PI: [redacted]	Title: [redacted]	
Received: [redacted]	FOA: [redacted]	Council: [redacted]
Competition ID: ADOBE-FORMS-B1	FOA Title: RESEARCH PROJECT GRANT (PARENT R01)	
[redacted]	Dual:	Accession Number: [redacted]
IPF: [redacted]	Organization: [redacted]	
Former Number:	Department: [redacted]	
IRG/SRG: [redacted]	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: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
[redacted]	[redacted]	PD/PI
[redacted]	[redacted]	Co-Investigator
[redacted]	[redacted]	Co-Investigator
[redacted]	[redacted]	Consultant
[redacted]	[redacted]	Consultant

OMB Number: [redacted]
Expiration Date: 0[redacted]

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE

State Application Identifier

1. * TYPE OF SUBMISSION

☐ Pre-application ☒ Application ☐ Changed/Corrected Application

2. DATE SUBMITTED

Applicant Identifier

4. a. Federal Identifier [redacted]

b. Agency Routing Identifier

5. APPLICANT INFORMATION

*** Organizational DUNS:** [redacted]

* Legal Name: [redacted]

Department: [redacted] Division:

* Street1: [redacted]

Street2:

* City: [redacted] County / Parish:

* State: [redacted] Province:

* Country: [redacted] * ZIP / Postal Code: [redacted]

Person to be contacted on matters involving this application

Prefix: * First Name: [redacted] Middle Name: M

* Last Name: [redacted] Suffix:

* Phone Number: [redacted] Fax Number: [redacted]

Email: [redacted]

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* [redacted]

7. * TYPE OF APPLICANT: [redacted]

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☐ New ☒ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: [redacted]

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE: Research Project Grant [redacted]

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT: [redacted]

12. PROPOSED PROJECT:

* Start Date [redacted] * Ending Date [redacted]

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT** [redacted]

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: [redacted] * First Name: [redacted] Middle Name: [redacted]

* Last Name: [redacted] Suffix:

Position/Title: [redacted]

* Organization Name: [redacted]

Department: [redacted] Division: [redacted]

* Street1: [redacted]

Street2:

* City: [redacted] County / Parish:

* State: [redacted] Province:

* Country: [redacted] * ZIP / Postal Code: [redacted]

* Phone Number: [redacted] Fax Number:

* Email: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	1,912,500.00
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	1,912,500.00
d. Estimated Program Income	0.00

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

18. SFLLL or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: * First Name: [redacted] Middle Name: [redacted]

* Last Name: [redacted] Suffix:

* Position/Title: [redacted]

* Organization: [redacted]

Department: [redacted] Division:

* Street1: [redacted]

Street2: [redacted]

* City: [redacted] County / Parish: [redacted]

* State: [redacted] Province:

* Country: [redacted] * ZIP / Postal Code: [redacted]

* Phone Number: [redacted] Fax Number: [redacted]

* Email: [redacted]

*** Signature of Authorized Representative** [redacted]

*** Date Signed** [redacted]

20. Pre-application Add Attachment | Delete Attachment | View Attachment

424 R&R and PHS-398 Specific
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OMB Number: [redacted]
Expiration Date: [redacted]

Project/Performance Site Location(s)

Project/Performance Site Primary Location

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: [redacted]

DUNS Number: [redacted]

* Street1: [redacted]

Street2:

* City: [redacted]

County:

* State: [redacted]

Province:

* Country: [redacted]

* ZIP / Postal Code: [redacted]

* Project/ Performance Site Congressional District: [redacted]

Project/Performance Site Location 1

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:

DUNS Number:

* Street1:

Street2:

* City:

County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code:

* Project/ Performance Site Congressional District:

Additional Location(s)

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ No

1.a If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☒ No

IACUC Approval Date: [redacted]

Animal Welfare Assurance Number [redacted]

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ 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? ☐ Yes ☐ No

4.d. If yes, please explain:

5. * Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. * Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. * Project Summary/Abstract 7__Summary_11_3.pdf Add Attachment Delete Attachment View Attachment

8. * Project Narrative 8__Narrative_11_1.pdf Add Attachment Delete Attachment View Attachment

9. Bibliography & References Cited 5_Literature_Cited_new_1.pdf Add Attachment Delete Attachment View Attachment

10. Facilities & Other Resources 9_Resources_11_1.pdf Add Attachment Delete Attachment View Attachment

11. Equipment 10_Equipment_11_1.pdf Add Attachment Delete Attachment View Attachment

12. Other Attachments Add Attachments Delete Attachments View Attachments

Summary

Human reproduction is complex and not very efficient. More than 30% of conceptions result in spontaneous abortion with most losses occurring around the time of implantation due to an inadequate uterine milieu. Unwanted pregnancy loss is a major psychological, economical and clinical problem. One prerequisite for implantation in placental mammals is an effective two-way interaction between an implantation-competent blastocyst and the receptive uterus. The blastocyst will implant only when this molecular dialogue is established. The underlying mechanism by which a uterus transits from the prereceptive to the receptive to the nonreceptive phase remains unknown. We hypothesize that two highly conserved genes (Msx1 and Msx2) of the muscle segment homeobox (Msh) family have key roles in uterine receptivity and nonreceptivity to implantation. In the proposed study, we will test the hypothesis that these morphogenetic genes, critical for epithelial-mesenchymal interactions during development, also play crucial roles in implantation by altering the epithelial cell polarity and integrity via a non-canonical Wnt signaling involving E-cadherin-β-catenin complex formation. To test our hypothesis, we will pursue two specific aims in mice. The first specific aim will test the hypothesis that while Msx1 is a major critical factor in implantation, Msx2 has a compensatory role if Msx1 is missing. The second specific aim will test the hypothesis that Msx1 and/or Msx2 direct implantation by influencing the epithelial cell polarity and integrity. The overall goal of this proposal is to better understand the mechanisms that direct uterine receptivity and nonreceptivity with the aim of improving female fertility. We will use conditionally gene-deleted mouse models to address the molecular basis of these events, since these models provide mechanistic information relevant to female fertility which cannot be pursued in humans due to ethical restrictions. However, we will collaborate with clinician scientists to determine clinical correlates of our findings in mice.

Narrative

The underlying mechanism by which the uterus transits from the prereceptive to receptive to non-receptive phases in the context of embryo implantation is not clearly understood. The proposed study will explore the role and mechanism of Msx1 and/or Msx2, two homeotic transcription factors, in this transition. The results may help developing novel strategies to improve pregnancy rates in women with poor fertility.

Resources

[redacted] Sciences laboratories are located on the [redacted] of the newly built state-of-the-art [redacted] (designated as [redacted]) at [redacted], with a total research space of 415,000 sq. ft. [redacted] has been allocated space on the [redacted] to start a new division of [redacted] Sciences. The total space available is approximately 5,000 sq. ft., including five bays with an open-lab setup, offices and work stations. This facility is equipped with two dark rooms, fume hoods, tissue culture rooms with hoods, several incubators and an autoclave. This facility also has a cold room and an ice-maker.

Offices of PIs are located adjacent to the laboratory space and fully equipped with computers, printers, scanner, telephones and fax machines. An administrator and a business director handle day to day operation of the center. IT Services maintains group-used software and shared drives. PIs have access to the Hospital's computer facility and literature searches through their licenses.

Veterinarian supervised barrier facilities are available for housing mice at [redacted]. Many procedure rooms are available within the facility, allowing for onsite tissue collection and surgical procedures. The facility is [redacted] approved and PHS animal welfare assurance is on file. This facility is in the [redacted] to the laboratories of PIs.

[redacted] has many core facilities, including transgenic, imaging, bioinformatics & biostatistics, histology, small animal imaging, vector preparation, laser capture microdissection and microarray, and flow cytometry. Cores are in the same building as labs of PIs or in adjoining buildings. The [redacted] core is under the joint supervision of [redacted] and [redacted] and provides cryopreservation of sperm and embryos. Division of [redacted] provides excellent services for repair and maintenance of lab equipment. Division of [redacted] is a part of the newly created [redacted] Institute, a source of intellectual enrichment and a variety of expertise. [redacted] has a joint appointment with the [redacted] and [redacted] Department and as such has access to their proteomics, drug discovery and microscopy cores, and to the state of the art [redacted]. The recently created joint [redacted] Center by [redacted] and [redacted] is a clear evidence for the commitment of cancer research in the region.

Equipment

In PI's laboratory, major items include 2 cytocentrifuges, 3 microcentrifuges, 2 vacuum evaporators, several water baths, 3 dissecting microscopes (1 with camera), 2 compound microscopes with digital cameras, One confocal microscope, 4 refrigerators, 4 freezers (-20 & -80 F), 3 balances, 2 incubators, a recording spectrophotometer, a Beckmann ultracentrifuge, a Sorval high-speed centrifuge and a cold centrifuge, two pH meters and several protein, RNA and DNA gel electrophoresis systems with power supplies. Two cryostats for frozen sections and an embedding station with a microtome and processor are fully functional in the lab. Three thermal cyclers are also functional in the laboratory. A darkroom equipped with a Kodak developer and another darkroom for in situ hybridization are located adjacent to the laboratories of PI and Co-Is. Two tissue culture rooms with hoods and incubators are attached to the labs. PI's office and laboratory are also adjacent to Co-Is and close to other investigators in the division. As a group, they welcome cooperative use of equipment and space. Various other facilities and cooperation are always provided by the other investigators in the building.

OMB Number: [redacted]
Expiration Date: 0[redacted]

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: [redacted] * First Name: [redacted] Middle Name: [redacted]
* Last Name: [redacted] Suffix:
Position/Title: [redacted] Department: [redacted]
Organization Name: [redacted] Division:
* Street1: [redacted]
Street2:
* City: [redacted] County/ Parish:
* State: [redacted] Province:
* Country: [redacted] * Zip / Postal Code: [redacted]
* Phone Number: [redacted] Fax Number:
* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** [redacted] **Other Project Role Category:**

Degree Type: [redacted]
Degree Year:

***Attach Biographical Sketch** [redacted]iosketch_11[redacted] Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 1

Prefix: * First Name: [redacted] Middle Name:
* Last Name: [redacted] Suffix:
Position/Title: [redacted] Department: [redacted]
Organization Name: [redacted] Division:
* Street1: [redacted]
Street2:
* City: [redacted] County/ Parish:
* State: [redacted] Province:
* Country: [redacted] * Zip / Postal Code: [redacted]
* Phone Number: [redacted] Fax Number:
* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** [redacted] **Other Project Role Category:**

Degree Type:
Degree Year:

***Attach Biographical Sketch** [redacted]biosketch_[redacted].pdf Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 2

Prefix: * First Name: Middle Name:
* Last Name: Suffix:
Position/Title: Department:
Organization Name: Division:
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** **Other Project Role Category:**

Degree Type:
Degree Year:

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 3

Prefix: * First Name: Middle Name:
* Last Name: Suffix:
Position/Title: Department:
Organization Name: Division:
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** **Other Project Role Category:**

Degree Type:
Degree Year:

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 4

Prefix: * First Name: [redacted] Middle Name:

* Last Name: [redacted] Suffix:

Position/Title: [redacted] Department: [redacted]

Organization Name: [redacted] Division:

* Street1: [redacted]

Street2: [redacted]

* City: [redacted] County/ Parish:

* State: [redacted] Province:

* Country: [redacted] * Zip / Postal Code: [redacted]

* Phone Number: [redacted] Fax Number:

* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** [redacted] **Other Project Role Category:**

Degree Type: [redacted]

Degree Year:

***Attach Biographical Sketch** 1[redacted] Add Attachment Delete Attachment View Attachment

Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

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
[redacted]	[redacted]
eRA COMMONS USER NAME (credential, e.g., agency login)	
[redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
[redacted]	[redacted]	[redacted]	[redacted]

A. Personal Statement

[redacted]

B. Positions and Honors.

Professional Experience

[redacted]

[redacted]

Honors

[redacted]

Memberships

[redacted]

B. Selected peer-reviewed publications (Representative of [redacted] publications).
Most relevant to the current application

[redacted]

[redacted]

[redacted]

C. Research Support

[redacted]

[redacted].

