the transfer number small. Transferring multiple numbers of embryos increases the pregnancy rate, but also increases the risk of multiple gestations with consequent obstetrical morbidity (Geva et al., 1998). Therefore, it is important to address the following questions: What characteristics do viable oocytes/embryos have? How does one select for viable oocytes/embryos, doing so in a manner that does not compromise their health? Identification of variables that are linked with IVF success would be beneficial in predicting pregnancy outcome, thus enabling modification of IVF strategy in such a manner as to maximize the odds for success.

2.2 Markers of Oocyte and Embryo Quality

The most successful methods of assessing oocyte/embryo quality in an IVF setting, without compromising oocyte/embryo health, are non-invasive. Non-invasive methods of assessment, both qualitative and quantitative, have been used to investigate predictive variables in follicular fluid, cumulus complexes and embryos. The majority of

non-invasive methods employed in IVF clinics, at present, are qualitative, based upon the morphologic assessment of the oocyte/embryo, as evaluated under a stereomicroscope.

2.2.1 Follicular Fluid Components

Several follicular parameters have been investigated for their association with predicting oocyte fertilization and embryo health, leading to pregnancy. An interesting prospect has been the use of Doppler ultrasonography technology, with which a physiological relationship between blood velocity, oocyte recovery, and recovery of high-quality preimplantation embryos has been shown (Nargund et al., 1996), along with an association between high follicular vascularity and pregnancy rate (Chui et al., 1997). Also, an aspect of follicle morphology that has been associated with the developmental capacity of oocytes/embryos is follicular volume and number, although results to the contrary have also been published (Salha et al., 1998). Large follicular number and high mean follicular volume related positively to pregnancy outcome, and successful oocyte retrieval and fertilizability, and high-grade embryos were strongly correlated with increasing individual follicular volume (Arnot et al., 1995; Nayudu et al., 1987).

In terms of biochemical markers, several studies have attempted to associate molecules found in follicular fluid with oocyte/embryo quality, fertilizability and pregnancy outcome. Estradiol (E_2) and progesterone (P_4) in follicular fluid, as well as in serum (Burns et al., 1994), have attracted great attention as possible predictors for pregnancy. The association between these follicular steroids and oocyte fertilizability has been attempted, where it has been found that follicles yielding oocytes that result in pregnancy are characterized by relatively high P_4 content and a high P_4/E_2 ratio (Basuray

et al., 1988). Follicular steroids remain one of a few reliable predictors of pregnancy outcome. Follicular fluid components α-antitrypsin, complement C_3, immunoglobulin (Ig) G 2, and total protein have been associated with normal pregnancy (Nayudu et al., 1987). A variety of other follicular fluid components have been tested for an association with oocyte/embryo grade, fertilizability, and pregnancy. Follicular fluid proteoglycan hyaluronic acid showed no correlation, whereas follicle-stimulating hormone (FSH) did show an association with cumulus complex morphological quality and fertilizability of oocytes (Eriksen et al., 1997; Suchanek et al., 1994). In contrast, the presence of low levels of the proteoglycan chondroitin sulfate were associated with higher follicular volumes and greater fertilization rates and embryo transfer (Eriksen et al., 1997). The presence of anti-zona pellucida antibodies in follicular fluid has been shown to correlate positively to the degree of fertilization failure (Papale et al., 1994). In addition, c-*kit* and its ligand (KL), which play important roles in primordial germ cell survival and proliferation and follicular growth, have been measured in follicular fluid and found to correlate positively with follicular fluid volume and steroids, and with successful IVF pregnancies (only KL) (Smikle et al., 1998; Tanikawa et al., 1998).

2.2.2 Oocyte Markers

From the outset of the search for predictive markers, researchers realized the potential value of looking directly at cumulus complex or oocyte morphology as a gauge for reproductive success. One of the first studies to use morphology in assessing oocyte developmental capability reported a correlation between extent of cumulus expansion and oocyte quality/fertilizability, where the incidence of IVF was analyzed with respect to the

degree of cumulus expansion at the time of oocyte recovery (Testart et al., 1983). However, the results of recent studies have suggested that there is no correlation between cumulus complex morphology and nuclear maturity nor fertilizability (Balaban et al., 1998; Rattanachaiyanont et al., 1999), bringing into question the efficacy of using this wide-spread 'predictive' parameter in IVF centres. Oocyte score, a grade based upon growth and morphology, has been significantly correlated to two important physiological parameters of oocytes: nuclear maturity and fertilization rate (Mahadevan and Fleetham, 1990; Veeck, 1988). The predictive value of second polar body extrusion has also been examined (Van den Bergh et al., 1995), and it has been found that second polar body extrusion is a valuable predictor of oocyte fertilization. In terms of granulosa cell-secreted factors, relaxin secretion has been correlated with implantation success in IVF-embryo transfer cycles (Stewart and VandeVoort, 1999).

2.2.3 Embryo Markers

A variety of markers that involve embryo assessment has been investigated for their ability to predict IVF success. Most grading systems focus on day 2 or 3 (4-8 blastomere stage) embryos, although embryos can be assessed at any time during the pre-implantation period. At present, embryos are usually selected for transfer on the basis of their gross morphology, cleavage rate and growth in culture. The most frequently used variables have been blastomere number, incidence of fragmentation, cytoplasmic darkness and granularity, presence of vacuoles, blastomere size and symmetry, and zona thickness and regularity. Positive correlations have been found between morphological scoring of embryos and pregnancy outcome (Shulman et al., 1993; Visser and Fourie,

1993). When taking the metabolic/biochemical approach to assessing embryo viability, the following parameters have shown much promise in the prediction of pregnancy: O_2 consumption (Magnusson et al., 1986), pyruvate uptake (Conaghan et al., 1993; Hardy et al., 1989; Leese et al., 1986), glucose uptake and lactate production (Hardy et al., 1989), and immunosuppressive activity (Jones et al., 1992). Embryo-derived PAF has been detected by a bioassay and shown to be strongly correlated with the viability of embryos and their pregnancy potential (O'Neill and Saunders, 1984; O'Neill et al., 1987).

Although research has shown that some variables (e.g., PAF) correlate with embryo quality, they have not yet been implemented in clinical assays. Due to the recognition that numerous factors are collectively involved in the successful growth of the oocyte and embryo, researchers are now acknowledging the need to use multivariate testing in order to ensure the accurate assessment of oocyte and embryo quality. Therefore, the use of known quantitative, non-invasive parameters along with oocyte/embryo-secreted factors would significantly improve reproductive technologies by reducing costs associated with poor oocyte and embryo selection and reducing the risk of multiple pregnancies associated with the current practice of multiple embryo transfer.

3. Oocyte and Embryo Secreted Factors

The fate of all ovarian follicles, the fundamental functional unit of the ovary, relies on the oocyte enclosed within the ovarian follicle. The development of the ovary is a sophisticated communication-dependent process, with elaborate cellular interactions between the germ cells and somatic cells. Communication between the germ cells (oocytes) and somatic cells (e.g. thecal cells, granulosa cells) occurs via both membrane gap junctions (Heller and Schultz, 1980; Heller et al., 1981; Moor et al., 1980) and paracrine factors (Driancourt and Thuel, 1998). For decades, the oocyte was thought to be a passive cell sitting under the control of surrounding somatic cells, which, supposedly, dictated its fate. However, in the past thirty years the role of the oocyte in follicular development has changed from that of a passive cell, to that of an active participant in its fate.

Of particular interest, in terms of somatic cell and germ cell interaction, is the secretion of paracrine factors by the oocyte that mediate several key functions. Two factors which have been recently described to be involved in cumulus expansion and steroid regulation are cumulus expansion-enabling factor (CEEF) and steroid regulatory factor (Buccione et al., 1990; Vanderhyden et al., 1993). Although the importance of the oocyte in inducing the differentiation and proliferation of granulosa cells has been shown, the identification of these paracrine factors has been elusive. However, several potential candidates have been proposed to be the oocyte-secreted factor (Alak et al., 1998; Dong et al., 1996) and these will be described in Section 3.1.

In terms of embryo-secreted factors, the embryo has been shown to secrete a variety of cytokines that may be acting in an autocrine fashion or on the endometrium to

influence its receptivity. Several growth factors known to be secreted by the human embryo are interleukin-1, interleukin-6, colony-stimulating factor-1, and tumor necrosis factor-α (Witkin et al., 1991; Zolti et al., 1991). Also, a more recent study by Seifer et al. (1995) has shown that human embryos secrete a factor that regulates granulosa cell steroidogenesis. Due to the limited work being done in this area, the specific roles of embryo secreted-factors in preimplantation development, particularly in the human, remains somewhat obscure.

3.1 Cumulus Expansion-Enabling Factor (CEEF)

The specialized granulosa cells that surround, and are metabolically coupled to, antral follicle oocytes are called cumulus cells (Anderson and Albertini, 1976; Gilula et al., 1978). Upon the preovulatory release of LH, the cumulus cells undergo a dramatic conformational change called cumulus expansion. The LH surge induces the production of hyaluronic acid, a nonsulfated glycosaminoglycan, by cumulus cells, which, in turn, disperses these same cells, embedding them in a gelatinous matrix.

In vitro, cumulus expansion of oocyte-cumulus cell complexes (OCC) can be stimulated by FSH (Ball et al., 1985; Dekel and Kraicer, 1978; Eppig, 1979b; Hillensjö and Channing, 1980; Thibault, 1972) via a mechanism that appears to be mediated by cAMP (Dekel et al., 1978; Eppig, 1979a). Interestingly, microsurgical removal of the oocyte (oocytectomy) resulted in the inability of the oocytectomized cumulus complex (OOX) to produce hyaluronic acid and to undergo cumulus expansion in response to FSH (Buccione et al., 1990). However, expansion of oocytectomized cumulus complexes could be induced by denuded oocytes in the presence of FSH, EGF, or the cAMP

analogue 8-bromo cyclic adenosine monophosphate (8Br-cAMP), to the same extent as occurred in intact cumulus complexes, demonstrating the secretion of a factor by oocytes that enables hyaluronic acid secretion by cumulus cells. The influence of the oocyte occurred downstream from the elevation of cAMP levels in the cumulus cells, as no difference in cAMP levels were detected between intact and oocytectomized complexes stimulated with FSH, and 8Br-cAMP did not stimulate expansion in oocytectomized complexes (Buccione et al., 1990). Mouse and rat oocytes were shown to secrete this CEEF, necessary for cumulus cells to undergo expansion in response to FSH, EGF, or dibutyryl cyclic adenosine monophosphate (dbcAMP), although rat oocytectomized complexes did undergo limited expansion in the absence of CEEF (Vanderhyden, 1993). However, pig cumulus expansion did not appear to be dependent upon this factor, although pig oocytes can secrete CEEF (Vanderhyden, 1993). There seem to be cross-species similarities in CEEF, as pig oocyte-conditioned media enabled the FSH-stimulated expansion of mouse and rat oocytectomized complexes (Vanderhyden, 1993).

In terms of the developmental pattern of secretion of CEEF, growing meiotically incompetent oocytes do not produce detectable amounts of factor, whereas fully grown meiosis-arrested oocytes, maturing oocytes, and metaphase II oocytes do (Vanderhyden et al., 1990). The ability of complexes to undergo expansion in response to FSH and CEEF has been correlated with the acquisition of oocyte competence to undergo germinal vesicle breakdown. In addition, detectable amounts of CEEF were produced by zygotes, but not by embryos at the two-cell to morula stages of development (Vanderhyden et al., 1990).

Preliminary characterization of CEEF from both mouse and pig oocytes has revealed it to be heat labile (65°C for 15 min), and proteinase K sensitive (Eppig et al., 1993; Vanderhyden, 1993) and its activity was retained in the fraction with molecular mass > 100 kDa during fractionation (Vanderhyden, 1993). Potential candidates for CEEF have been suggested and several studies have produced interesting leads. As FBS is one of the three components, along with CEEF and FSH, necessary for cumulus expansion, Singh et al. (1993) investigated and provided evidence that insulin-like growth factor-1 (IGF-1) is a component of FBS that is responsible for its cumulus expansion-enabling activity *in vitro*. TGF-β has also been shown to induce cumulus expansion by substituting for the oocyte-secreted factor (Salustri et al., 1990). However, it is less effective than the oocyte factor, and neutralizing antibodies against TGF-β did not inhibit the response of granulosa cells to the oocyte factor indicating that these two factors are different. The two factors do show additive effects at suboptimal concentrations and they show identical temporal patterns of induction (Tirone et al., 1997). It seems likely that the oocyte-secreted factor belongs to the TGF-β superfamily. Fortuitously, a new member of the TGF-β superfamily has recently been discovered, growth differentiation factor-9 (GDF-9) (McPherron and Lee, 1993), which is localized specifically to the oocyte in mice and humans (McGrath et al., 1995). GDF-9 has been shown to stimulate cumulus expansion of oocytectomized complexes in the presence of FSH (Elvin et al., 1999b); however, studies using GDF-9 antibodies to confirm that it is CEEF remain to been done.

3.2 Steroid Regulatory Factor

The first positive suggestion that granulosa cells are dependent upon the oocyte for development and function resulted from a study in which granulosa cell luteinization occurred following rabbit follicle ovectomy (El-Fouly et al., 1970). This same group was the first to suggest a role for the oocyte in regulating granulosa cell steroidogenesis, and suggested that the oocytes may be secreting a substance which is preventing the luteinization of the granulosa lining. Additional evidence for such a factor has been provided by *in vivo* and *in vitro* experiments in rats and rabbits, which show that removal, absence, or destruction of oocytes leads to granulosa cell luteinization (Hubbard and Erickson, 1988; Nekola and Nalbandov, 1971; Stoklosowa and Nalbandov, 1972).

Further research on the growth, development and function of ovarian granulosa cells has been conducted by culturing the cells as monolayers in the presence of growth factors and hormones. Because the cells are not in their original spatial conformation as found within the follicle, there is disruption of intercellular communication provided by gap junctions between granulosa cells and changes in cytoskeletal structure due to loss of contact with certain components of the basal lamina; thus, the interpretation of these studies may be somewhat misleading (Ben-Ze'ev and Amsterdam, 1986; Carnegie et al., 1988).

With the development of the technique of oocytectomy (Buccione et al., 1990), the microsurgical removal of the oocyte from the cumulus complex, the three-dimensional organization of the complex is retained and the role of the oocyte in regulating granulosa cell steroidogenesis can be elucidated with greater similarity to *in vivo* conditions. In the first of such experiments using oocytectomy, mouse oocytes were

found to regulate both FSH-induced progesterone and estradiol production by granulosa cells (Vanderhyden et al., 1993). Oocytectomized cumulus complexes produced significantly greater amounts of progesterone than intact cumulus complexes; however, this increase was reduced in the presence of oocyte-conditioned medium, suggesting the presence of an oocyte-secreted progesterone-inhibitory factor (PIF) (Vanderhyden et al., 1993). Also, removal of the oocyte reduced estradiol production, indicating that the oocyte was secreting a steroid-regulatory factor that stimulates granulosa cell estradiol production. The involvement of the oocyte in the regulation of progesterone production suggests a potentially important role for the oocyte in the prevention of premature luteinization of the follicle.

In subsequent studies, with the use of non-aromatizable substrates and aromatase inhibitors, it was shown that estradiol and progesterone are regulated independently by the oocyte (Vanderhyden and Tonary, 1995). The observation that estradiol and progesterone production are independently regulated by the oocyte-secreted factor suggests that this factor(s) may act on several enzymes in the steroidogenic pathway. These could include 1) the aromatase enzyme, to enhance estradiol production; 2) the enzymes in the progesterone production pathway e.g., P450 side chain cleavage enzyme, to inhibit the conversion of cholesterol to pregnenolone, and/or 3β-hydroxysteroid dehydrogenase, to inhibit the conversion of pregnenolone to progesterone; and 3) the enzymes involved in the metabolism of progesterone, e.g., 20α-hydroxysteroid dehydrogenase, to stimulate the conversion of progesterone to its metabolites (Figure 2).

