

REI R01

PI:	Title: Novel biomarkers for oocyte quality	
Received: 10/19/2007	FOA: PAR07-350	Council: 05/2008
Competition ID:	FOA Title: FEMALE HEALTH AND EGG QUALITY (R01)	
1 R01 R01	Dual: AA	Accession Number: 3034140
IPF: 6144650	Organization:	
Former Number:	Department:	
IRG/SRG: ZRG1 EMNR-L (50)R	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 250,000 Year 2: 250,000 Year 3: 250,000 Year 4: 250,000 Year 5: 250,000	Animals: Y Humans: Y Clinical Trial: N Exemption: 30 HESC: N	New Investigator: N
<i>Senior/Key Personnel:</i>	<i>Organization:</i>	<i>Role Category:</i>
		PD/PI
		MPI
		Other (Specify)-Co-Investigator
		Other (Specify)-Collaborator
		Other (Specify)-Collaborator
		Other (Specify)-Collaborator

SF 424 (R&R)

		2. DATE SUBMITTED	Applicant Identifier
		3. DATE RECEIVED BY STATE	State Application Identifier
1. * TYPE OF SUBMISSION		4. Federal Identifier	
<input type="radio"/> Pre-application <input checked="" type="radio"/> Application <input type="radio"/> Changed/Corrected Application			
5. APPLICANT INFORMATION			
* Legal Name:			* Organizational DUNS:1
Department: Office for Sponsored Research		Division:	
* Street1:		Street2:	
* City:		* State:	
Province:		* Country: USA: UNITED STATES	
		* ZIP / Postal Code: 00000	
Person to be contacted on matters involving this application			
Prefix:	* First Name:	Middle Name:	* Last Name:
Dr.			Suffix:
* Phone Number:	Fax Number:	Email:	
6. * EMPLOYER IDENTIFICATION NUMBER (EIN) or (TIN):		7. * TYPE OF APPLICANT	
		<input type="radio"/> Private Institution of Higher Education	
8. * TYPE OF APPLICATION:		Other (Specify):	
<input checked="" type="radio"/> New <input type="radio"/> Resubmission <input type="radio"/> Renewal <input type="radio"/> Continuation <input type="radio"/> Revision		Small Business Organization Type <input type="radio"/> Women Owned <input type="radio"/> Socially and Economically Disadvantaged	
If Revision, mark appropriate box(es).		9. * NAME OF FEDERAL AGENCY:	
<input type="radio"/> A. Increase Award <input type="radio"/> B. Decrease Award <input type="radio"/> C. Increase Duration <input type="radio"/> D. Decrease Duration <input type="radio"/> E. Other (specify):		National Institutes of Health	
* Is this application being submitted to other agencies? <input type="radio"/> Yes <input checked="" type="radio"/> No		10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:	
What other Agencies?		TITLE:	
11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:			
Novel biomarkers for oocyte quality			
12. * AREAS AFFECTED BY PROJECT (cities, counties, states, etc.)			
N/A			
13. PROPOSED PROJECT:		14. CONGRESSIONAL DISTRICTS OF:	
* Start Date	* Ending Date	a. * Applicant	
08/01/2008	07/31/2013	b. * Project	
15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION			
Prefix:	* First Name:	Middle Name:	* Last Name:
Dr.			Suffix:
Position/Title: Associate Professor	* Organization Name:		
Department: Obstetrics and Gynecology	Division:		
* Street1:	Street2:		
* City:	* State:		
Province:	* Country: USA: UNITED STATES		
* Phone Number:	* ZIP / Postal Code: ...		
	Fax Number:		
	* Email:		

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RESEARCH & RELATED Project/Performance Site Location(s)

Project/Performance Site Primary Location

Organization Name:

* Street1:

Street2:

* City:

County:

* State:

Province:

* Country: USA: UNITED STATES

* Zip / Postal Code:

Project/Performance Site Location 1

Organization Name:

* Street1:

Street2:

* City:

County:

* State:

Province:

* Country: USA: UNITED STATES

* Zip / Postal Code:

File Name

Mime Type

Additional Location(s)

RESEARCH & RELATED Other Project Information

1.	* Are Human Subjects Involved?	<input checked="" type="radio"/> Yes	<input type="radio"/> No	
	1.a. If YES to Human Subjects			
	Is the IRB review Pending?	<input checked="" type="radio"/> Yes	<input type="radio"/> No	
	IRB Approval Date:			
	Exemption Number:	__ 1 __ 2 __ 3 __ 4 __ 5 __ 6		
	Human Subject Assurance Number			
2.	* Are Vertebrate Animals Used?	<input checked="" type="radio"/> Yes	<input type="radio"/> No	
	2.a. If YES to Vertebrate Animals			
	Is the IACUC review Pending?	<input checked="" type="radio"/> Yes	<input type="radio"/> No	
	IACUC Approval Date:			
	Animal Welfare Assurance Number			
3.	* Is proprietary/privileged information included in the application?	<input type="radio"/> Yes	<input checked="" type="radio"/> No	
4.a.*	Does this project have an actual or potential impact on the environment?	<input type="radio"/> Yes	<input checked="" type="radio"/> No	
	4.b. If yes, please explain:			
	4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	<input type="radio"/> Yes	<input type="radio"/> No	
	4.d. If yes, please explain:			
5.a.*	Does this project involve activities outside the U.S. or partnership with International Collaborators?	<input type="radio"/> Yes	<input checked="" type="radio"/> No	
	5.b. If yes, identify countries:			
	5.c. Optional Explanation:			
6.	* Project Summary/Abstract	7767-Abstract.pdf		Mime Type: application/pdf
7.	* Project Narrative	2613-Narrative.pdf		Mime Type: application/pdf
8.	Bibliography & References Cited	5582-Bibliography.pdf		Mime Type: application/pdf
9.	Facilities & Other Resources	2296-Resources.pdf		Mime Type: application/pdf
10.	Equipment	3530-Equipment.pdf		Mime Type: application/pdf

A healthy oocyte (egg) is required for conception, development and birth of a healthy child. Deterioration of oocyte quality occurs with advancing maternal age and may partially account for the age-related decline in fertility. The effect of aging on oocyte quality is likely exacerbated or mediated by cumulative exposure to environmental pollutants, smoking or drug/alcohol abuse. The low efficiency of infertility treatment by in vitro fertilization (IVF), in terms of the number of babies born per the number of oocytes obtained for IVF, can be largely attributed to poor oocyte quality. Assessing oocyte health, particularly in the human, is hampered by the lack of reliable, non-destructive methods of determining oocyte quality. **Our long-term goals are to identify biomarkers of oocyte quality and to use these markers, not only as research tools for investigating the regulation of oocyte development, but also as diagnostic tools to improve women's reproductive health.** Oocyte development occurs concurrently with follicular development and both are regulated via paracrine signaling between the oocyte and its surrounding somatic cells, particularly cumulus granulosa cells. Alterations in gene expression in cumulus cells may cause or occur as a result of abnormalities in the oocyte. **We hypothesize that certain cumulus cell genes may be useful for predicting oocyte quality.** In an earlier study, we collected cumulus cells during IVF treatment from oocytes that either did not fertilize or were fertilized but did not develop well in vitro. We identified a number of differentially expressed genes between the two groups of cells. We plan to extend these findings by studying cumulus cells classified according to the patients' age and the ability of the oocyte to implant in vivo after embryo transfer. Markers of oocyte quality will be selected by screening a list of genes with differential expression among these different groups of cumulus cells. Using a large sample of women, the predictive value of selected cumulus cell biomarkers for determining oocyte capacity for fertilization and successful implantation will be further validated. Parallel studies will be carried out in a mouse model to identify additional candidate marker genes for validation in the human, and to develop model systems to study the mechanistic roles of these biomarkers in oocyte development. This research attempts to develop non-destructive methods to predict oocyte quality through examination of cumulus cells. It is our hope that such methods will improve the efficiency of IVF, avoid the creation of extra embryos, reduce the risk associated with multiple gestations, and lead ultimately to the development of new diagnostic, therapeutic and preventative strategies for infertility, miscarriage and birth defects.

We propose to develop non-destructive methods to predict oocyte quality through examination of cumulus cells. It is our hope that such methods will improve the efficiency of IVF, avoid the creation of extra embryos, reduce the risk associated with multiple gestations, and lead ultimately to the development of new diagnostic, therapeutic and preventative strategies for infertility, miscarriage and birth defects.

RESOURCES

Collection, storage and processing of human cumulus cells:

Collection, storage and processing of human cumulus cells for experiments in Aims 1 and 3 will take place at the Reproductive Endocrinology and Infertility (REI) Division, the clinical practice of the Department of Obstetrics and Gynecology, at the University of Michigan Medical Center. The Laboratory Director of the REI Division, Dr. [redacted], one of the two PIs, is the Laboratory Director of [redacted] and Dr. [redacted], Co-PI, is the Division Director.

Physical facilities:

The REI Division occupies approximately 10,000 square foot of space on the same floor of a high-rise, ambulatory-care building connected by enclosed pedestrian bridge to research buildings of the Department of Obstetrics and Gynecology, where one of the two PIs, Dr. [redacted], has 1200 square foot of lab space for research.

The Division's IVF lab has numerous dissection microscopes, two of which are placed in environmentally-controlled chambers for identify oocytes from follicular aspirates during oocyte-retrieval procedures and for removing cumulus cells after oocytes are identified. The lab is accredited by American College of Pathologists, in compliance with CLIA 88 and the practice guidelines of American Society for Reproductive Medicine.

Personnel for IVF lab:

There are five board-certified physicians in Division. The IVF has five full-time technologists, in addition to the Laboratory Director. Removing cumulus cells from the oocytes is one of the routine tasks for the technologists. For this research, a research technician, [redacted], will collect the cumulus cells after they are removed from oocytes by the IVF Lab Technologists, and will store or process the cells for this research. [redacted] has been successfully trained for all the techniques required for storing and processing of the cells and for RNA and DNA extraction. She is trained with respect to HIPPA regulations.

Patient volume:

Approximately 1000 new female patients each year attend the Division for Reproductive Endocrinology-related disorders. Approximately 500 – 600 oocyte-retrieval procedures are performed each year in the Division's IVF Laboratory. The following table summarizes the patient populations relevant to this research, from January 2005 to December 2006:

Table 1.1. Some statistics of the IVF cycles corresponding to the four populations of the cumulus cell donors at

	No. IVF cycles	No. Pregnancies	No. pregnancies with 100% implantation rate	No. oocytes fertilized/cycle	8-cell formation rate	Implantation rate
Oocyte donors	111	70	21	13	33.1%	39%
<35 years old	354	142	14	12	29.3%	33%
35 – 37 years old	200	79	7	8	31.3%	23%
>38 years old	422	111	4	7	31.2%	13%

Laboratory:

Dr. lab is affiliated with Division of Reproductive Biology Research within Department of Obstetrics and Gynecology. The division consists of three other labs –Dr. Dr. and Dr. All four labs carry out active molecular and cellular biology research in the field of reproductive science and the labs within the division interact actively. Dr. , a well-regarded oocyte biologist, recently has just joined department of Obstetrics and Gynecology to lead the Division of , which will focus on fertility preservation for female cancer patients. Dr. lab will move from campus to downtown campus soon, into the same building as ours. Proximity to such a strong oocyte and endocrinology group will further enhance our interaction with oocyte researchers in Dr. lab. Such interaction with Dr. and associates will be very helpful for this project.

Dr. lab has 1200 sq ft laboratory space located on the 7th floor of the brand-new Research Building. The Research Building is a state of the art modern biomedical research complex combining basic research, translational research and clinical research with a suite of core supporting facilities and mouse housing space all under one roof. The research space is an open lab design, providing ample opportunities for interactions and exchanges as well as easy access to common core equipments such as cold room, ultracentrifuge, automatic X-ray film developer, dark room, etc. Dr. lab is also part of the Cancer Center, enjoying access to many core facilities such as pathology core, genomics core, and bioinformatics core. The PI's lab is affiliated with the and is physically located on the floors of labs. Among the labs occupying the floors of are several genetics lab working on model organism such as the labs of Drs. and mouse genetics and developmental biology labs such as laboratories of Drs. In particular, our neighbor on the same floor--Dr. lab has been working in the reproductive endocrinology for more than twenty years and his lab works on both male and female reproductive mutations as well as the genetic mechanism of sex-reversal. Members from our two groups interact actively and share our resources and technical expertise.

lab is also a member of Center University consisting of faculty in departments working in the different aspects of reproductive sciences at . The helps to bring together faculty with diverse individual interest, leading to coordinated programs of cooperative investigation that enhance the overall quality of research in the reproductive sciences at . Other than regular interactions and sharing of the reagents, there is also an annual mini-symposium on Reproductive Biology, where students and postdoctoral fellows present their research, and seminar series in reproductive biology.

Animal: Extensive animal facilities are located in the basement of the building and are easily accessible through elevators. All mouse colonies maintained by the PI are housed in full barrier facilities. The animal facilities are accredited by AAALAC with administrative oversight by the and an Institutional Animal Care and Use Committee. There are 7 veterinarians available for consultation and a detailed training program is available to assure compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Core Facilities at Both PI labs have access to state-of-art core facilities such as Genomics Core, Imaging Core, Transgenic Mice Core, Pathology Core which provide services from gene expression profiling including multiple platforms, SNP analysis, histology, Confocal Microscopy, Electronic Microscopy etc.

MAJOR EQUIPMENT: The PI's laboratory contains table-top and floor-top centrifuges which can be used for different sizes of tubes and plates, one -80° C freezers, a computerized BioRad ImageAnalysis System, two biological safety culture hoods, two incubators, one chemical safety hoods, an upright Leica Fluorescent Compound Microscope. We also have the access to the major equipments in the Reproductive Biology Research Division such as inverted compound Microscopes, an ABI7000 real-time PCR apparatus, one immunofluorescent microscope, a developer, a Phosphor-Imager system and various software programs for molecular biological analyses. Additionally, the Cancer Center and the have multiple state-of-the-art core facilities. Some of these cores are flow cytometry core, Laser-capture micro-dissection core, Affymetrics genomics facility, ABI7900 real-time PCR machines, proteomics core, cell imaging core, DNA and peptide sequencing facility, structural biology core and transgenic animal core.

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator				
Prefix Dr.	* First Name	Middle Name	* Last Name	Suffix PhD
Position/Title: Associate Professor		Department:		
Organization Name:		Division:		
* Street1:		Street2:		
* City:	County:	* State:	Province:	
* Country: USA: UNITED STATES	* Zip / Postal Code:			
*Phone Number		Fax Number	* E-Mail	
Credential, e.g., agency login:				
* Project Role: PD/PI		Other Project Role Category:		
*Attach Biographical Sketch		File Name	Mime Type application/pdf	
Attach Current & Pending Support				

PROFILE - Senior/Key Person				
Prefix Dr.	* First Name	Middle Name	* Last Name	Suffix PhD
Position/Title: Assistant Professor		Department:		
Organization Name:		Division:		
* Street1:		Street2:		
* City:	County:	* State:	Province:	
* Country: USA: UNITED STATES	* Zip / Postal Code:			
*Phone Number		Fax Number	* E-Mail	
Credential, e.g., agency login:				
* Project Role: PD/PI		Other Project Role Category:		
*Attach Biographical Sketch		File Name	Mime Type application/pdf	
Attach Current & Pending Support				

PROFILE - Senior/Key Person				
Prefix Dr.	* First Name	Middle Name	* Last Name	Suffix MD
Position/Title: Professor		Department:		
Organization Name:		Division: I		
* Street1:		Street2:		
* City:	County:	* State:	Province:	

* Country: USA: UNITED STATES * Zip / Postal Code:		
*Phone Number	Fax Number	* E-Mail
Credential, e.g., agency login:		
* Project Role: Other (Specify)		Other Project Role Category: Co-Investigator
*Attach Biographical Sketch Attach Current & Pending Support		File Name Mime Type application/pdf

PROFILE - Senior/Key Person				
Prefix Dr.	* First Name	Middle Name	* Last Name	Suffix PhD
Position/Title: Professor		Department:		
Organization Name:		Division:		
* Street1:		Street2:		
* City:	County: I	* State:	Province:	
* Country: USA: UNITED STATES		* Zip / Postal Code:		
*Phone Number	Fax Number	* E-Mail		
Credential, e.g., agency login:				
* Project Role: Other (Specify)		Other Project Role Category: Collaborator		
*Attach Biographical Sketch Attach Current & Pending Support		File Name Mime Type application/pdf		

PROFILE - Senior/Key Person				
Prefix Dr.	* First Name	Middle Name	* Last Name	Suffix PhD
Position/Title: Assistant Professor		Department: I		
Organization Name:		Division:		
* Street1:		Street2:		
* City:	County:	* State:	Province:	
* Country: USA: UNITED STATES		* Zip / Postal Code:		
*Phone Number	Fax Number	* E-Mail		
Credential, e.g., agency login:				
* Project Role: Other (Specify)		Other Project Role Category: Collaborator		
*Attach Biographical Sketch Attach Current & Pending Support		File Name Mime Type application/pdf		

PROFILE - Senior/Key Person				

Prefix Dr.	* First Name	Middle Name	* Last Name	Suffix PhD
Position/Title: Research Assistant Professor		Department:		
Organization Name:		Division:		
* Street1:		Street2:		
* City:	County: Cook	* State:	Province:	
* Country: USA: UNITED STATES	* Zip / Postal Code:			
*Phone Number		Fax Number		* E-Mail
Credential, e.g., agency login:				
* Project Role: Other (Specify)		Other Project Role Category: Collaborator		
*Attach Biographical Sketch		File Name	Mime Type	
Attach Current & Pending Support			application/pdf	

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

Additional Senior/Key Person Form Attachments

When submitting senior/key persons in excess of 8 individuals, please attach additional senior/key person forms here. Each additional form attached here, will provide you with the ability to identify another 8 individuals, up to a maximum of 4 attachments (32 people).

The means to obtain a supplementary form is provided here on this form, by the button below. In order to extract, fill, and attach each additional form, simply follow these steps:

- Select the "Select to Extract the R&R Additional Senior/Key Person Form" button, which appears below.
- Save the file using a descriptive name, that will help you remember the content of the supplemental form that you are creating. When assigning a name to the file, please remember to give it the extension ".xfd" (for example, "My_Senior_Key.xfd"). If you do not name your file with the ".xfd" extension you will be unable to open it later, using your PureEdge viewer software.
- Using the "Open Form" tool on your PureEdge viewer, open the new form that you have just saved.
- Enter your additional Senior/Key Person information in this supplemental form. It is essentially the same as the Senior/Key person form that you see in the main body of your application.
- When you have completed entering information in the supplemental form, save it and close it.
- Return to this "Additional Senior/Key Person Form Attachments" page.
- Attach the saved supplemental form, that you just filled in, to one of the blocks provided on this "attachments" form.

Important: Please attach additional Senior/Key Person forms, using the blocks below. Please remember that the files you attach must be Senior/Key Person Pure Edge forms, which were previously extracted using the process outlined above. Attaching any other type of file may result in the inability to submit your application to Grants.gov.