[redacted]

[redacted]

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/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
[redacted]	[redacted]
eRA COMMONS USER NAME (credential, e.g., agency login)	
[redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
[redacted]	[redacted]	[redacted]	[redacted]

A. Personal statement.

[redacted]

B. Positions and Honors.

Positions and Employment

[redacted]

Other Experience and Professional Memberships

[redacted]

Selected peer-reviewed publications.

Most relevant to the current application

[redacted]

Additional recent publications of importance to the field

[redacted]

C. Research Support.

On going

[redacted].

[redacted]

[redacted]

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
[redacted]	[redacted]
eRA COMMONS USER NAME (credential, e.g., agency login)	
[redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
[redacted]	[redacted]	[redacted]	[redacted]

A. Personal Statement

[redacted]

B. Positions and Honors

Positions and Employment

[redacted]

[redacted]

Other Experience and Professional Memberships

Member: [redacted]

Grant Review: [redacted]

C. Selected Peer-reviewed publications (Selected from [redacted] peer-reviewed publications)

Most relevant to the current application

[redacted]

[redacted]

D. Research Support.

Ongoing Research Support

[redacted]

[redacted]

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
[redacted]	[redacted]
eRA COMMONS USER NAME (credential, e.g., agency login)	
[redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
[redacted]	[redacted]	[redacted]	[redacted]

A. Personal Statement

[redacted]

[redacted] and Honors

Positions and Employment

[redacted]

[redacted]

Honors

[redacted]

C. Selected Peer-reviewed Publications (selected from [redacted] peer-reviewed publications)

[redacted]

[redacted]

D. Research Support

Ongoing Research Support

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

Completed Research Support

[redacted]

Program Director/Principal Investigator (Last, First, Middle):

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/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 [redacted]	POSITION TITLE [redacted]
eRA COMMONS USER NAME (credential, e.g., agency login) [redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	

A. Personal Statement

[redacted]

B. Positions and Honors

(i) Academic positions held

[redacted]

(ii) Honorary and advisory appointments

[redacted]

Program Director/Principal Investigator (Last, First, Middle):

[redacted]

C. Selected Peer-reviewed Publications (papers from the period [redacted])

[redacted]

Program Director/Principal Investigator (Last, First, Middle):

[redacted]

D. Research Support

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

PHS 398 Cover Page Supplement

OMB Number:

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

Prefix:

* First Name:

Middle Name:

* Last Name:

Suffix:

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/Parish:

* State:

Province:

* Country:

* Zip / Postal Code:

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

PHS 398 Modular Budget, Periods 1 and 2

OMB Number: [redacted]

Budget Period: 1

Start Date: [redacted] End Date: [redacted]

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	53	250,000.00	132,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) [redacted]

Indirect Cost Rate Agreement Date [redacted]

Total Indirect Costs 132,500.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) 382,500.00

Budget Period: 2

Start Date: [redacted] End Date: [redacted]

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	[redacted]	53	250,000.00	132,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) [redacted]

Indirect Cost Rate Agreement Date [redacted]

Total Indirect Costs 132,500.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) 382,500.00

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3

Start Date: 1[redacted] End Date: [redacted]

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	[redacted]	53	250,000.00	132,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) [redacted]

Indirect Cost Rate Agreement Date [redacted]

Total Indirect Costs 132,500.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) 382,500.00

Budget Period: 4

Start Date: [redacted] End Date: [redacted]

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	[redacted]	53	250,000.00	132,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) [redacted]

Indirect Cost Rate Agreement Date [redacted]

Total Indirect Costs 132,500.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) 382,500.00

PHS 398 Modular Budget, Periods 5 and Cumulative

Budget Period: 5

Start Date: [redacted] End Date: [redacted]

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	0.00
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	[redacted]	53	250,000.00	132,500.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) [redacted]

Indirect Cost Rate Agreement Date [redacted]

Total Indirect Costs 132,500.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) 382,500.00

Cumulative Budget Information

1. Total Costs, Entire Project Period

*Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$ 1,250,000.00
Section A, Total Consortium F&A for Entire Project Period	$ 0.00
*Section A, Total Direct Costs for Entire Project Period	$ 1,250,000.00
*Section B, Total Indirect Costs for Entire Project Period	$ 662,500.00
*Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$ 1,912,500.00

2. Budget Justifications

Personnel Justification	[redacted]Just_1[redacted].pdf	Add Attachment	Delete Attachment	View Attachment
Consortium Justification		Add Attachment	Delete Attachment	View Attachment
Additional Narrative Justification		Add Attachment	Delete Attachment	View Attachment

Budget Justification

Personnel

[redacted] (2.4 calendar months, 20% effort), jointly with [redacted] (1.2 calendar months,10% effort), and [redacted] (3.0 calendar months, 25% effort) has planned this project. [redacted], and [redacted] will serve as consultants/collaborators on this grant with no salary support. PI will supervise the overall project. [redacted] together with the co-investigators and research associate will be responsible for designing experiments, analyzing data, manuscript preparation, annual report, and exchanging their findings with other investigators.

The proposed research is complex and requires diverse technical expertise. Inclusion of experienced investigators likes [redacted] and [redacted] as co-investigators, and [redacted] and [redacted] as [redacted] fulfills this objective. The molecular and cell biological expertise of [redacted] and [redacted] are great strengths to this proposal. PI has a long-standing mutually benefitting scientific relationship with these excellent investigators. [redacted] and [redacted] are experienced molecular reproductive biologists. Their expertise in molecular biology includes cloning, sequencing, Northern, Southern and in situ hybridization, RT-PCR, Chip assay and EMSA, and protein biochemistry. [redacted] and [redacted] have a very cordial relationship which is based on mutual interest in their research pursuits and have many publications together. [redacted] is well-known for his work on Msx1 and Msx2 on development. [redacted] laboratory was the first to generate floxed *Msx1* and *Msx2* and *Msx1/Msx2* double floxed mice and has provided us with the floxed *Msx1* and double floxed *Msx1/Msx2* mice. [redacted] is an internationally known expert in cadherin mediated actin assembly and cell polarity during development. The participation of these individuals is an extraordinary strength to the proposed investigation ([redacted]).

Other personnel

The proposed research is complex, tedious, time consuming and requires patience and precision. For example, the time required just to produce one *in situ* hybridization experiment, beginning from tissue processing but not including animal preparation, takes about 3-4 weeks. Furthermore, sectioning of implantation sites at 10 µm slices containing tiny blastocysts (50-75 µm) for *in situ* hybridization and immunocytochemistry are labor intensive and extremely time-consuming. Each paraffin or frozen section has to be examined under a microscope for the presence of blastocysts before mounting onto the slide for histology, immunohistochemistry or in situ hybridization. Breeding, maintenance and genotyping of conditionally knock-out mice are also a huge responsibility. Due to the tedious and time-consuming nature of the project, one full-time postdoctoral fellow and one graduate student with 100% effort (12 calendar months) are included in the budget.

[redacted] is currently working in [redacted] laboratory as a [redacted] and is on this project, since he has been involved with the project from the beginning. [redacted] is well-trained to pursue research on this project using physiological, molecular and cellular approaches and has been very productive during [redacted] tenure in the lab. [redacted] is also well-trained in mice breeding and handling, and has gained extensive expertise and knowledge in characterizing reproductive phenotypes of mutant mice. [redacted] has become an expert in confocal microscopy and, in fact, the confocal images presented in this application were performed by [redacted]. [redacted] has developed a good working and professional relationship with [redacted] and [redacted] and is closely participating in several ongoing experiments. [redacted] has [redacted] with [redacted]. [redacted] will be working with [redacted] and the collaborators for various experiments especially involving

phenotype characterization, in situ hybridization, Northern and Western blotting, confocal microscopy, and embryo transfer experiments. [redacted] will also participate in fostering new ideas, writing manuscripts, preparing materials for preparation of manuscripts, teaching and orienting newly recruited research assistants and graduate students devoted to this project. A [redacted] of [redacted] caliber and qualifications is essential for smooth conductance of the proposed research. [redacted] will devote 100% effort (12 calendar months) on this project.

[redacted], an [redacted] student ([redacted]), has been working in [redacted] lab to pursue [redacted] in [redacted]. [redacted] is a very bright [redacted] and already mastered many techniques. [redacted] has completed all course work and is fully engaged in research activities. [redacted] is also very proficient in handling, breeding and geneotyping of gene-deleted mice. [redacted] will be working on this project directly under the mentorship of [redacted]. [redacted] will be responsible for in situ hybridization, Western blotting, Northern hybridization, immunofluorescence, immunoprecipitation and other needed techniques for the successful completion of the project. [redacted] will also be responsible for breeding and genotyping floxed and deleted *Msx2* and *Msx1/Msx2* mice. [redacted] will also work with [redacted] on ChiP assays. [redacted] will devote 100% (12 calendar months) on this project.

All salaries are based on the current salary structure of the institution and are anticipated to increase by 3.5 % annually.

OMB Number: [redacted]

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is 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_Introduction_11_3.pdf	Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	3_Specific_Aims_11_1.pdf	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	4_Strategy_11_final_1.pdf	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects		Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals	6_Vertebrate_Animals_11_2.p	Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research		Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
14. Letters of Support	22__Ltr_Suppt_msx11_1.pdf	Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)	12_Resource_Sharing_Plan_11_	Add Attachment	Delete Attachment	View Attachment

16. Appendix Add Attachments Remove Attachments View Attachments

Introduction. [redacted]

Specific Aims

An effective two-way interaction between an implantation-competent blastocyst and the receptive uterus is prerequisite for implantation in eutherian mammals. The blastocyst will implant only when this molecular dialogue is established. The underlying mechanism by which a uterus transits from the prereceptive to the receptive to the nonreceptive phase is far from clear. Although a few signaling pathways are known to be critical for uterine receptivity in mice, almost nothing is known as to how the uterus spontaneously transits from a receptive to a nonreceptive (refractory) state both in mice and humans. In mice, the uterus becomes receptive on day 4 (day of implantation), while by day 5 it becomes refractory to implantation as determined by blastocyst transfer experiments. These uterine phases can be induced in ovariectomized mice by appropriate P_4 and estrogen treatment and also in delayed implanting mice. In delayed implanting mice, the uterus becomes non-responsive to implantation when exposed to P_4 alone with blastocysts undergoing dormancy. However, the delayed uterus responds to the presence of blastocysts for implantation only if exposed to estrogen after 24-48 h of P_4 priming. Even so, the induced window of receptivity only lasts for a limited period (~24 h) after estrogen treatment. The uterus then spontaneously proceeds to the refractory phase [**1, 2**]. The difference between receptive and delayed uteri is that the receptive phase is transient and spontaneously proceeds to the refractory phase, while the delayed state can be extended with continued P_4 treatment. Implantation can be initiated in the delayed state, but not in the refractory phase, by an estrogen injection.