The oocyte-secreted steroid regulatory factor seems to be acting downstream of the generation of cAMP to inhibit progesterone production, as replacement of FSH with

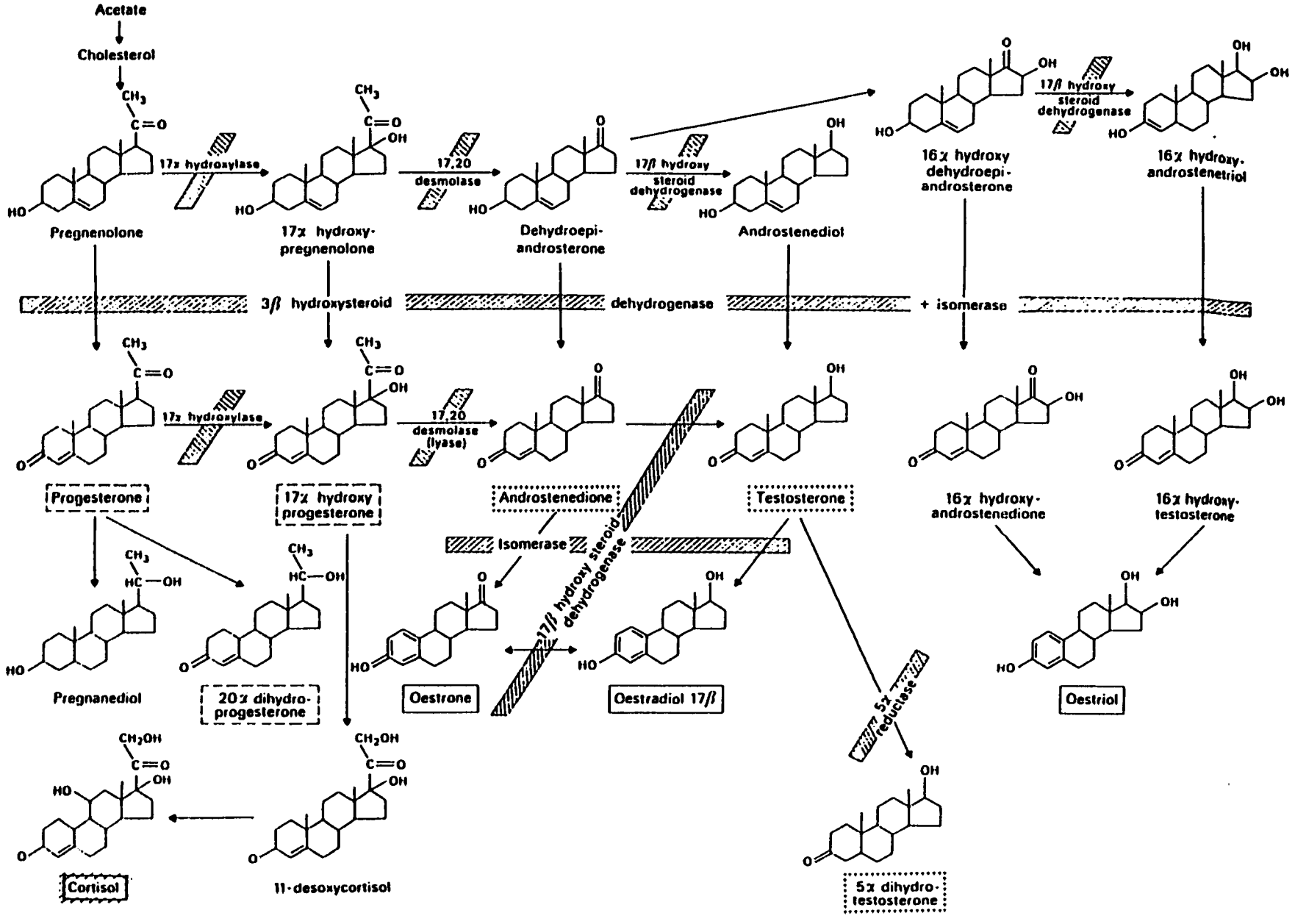

Acetate
Cholesterol
17α hydroxylase
17,20 desmolase
17β hydroxy steroid dehydrogenase
16α hydroxy dehydroepi-androsterone
17β hydroxy steroid dehydrogenase
16α hydroxy-androstenetriol
Pregnenolone
17α hydroxy-pregnenolone
Dehydroepi-androsterone
Androstenediol
3β hydroxysteroid
dehydrogenase
+ isomerase
17α hydroxylase
17,20 desmolase (lyase)
Progesterone
17α hydroxy progesterone
Androstenedione
Testosterone
16α hydroxy-androstenedione
16α hydroxy-testosterone
Isomerase
17β hydroxy steroid dehydrogenase
5α reductase
Pregnanediol
20α dihydro-progesterone
Oestrone
Oestradiol 17β
Oestriol
Cortisol
11-desoxycortisol
5α dihydro-testosterone

FIGURE 2: ***Pathway of Interconversion of Steroids***

The conversion of cholesterol to pregnenolone is the initial step shared in the formation of the major steroid hormones (e.g., progesterone, estradiol) and involves the specialized enzyme P-450 side-chain-cleavage, a cytochrome (not shown). The interconversion of steroids is undertaken by a series of enzymes. Progesterone function is characterized by activities that prepare for pregnancy and maintainance, thus is of importance in the luteal phase of the ovulatory cycle. Estradiol is associated with the development and maintenance of feminine traits (e.g., preparation for egg production, fertilization).

Johnson and Everitt, 1980

dibutyryl cAMP resulted in no change in steroid production and the addition of oocyte-conditioned media still resulted in an inhibition of granulosa cell progesterone production. In terms of preliminary characterization, the steroid-regulatory factor is heat stable and is removable by charcoal extraction, suggesting that it is a small molecule, possibly a steroid (Coskun et al., 1995).

Observations have been made for porcine oocyte-granulosa cell paracrine interactions, where an oocyte-secreted factor inhibited porcine granulosa cell progesterone production. However, porcine oocytes secreted a steroid-regulatory factor that also inhibited estradiol production by granulosa cells, in contrast to the mouse model (Coskun et al., 1995). Inhibition of progesterone production was found to be dependent upon oocyte number: an increase in oocyte number resulted in greater inhibition of progesterone production, indicative of a greater concentration or activity of steroid-regulatory factor (Coskun et al., 1995).

Coculture of human zygotes with granulosa cells results in a decrease in progesterone and estradiol production, suggesting that human zygotes also secrete a steroid-regulatory factor(s) (Seifer et al., 1996). That human embryos affect luteinized granulosa cell activity is contrary to the corpora luteal production of progesterone that normally occurs during early embryo development. However, it has been shown that mouse oocytes produce progesterone-inhibitory factor (PIF) at several stages of development, from midgrowth to full-grown stages as well as ovulated oocytes and zygotes (Vanderhyden and Macdonald, 1998) (Figure 3). Thus, the production of the steroid-regulatory factor by human embryos may just be a continuation of the constitutive production of this factor during oocyte development. The secretion of steroid-regulatory

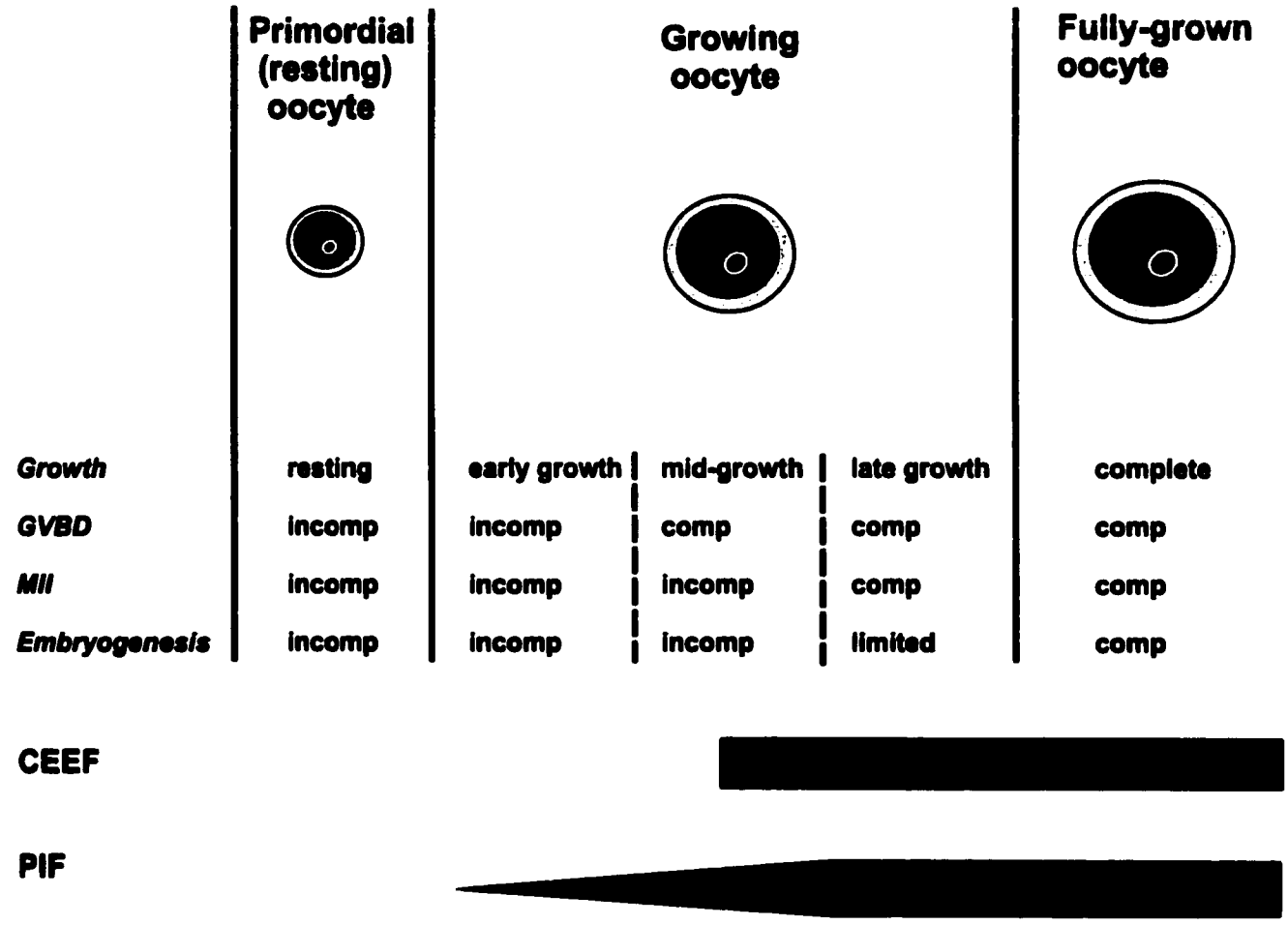

Primordial (resting) oocyte
Growing oocyte
Fully-grown oocyte
Growth
GVBD
MII
Embryogenesis
resting
incomp
incomp
incomp
early growth
incomp
incomp
incomp
mid-growth
comp
incomp
incomp
late growth
comp
comp
limited
complete
comp
comp
comp
CEEF
PIF

FIGURE 3: ***Oocyte Growth and the Secretion of Cumulus Expansion-Enabling Factor (CEEF) and Progesterone-Inhibitory Factor (PIF)***

Oocytes acquire meiotic competence towards mid-growth, characterized by germinal vesicle breakdown (GVBD) but actually resume meiosis in the late growth period, arresting at metaphase II (MII) until fertilization. Oocytes begin secreting PIF early on in their growth in progressively greater amounts, whereas CEEF is not secreted until meiotic competence is achieved.

factors by both oocytes and embryos raises the possibility of using these factors as markers for embryo quality and developmental competence.

4. Growth Differentiation Factor-9 (GDF-9)

4.1 Discovery

A molecule of particular interest recently within the context of follicular development is GDF-9, a member of the TGF-β superfamily. GDF-9 was recently identified by using degenerate oligonucleotides corresponding to conserved regions among known family members. The GDF-9 gene is encoded by two exons separated by a 2.9 kb intron (Incerti et al., 1994). Analysis of cDNA sequences revealed that the GDF-9 polypeptide contains a potential signal sequence for secretion (suggested by presence of a core of hydrophobic amino acids near the NH2 terminus), a putative tetrabasic proteolytic processing site, and a COOH-terminal region that shows significant homology to the known family member of the TGF-β superfamily. A peculiar feature of GDF-9 is that, unlike all previously described members of the family, it lacks the conserved cysteine residue that is believed to form the only disulfide linkage between subunits in other family members (cysteine 7) (McPherron et al., 1993). It is possible that GDF-9 does not form dimers or it may form noncovalently linked dimers in which the subunit interaction is dynamic and subject to regulation.

4.2 Localization and Expression of GDF-9

In the first study of GDF-9 expression, mRNA samples from a variety of adult mouse tissues were screened by Northern analysis and GDF-9 was found to be expressed exclusively in the ovary [1.7kb: (McPherron et al., 1993)]. Thus, it is the only member of the TGF-β superfamily to date which is ovary-specific. In terms of ovarian cell-type, GDF-9 mRNA was localized exclusively to oocytes in both neonatal and adult mouse

ovaries (Fitzpatrick et al., 1998; Laitinen et al., 1998) and to ovine and bovine oocytes (Bodensteiner et al., 1999). Using antibodies to the mature region of GDF-9, it was shown that mature GDF-9 protein was present in the oocytes of primary, secondary and larger follicles, indicating that GDF-9 transcripts are being translated into proteins (Hayashi et al., 1999). GDF-9 was also shown to be synthesized by oocytes of large antral and preovulatory follicles (Elvin et al., 1999b). The onset of expression during ovarian development appears to coincide with the formation of primary follicles in the mouse, although GDF-9 mRNA is also expressed in primordial follicles of ovine and bovine ovaries (Bodensteiner et al., 1999). GDF-9 mRNA expression continues throughout follicular development as well as after ovulation but disappears by day 1.5 after fertilization (Fitzpatrick et al., 1998).

The relatively long window of GDF-9 expression suggests that it may be playing various regulatory roles in both follicular and early embryonic development. In the capacity of an autocrine factor, it may be regulating oocyte maturation; whereas, as a paracrine factor, GDF-9 may play a role in regulating the proliferation and/or differentiation of granulosa cells. Either case is supported by the presence of a putative signal sequence for secretion in the GDF-9 cDNA sequence.

4.3 GDF-9 Knockout Mice

In order to fully understand the function and role of GDF-9 in mammalian development, knockout mice were recently engineered. A targeted deletion of exon 2, encoding the mature region of GDF-9, was generated using embryonic stem cell technology (Dong et al., 1996). The deletion targeting vector generated a 1.1-kb deletion.

Because homozygous (-/-) GDF-9 mutant female mice were found to be infertile, it was necessary to intercross the viable and fertile heterozygous (+/-) female mice with homozygous (-/-) males to maintain the GDF-9 breeding colony.

Analysis of homozygous (-/-) GDF-9 female mice ovaries revealed that they were smaller in size. In addition, they failed to respond to pregnant mares' serum gonadotropin (PMSG) superovulation treatment. Closer inspection of homozygous ovaries showed a lack of both corpora lutea and normal follicular growth beyond the primary one-layer follicle stage; follicles beyond the one-layer primary follicle were abnormal and contained an asymmetrical arrangement of 'distorted' granulosa cells. Numerous primordial and primary follicles were visible, each primary follicle having an oocyte that had achieved normal size and formed a zona pellucida. Strangely enough, GDF-9 mutant (-/-) ovaries, in contrast to control, did not exhibit any signs of follicular atresia, suggesting that a block in folliculogenesis caused by the absence of GDF-9 may be affecting the ability of the granulosa cells to become competent to undergo atresia (Dong et al., 1996).

In terms of oocyte development, GDF-9 homozygous (-/-) oocytes rarely contained cortical granules, and most organelles were clustered around the germinal vesicle. With respect to other ultrastructural features (e.g., dispersed Golgi complexes, vacuolated mitochondria), development appeared normal. Oocytes obtained from 12-week old GDF-9 homozygous (-/-) mice were slightly larger than the heterozygous controls and, upon culture, seemed to be defective in their ability to acquire meiotic competence (Dong et al., 1996). In addition, the rate of oocyte growth relative to follicle

size was accelerated in GDF-9-deficient mice when compared to heterozygous controls (Carabatsos et al., 1998).

Serum FSH and LH levels in the GDF-9 homozygous females were elevated threefold and twofold, respectively; thus, these mice were hypogonadal and hypergonadotropic, although LH receptor, FSH receptor and activin type II receptor mRNA levels seemed unaltered in the homozygous (-/-) GDF-9 female mice. However, cytochrome P450 aromatase mRNA levels were reduced by 50%. It seems that the alteration in negative feedback by gonadal steroids and peptides in the absence of GDF-9 could be due to the presence of abnormal granulosa cells, resulting in insufficient steroid production (Dong et al., 1996).

4.4 Function of GDF-9

With the generation and analysis of GDF-9 knockout mice, it was apparent that GDF-9 plays an integral role in the development of the ovary. However, its exact function and role in this elegant and sophisticated process remains unknown. Hayashi et al. (1999) recently generated a recombinant GDF-9 protein to study its physiological role. With the use of preantral follicles and neonatal explants from rats, they were able to demonstrate the stimulatory ability of GDF-9 on preantral follicle growth and inhibin-α production by early follicles.

Further functional studies have demonstrated that there is an upregulation of KL and inhibin-α in GDF-9 deficient mice (Elvin et al., 1999a). The observation of dramatically elevated KL suggests that GDF-9 may be one of the paracrine factors that negatively regulates KL expression. Also, the observation that GDF-9 deficient oocytes

grew more rapidly and to a greater size (15%) than controls (Carabatsos et al., 1998) further confirms the previous suggestion that KL stimulates oocyte growth (Packer et al., 1994).

GDF-9 action includes the regulation of the gene expression of hyaluronan synthase 2 (HAS-2), cyclooxygenase-2 (COX-2), steroidogenic acute regulator protein (StAR), urokinase plasminogen activator (uPA), and luteinizing hormone receptor (LH) (Elvin et al., 1999a). Interestingly, recombinant GDF-9 has been shown to enable cumulus expansion of oocytectomized cumulus complexes (Elvin et al., 1999b). That GDF-9 can enable cumulus expansion in the absence of the oocyte, can upregulate HAS-2, the major hyaluronic acid synthase involved in cumulus expansion (Spicer et al., 1996), and inhibits the protease uPA, an enzyme suppressed during production of the hyaluronic acid extracellular matrix, clearly implicates GDF-9 as the cumulus expansion-enabling factor.