- 1) Please attach Attachment 1
- 2) Please attach Attachment 2
- 3) Please attach Attachment 3
- 4) Please attach Attachment 4

	Filename
ADDITIONAL SENIOR/KEY PERSON PROFILE(S)	MimeType

	Filename
Additional Biographical Sketch(es) (Senior/Key Person)	MimeType

	Filename
Additional Current and Pending Support(s)	MimeType

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME	Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
	B.Sc.		Animal Science
	Ph.D.		Reproductive Biology
	Post-doctor Fellow		Reproductive Biology

A. Positions and Honors.

Academic Positions:

Clinical Positions:

Honors and Awards:

B. Peer-reviewed publications (in chronological order).

Principal Investigator/Program Director (Last, first, middle): .

Principal Investigator/Program Director (Last, first, middle):

.....
C. Research Support
None.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE Assistant Professor		
eRA COMMONS USER NAME			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
	B.S. M.S. PhD. Postdoc Postdoc		Biology Medical Genetics Genetics Developmental Biology Human and mouse genetics

Academic Appointments

Honors and Awards

Publications

Principal Investigator/Program Director (Last, first, middle):

Research Support—ongoing research

NIH/

Overlap: None

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
eRA COMMONS USER NAME		Professor of Obstetrics and Gynecology	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
	B.A. M.D.		Physics Medicine Internship and Residency in Obstetrics and Gynecology

Professional Experience:

Honors and Awards:

Selected Manuscripts:

Other Support

None.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
eRA COMMONS USER NAME		Professor	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
	MS PhD Post- Doctoral		Chemical Engineering Organic Chemistry Neuroendocrinology

A. Positions and Honors.**Professional Activities:****B. Selected peer-reviewed publications**

Principal Investigator/Program Director (Last, first, middle):

C. Research Support

Active:

R21
NIH/NIMH

R01
NIH/NIAA

R21
NIH/HD

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
eRA COMMONS USER NAME		Assistant Professor	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
	B.S.		Applied Mathematics
	M.S.		Statistics
	Ph.D.		Biostatistics

Positions and Professional Experience**Publications**

Principal Investigator/Program Director (Last, first, middle):

Research Support—ongoing research

1 R01
NIH/NHLBI

Role: PI

R01
NIH/NHLBI

Role: co-Investigator

U01
NIH/NCI

Role: subcontract PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on for each person. (See attached sample). **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
		Research Assistant Professor Director,	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
	B.S. PhD Post-doc Masters		Medical Technology Laboratory Medicine Infectious Diseases Biotechnology

A. Positions and Honors.**Positions and Employment****Other Experience**

Membership

Honors & Awards

B. Selected peer-reviewed publications (in chronological order).

C. Research Support

None.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Project Director / Principal Investigator (PD/PI)

Prefix: * First Name:

Middle Name:

* Last Name:

Suffix:

* New Investigator? No Yes

Degrees:

2. Human Subjects

Clinical Trial? No Yes

* Agency-Defined Phase III Clinical Trial? No Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix: * First Name:

Middle Name:

* Last Name:

Suffix:

* Phone Number: Fax Number:

Email:

* Title:

* Street1:

Street2:

* City:

County:

* State:

Province:

* Country: * Zip / Postal Code:

PHS 398 Modular Budget, Periods 1 and 2

Budget Period: 1			
Start Date: <input style="width: 80%;" type="text" value="08/01/2008"/>	End Date: <input style="width: 80%;" type="text" value="07/31/2009"/>		
A. Direct Costs			Funds Requested (\$)
* Direct Cost less Consortium F&A			<input style="width: 80%;" type="text" value="250,000.00"/>
Consortium F&A			<input style="width: 80%;" type="text"/>
* Total Direct Costs			<input style="width: 80%;" type="text" value="250,000.00"/>
B. Indirect Costs			
	Indirect Cost Rate (%)	Indirect Cost Base (\$)	* Funds Requested (\$)
1. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text" value="51.00"/>	<input style="width: 80%;" type="text" value="250,000.00"/>	<input style="width: 80%;" type="text" value="127,500.00"/>
2. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>
3. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>
4. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>
Cognizant Agency (Agency Name, POC Name and Phone Number)			<input style="width: 90%;" type="text"/>
Indirect Cost Rate Agreement Date			<input style="width: 80%;" type="text" value="04/24/2007"/>
			Total Indirect Costs <input style="width: 80%;" type="text" value="127,500.00"/>
C. Total Direct and Indirect Costs (A + B)			Funds Requested (\$) <input style="width: 80%;" type="text" value="377,500.00"/>
Budget Period: 2			
Start Date: <input style="width: 80%;" type="text" value="08/01/2009"/>		End Date: <input style="width: 80%;" type="text" value="07/31/2010"/>	
A. Direct Costs			Funds Requested (\$)
* Direct Cost less Consortium F&A			<input style="width: 80%;" type="text" value="250,000.00"/>
Consortium F&A			<input style="width: 80%;" type="text" value="0.00"/>
* Total Direct Costs			<input style="width: 80%;" type="text" value="250,000.00"/>
B. Indirect Costs			
	Indirect Cost Rate (%)	Indirect Cost Base (\$)	* Funds Requested (\$)
1. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text" value="51.00"/>	<input style="width: 80%;" type="text" value="250,000.00"/>	<input style="width: 80%;" type="text" value="127,500.00"/>
2. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>
3. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>
4. <input style="width: 90%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>	<input style="width: 80%;" type="text"/>
Cognizant Agency (Agency Name, POC Name and Phone Number)			<input style="width: 90%;" type="text"/>
Indirect Cost Rate Agreement Date			<input style="width: 80%;" type="text" value="04/24/2007"/>
			Total Indirect Costs <input style="width: 80%;" type="text" value="127,500.00"/>
C. Total Direct and Indirect Costs (A + B)			Funds Requested (\$) <input style="width: 80%;" type="text" value="377,500.00"/>

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3				
Start Date: <input style="width: 80%;" type="text" value="08/01/2010"/>	End Date: <input style="width: 80%;" type="text" value="07/31/2011"/>			
A. Direct Costs				
			Funds Requested (\$)	
* Direct Cost less Consortium F&A			<input style="width: 80%;" type="text" value="250,000.00"/>	
Consortium F&A			<input style="width: 80%;" type="text" value="0.00"/>	
* Total Direct Costs			<input style="width: 80%;" type="text" value="250,000.00"/>	
B. Indirect Costs				
	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base (\$)	* Funds Requested (\$)
1.		<input style="width: 40%;" type="text" value="51.00"/>	<input style="width: 40%;" type="text" value="250,000.00"/>	<input style="width: 40%;" type="text" value="127,500.00"/>
2.		<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>
3.		<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>
4.		<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>
Cognizant Agency (Agency Name, POC Name and Phone Number)				
Indirect Cost Rate Agreement Date <input style="width: 80%;" type="text" value="04/24/2007"/>				Total Indirect Costs <input style="width: 80%;" type="text" value="127,500.00"/>
C. Total Direct and Indirect Costs (A + B)				Funds Requested (\$) <input style="width: 80%;" type="text" value="377,500.00"/>
Budget Period: 4				
Start Date: <input style="width: 80%;" type="text" value="08/01/2011"/>		End Date: <input style="width: 80%;" type="text" value="07/31/2012"/>		
A. Direct Costs				
			Funds Requested (\$)	
* Direct Cost less Consortium F&A			<input style="width: 80%;" type="text" value="250,000.00"/>	
Consortium F&A			<input style="width: 80%;" type="text" value="0.00"/>	
* Total Direct Costs			<input style="width: 80%;" type="text" value="250,000.00"/>	
B. Indirect Costs				
	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base (\$)	* Funds Requested (\$)
1.		<input style="width: 40%;" type="text" value="51.00"/>	<input style="width: 40%;" type="text" value="250,000.00"/>	<input style="width: 40%;" type="text" value="127,500.00"/>
2.		<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>
3.		<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>
4.		<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>	<input style="width: 40%;" type="text"/>
Cognizant Agency (Agency Name, POC Name and Phone Number)				
Indirect Cost Rate Agreement Date <input style="width: 80%;" type="text" value="04/24/2007"/>				Total Indirect Costs <input style="width: 80%;" type="text" value="127,500.00"/>
C. Total Direct and Indirect Costs (A + B)				Funds Requested (\$) <input style="width: 80%;" type="text" value="377,500.00"/>

PHS 398 Modular Budget, Period 5 and Cumulative

OMB Number: 0925-0001
Expiration Date: 9/30/2007

Budget Period: 5	Start Date: <input type="text" value="08/01/2012"/>	End Date: <input type="text" value="07/31/2013"/>
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A. Direct Costs	Funds Requested (\$)
* Direct Cost less Consortium F&A	<input type="text" value="250,000.00"/>
Consortium F&A	<input type="text" value="0.00"/>
* Total Direct Costs	<input type="text" value="250,000.00"/>

B. Indirect Costs			
	Indirect Cost Rate (%)	Indirect Cost Base (\$)	* Funds Requested (\$)
1. <input style="width: 90%;" type="text"/>	<input type="text" value="51.00"/>	<input type="text" value="250,000.00"/>	<input type="text" value="127,500.00"/>
2. <input style="width: 90%;" type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
3. <input style="width: 90%;" type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
4. <input style="width: 90%;" type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Cognizant Agency (Agency Name, POC Name and Phone Number) <input style="width: 80%;" type="text"/>			
Indirect Cost Rate Agreement Date <input type="text" value="04/24/2007"/>		Total Indirect Costs <input type="text" value="127,500.00"/>	

C. Total Direct and Indirect Costs (A + B)	Funds Requested (\$) <input type="text" value="377,500.00"/>
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Cumulative Budget Information

1. Total Costs, Entire Project Period

* Section A, Total Direct Cost less Consortium F&A for Entire Project Period	\$ <input type="text" value="1,250,000.00"/>
Section A, Total Consortium F&A for Entire Project Period	\$ <input type="text" value="0.00"/>
* Section A, Total Direct Costs for Entire Project Period	\$ <input type="text" value="1,250,000.00"/>
* Section B, Total Indirect Costs for Entire Project Period	\$ <input type="text" value="637,500.00"/>
* Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	\$ <input type="text" value="1,887,500.00"/>

2. Budget Justifications

Personnel Justification	<input type="text" value="5387-PersonnelJustification.pdf"/>
Consortium Justification	<input type="text"/>
Additional Narrative Justification	<input type="text"/>

Attachments

PersonnelJustification_attDataGroup0

File Name

5387-PersonnelJustification.pdf

Mime Type

application/pdf

ConsortiumJustification_attDataGroup0

File Name

Mime Type

AdditionalNarrativeJustification_attDataGroup0

File Name

Mime Type

Budget Justification

Personnel:

, Ph.D.

Role: Principal Investigator (2.4 calendar person month effort)

has an appointment with and with the affiliated The institutional base salary used in this application represents the combined salary from both and which is paid by under a common paymaster. The number of person months in this application represents effort on the proposed project in relation to professional effort encompassed by the dual appointments.

Responsibility: will be responsible for planning experiments involving human cumulus cells studies and supervising the execution of the experiments. will be coordinating with physician colleagues in obtaining informed consent from oocyte donors and female patients attending the and will make himself available to discuss the research with patients if the patients request.

will be responsible for data collection and interpretation on experiments involving human cumulus cells (Aim 1 and 3) and communicates with Dr. the other PI, on experimental planning, execution, data interpretation, and trouble-shooting on the entire research project on human and mouse.

Ph.D.

Role: Principal Investigator (2.4 calendar person month effort)

Responsibility:

primary appointment is in the also has a secondary appointment in the where lab is located. has listed an effort of 20% throughout the grant period. has been working on genetic regulation of germ cell development using animal models. has been collaborating with to apply mouse ES gene trap technology to reproductive research and have successfully established a system in his lab from mining gene trap database to establishing ES cell lines to generating mutant mice. Characterizing the function of highly conserved protein- in mouse is one of the main focuses in lab. For this grant will be supervising the activities of postdoc and graduate student and discuss the experimental design and plan, data interpretation (Aim 2 and 4). will also be responsible for communicating with Dr. to coordinate our efforts in identifying conserved biomarkers for egg quality prediction (Aim 1, 2, 3 and 4).

M.D.

Role: Co-Investigator (0.24 calendar month effort with no salary requested)

Responsibility: Physician liaison. primary responsibility is to provide the research team (PIs) with clinical consultation with regard to all patients-related issues, such as clinical data interpretation, answering patients' questions on the research and consent. will devote 2% of his effort to this research.

B.S.

Role: Research technician (12 calendar month effort)

Responsibility: has a Bachelor of Science degree, majoring in Biology. responsibility will be to assist clinical lab staff to collect cumulus cells, and to store/process the cells to extract RNA or protein, to coordinate with the Dr. and staff, to carry out real time PCR and

microarray analyses. will also be involved developing and carrying out immunoassays described in Aim 3. will devote 100% of effort to this research.

B.S.

Role: Graduate student (12 calendar person month effort)

Responsibility: is a second-year graduate student in the . Although is a junior graduate student, level of experience exceeds that of classmates and most graduate students in general. has worked in a fly lab for three years as an undergraduate, including the completion of an honor thesis. After graduation, took a technician position in a mouse developmental biology lab and learned many different techniques as well as being in charge of a project in the lab. experience and skill in both genetics and mouse developmental biology makes well-qualified for participation in this project. will be responsible for alcohol effect on egg quality in Aim 2 and establishing new gene trap mouse lines in Aim 4.

Ph.D.

Role: Postdoctoral Fellow (12 calendar person month effort)

Responsibility: is a very experienced cell biologist. has extensive experience in working with embryonic stem cells and embryonic germ cells. is also a versatile molecular biologist who could independently learn and perform many molecular biology experiments. recently gained a lot of experience in performing IVF on the characterization of mouse mutants defective in such early stage. will be responsible for gene profiling of cumulus cells from different age groups and alcohol-treated or non-treated as well as characterization of Pum function (Aim 2 and Aim 4).

M. S.

Role: Biostatistician and microarray analyst (2.4 calendar month effort)

Responsibility: under supervision of our collaborator Dr. will be responsible for our microarray data analysis under Aim 1 and 2. will also provide statistical help in our data analysis.

Ph.D.

Role: Collaborator for Aim 2

Responsibility: is a well-known mouse behavior biologist who is an expert on alcohol's effect on pregnant rats and mice. group recently has uncovered a novel molecular mechanism underlying the reduced litter sizes due to alcohol consumption in mother rats. Dr. provides expertise and assistance in our experiment with alcohol-treated mice versus non-treated mice under Aim 2. will also be involved in the discussion of our results and interpretations.

Ph.D.

Role: Collaborator for Aim 1, 2 and 3

Responsibility: is a local expert on biostatistics and microarray data analysis and interpretation. will provide advice as to the method and procedure for the analysis of our microarray data in Aim 1 and 2. Dr. also will supervise a biostatistics analyst (20% effort) to work on microarray data analyses in Aim 1, 2 and 3.

Principal Investigator/Program Director (Last, first, middle):

, Ph.D.

Role: Collaborator for Aim 1 and Aim2

Responsibility: microarray experiments in core facility. is the director of core facility and will provide advice and help in the microarray experiments for both human and mouse cumulus cells (RNA labeling, hybridization and washing).

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page and PHS398 Checklist. The responses provided on these pages, regarding the type of application being submitted, are repeated for your reference, as you attach the appropriate sections of the research plan.

*Type of Application:

- New
- Resubmission
- Renewal
- Continuation
- Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

- 1. Introduction to Application
(for RESUBMISSION or REVISION only)
- 2. Specific Aims
- 3. Background and Significance
- 4. Preliminary Studies / Progress Report
- 5. Research Design and Methods
- 6. Inclusion Enrollment Report
- 7. Progress Report Publication List

Human Subjects Sections

Attachments 8-11 apply only when you have answered "yes" to the question "are human subjects involved" on the R&R Other Project Information Form. In this case, attachments 8-11 may be required, and you are encouraged to consult the Application guide instructions and/or the specific Funding Opportunity Announcement to determine which sections must be submitted with this application.

- 8. Protection of Human Subjects
- 9. Inclusion of Women and Minorities
- 10. Targeted/Planned Enrollment Table
- 11. Inclusion of Children

Other Research Plan Sections

- 12. Vertebrate Animals
- 13. Select Agent Research
- 14. Multiple PI Leadership
- 15. Consortium/Contractual Arrangements
- 16. Letters of Support
- 17. Resource Sharing Plan(s)

18. Appendix

Attachments

IntroductionToApplication_attDataGroup0

File Name

Mime Type

SpecificAims_attDataGroup0

File Name

7749-SpecificAims.pdf

Mime Type

application/pdf

BackgroundSignificance_attDataGroup0

File Name

6929-BackgroundSignificance.pdf

Mime Type

application/pdf

ProgressReport_attDataGroup0

File Name

5687-Prelimstudies.pdf

Mime Type

application/pdf

ResearchDesignMethods_attDataGroup0

File Name

7008-ResPlan.pdf

Mime Type

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InclusionEnrollmentReport_attDataGroup0

File Name

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ProgressReportPublicationList_attDataGroup0

File Name

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ProtectionOfHumanSubjects_attDataGroup0

File Name

3995-Human_Subjects.pdf

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InclusionOfWomenAndMinorities_attDataGroup0

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8093-WomenMinorities.pdf

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TargetedPlannedEnrollmentTable_attDataGroup0

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5353-EnrollmentTable.pdf

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7547-Children.pdf

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VertebrateAnimals_attDataGroup0

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6815-Vertebrate_Animals.pdf

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Mime Type

MultiplePILeadershipPlan_attDataGroup0

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2805-MultiplePI.pdf

Mime Type

application/pdf

ConsortiumContractualArrangements_attDataGroup0

File Name

Mime Type

LettersOfSupport_attDataGroup0

File Name

Mime Type

Principal Investigator/Program Director (Last, first, middle):
3529-Support_Letter.pdf

application/pdf

ResourceSharingPlans_attDataGroup0

File Name

9035-ResourceSharing.pdf

Mime Type

application/pdf

Appendix

File Name

Mime Type

A. SPECIFIC AIMS

Oocyte quality is required for conception and birth of a healthy child. Oocyte quality deteriorates as maternal age advances, an effect that may be exacerbated by exposure to environmental factors such as smoking and pollutants. In addition, alcohol is detrimental to fetal development but its effect on oocyte quality is not well studied. Furthermore oocyte quality is an important determinant for the success of infertility treatment by in vitro fertilization (IVF).