We hypothesize that the muscle segment homeobox gene (Msh) family members Msx1 and Msx2, two highly conserved transcription factors, have key roles in uterine receptivity and nonreceptivity related to implantation. *Msx1* and *Msx2* show overlapping expression patterns during development and are critical for craniofacial patterning and other developmental events. While mice with systemic deletion of the *Msx1* gene die shortly after birth due to craniofacial defects, *Msx2*$^{-/-}$ null females show defects in tooth and hair follicle development and seizures [**3, 4**]. However, *Msx2*$^{-/-}$ mice are fertile, if provided liquid or powdered food for survival [**4**]. To circumvent these developmental defects, we are using mice with conditional uterine deletion of these genes to define their roles in uterine receptivity and nonreceptivity. Our preliminary results show that while *Msx1* is robustly expressed in the epithelium of the receptive uterus on day 4 of pregnancy, *Msx2* expression is barely detectable (**Figs. 2A & 4A**). Our preliminary results also show that while Msx1 is the primary critical factor in pregnancy success, Msx2 has a compensatory role. For example, *Msx2* expression is upregulated in the uterus lacking Msx1 and uterine deletion of both *Msx1* and *Msx2* results in complete female infertility due to implantation failure (**Fig. 5 & 9**). We propose that these genes have key roles in uterine receptivity and its transition from a receptive to a nonreceptive state. We also propose that persistent expression of *Msx* genes is critical for conferring the uterus a state of readiness to implantation that we see in P_4-primed delayed implanting wild-type (WT) mice (**Fig. 3**). Using the mouse as a model organism, our continuing goal is to better understand the mechanisms that direct various phases of uterine sensitivity to implantation with the aim of improving female fertility and developing novel non-hormonal contraceptives. **In the proposed study, we will test the hypothesis that the Msh family of homeobox genes (*Msx1* and *Msx2*), critical for epithelial-mesenchymal interactions during development [5, 6], also play crucial roles in implantation by altering the luminal epithelial cell polarity and integrity via a non-canonical Wnt signaling involving E-cadherin-β-catenin complex formation at the adherence junctions (Fig.1). To test our hypothesis, we will pursue the following specific aims in mice.**

Specific Aim 1. This aim will test the hypothesis that *Msx* genes (Msx1 and Msx2) have critical roles in uterine receptivity and non-receptivity to implantation. We will use ***Msx1***$^{loxP/loxP}$ and/or ***Msx2***$^{loxP/loxP}$ (***Msx1***$^{f/f}$ and/or ***Msx2***$^{f/f}$) mice and cross them with *PR*$^{Cre/+}$ mice to generate mice with uterine deletion of *Msx1* and/or *Msx2* (***Msx1***$^{d/d}$**,** ***Msx2***$^{d/d}$ or ***Msx1/Mx2***$^{d/d}$). These mice will undergo in-depth assessment for uterine receptivity and non-receptivity events during early pregnancy. The results will show their relative, overlapping and/or compensatory roles in implantation.

Specific Aim 2. **This aim will test the hypothesis that *Msx1* and/or *Msx2* genes direct implantation by influencing epithelial cell polarity and integrity.** We will examine whether Msx1 and/or Msx2 influence noncanonical Wnt5a signaling as a downstream target involving Ca^{++}-dependent E-cadherin and β-catenin to impact epithelial cell polarity, adhesion and cytoskeleton dynamics in the context of implantation. To fulfill this objective, we will use mice with conditional deletion of uterine *Msx* genes. The results will tell us whether Msx1 and/or Msx2 impact early pregnancy events by altering the epithelial cell polarity and integrity.

These studies are clinically relevant because human endometria show *Msx1* expression very similar to that observed in mice during the prereceptive, receptive and nonreceptive phases (**Figs. 2 vs. 13**). Further, human studies will be pursued to correlate mouse studies in collaboration with [redacted] and [redacted].

Strategy

Significance. The proposed study is highly significant in that we are exploring a genetic and molecular basis by which the uterus transits from the prereceptive to the receptive to the nonreceptive phase. An answer to this unaddressed question will exert a powerful influence in the field of female fertility regulation. We are now poised to pursue this question with the use of conditional single and double knockout mouse models of *Msx* genes. Human reproduction is complex, but not very efficient. Over 30% of conceptions result in spontaneous abortion with most losses occurring around the time of implantation. Early pregnancy loss in humans, which often occurs due to defects that arise before, during, or immediately after implantation, is a worldwide social, psychological, economic, and clinical concern. While the causes of pregnancy loss are multifaceted, inappropriate uterine milieu is a major cause of early pregnancy failure. Although the human population is growing rapidly and perhaps will reach 9 billion by 2050, 15% of couples worldwide are childless because of infertility. Many underlying causes of human infertility have been overcome by *in vitro* fertilization and embryo transfer (IVF-ET), yet implantation rates remain disappointingly low. One major cause of this low implantation rate is the transfer of embryos into nonreceptive uteri. There is a serious need to unravel the complexities of uterine receptivity and nonreceptivity to address two contrasting global issues: improving fertility and developing novel contraceptives. Since our preliminary results (**Fig. 13**) and gene array analysis have shown that higher *Msx1* and *Msx2* levels are downregulated during the receptive phase in humans (1-3), and since ethical restrictions preclude performing mechanistic studies in humans, it is scientifically prudent to gather mechanistic evidence as to the critical roles of *Msx* genes in implantation using genetically altered mouse models.

Innovation. This proposal presents an intriguing hypothesis that has not been previously addressed. Our preliminary findings that Msx genes, which are known for their critical roles in craniofacial and other developmental events, have such profound effects on uterine biology and implantation are truly novel and exciting. The idea that these genes govern implantation events by altering the luminal epithelial cell polarity and integrity via a noncanonical Wnt pathway (involving E-cadherin and β-catenin complex formation at the adherence junctions) is a brand new concept in implantation biology (Fig. 1). Our preliminary results using mice with conditional deletion of uterine *Msx1* and/or *Msx2* have created an extraordinary opportunity to generate molecular and genetic information on potential mechanisms by which the uterus becomes receptive and refractory and maintains the state of readiness. If we prove that *Msx* genes impact implantation by altering epithelial cell polarity via a noncanonical Wnt pathway, the finding will be a milestone step forward in our understanding of uterine receptivity and nonreceptivity. It is still not understood how the uterus spontaneously proceeds from the prereceptive to the receptive to the nonreceptive state. Our preliminary findings lead us to believe that *Msx* genes are not only critical for uterine receptivity, but also for maintaining the uterus in a state of preparedness for an implantation-inducing stimulus, similar to P_4-primed delayed implanting mice which readily respond to estrogen for implantation (**Fig. 3 & Table 1**). If our preliminary results are confirmed, we may be able to maintain uterine Msx1 expression levels to confer a similar extended state of responsiveness to implantation in humans. As humans and mice share over 90% of their genes, many features of human biology are similar to those of mice, including events of early development. Exploring the link between Msx genes and noncanonical Wnt signaling will generate valuable information on the role of a homeobox-noncanonical Wnt signaling axis in guiding various uterine phases in the context of implantation. The proposed study is clinically relevant, since *Msx1* and *Msx2* are also downregulated during the window of implantation (receptive phase) in humans (1-3), similar to that which occurs in mice (**Figs. 2 vs. 13**). Findings that Msx genes are critical to uterine receptivity and maintaining uterine readiness to implantation are of high relevance to female fertility as clinicians may be able to develop new strategies to improve implantation rates in IVF-ET programs by temporarily increasing uterine Msx levels to extend the

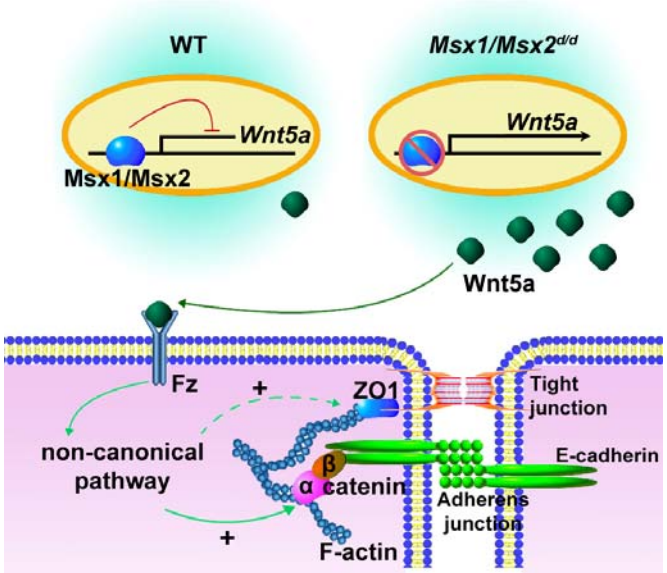

Figure 1. A proposed working model to define the role of Msx genes in implantation via noncanonical Wnt5a signaling, altering cell polarity by influencing E-cadherin/β-catenin complex formation.

uterine responsiveness to implantation prior to embryo transfer. In the same vein, uncovering the role of Msx genes may aid in developing non-hormonal contraceptives. This could be accomplished by screening and developing small molecules to intervene targets of choice. Thus, basic research to better understand the various signaling pathways integral to successful implantation will help alleviate problems of female infertility and develop novel contraceptives.

Approach

Preface. Since the application has been shortened from 25 to 13 pages, a significant amount of preliminary results are only described, without showing the data, and study designs have been shortened.

Specific Aim 1. This aim will test the hypothesis that *Msx* genes (Msx1 and Msx2) have critical roles in uterine receptivity and non-receptivity to implantation. The proposed experiments in this aim will use *Msx1*$^{loxP/loxP}$, *Msx2*$^{loxP/loxP}$ and *Msx1*$^{loxP/loxP}$/*Msx2*$^{loxP/loxP}$ mice bred with *PR*$^{Cre/+}$ mice, generating mice with uterine deletion of *Msx1* (***Msx1***$^{d/d}$ = *Msx1*$^{loxP/loxP}$/*PR*$^{Cre/+}$), *Msx2* (***Msx2***$^{d/d}$ = *Msx2*$^{loxP/loxP}$/*PR*$^{Cre/+}$) and both *Msx1* and *Msx2* (***Msx1/Msx2***$^{d/d}$ = *Msx1*$^{loxP/loxP}$/*Msx2*$^{loxP/loxP}$/*PR*$^{Cre/+}$). Littermate floxed *Msx1* (***Msx1***$^{f/f}$ = *Msx1*$^{loxP/loxP}$/*PR*$^{+/+}$), *Msx2* (***Msx2***$^{f/f}$ = *Msx2*$^{loxP/loxP}$/*PR*$^{+/+}$) and ***Msx1/Msx2***$^{f/f}$ (*Msx1*$^{loxP/loxP}$/*Msx2*$^{loxP/loxP}$/*PR*$^{+/+}$) mice without Cre expression will be used as controls. We obtained *Msx1*$^{f/f}$ and *Msx1/Msx2*$^{f/f}$ mice from [redacted], [redacted], [redacted]) (4). ***Msx1***$^{f/f}$, ***Msx2***$^{f/f}$ and ***Msx1/Msx2***$^{f/f}$ mice are being bred with *PR*$^{Cre/+}$ mice (provided by [redacted] and [redacted], [redacted]). The following experiments will characterize female fertility phenotypes in the absence of *Msx1*, *Msx2*, or both in the context of implantation.