GDF-9 has also been shown to play an integral role in the regulation of granulosa cell steroidogenesis. The presence of recombinant GDF-9 with granulosa cells resulted in a 2-5 fold induction of StAR mRNA. StAR is the protein responsible for allowing P-450 side-chain-cleavage enzyme access to cholesterol for conversion to pregnenolone in the synthesis of progesterone (Figure 2). Elvin et al. (1999) observed that recombinant GDF-9 also significantly stimulated the production of progesterone by murine mural granulosa cells. It seems that, like FSH, GDF-9 is a growth and differentiation factor for early follicles, although the exact mechanism remains to be elucidated.

5. Rationale

Reproductive technologies could be markedly improved by the development of methods to evaluate oocyte and/or embryo quality in a non-invasive, quantitative manner. Hence, the overall purpose of the research presented within this treatise was to identify an embryo-secreted factor(s) and develop a novel and non-invasive method to detect and measure the amount of factor activity in human embryo-conditioned (HEC) media and to correlate this activity with embryo health and developmental capacity. Therefore, the following were the objectives of this research project:

(1) To establish a non-invasive, interspecies bioassay. This entailed that the following specific objectives were met:

   (i) To determine if the freezing of HEC media and its small culture volume would pose potential limitations in the establishment of the bioassay.

   (ii) To detect the presence of CEEF in HEC media

   (iii) To detect the presence of an estradiol-regulatory factor in HEC media

   (iv) To detect the presence of progesterone-inhibitory factor (PIF) in HEC media

(2) To determine if the presence of the PIF in HEC media correlates with the health and developmental capacity of the embryos.

(3) To identify the role of GDF-9 in cumulus expansion and steroid regulation and to determine its potential use as a non-invasive marker of embryo quality and developmental capacity.

Identification of variables that are linked with IVF success would be beneficial in predicting pregnancy outcome and for enabling modification of IVF strategies in such a manner as to maximize odds for success. It is important to select the most viable oocytes/embryos, i.e. those most likely to develop into healthy fetuses and reach term, for transfer and to keep the transfer number small, in order to increase pregnancy rates, and decrease risk of multiple gestations with consequent obstetrical morbidity. Identification of such factors will enable the quantitative assessment of embryo quality and may improve embryo culture conditions or the receptivity of the uterus for implantation.

MATERIALS & METHODS

1. Human Embryo-Conditioned (HEC) Media Samples

HEC samples were obtained from embryos produced by the IVF program of the Ottawa Hospital. Consent was obtained from patients prior to obtaining and using HEC samples. The IVF procedure involved the collection of OCCs from follicular aspirates and culturing them singly for 4 h in 1 ml Human Tubal Fluid media (HTF: Meditech IST, Montreal, Quebec, Canada) containing 0.5% w/v bovine serum albumin (BSA: Bayer, Kankakee, IL) at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. After the initial 4 h incubation, the OCCs were inseminated singly in 50 μl drops of HTF-BSA with ~6250 motile spermatozoa, for approximately 16-18 h, whereupon the OCCs were stripped of their cumulus cells and assessed for fertilization by the presence of 2 pronuclei. Fertilized oocytes were then individually cultured for an additional 48 h in 20 μl drops of HTF-BSA.

For oocytes fertilized by intracytoplasmic sperm injection, OCC were collected from follicular aspirates and cultured for 2-3 h in the same conditions as for IVF. The oocytes were subsequently denuded of cumulus cells, and their maturity was assessed immediately under a stereomicroscope. The oocytes were further cultured in 20 μl drops of HTF-BSA for 1-2 h [oocyte-conditioned media (OCM)], and then ICSI was performed on all MII oocytes (as assessed by absence of the germinal vesicle and presence of the polar body) using motile spermatozoa isolated by density gradient centrifugation. Injected oocytes were assessed 18 h after injection for fertilization and were individually cultured for 42 h before transfer (Van Steirteghem et al., 1993).

The use of a composite embryo score, consisting of the product of cell number and morphological quality (cell number X morphological quality=score), has been reported previously as a tool to select high-grade embryos (Joesbury et al., 1998) and, therefore, a similar composite score was used in this study. The morphological quality and stage of embryo growth were assessed qualitatively by IVF technicians at 42 h after insemination. Embryo morphology was graded with a score of 1-5 according to blastomere quality and level of fragmentation: grade 5 = regular blastomeres and no anucleated fragments; grade 4 = regular blastomeres and <25% fragmentation; grade 3 = regular or irregular blastomeres and <25% fragmentation; grade 2 = regular or irregular blastomeres and 25-50% fragmentation; and grade 1 = regular or irregular blastomeres and >50% fragmentation (Rattanachaiyanont et al., 1999). Points for embryo cell number (growth) were given according to the number of cells observed up to the 4-cell stage, (e.g., 2-cell embryo was given a score of 2). Embryos with cell number of 5 were given 3 points and those embryos with a number of cells greater than 6 were given scores of 2. Embryos of good quality were considered those that had achieved at least a 4-cell stage with a morphological quality of at least grade 3 (e.g., Score=12). The minimum score that could be given was 1, poorest quality, and the maximum was 20, greatest quality.

Embryo transfers occurred 48 h post-retrieval and embryos were selected on the basis of the combined embryo score of morphological quality and growth. The remaining spent media (HEC) were immediately frozen at –20°C until use in experiments. HEC media from both IVF and ICSI fertilized oocytes were used in experiments. For all experiments, control media refer to fresh aliquots of HTF-BSA media frozen for approximately the same length of time as the HEC media before use in the experiments.

2. Harvest of Intact Oocyte Cumulus Complexes (OCC) and Oocytectomized Cumulus Complexes (OOX)

2.1 Collection of mouse OCC and denuded oocytes

Animals were maintained and handled according to the Guidelines for the Care and Use of Animals established by the Canadian Council on Animal Care. Ovaries were isolated from 22- to 26-day-old (C57BL/6 X Balb/C) F1 mice 44-48 h after injection with 5 IU pregnant mares' serum gonadotropin (PMSG: Folligon; Intervet Canada Inc., Whitby, ON, Canada). The superovulation of the ovaries yielded approximately 40-50 OCC per mouse. On the day of the experiment, the ovaries were removed from the animals and the antral follicles were punctured with 25-gauge needles in Waymouth MB752/1 culture medium (WAY) supplemented with 75 mg/ml penicillin-G, 50 mg/ml streptomycin, 25 mg/ml sodium pyruvate (all from Sigma Chemical Co., Milwaukee, WI), and 5% fetal bovine serum (FBS: Hyclone Laboratories Inc., Logan, UT). After isolation, the complexes were washed twice in fresh WAY-FBS. For positive controls for cumulus expansion experiments, denuded mouse oocytes were used. OCC were isolated from the antral follicles of PMSG-treated mice and oocytes were denuded by repeated pipetting with a Pasteur pipette. Denuded oocytes were then washed twice in WAY-FBS.

2.2 Oocytectomy

Oocytectomy, a microsurgical technique, was used to remove the oocyte from isolated oocyte-cumulus complexes. The oocyte was microsurgically removed with the use of two Leitz micromanipulators and a Leitz Labovert inverted microscope using a technique described previously (Buccione et al., 1990; Vanderhyden et al., 1990). Each

intact complex was held with a holding pipette. A lancing pipette was then pushed through the entire complex and into the holding pipette. The lancing pipette was withdrawn, upon which the negative pressure within the holding pipette aspirated the oocyte, causing the zona pellucida to collapse. The complex was then released from the holding pipette, resuming its original three-dimensional conformation within 2-3 minutes (Figure 4).

2.3 Culture of OCC and OOX complexes

2.3.1 Cumulus Expansion

To assess the presence of CEEF in HEC media, OCCs and OOXs were cultured in 50 µl drops of frozen-thawed HTF (control) or HEC medium supplemented with 5% FBS and FSH (NIADDK-oFSH-19; 300 ng/ml) under washed mineral oil (Fisher Scientific, Fair Lawn, NJ) in 35 mm petri dishes (Falcon: Becton Dickinson and Company, Franklin Lakes, NJ). Since the embryos were cultured in only 20 µl, the HEC media samples were diluted 2.5-fold in HTF to generate the 50 µl drops. To confirm the ability of OOX complexes to expand in the presence of CEEF, some drops of OOX included 10 denuded mouse oocytes. The complexes were cultured for 16 h at 37°C in 5% CO_2: 5% O_2: 90% N_2 and then assessed for the degree of cumulus expansion using a subjective scoring system, described in 3.1, that ranges from 0 (no response) to +4 (maximal expansion) as described previously (Buccione et al., 1990) (Figure 4).

FIGURE 4: ***Oocytectomy and Cumulus Expansion***

Upper Panel: Oocytectomy is the microsurgical removal of the oocyte from a cumulus complex. Photograph **a** shows the holding pipette grasping the intact (OCC) complex. Arrowhead is pointing at germinal vesicle. The lancing pipette is then pierced through the entire complex into the holding pipette (**b**). The negative pressure within the holding pipette aspirates the oocyte, causing the complex to collapse (**c**). The oocytectomized complex resumes the original three-dimensional conformation in 2-3 minutes (**d**). Arrowhead is pointing at intact zona pellucida. (From Buccione et al., 1990)

Lower Panel: The assessment of cumulus expansion is done according to a subjective scoring system. Complexes with granulosa cell plating and no detectable response are given a score of 0 (**1a**). +1 indicates minimal expansion (**1b**). +2 indicates expansion of the outermost layer of the cumulus oophorus (**1c**). A score of +3 is given to those complexes with expansion of all cumulus layers except for the corona radiata (**1d**). Cumulus complexes with maximal expansion of the cumulus oophorus are given a score of +4 (**1e**). (From Vanderhyden et al., 1990)

2.3.2 Steroid Regulation

Since HEC media samples were frozen at the source, initial experiments were conducted to ensure retention of the ability of granulosa cells to produce progesterone in frozen-thawed media. Two OCCs or OOXs were cultured in 50 µl drops of HTF-FBS per well of 96-well plates (Costar: Corning Costar Corp., Cambridge, MA). In addition, limited volumes of HEC media (20 µl per embryo) were available; thus, the ability to detect differences between OCCs and OOXs needed to be established by determining appropriate culture conditions. To determine the minimum culture volume at which PIF activity could be detected, 2 OCCs or OOXs were cultured in a volume of 20 to 350 µl of HTF-FBS per well of 96-well plates. To determine the optimal number of complexes to be cultured in the appropriate volume of medium, a range of 1 to 10 OCCs and OOXs were cultured in 100 µl of HTF-FBS media. For all experiments measuring steroid production, complexes were cultured in the presence of FSH (150 ng/ml) and testosterone (Sigma; 500 nM) to promote the production of progesterone and estrogen. After 48 h, the culture media were collected and stored at –20°C until assayed for progesterone or estradiol using radioimmunoassays (RIA) (Daniel and Armstrong, 1984; Leung and Armstrong, 1979) (see section 3.2). In the initial experiments, the optimal culture conditions for the bioassay were determined to be the culture of 2 complexes in 100 µl of culture medium; therefore, these conditions were then used to bioassay the HEC media for progesterone inhibitory activity. Due to the inability to detect estradiol using these culture media conditions, culture media conditions were altered such that a combination of either HTF, WAY, or HTF/WAY (1:4) was employed to determine the optimal culture medium that could be used to detect granulosa cell estradiol production. Upon

establishment of the optimal culture medium, HEC medium was assayed for the presence of an estradiol-regulatory factor, using the same culture conditions as for the PIF bioassay, save culture medium. After the 48 culture, media were stored at –20°C until assayed for estradiol.

To assess the effects of HEC medium on progesterone production, as well as the effectiveness of HEC media following 5-fold dilution, 2 OOXs were added to either 100 µl of pooled HEC media (same patient) or 20 µl of pooled HEC media diluted to 100 µl with HTF. These media were supplemented with 5% FBS, 150 ng/ml FSH and 500 nM testosterone and the complexes were cultured for 48 h. The culture media were then stored at –20°C until assayed for progesterone. Progesterone accumulation in the HEC media were compared with progesterone production by 2 OOXs cultured in frozen-thawed HTF-FBS media under identical conditions, and the degree of HEC media-induced changes in progesterone production were compared to the ability of the oocytes/embryos that were cultured in that medium to be fertilized, to undergo cleavage, to develop into morphologically healthy embryos and to establish pregnancy.

3. Assays

3.1 Cumulus Expansion Assessment

For the assessment of cumulus expansion, a subjective scoring system was employed. Complexes with no detectable response were given a score of 0: the cumulus cells have plated down on the substratum and migrated away from the complex to form a monolayer. +1 indicates minimal expansion, characterized by the glistening appearance of cumulus cells: very few cumulus cells have plated down. A score of +2 was given to

complexes with expansion of the outer most layer of the cumulus oophorus, and a score of +3 to those complexes with expansion of all layers save the corona radiata. +4 indicates maximal expansion of all layers of cumulus cells surrounding the oocyte (Figure 4).

3.2 Steroid Radioimmunoassay

All media for steroid experiments were assayed for progesterone and estradiol-17β using RIAs that have been described and validated for direct measurements (Leung et al., 1979; Daniel et al., 1984). Progesterone and estradiol concentrations were measured using high specific activity tritiated hormones (progesterone 1, 2, 6, 7, 16, 17-^{3}H(N), and estradiol 1, 2, 6, 7-^{3}H(N): Dupont NEN, Dupont Co., Biotechnology Systems, Wilmington, DE) and goat anti-progesterone or anti-estradiol provided by David T. Armstrong's laboratory (Department of Obstetrics and Gynecology, University of Western Ontario, London, Ontario). Appropriate blanks were also included and subsequently subtracted from the measured values. The limit of detection for the progesterone assay was 12.5 pg and was 5 pg for the estradiol assay. Cross-reactivity of the progesterone antibody with pregnenolone is 0.13%, and with testosterone is 0.05%.

4. Genotyping of GDF-9 Mice

4.1 GDF-9 Knockout Mice

GDF-9 knockout mice were provided by Dr. Martin M. Matzuk (Baylor College of Medicine, Houston, Texas, USA). They were generated by a 1.1kb targeted deletion of exon 2, which encodes the majority of the propeptide sequence (174 amino acids) and

the mature portion of GDF-9 (134 amino acids), using embryonic stem (ES) cell technology (Dong et al., 1996). Heterozygous (+/-) female mice were viable and fertile and were intercrossed with homozygous (-/-) male mice to obtain the infertile homozygous (-/-) GDF-9 female mice. We received 600 ng of pGDF-9-202-1 plasmid, containing the GDF-9 5' external probe (270 bp) from Dr. Martin M. Matzuk, which was used to probe the Southern blots.

4.2 GDF-9 Colony Maintainance

The GDF-9 colony that was generated was housed in the University of Ottawa Animal Care Facility. New breeding pairs were established every six months. GDF-9 pups were weaned at 20 days of age, whereupon the females were tagged and their tails clipped for genotyping via Southern analysis. The males were disposed of until needed for establishing new breeding pairs, whereupon they were kept in separate cages, genotyped, and eventually paired with (+/-) females.

4.3 Southern Hybridization

4.3.1 Extraction and Isolation of DNA

GDF-9 mice were genotyped using Southern blot analysis of DNA derived from mouse tails (Sambrook et al., 1989). Mouse tails were digested overnight at 55°C in DNA extraction buffer containing proteinase K (500 μg/ml). DNA was purified the following day starting with the addition of NaCl, subsequent vortexing (15 sec.) and centrifugation at 3000 rpm for 10 min. The supernatant was transferred to fresh eppendorf tubes followed by the addition of isopropanol (100%) with subsequent mixing.

The samples were then spun at 14,000 rpm for 5 min. at 4°C. The supernatant was decanted and the pellets washed with 70% ethanol. The samples were spun again at 14,000 rpm for 10 min. at 4°C. The tubes were decanted and the pellet dried for 2-3 h. The pellets were then resuspended in 300 µl TE (Tris•Cl, EDTA) buffer and kept at 4°C overnight.

4.3.2 Digestion of DNA

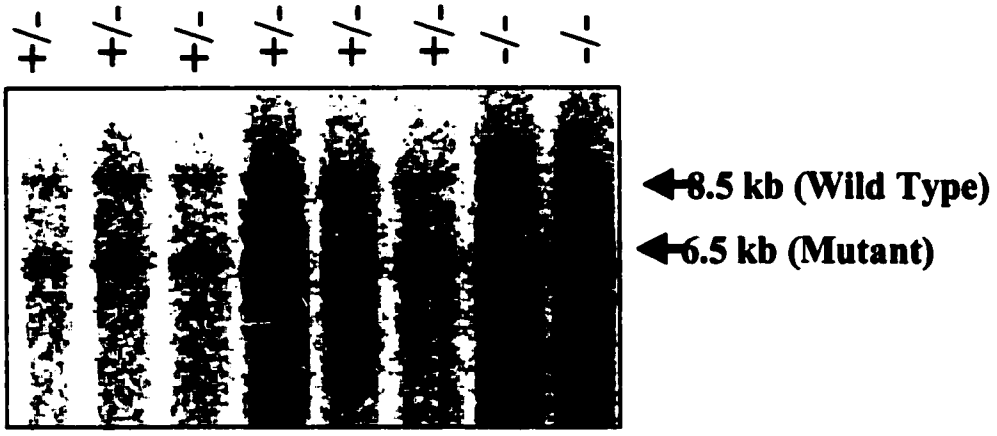
After 24 h dissolution of DNA in TE buffer, 60 µl of the total sample volume was added to fresh eppendorf tubes (Sambrook et al., 1989). 40 µl of a Master-Mix solution [*Eco*RV and *Bam*H1 (New England Biolabs Inc., Beverly, MA), Reaction Buffer 2, BSA (Bovine Serum Albumin), distilled water] was added to the sample volumes and incubated at 37°C overnight. The following day sodium acetate and cold ethanol (100%) were added to the DNA digests and placed on dry ice for 20 min. The samples were subsequently spun at 14,000 rpm for 20 min. at 4°C, after which the supernatant was discarded and the pellet dried for 1-2 h.