Significant progress has been made in understanding the molecular events involved in oocyte development and the factors that may determine oocyte quality in model organisms, but very little is known about similar mechanisms in the human. Studies of oocyte development and markers of oocyte health in the human, as well as in many other species, are hampered by the lack of reliable, non-destructive methods. **Our long-term goals are to identify biomarkers of oocyte quality and to use these markers, not only as research tools for investigating the regulation of oocyte development, but also as diagnostic or therapeutic tools to improve women's reproductive health.**

During oocyte growth and maturation, the oocyte is under the influence of its surrounding environment, particularly granulosa/cumulus cells. In turn, the oocyte regulates granulosa/cumulus cell activity during the course of follicular development. Thus, alterations in gene expression patterns in cumulus cells may either cause, or occur as a consequence of, abnormalities in the oocyte. **We hypothesize that negative effects of aging and other factors affecting oocyte quality are mediated by and/or reflected in changes in gene expression patterns in cumulus cells; accordingly, gene expression changes in cumulus cells may be useful for predicting oocyte quality.**

Unused cumulus cells collected during IVF provide an excellent opportunity for studying oocyte quality in the human. We have compared gene expression profiles between cumulus cells from good quality and poor quality oocytes from our IVF patients, and have identified a number of differentially expressed genes. We further assessed two of the differentially expressed genes, *PENTRAXIN-3 (PTX3)* and *PUMILIO1 (PUM1)*. We generated a transgenic mouse model carrying a lacZ reporter under control of the *Pum1* promoter and found that *Pum1* is expressed strongly in mouse cumulus cells. To extend these studies and achieve our long-term goals, we propose the following specific aims:

AIM 1. To identify differentially expressed genes in cumulus cells that correlate with the oocyte's potential to implant and with patient age (

A list of candidate genes will be analyzed for their relative mRNA abundance by quantitative real-time PCR (qRT-PCR) in four groups of cumulus cells collected from two populations of female subjects attending the Northwestern University IVF Program (Table A.1).

Table A.1: Grouping Cumulus Cells for Evaluating Potential Oocyte Quality Biomarkers

Age of subjects	Oocytes resulting in implanted embryos	Oocytes resulting in poor quality embryos in vitro
Women under 35 years of age*	Cumulus Group A	Cumulus Group B
IVF patients, >37 years of age	Cumulus Group C	Cumulus Group D

*Egg donors under 35 years of age will be included with IVF patients this group.

The list of candidate genes will be developed based on (1) our preliminary data on human cumulus cells; (2) findings published by other investigators in human and mouse models, and (3) conserved genes identified from mouse studies described in **Aim 2**.

Relative levels of candidate gene expression (mRNA) will be compared between the four groups of cumulus cells by standard analysis of variance and multiple range test methods. Variations in gene expression between Groups A and C should be small. Differences in gene expression between Groups A and B may reveal biomarkers related to non-age related adverse effects on oocyte quality, such as ovarian hyperstimulation in IVF treatment. Comparisons of gene expression in Groups A, C and D could reveal effects of age-related deterioration in oocyte quality, in addition to hyperstimulation-related effects. A sample size of 15 is needed to detect a difference equal to or greater than the standard deviation, with a power of 0.90 and a confidence interval of 95% ($\alpha=0.05$) (SigmaStat 1997, SPSS, Chicago, IL). With this sample size, a gene will be arbitrarily qualified as a marker if the relative abundance of its mRNA is different between Group A and another group with a p value equal to or less than 0.05, and will be further tested/validated in **Aim 3**.

AIM 2. To identify conserved oocyte-quality predictor genes in mice (

Oocyte maturation is a highly conserved physiological process present in all mammals and many vertebrates, hence signaling pathways between oocyte and cumulus cells are likely to be conserved. We

hypothesize that gene expression profile changes associated with variations in human oocyte quality will also occur in mouse cumulus cells. Animal models will allow us to set up controlled experiments to determine gene expression changes in oocytes from young and old mice, from mice exposed to alcohol, from superovulated ovaries, and from normal control mice. Ultimately, we will compare gene expression profiles from each of these mouse models with changes in human cumulus gene expression from **Aim 1** in order to identify additional genes with conserved expression profile changes. We expect to generate a list of biomarkers whose expression correlates with the decline of oocyte quality in both humans and mice caused by aging as well as by external factors such as alcohol exposure or superovulation.

AIM 3: Validate predictive values of the selected biomarkers in IVF patients ().

If mRNA levels of a candidate biomarker gene in cumulus cells in Group A (oocytes resulting in embryo implantation) are different from any other cumulus cell groups with a $n=15$ and $p \leq 0.05$ (Turkey's multiple range test), the expression products (mRNA and possibly protein) of this gene are likely to predict the implantation potential of an oocyte and may further distinguish the factors that affect oocyte quality, such as age, prior exposure to environmental pollutants, or excessive alcohol consumption. The list of candidate markers may be shortened to a few genes based on the findings of studies in **Aims 1 and 2** in order to construct a core panel of biomarker genes. The mRNA and protein products of genes in this panel will be determined in individual cumulus complexes from all consenting IVF patients (without grouping) via qRT-PCR or immunolinked assays. Correlation between the amount of biomarker mRNA or protein and oocyte quality (implanted or not implanted) will be quantified by logistic regression analysis, and a cut-off value for predicting oocyte quality will be estimated by Receiver-Operator Characteristics (ROC) curve analysis. Correlations between the levels of mRNA or protein of the marker genes and patient age and/or alcohol consumption will be determined by linear regression analysis.

AIM 4: To establish and characterize mouse biomarker reporter lines for egg quality ().

In order to further examine the utility of candidate genes as predictors of egg quality, we will characterize gene expression patterns by establishing reporter mice using gene trap technology. We hypothesize that mouse gene trap lines will allow us to visualize directly the cumulus expression of candidate biomarker genes and reveal those with the greatest predictive value for future testing in clinical trials. The advantages of generating gene trap lines are two-fold: first, the animal models can be exposed to environmental factors in order to identify other potential biomarkers of oocyte health; second, mutations that often occur in gene trap insertions may help uncover the function of candidate biomarkers in oocyte development. Further characterization of such mouse models could provide insight into mechanisms of oocyte-cumulus communication and their roles in determining oocyte quality.

This research is innovative because it addresses the long-standing lack of oocyte quality predictors using a multi-faceted head-on approach to identifying biomarkers in human cumulus cells. Animal models will then be used to refine the list of candidate genes to a robust set of core conserved biomarkers predictive of oocyte quality that can be validated in both humans and mice. Furthermore, by generating mouse reporter lines for the core biomarkers, we can study their physiologic role in mediating the effects of aging and environmental pollutants on oocyte quality.

B. BACKGROUND AND SIGNIFICANCE

Oocyte quality deteriorates as women age.

The age-related decline in women's fertility is caused by a depletion of oocytes and the deterioration of oocyte quality (1-5). Clinical evidence suggests that the age-related decline in oocyte quality is due to an increased incidence of meiotic division errors in the oocytes of older women, which leads to more frequent miscarriages and birth defects (6, 7). After IVF, embryos created using oocytes of older women are much less likely to implant than those created using oocytes of younger women (5, 8, Table C.1 in Preliminary Studies).

Oocyte depletion continues throughout life, and is known to be mediated by apoptotic processes at the cellular and molecular levels (8, 9). In contrast, the cellular or molecular mechanisms underlying the deterioration of oocyte quality are poorly understood. The molecular processes that cause the increase in meiotic errors remain unclear (6, 7). Most, if not all, of the oocytes present in an adult ovary are formed before birth. It is possible that oocyte quality deteriorates simply due to the prolonged exposure to unfavorable factors, such as metabolic byproducts and locally generated Reactive Oxygen Species (ROS).

Mitochondria are the sites of ROS production and are also the first line of cellular defense against toxic chemicals. Increased mutation rates in mitochondrial DNA in the oocytes from older women were observed in some studies (10), but not in others (11, 12). Telomere length is shortened both as a result of increased number of cell divisions and ROS assault (13). It has been suggested that the shortened telomeres in oocytes from older women is associated with age-related meiotic errors (14).

Other factors that may affect oocyte quality.

Involuntary exposure to environmental pollutants may damage the oocyte, also by eliciting ROS production. Certain unhealthy behaviors, such as cigarette smoking and excessive consumption of alcohol, may also decrease oocyte quality. In addition, certain medications may have a negative impact on oocyte quality. For example, cytotoxic chemotherapy drugs for cancer treatment are known to be detrimental to oocytes. It is well documented in a number of animal species that superovulatory doses of gonadotropins are detrimental to oocyte quality, resulting in reduced fertilization rates and compromised embryo implantation and development (15-19). The negative impact of gonadotropin stimulation on oocyte quality is largely due to the induced asynchrony of follicle development and oocyte maturation (20) and interference with the balance of steroid hormones in the follicular milieu (21). Despite the extensive use of gonadotropins in women for ovarian stimulation, information on its direct impact on oocyte quality is limited, and the negative effect of high doses of gonadotropins on oocyte quality may partially explain the poor implantation rates seen with IVF treatment. Studies of this phenomenon in IVF patients are usually complicated by indirect effects of high doses of gonadotropins on uterine receptivity (22, 23).

Alcohol consumption in pregnant mothers is known to have detrimental effect on fetal development, often inducing anatomical defects of the human Fetal Alcohol Syndrome (FAS) (24-26). FAS occurs in a substantial proportion of infants born to mothers who are chronic heavy daily drinkers (27). Extensive studies have been carried out to reveal the mechanism of alcohol consumption by the mother on fetal development, yet much less is known about the susceptibility of oocyte development and preimplantation stage embryo development to maternal alcohol exposure. Among the few studies investigating the relationship between oocyte quality and alcohol consumption, Cebal *et al.* reported that chronic moderate ethanol intake by prepubertal female mice reduces the ovulatory response and impairs *in vitro* fertilization and *in vitro* embryo preimplantation development (28-30). These data suggest that chronic alcohol consumption may reduce the quality of oocyte, but further studies are needed in order to understand the mechanisms underlying this effect, i.e., if the mechanisms are similar to those involved in age-related decline in oocyte quality. Identification of biomarkers of oocyte quality could help elucidate the mechanisms of alcohol-related oocyte quality decline.

Cumulus cells may contain biomarkers that reflect oocyte quality.

Close communication and interdependence exist between the oocyte and its surrounding somatic cells throughout the course of oocyte growth and maturation and the accompanying follicular development (31-33). The oocyte acquires competence for maturation after it completes the growth phase, while the follicle transitions from the primordial stage to the primary follicle stage. Normal oocyte growth requires the presence of granulosa cells (34). In response to the preovulatory surge of Luteinizing Hormone (LH), the oocyte in a follicle destined to ovulate enters the maturation phase by resuming and completing the first meiotic division. The oocyte does not express receptors for gonadotropins; thus, the resumption of meiotic division triggered by LH must be mediated indirectly by granulosa/cumulus cells that surround the oocyte within an antral follicle, (35, 36). Oocytes can resume first meiotic division, also known as *nuclear* maturation, independent from LH control, if they are isolated from the maturation inhibiting environment in the follicle. However, without

granulosa/cumulus support, these mature oocytes have limited potential to give rise to viable embryos. This suggests that granulosa/cumulus cells also play an important role in supporting *cytoplasmic* maturation in the oocyte (4, 37). In addition to their supporting role in oocyte development, cumulus cells are also required for the natural depletion of oocytes and follicles, a process called atresia. Cumulus cells mediate the apoptotic process in the oocyte by transmitting apoptotic signals via gap junctions (38).

The oocyte is not a just passive recipient of granulosa/cumulus regulation, however. The oocyte can regulate follicular development. The formation of the primordial follicle is dependent on the presence of a viable oocyte. Further development of the follicle also requires signals from the oocyte. For example, the differentiation of the theca component of the follicle is regulated by a growth factor from the oocyte, Gonadal-Derived Factor-9 (GDF-9), which is produced by the oocyte during its growth phase and declines only after ovulation. Together with gonadotropins, GDF-9 from the oocyte modulates steroid production in granulosa cells. The expansion of cumulus cells during oocyte maturation is also controlled by oocyte-derived GDF-9 (32, 33).

Taken together, we hypothesize that changes in activities of cumulus cells, as reflected in alterations in gene expression and protein production, may either result from or cause abnormal growth, development and maturation of the oocyte. Identification of changes in cumulus cell gene expression may reveal biomarkers that can predict oocyte quality.

Possible biomarker candidates in cumulus cells.

Candidates for oocyte quality biomarkers may include several functional groups:

Mediators of LH-triggered oocyte maturation.

Early in vertebrate oogenesis, germ cells enter the meiotic cell cycle and then arrest in prophase of meiosis I for extended periods of time, up to 40-50 years in humans, for example (39). During this time, the oocyte grows and accumulates crucial macromolecular components for supporting later development (40). Release from meiotic arrest marks the initiation of oocyte maturation, an essential cellular event that prepares the egg for fertilization and early embryonic development (39). In most mammalian species studied, meiotic arrest is maintained by cAMP and, thus, cumulus cells may mediate the induction of maturation by lowering or neutralizing cAMP signaling in the oocyte. Steroid hormones and prostaglandins produced by granulosa/cumulus cells have been implicated in mediating LH-stimulated oocyte maturation (41-43). Thus, hormones or paracrine factors produced in the cumulus cells may be natural candidates for biomarkers of oocyte quality. The level of steroid hormones produced by follicular cells may predict oocyte quality (44-46). However, granulosa cells transition to luteal cells during the preovulatory period, a time marked by dynamic changes in steroid hormone production profiles. The impact of individual steroid hormone levels at a single time point is difficult to interpret and these factors have not proven to be reliable and sensitive predictors of oocyte quality.

Factors associated with ovulation.

Apart from steroid hormones, granulosa/cumulus cells employ a variety of molecules to prepare the follicle for ovulation, including growth factors, proteins and enzymes that are involved cell division, cell differentiation, metabolism, apoptosis and tissue remodeling (47, 48). Because of the complexity of cellular events associated with ovulation, a great deal of effort would be required to sort through the ever-increasing number of regulatory molecules to determine which are predictive of oocyte quality. Numerous studies have assessed the predictive values of some of the cellular events or have measured specific regulating factors (e.g. (49-52)). However, these studies have not led to the development of reliable biomarkers because, among other reasons, the sample sizes of these studies were not large enough to produce conclusive, reproducible results.

Factors regulated by oocytes.

Molecules and cellular events within cumulus cells that are affected by the oocyte would be natural candidates for predicting oocyte quality. The identification of oocyte-derived regulator molecules, such as GDF-9 and Bone Morphogenic Protein-15 (BMP-15), has led to the identification of many novel target molecules in the cumulus cells, including Pentraxin-3, a protein implicated in inflammatory responses (53). Some GDF-9-regulated genes in cumulus cells have been studied for their value in predicting oocyte quality (54, 55) but other molecules have yet to be evaluated (56, 57). Oocyte-secreted factors can also be measured directly in the follicular fluid and their levels have been investigated as predictors of oocyte quality (58)

Novel candidates from gene expression profiling.

DNA microarray has been used to efficiently and quickly profile changes in the expression levels of novel genes in cumulus cells that are associated with oocyte quality (55) or follicular development (59-61). The

expression of these novel genes may predict oocyte quality. Gene expression profiling of mice with known fertility defects may also reveal novel genes as candidates for oocyte quality markers (53, 62).

Conservation of molecular mechanisms of ovarian development including oocyte-cumulus communication in mammals.

The major function of the female gonad is the development and release of the mature oocyte for fertilization. In mammals, follicles develop through primordial, primary and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, while a few respond to gonadotropin stimulation and reach the preovulatory stage (63-65). These large, preovulatory Graafian follicles are the major source of ovarian estrogens in women of reproductive age. In response to a preovulatory gonadotropin surge, the dominant Graafian follicle ovulates to release a mature oocyte competent for fertilization. The remaining theca and granulosa cells undergo transformation to become the corpus luteum.

The pool of oocytes in the mammalian ovary is fixed early in life; thus, ovarian senescence is linked to the dwindling supply and eventual exhaustion of the pool of primordial follicles. Cyclic recruitment and selection of follicles represents a continuous process, eventually leading to the emergence of the preovulatory follicle(s). Important landmarks of ovarian development in rodents are similar to those in the human; however, the timing is greatly compressed in rodents and multiple follicles become dominant during each estrous cycle (40, 65). A rapidly growing body of evidence from rodents, in particular mice, has revealed multiple, complex molecular pathways and gene products that direct ovarian development and maturation and oocyte-cumulus communication in the developing follicle (32, 39; 59, 66). Human homologs of many of these important ovarian genes have been identified and some have been localized to the human ovary (55). Several of the mouse and human homologs have conserved functions (67), while others do not (68). The fundamental mechanisms that regulate oocyte-cumulus communication and oocyte maturation are likely to be conserved; indeed, ovulation and the response to gonadotropins are conserved processes in mammals and lower vertebrates. Identification of genes involved in oocyte-cumulus communication whose expression in cumulus cells correlates with oocyte quality in both humans and mice could lead to the discovery of robust and reliable egg quality biomarkers.

Gene trap mutagenesis is an efficient way to mutate reproductive genes and to generate a reporter mouse model.

Animal models have played critical roles in improving our understanding of mechanisms of disease pathogenesis. Mouse knockout models have often provided highly needed functional validation of genes implicated in human diseases or potential biomarkers for human disease conditions. Gene trapping is a high-throughput approach for generating mutations in murine embryonic stem (ES) cells through vectors that simultaneously disrupt and report the expression of the endogenous gene at the point of insertion. In a single transfection, hundreds to thousands of random insertional mutations can be recovered, each of which is immediately accessible by molecular characterization. Gene trap vectors result in the production of a reporter-gene fusion transcript after insertion of the trapping vector into endogenous transcription units. The expression of the trapped gene is tagged by the reporter, and a portion of the target gene can be cloned directly from the fusion transcript (69-72). Thus, the rate at which new mutations can be produced with gene-trapping techniques exceeds, by two to three orders of magnitude, that obtainable by conventional gene-targeting approaches. Moreover, unlike the vast majority of mice that have been created by gene targeting, all of the knockouts produced by this technique result in the production of a visible marker for histological studies.

Various laboratories worldwide have conducted large-scale efforts to generate gene trap ES cell lines, with the ultimate goal of trapping every gene in the murine genome (69-71, 73-84). In an effort to consolidate this data, the International Gene Trap Consortium (IGTC) was created with the goal of providing a free library of all publicly-available gene trap cell lines from its members (80): BayGenomics, Centre for Modeling Human Disease, Embryonic Stem Cell Database, Exchangeable Gene Trap Clones, German Gene Trap Consortium, Sanger Institute Gene Trap Resource, Soriano Lab Gene Trap Database, and TIGEM-IRBM Gene Trap. This effort saves researchers the tedious and sometimes challenging tasks of making knockout vectors and screening ES cell colonies by providing researchers with an ES cell clone that carries the mutation of the gene of interest. To date, the IGTC has generated more than 57,000 cell lines, representing over 9,000 known mouse mutations or approximately 40% of known mouse genes (81). The sequence tags for the trapped cell lines are fully annotated in a user-friendly, searchable website located at the IGTC website (<http://www.genetrapp.org>), which also contains features such as useful documentation, the ability to view trapped genes within biological pathways, and the ability to search by expression profile (80). In addition, the sequence tags are mapped on the Ensemble mouse genome browser (http://www.ensembl.org/Mus_musculus) under the DAS source "Gene Trap" (79). The development of the IGTC website marks a significant advance by providing the research community with the data and tools

necessary to utilize public gene trap resources for the large-scale characterization of mammalian gene function.