Experiment 1. Determine whether uterine Msx1 and/or Msx2 are critical to implantation events. Our preliminary results show that more that 40% of *Msx1*$^{d/d}$ females are totally infertile and the remaining females have significantly reduced litter sizes compared to *Msx1*$^{f/f}$ females (**Fig. 5**). This reduced fertility is not due to defects in ovulation, fertilization, and/or preimplantation embryo development nor is it due to aberrant steroid levels or uterine ER or PR expression (**data not shown here, but were presented at** [redacted]). These preliminary results show that while uterine deletion of *Msx1* significantly interferes with the implantation process and compromises pregnancy outcome, the infertility is not complete. **In contrast, uterine deletion of both *Msx1* and *Msx2* imposes total infertility (Fig. 9)**. Although these results suggest that uterine *Msx* genes are critical to pregnancy success, it is not clear at what stage of pregnancy they are critical. *Msx1* expression is robust in the epithelium on the morning of day 4 of pregnancy, remarkably downregulated with approaching blastocyst attachment on day 4 evening, and disappears with the progression of implantation from day 5 onwards. This transient expression suggests that while *Msx1*'s expression is important for uterine receptivity, its downregulation is important for blastocyst attachment and implantation. In contrast, *Msx2* expression is very low to undetectable on days 1-4 of pregnancy, but it is upregulated in the epithelium in the absence of Msx1 on day 4. This is consistent with our preliminary results that Msx2 plays an important compensatory role in the absence of *Msx1* in partially rescuing implantation. We propose that the lack of uterine Msx genes confers aberrant uterine receptivity and implantation, compromising pregnancy outcome. To address their roles in early pregnancy, we propose the following experiments using mice lacking uterine *Msx1*, *Msx2*, or both.

In mice, increased endometrial vascular permeability at the site of blastocyst apposition coincides with initial blastocyst attachment with the luminal epithelium (5). This process can be visualized by an intravenous injection (0.1 ml/mouse) of a Chicago Blue dye solution and killing mice three minutes later. Implantation sites (**IS**) are demarcated by discrete blue bands (increased vascular permeability) along the uterus (6). If no blue bands are observed, uteri are flushed with saline to recover blastocysts to confirm that the dams ovulated, their eggs were fertilized, and embryos developed normally. The initiation of blastocyst attachment with the luminal epithelium normally occurs on the evening of day 4 (2000-2400h).

We will examine the initiation of blastocyst attachment on the evening of day 4 in *Msx1*$^{d/d}$, *Msx2*$^{d/d}$, and *Msx1/Msx2*$^{d/d}$ females and compare them with those in *Msx1*$^{f/f}$, *Msx2*$^{f/f}$, and *Ms1/Msx2*$^{f/f}$ females after mating them with WT males. We know a short delay in implantation elicits an adverse ripple effect throughout the course of pregnancy, compromising pregnancy outcome (7-9). We will determine whether the attachment reaction, if it occurs, is normal with uterine deletion of *Msx* genes. For these experiments, we will examine the expression of implantation-specific genes *Hb-egf* and *Cox2* at the site of blastocyst apposition along with histological analysis. We will then examine the expression of decidualization-specific genes *Bmp2* and *Hoxa10* on days 5-8 to assess the decidualization status. Results of these experiments will dictate the characterization of other pregnancy events from day 9 onwards. We will particularly focus on placentation using appropriate markers of specific placental zones as we have reported (10).

To ensure that the ovarian endocrine status is normal, we will compare peripheral blood levels of P_4 and E_2 and expression profiles of their receptors in uteri of these deleted and floxed mice during early pregnancy as we have done in our preliminary results for *Msx1*$^{d/d}$ mice (**data shown in Introduction**) and other studies (11, 12). To be absolutely sure that uterine Msx1 and/or Msx2 are critical to uterine receptivity and implantation, we will perform reciprocal blastocyst transfer experiments. Day 4 WT blastocysts will be transferred into uteri of day 4 pseudopregnant deleted and floxed recipients and IS examined on days 5-8 of pregnancy. Uteri prepared with P_4 and E_2 will also be used for blastocyst transfer experiments.

Experiment 2. Determine whether implantation is induced in P_4-primed delayed implanting *Msx1*$^{d/d}$, *Msx2*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ mice after estrogen treatment. Persistent expression of *Msx1* in P_4-primed delayed implanting WT uteri, but its downregulation with resumption of implantation after an estrogen injection, suggests a role for *Msx* genes in governing uterine responsiveness to implantation.

To test whether *Msx* genes are required to maintain uterine responsiveness to implantation, we will use the delayed implantation model as described in the preface to the Specific Aims and previously reported (13-15). Pregnant mice will be ovariectomized on the morning of day 4 (0830-0900 h) and maintained by daily P_4 injections (2 mg/mouse) from day 5 until killed. To initiate implantation, P_4-primed females will receive an injection of E_2 (25 ng/mouse) on day 7. Mice will be killed 24 h and 48 h later, and the number of IS will be recorded. Our preliminary results show that mice with uterine deletion of *Msx1* have implantation failure (**Table 1**). This suggests that Msx1 plays a critical role in maintaining uterine readiness to an implantation-inducing stimulus. Since this is an important finding, these experiments must be confirmed beyond any doubt by increasing the number of mice and expanding to *Msx2*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ mice.

Experiment 3. Determine whether Msx1/Msx2 influence uterine responsiveness to steroid hormones. Our preliminary observation of failure of implantation in P_4-primed delayed implanting *Msx1*$^{d/d}$ mice after estrogen injection suggests that uterine responsiveness to estrogen and/or P_4 is compromised in the absence of Msx1. Estrogen and P_4 are the primary mediators that confer various phases of uterine sensitivity to implantation (16). Therefore, it is important to assess uterine responsiveness to steroid hormones in order to correctly define reproductive phenotypes resulting from gene deletions. Msx genes influence epithelial-mesenchymal interactions by interacting with BMP, Wnt, and hedgehog signaling (17-22). Since uterine events also involve epithelial-mesenchymal interactions, we speculate that *Msx* genes play a role in uterine biology via one or more of these inductive factors (17-23). Indian hedgehog (**IHH**) is a secreted signaling molecule that is critical for uterine receptivity and implantation (24, 25). However, our preliminary results show that uterine *Ihh* expression is not altered in *Msx1*$^{d/d}$ females (**Fig. 6**).

To determine whether mice with conditional deletion of uterine *Msx* genes show reduced responsiveness to steroid hormones during pregnancy, we will first examine known estrogen and P_4 regulated genes on days 1 and 4 of pregnancy, respectively. On day 1, the uterus is under the influence of a preovulatory estrogen surge, while on day 4 the uterus is primed with rising P_4 levels from the newly formed corpora lutea and is superimposed with a small amount of preimplantation ovarian estrogen secretion. *Floxed* and deleted females will be mated with WT males, and uteri will be collected and snap-frozen. Our preliminary results show that day 4 pregnant *Msx1*$^{d/d}$ mice have normal uterine expression of known P_4-responsive genes *Hoxa10* and *Ihh*. We will expand these experiments in *Msx2*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ mice. Since uterine *Lif* (*leukemia inhibitory factor)*, an estrogen-responsive gene, is critical to implantation in mice (26, 27), and expressed in the luminal epithelium on day 1 and glandular epithelium on day 4, we will examine its expression in days 1 and 4 pregnant uteri by Northern and in situ hybridization. These experiments will be followed by assessment of uterine cell-specific proliferation by Ki-67 staining and BrdU labeling, and ER and PR expression profiles. Our preliminary immunohistochemistry results show that ER and PR expression profiles are comparable in *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ uteri on day 4 (**data shown in the Introduction**). Day 5 pregnant uteri will also be assessed for uterine histology, cell proliferation, and ER/PR immunostaining. These results will tell us whether *Msx* genes participate in uterine preparation to the receptive state under coordinated actions of ovarian steroids. We will also more precisely examine uterine responsiveness to steroid hormones in ovariectomized mice deleted of *Msx1* and/or *Msx2*. Littermate floxed and deleted females will be ovariectomized and rested for 10 days. They will then be injected with either oil (vehicle control), E_2 (25 ng/mouse), P_4 (2 mg/mouse), or E_2 + P_4. Mice will be killed tentatively 6, 12, 24, and 48 h later. Uteri will be analyzed by Northern and in situ hybridization for *Lif* (E_2 responsive) and *Ihh* and *Hoxa10* (P_4-responsive).

Our preliminary results show that *Lif* expression is downregulated in *Msx1*$^{d/d}$ uteri on day 4 of pregnancy (**Fig. 10**), suggesting that Msx1's inhibitory role in implantation could be mediated by LIF. However, we now have data to show that implantation fails in *Lif*$^{-/-}$*/Msx1*$^{d/d}$ females after transfer of day 4 WT blastocysts on day 4 of pseudopregnancy in spite of the recipients receiving recombinant LIF (rLIF); unimplanted blastocysts were

recovered. In contrast, a similar injection of rLIF induced implantation in pseudopregnant *Lif*$^{-/-}$/*Msx1*$^{f/f}$ females receiving similar blastocyst transfers (**Table 2**). The results suggest that not only *Lif* expression, but uterine responsiveness to LIF that is normally maintained in *Lif*$^{-/-}$ uteri is also profoundly disturbed in the absence of *Msx1*. These results are exciting and strongly suggest a critical role for *Msx* genes in uterine receptivity independent of LIF, although it is possible that LIF failed to function because of aberrant LIFR and/or gp130 activity in the uterus missing Msx1.

Experiment 4. Determine whether decidualization is normal in uteri deleted of *Msx* genes. Normally, blastocyst attachment triggers decidualization, which is critical for pregnancy success in many species. Various exogenous stimuli, including intraluminal oil infusion, if applied on day 4 (the day of uterine receptivity), also induce many aspects of the decidual response in pseudopregnant mice (28, 29), although uterine gene expression differs in decidua induced by blastocysts from deciduoma induced experimentally (30). We also showed that implantation with accompanying decidualization occurs in CD1 *Fkbp52*$^{-/-}$ females receiving exogenous P_4, but oil-induced decidualization is severely compromised (11). Thus, it is important to know whether decidualization occurs in pseudopregnant uteri deleted of *Msx* genes after intraluminal oil infusion.

Floxed and deleted females will be mated with vasectomized WT males to induce pseudopregnancy. On day 4, one uterine horn will intraluminally receive sesame oil (10 µl) and the non-infused contralateral horn will serve as a control. Mice will be killed on days 5, 6, and 8 of pseudopregnancy, respectively; maximal decidual response occurs on day 8. Fold increases in uterine weights between oil-infused and non-infused horns are used as an index of decidualization. If decidualization is compromised in the absence of *Msx* genes, we will examine the expression of decidualization-specific genes, *Bmp2* and *Hoxa10*. Because *Msx* genes are homeotic transcription factors, their deletion could alter the expression of other conserved developmental genes. Notably, *Msx* genes are not expressed in WT stroma (data not shown). If deciduoma formation is defective, it may be due to defective luminal epithelial function as has previously been shown (31).

Specific Aim 2. This specific aim will test the hypothesis that *Msx* genes direct the implantation process by influencing the epithelial cell polarity and integrity.