4.3.3 Gel Electrophoresis

The DNA pellets were resuspended in 20 µl TE buffer and the absorbance of the DNA was measured at 260 nm and 280 nm to ensure purity of the DNA (Sambrook et al., 1989). (The ratio of A_{260} to A_{280} should be greater than 1.75). Loading dye was added to all samples prior to overnight electrophoresis in 0.9% agarose gels at 23 V.

4.3.4 Gel Transfer and Hybridization

After overnight electrophoresis, the DNA was denatured in situ by washes in 0.25 N HCl (15 min.) and then 0.4 N NaOH (15 min.) (Sambrook et al., 1989). The gel was subsequently placed in a gel-transfer apparatus to be transferred for 3 h in 2X saline sodium citrate (SSC) to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL). The nylon membrane was then placed in a prehybridization solution [50% w/v formamide, 5X Denhardt's, 5X SSPE (NaCl, $NaH_2PO_4 \cdot H_20$, EDTA), 1% SDS (sodium dodecyl sulfate)] at 42°C overnight. The next day, DNA attached to the membrane was hybridized to a ^{32}P-radiolabelled 5' external DNA (GDF-9) probe (270 bp). The probe was prepared by the addition of 16 µl distilled water to 4 µl of DNA (5' external probe). This was boiled at 100°C for 10 min. and quick-cooled on ice before the addition of labelling mix (5 µl) and Klenow enzyme (2 µl). 5 µl ^{32}P-dCTP was subsequently added and the mixture placed at 37°C for 1 hr. The probe was purified of free nucleotides by centrifugation through Sephadex G50 fine spin-columns and denatured at 100°C for 4-5 min, quick-cooled, and immediately added to the prehybridization tube for overnight incubation at 42°C. The Southern blots were washed twice in 2X SSC for 15 min. Hybridization signals on the membranes were analyzed using a Molecular Dynamics Phophoimager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The presence of a single ~6.5 kb fragment in a lane indicated a homozyous mutant genotype. The presence of a single ~8.5 kb fragment in a lane indicated a wild type (+/+) genotype, and the heterozygote genotype was characterized by the presence of both alleles (Figure 5).


+/-
+/-
+/-
+/-
+/-
+/-
-/-
-/-
8.5 kb (Wild Type)
6.5 kb (Mutant)

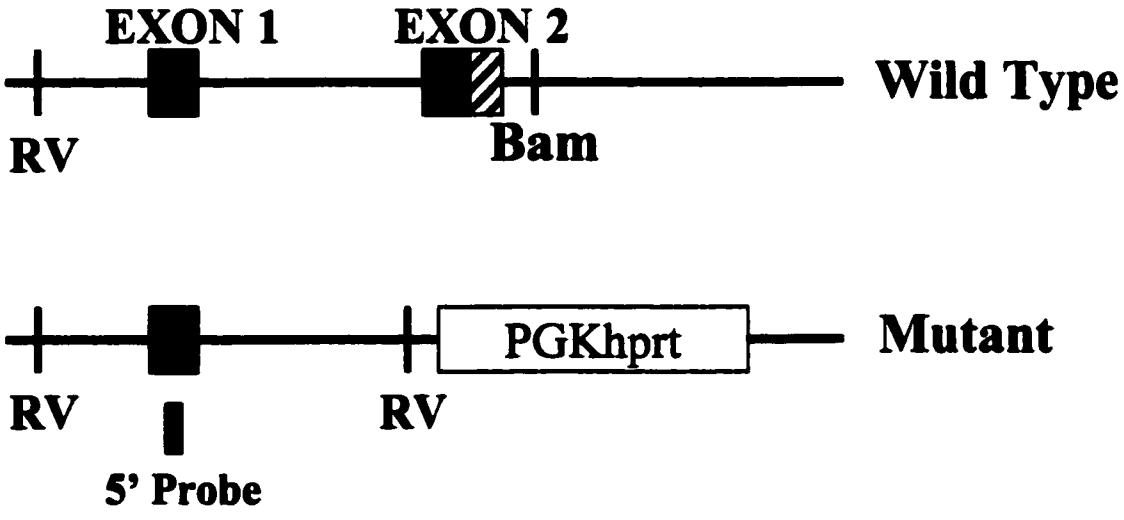

EXON 1
EXON 2
Wild Type
RV
Bam
PGKhprt
Mutant
RV
RV
5' Probe

FIGURE 5: ***GDF-9: Southern Hybridization***

Upper Diagram: The following Southern blot illustrates the banding pattern for heterozygous (+/-) and homozygous (-/-) GDF-9 mice, used for GDF-9 mice genotyping. Genomic DNA from mouse-tails was digested with *Eco*RV and *Bam*H1. A 5' external probe (270 bp) was used to probe GDF-9 Southern blots. The ~6.5 kb fragment indicates the GDF-9 mutant allele genotype, whereas the ~8-9 kb fragment corresponds to the GDF-9 wild-type allele (+/-). The heterozygous genotype is characterized by the presence of both alleles.

Lower Diagram: The 5' external probe recognizes both the wild-type allele and the mutant recombinant deleted allele, which lacks exon 2. **RV** = *Eco*RV, **Bam** = *Bam*H1 PGKhprt = deletion targeting vector

5. GDF-9 Experiments

5.1 Isolation and Culture of GDF-9 Oocytes

Upon identification of the genotype of each GDF-9 mouse, the animals, along with C57Bl/6 X Balb/C control mice, were each injected with 5IU PMSG 48 hours prior to the experiment to stimulate follicular development. On the day of the experiment the GDF-9 mice were sacrificed (cervical dislocation) and their ovaries surgically removed and placed in WAY medium supplemented with FBS (5%). GDF-9-deficient (-/-) ovaries were placed in WAY/FBS + collagenase (3 mg/ml) for ~45 min, with intermittent pipetting with a Pasteur pipette to denude the oocytes of granulosa cells. Control and +/- denuded oocytes were collected as described previously (section 2.1) Denuded oocytes were then washed thrice in WAY/FBS.

5.2 GDF-9 Oocyte Size and Morphology

Oocytes were cultured in WAY/FBS for 5-6 hours, whereupon the morphology of GDF-9 oocytes was assessed under a stereo microscope (Wild Heerbrugg: Wild Leitz Canada Ltd., Willowdale, Ontario). Oocytes were separated based upon meiotic competence (indicated by presence or absence of germinal vesicle), and photographs were taken of each oocyte group [heterozygous meiotically competent (MC), homozygous MC or homozygous meiotically incompetent (MI)] using a camera (Wild Leitz Canada Ltd., Willowdale, Ontario) attached to an inverted microscope (Leica DMIL: Leica Canada, Willowdale, Ontario). To measure the size of oocytes, a photograph of a hemocytometer (Brightline Counting Chamber: Hausser Scientific Co., Horsham, PA) was taken at the same magnification to be the reference measurement. The hemocytometer is divided into

squares that form a group of 25 squares, each of those squares being further subdivided into 16 smaller squares: dimensions of each of the smaller squares is 0.05 X 0.05 mm.

5.3 Cumulus Expansion

To assess the ability of GDF-9 deficient oocytes to enable cumulus expansion, 2 OOX complexes were cultured in the presence of either 25 heterozygous (+/-) or homozygous (-/-) meiotically competent (MC) or incompetent (MI) denuded oocytes. The OCC and OOX controls were included in these experiments. Cumulus complexes and denuded oocytes were cultured in 50 µl drops of WAY/FBS under washed oil in 35 mm petri dishes, as for HEC media cumulus expansion experiments. The complexes were cultured for 16 h at 37°C in 5% CO_2: 5% O_2: 90% N_2 and then assessed for the degree of cumulus expansion using a subjective scoring system that ranges from 0 (no response) to +4 (maximal expansion) as described in section 3.1.

5.4 Steroidogenesis

To assess the ability of GDF-9 deficient oocytes to regulate granulosa cell progesterone and estradiol production, 2 OOX complexes were cultured with either 75 heterozygous (+/-) or homozygous (-/-) meiotically competent or incompetent denuded oocytes. The OCC and OOX controls were included in these experiments. Denuded oocytes were cultured for 24 hrs in 100 µl WAY/FBS prior to the addition of cumulus complexes. Cumulus complexes were added to the 96-well culture plates that had been prepared 24 hrs earlier and were supplemented with FSH and testosterone for 48 hrs at

37°C in 5% CO_2: 5% O_2: 90% N_2 (Figure 6). The culture media were stored at –20°C until assayed for progesterone and estradiol-17β by RIA.

6. Statistical analysis

All experiments were performed at least three times, with different pools of ovaries and at least 2 replicates per pool. When comparing multiple groups, data were expressed as mean ± standard error of the mean, and statistical comparison was made using analysis of variance (ANOVA) with Newman-Keuls test for multiple comparisons. Statistical comparisons of two groups were made using unpaired, two-tailed *t*-tests for normal distributions and non-parametric, Mann-Whitney *t*-tests for non-Gaussian distributions (e.g., non-fertilized vs. fertilized, non-pregnancy vs. pregnancy). Statistical significance was inferred at $p < 0.05$.

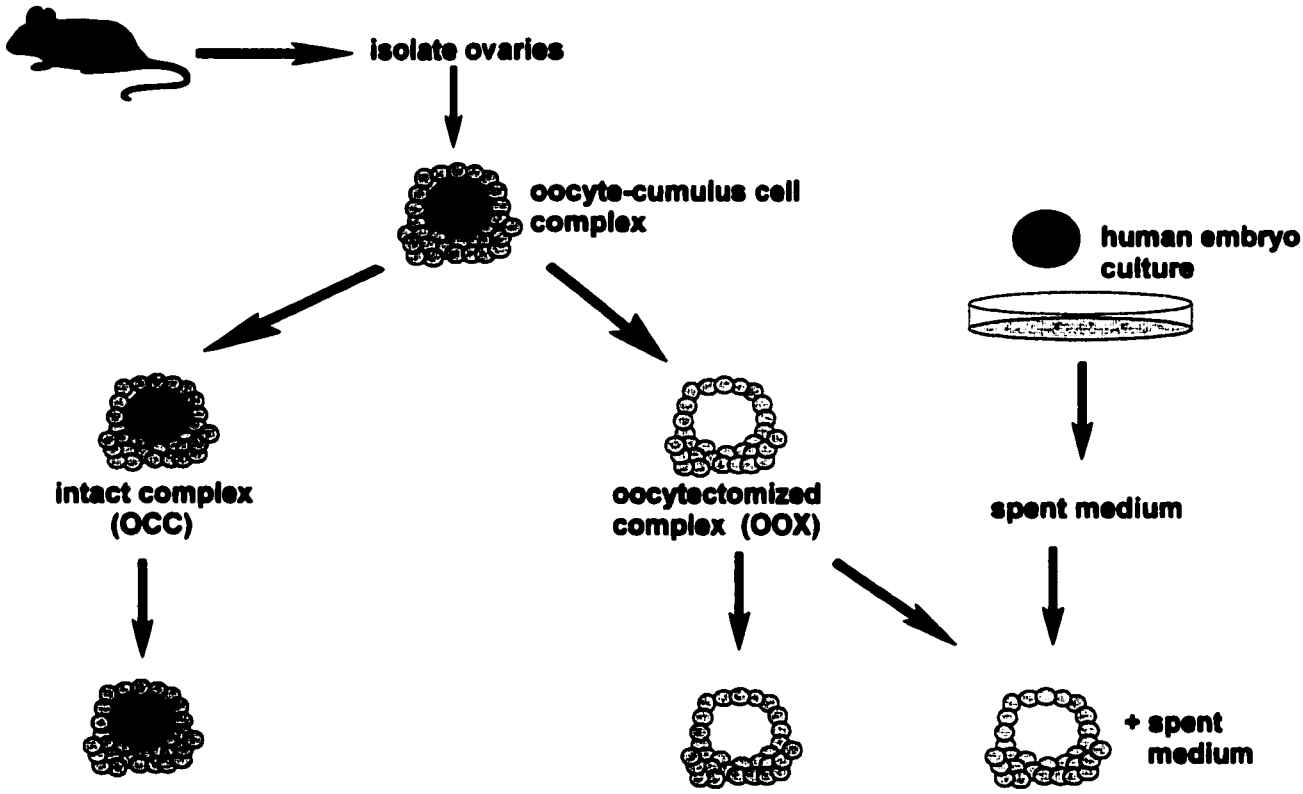

isolate ovaries
oocyte-cumulus cell complex
human embryo culture
intact complex (OCC)
oocytectomized complex (OOX)
spent medium
+ spent medium

FIGURE 6. ***Protocol for the Culture of Cumulus Complexes***

Mice were injected with PMSG 48 hrs prior to the experiment. Upon removal of ovaries, cumulus complexes were isolated for oocytectomy and for use as OCC controls. Cumulus complexes were cultured as controls or in specific treatment groups (e.g., HEC media, GDF-9 deficient (-/-) oocytes) for 16-48 hours with various substrates (e.g., FSH, Testosterone) or to determine the ability of cumulus cells to undergo expansion and to produce estradiol or progesterone.

RESULTS

The overall goal of this research project was to identify an oocyte/embryo-secreted factor that could be used in a bioassay to assess embryo health and developmental competence. The majority of the project dealt with the attempt to detect the presence of a steroid-regulatory factor in HEC media and to develop an experimental bioassay that would measure this factor's activity in HEC media. Upon measurement of progesterone-inhibitory factor (PIF) activity in all HEC media samples, an attempt was made to find a correlation between the PIF activity in each individual sample and the development and health of the respective embryo that had conditioned the same media sample.

The latter part of the project dealt with the newly discovered GDF-9 protein and its role in regulating granulosa cell differentiation. GDF-9 knockout mice were bred to harvest oocytes which were subsequently used to observe if GDF-9 plays a role in enabling expansion of cumulus granulosa cells and regulating granulosa cell progesterone and estradiol production. GDF-9 could potentially be used as a marker of oocyte and embryo health and developmental capacity.

1. Human Embryo-Conditioned (HEC) Media Samples

1.1 Assaying for Cumulus Expansion-Enabling Factor (CEEF)

To test for the presence of CEEF in HEC media, mouse OCCs and OOXs were stimulated with FSH in 50 μl drops of HTF or HEC (diluted 2.5 fold: 20 μl HEC media brought up to 50 μl with HTF) media (Table 1). As expected, OCCs underwent cumulus expansion to near maximal levels (3.5 ± 0.1), whereas OOXs cultured in control HTF-

FBS did not expand. Although OOXs could expand in the presence of denuded oocytes (2.2 ± 0.3), HEC media failed to support expansion of the OOX complexes. With this inability to detect CEEF in HEC media under these conditions, further experiments were conducted on human oocyte-conditioned media (OCM). To test for the presence of CEEF in OCM, the same culture conditions were used as for HEC media. Cumulus expansion was observed only for OCCs (3.4 ± 0.1) and OOXs cultured with denuded oocytes (3.2 ± 0.2); however, OCM failed to enable the expansion of cumulus complexes (Table 2). Due to the inability to detect CEEF in either HEC media or OCM, the remaining experiments focussed on the detection of PIF and estradiol-regulatory factor in the HEC media.

TABLE 1: Cumulus expansion of FSH-stimulated OCC and OOX complexes cultured in the absence or presence of human embryo-conditioned (HEC) media

TREATMENT GROUP	NUMBER OF COMPLEXES ASSESSED	NUMBER OF HEC MEDIA SAMPLES	DEGREE OF EXPANSION (MEAN ± SEM)
OCC	50	0	3.5 ± 0.1
OOX	50	0	0
OOX + DO	25	0	2.2 ± 0.3
OOX + HEC	65	13	0

OCC and OOX were cultured in 50 µl drops of frozen-thawed HTF (control) or HEC medium supplemented with 5% FBS and FSH (300 ng/ml) for 16 h at 37°C in 5% CO_2: 5% O_2: 90% N_2. HEC media samples were diluted 2.5-fold in HTF to generate the 50 µl drops. Cumulus expansion was only observed for OCC complexes and OOX complexes cultured with denuded oocytes (DO). Experiments were done three times, at least in duplicate. Values represent the mean ± SEM of the three experiments.