The ability to efficiently trap, sequence and detect the expression of genes, regardless of their transcriptional activity, has made gene trapping an exceptional tool for gene discovery, especially with the establishment of the IGTC. Moreover, the versatility of this approach has been improved by the development of vectors that include recombination or promoter sites (82, 85). Gene trapping has been utilized in many areas of research, including apoptosis, neurology and hematopoiesis (83, 84, 86). Reproductive research also has great potential to benefit from this method of mutagenesis, evidenced by increasing reports of reproductive mutants induced by gene trap mutations (87-100). and his colleagues have been actively evaluating the efficacy of gene trapping with the goal of using this resource to perform functional analyses of genes involved in reproduction. Our group has generated about a dozen lines of mice from gene trap ES cell lines that contain insertions in reproductive genes (97, 100, 101). Furthermore, we have evaluated the efficacy of gene trapping for reproductive research using in-house software mining the entire IGTC database with recent microarray and proteomics data from human and mouse reproductive organs. Our research clearly shows that gene trap techniques can be used to target genes expressed in reproductive tissues and can be an important tool for reproductive research (Preliminary Studies).

***Pumilio* gene family members are highly conserved reproductive regulators that play important roles in ovarian development and oocyte-cumulus interaction.**

Pumilio homologs encode conserved RNA binding proteins that may play important roles during different stages of development in diverse organisms (102-121). The first *Pumilio* homolog was identified in *Drosophila*, in a screen for maternal factors that are required for embryo development; in this case, for anterior-posterior patterning of embryos (122, 123). Loss of function of *Pumilio* in *Drosophila* results in the disruption of the anterior-posterior gradient of hunchback protein; subsequently, mutant embryos fail to develop abdominal segments (117). Later in development, *Drosophila Pumilio* is required for development and migration of the embryonic primordial germ cells, regulation of germ line stem cell renewal inside the ovary, differentiation and neuronal development (102, 103, 124). In the evolutionarily-distant nematode, *Caenorhabditis elegans* (*C. elegans*), *Pum* homologs regulate the sperm-oocyte switch as well as the maintenance of germ line stem cells and the survival, migration and mitotic proliferation of germ cells (109, 125). Recently, the frog homolog of *Pum* protein has been shown to play a key role in meiotic maturation (119). Thus, although in different lineages, *Pum* homologs have diverse functions in multiple compartments, in particular reproductive development, and the ancestral function of this highly conserved *Pum* family is thought to be critical for development of the germ line, possibly in the stem cells of the germ cell lineage (126).

Much less is known about the function of *Pum* homologs in mammals. In humans and mice, two highly-conserved *Pum* homologs (*Pum1* and *Pum2*) have been identified and characterized (127-131). We have shown that murine *Pum2* is highly expressed in reproductive tissues and its expression pattern is developmentally regulated in both the testis and ovary (100). Expression is not limited to germ cells, with *Pum2* localizing to somatic cells of the testis and ovary, including both granulosa and theca cells. Recently Vessey *et al.* (132) showed that *Pum2* mRNA is expressed during neuronal development and the protein is found in stress granules in neurons during metabolic stress. The role of *Pum* proteins in the stress response is novel for mammalian *Pum* proteins, but has been reported for the yeast homolog of *Pum*—Mst5p (121, 133-135). Cytoplasmic RNA granules in germ cells (polar and germinal granules), somatic cells (stress granules and processing bodies, and neuronal granules) have emerged as important players in the posttranscriptional regulation of gene expression (136, 137). The fact that *Pum* homologs are found in germ cell granules in fly and worm and in stress granules in mammalian neurons and that the key functional domain --RNA binding domains are extraordinarily conserved raise an interesting possibility that the *Pum* protein maintains a highly conserved function in stress response in eukaryotes.

During our investigation of *Pum* protein expression, we observed a granular staining pattern in reproductive tissues including granulosa cells (**Preliminary Studies**). Furthermore, *PUM1* has been found to be highly expressed in human cumulus cells from poor quality oocytes when compared with cumulus cells from high quality oocytes. Thus, we propose the experiments outlined in our Research Plan to test the hypothesis that *Pum* protein is a stress-response protein that may be a useful biomarker to predict oocyte quality.

Significance of identifying biomarkers of oocyte quality in cumulus cells.

Oocyte quality is essential for successful reproduction. Poor quality oocytes may have a decreased potential for fertilization, implantation or further development. The preimplantation development of the embryo is largely driven by the maternal genome inherited in the oocyte. Meiotic division errors common in poor quality

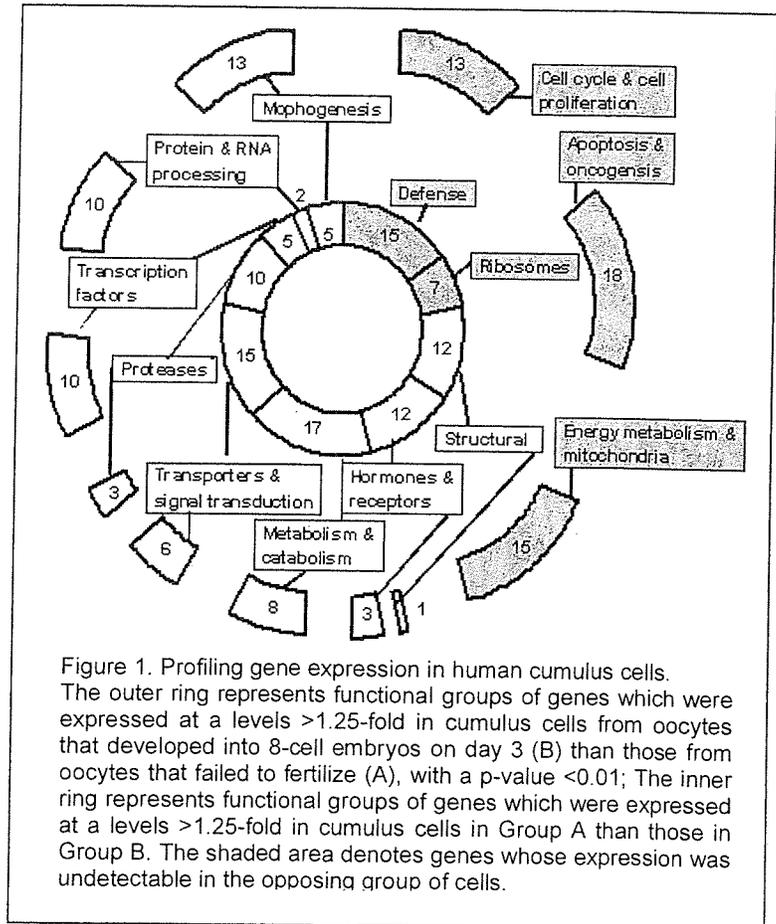
oocytes could lead to a higher frequency of miscarriage or birth defects. The mechanisms regulating oocyte quality are poorly understood at the molecular level. A major technical difficulty that hampers our understanding of the molecular events underlying oocyte quality is the sparse material available for studies. In addition, there are no non-destructive methods for determining oocyte quality, and as such, ethical and social issues currently present barriers to obtaining oocytes for scientific manipulation and research. Identifying biomarkers of oocyte quality in cumulus cells will have the following clinical significance:

- 1) Provide important tools for scientific investigation of oocyte development and quality. Biomarkers in cumulus cells associated with oocyte quality could provide important insights into the cellular and molecular pathways essential for normal oocyte development and maturation.
- 2) Provide important tools for clinical management of women's reproductive health. Cumulus cells are abundant and are disposable byproducts of oocyte retrieval for IVF treatment. Collection and analysis of cumulus cells does not harm the oocyte. Biomarkers from cumulus cells can be used for diagnosis of infertility or reproductive toxicity. They can be used to improve the efficiency and efficacy of infertility treatments by identifying the most viable oocytes for fertilization in vitro. This will reduce the need for, and side effects associated with, hyperstimulation of ovarian development during IVF. By limiting the number of oocytes needed for fertilization, fewer extra embryos will be generated, which avoids the ethical and social complications of handling and disposing of these extra embryos. It will also reduce the number of embryos transferred, thus reducing the risk associated with high-order multiple gestations.

C. PRELIMINARY STUDIES

Our hypothesis is that the gene expression profile of cumulus cells reflects the quality of the oocyte they enclose. We also propose that some of these signature profile changes are conserved in mice and humans. Therefore, we propose to identify genetic changes in both human and mouse cumulus cells in order to characterize the conserved signature profile reflecting oocyte quality. Furthermore, we will establish reporter mouse models that carry lacZ reporters downstream of validated genes predictive of oocyte quality. These animal models will allow us to carry out further mechanistic studies of factors involved in modifying oocyte quality decline, including normal aging and environmental assaults such as alcohol exposure. Our hypothesis was developed through interdisciplinary discussions between researchers in the fields of embryology, molecular biology, human oocyte biology and mouse ovarian biology. Preliminary data were collected in both Dr. and Dr. laboratories and provide further support for their hypothesis. In summary, we have made the following advances in our research:

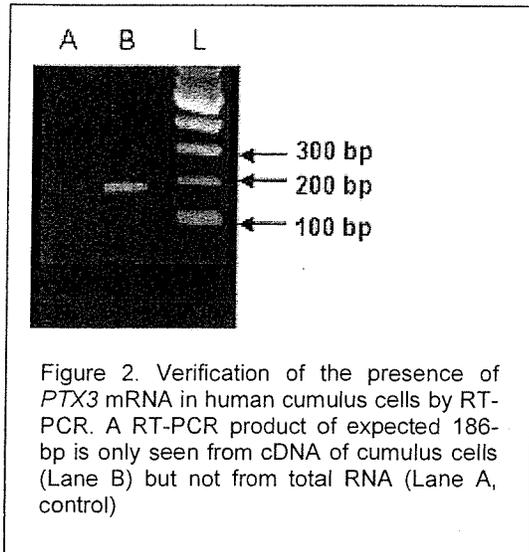
- Global gene expression profiling of human cumulus cells from oocytes used in successful or unsuccessful IVF
- Identification of 160 significantly upregulated or downregulated genes in cumulus cells from oocytes used in successful or unsuccessful IVF
- Demonstration of pentraxin-3 (Ptx3) expression in human cumulus cells and the correlation between Ptx3 expression and oocyte potential for successful IVF. This finding also supports the existence of conserved mechanisms of cumulus-oocyte communication in humans and mice.
- Demonstration of the conservation of a reproductive regulator, *BOULE*, in insects and mammals, making a clear case for the functional conservation of reproductive regulators across large evolutionary distances
- Reconstruction of the evolutionary history of a human reproductive gene family that originated with a single meiotic regulator, and gave rise to three members with distinct yet overlapping functions, thus providing clear evidence that not all reproductive genes evolve rapidly, as well as providing an example of how a human reproductive gene family has been derived evolutionarily
- Demonstration of the feasibility of gene trap mutagenesis for reproductive research
- Establishment of protocols for generating mice from ES gene trap cell lines and use of bioinformatic software for large-scale mining of the IGTC ES database for functional genomics in reproductive research
- Generation of several mouse gene trap mutations affecting the highly conserved germ cell regulators *Pumilio1*, *Pumilio2*, *Mouse vasa homologue (Mvh)*, *Outer dense fiber 2 (Odf2)*, and *DAZ interacting protein 2 (Dazap2)*
- Functional characterization of murine homologues of highly conserved RNA binding proteins *Pum1* and *Pum2*, suggesting that *Pum1* is required for early embryonic development and that *Pum2* is not essential for viability but is required for normal fertility in males
- Determination of the gene expression patterns of *Pum1* and *Pum2* during embryonic development and in adult testes and ovaries



Our previous work is discussed in more details below.

Differential gene expression in cumulus cells is associated with oocyte quality.

Dr. (PI) and colleagues carried out a study to search for differentially expressed genes in cumulus cells from IVF patients that could be associated with oocyte quality. In the first experiment, global gene expression profiles of cumulus cells from oocytes that failed to fertilize in vitro (Group A) and those that developed into normal-appearing embryos on day 3 (Group B) were obtained by DNA microarray analyses and compared between the two groups of cells. The comparison identified 160 upregulated or downregulated genes, including *PENTRAXIN-3* (*PTX3*, upregulated), a target gene in mouse cumulus cells under oocyte



regulation that was required for oocyte fertilization (Figure 1). The next experiment confirmed the presence of *PTX3* mRNA in human cumulus cells by reverse transcription-PCR analysis (Figure 2). In the third experiment on a small number of cumulus complexes, quantitative analyses by real-time PCR demonstrated a significant difference in the relative abundance of *Ptx3* mRNA between cumulus cells from good quality and poor quality oocytes (based on development in vitro; Figure 3) (56).

This study (1) demonstrated the technical feasibility of analyzing gene expression in human cumulus cells, "byproducts" of oocyte retrieval procedures in IVF treatment for infertility, (2) identified candidate genes in cumulus cells that may be biomarkers for oocyte quality, and (3) provided confirmative evidence that in human cumulus cells, as in the mouse model, *Ptx3* and possibly other genes are regulated by the oocyte through oocyte-secreted factors, such as GDF-9.

Several weaknesses/shortcomings of this study will be addressed by the current proposal. First, because a large proportion of embryos with good morphological scores fail to implant after embryo transfer in IVF treatment for infertility, the ability to implant is a better end-point for oocyte quality than morphological score of embryo development in vitro. Second, the source of cumulus cells was heterogeneous in the earlier study because it included patients of different ages and causes for infertility. Lastly, the small sample size and its heterogeneity likely severely reduced the "resolution" of the search for an accurate oocyte quality marker, since the differences in gene expression related to oocyte quality could have been lost due to intersample variation or offset by differences in the women's ages or their causes of infertility.

In addition to *PTX-3*, we have also observed the significant change in expression of another protein -- *PUMILIO1* (*PUM1*), a member of highly conserved *PUF* (*Pumilio* and *FBF*) gene family. *PUM1* was one of the

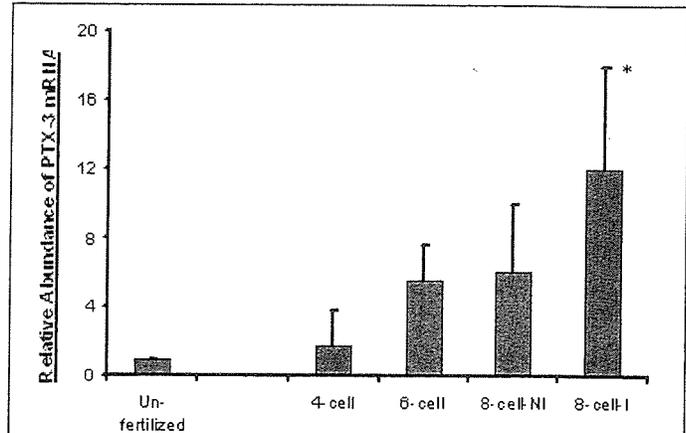


Figure 3. Relative abundance of *PTX3* mRNA in human cumulus cells.

Total RNA was extracted from individual cumulus complexes and reverse transcribed to obtain cDNA which was then subjected to real-time PCR. The relative abundance of *PTX3* mRNA was the difference between the number of cycles of amplification for *PTX3* to reach an arbitrary threshold and the number of cycles of amplification for 18s RNA to reach this threshold in the same sample. The relative abundance of *PTX3* in cumulus cells from fertilized oocytes is expressed as the number of folds changes from that of cumulus cells of unfertilized oocytes. Embryos in "8-cell-NI" were those in an embryo cohort that were transferred but did not establish a pregnancy; and embryos in "8-cell-I" were in an embryo cohort that were transferred and led to the establishment of clinical pregnancy.

* Compared with cumulus cells from unfertilized oocytes, $p < 0.01$ (*t*-test)

downregulated genes identified in the earlier microarray study (56, 126). PUF family has been shown to be important for the regulation of reproductive development in diverse species from invertebrates to vertebrates. And recently [redacted] has shown that murine homologs of Pumilio are highly expressed in the ovary, in particular in granulosa cells (see below Figure 11) (101). We have further confirmed the expression of human *PUM1* expression in cumulus cells from individual follicles by quantitative real-time PCR (Figure 4).

Oocyte quality, determined by the ability to implant after in vitro fertilization, is highly age-dependent, whereas embryo cleavage in vitro is less affected by age.

Analysis of recent clinical data from IVF patients at [redacted] confirmed that oocyte quality deteriorates as a woman's age advances, and such deterioration is reflected in implantation rates rather than in vitro fertilization or embryo cleavage rates. (Table C.1).

Table C.1. Selected statistics of the IVF cycles corresponding to the two populations of cumulus cell donors at the

Age	IVF cycles	Pregnancies	Pregnancies with 100% implantation rate	Oocytes/cycle (mean \pm s.d.)	Fertilization rate	8-cell formation rate	Implantation rate
<35 years*	446	235	68	16 \pm 6	67%	31%	31%
>37 years	582	150	14	10 \pm 3	61%	30%	10%

*Including oocyte donors.

Efficacy and feasibility of using ES gene trap to target genes expressed in reproductive development.

Gene trapping is a high-throughput approach to generating mutations in murine ES cells using vectors that simultaneously disrupt and report the expression of the endogenous gene at the point of insertion. Gene

trapping has been shown to be effective in generating mutations affecting developmental processes in early embryonic development (139-141), but its application in reproductive research remains largely unexplored. We evaluated the efficiency of gene trapping in comparison with traditional knockout methods and used an in-house software program—

([redacted])—to screen the IGTC database for existing cell lines with possible mutations in genes expressed in various reproductive tissues. The advantage of our SpiderGene program is that it is able to conduct a large-scale search of the IGTC Web site rather than a single query at a time. The second advantage of this program is that it allows rapid BLAST searches of mouse cDNA sequences in the IGTC database. Search using cDNA sequences is preferred over gene names. Gene symbols are somewhat variable and referencing them is an unreliable way to search the IGTC database. Moreover, a substantial amount of time and effort is required to obtain a FASTA-formatted list of DNA sequences, perform a BLAST search on the IGTC database, and sort through the results to identify valid gene trap lines.

To compare the efficacy of gene trapping against other mutagenesis strategies in reproductive research, we used a list of 202 reported mouse reproductive mutations (142) and asked how many of those genes with reproductive function have been targeted in the current gene trap ES cell database. These gene mutations cause various reproductive defects and mouse models were generated by various mutagenesis schemes, including spontaneous mutations, fortuitous transgenic integration, retroviral infection of embryonic stem cells, ethylnitrosurea (ENU) mutagenesis and gene

targeting technologies (142). After processing these genes with [redacted] results revealed that 76 genes, or 38% of the list, had been trapped using gene trap mutagenesis. Gene trapping technology alone has targeted more than one third of known reproductive genes in mice, which have been mutated using various mutagenesis methods in the past several decades. Thus, we concluded that gene trapping is a valid way to approach functional analysis in reproductive research, and that the percentage of gene trapped reproductive genes will continue to increase with next generation vectors and improved efficiency, as well as rapidly expanding cell lines.