Experiment 1. Msx genes regulate uterine luminal epithelial cell polarity and cytoskeleton reorganization by controlling Wnt5a expression. The objective here is to explore the potential mechanism by which Msx genes direct cell polarity and epithelial-mesenchymal interactions which are critical for various phases of uterine sensitivity to implantation. *Wnt5a* is expressed in the epithelium and subepithelial stroma. It is critical for uterine patterning and gland formation during development in mice (32) and in cell polarity and intracellular adhesion in other systems (33). Our preliminary results show that *Wnt5a* is upregulated in *Msx1*$^{d/d}$ uteri, particularly in epithelial cells, on day 4 of pregnancy (**Fig. 11**), suggesting that Wnt5a is a downstream target of Msx1. This is consistent with the presence of multiple Msx1 binding sites in the *Wnt5a* gene and Msx1's role as a transcriptional repressor (19). This is very interesting since aberrant luminal epithelial architecture and heightened epithelial cell polarity are evident in both *Msx1*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ females with implantation failures (**Figs. 5, 9, & 12**). Thus, we will focus on the impact of *Msx* genes on Wnt5a as a downstream target for non-canonical signaling, since Wnt5a regulates epithelial-mesenchymal interactions and cell polarity by promoting β-catenin/E-cadherin complex formation via a non-canonical pathway in other systems (33, 34).

First, we will examine uterine *Wnt5a* expression in floxed and deleted *Msx* females on day 4 morning (10:00-11:00 h) and afternoon (17:00-18:00 h), and at the site of blastocyst apposition on days 5 and 6 by Northern and in situ hybridization, RT-PCR, immunohistochemistry and Western blotting, as appropriate. Since Wnt5a interacts with Frizzed (**fz**) receptors to mediate its function and since Fz2 and Fz6 are expressed in the mouse uterine epithelium and stroma (35, 36), we will initially examine uterine *Fz2* and *Fz6* expression in these mice on similar days as above by Northern and in situ hybridization.

The overlapping uterine expression pattern of *Msx1* and *Wnt5a*, as well as upregulation of *Wnt5a* with the loss of *Msx1* on day 4 (**Fig. 11**), suggest their cooperative role in uterine function. Since the *Wnt5a* gene has multiple Msx1 binding sites (19), we are interested to examine in *vivo* gene transactivation of Msx1 on the *Wnt5a* gene by chromatin immunoprecipitation (ChIP) assay as described by [redacted]. Uterine tissues will be cross-linked by 1% formaldehyde and subjected to cell lysis followed by chromatin fragmentation as described (37). The final extracts will be used for immunoprecipitation using Msx1 antibody or normal IgG (as control). The bound protein-DNA complex will be reverse cross-linked, and DNA will be purified and analyzed by PCR. Based on the published report (19) and computer search analysis, two distinct regions are likely to be the sites of Msx1's interaction with the *Wnt5a* gene sequences. A pair of primers encompassing each region will be designed, while an arbitrary region located upstream with no recognizable binding

sequences for interaction will be chosen as a negative control for PCR amplification. PCR products will be resolved on agarose gels and examined by ethidium bromide staining. These results will show if Msx1 has direct interaction with *Wnt5a* in vivo. If the above experiments are successful, time and funds permitting, we will also explore uterine global gene targets that are under the control of Msx1's interactions on gene promoters using ChIP-Chip studies.

Experiment 2. *Msx* genes regulate uterine luminal epithelial cell polarity by noncanonical Wnt signaling. Wnt5a enhances β-catenin/E-cadherin complex formation which functions as adherence junctions to influence cell polarity (33, 34). We will use immunofluorescence to visualize cell-specific localization of Wnt5a, E-cadherin, β-catenin, and α-catenin by confocal microscopy in uteri of floxed and deleted mice on days 4-6 of pregnancy. The results will show us the status of epithelial cell membrane association of β-catenin and E-cadherin in the presence and absence of *Msx* genes. These experiments will be followed by co-immunoprecipitation (**IP**) experiments with β-catenin/E-cadherin in extracts of uterine luminal epithelium of these mice on day 4 to further confirm complex formation between these molecules. The epithelium will be isolated by stripping as we previously reported (40).

Wnt5a signals through the noncanonical pathway in many systems via stimulation of intracellular Ca^{2+} release, and activation of PKC and CaMKII (41, 42). To see if Wnt5a is operating through the noncanonical pathway, we will examine the status of PKC and CaMKII in uteri of floxed and deleted females on day 4 of pregnancy prior to and during the attachment reaction. We will use confocal microscopy, IP and Western blotting to assess the status of PKC and CaMKII. Notably, several isoforms of PKC and CaMKII are expressed in the rodent uterus (43, 44). We will first use an antibody to pCaMKII (V111A, Promega), which has affinity to $pTyr^{286}$ CaMKII that is conserved in all isoforms, and to PKC (Ab23511, Abcam) which has affinity to PKCα/β/γ. Depending on our initial results, we will examine the status of the isoforms individually.

Since we speculate that *Msx* deleted luminal epithelial cells remain highly polarized, it is possible that the landscape of the tight junction molecules (claudins, occludins, and tricellulin) is also altered in these cells. Initially, we will compare localization of Claudin-1 and occludin by confocal microscopy. These experiments will be followed by IP and Western blotting experiments. Zona occludins are part of the tight junctions and interact with actins to maintain or reorganize the cytoskeleton architecture. We will compare the localization patterns of these molecules by confocal microscopy and their association and quantitation by IP and Western blotting. F-actin localization will be performed by Phalloidin conjugated with Rhodamin. The results of these experiments will show whether failure of implantation or aberrant implantation in *Msx* deleted mice are due to the greater cell polarization, and perhaps greater adhesion. [redacted], is an internationally known developmental biologist and is widely recognized for his work on cadherin assembly of actin filaments during development. [redacted] will serve as a c[redacted] on this aspect of the project [redacted].

Future Clinical correlates of the proposed research. To determine the translational applications of our preclinical work, we will continue our collaboration with [redacted] and [redacted], [redacted], which has generated preliminary results on human samples (**Fig. 13**) and a joint publication ([redacted]) on FKBP52's roles in endometriosis. This group has access to human endometrial samples from various stages of the menstrual cycle, from fertile and infertile patients, and from patients with and without endometriosis. In addition, they have human epithelial cell lines and primary cultured cells. We are well positioned to collaborate with this group on Msx genes' roles in human female fertility. We will examine the status of cell-specific expression of *Msx1* and *Msx2* in endometria of women with recurrent implantation failure, and with and without endometriosis. These experiments will use in situ hybridization, immunohistochemistry, and quantitative RT-PCR. We will also use *Msx1* and *Msx2* siRNAs to knockdown *Msx1* and *Msx2* in cultured primary endometrial epithelial cells on matrix coated inserts to evaluate cell polarity by examining E-cadherin/β-catenin complex formation in the context of Wnt5a. The results will reveal critical roles of Msx1/Msx2 in cell polarity related to human implantation.

Anticipated results, interpretation and alternative approaches. Our preliminary results provide evidence that while Msx1 is the major player in directing uterine receptivity, implantation, and nonreceptivity, Msx2 has an important compensatory role in the absence of Msx1. *Msx2* expression, which is normally undetectable, is upregulated in the absence of *Msx1*, and uterine deletion of both *Msx1* and *Msx2* results in complete implantation failure and infertility. These results are extraordinary in the sense that these highly conserved developmental genes are so crucial for preserving critical uterine function for implantation. Although Msx2 has an important compensatory role, we believe that Msx2 is not a major player under normal conditions, since mice constitutively deleted of *Msx2* are fertile, although they have other developmental deficiencies (45). This

speculation needs to be confirmed by characterizing and comparing uterine phenotypes in *Msx2*$^{d/d}$ females compared to *Msx1*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ females as proposed.

PR-driven Cre can delete floxed genes in other PR-expressing tissues, such as the ovary. It is important to ask whether the significant implantation defect in *Msx1*$^{d/d}$ females or complete implantation failure in *Msx1/Msx2*$^{d/d}$ females is specific to uterine deficiencies independent of any aberration in ovarian steroid hormone levels or uterine expression of their receptors. Our preliminary results show that *Msx1* is not expressed in the ovary, and P_4 and E_2 levels as well as expression profiles of their nuclear receptors (PR and ERα) are comparable in *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ females during early pregnancy (**data shown in the Introduction**). However, these parameters need to be assessed in *Msx2*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ females during early pregnancy as proposed. To circumvent any subtle changes in P_4 and E_2 levels or their receptors, we will perform reciprocal embryo transfer experiments in ovariectomized recipients treated with P_4 and E_2 suitable for implantation.

One possibility is that uteri lacking *Msx1* or *Msx1/Msx2* have reduced responsiveness to P_4 and E_2. However, *Ihh* and *Hoxa10*, two critical P_4-responsive genes that are expressed in the epithelium and stroma, respectively, are normal in *Msx1*$^{d/d}$ females on day 4 of pregnancy (**Fig. 6**). Is this the cause for the preservation of some fertility in *Msx1*$^{d/d}$ females? The answer awaits results from our proposed experiments in *Msx1/Msx2*$^{d/d}$ females in which complete failure of implantation is noted. Normal expression profiles would suggest that the effects of *Msx* genes on uterine receptivity are not mediated by IHH and/or Hoxa10. In contrast, their aberrant expression may suggest that IHH and/or Hoxa10 are downstream targets of Msx genes. Alternatively, their aberrant expression could be the consequence of the failure of the uterus to achieve receptivity in the absence of *Msx1/Msx2*. We would also examine estrogen-responsive genes lactoferrin and *Lif* in these uteri. We believe that implantation failures in *Msx1*$^{d/d}$ or *Msx1/Msx2*$^{d/d}$ females are not due to the failure of ovulation, fertilization, or preimplantation embryo development, since we recover comparable numbers of healthy blastocysts in mice not showing implantation.

We think that one major role of Msx1 is to make the uterus receptive for on-time implantation, since *Msx1* expression is robust on the morning of day 4 of pregnancy. Second, downregulation of *Msx1* appears to be critical to implantation, since *Msx1* expression is remarkably downregulated approaching and following implantation, but persists in P_4-primed delayed implanting uteri with blastocyst dormancy until implantation is induced by estrogen administration. Third, we think that Msx1 is required for sustaining uterine readiness to an implantation stimulus. This is supported by our preliminary observation of downregulation of sustained *Msx1* expression and implantation in P_4-primed delayed implanting WT uteri by E_2, as opposed to E_2's failure to induce implantation under similar conditions in *Msx1*$^{d/d}$ mice. It would be interesting to see whether we obtain similar results from our proposed experiments with *Msx1/Msx2*$^{d/d}$ mice.

Implantation failure and poor implantation (weak blue bands) that are seen in *Msx1*$^{d/d}$ females and complete implantation failure in *Msx1/Msx2*$^{d/d}$ mice examined on day 5, suggest that *Msx* genes are required for optimal uterine receptivity with appropriate cell polarity conducive to normal blastocyst attachment. This is consistent with our observations that: 1) blastocyst attachment occurs at aberrant sites within uterine lumens of *Msx1*$^{d/d}$ mice, and 2) uterine lumens fail to assume slit-like structure and have altered cell polarity (**Figs. 7 & 12**). The deferred on-time attachment or abnormal implantation in *Msx1*$^{d/d}$ uteri leads to adverse ripple effects during subsequent pregnancy events, compromising pregnancy outcomes as we and others have found in other gene deleted mice (7-9). We believe that defective attachment of the blastocyst trophectoderm to the luminal epithelium in those *Msx1*$^{d/d}$ mice with weak blue reactions led to poor vascular permeability and stromal cell proliferation and differentiation in decidualization. That in turn led to poor placentation and fetal growth, ultimately compromising pregnancy outcome. Our proposed experiments to examine the pregnancy status in *Msx1*$^{d/d}$, *Msx2*$^{d/d}$, and *Msx1/Msx2*$^{d/d}$ mice during the initiation of implantation, decidualization, and placentation will address these issues.