TABLE 2: Cumulus expansion of FSH-stimulated OCC and OOX complexes cultured in the absence or presence of human oocyte-conditioned media (OCM)

TREATMENT GROUP	NUMBER OF COMPLEXES ASSESSED	NUMBER OF HEC MEDIA SAMPLES	DEGREE OF EXPANSION (MEAN ± SEM)
OCC	45	0	3.4 ± 0.1
OOX	45	0	0
OOX + DO	45	0	3.2 ± 0.2
OOX + OCM	45	13	0

OCC and OOX were cultured in 50 µl drops of frozen-thawed HTF (control) or OCM medium supplemented with 5% FBS and FSH (300 ng/ml) for 16 h at 37°C in 5% CO_2: 5% O_2: 90% N_2. OCM media samples were diluted 2.5-fold in HTF to generate the 50 µl drops. Cumulus expansion was only observed for OCC complexes and OOX complexes cultured with denuded oocytes (DO). Values represent the mean ± SEM of the three experiments.

1.2 Establishment of Experimental Bioassay

1.2.1 Potential Limitations

1.2.1.1 Fresh Versus Freeze-Thawed Media

The first potential limitation in testing the human embryo-conditioned media was the necessity to freeze the HEC, which could impact on the ability of the media to support steroidogenesis. OCCs produced significantly less progesterone compared to OOX complexes for both media groups (Figure 7). There was no significant difference in progesterone production between the fresh and frozen-thawed media groups. Therefore, freezing the HTF medium did not impair the medium's ability to successfully support the steroidogenesis of murine cumulus complexes.

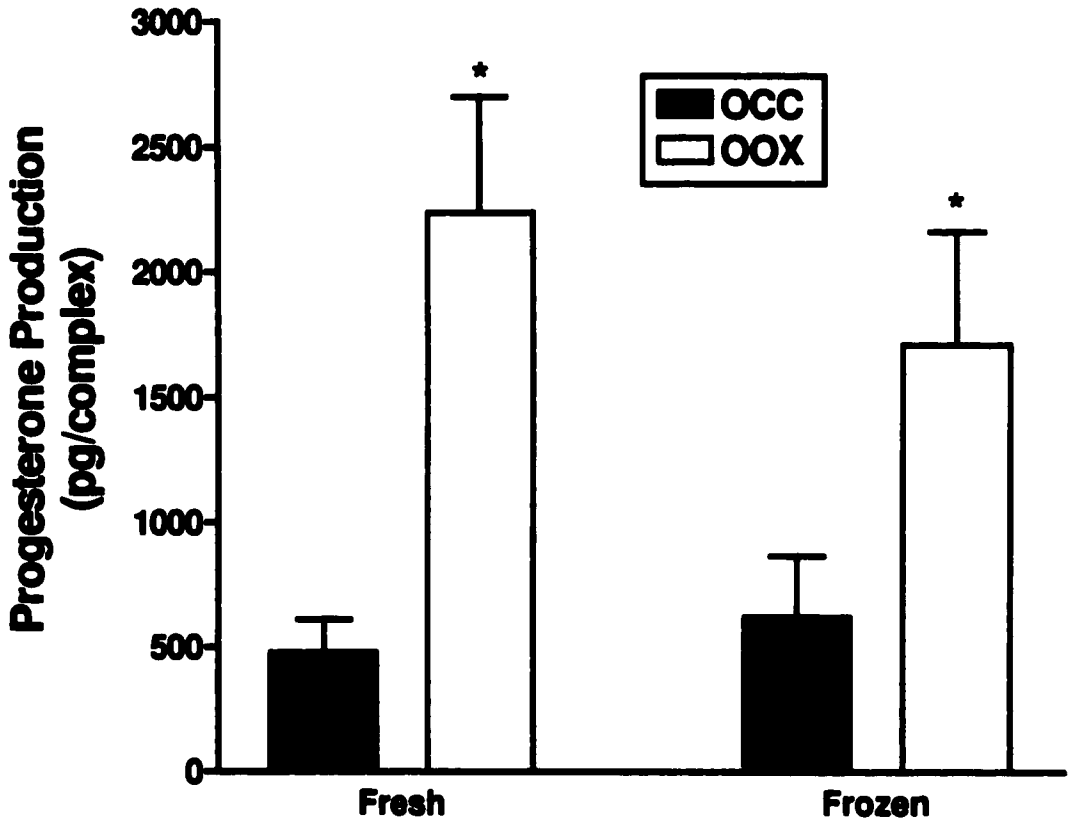

Progesterone Production
(pg/complex)
3000
2500
2000
1500
1000
500
0
OCC
OOX
*
*
Fresh
Frozen

FIGURE 7: ***Fresh versus frozen-thawed media***

Progesterone production by mouse OCC and OOX complexes cultured in fresh and frozen-thawed HTF media. Complexes were cultured in the presence of 5% FBS, 150 ng/ml FSH and 500 nM testosterone for 48 h. Values are mean ± SEM for 3 experiments. *Significantly different ($p<0.05$) than OCC.

1.2.1.2 Culture Volume and Cumulus Complex Number

The second potential limitation involved in the assessment of the HEC media was culture volume. Due to the small volumes of HEC media (20 μl) used to culture each embryo, it was necessary to determine the minimal culture volume that could be used to avoid significant dilution of the HEC, and without effecting the health, and as a consequence, steroidogenic ability of OCCs and OOXs. In order to identify the minimum culture volume, 2 OCCs or OOXs per well were cultured in a range of volumes from 20 μl to 350 μl HTF media for 48 h (Figure 8). Concurrent with the increase in culture volume was a dramatic (5-fold) increase ($p<0.05$) in FSH + testosterone-stimulated progesterone production by both OCCs and OOXs (OCC: from 1.7 ± 0.3 to 8.3 ± 1.1 ng/complex; OOX: from 5.3 ± 1.2 to 26.3 ± 6.7 ng/complex). However, for each culture volume, OOXs produced significantly greater amounts of progesterone than OCCs. Since cultures in 100 μl generated a large difference between OCCs and OOXs in their progesterone production with a relatively low level of variability, this volume was determined to be the optimal culture volume.

Upon finding the ideal volume to culture cumulus complexes, it was necessary to determine the optimal number of complexes that could be cultured in the same volume. To establish the optimal number of cumulus complexes to use in the bioassay to test HEC media, 1 to 10 OCCs or OOXs were cultured in 100 μl of frozen-thawed HTF media for 48 h (Figure 9). FSH- and testosterone-stimulated progesterone production for OCCs and OOXs dramatically decreased as the number of complexes per well increased; however, the production of progesterone by OOX was significantly greater ($p<0.05$) than OCC with all complex numbers. The culture of 2 complexes in 100 μl culture media yielded a

significant difference between FSH- and testosterone-stimulated progesterone production of OOXs and OCCs with relatively low variability; therefore, the bioassay was established as the culture of 2 complexes per 100 μl medium.

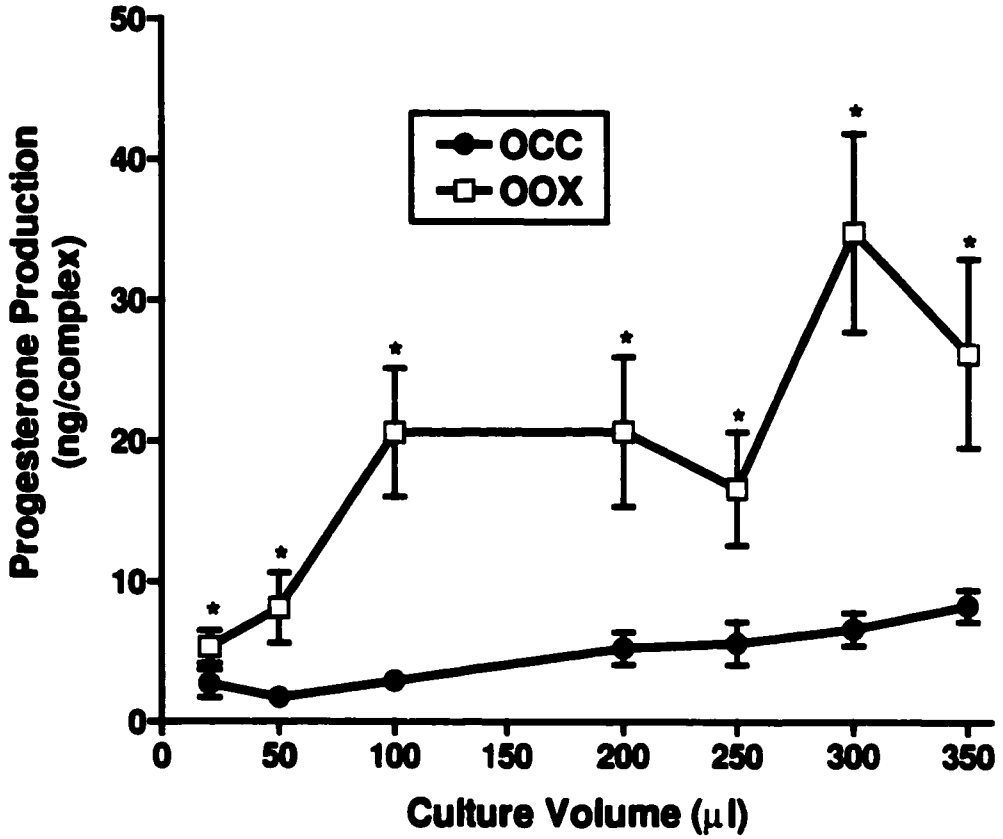

Progesterone Production
(ng/complex)
50
40
30
20
10
0
OCC
OOX
*
0
50
100
150
200
250
300
350
Culture Volume (µl)

FIGURE 8: ***Progesterone Production for a Range of Culture Volumes***

Progesterone production by OCC and OOX complexes cultured in a range of culture volumes from 20 μl to 350 μl. Cells were cultured for 48 h in the presence of FSH (150 ng/ml) and testosterone (500 nM). Each value is the mean ± SEM of 3 experiments. *Significantly different ($p<0.05$) than OCC in the same volume.

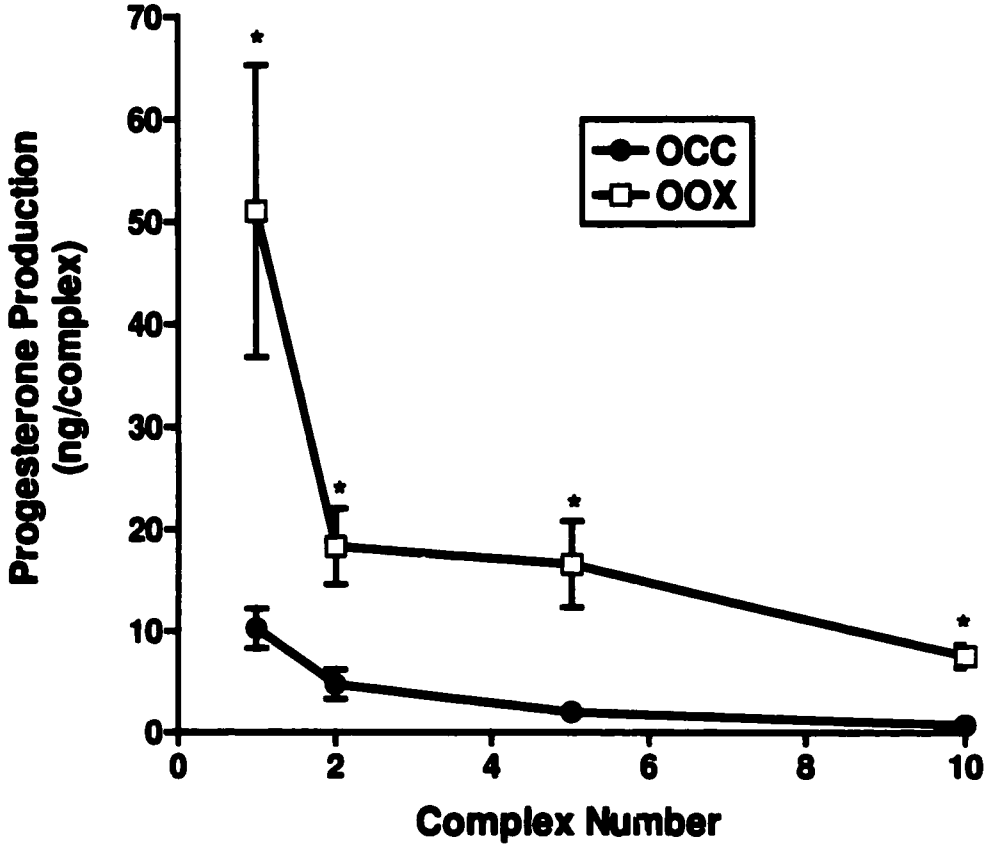

Progesterone Production
(ng/complex)
70
60
50
40
30
20
10
0
0
2
4
6
8
10
Complex Number
OCC
OOX
*
*
*
*

FIGURE 9: ***Progesterone Production for a Range of Cumulus Complex Number***

Accumulation of progesterone by varying numbers of OCC and OOX complexes. Complexes were stimulated with 150 ng/ml FSH and 500 nM testosterone for 48 h. Values are mean ± SEM for a minimum of 3 experiments. *Significantly different ($p<0.001$) than OCC complexes with same number of cumulus complexes.

1.3 Establishing Culture Conditions to Detect Estradiol Regulatory Factor in HEC Media Samples

Since the bioassay established for the detection and measurement of PIF in HEC media was insufficient for detecting an estradiol regulatory factor in HEC media samples (data not shown), an attempt was made to alter the culture media conditions in order to make the culture environment more conducive to the production of estradiol by granulosa cells. Three different media were prepared [HTF, WAY, and HTF/WAY (1:4)] and the steroid experiments were repeated as per the progesterone complex number protocol to test the optimal conditions for granulosa cell estradiol production, and subsequent detection (Figure 10). HTF/WAY medium supported the greatest amount of estradiol production ($p<0.05$). For all types of media, OCC complexes produced significantly greater estradiol than OOX complexes for each complex number group, with estradiol production decreasing with an increase in the number of complexes cultured per well ($p<0.05$). Production of estradiol by OCC complexes only is indicative of the secretion of an estradiol stimulatory factor (ESF) by the oocyte.

Using the HTF/WAY medium, an estradiol-specific bioassay was performed on HEC samples to detect granulosa cell estradiol production. Estradiol production was only observed for OCC complexes (255 ± 42 pg/complex) (Figure 11). Culture of OOX complexes with denuded oocytes failed to stimulate estradiol production, remaining at the same basal levels as for the OOX complex control. OOX complexes cultured with HEC media and HEC media with denuded oocytes also produced basal levels of estradiol. The inability to measure an estradiol-regulatory factor in HEC media with the established bioassay led to the focus of all remaining HEC experiments on PIF detection.

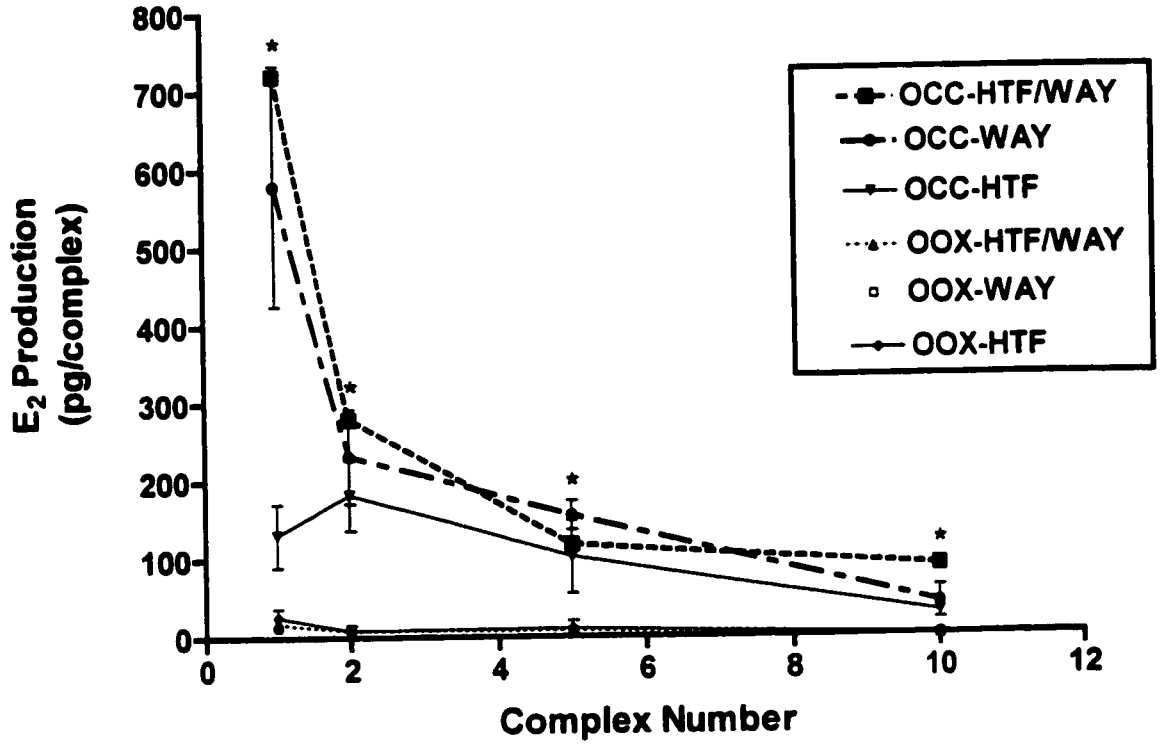

800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
E2 Production (pg/complex)
Complex Number
OCC-HTF/WAY
OCC-WAY
OCC-HTF
OOX-HTF/WAY
OOX-WAY
OOX-HTF

FIGURE 10: ***Establishing Optimal Media Conditions to Detect Granulosa Cell Estradiol Production***

In order to determine which medium would allow for the optimal production and detection of estradiol, three different media were selected (HTF, WAY, and HTF/WAY): steroid experiments were repeated in the same manner as for progesterone production, supplemented with FSH and testosterone, and cultured for 48 hours at 37°C. Values represent mean ± SEM, with the experiment being repeated three times (n=3). *Significantly different ($p<0.05$) than OOX complexes for same medium.