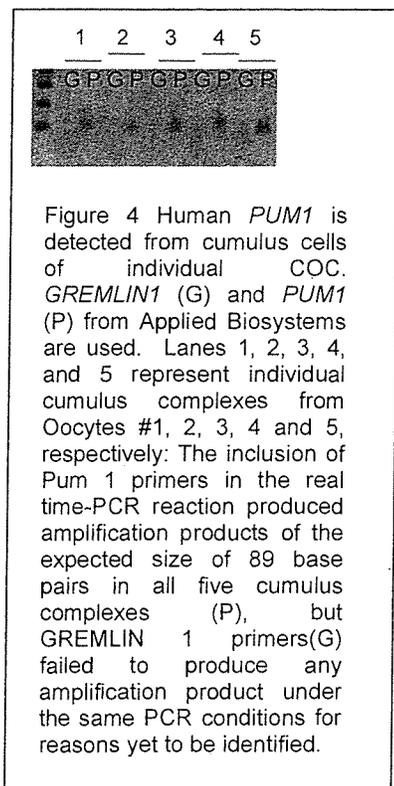


Figure 4 Human *PUM1* is detected from cumulus cells of individual COC. *GREMLIN1* (G) and *PUM1* (P) from Applied Biosystems are used. Lanes 1, 2, 3, 4, and 5 represent individual cumulus complexes from Oocytes #1, 2, 3, 4 and 5, respectively: The inclusion of Pum 1 primers in the real time-PCR reaction produced amplification products of the expected size of 89 base pairs in all five cumulus complexes (P), but *GREMLIN1* primers(G) failed to produce any amplification product under the same PCR conditions for reasons yet to be identified.

A total of ~3000 genes expressed in the testis have been trapped by at least one ES gene trap line. About 17% of premeiotic genes have been trapped. Even for subcellular organelles as specific as the acrosome, 30% of its known protein components exist as a gene trap line. We then focused on genes reported to be expressed in human and mouse ovary. On average, at least 50% of genes highly expressed in either human or mouse ovary have at least one hit in the IGTC ES database (Table C.2). These systematic analyses provide strong evidence that ES gene trap can efficiently mutate and localize a large number of genes expressed in reproductive tissues, thus potentially providing an efficient mouse reporter to monitor gene expression in reproductive tissues.

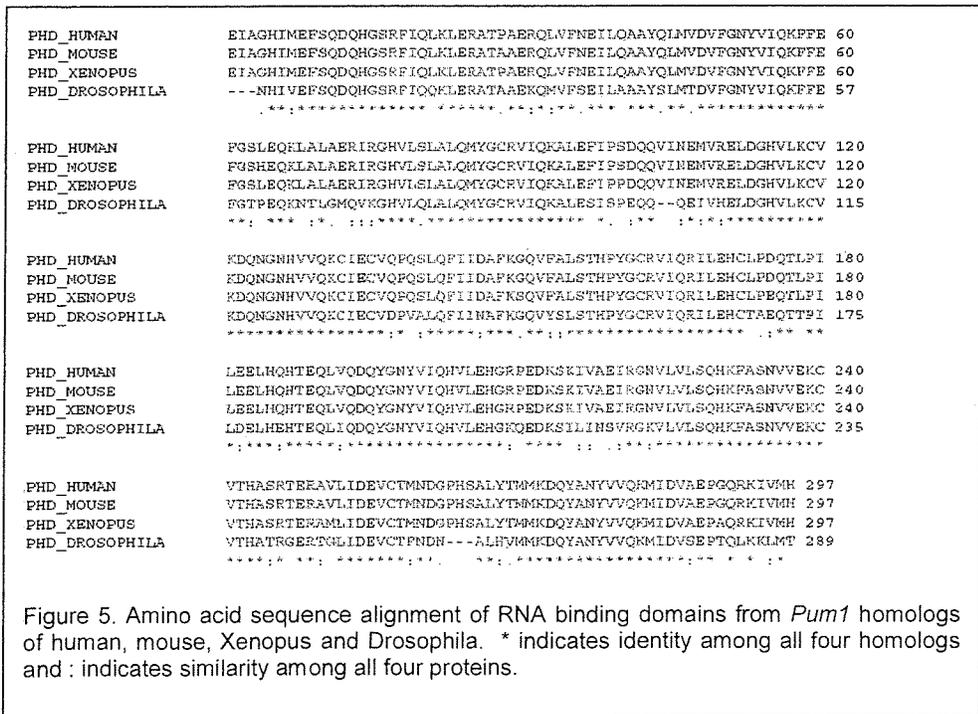
Table C.2. Percentage of Gene Trap Hits for genes expressed in human and mouse ovaries

Tissue	# of Genes Searched	# of Hits Found	Percentage of Hits Found	# of Genes with Restricted Expression	Reference
Ovary (mouse)	3267	1797	55%	8	(143)
Ovary (human)	7142	3476	49%	2	(143)

The validity of a "hit" is based on a user-defined criterion, and we decided on an arbitrary minimum hit score of 100. "Restricted expression" is also a user-defined criterion: based on Unigene's expression profile, at least 50% of the gene's expressed sequence tags (EST) must be expressed in male or female reproductive tissue. Additionally, the gene must have at least 100 transcripts per million in the reproductive tissue.

Mammalian *Pum1* is a highly conserved RNA binding protein expressed in male reproductive organs in mice.

We compared mouse and human RNA binding domains, also called the *Pumilio* homology domain (PHD), in mouse *Pum1* and human *PUM1* with those of fly and frog homologs. *Drosophila* diverged from mammals at



least 600 million years ago, but the alignment of identical amino acids in *Pum1* protein from fly to human remaining is striking. It is clear that the RNA binding protein is highly conserved (Figure 5). Previously, it has been reported that the PHD domain is necessary and sufficient for *Pumilio* function during embryonic development. Such extraordinary conservation of a key functional domain suggests that the mammalian *Pum* protein plays a conserved key regulatory role. We hypothesized that human and mouse *Pum1* regulate reproductive development

through posttranscriptional regulation by binding to a number of mRNA transcripts of genes important for differentiation. We first examined *Pum1* protein expression in the mouse male reproductive system to determine the cellular and subcellular localization pattern. We found that *Pum1* is highly expressed in the testis, specifically, the *Pum1* protein localized to both germ cells and somatic cells of the testis (Figure 6). The strongest levels of *Pum1* expression localized to postmeiotic spermatids and interstitial somatic cells, the Leydig cells.

Generation and characterization of two mouse gene trap lines for *Pum1* gene.

The importance of Pum family proteins in reproductive development is illustrated by the widespread and conserved roles across diverse species such as fly, worm and frog. Microarray profiling of human cumulus cells by Zeng et al identified *Pum1* as one of genes whose expression was significantly different in cumulus cells associated with oocytes of good or poor quality (56). We hypothesized that the increase in *Pum1* gene expression occurs in response to cumulus cell stress. Enhanced transcription of RNA binding proteins such as Pum then leads to sequestration of bound mRNA in granules similar to stress granules observed in neurons. Thus, an increase in *Pum1* expression in cumulus cells may be a good predictor of stress level in the Cumulus-Oocyte-Complex (COC) complex and a predictive marker of oocyte quality. We decided to use gene trap to generate mouse reporter lines for the *Pum1* gene to characterize its function and expression patterns.

We identified two gene trap lines from the IGTC database and confirmed the insertion in *Pum1* genes by RT-PCR from ES cells. We also mapped the insertion points of both lines into the specific location of the genomic region of *Pum1* (Figure 7). In *Pum1*^{XB063}, the gene trap vector is inserted between exon 18 and 19, while *Pum1*^{XE002} contains the gene trap vector within the N-terminal of the protein, between exon 2 and 3

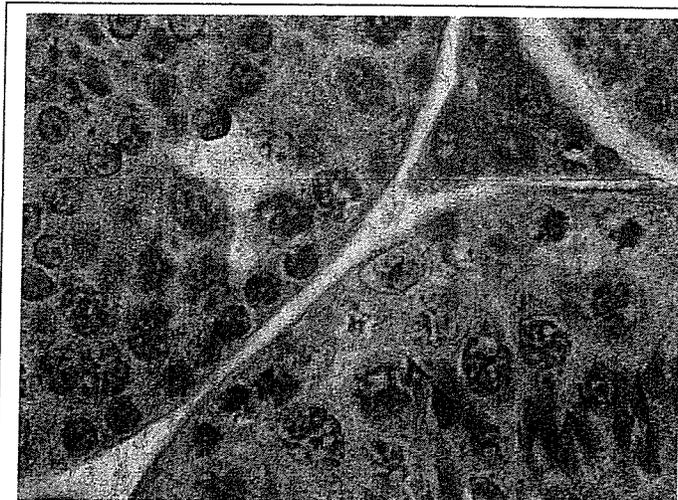


Fig. 6 *Pum1* protein is highly expressed in mouse germ cells and somatic cells in the testis. Immunostaining shows that highest expression of Pum is seen in postmeiotic spermatids and Leydig cells in the interstitial region.

(Figure 7). It is necessary to determine the precise location of the vector in the intron in order to design mutation-specific genotyping primers. This is achieved by using long-range PCR with primers spanning the entire intron where the insertion is located, with primers on the vector. The amplified PCR product is sequenced and the breakpoint between the vector and *Pum* intron is determined. We have observed up to 500-bp deletions in the vector at the insertion point.

We have established genotyping methods that allow us to successfully distinguish between the mutant allele and wild type allele. We further characterized each gene trap line by Southern hybridization, RT-PCR and immunoblot analysis. We first probed a Southern blot of a *Sac* I and *Sph* I genomic digestion from heterozygotes of *Pum1*^{XE002} and wild type lines. We detected an expected wild type band at 4.9 kb for *Sac* I digestion and 5.6 kb for *Sph* I digestion for both heterozygotes and the wild type (Fig 8 A and B). However, we

also detected expected 7.6 kb *Sac* I and 8.9 kb *Sph* I mutant fragments, which were only present in the heterozygotes. The same 8.9 kb *Sph* I fragment was also detected by a *LacZ* probe, confirming that it is a chimeric fragment between the vector and *Pum1* genomic DNA. The fact that there was only one *lacZ* band indicates that *Pum1*^{XE002} only contains a single gene trap vector inserted in the *Pum1* gene (Figure 8). The specific gene insertion was further confirmed by RT-PCR of exons spanning the insertion point and immunoblot analysis with *Pum1*

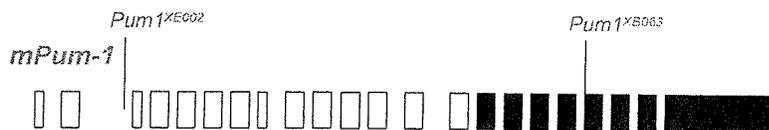


Figure 7 Two gene trap lines have been generated with the vectors insert at two different part of *Pum1* gene. Each box (open or filled) represents an individual exon of *Pum1* which consists of 22 exons. The filled boxes represent the highly conserved RNA binding domain. *Pum1*^{XE002} is inserted between exon 2 and exon3 while *Pum1*^{XB063} is inserted between exon18 and 19, which is inside the PHD domain.

antibody (Figure 8 C and D). Similar analyses have been carried out on *Pum1*^{XB063} to confirm the insertion and the formation of chimeric protein (unpublished). We generated two independent gene trap lines with insertion in two different parts of *Pum1* gene. In addition, we generated two gene trap lines for the other member of *Pum* family—*Pum2*. Using these lines, we will be able to examine the gene expression pattern of the entire *Pum* family in the developing ovary and granulosa cells of ovulated eggs.

Use of lacZ reporters to detect gene expression in reproductive tissues from gene trap ES cell-derived mice.

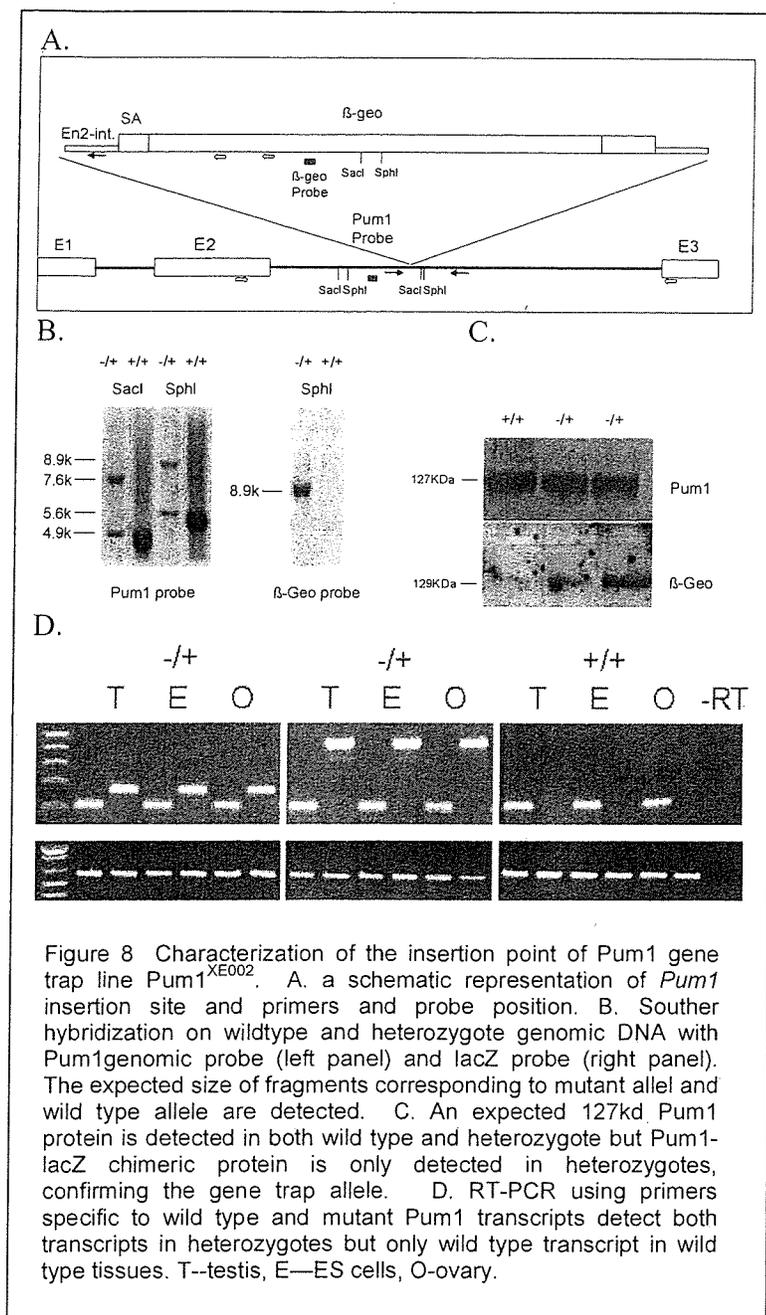


Figure 8 Characterization of the insertion point of *Pum1* gene trap line *Pum1*^{XE002}. A. a schematic representation of *Pum1* insertion site and primers and probe position. B. Southern hybridization on wildtype and heterozygote genomic DNA with *Pum1* genomic probe (left panel) and lacZ probe (right panel). The expected size of fragments corresponding to mutant allele and wild type allele are detected. C. An expected 127kd *Pum1* protein is detected in both wild type and heterozygote but *Pum1*-lacZ chimeric protein is only detected in heterozygotes, confirming the gene trap allele. D. RT-PCR using primers specific to wild type and mutant *Pum1* transcripts detect both transcripts in heterozygotes but only wild type transcript in wild type tissues. T--testis, E—ES cells, O-ovary.

We further determined that the lacZ assay can be used to reveal gene expression patterns of *Pum* family members. We first examined a gene trap line we established in the lab containing a lacZ insert in the *Pum2* gene in the testis (Figure 9). We characterized the localization patterns of the *Pum2* gene by tracking lacZ reporter expression during developmental changes in prepubertal and adult testes. As shown in Figure 9, lacZ staining was prevalent in both germ cells inside the seminiferous tubules and somatic cells in the interstitial area on postnatal day 1. The perinuclear localization pattern is strong in germ cells. In day 10 testes, many germ cells have migrated towards the basement of the tubule and lacZ staining is strongest in a subset of cells at the basement membrane. In adult testes, the somatic staining of lacZ disappears. Inside the tubules, only a small subset of germ cells on the basement membrane stain strongly while other germ cells stain weakly or not at all. These studies demonstrate the feasibility of using lacZ to characterize changes in gene expression patterns in gene trap ES cell-derived mice, and support the possibility of establishing a mouse oocyte quality reporter line.

***Pum1*-lacZ expression patterns in reproductive tissues of *Pum1* gene trap ES cell-derived mice.**

We first examined the *Pum1*-lacZ expression pattern in the testis and ovary of *Pum1*^{XE002} heterozygotes. There was clear lacZ staining in both testis and ovary sections (Figure 10), consistent with our previous finding that *Pum1* is expressed in both testis and ovary. There was also a developmental change in *Pum1*-lacZ expression in different stages of spermatogenesis (Fig 10 A and B),

suggesting that *Pum1* expression is developmentally regulated throughout spermatogenesis. In the ovary sections, lacZ staining was weaker but clearly present in granulosa cells and thecal cells (Figure 10C and D). Although granulosa cells from different follicles stained at different intensities, it remains to be determined if differences in lacZ staining correlate with normal developing follicles and follicles undergoing atresia.

We further examined lacZ staining in ovaries from a *Pum2* gene trap mouse line and determine if *Pum1*-lacZ could be detected in granulosa cells. Female gonads of 1-day old mice demonstrated strong staining in both the germ cells and somatic cells; by day 10, expression of the *Pum2* reporter was observed in both oocytes and theca cells of developing follicles. By day 14, the ovaries had a large number of primary and secondary follicles and staining was most concentrated in theca cells with some expression in granulosa cells. Finally, in the 21-day or older adult ovaries, reporter staining was most notable in the somatic granulosa cells

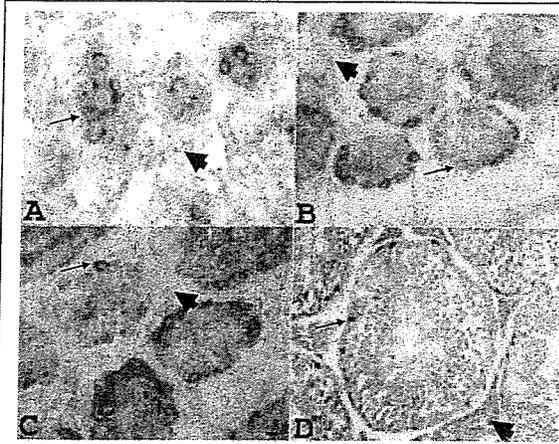


Figure 9 lacZ staining of gene trap line for Pum gene in the prepubertal (A, B, C) and adult testis (D). Expression is stage specific and cell-specific. A-Day 1 postnatal testis section, B and C are day 10 testis, D is 3 month old testis. Thin arrow points to lacZ-positive germ cells inside seminiferous tubules; note that the staining is specific to certain germ cells and pattern changes. Short arrow points to somatic cells which are positive in prepubertal testis but negative in adult.

and theca cells with occasional strong dot-like staining inside the oocyte (Figure 11A). At higher magnification (Figure 11B), strong lacZ staining in granulosa cells was clear.