Our preliminary findings showing that implantation largely fails in P_4-primed delayed implanting *Msx1*$^{d/d}$ females after an E_2 injection strongly implies a role for Msx1 in maintaining uterine readiness to implantation. We speculate that the loss of *Msx1* confers uterine refractory phase. On the other hand, persistent expression of Msx1 in the delayed uterus or in pregnant *Lif*$^{-/-}$ uteri followed by its disappearance with the initiation of implantation by E_2 (**Fig. 3**) or LIF injection (46) suggests that Msx genes maintain uterine readiness for implantation. We will repeat and expand these experiments in mice with uterine deletion of *Msx1*, *Msx2*, or both *Msx1* and *Msx2*. The robust expression of *Msx1* on day 4 morning of pregnancy suggests that P_4 and E_2 regulate uterine *Msx1* expression.

Our finding of LIF's failure to rescue implantation in mice deleted of both *Lif* and *Msx1* suggests Msx1's role is independent of LIF (**Table 2**). It is possible that not only Lif, but also its receptors LIFR and gp130 are

aberrant in the absence of Msx1, thereby rendering the *Lif*$^{-/-}$ uterus non-responsive to LIF. We have mice with conditional deletion of gp130 and Stat3 and with progress on the grant we will pursue these experiments.

Wnt5a is a potential downstream target of Msx1, since Msx1 can bind the *Wnt5a* gene at multiple sites (19). Our finding of upregulation of uterine *Wnt5a*, particularly in the epithelium, in *Msx1*$^{d/d}$ uteri on day 4 (**Fig. 11**) is consistent with this finding and Msx1's known role as a transcriptional repressor. This preliminary finding has huge implication, since Wnt5a can direct cell polarity and cytoskeleton reorganization by influencing β-catenin/E-cadherin complex formation via a noncanonical pathway (33, 34, 47). Since luminal epithelial cells transit from a more polar to a less polar state prior to blastocyst attachment, the Wnt5a-β-catenin/E-cadherin signaling cascade is a potential downstream signaling target for *Msx* genes to influence the implantation process (**Fig. 1**). Our preliminary results also attest to this proposition. We are hopeful that our proposed experiments to examine *in vivo* gene transcription of *Wnt5a* by Msx1 using ChIP assay will show if there is direct interaction between Msx1 and Wnt5a. [redacted] will be responsible for these experiments, since he has considerable experience in ChiP assays. We will also try ChiP assays in uterine cell lines expressing these two genes. We have an excellent antibody against Msx1 that we will use for ChiP assays. Our proposed study does not rule out a role for canonical Wnt signaling in implantation, but our preliminary results have directed us to focus in this proposal to explore the role of non-canonical Wnt signaling pathway.

We will also use human epithelial cell lines to more precisely determine the role played by Msx1 in cell polarity and cytoskeleton distribution via Wnt5a-β-catenin/E-cadherin by overexpressing or neutralizing Msx1 or adding Wnt5a in these cells. We have generated an overexpressing *Msx1* construct and plan to use the RNAi approach to neutralize *Msx1* expression. Recombinant Wnt5 is commercially available.

We will test the hypothesis that *Msx* genes, through Wnt5a-β-catenin-E-cadherin signaling pathways, alter the luminal epithelial architecture by modulating cell polarity and cytoskeleton status (33, 34), thus influencing epithelial-stromal interactions conducive to implantation. My 40 years of research experience and focus on implantation has given me the confidence and resolve to successfully direct the course of this project even in the face of experimental difficulties. I hope the Study Section has confidence in my ability to judge how to characterize female reproductive phenotypes in mice with specific gene mutations or overexpression, and the mechanisms underlying the observed changes in order to build a robust roadmap to implantation.

Statistical analysis. Each experimental group will consist of at least 10 mice and each assay will be performed at least 4-6 times with independent samples with careful analysis of the consistency of results. These numbers are necessary to obtain sufficient confidence ($P<0.05$) in data obtained. We will use the SPSS program (SPSS, Inc., Chicago, IL) to perform statistical analyses. Differences among individual sample means will be assessed by Student's t test, and groups will be analyzed by ANOVA with posthoc analysis by Dunnett's test for multiple comparisons. We have employed this program for statistical analysis in our previous studies. Moreover, we always consult with biostatisticians in the [redacted].

Anticipated time table. The proposed research is anticipated to require 5 years to complete. The reasons necessitating this time schedule are: [1] studies on implantation are slow due to the necessity of obtaining timed pregnancy or pseudopregnancy, [2] experiments on preimplantation embryos, delayed implantation, in situ hybridization, and knockout mice with reduced fertility are all tedious and time consuming, and [3] studies with conditional gene knockout mice are time consuming due to several rounds of breeding to obtain mice of experimental choice. We anticipate that experiments in Specific Aim 1 will require about 2.5 years, while experiments in Specific Aim 2 will require another 2.5 years to complete. Experiments in both Specific Aims will be pursued simultaneously. Most cross-breedings of mouse lines are already in progress.

Preliminary results and scientific accomplishments: This a revision of a new grant (A1), replacing my [redacted] which received [redacted] percentile during the last submission, but was below the funding level. My life-long objective has been to define the roadmap of embryo-uterine interactions during implantation. Since the inception of this grant, we have published [redacted] reviews and [redacted] peer-reviewed articles with major contributions in the field; many of our findings have direct applications to human fertility. [redacted] We published [redacted] peer-reviewed articles, [redacted] review articles, [redacted] articles, 2 book reviews, and 1 book chapter. **The [redacted] peer-reviewed articles and 2 review articles directly related to [redacted], with an average impact factor of 10.6, are listed below. Three of our publications have been highlighted with cover displays. Our recent paper in [redacted] was highlighted in [redacted]. In addition, we wrote a documentary article on [redacted]" in [redacted] and I served as a [redacted] for the [redacted].**

Peer-reviewed articles during the last grant period

[redacted]

[redacted]

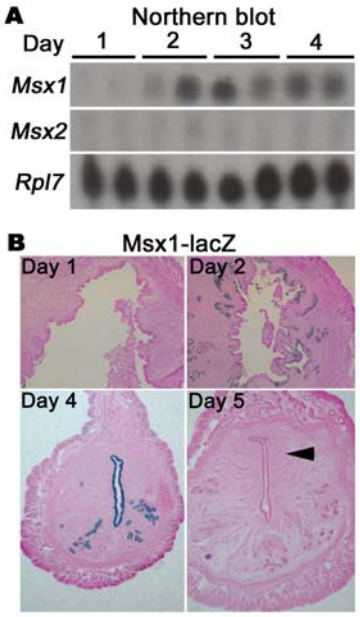

Figure 2. Uterine *Msx1* and *Msx2* expression. A. Northern Blot. **B.** LacZ staining (x100).

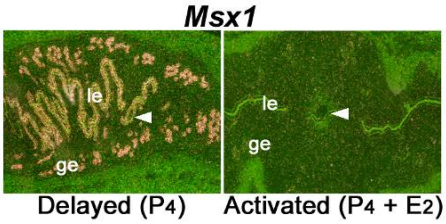

Figure 3. In situ hybridization of Msx1 in WT delayed vs. activated uteri. Arrowheads denote locations of blastocysts. 40x.

Peer-reviewed review articles

[redacted]

Preliminary results directly related to the grant.

***Msx1* expression is spatiotemporal in the preimplantation uterus.** Msx1 in luminal and glandular epithelia starts appearing from day 2, peaking expression on day 4 morning, and disappears with approaching implantation on day 4 evening and after implantation on day 5 (46). We confirmed this expression pattern using transgenic mice (gift of [redacted]) in which nuclear *LacZ* is expressed as a readout for *Msx1* expression (48) & (**Fig. 2**). *Msx1* expression is not dependent on the presence of blastocysts, since it is also expressed in pseudopregnant uteri on day 4 with its disappearance on day 5 (46).

***Msx1* expression persists during delayed implantation.** In ovariectomized, P_4-primed delayed implanting uteri, blastocysts remain dormant until an $\mathbf{E_2}$ injection induces their activation and attachment. *Msx1* persists in delayed uteri, but disappears with embryo attachment (**Fig. 3**).

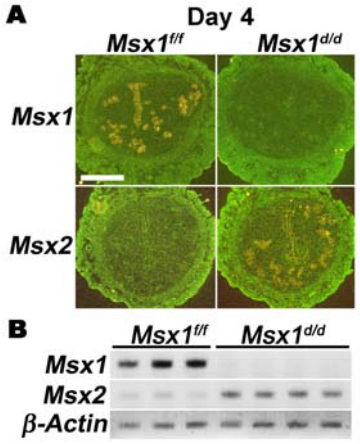

Figure 4. Msx2 can partially compensate for Msx1 deletion. A. In situ hybridization. Bar, 400 μm. **B.** RT-PCR.

Uterine *Msx1* is efficiently deleted in *Msx1*$^{d/d}$ mice. *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ females were mated with WT males, and killed on day 4 morning. We confirmed efficient deletion of uterine *Msx1* in *Msx1*$^{d/d}$ females (**Fig. 4**).

Uterine *Msx2* expression is undetectable in *Msx1*$^{f/f}$, but is upregulated in *Msx1*$^{d/d}$ uteri. To see compensatory upregulation of *Msx2* occurs in the absence of *Msx1*, *Msx2* expression was examined by in situ hybridization and RT-PCR. We found that *Msx2* is not detectable in floxed (WT) uteri, but is upregulated in*Msx1*$^{d/d}$ uteri (**Figs. 2 A & 4**).

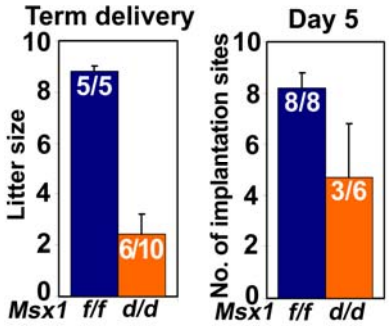

Figure 5. *Msx1*$^{d/d}$ mice have reduced fertility. Term delivery. 40% of *Msx1*$^{d/d}$ mice did not produce any pups and litter size was very low. **Implantation on day 5.** Only 50% of *Msx1*$^{d/d}$ mice showed weak blue reaction compared to *Msx1*$^{f/f}$ mice. Blastocysts were recovered from *Msx1*$^{d/d}$ mice without blue bands.

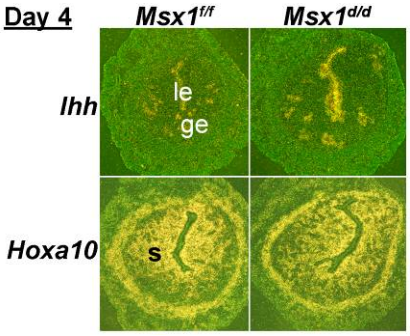

Figure 6. In situ hybridization of *Ihh* and *Hoxa10* on day 4 of pregnancy. 40X.