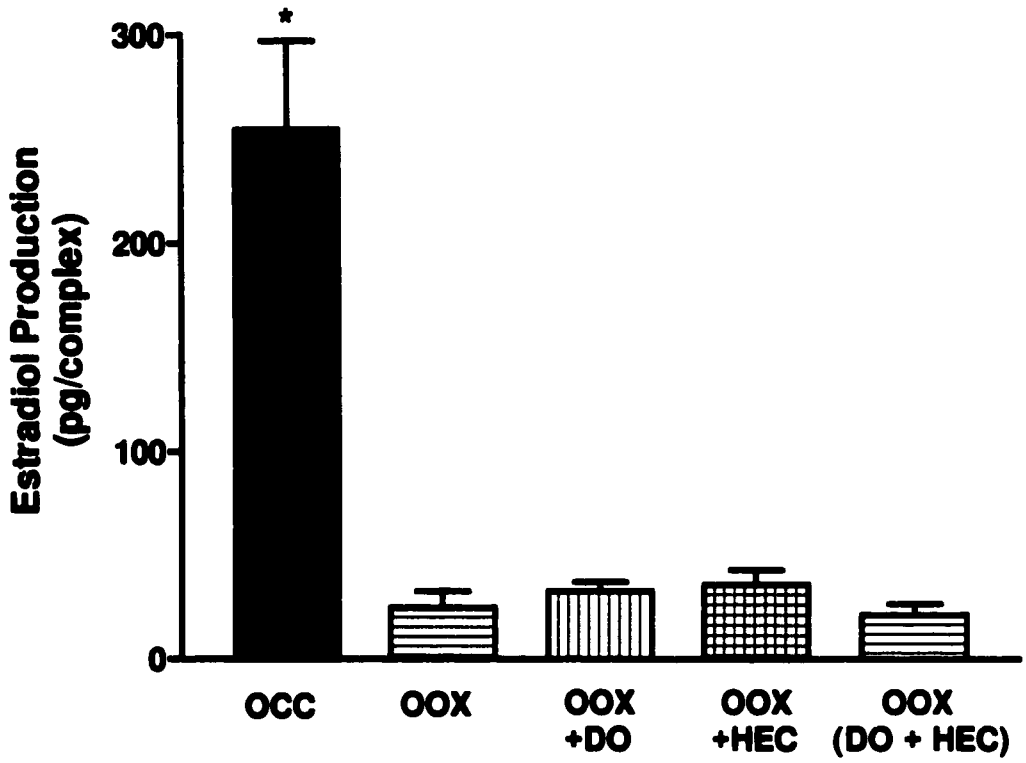

*
300
200
100
0
Estradiol Production
(pg/complex)
OCC
OOX
OOX
+DO
OOX
+HEC
OOX
(DO + HEC)

FIGURE 11: ***Effect of HEC Media on Granulosa Cell Estradiol Production***

Estradiol production by OCC or OOX complexes cultured alone or in the presence of HEC media or denuded mouse oocytes, supplemented with FSH and testosterone and cultured for 48 hours at 37°C. Values are presented as mean ± SEM where each data point was derived from 3 individual experiments (n=3), performed in duplicate. *Significantly different ($p<0.05$) than OOX complexes.

1.4 Correlating PIF Activity to the Health and Developmental Potential of Human Embryos

1.4.1 Dilution of HEC Media Samples

To test the effects of dilution of HEC media on murine cumulus cell progesterone production, 2 OOXs were cultured in a total volume (100 μl) of either pooled (same patient) or diluted (20 μl of the same pooled samples diluted to 100 μl with HTF media) HEC media for 48 h. Control OOXs, cultured in 100 μl HTF, produced significantly more progesterone per complex than OCCs, which were 20% of OOX control. For three patients (A, B, C) HEC media inhibited progesterone production from 22% to 99%, with pooled HEC media containing greater inhibitory activity than diluted HEC media (Figure 12). The results suggested that human embryos secreted a factor that inhibits progesterone production. With the observation of variable, and measurable, steroid-regulatory activity exerted by diluted HEC media upon murine OOXs, the remaining HEC media samples were tested for their ability to regulate progesterone production by murine OOXs.

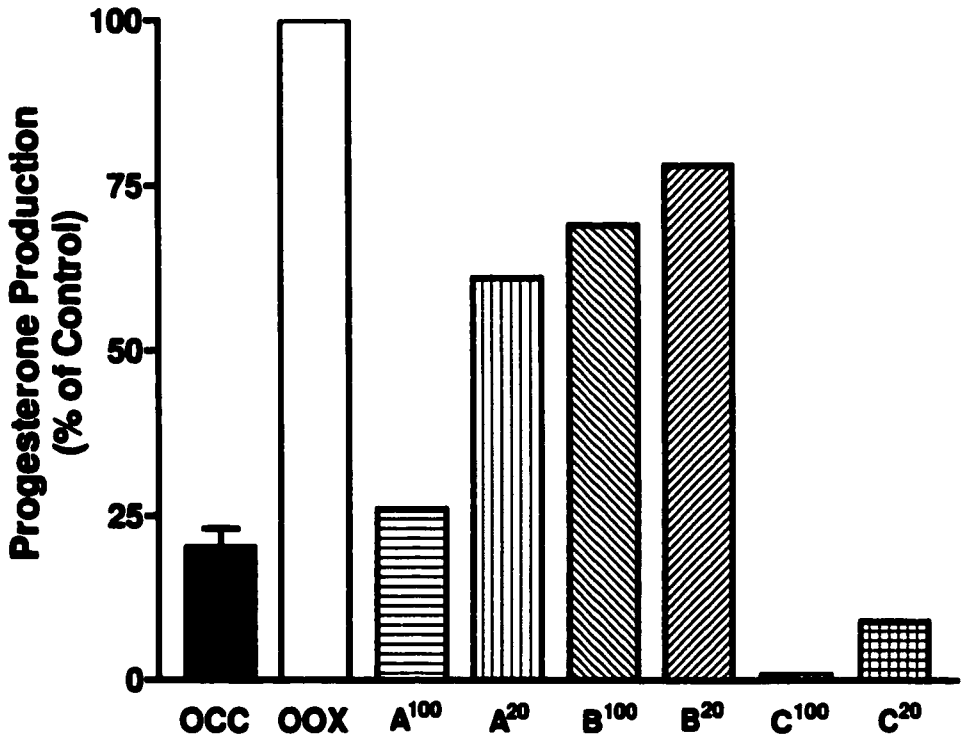

Progesterone Production
(% of Control)
100
75
50
25
0
OCC
OOX
A100
A20
B100
B20
C100
C20

FIGURE 12: ***Dilution of HEC Media Samples***

Progesterone production by OOX complexes cultured in 100 μl pooled HEC (e.g. A^{100}) media or in 20 μl of the pooled HEC media diluted to 100 μl (eg. A^{20}). HEC media were obtained from 3 different patients (A, B and C). OCC and OOX cultured in HTF were used as controls. Cells were cultured for 48 h, supplemented with 5% FBS, 150 ng/ml FSH and 500 nM testosterone. Values represent data from one experiment.

1.4.2 Correlating PIF Activity to Embryo Score

There was tremendous variability in progesterone regulatory activity among HEC media samples (n=91), with the effects ranging from stimulation (182% of progesterone production by OOX complexes in control medium) to the greatest inhibition at 11% of control. However, the vast majority (96%) of samples elicited some degree of inhibition, whereas only 4 samples showed stimulation. No correlation ($r = -0.013$) was found between the level of progesterone production by OOXs in HEC media from cultured embryos and the cumulative embryo score for the embryos cultured in that media (Figure 13). The embryo scores, derived from assessment of embryo morphology and growth, ranged from a minimum score of 1 to a maximum of 20. The degree of variability for progesterone inhibition by HEC media was similar for all embryo scores, suggesting that PIF activity does not change with improved embryo quality. A comparison of embryo morphology or cell number independently with PIF activity found no correlation, indicating that PIF activity is not influenced by embryo morphology or growth (data not shown).

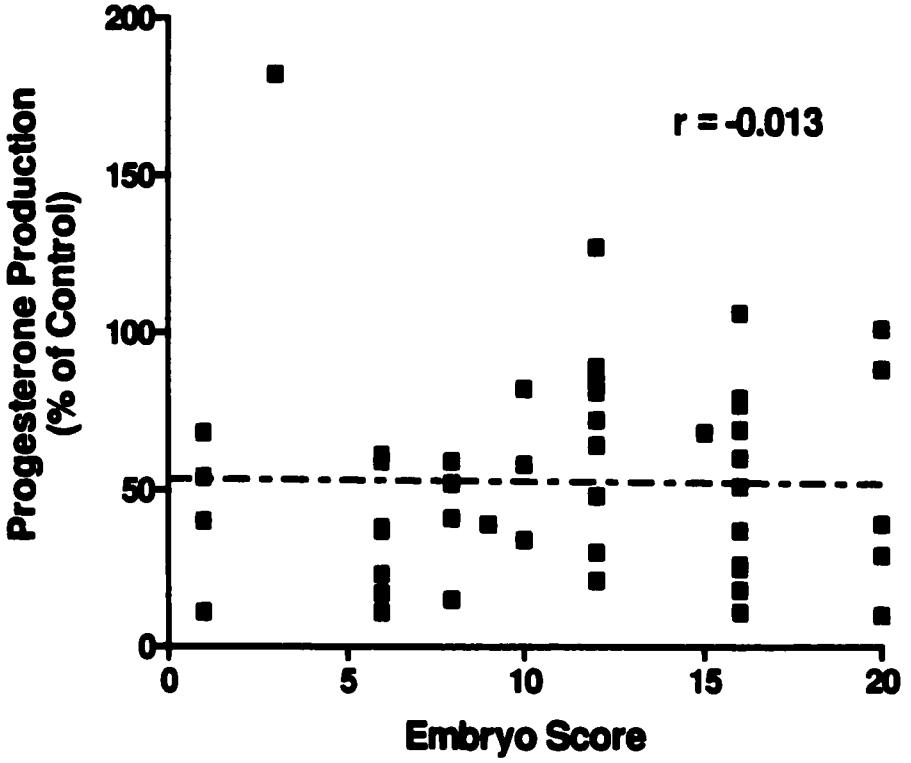

Progesterone Production
(% of Control)
200
150
100
50
0
r = -0.013
0
5
10
15
20
Embryo Score

FIGURE 13: ***Correlating PIF Activity to Embryo Score***

PIF (progesterone-inhibitory factor) activity in HEC media of fertilized embryos assessed as a percentage of progesterone production relative to control (OOX in HTF medium). OOX complexes were cultured in the presence of FBS, FSH and testosterone for 48 h. Progesterone production by individual HEC media samples are plotted with respect to cumulative score of that embryo. The line indicates linear regression analysis (r=-0.013).

1.4.3 Correlating PIF Activity to Fertilization Outcome and Pregnancy Outcome

Figure 14 shows the progesterone production (% of control) of OOX complexes cultured in HEC media grouped according to the absence or presence of fertilization of the oocytes. Using a non-parametric (Mann-Whitney) *t*-test, no significant difference was found between the ability of media from non-fertilized (n=44) and fertilized oocytes (n=47) to inhibit cumulus cell progesterone production (median: 39.5% and 51%, respectively). Of the total number of embryos that were transferred (n=37), 8 resulted in pregnancy. A comparison of HEC media samples from embryos that did not result in pregnancy to those that did revealed no significant difference in their ability to inhibit murine cumulus cell progesterone production (median: 58% and 35.5%, respectively; Figure 15).

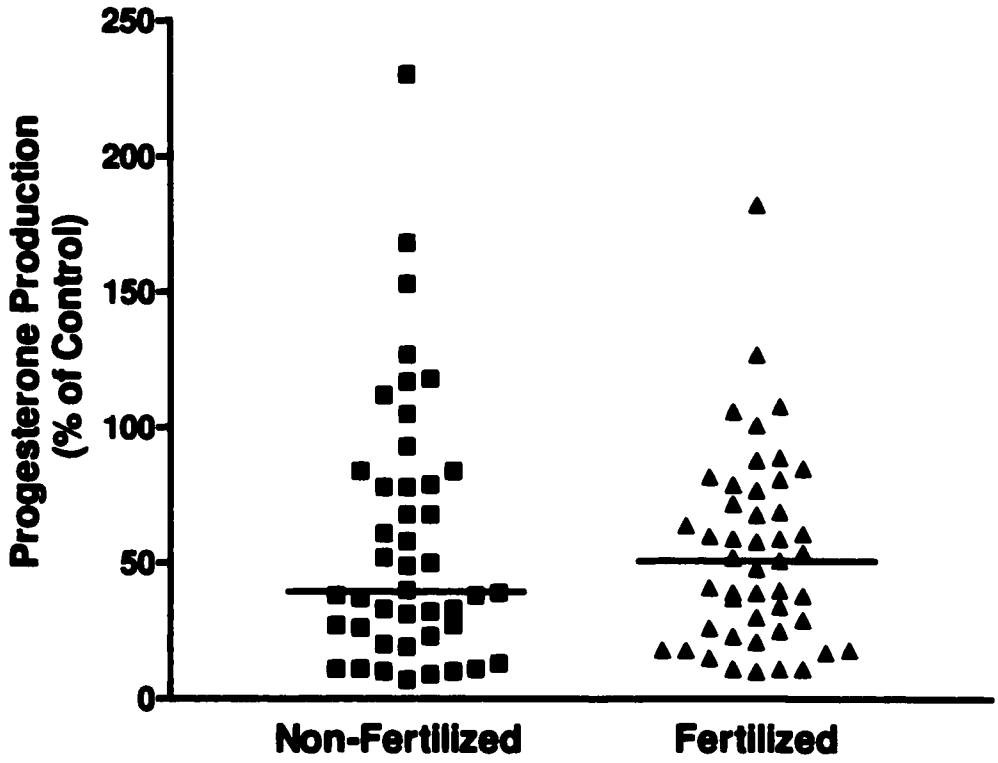

Progesterone Production
(% of Control)
250
200
150
100
50
0
Non-Fertilized
Fertilized

FIGURE 14: ***Correlating PIF Activity to Fertilization Outcome***

Progesterone production, as a percentage of control (OOX in HTF medium), by OOX cultured in HEC media from non-fertilized and fertilized oocytes. Complexes were treated with 150 ng/ml FSH and 500 nM testosterone for 48 h. Each data point is the accumulation of progesterone by OOX complexes cultured in HEC media samples from individually-cultured embryos. The lines represent the median of all values in that group.

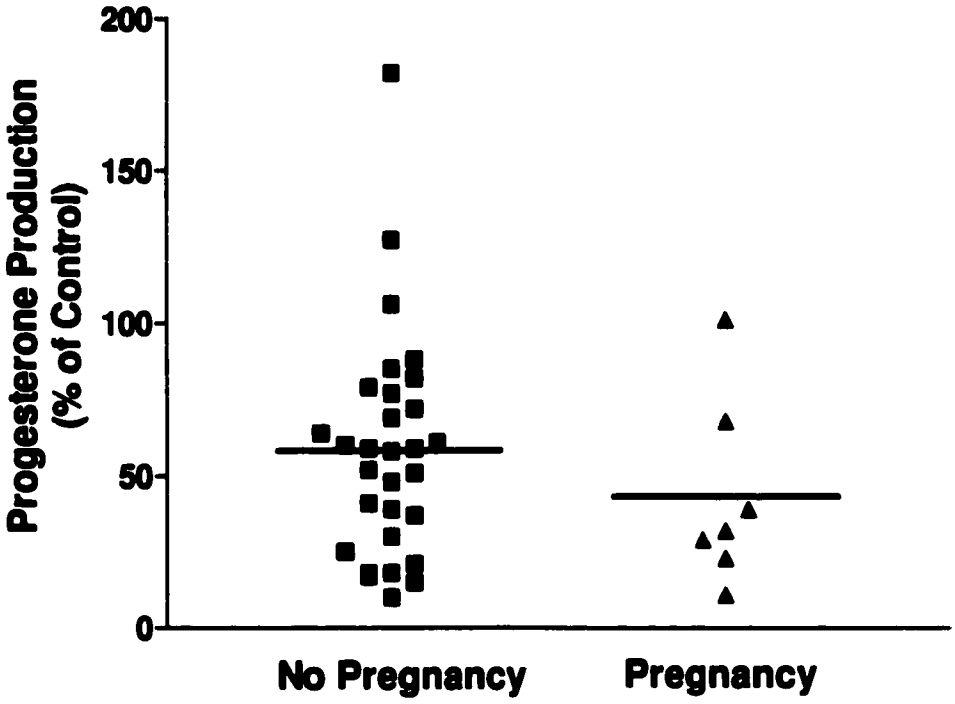

Progesterone Production
(% of Control)
200
150
100
50
0
No Pregnancy
Pregnancy

FIGURE 15: ***Correlating PIF Activity to Pregnancy Outcome***

Comparison of progesterone production by OOX complexes cultured in HEC media samples from embryos that resulted in no pregnancy or pregnancy. Complexes were stimulated with FSH (150 ng/ml) and testosterone (500 nM) for 48 h. Each data point is a measurement of progesterone produced by OOX complexes cultured in HEC media samples from individually-cultured embryos. The lines represent the median of all values in that group.