Our goal is to use Pum1-lacZ to visualize Pum gene expression in ovulated follicles, which are unable to be sectioned. Thus, we decided to test if our lacZ assay would work with whole mount tissues, such as embryos or whole ovary. We isolated 3.5-day-old blastocyst embryos from heterozygote mothers crossed with heterozygote fathers, as well as blastocysts from wild type mothers mated with wild type males. Heterozygote embryos showed clear lacZ-positive cells but no lacZ staining was observed in wild type embryos (Figure 12A and B). We also stained the entire ovary from *Pum1^{XE002}* females and found strong staining (Figure 12C). Pum1 protein was also detected from cell extracts from ovaries of wild type and heterozygote of *Pum1^{XE002}* animals, but Pum1-lacZ chimeric protein could only be detected in heterozygotes, and not wild type (Figure 12D and E). These studies confirm that the lacZ signal detected in *Pum1^{XE002}* ovaries is indeed from the Pum1-lacZ chimeric protein controlled by the endogenous *Pum1* promoter. LacZ signal from whole ovary and COCs should reflect the expression of *Pum1*.

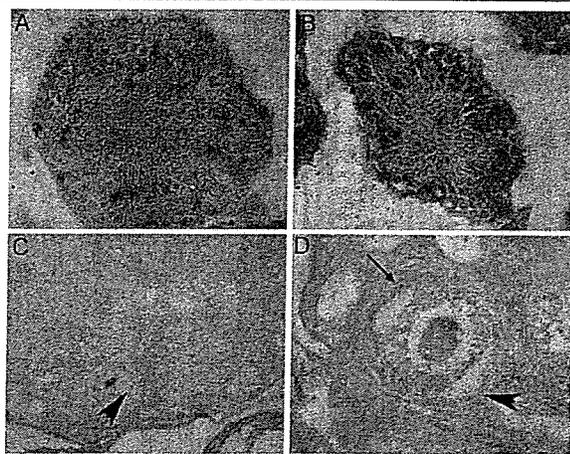


Figure 10 LacZ expression pattern in the testis and ovary sections of *Pum1^{XE002}*. A and B are two seminiferous tubules at different stage of epithelial cycle demonstrating stage-dependent *Pum1*-lacZ expression pattern. C is lower magnification of ovary section and D shows a single follicle with lacZ positive cells. Arrowhead points to some weakly stained granulosa cells. lacZ Theca cells are also positive for lacZ (arrow).

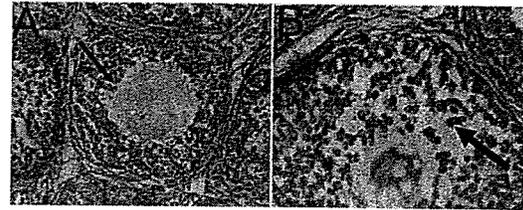
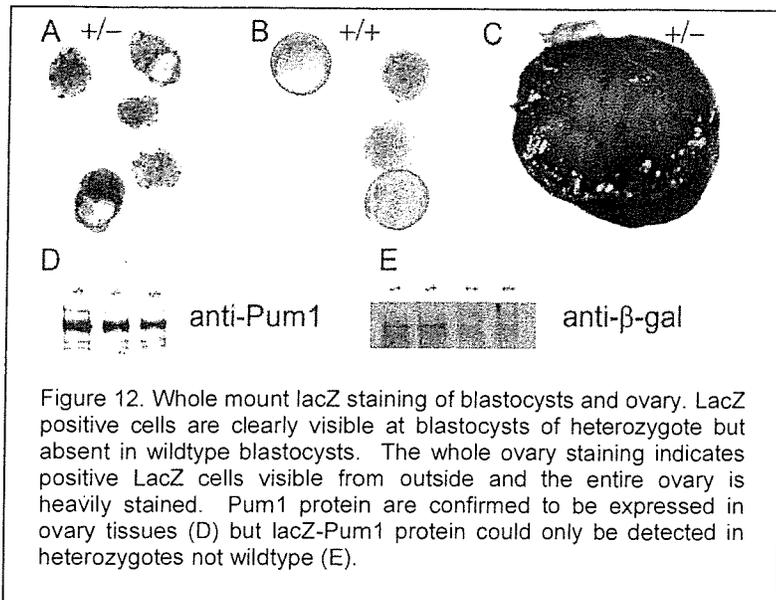


Figure 11. LacZ is detected in oocyte and many granulosa cells in the ovary of *Pum2* gene trap line XE772. A. arrow points to lacZ positive granule in oocyte. B. LacZ positive granulosa cells (arrow) in another follicle.



D. RESEARCH DESIGN AND METHODS

SPECIFIC AIM 1. *To identify differentially expressed genes in cumulus cells that correlate with the oocyte's potential to implant and with patient age*

AIM 1: Hypothesis.

The ability of an embryo to implant is determined by the quality of the fertilized oocyte. The quality of oocytes retrieved for IVF is affected by several factors, including age, exposure to exogenous hormones used for ovarian stimulation, prior exposure to environmental pollutants, or excessive alcohol consumption. The effect of these factors on oocyte quality may be mediated by and/or reflected in changes in gene expression in the cumulus cells.

Aim 1: Objective.

The objective of the studies in **Aim 1** is to evaluate these genes as candidate biomarkers of oocyte quality by qRT-PCR in four populations of cumulus cells classified by donor type and oocyte fate.

AIM 1: Experimental Design.

Collection and classification of cumulus cells.

During IVF treatment, oocytes are aspirated from antral follicles 36 hours after the preovulatory administration of hCG. The oocytes are usually aspirated together with surrounding cumulus cells. Excessive cumulus cells are then trimmed from the oocytes with fine needles before the oocytes are placed in culture for IVF. For this study, the trimmed cumulus cells will be saved by freezing in liquid nitrogen and stored at -80°C for further processing.

Cumulus cells will be divided into four groups according to the age of the subjects (cumulus cell donors) and the developmental fate of the oocytes (**Table A.1**).

Groups A and B: cumulus cells in these two groups will be collected from young women (<35 years of age) who are healthy but whose partners have fertility problems (male factor infertility), or from oocyte donors who are usually younger than 30 years of age or proven fertile.

Groups C and D: cumulus cells in these two groups will be collected from women seeking infertility treatment who are over the age of 37, whose primary cause of infertility is not pathological changes in the ovary, such as polycystic ovary syndrome. Depletion of ovarian follicles is usually the primary cause of infertility in women of this age group. The number of retrieved oocytes and implantation rates are significantly lower than women donating cumulus cells to Groups A and B. Prognosis for infertility treatment is poor with a significantly reduced pregnancy rate (**Table C.1**).

Groups A and C: cumulus cells in these two groups are removed from "**Good-Quality**" oocytes, those that are fertilized in vitro, and the resultant embryos are confirmed to have implanted after embryo transfer in pregnancies with the number of fetuses equal to the number of embryos transferred (i.e. 100% implantation). Monozygotic twin pregnancies will not be included.

Groups B and D: cumulus cells in these two groups are removed from "**Poor Quality**" oocytes, those that are fertilized but fail to reach the 8-cell stage by day 3 or develops cellular fragmentation. Oocytes that are not fertilized will not be included because, apart from oocyte quality, sperm defects can result in fertilization failure.

Comparisons in candidate biomarker gene expression from cumulus cells between Group A and Group B may reveal adverse effects associated with non-age related factors, such as hyperstimulation of ovarian follicle development during IVF treatment. Comparison of biomarker gene expression in cumulus cells between Group A and Groups C or D could reveal both age-related and hormonal hyperstimulation-related effects on oocyte quality.

Experiment 1-1: Quantitative RT-PCR to assess expression levels of cumulus cell candidate biomarkers of oocyte quality.

To quantify the abundance of mRNA transcripts of candidate biomarker genes in cumulus cells collected from each of the 4 donor groups, total RNA will be extracted from individually collected cumulus complexes with Tri-agent (Sigma, St. Louis, MO), followed by reverse transcription to obtain cDNA. The cDNA will then be analyzed by qRT-PCR with specific Taqman[®] probes in triplicate reactions, using an Applied Biosystems

(Foster City, CA) automated DNA Sequence Detection System (PRISM[®] 7900HT). The reagents for real-time PCR, including the PCR primers and Taqman probes, will be purchased from Applied Biosystems.

Relative mRNA abundance will be calculated per the recommendations of Applied Biosystems. After RT-PCR has been completed, the accumulation of amplification product will be plotted against the number of amplification cycles. A threshold cycle number will be selected in the linear region of the accumulation curve for the amplification product. The relative abundance of mRNA of each gene of interest will be the difference between the number of cycles of amplification for this gene to reach the threshold and the number of cycles of amplification for 18s RNA to reach the threshold in the same sample. The use of 18s RNA as a reference has been validated in our pilot trials, which demonstrated little variation between cumulus complexes, with a standard deviation less than 2% of the mean for 36 complexes from five patients (unpublished data).

Candidate genes to be tested by qRT-PCR will include those genes listed in **Table D.1**.

Table D.1. List of candidate biomarker genes for qRT-PCR.

Gene	Functional relevance to oocyte quality	Anticipated correlation with good oocyte quality	References
<i>PTX3</i>	Target of oocyte GDF-9 regulation; required for fertilization	upregulated	(53, 55, 143)
<i>PCNA</i>	Target of oocyte GDF-9 regulation; associated with preovulatory follicular development	upregulated	(144-146)
<i>HAS2</i>	Target of oocyte GDF-9 regulation; associated with cumulus expansion	upregulated	(53, 54, 144)
<i>PTGS2</i>	Target of oocyte GDF-9 regulation; associated with ovulation	upregulated	(53, 54, 144)
<i>GREM1</i>	Target of oocyte GDF-9 regulation	upregulated	(53, 54, 144)
<i>BDNF</i>	Associated with oocyte quality; functions unclear	upregulated	(60)
<i>Inhibin beta B</i>	Involved in apoptosis and ovulation	downregulated	(147-149)
<i>Pum1</i>	Unknown	downregulated	(55)
<i>PR</i>	Involved in preovulatory follicular development	downregulated	(150-152)
<i>AhR</i>	Mediates cytotoxicity of common environmental pollutants	unknown	(153, 154)
<i>Cytochrome P450 2E1</i>	Metabolizes common environmental pollutants and alcohol	unknown	(155, 156)

In our previous study, we identified over 100 differentially expressed genes in cumulus cells associated with oocytes having different developmental fates (55). *PTX3* was one of the upregulated genes identified in cumulus cells from oocytes of good quality, i.e., oocytes that were successfully fertilized and developed to 8-cell embryos on day 3. *PTX3* expression in cumulus cells was verified by RT-PCR. Subsequent quantitative RT-PCR (qRT-PCR) suggested that the relative abundance of *PTX3* mRNA was positively associated with the rate of successful in vitro embryo development. In mice, *PTX3* expression in cumulus cells is regulated by oocyte GDF-9 and is required for cumulus expansion during oocyte maturation and fertilization (53, 143).

Proliferating cell nuclear antigen (PCNA) is another upregulated gene identified in our earlier microarray study. The expression of *PCNA* in granulosa cells is associated with follicular growth and maturation, and is under the regulation of oocyte *GDF9* (144-146).

... et al (2004) (54) examined human cumulus cells for a possible relationship between oocyte quality and expression of **hyaluronic acid synthase 2 (HAS2)**, **cyclooxygenase 2 (PTGS2)** and **gremlin (GREM1)**, genes that are known to be regulated by GDF-9; they reported that these genes were highly expressed in cumulus cells from oocytes that later gave rise to good quality embryos in vitro

... et al (2005) (60) analyzed gene expression profiles in preovulatory mouse ovaries and found that the expression of *Brain-derived neurotrophic factor (Bdnf)* in cumulus cells was positively correlated with oocyte quality.

Certain genes have been shown to be downregulated at the time of oocyte maturation and ovulation. Inclusion of such negatively correlated genes in our candidate gene panel will help validate the predictive value of oocyte quality biomarkers. Our microarray data indicate that *Inhibin beta B* and *PUMILIO1 (Pum1)* are low in cumulus cells from good quality oocytes. The functional dimer of inhibin beta B subunits, inhibin B, is secreted by granulosa cells during follicular development and its serum level peaks at the mid-follicular phase (147, 149). A reduction in *in vitro* secretion of inhibin B by cumulus cells from IVF patients is positively correlated with the size of the originating follicles (148).

Pum1 is a member of the highly conserved *Pum* family of RNA binding proteins. *Pum* homologs have been shown to be important regulators in reproductive development in model organisms, such as oogenesis in *Drosophila* and meiotic maturation in the frog (L. and C. ... paper). Recently, mammalian homologs of *Pumilio* have been identified and mouse *Pum* is expressed at high levels in both the testis and ovary. In the ovary, *Pum* is expressed in mouse granulosa cells and thecal cells ((100) and **Preliminary Studies**). However, its role in follicle and oocyte development is unknown.

Progesterone receptor (PR) is expressed in human cumulus cells and its level of expression increases as antral follicles increase in size (152). The level of PR decreases in porcine cumulus cells 12 hours after the preovulatory LH surge (Shimada et al 2004) and is found to be downregulated in cumulus from oocytes that give rise to good quality embryos in vitro in women undergoing IVF treatment (150).

Aryl hydrocarbon receptor (AhR) mediates the toxic actions of most hydrocarbon compounds, including common environmental pollutants, cigarette smoking and industrial waste. AhR is expressed in granulosa cells and may be involved in the oocyte maturation process (153, 154). Some isoforms of the cytochrome P450 enzyme system, such as CYP2E1, are the main metabolizing enzymes involved in detoxification of small-molecule chemicals, including alcohol, and have been shown to be upregulated in mouse granulosa cells after *in vivo* exposure to reproductive toxicants (155, 156)

Experiment 1-2: Identification of differentially expressed genes by DNA microarray analysis to provide more candidate genes

Gene expression patterns will be examined by DNA microarray on pooled cumulus complexes (15-20 complexes) from each of four groups (**Table A.1**). Our earlier study profiled gene expression in two groups of cumulus cells according to the *in vitro* development of the oocytes, those not fertilized and those fertilized that developed into good quality embryos on day 3 (day 0 = day of oocyte retrieval and insemination). The "resolution" of the results in that study was low because (1) not all good quality embryos can implant, (2) fertilization failure could be the result of multiple factors. In the current proposal, resolution of gene expression profiling by microarray will be improved by (1) using cumulus cells from oocytes that are confirmed to be implanted after transfer, and (2) grouping cumulus cells according to cell donor age, a well-established primary factor determining oocyte quality.

Based on our previous experience, 5 to 10 μ g of total RNA will be required as template to obtain double-stranded cDNA using the MessageAMP kit (Ambion Inc., Austin, Texas). The cDNA sample is applied to a filter cartridge provided in the MessageAMP kit to remove RNA, and used as template in an *in vitro* transcription reaction to obtain labeled antisense RNA (aRNA), in the presence of Biotin-16-UTP (Roche Molecular Biochemicals, Nutley, New Jersey) and Biotin-11-CTP (Perkin Elmer Life Science, Boston, Massachusetts). Twenty μ g of the biotin-labeled aRNA will be used for hybridization to U133A chips from Affymetrix (Santa Clara, California) following Affymetrix protocols. Each U133A chip contains 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes.

Expression analysis will be carried out using the open-source Bioconductor version 1.5, <http://www.Bioconductor.org> (157) (Collaborator-Dr. ...). Raw expression numbers (probe level data) will be corrected, normalized and modeled using the RMA function with AFFYpackage (version 1.5.8) and Limma (version 1.8.6) software packages (158). Limma uses an empirical Bayesian method to control standard errors of the estimated log-fold changes between samples and provides more stable inference and improved power, especially for experiments with a small number of arrays. Genes of interest will be selected if the t-test

P values, difference probability (>90%), and fold change of at least 3-fold (upregulated or downregulated) are true for the comparison of different patient groups.

Candidate marker genes will be selected after the microarray analysis to be tested in **Experiment 1-1**. Since 5 to 10 μ g total RNA are required for one replicate of microarray analysis, collection of sufficient RNA for two replicates of microarray analyses would take approximately 24-30 months.

AIM 1: Expected Outcomes

Experiment 1-1: A sample size of 15 is needed to detect a difference equal to or greater than the standard deviation, with a power of 0.90 at a 95% confidence interval (SigmaStat 1997, SPSS, Chicago, IL). With this sample size, a gene will be arbitrarily qualified as a marker if its mRNA level is different between Group A and another group with a p value equal to or less than 0.05., and will be further tested/validated in **Aim 3**.

For each cumulus complex, approximately 0.5 to 2 μ g total RNA can be obtained. One quarter to one half of the RNA collected will be needed for qRT-PCR to analyze 5 candidate biomarker genes. Variation in gene expression between Groups A and C is expected to be small. Therefore, cumulus cells in Group A are the limiting factor for sample size since the only sources of cumulus cells in this group are women under 35 years of age who are pregnant with a 100% implantation and patients conceived with a 100% implantation of embryos resulting from donor oocytes. Most of such pregnancies are twin pregnancies and, therefore, each pregnant patient can donate two cumulus complexes to Group A. Based on our experience, 75% of patients will consent to participating in this study. Assuming our patient population in the next five years is comparable to that of the past three years (**Table C.1**), we estimate that it will take 30 to 36 months to evaluate 15 to 20 candidate genes.

Experiment 1-2: By grouping cumulus cells according to oocytes' capability to implant in vivo and according to patients' age, this gene expression profiling study by microarray should reveal differentially expressed genes that can better predict oocyte quality than our previous study. Subjecting candidate genes from this analysis to testing in Experiment 1-1 will be more likely to result in predictive biomarkers for oocyte quality.

AIM 1: Alternative Approaches/Anticipated Pitfalls.

The 11 candidate biomarker genes listed in Table 1.1 are selected based on our own studies and studies of others in human or mouse models, and their up- or downregulation is expected to correlate with implantation. The technique of qRT-PCR is well developed and the PIs have extensive first-hand experience with these techniques and in interpretation of results.

Although the 11 genes were selected because they are associated with oocyte in vitro development or preovulatory changes in the follicle, they may not be associated with the embryo's ability to implant, which is a more stringent measure of oocyte quality. In anticipation for the possibility that none of the selected genes predict implantation, we will set aside sufficient amounts of cumulus RNA for Experiment 1-2 to profile gene expression patterns by microarray analysis, which should provide us with additional candidate genes for further testing. Another "insurance" measure is the screening studies described **Aim 2** using the mouse model, which will also provide additional candidate genes to be tested as oocyte quality biomarkers.

SPECIFIC AIM 2: To identify conserved oocyte-quality predictor genes in mice ().

AIM 2: Hypothesis.

We hypothesize that the molecular mechanisms underlying oocyte-cumulus communication is conserved between humans and mice and identifying genes that are biomarkers of both human and mouse oocyte quality will select for a robust, core panel of oocyte quality predictor genes.

Aim 2: Rationale and Objectives.