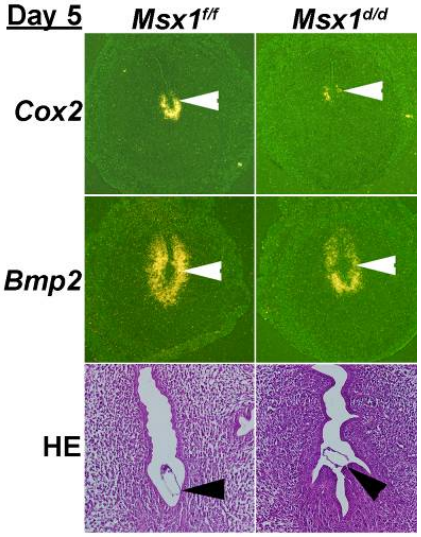

Figure 7. In situ hybridization of *Cox2* and *Bmp2* on day 5 of pregnancy (upper panels). Hematoxylin and Eosin (HE) staining (bottom panel). Arrowheads indicate the location of blastocysts. 40X.

Uterine deletion of *Msx1* adversely affects implantation. *Msx1*$^{d/d}$ and *Msx1*$^{f/f}$ females were mated with WT males. We first confirmed that *Msx1*$^{f/f}$ females have normal fertility (**Fig. 5**) and *Msx1* expression (**Fig. 4**). *Msx1*$^{d/d}$ mice have normal ovulation, fertilization, and preimplantation development (data not shown). We examined implantation sites (**IS**) on day 5 by the blue dye method (6) and found that 40% of plug-positive *Msx1*$^{d/d}$ mice failed to show blue bands and others showed very weak blue bands. The results suggest that implantation is defective in *Msx1*$^{d/d}$ mice (**Fig. 5**). We recovered blastocysts from uteri with no blue bands. *Ihh* and *Hoxa10* are P_4- responsive genes and critical to uterine receptivity. We found that their expression patterns are similar in *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ mice on day 4 (**Fig. 6**), implying that Msx1's effects on uterine receptivity are not mediated by these genes. We also examined *Cox2* and *Bmp2* expression at the site of blastocysts on day 5; they are critical for implantation and decidualization. Both *Cox2* and *Bmp2* expression, especially *Cox2*, was much lower surrounding the blastocysts in *Msx1*$^{d/d}$ uteri with weak blue bands (**Fig. 7**). Histological examination showed notable differences between *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ implantation sites. Blastocysts normally attach at the end of invaginated slit-like lumens in crypts at the antimesometrial pole. In *Msx1*$^{d/d}$ females, blastocysts attached laterally in the middle of the lumen without forming a typical crypt (**Fig. 7**). The lumen was not slit-like and lacked a well-defined crypt (nidus) for homing and attachment of the blastocyst. Moreover, the stromal bed surrounding the blastocyst had reduced edema (poor vascular permeability demarcated by a dense purple zone due to compact stromal cell layers) in *Msx1*$^{d/d}$ mice. This explains why blue reaction was so weak in *Msx1*$^{d/d}$ females.

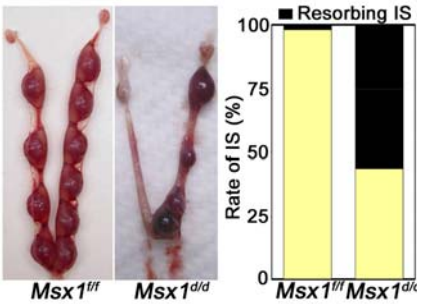

Figure 8. Implantation sites in *Msx1*$^{d/d}$ undergo resorption (Day 12 of pregnancy).

Defective implantation in *Msx1*$^{d/d}$ mice leads to adverse ripple effects. Defective implantation leads to adverse ripple effects throughout the course of pregnancy, including abnormal embryo spacing, retarded fetoplacental growth, and increased resorption sites (7, 9). Since we saw poor and abnormal implantation in *Msx1*$^{d/d}$ uteri, we examined whether pregnancy was sustained in *Msx1*$^{d/d}$ mice. On day 12, significantly increased numbers of resorption sites (60%) were noted in *Msx1*$^{d/d}$ mice (**Fig. 8**). We attribute these effects to defective implantation since *Msx1* is not expressed in the uterus on these later days of pregnancy (**data not shown**). These results suggest that defective implantation in *Msx1*$^{d/d}$ uteri adversely affects subsequent development.

Uterine deletion of *Msx1* fails to induce implantation in delayed implanting mice receiving estrogen. Pregnant *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ mice were ovariectomized on day 4 (0900 h) and given daily P_4 injections (2 mg/mouse, sc) from days 5-7 to induce delayed implantation. To initiate implantation, mice received an

injection of P_4 plus E_2 (10 ng/mouse) on day 7. IS was recorded by the blue dye method on day 8. Only one *Msx1*$^{d/d}$ mouse showed one weak IS (**Table 1**), but ng blastocysts were recovered from *Msx1*$^{d/d}$ uteri. This finding clearly suggests a role for Msx1 in maintaining uterine readiness to implantation.

Table 1. P_4-primed delayed implanting *Msx1*$^{d/d}$ females fail to show implantation in response to estrogen.

Genotype	No. of mice	No. of mice with IS (%)	No. of IS	No. of blastocysts recovered
Msx1$^{f/f}$	4	4	7.3 ± 0.6	n/a
Msx1$^{d/d}$	6	1	1	20[a]

[a]20 blastocysts were recovered from *Msx1*$^{d/d}$ mice without IS (mean ± SEM). n/a, not applicable.

Mice with uterine deletion of both *Msx1* and *Msx2* are totally infertile.
Since *Msx2* is upregulated in *Msx1*$^{d/d}$ uteri, we checked implantation and pregnancy outcome in *Msx1/Msx2*$^{d/d}$ females mated with WT males. We found complete implantation failure and total infertility in these mice (**Fig. 9**), suggesting a compensatory role for Msx2 in the absence of Msx1. Of note, constitutively deleted *Msx2* mice have normal fertility (45), but this needs to be examined for conditionally deleted *Msx2*$^{d/d}$ mice.

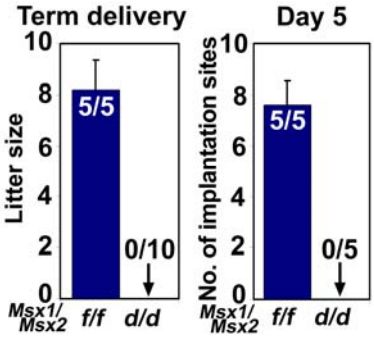

Figure 9. *Msx1/Msx2*$^{d/d}$ mice are infertile.

Peripheral ovarian hormone levels and uterine ER and PR expression are normal in *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$pregnant mice. Since PR-*Cre* can delete *Msx1* in other PR-expressing tissues, such as the ovary, adverse effects on early pregnancy in *Msx1*$^{d/d}$ females could be secondary to alterations in ovarian E_2 and P_4 levels or their uterine receptors. First, *Msx1* is not expressed in the ovary, and our preliminary results show that serum levels of P_4 and E_2 and uterine expression of ERα and P_4 are normal in *Msx1*$^{d/d}$ pregnant mice (data not shown). More importantly, implantation failure persists in *Msx1*$^{d/d}$ uteri primed with P_4 and E_2 (**Table 1**). These results suggest that Msx1's role in early pregnancy is not due to altered levels of P_4 or E_2 or their receptors. However, these parameters need to be examined in *Msx1*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ females.

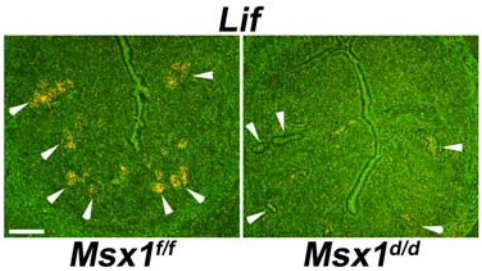

Figure 10. Uterine *Lif* expression is drastically reduced in *Msx1*$^{d/d}$ uteri on day 4 of pregnancy. Arrowheads indicate the location of glands. Bar, 500 μm.

Uterine *Lif* expression is downregulated in *Msx1*$^{d/d}$ uteri on day 4 of pregnancy. Since uterine Lif expression on day 4 is critical to implantation, we thought that defective implantation in *Msx1*$^{d/d}$ could be due to downregulation of *Lif*. Indeed, *Lif* expression is remarkably downregulated in *Msx1*$^{d/d}$ uteri (**Fig. 10**). These results suggest an interaction between Msx1 and Lif in regulating uterine receptivity and implantation.

Table 2. LIF injection fails to induce implantation in *Lif*$^{-/-}$/*Msx1*$^{d/d}$ females.

Genotype		No. of blastocysts transferred	No. of recipients	No. of mice with IS	No. of IS	No. of blastocysts recovered
Donor	Recipient					
WT	*Lif*$^{-/-}$/*Msx1*$^{f/f}$	33	3	3	7.0 ± 2.0	N/A
WT	*Lif*$^{-/-}$/*Msx1*$^{d/d}$	50	4	0	0	18

WT day 4 blastocysts were transferred into day 4 pseudopregnant *Lif*$^{-/-}$/*Msx1*$^{f/f}$ or *Lif*$^{-/-}$/*Msx1*$^{d/d}$ uteri. Recipients were given an ip injection of rLIF (20 μg/mouse) and implantation sites (IS) were examined on day 6 by blue dye injection. If blue bands were not present, uteri were flushed to recover blastocysts to confirm successful transfer. Results are mean ± SEM.

LIF administration fails to induce implantation in *Lif*$^{-/-}$/*Msx1*$^{d/d}$ recipients. If Msx1 works through LIF as a downstream target, then LIF administration in ***Lif*$^{-/-}$/*Msx1*$^{d/d}$** mice should rescue implantation. Surprisingly, we found that WT blastocysts transferred into uteri of pseudopregnant *Lif*$^{-/-}$/*Msx1*$^{d/d}$ recipients failed to implant even after injection of recombinant LIF (rLIF); unimplanted blastocysts were recovered from these mice. In contrast, a similar injection of rLIF induced implantation in pseudopregnant *Lif*$^{-/-}$/*Msx1*$^{f/f}$ females receiving similar transfers of WT blastocysts (**Table 2**). These results suggest that the uterine responsiveness to LIF that is normally maintained in *Lif*$^{-/-}$ uteri is profoundly disturbed in the absence of *Msx1*.

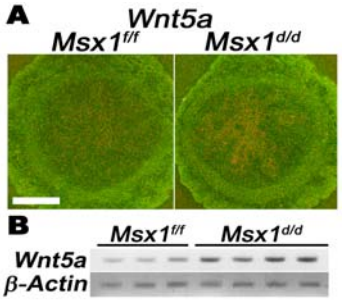

Figure 11. Uterine *Wnt5a* expression on day 4 of pregnancy. A. In situ hybridization. Bar, 400 μm. **B.** RT-PCR.

***Wnt5a* is upregulated in *Msx1*$^{d/d}$ uteri on day 4.** The *Wnt5a* gene has multiple *Msx1* binding sites and Msx1 can bind to *Wnt5a* (19). Normally, *Wnt5a* is expressed in the subepithelial stroma and, at lower levels, in the epithelium (35,

47). Our preliminary results show that that *Wnt5a* is considerably upregulated in *Msx1*$^{d/d}$ uteri, particularly in the epithelium (**Fig. 11**). This correlates with Msx1's role as a transcriptional repressor.