2. Effect of GDF-9 on Granulosa Cell Differentiation

In order to assess the role of GDF-9 in follicular development and function, GDF-9 knockout mice were engineered (Dong et al., 1996). With the absence of GDF-9 from oocytes, and their use in cumulus complex culture, the role of GDF-9 in granulosa cell function could be readily examined.

2.1 GDF-9 Oocyte Morphology and Size

Although (-/-) GDF-9 mice were primed with PMSG 48 hrs prior to the experiment, their ovaries failed to respond to superovulation treatment. Upon further inspection, homozygous ovaries were found to be dramatically smaller in comparison to wild-type and heterozygous ovaries, and were embedded in large quantities of adipose tissue (data not shown). Following a period of culture, the majority of the heterozygous oocytes had matured, characterized by the disappearance of the germinal vesicle during a 5 hr incubation. However, approximately half of the homozygous oocytes were MI. In addition, many of the oocytes from the homozygous ovaries were deformed in appearance: zona pellucidae were non-uniform in thickness, oocytes looked darker and more granular, and oocytes were oblong shaped as opposed to spherical.

Photographs were taken for morphological comparison of size upon isolation of oocytes from heterozygous and homozygous animals (Figure 16). In terms of oocyte size, heterozygous MC oocytes (74 ± 1 μm) were significantly smaller in diameter than homozygous oocytes (80 ± 1 μm); $p<0.05$ (Figure 17). Homozygous MI oocytes were significantly smaller (63 ± 2 μm) than both heterozygous and homozygous MC oocytes

($p < 0.05$). These observations suggest that GDF-9 is involved in oocyte growth: absence of GDF-9 in MC oocytes supports an increase in oocyte size.

FIGURE 16: ***Photographs of GDF-9 Heterozygous and Homozygous Oocytes***

Photographs were taken of GDF-9 meiotically competent (MC) heterozygous oocytes (**A**) and GDF-9 MC (**B**) and incompetent (MI) (**C**) homozygous oocytes (-/-) using a Leitz inverted microscope at a magnification of 50X. MC heterozygous oocytes were smaller (74 ± 1 μm) in size than MC homozygous oocytes (80 ± 1 μm) , while MI homozygous oocytes were smaller (63 ± 2 μm)than both MC heterozygous and homozygous oocytes. Greater numbers of abnormal oocytes were observed for GDF-9 homozygous animals. Also, the majority of GDF-9 heterozygous oocytes were MC, whereas approximately half of all GDF-9 oocytes were MC.

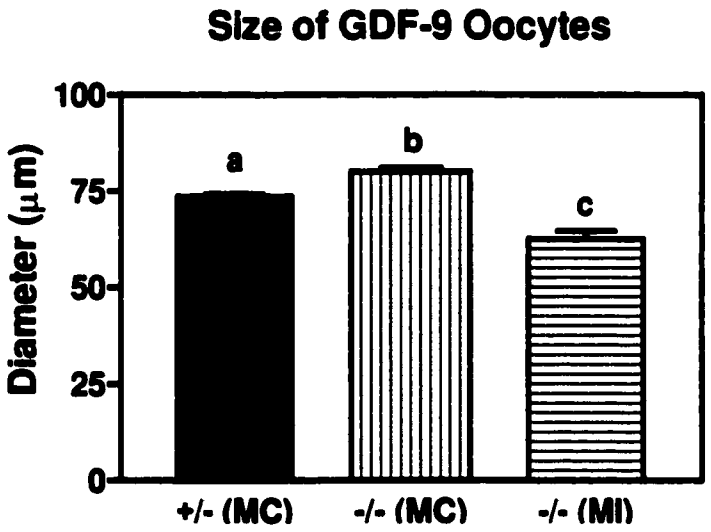

Size of GDF-9 Oocytes
Diameter (μm)
100
75
50
25
0
a
b
c
+/- (MC)
-/- (MC)
-/- (MI)

FIGURE 17: ***GDF-9 Oocyte Size Relative to Genotype***

The growth of GDF-9 heterozygous and homozygous oocytes was compared according to oocyte size (diameter). Heterozygous meiotically competent (MC) oocytes (N=43) were significantly smaller in size (74 ± 1 μm) than homozygous MC oocytes (N=20; 80 ± 1 μm). Homozygous meiotically incompetent (MI) oocytes (N=46) were significantly smaller (63 ± 2 μm) than both heterozygous and homozygous MC oocytes ($p < 0.05$). Complexes were cultured in WAY/FBS and supplemented with FSH and testosterone.

2.2 Cumulus Expansion

2.2.1 Ability of GDF-9 Deficient Oocytes to Enable Cumulus Expansion of OOX complexes

To determine if GDF-9 is CEEF, enabling the expansion of cumulus granulosa cells, GDF-9-deficient oocytes were co-cultured with OOX complexes. The appropriate controls of OCC, OOX, and OOX complexes co-cultured with GDF-9 heterozygous oocytes were included in the experiment (Table 3). OCC complexes expanded (3.3 ± 0.3), whereas no expansion was observed for OOX complexes cultured alone. GDF-9 heterozygous oocytes enabled the expansion of OOX complexes to near maximal levels (3.3 ± 0.2), whereas OOX complexes cultured with meiotically competent and incompetent GDF-9-deficient oocytes did not expand.

TABLE 3: Effect of GDF-9 Denuded Oocytes (+/-, -/-) on OOX Cumulus Expansion

CONTROL/ TREATMENT GROUP	NUMBER OF OOCYTES USED FOR CONDITIONING	NUMBER OF COMPLEXES ASSESSED	DEGREE OF EXPANSION
OCC	0	15	3.3 ± 0.3
OOX	0	15	0
OOX + GDF-$9^{+/-}$ MC	25	20	3.3 ± 0.2
OOX + GDF-$9^{-/-}$ MC	25	20	0
OOX + GDF-$9^{-/-}$ MI	25	25	0

OOX complexes were cultured in the presence of GDF-9 heterozygous and homozygous oocytes. Control and treatment groups were cultured in 50 µl WAY/FBS under washed mineral oil for 16 hours at 37°C in an atmosphere of 5% CO_2: 5% O_2: 90% N_2: cultures were supplemented with FSH (300 ng/ml). Cumulus expansion was observed only for OCC complexes and OOX complexes cultured with heterozygous oocytes. Values represent the mean ± SEM of the three experiments.

2.3 Steroidogenesis

2.3.1 Ability of GDF-9 Deficient Oocytes to Regulate Granulosa Cell Steroidogenesis

To investigate the role of GDF-9 in regulating granulosa cell steroid production, GDF-9-deficient MC and MI oocytes were cultured in the presence of OOX complexes. Steroid experiments included the control cultures of OCC, OOX, and GDF-9 heterozygous oocytes co-cultured with OOX complexes.

2.3.1.1 Regulation of Progesterone Production

OCC complexes produced progesterone at 12 ± 1.6% of OOX control, indicative of the secretion of a progesterone-inhibitory factor by the oocyte (Figure 18). Similar levels ($p>0.05$) of progesterone production were observed for OOX complexes co-cultured with (C57Black/6 X Balb/C)F1 oocytes (27 ± 1%) and GDF-9 wild type (+/+) oocytes (41%). Significantly greater progesterone production ($p < 0.05$) was observed for OOX complexes co-cultured with GDF-9 heterozygous MC oocytes (70 ± 15.5%) and GDF-9 deficient (-/-) MC (94 ± 16.4%) and MI (84 ± 8%) oocytes. These results indicate a progesterone-inhibitory effect of GDF-9, and suggest that GDF-9 plays an integral role in the regulation of granulosa cell progesterone production.

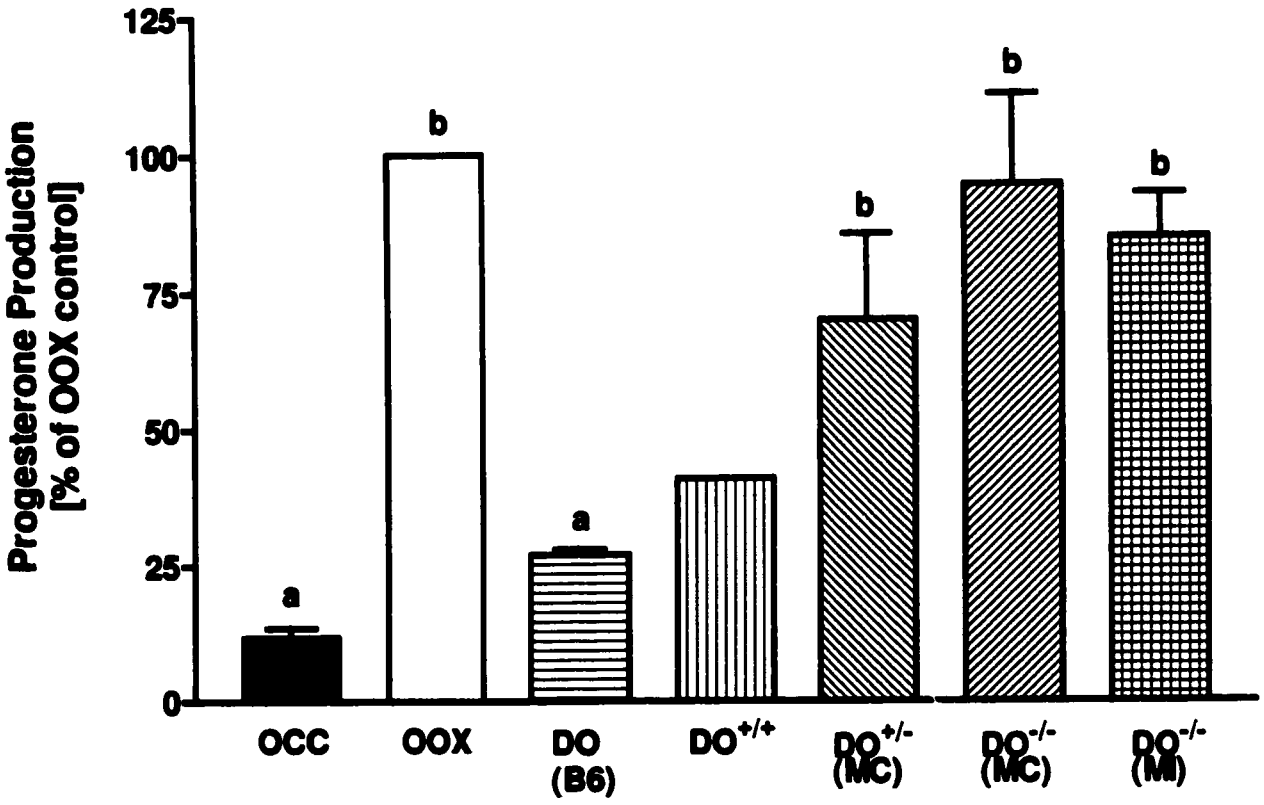

Progesterone Production
[% of OOX control]
125
100
75
50
25
0
a
b
a
b
b
b
OCC
OOX
DO
(B6)
DO+/+
DO+/-
(MC)
DO-/-
(MC)
DO-/-
(MI)

FIGURE 18: ***Ability of GDF-9 Deficient Oocytes to Regulate Progesterone Production***

GDF-9 (+/+, +/-, -/-) oocytes were co-cultured with OOX complexes for 48 hours in the presence of FSH and testosterone. Control OCC complexes produced significantly less progesterone than control OOX complexes. OOX complexes co-cultured with GDF-9 heterozygous meiotically competent (MC) and homozygous MC and meiotically incompetent (MI) denuded oocytes produced significantly greater amounts of progesterone, approaching control OOX levels, compared with OOX complexes co-cultured with control denuded (C57Bl/6 X Balb/C)F1 oocytes and GDF-9 wild-type denuded oocytes co-cultured with OOX complexes. (N=4: $p < 0.05$)

2.3.1.2 Regulation of Estradiol Production

In contrast to progesterone regulation, the murine oocyte secretes a factor that stimulates granulosa cell estradiol production, as deduced from the significantly lower production of estradiol by OOX complexes (24 ± 4.5%) in comparison to the OCC control (100%) (Figure 19). The co-culture of OOX complexes with GDF-9 heterozygous oocytes resulted in a dramatic increase in estradiol production to 89% of control levels. Reduced levels of estradiol production were observed when OOX complexes were co-cultured with GDF-9 homozygous MC (18 ± 5%) and MI (25 ± 4%) oocytes. These results suggest that GDF-9 is either acting as the estradiol-regulatory factor or is playing a key role in regulating granulosa cell estradiol production.

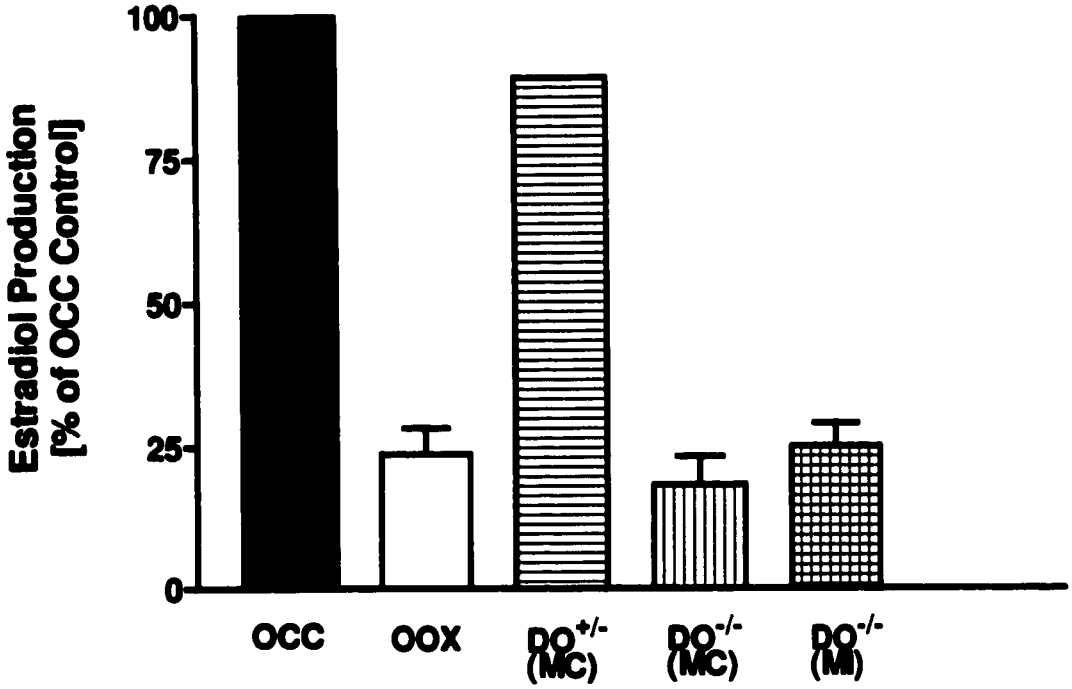

Estradiol Production
[% of OCC Control]
100
75
50
25
0
OCC
OOX
DO+/-
(MC)
DO-/-
(MC)
DO-/-
(MI)

FIGURE 19: ***Ability of GDF-9 Deficient Oocytes to Regulate Estradiol Production***

GDF-9 (+/-, -/-) oocytes were co-cultured with OOX complexes for 48 hours in the presence of FSH and testosterone. OCC complexes produced significantly greater quantities of estradiol than OOX complexes. Co-culture of OOX complexes with denuded oocytes from meiotically competent (MC) GDF-9 animals resulted in a dramatic increase in granulosa cell estradiol production, in comparison to the basal level of estradiol production by OOX complexes co-cultured with GDF-9 MC/MI homozygous denuded oocytes. These observations are from one experiment performed in triplicate.

DISCUSSION

The experiments reported here identify the optimal conditions for a bioassay that can be used to determine quantitatively the activity of the progesterone inhibitory factor produced by human oocytes and embryos. Despite the potential restrictive limitations of very small volumes of culture media to evaluate, and the need to freeze this media at the source, the bioassay could be used to measure the progesterone suppressive activity in human oocyte/embryo-conditioned media. Most embryos produced the factor(s) that inhibited progesterone production, but the degree of inhibition did not correlate with the ability of the oocytes to be fertilized nor with embryo morphology or their ability to cleave or develop after transfer. The role of GDF-9, a recently discovered growth factor, in granulosa cell differentiation has also been studied and now poses as a potential marker for assessing embryo health and development capacity.