The major function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species. Oocyte maturation and oocyte ovulation are highly conserved processes present in many animal species. Cyclic recruitment of early antral follicles and selection of dominant follicles in rodent is similar to that of primates, including humans, with the major exception that multiple follicles become dominant during each estrous cycle. Extensive evidence also suggests that molecular mechanisms underlying follicular development are likely to be conserved between the human and rodent (32, 34, 39, 65). We hypothesize that a common gene expression signature exists within cumulus cells of both humans and mice that reflects the developmental and fertilization potential of oocytes. Identification of conserved biomarkers associated with egg quality could refine the list of candidate biomarkers. We will test our hypothesis by examining the expression changes of murine homologs of candidate human biomarkers

identified in **Aim 1** among cumulus cells collected from control mice, older mice, alcohol-treated mice and mice that have undergone superovulation. The goal is to identify a gene expression signature associated with good quality eggs that is conserved between humans and mice to refine the list of genes predictive of oocyte quality.

AIM 2: Experimental Design.

Experiment 2-1: Compare changes in gene expression in mouse cumulus cells from young oocytes and old oocytes (Collaborators-_____).

We previously observed a correlation in gene expression in human cumulus cells with oocyte quality, and we will extend our analysis to the mouse, in which we are able to carry out more controlled comparative analyses. We hypothesize that most of the gene expression changes we observed in cumulus cells of human oocytes from older women will also occur in mice. We will compare the age-related changes in cumulus cell gene expression with human data from **Aim 1** to identify conserved biomarker gene candidates.

Collection of COCs and separation of cumulus cells for RNA extraction.

We will quantify gene expression changes in cumulus cells from oocytes collected from 7-week-old (n=15), 20-week-old (15) and 48-week-old (15) ICR mice purchased from Taconic (Albany, NY). We will collect cumulus cells from mice of each age group following superovulation with 10 IU of equine chorionic gonadotrophin (eCG) followed by 10 IU of human chorionic gonadotrophin (hCG) 48 hours later. COCs will be collected from the oviducts 16 hours after hCG injection. COCs will be denuded of cumulus cells by a 1-minute incubation in 80 IU/ml hyaluronidase (Sigma). Cumulus cells will be stored at -80°C for later RNA extraction. Total RNA will be isolated from pooled cumulus cells collected from the COCs from eCG-primed mice (15). Pooling CCs is necessary to obtain sufficient RNA but also provides a means to reduce variation among individual mice in each age group. We will also collect granulosa cells released from follicles in the ovaries by needle puncture method. Granulosa cells will be centrifuged and frozen at -80°C for later RNA extraction. The experiments will be repeated to provide two independent cumulus and granulosa cell pools from the three different age groups.

Characterize gene expression profiles of cumulus cells from young vs old mice.

RNA will be isolated using the RNeasy mini kit (Qiagen Sciences, Germanytown, MD). The quality of RNA will be analyzed by an Agilent Bioanalyzer 2100. Total RNA will be labeled and hybridized to Affymetrix Mouse Genome 430 2.0 array gene chips by the _____

(collaborator-Dr. _____). Expression analysis will be carried out using the open-source Bioconductor (collaborator-Dr. _____). Genes of interest will be called if the t test P values, difference probability (>90%), and fold change at least 3-fold (upregulated or downregulated) are true for the comparison between different age groups. After identifying a list of genes, we will confirm our analysis using RT-PCR with primers to a select list of age-related upregulated and downregulated genes.

Experiment 2-2: Establish conserved gene expression signature profile associated with mammalian oocyte aging (Collaborator—_____)

We will first determine the gene expression and possible pathways affected during oocyte aging by analyzing the gene expression profiles in cumulus cells from the three different age groups. The list of genes showing upregulation or downregulation will then be compared with list of genes analyzed in human cumulus cells in **Aim 1**. Homologous genes with conserved patterns of expression changes associated with oocyte aging will be identified. This information will then be compared with gene expression data from mouse granulosa cells and from human cumulus cells obtained in **Aim 1**. In this manner, we will derive a list of conserved egg quality biomarkers whose expression changes with oocyte age in both humans and mice.

Experiment 2-3: Determine gene expression profiles in cumulus cells of mice exposed or not exposed to alcohol (Collaborator—Dr. _____).

Determine the effect of alcohol consumption on oocyte quality and embryo quality from IVF.

We hypothesize that environmental factors such as alcohol consumption contribute to a decline in oocyte quality, and that cumulus cell gene expression changes will be observed in mice exposed to alcohol. We will

test this hypothesis in mice by first examining embryo quality from oocytes collected in IVF from alcohol-treated mice versus control mice.

ETOH will be given to experimental animals via a liquid diet in a pair-feeding protocol with control animals. In brief, 6-8 week-old female C57BL/6 mice will be assigned at random to one of three diet groups: (1) an ad libitum Lieber-Decarli liquid diet (catalog number 710262; Dyets, Bethlehem, PA) containing 5% (vol/vol) ETOH and providing 35% ETOH-derived calories; (2) an isocaloric Lieber-Decarli control diet (catalog number 710029; Dyets) for pair-fed mice; (3) laboratory chow and water ad libitum. The third group is included to control for the effects of a liquid diet. The volume of diet consumed daily by each ETOH-fed mouse will be determined, and a volume of the control diet equal to the mean of the volume consumed by the ETOH-fed group from the previous day will be fed to each of the pair-fed mice. Animals will be maintained on the appropriate diet for ≤ 8 days to avoid terminal withdrawal from ETOH.

We plan to compare embryo quality from 15 alcohol-treated and 15 control mice. At the end of 8 days of alcohol feeding, we will prime the mice with eCG for 48 hours followed by hCG injection. IVF will be carried out using COC from 12 treated and 12 untreated females with sperm from wild type C57B6 males. We will evaluate the IVF outcomes at day 2, day 3 and day 5 post-fertilization, based on the percentage of 2-cell embryos, 4-cell embryos, compacted morulae and blastocysts.

Characterize gene expression profiles from cumulus cells collected from alcohol-treated and control mice.

To identify candidate biomarkers of oocyte quality decline associated with alcohol consumption, we will isolate cumulus cells from COC collected from the oviducts of eCG primed, alcohol-treated or control mice ($n=3$). The COC will be denuded and cumulus cells will be collected and stored at -80°C . RNA will be extracted with RNeasy Qiagen kit and amplified for one round to achieve enough RNA for use in microarray experiments. cDNA will be synthesized. Real-time PCR using primers representing the list of conserved egg quality predictor genes will be carried out to identify cumulus cell genes whose expression levels differ between alcohol-treated and control mice. Our hypothesis is that alcohol consumption affects egg quality, which will result in similar cumulus cell gene expression profile changes as those seen with oocyte aging. We have designed primers for all 11 genes and will create additional primers once we have established a list of conserved candidate egg quality predictors from **Aim 1**.

Experiment 2-4: Compare the quality of superovulated and normally ovulated oocytes.

Treatment of human infertility typically involves the administration of exogenous gonadotropins to aggressively stimulate the ovaries to produce the maximal number of mature oocytes, which are then harvested and fertilized in vitro. The negative effects of ovarian stimulation on oocyte quality and embryo development have been documented in animals (159, 160) and similar detrimental effects may occur in human assisted reproduction. We hypothesize that molecular changes associated with egg quality decline in superovulated eggs are similar to those due to aging, and that some of the biomarkers established from our list of conserved biomarkers of oocyte quality could also be used to predict the decline in oocyte quality due to ovarian hyperstimulation.

We will collect COCs from 6-7-week-old mice superovulated with eCG followed by hCG 48 hours later. Cumulus cells will be collected and stored at -80°C for later RNA extraction. COC from the oviducts of normally ovulating mice will also be collected and stored. Real-time PCR using primers representing the list of conserved egg quality predictor genes will be carried out to identify cumulus cell genes whose expression levels differ between superovulated COC and naturally ovulated COC.

Experiment 2-5: Establish a core list of oocyte quality biomarker genes in mice.

The objective of this proposal is to identify novel biomarkers for monitoring egg quality. We hypothesize that the decline in egg quality due to aging or environmental factors causes changes in the expression of a key set of genes in cumulus cells. The goal of this aim is to identify a list of genes whose expression changes similarly as a consequence of aging or environmental assaults such as alcohol or hormone exposure. Specifically, we will perform an extensive bioinformatics comparison among gene expression data collected from our aging analyses in both humans and mice and from our alcohol-treatment experiments. We will assemble a list of conserved candidate biomarkers for egg quality that show consistent cumulus cell expression profile changes between humans and mice, and among aged mice, alcohol-treated mice and superovulated mice. This refined list will represent biomarkers for egg quality we will further validate in the human in **Aim 3** and in future clinical trials. Also we will investigate the role of these genes in normal oocyte physiology and the mechanisms underlying their association with oocyte quality in **Aim 4** using mouse model.

AIM 2: Expected Outcomes.

We expect that changes in the expression of several of the human cumulus cell genes tested in Aim 1 will also be observed for the murine homologs. By comparing the results of **Aims 1 and 2**, we will be able to establish a list of conserved egg quality biomarkers for oocyte aging between humans and mice. Changes in cumulus cell gene expression from aging mice and mice treated with alcohol are expected to overlap to some degree. We will identify those genes to refine the list of biomarkers for egg quality. This list of biomarkers will be further refined when compared with those that show expression differences between superovulated or normally ovulating mice. Only the biomarkers whose changes in expression can be consistently correlated with declines in oocyte quality due to aging, alcohol exposure and superovulation will be selected as our candidate egg quality predictors for the further validation in human cumulus cells in **Aim 3**.

AIM 2: Alternative Approaches/Anticipated Pitfalls.

One potential pitfall under this Aim is that alcohol treatment elicits gene expression profile changes with little overlap with gene expression profile changes due to aging. Although this is possible, we think it is unlikely because a decline in egg quality results in the same poor outcomes regardless of the cause: decreased fertilization, embryo growth retardation, cleavage arrest, impaired blastocyst hatching, embryo loss by fragmentation and oocyte fragmentation (29, 30, 51, 161, 162). Molecular and physiological events leading to such common physiological abnormalities in oocyte and cumulus cells are likely to be shared at least to some extent in aged oocytes and alcohol-treated oocytes. It is expected those common molecular changes will bring about identifiable common signature gene expression profile changes associated with egg quality decline. In the event that we are not able to identify common gene expression profile changes among aging mice and those exposed to alcohol or undergoing superovulation, we will proceed with Aim 3 experiments with a slightly longer list of candidate biomarkers, such as those associated with age-related decline in oocyte quality. Such a list is still very important as oocyte aging is the primary cause of poor egg quality and low IVF success rate.

The second potential pitfall is that our alcohol treatment will be too mild to have any effect on IVF and embryo quality. However, we may still see gene expression changes in this case, and such changes could signify molecular and cellular changes that would be amplified with higher concentrations of or longer exposures to alcohol. The data will be still important to collect and compare with the results of our aging experiments. We will discuss the need to modify the treatment dose or time course with our collaborator, Dr.

SPECIFIC AIM 3: Validate predictive values of the selected biomarkers in IVF patients (Zhang).

AIM 3: Hypothesis.

If mRNA levels of a gene in the cumulus cells Group A (oocytes resulting in embryo implantation, **Table A.1**) are different from those in any other cumulus cell groups, this gene's transcription (and possibly translation) products are likely to predict prospectively the implantation potential of a fertilized oocyte, and may further identify factors mediate changes in oocyte quality caused by age, prior exposure to environmental pollutants, or excessive alcohol consumption.

AIM 3: Rationale and Objectives.

The list of candidate biomarkers for oocyte quality will be refined based on the results of experiments described in **Aims 1 and 2** to establish a core "panel" of marker genes for further validation. The prognostic and/or diagnostic value of the genes within this panel will be tested in order to develop a reliable, practical, and non-destructive assay system for assessing oocyte quality using biomarkers in cumulus cells.

AIM 3: Experimental Design.

Experiment 3-1: Quantitative RT-PCR to validate oocyte quality biomarkers.

Individual cumulus complexes will be collected and RNA extracted for qRT-PCR. The same methodology described in **Experiment 1-1** will be used here, except that all cumulus cells from all consenting patients will be included in the analysis. We anticipate that this study will be carried out over a two-year period, and will include between 400 and 800 IVF patients (if a patient undergoes more than one IVF treatment cycle in the two year period, only the first treatment will be included in the study to minimize bias). This large sample size will allow us to verify correlations between the relative abundance of the mRNA of each candidate biomarker and oocyte

quality (implanted or not implanted) by logistic regression analysis, and to determine a cut-off value for predicting oocyte quality by receiver-operator characteristics (ROC) curve analysis. The large sample size will also allow us to establish a correlation between the relative abundance of biomarker mRNA and patient's age and alcohol consumption by linear regression analysis.

Experiment 3-2: Developing ELISA assays for biomarker proteins.

Immunoassays (e.g., ELISA) will be developed to study protein products of the cumulus cell biomarker genes selected based on the studies described in **Aims 1 and 2**.

Antibodies

We will check the availability of antibodies for biomarker gene protein products with commercial vendors. For example, Pentraxin-3 antibody is available commercially from two vendors, Upstate (Billerica, MA) and Alpha Diagnostics (San Antonio, TX). If antibodies are not commercially available, short peptide antigens will be synthesized to produce antibodies. Although Dr. [redacted] developed proficiency in raising antibodies in rabbits during his Ph.D. studies of uterine-secreted proteins, it is more efficient and economical to contract specialized companies to synthesize the peptides and produce antibodies. The efficacy of the antibodies will be confirmed by immunoblot analysis. Protein will be prepared from cumulus cells by lysing in the presence of a detergent (Nonidet P-40 or Triton X-100) and protease inhibitors to minimize protein degradation. If the protein is secreted, cumulus cells will be cultured for 2 to 18 hours and the conditioned culture medium will be collected for protein extraction. Protein will be extracted by precipitation with deoxycholate and trichloroacetic acid, and analyzed by a standard, non-radioactive immunoblot to determine the presence and molecular mass of the target protein. Once the antibody and the presence of protein in cumulus cells or their secretions are confirmed, the antibodies will be used to develop sensitive ELISA assays. Again, to save time and money, commercial assay developers such as US Biological (Swampscott, MA) will be contracted to develop these assays.

Cell lysates from individual cumulus complexes collected in **Experiment 3-1**, or their secreted proteins collected after in vitro culture, will be subjected to the ELISA assays to determine the correlation of the biomarker protein with oocyte quality (logistic regression and ROC curve analyses) and with patients' age and their alcohol consumption (linear regression analysis).

AIM 3: Expected Outcomes.

A qRT-PCR assay or protein ELISA assay may be developed to reliably predict an oocyte's potential to result in implantable embryos, or to diagnose the cause of poor oocyte quality, such as age or alcohol consumption.

AIM 3: Alternative Approaches/Anticipated Pitfalls.

1) The marker genes selected in Aims 1 and 2, due to the small sample sizes, may not be able to effectively predict oocyte quality due to large variations in a large size of validation sample. One such variation may arise from uterine factors, because an embryo may fail to implant, not because of the oocyte quality, but because of a poor uterine environment. If no correlation between the levels of biomarker mRNA or protein and oocyte quality can be established, patients may be grouped to control for variations. For example, to minimize bias from uterine factors, patients who failed to achieve pregnancy repeatedly after transferring embryos with good morphology will be excluded from the analysis.

2) The qRT-PCR assay for determining the relative abundance of mRNA is advantageous because it requires only a small number of cells. However, technical challenges, such as the need for costly equipment, experience in preventing RNA degradation and interpretation of results may limit its clinical application. In this case, the ELISA assay may be good alternative because it is technically easier and requires less expensive equipment. An ELISA assay will be developed concurrently with the qRT-PCR assay to anticipate this scenario. However, there are potential pitfalls with protein assays, including (1) discordance in gene expression and protein levels, which would make mRNA levels a more appropriate marker for oocyte quality, and (2) difficulty in obtaining authentic antibodies. If the protein does not exist in tissue in sufficient quantities to allow easy purification for antibody production, synthetic peptides can be used as antigens, though the resultant antibody may not recognize the protein in its natural form. This may require testing of multiple peptides corresponding to different sections of the protein. Another alternative will be to purify target protein from natural sources, which may be time consuming.

SPECIFIC AIM 4: *To establish and characterize mouse biomarker reporter lines for egg quality (. . .*

AIM 4: Hypothesis.

We hypothesize that establishment of animal models carrying reporter genes under the control of the promoters for the identified novel biomarkers for oocyte quality will help to further define the utility of those biomarkers and lead to an efficient assay system for future screening of environmental factors detrimental to the oocyte's potential to implant. We further hypothesize that generation of mouse mutations that disrupt murine homologs of these novel biomarkers could provide animal models for the study of the molecular and biochemical mechanisms that determine oocyte quality.

AIM 4: Rationale and Objectives.

The clinical significance of successful identification of novel biomarkers for female oocyte quality is two-fold: first, it will lead to the development of effective laboratory assays for ART product screening and method improvement, and provide reliable predictors of ART responsiveness so that we will be able to achieve higher success rates; second, such novel molecular markers provide excellent entry points to a better understanding of the mechanisms that underlie the decline in oocyte quality, such as how aging or environmental factors affect oocyte potential to fertilize and to implant. The list of conserved egg quality predictors from **Aims 1 and 2** will be tested in the prospective assay in **Aim 3**, and we expect to identify a short list of biomarkers that are consistent indicators of egg quality in humans and mice. The next step will be to generate animal models that will allow detailed study the expression and function of those biomarkers in egg quality decline. Animal reporter lines that reveal the expression patterns of biomarkers could be used to establish animal assays for testing IVF reagents and techniques. We will use mouse gene trap technology to establish mouse reporter lines of the murine homologs of oocyte quality biomarkers and study the expression of those biomarkers using the lacZ reporter in the gene trap vector. Our objective is to establish 5 to 8 mouse gene trap lines targeting the identified novel biomarkers for expression analysis in ovarian follicles using lacZ reporter, and to carry out detailed functional analyses on two biomarkers using the animal models.

AIM 4: Experimental Design.

Experiment 4-1: Establish and characterize the *Pum1* gene trap reporter lines.

Characterization of *Pum1* gene trap lines.

Preliminary experiments have identified a list of genes with significant changes in expression levels in human cumulus cells between good quality and bad quality oocytes. One of the genes whose expression was significantly higher in good quality oocytes is a highly conserved translational regulator, *Pum1*. The *Pum 1* gene is of particular interest because posttranscriptional regulation has been recognized as an important regulatory mechanism in oocyte development (35, 119, 163). Recently, *Pum* proteins have been shown to be part of stress granules found in neurons (132). Stress granules are thought to be RNA granules that protect and store mRNA in case of stress. The formation of such granules is one of the cellular responses to an adverse environment. Although it is not known if stress granules exist in the ovary, RNA granules are known to be abundant in germ cells such as the chromatoid body, nuage. We hypothesize that *Pum1* expression in granulosa/cumulus cells increases in response to adverse factors, such as aging or environmental assaults, to facilitate the formation of RNA granules. The expression level of *Pum1* could be an indicator of the stress level of ovulated ovarian follicles and thus a biomarker for oocyte quality. We decided to investigate the expression of *Pum1* in ovary and ovulated follicles, in particular the expression change of *Pum1* in cumulus cells, to determine the utility of *Pum1* as a potential biomarker of oocyte quality.