Both human oocyte and embryo culture media failed to enable cumulus expansion of murine OOX cumulus complexes. CEEF has been shown to be secreted by meiotically-competent oocytes (Vanderhyden, 1993) of various mammalian species, (e.g., mice, rats, pigs) (Buccione et al., 1990; Vanderhyden, 1993) and the factor appears to act across species; i.e. pig and rat oocytes secrete a factor that enables the expansion of mouse OOX complexes (Vanderhyden, 1993). Murine zygotes have also been shown to secrete CEEF, although the degree of expansion that they elicit is below maximal (Vanderhyden et al., 1990). The absence of any response from HEC media upon OOX complex expansion may be explained by an insufficient amount of CEEF, since only one embryo conditioned 20 μl of medium, whereas previous studies have used 2 oocytes/embryos to condition each μl of medium (El-Fouly et al., 1970; Vanderhyden et

al., 1990). It is also possible that the human CEEF, if one exists, is unable to enable the expansion of murine cumulus cells, due to species differences in CEEF structure and activity. Fresh samples of HEC and oocyte-conditioned media were also tested (data not shown) to address the possibility of a loss of CEEF activity due to freeze-thawing effects. The fresh samples elicited the same response as frozen samples: no cumulus expansion. Interestingly, preliminary characterization by Eppig et al. (1993) found the murine CEEF to be very unstable, with activity being lost within a few hours after removal of the oocytes (Hosono et al., 1992). However, porcine CEEF is considerably more stable as observed by the retention of CEEF activity even after freezing (data not published). Although this assay system was unable to detect CEEF, future identification of the biochemical nature of CEEF may permit the detection of CEEF in HEC or oocyte–conditioned media with the potential to develop assays of greater sensitivity.

Freezing the culture media (for up to 8 weeks) had no effect on the ability of the media to support progesterone production by murine OOX complexes. No significant difference was found between the accumulation of progesterone by OOX complexes in fresh and frozen-thawed media. In both media, OOX complexes produced significantly greater amounts of progesterone than OCC, confirming previous findings from this lab (Vanderhyden et al., 1993; Vanderhyden et al., 1995) that showed murine oocytes secrete a factor that inhibits progesterone production by granulosa cells.

The optimal bioassay conditions were determined by identifying the minimum culture volume and minimum number of OOX complexes that could be used while retaining the ability to produce progesterone at levels that could be influenced by the presence of oocytes or oocyte-conditioned media. For volume experiments, a 17-fold

increase in culture volume resulted in a 5-fold increase in progesterone accumulation by both OCCs and OOXs. All culture volumes enabled a significant difference between OCCs and OOXs in their progesterone production, although a large difference, along with a low variability, was observed for 100 μl, identifying that volume as optimal for the bioassay. An increase in the number of cumulus complexes per well resulted in an overall decrease in progesterone production per complex. Bar-Ami and Khoury (1994) demonstrated a similar trend when changing the cell-plating density of cumulus cells in culture, and found that progesterone production decreased with a concurrent increase in cell-plating density. These results suggest the possible presence of a progesterone concentration threshold at which negative feedback regulation of progesterone production by cumulus granulosa cells occurs. By looking at progesterone concentration (ng/μl) per well relative to culture volume (data not shown) it was observed that as culture volume increased the concentration of progesterone per well decreased. That progesterone concentration was significantly greater at smaller volumes suggests that a threshold of inhibition may be reached more quickly in smaller volumes, thus limiting the amount of progesterone produced per complex. It is also possible that a substrate(s) may be exhausted or toxin(s) have accumulated in the medium, thus compromising the ability of granulosa cells to regulate steroid production resulting in lower progesterone production per complex at smaller volumes.

All numbers of cumulus complexes cultured in 100 μl yielded a significant difference in progesterone production between OCCs and OOXs; however, 2 complexes were chosen to be the optimal number of complexes for use in the bioassay. The particular culture conditions chosen were due to the relatively low variability observed in

combination with a large difference between OOX and OCC progesterone production, thus allowing for a large range within which progesterone inhibition could be observed and a minimal dilution of HEC media. Thus, the established experimental bioassay consisted of the culture of 2 OOXs for 48 hours in HEC media diluted 5-fold to 100 µl.

In these studies, PIF activity was detected in HEC media, suggesting that human oocytes and embryos are capable of secreting a factor that can inhibit murine granulosa cell steroidogenesis. A previous study in this lab found that PIF was secreted by human zygotes and was involved in the regulation of granulosa cell progesterone production. Cocultures of zygotes on monolayers of human granulosa cells were shown to suppress granulosa cell progesterone and estradiol production (Seifer et al., 1996), suggesting human germ cell influence on granulosa cell activity. In mice, oocytes have been found to secrete PIF throughout follicular development, from mid-growth to full-grown stages as well as after ovulation and fertilization to yield zygotes (Vanderhyden et al., 1998), although the later stages of embryonic development have not yet been examined. It is possible that the secretion of PIF by human embryos is the continuation of its constitutive production during oocyte development.

Due to the inability to detect estradiol-regulatory factor in HEC media using the bioassay established for progesterone, a modified bioassay was developed by altering the media used to culture the cumulus complexes. Finding HTF/WAY to be the optimal culture media for use in the estradiol-specific bioassay, OCC complexes were observed to produce significantly greater amounts of estradiol (255 ± 42 pg/complex) than OOX complexes, confirming previous findings (Vanderhyden et al., 1993; Vanderhyden et al., 1995). However, the co-culture of denuded oocytes with OOX complexes did not

stimulate estradiol production, contrary to what has previously been shown (Vanderhyden et al., 1993; Vanderhyden et al., 1995). Murine oocytes have been shown to secrete an estradiol-regulatory factor that stimulates estradiol production by murine cumulus granulosa cells. However, in both pigs (Coskun et al., 1995) and humans (Seifer et al., 1996) the estradiol-regulatory factor inhibits estradiol production by granulosa cells. Because the *in vitro* production of estradiol by granulosa cells is notoriously variable and was unreliable in this bioassay, the remainder of HEC experiments focussed on the detection and use of PIF.

The role of oocyte-secreted factors during follicular development is becoming increasingly clear. The oocyte first came under suspicion for playing an active role in regulating its environment when researchers observed the luteinization, and eventual degeneration, of granulosa cells upon destruction of the oocyte. Progesterone concentration had increased significantly, and a substance secreted by the oocyte was suspected to play a role in regulating levels of this steroid (El-Fouly et al., 1970). This led to studies investigating the sporadic interactions between germ cells and somatic cells and insightful statements asserting the 'directing' influence of the oocyte upon follicular development (Hubbard et al., 1988; Wallace, 1983). Recent research has confirmed the secretion of steroid-regulatory factors by mouse oocytes that regulate both progesterone (inhibitory) and estradiol (stimulatory) production by granulosa cells (Vanderhyden et al., 1993), acting by independent mechanisms (Vanderhyden et al., 1995). Porcine oocytes have been documented to produce steroid-regulatory factors as well (Coskun et al., 1995). The secretion of PIF by oocytes is thought to inhibit premature luteinization of granulosa cells since the presence of PIF inhibits progesterone production by granulosa

cells and the induction of LH receptors (Eppig et al., 1997). Previous research has shown that follicular fluid contains a factor that prevents luteinization of granulosa cells (Ledwitz-Rigby and Rigby, 1979), suggesting that measurement of this factor in follicular fluid may be a viable alternative to the bioassay described in this study. It is possible that defects in oocyte or embryo function could impair the secretion of PIF, thus contributing to conditions that potentially cause infertility, such as unruptured follicle syndrome or unexplained anovulatory cycles.

Further evidence supporting the integral role of the oocyte in follicular development was provided by studies on male germ cell-somatic cell interactions. At all stages of sexual maturation, male germ cells and Sertoli cells have also been shown to develop intricate sets of communication pathways, which act to regulate attachment, displacement, cell shaping, and cell-cell transfer of molecules and cellular materials. In fact, it is the unique position of the Sertoli cells, as is the case with female granulosa cells, which allows them to receive, integrate, and emit all signals required for spermatogenesis (Jégou et al., 1993). Much of the communication between germ cells and somatic cells seems to be paracrine in nature, as evidenced by a large number of studies showing the interaction of ligand-receptor systems in mediating cell function. For example, male germ cells have been shown to produce a factor that regulates the expression of the preproenkaphalin (Ppenk) gene, an opioid gene, expressed in Sertoli cells (Fujisawa et al., 1992). In addition, several Sertoli cell products, such as activin and inhibin, have been shown to affect spermatogenesis (Mather et al., 1990; van Dissel-Emiliani et al., 1989), revealing the close interaction between male germ cells with

Sertoli cells. Persson *et al.*, (1988), showed that nerve growth factor was being produced by male germ cells and has a receptor on Sertoli cells.

Perhaps the most studied ligand-receptor relationship in germ cell-somatic cell interactions has been the Kit tyrosine-kinase receptor, c-*kit*, and its ligand, KL. Since the discovery of c-*kit* (Besmer et al., 1986) and KL (Huang et al., 1990), studies have confirmed the expression of c-*kit* by both male and female germ cells, and expression of KL by their surrounding somatic cells. Knockout studies of c-*kit* (product of *W* gene locus) have revealed its importance in hematopoiesis, gametogenesis, and melanogenesis (Chabot et al., 1988; Geissler et al., 1988). Similar studies with KL, which maps to the Steel (*Sl*) gene locus, have identified its involvement in pigmentation, and primordial stem cell migration and growth (Witte, 1990). The research involving male c-*kit*/KL interactions is not as advanced with respect to its elucidation in females, although all emerging observations indicate that a parallel system exists in both genders. C-*kit* has been found to be expressed in male germ cells (Sandlow et al., 1997; Sorrentino et al., 1991) and Leydig cells, whereas KL is expressed in both Sertoli cells and weakly in Leydig cells. Thus, it seems apparent that the relationship and interactions between germ cells and somatic cells in both males and females are quite similar, if not parallel. These observations lend to the ability of applying the research from one gender to the other, thus facilitating the identification of the paracrine factors involved in germ cell-somatic cell interaction.

The role embryo-secreted PIF is unclear since the ovarian production of progesterone *increases* during the preimplantation stages of human embryo development. Several studies have shown that high levels of progesterone adversely affect embryo

quality and IVF pregnancy rates. Schoolcraft *et al.* (1991) have observed that premature luteinization with increased progesterone levels negatively affects oocytes, leading to a reduction in their fertilizability. Also, a subtle rise in serum progesterone concentrations during the follicular phase has been associated with reduced implantation rates (Harada et al., 1995). Embryonic secretion of progesterone inhibitory factors may be a mechanism to maintain at least local concentrations of progesterone at reduced levels.

Regardless of its possible functions, the demonstration of PIF secretion by human embryos enabled the establishment of a non-invasive bioassay with the aim to correlate the amount of factor secreted by embryos to their developmental capacity. HEC media samples exhibited tremendous variability in progesterone regulatory activity ranging from various levels of inhibition in the majority of samples (96%) to stimulation in a few samples (182%). However, no correlation ($r = -0.013$) was found between the embryo score and OOX progesterone production in HEC media. Although each embryo sample exhibited a unique and varied level of PIF activity, it was not related to its morphology or growth. The comparison of non-fertilized and fertilized embryo PIF activity yielded no significant difference, as was the case with comparing embryos that did not result in pregnancy to those that did, although the latter had a substantially lower level (35.5%) of progesterone production indicating greater PIF activity. Although not significant, the greater PIF activity of embryos that lead to pregnancy may be indicative of greater health and capacity to develop.

With the timely arrival of GDF-9 knockout mice to our laboratories, a final group of experiments were developed to look at the role of this recently discovered growth factor as a paracrine factor secreted by the oocyte and its possible use as a quantitative

marker of embryo quality. In terms of development, GDF-9 ovaries were arrested at the primary one-layer follicle stage of follicular development, as indicated by the sole presence of primordial and primary one-layer follicles, and confirming the findings of Dong et al. (1996). The GDF-9 homozygous MC oocytes were larger in diameter than GDF-9 heterozygous MC oocytes (80 ± 4.8 μm and 74 ± 4.5 μm, respectively), whereas MI GDF-9 homozygous oocytes were significantly smaller than both (63 ± 14 μm). This indicates that GDF-9 deficient oocytes grow more rapidly than control oocytes. This observation was also made by Carabatsos et al. (1998) who found that the rate of oocyte growth relative to follicle size was accelerated in GDF-9-deficient mice when compared to heterozygous controls. One factor that contributes to oocyte growth has been shown to be KL (Packer et al., 1994). KL is produced by granulosa cells and acts on the c-*kit* receptor, which is expressed on the surface of the oocyte (Horie et al., 1991). It seems that KL is acting as the positive regulator of oocyte growth, yet its actions may need to be checked by the presence of a negative regulator to ensure synchronous growth of the oocyte and follicle (Carabatsos et al., 1998). It is more than likely that GDF-9 is playing an active role in the negative feedback pathway that regulates oocyte growth; this suggestion is supported by the discovery that KL mRNA levels are upregulated in granulosa cells of GDF-9-deficient mice (Elvin et al., 1999a).

The function of GDF-9 in the mechanism of cumulus expansion was explored by culturing GDF-9 denuded oocytes with OOX complexes. It is well established that CEEF, secreted by the oocyte, is required along with FSH to enable the expansion of the cumulus oophorus (Buccione et al., 1990; Vanderhyden et al., 1990) however, GDF-9 homozygous oocytes (both MC and MI) failed to enable cumulus expansion, in contrast

to the cumulus expansion elicited by GDF-9 MC heterozygous oocytes and OCC controls. That GDF-9-deficient (-/-) oocytes do not enable cumulus expansion suggests that GDF-9 plays an integral role in cumulus expansion either as the CEEF or in the downstream pathway that mediates the CEEF signal. Recently, recombinant GDF-9 has been demonstrated to enable the expansion of oocytectomized cumulus complexes (Elvin et al., 1999b), although these results do not conclusively determine if GDF-9 is the CEEF. GDF-9 could be working in the same manner as TGF-β, which also has expansion-enabling abilities but was shown not to be the CEEF by demonstrating the ability of oocytes to enable cumulus expansion in the presence of TGF-β antibodies (Tirone et al., 1997). However, combined with our observations using GDF-9-deficient oocytes, there is stronger evidence that GDF-9 is either the CEEF or is integral in mediating the cellular signals that enable cumulus expansion. In further support of GDF-9's role in cumulus expansion, recombinant GDF-9 was shown to upregulate the expression of HAS-2, the enzyme responsible for hyaluronic acid production, and inhibit the synthesis of protease uPA (Elvin et al., 1999b). uPA synthesis has also been shown to be inhibited by CEEF (Canipari et al., 1995).

The role of GDF-9 in regulating granulosa cell steroidogenesis was also investigated. GDF-9 heterozygous and –deficient oocytes were both shown to lack the ability to inhibit cumulus granulosa cell progesterone production, whereas control (C57Bl/6 X Balb/C)F1 and GDF-9 wild-type (+/+) oocytes co-cultured with OOX complexes produced significantly lower levels of progesterone, indicating active inhibition of progesterone production by PIF. In terms of estradiol regulation, GDF-9 heterozygous oocyte/OOX co-cultures produced greater amounts of estradiol than GDF-

9-deficient oocyte/OOX co-cultures, indicative of GDF-9 having a stimulatory effect on granulosa cell estradiol production. From these results, GDF-9 seems to be involved in the regulation of both progesterone and estradiol production. The addition of recombinant GDF-9 to murine mural granulosa cell cultures has been shown to stimulate progesterone production and induce a 2-5 fold increase in StAR, steroidogenic acute regulator protein, mRNA (Elvin et al., 1999b). Although GDF-9 has been shown to stimulate progesterone production in mural granulosa cells (Elvin et al., 1999b), we have shown that GDF-9 plays a role in the inhibition of cumulus granulosa cell progesterone production. However, mural and cumulus granulosa cells are two distinct cell types that may be regulated differentially with regard to the production of steroid hormones. Cumulus and mural granulosa cells have been shown to differ in their distribution of LH receptors (Amsterdam et al., 1975) and steroidogenic capabilities (Hillensjö et al., 1981; Zoller and Weisz, 1979). These results, combined with recent research studies on the actions of recombinant GDF-9, suggest that GDF-9 is functioning to regulate cumulus granulosa cell progesterone and estradiol production in murine ovaries. Because preliminary characterization of the steroid-regulatory factor suggests that it is a steroid (Coskun et al., 1995), it is unlikely that GDF-9, a protein, is the progesterone-inhibitory or estradiol-regulatory factor. However, the evidence suggests that GDF-9 is playing an integral role, i.e. intracellular signaling, in the overall process of steroidogenesis.

In closing, no correlation was found between the presence of PIF and human embryo developmental capacity, although the discovery of a potential paracrine factor, GDF-9, poses as a potential non-invasive marker for oocyte and embryo quality. Further studies aimed at elucidating the identity and mechanism of action of oocyte- and embryo-

secreted factors will be of tremendous benefit in developing accurate, non-invasive methods to evaluate oocyte and embryo maturity, health and developmental potential. Identification of such factors as GDF-9 will enable the quantitative assessment of embryo quality and may improve embryo culture conditions or the receptivity of the uterus for implantation. That most current research is now acknowledging the need to use multivariate tests to assess oocyte and embryo quality is indicative of the many different factors and conditions required for the production of oocytes and embryos of great quality and the difficulty in employing only one select factor as an accurate gauge. Oocyte and embryo development are elegant and stunningly sophisticated processes, involving a myriad of developmental pathways and factors that work harmoniously to orchestrate the desired aim of Mother Nature: the birth of a healthy baby.

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