We established two mouse *Pum1* reporter lines with lacZ reporter inserted at two different locations in the gene so that we could ensure gene expression detection from all possible splice variants. The insertion points have been determined by PCR and Southern hybridization (**Preliminary results**) and heterozygotes from both lines are viable and fertile. We will examine the expression of *Pum1* in ovarian development by lacZ staining of ovary sections and ovulated follicles from oviducts. Since lacZ staining is indicative of chimeric *Pum1* and lacZ protein, lacZ expression will be a good indicator of *Pum1* transcription but does not necessarily reveal the *Pum1* protein localization pattern. We will examine the protein expression pattern of *Pum1* by immunoblot analysis. We will also perform immunostaining of the ovary and COC to determine the specific localization of *Pum1* protein using antibodies described in **Aim 3**. We will further determine whether *Pum1* is present in granules inside granulosa cells, or whether it colocalizes with other stress granule components in the ovary.

We will use antibodies against two known components of stress granules, Staufen and p-eIF2alpha (136, 164) to determine if stress granules exist in granulosa cells.

Determine changes in Pum1-lacZ reporter expression during aging and with alcohol treatment.

We will compare Pum1-lacZ expression in cumulus cells from Pum1^{XE002} and Pum1^{XB063} females at 7 weeks, 20 weeks, 30 weeks and 48 weeks to assess the impact of age on biomarker levels and localization. We expect that *Pum1* expression will increase with aging and the decline in oocyte quality. Similarly, we will perform alcohol and control pair-feeding of *Pum1*^{XE002} mice for seven days and extract COC from superovulated alcohol-treated mice and control mice. Expression of Pum1-lacZ in cumulus cells will be compared to determine to what extent Pum1 is upregulated upon alcohol exposure.

Experiment 4-2: Identify ES gene trap cell lines and confirm the insertion site and reporter expression in ES cells.

After we acquire a validated list of biomarkers of oocyte quality from **Aims 1, 2 and 3**, we will search the IGTC to identify all the ES gene trap cell lines available for those genes. We will perform bioinformatic analysis to determine the insertion site, potential impact on the protein and vectors used in all positive ES cell lines to generate a list of mouse ES cell lines that could potentially be useful oocyte quality reporter lines. We will then request ES cells directly for culture in the lab. RT-PCR will be performed to confirm the insertion in the reporter genes and lacZ staining of ES cells will be performed to determine the reporter's signal strength. We will design primers that cover the entire intron where the gene trap vector is inserted and perform PCR and long-range PCR to determine the specific insertion site of the gene trap vector. The PCR products between the vector primers and genomic primers will be sequenced and the insertion site will be determined based on the sequencing results. ES cell line-specific genotyping primers will be designed to establish genotyping methods for each individual line.

Experiment 4-3: Generate potential oocyte quality reporter mouse lines.

We will send the biomarker-positive ES cells to our Transgenic Core facility to perform blastocyst injection to generate chimera. Germline transmission will be confirmed by line-specific genotyping and Southern hybridization with lacZ and gene-specific probes. We plan to establish 5 to 8 mouse gene trap lines representing animal models for the identified novel oocyte quality predictors. The mice will be then used to first establish the lacZ expression profiles of each biomarker in cumulus cells of COC. The lacZ expression pattern will be compared with RNA in situ and protein immunostaining patterns of each biomarker in mouse cumulus and ovary sections. Only the lines consistently and reliably recapitulating the endogenous gene expression pattern of our validated biomarker genes will be chosen as the oocyte quality reporter lines for future assay.

Experiment 4-4: Determine cumulus cell biomarker expression patterns in aged and alcohol-treated heterozygote reporter mice.

Similar to **Experiment 4-1** in the *Pumilio* reporter line, we will examine lacZ reporter expression in cumulus cells from COC collected from mice of different ages and from alcohol-treated mice. We will select the lines that exhibit the most sensitive lacZ expression changes in response to aging or alcohol treatment as designated oocyte quality reporter line(s). Such line will be used for future assays to test the effect of other environmental factors on oocyte quality.

Experiment 4-5. Characterizing the function of two oocyte quality predictor genes in the reporter mouse lines. One advantage of the gene trap lines is that lacZ insertion can produce a disruption of protein function. We will generate homozygote mice by crossing heterozygotes for each gene trap line and determine the fertility of females. If females are infertile or subfertile, we will determine at what stage female reproduction is compromised. We will perform superovulation to determine if any eggs are ovulated. We will also perform H&E staining of ovarian sections to determine if all the stages of follicles are present. The characterization of reproductive defects, if any, in homozygote mutant females could reveal the potential function of those biomarkers in ovarian development, oocyte-cumulus communication or ovulation. The detailed study of the animal models for oocyte quality biomarkers could lead to a better understanding of the pathways involved in oocyte quality decline and reveal additional predictive biomarkers of oocyte quality.

AIM 4: Expected Outcomes. We expect to establish 5 to 8 mouse reporter lines for our validated human egg quality biomarkers. Among them, we will select one or two lines with the most sensitive lacZ expression

response to oocyte aging and environmental assaults (i.e., alcohol exposure). These lines will be used to test the effects of other factors on oocyte quality. Examination of reproductive defects in homozygotes for the gene trap mutations will also reveal the roles of those biomarkers in follicular development and ovulation. Such information could help us to understand why the identified biomarkers are good predictors of oocyte quality and identify other related components in the biomarker pathways.

AIM 4: Alternative Approaches/Anticipated Pitfalls. One potential pitfall that may affect the establishment of mouse oocyte quality reporter lines is that the gene trap mutation could have a dominant effect on female fertility or even viability as the insertion could inactivate one copy of the oocyte quality predictor gene. If a gene trap line is dominant-sterile or inviable, we will not be able to use this line for our study. However, dominant mutation is less common than recessive mutation. The existence of multiple gene trap lines for many mouse genes in IGTC database makes it possible to generate alleles of different strengths: some dominant, many recessive or no effect. We will derive two lines for each biomarker, if available, to reduce the chance of having a dominant gene trap line. Furthermore, we always analyze the insertion point and protein structure before choosing a specific ES cell line; such bioinformatic analysis will increase our chance that the gene of interest has been trapped. Since more than 70% of the mouse genome has been trapped and more gene trap lines are being created (79), we have a very good chance to obtain a gene trap line for most of our biomarkers. If we find that a gene trap line is not available for a particular biomarker, we will resort to gene targeting technology to generate an ES cell line with a gene trap vector inserted in an intron of the gene of interest. The process of gene targeting has been significantly shortened now with the recent development for gene targeting with the promoterless gene trap vector (165) and we will be able to create reporter lines with either lacZ or GFP.

HUMAN SUBJECTS RESEARCH:

This Human Subjects Research falls under Exemption 4.

1. Human Subjects Involvement and Characteristics

a) Inclusion of Human Subjects:

Women who have scheduled oocyte retrieval for in vitro fertilization at _____ will be informed about this research and consent for permission to use their cumulus cells. After the oocyte retrieval procedure, the cumulus cells will be removed from oocytes and stored in liquid nitrogen for further processing to extract RNA or protein for this research. IRB approval has been granted for this research. The consent will be revised to include the use of some personal and clinical information for this research and will be submitted to IRB for reevaluation and approval. The personal and clinical information used for this research will be limited to: age at the time of oocyte retrieval, diagnosis for infertility, alcohol consumption history, oocyte fertilization, embryo development score, and implantation status. No information that may reveal personal identity will be used for this research.

b) Subject population

Only women over the age of 21 will be included given the nature of the tissue collected. No men or children (ages 0-21) will be included in this study. This research has no inclusion or exclusion preference based on the race of the woman undergoing oocyte retrieval for in vitro fertilization.

c) Inclusion/Exclusion of subject population

Cumulus cells and certain personal and clinical information will be collected from women undergoing oocyte retrieval for in vitro fertilization at _____ No men or children (ages 0-21) will be included.

d) Vulnerable populations:

There is no involvement of vulnerable populations.

e) Collaborating sites:

All the research will be done in the PI's lab at _____ be performed at _____

All the oocyte retrieval procedures will be performed at _____

2. Sources of Materials:

a) Research material description:

Cumulus cells will be collected after the cumulus-oocyte complexes have been collected for in vitro fertilization. Limited information from the patient records will be required for the interpretation of experimental results. These are listed in b)

b) Data:

The personal and clinical information used for this research does not involve any new information specifically collected for this research and will be limited to: age at the time of oocyte retrieval, diagnosis for infertility, alcohol consumption history, oocyte fertilization, embryo development score, and implantation status. These are the only data that is required for proper interpretation of experimental results. No information that may reveal personal identity will be used for this research.

c) Access to Subjects:

One of the two PI, Dr. _____ and the Co-PI, Dr. _____ will have access to the subject identities. No other personnel on this research project will have access to subjects' personal or clinical records. Subjects will be de-identified from the samples by the use of coded identification numbers.

d) Collection of Data:

One of the PI, Dr. _____ and Co-PI, Dr. _____, have already been approved under a current IRB protocol.

3. Justification for Exemption:

a) The human subject research falls under exemption 4.

b) Justification for exemption 4:

We are using cells that will be normally discarded. We will not be actively recruiting subjects and subjects will not be directly identified. There is no risk to the subject, nor any direct benefit to the subject from this study.

Inclusion of women. All subjects will be women, since men do not undergo oocyte retrieval and do not have cumulus cells.

Inclusion of minorities. We will make every effort to include women of all racial backgrounds. These samples are going to be obtained from patients at _____ The estimated current makeup of these patients is:

Non-Hispanic Whites

Hispanics

African Americans

Asian

We expect a disproportionately higher representation of specimens from _____ represent the majority of our patient population at the _____ racial or ethnical differences in the regulation of oocyte development.

_____ subjects because they _____, but there is no evidence for

Targeted/Planned Enrollment Table

This report format should NOT be used for data collection from study participants.

Study Title: Novel Biomarkers for Oocyte Quality

Total Planned Enrollment: 900

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino			
Not Hispanic or Latino			
Ethnic Category: Total of All Subjects *	900		900
Racial Categories			
American Indian/Alaska Native			
Asian			
Native Hawaiian or Other Pacific Islander			
Black or African American			
White			
Racial Categories: Total of All Subjects *	900		900

* The "Ethnic Category: Total of All Subjects" must be equal to the "Racial Categories: Total of All Subjects."

Inclusion of children. Children will not be included in this research because all patients attending must be 21 years old or older.

F. VERTEBRATE ANIMALS

Mice

1) *Proposed Use of the Animals.* The proposed use of mice is for gene expression profiling of cumulus cells from aged mice versus young mice, alcohol-treated and superovulated mice versus normal control mice; the generation and characterization of mouse gene trap mutations from a list of highly conserved biomarkers for egg quality.

All studies for the preparation and use of transgenic mice and the breeding of double mutations will be reviewed and approved by _____; Animal Care and Use Committee in just-in-time fashion. This committee reviews all proposed animal studies. After approval, the written proposals are submitted to

_____ Committee on Animal Research for further review. Approved protocols are renewed every year, and they are subjected to an intensive review every 3 years. All of the proposed studies in the _____ have been approved by this committee. We consult routinely with the _____ animal care veterinary staff on animal care issues.

The pathogen-free mice to be used in the proposed studies include 129svJ, C57BL/6, and CD-1.

For Specific Aim 2: Gene expression profiling of cumulus cells collected from COC of 7 wk old, 20 wk old and 48 wk old mice as well as from alcohol-treated and superovulated mice versus control. In Aim2-1A, we will use 15 female for each group. The experiment will be repeated with the same number of animals one more time. For microarray data on alcohol-treated mice, we will use 3 pairs of mice within each group of liquid diet to collect COC and to extract RNA from cumulus cells. 3 pair is the minimal number of mice. 3 is the minimal number of animal needed to be statistically significant.

For Specific Aim 4: Gene trap ES cell lines will be recovered and injected into blastocyst to generate chimera. We plan to establish 5 to 8 mouse mutations this way. The total number of males needed for this experiment will be forty eight.

2) *Justification.* The number of animals to be used is described in the Research Plan and in above paragraph.

3) *Veterinary care.* All animals are housed in _____ mouse barrier facility in the basement of _____ and maintained by _____. The _____ animal facility is a recently-opened, state-of-the-art animal facility. The animals are monitored twice daily by skilled animal technicians, who check food and water intake. All animals are inspected weekly by _____ veterinary staff.

4) *Procedures.* No survival surgical procedure is planned.

5) *Euthanasia.* Mice will be euthanized by carbon dioxide at a pressure level causing no distress to the mice. It will be followed by cervical dislocation. These methods are consistent with the recommendation of the Panel on Euthanasia of the American Veterinary Medical Association.

Multiple PI leadership:

Dr. _____ will be supervising and is solely responsible for the work on human cumulus cells in Aim 1 and 3 while Dr. _____ will be supervising and solely responsible for the work involving animals in Aim 2 and Aim4. Dr. _____ and Dr. _____ will regularly communicate and coordinate to update each other on the progress in each side clearly and timely. Such communication is essential to the identification of conserved novel biomarkers of egg quality. Dr. _____ will be the contact PI but all the decisions with regard to this overall grant including changing of research direction should be approved by both PIs. Personnel changes or modification of budget in either Dr. _____ or Dr. _____ lab is encouraged to seek opinion of the other PI but will be ultimately decided by the supervising PI. Each of the PI is responsible for progress report for their respective Aims.

Dr. _____ will bring his expertise in oocyte/embryo physiology and clinical IVF to this research. Dr. _____ will bring his expertise in molecular genetics and developmental biology to this research. The expertise of both PIs compliments each other and forms a synergetic, resourceful team to ensure successful execution and completion of the proposed research.

In terms of budget, we would like to request separate budget for each PI from an annual module of \$250,000. We request 150,000 for Dr. _____ which will cover the budget for graduate student _____, Postdoctoral fellow _____ and 20 % of Dr. _____ salary, and supplier money including mouse cage cost with an estimate of 100 cages of gene trap mice at \$3000 each month. We request \$100,000 for Dr. _____ which will cover Research Technician's salary, 20% of Dr. _____ salary and supplier money. Both Dr. _____ and _____ will contribute to the 20% effort of microarray data analyst's salary. In case of budget cut, the budget cut percentage will be applied across the board for each PI.

Principal Investigator/Program Director (Last, first, middle):

E. BIBLIOGRAPHY

Principal Investigator/Program Director (Last, first, middle):

October 9, 2007

Re: National Institutes of Health "Novel biomarkers of egg quality"

Dear Dr.

I am pleased to help you on parts of your project to identify biomarkers for oocyte quality from human and mouse cumulus cells by DNA microarray analysis, and to validate biomarkers from a short list of candidate genes by quantitative real-time PCR.

The facility provides a wide range of services including: High, medium and low-throughput gene expression profiling and SNP analysis; high throughput DNA extraction; RNA quality control; quantitative real time-PCR and sequencing.

Our core is staffed by five technicians, a program assistant and a full-time Ph.D. level director. Our facility houses the Affymetrix 7G 30000GCS; the Illumina 500GDXW; a 2100 Bioanalyzer from Agilent; AutoPure Ls from Genra; the ABI 7900HT , 3730 and 3130XL DNA sequencers. For more information on our services and instruments, please visit our website at

We look forward to work with you on this exciting endeavor.

Sincerely,

October 9th, 2007

Dear Dr.

It is with great pleasure to express my support for your grant proposal entitled “Novel biomarkers for egg quality” as well as my desire to collaborate with you. Your proposed work on identifying novel biomarkers for egg quality prediction through comparative gene expression analyses of eggs of different age groups in human and mice is very exciting. I, with the assistance of the statistical analyst, would be happy to provide our bioinformatic expertise in gene data analysis and pattern recognition techniques to facilitate your study. Since 2000, I have been the subcontract PI in two large scale NCI-funded multi-center grant projects to develop and validate cancer biomarkers using gene expression profiles. The effort has identified more accurate diagnostic and prognostic gene signatures over current pathological parameters as well as novel genetic pathways associated with tumorigenesis. I am currently the PI of an NHLBI-funded grant project to identify biomarkers of sub-clinical atherosclerosis using gene expression profiles from peripheral blood leukocytes. The experiences gained therein will be uniquely applicable to your project.

My group has assisted several projects using microarrays from different vendors including Affymatrix and Lumina, and has developed a pipeline of software in large scale gene expression analysis. We are more than delighted to offer our help and expertise to this important endeavor into finding novel egg quality predictors. I am looking forward to a fruitful collaboration with you on your exciting adventure into identifying conserved gene expression signature during human and mice egg aging or quality decline due to environmental factors such as alcohol.

Respectfully,

Assistant Professor

September 23, 2007

Dear

This letter is written in strong support of your NIH R01 grant application entitled "Novel biomarkers for egg quality". I am very enthusiastic about your proposal; in particular, I am excited about your proposed work to examine the effect of alcohol on egg quality and the identification of novel biomarkers which could allow us to identify good quality eggs from bad quality eggs.

It has been long recognized that chronic alcohol consumption during pregnancy produces a variety of deleterious effects in developing embryos/fetus and active research is ongoing in multiple labs including my own to further our understanding on the biology of these effects. However, the susceptibility of developing eggs and the pre-implantation embryo to maternal alcohol exposure remain relatively unexplored. I am delighted that reproductive developmental biologist like you and human embryo and IVF expert - _____ team together to address this important question, and your research not only could provide highly needed biomarkers for egg quality but also makes inroads into mechanisms of egg quality degeneration due to aging or alcohol.

My lab has been actively investigating alcohol effect on pregnancy and fetal development in rat and mice, and we would be happy to share our expertise and experience with you in your experiments involving alcohol. I am looking forward to collaborating with you on your exciting endeavor and our collaboration will not only help to facilitate your research on alcohol's effect on egg but also beneficial to our research on alcohol-related embryo development in our projects.

Best of luck on this application, which is timely, important and innovative.

Sincerely,

Resource sharing:

We would like to share the research findings from our proposed project with the colleagues, reproductive scientists, women health care professionals and patients around the world through timely publication of our research findings in peer-reviewed journals and presentations in international and national conferences. One of the motivations for this proposal is to help infertility couples with more predictable outcome of their ART treatment. We are particularly interested in introducing our findings of novel biomarkers for egg quality to other IVF laboratories for large scale validation with an objective to develop one or two highly reliable and robust predictor for human oocyte quality.

The mouse reporter lines established for those biomarkers will also be available for research and clinical trials in non-profit institutions and profit institutions with a reasonable fee for cost-recovery.

PHS 398 Checklist

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

- New Resubmission Renewal Continuation Revision

Federal Identifier:

2. Change of Investigator / Change of Institution Questions

Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:

* First Name:

Middle Name:

* Last Name:

Suffix:

Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes No

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes No

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

Yes No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period *Anticipated Amount (\$)

*Source(s)

<input type="text"/>	<input type="text"/>	<input type="text"/>
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5. Assurances/Certifications (see instructions)

In agreeing to the assurances/certification section 18 on the SF424 (R&R) form, the authorized organizational representative agrees to comply with the policies, assurances and/or certifications listed in the agency's application guide, when applicable. Descriptions of individual assurances/certifications are provided at: <http://grants.nih.gov/grants/funding/424>

If unable to certify compliance, where applicable, provide an explanation and attach below.

Explanation:

Principal Investigator/Program Director (Last, first, middle): .

Attachments

CertificationExplanation_attDataGroup0

File Name

Mime Type