E-cadherin and β-catenin are highly expressed at apical junctional complexes in the luminal epithelium of *Msx1*$^{d/d}$ mice on day 4. Adhesion of epithelial cells is primarily regulated by cadherins. E-cadherin is a Ca^{++}-dependent transmembrane adhesion molecule that connects adjacent epithelial cells by linking to the cytoskeleton via β-catenin to maintain cell-cell adhesion and polarity (33). The loss of cell-cell adhesion and polarity resulting from downregulation of E-cadherin and/or β-catenin is seen in invasive tumors (34). The loosening of cell-cell adhesion in the mouse uterine epithelium after estrus or prior to blastocyst invasion has been associated with the cleavage of the extracellular domain of E-cadherin (49). There is also evidence that trophoblast adhesion to the epithelium involves changes in cell polarity; a transition from the polar to the non-polar state and focal adhesion disassembly are conducive to blastocyst implantation (50-52). Wnt5a is involved in cell polarity and participates in E-cadherin-β-catenin complex formation via a non-canonical pathway (33, 34). Since Wnt5a is upregulated in *Msx1*$^{d/d}$ uteri, we examined the status of E-cadherin and β-catenin in uteri of floxed and deleted mice on days 4 and 6 of pregnancy by confocal microscopy. Our preliminary results show that β-catenin and E-cadherin are more highly expressed at the apical junctional complexes of the luminal epithelium in *Msx1*$^{d/d}$ mice, suggesting greater polarization and, perhaps, greater adhesion (**Fig. 12A**). This highly polarized condition persists in *Msx1/Msx2*$^{d/d}$ mice even on day 6, leading to implantation failure (**Fig. 12B**). Immunoprecipitation experiments also showed that more E-Cadherin and β-catenin were physically associated in *Msx1/Msx2*$^{d/d}$ endometrial tissues (without myometrium) retrieved on the morning of day 5 of pregnancy (**Fig. 12C**). We believe one potential cause of failed or aberrant implantation in *Msx* gene deleted mice is a lack of transition of luminal epithelial cells from the polar to non-polar state. These experiments need to be repeated carefully under a variety of conditions to solidify our preliminary findings.

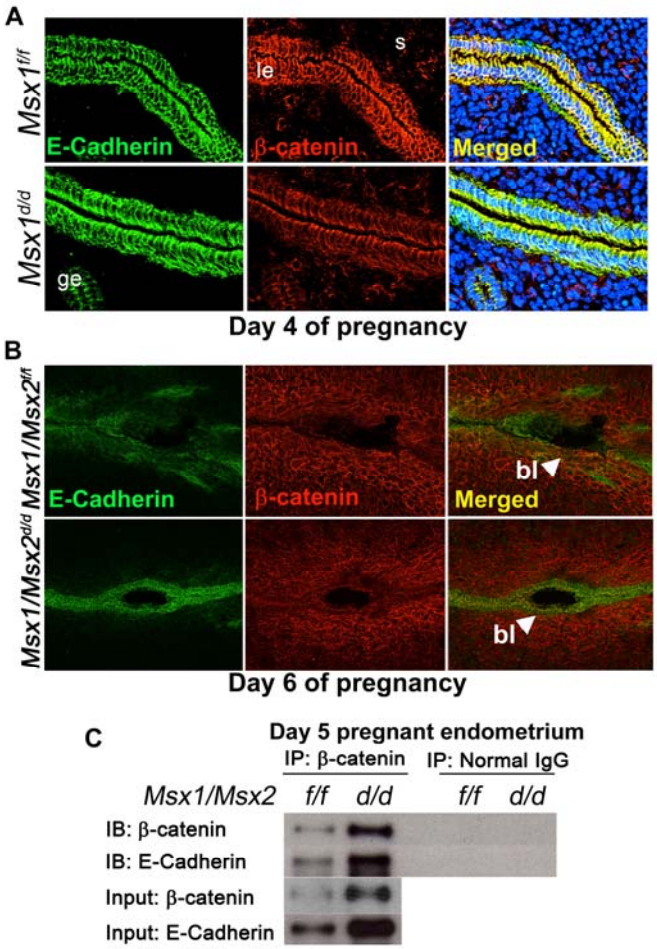

Figure 12. Co-localization and association of E-cadherin and β-catenin. Confocal image (A & B). A. *Msx1*$^{f/f}$ and *Msx1*$^{d/d}$ on day 4 of pregnancy. **B.** *Msx1/Msx2*$^{f/f}$ and *Msx1/Msx2*$^{d/d}$ on day 6 of pregnancy. **C. Immunoprecipitation.** *Msx1/Msx2*$^{f/f}$ and *Msx1/Msx2*$^{d/d}$ on day 5 of pregnancy **(New preliminary data).**

***Msx1* is expressed in the human endometrium.** Preliminary results, in collaboration with [redacted], show that *Msx1* is expressed in the human endometrium. Peak levels of *Msx1* during the ESP are preceded and followed by lower levels during the LPP and MSP (**Fig. 13**). These results are consistent with published gene array results and have similar parallels to what we see in mice, i.e. peak levels on day 4 morning followed by downregulation prior to blastocyst attachment on day 4 evening.

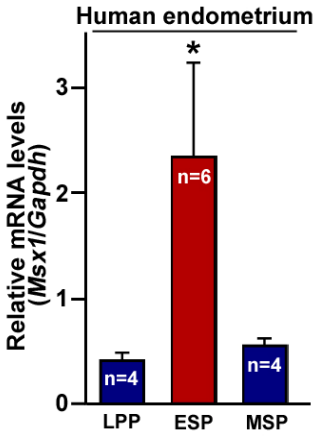

Figure 13. *Msx1* mRNA levels in human endometria quantitated by q-RT-PCR. LRP, late proliferation phase; ESP, early secretory phase; MSP, mid-secretory phase (receptive phase).

Human Subjects: None

Vertebrate Animals: Mouse

Justification and proposed use of animals. Mice will serve as a model to study the role of Msx genes during pregnancy for several reasons. **First**, there is no cell culture model that truly suffices for studies on pregnancy events in vivo. **Second**, there are certain similarities between mouse and human implantation physiology. For example, although embryo implantation in mice is eccentric as opposed to interstitial in humans, in both species implantation leads to stromal decidualization and embryos embed in the antimesometrial stroma. However in the mouse, the embryo is oriented with its inner cell mass (ICM) directed mesometrially, while in humans the ICM is directed antimesometrially. Moreover, both mice and humans have a hemochorial type of placentation. The implantation process is a two-way interaction between the embryo and the uterus. While uterine factors influence embryonic functions, conversely embryonic factors affect uterine events during implantation. Because it is not possible to use human embryos to study embryo-uterine interactions during implantation because of ethical reasons, mouse models fulfill this purpose and serve to provide more mechanistic approaches in defining the molecular basis of implantation. **Third,** tissue-specific inactivation or overexpression of target genes in mice provides the opportunity to study their physiological functions at the genetic and molecular level. **Fourth,** a considerable amount of information regarding the expression and role of various factors in pregnancy events is available in this species. **Additional reasons** for using mice are: (1) clearly defined timing of embryo development and implantation; (2) implantation can be delayed and re-initiated in a regulated manner; (3) pseudopregnancy can be easily induced in mice for embryo transfer and other experiments; (4) embryo culture and transfers are routine procedures in our lab; and (5) mouse-specific probes (antibodies and cDNAs) are well characterized in our lab.

Sexually mature (7-8 week old) C57BL6/129 wild-type, knockout and transgenic mice will be used in the proposed experiments. We will always use litter-mate floxed and deleted mice within the same set of experiments to ensure validity of our results. All mice will be housed in the Animal Care Facility at [redacted] according to the NIH and institutional guidelines on the care and use of laboratory animals under the surveillance of an Institutional Animal Care Committee. Mice will be observed on a daily basis and all veterinary care will be provided by veterinarians.

Adult females will be mated with fertile males according to the breeding schemes shown below. The morning of finding a vaginal plug is considered day 1 of pregnancy. Pseudopregnancy will be induced by mating with vasectomized males. Mouse usage will be kept to a minimum number; only sufficient numbers that provide statistically significant results will be included. Our projected estimate is that we will use about 600 mice per year. This estimate is based on the breeding schemes (see below) and pregnancy efficiency.

1. Breeding scheme to generate mice with uterine deletion of *Msx1*

$PR^{Cre/+}$ ♂ x $Msx1^{loxP/loxP}$ ♀ (P)

↓

$Msx1^{loxP/+}/PR^{Cre/+}$ ♂ (F1) **x** $Msx1^{loxP/loxP}$ ♀ (P)

↓

$Msx1^{d/d}$ ($Msx1^{loxP/loxP}/PR^{Cre/+}$) ♂ (F2) **x** $Msx1^{f/f}$ ($Msx1^{loxP/loxP}/PR^{+/+}$) ♀ (F2)

↓

$Msx1^{f/f}$ ♀ (F3) and $Msx1^{d/d}$ ♀ (F3) **for experiments**

We have successfully generated $Msx1^{f/f}$ and $Msx1^{d/d}$ mice as evident from our preliminary data. We predict that we will use about 20 males (20 cages) per year for breeding purposes. We will set up one male with 3 females to generate a maximum number of litters. This will require 60 females (15 cages). Pregnant dams will be separated before delivery, requiring an estimated total of about 25 cages for breeding purposes. Every 6-8 months, males are exchanged to ensure optimal fertility. Litters will be weaned at 19-21 days and genotyped, with $Msx1^{f/f}$ and $Msx1^{d/d}$ females then being separated. Genotyping is conducted within 5 days of weaning. For various experiments, 20 cages of $Msx1^{f/f}$ and $Msx1^{d/d}$ females each (40 cages) will be used. This would amount to approximately 100 cages of mice a year. These are projected estimates.

2. Breeding scheme to generate mice with uterine deletion of *Msx2*

$PR^{Cre/+}$ ♂ x $Msx2^{loxP/loxP}$ ♀ (P)

↓

$Msx2^{loxP/+}/PR^{Cre/+}$ ♂ (F1) **x** $Msx2^{loxP/loxP}$ ♀ (P)

↓

$Msx2^{d/d}$ ($Msx2^{loxP/loxP}/PR^{Cre/+}$) ♂ (F2) **x** $Msx2^{f/f}$ ($Msx2^{loxP/loxP}/PR^{+/+}$) ♀ (F2)

↓

$Msx2^{f/f}$ ♀ (F3) and $Msx2^{d/d}$ ♀ (F3) **for experiments**

We have successfully generated $Msx2^{f/f}$ and $Msx2^{d/d}$ mice and the colony is now being expanded. We predict that we will use about 20 males (20 cages) per year for breeding purposes. We will set up one male with 3 females to generate a maximum number of litters. This will require 60 females (15 cages). Pregnant dams will be separated before delivery, requiring an estimated total of about 25 cages for breeding purposes. Every 6-8 months, males are exchanged to ensure optimal fertility. Litters will be weaned at 19-21 days and genotyped, with $Msx2^{f/f}$ and $Msx2^{d/d}$ females then being separated. Genotyping is conducted within 5 days of weaning. For various experiments, 20 cages of $Msx2^{f/f}$ and $Msx2^{d/d}$ females each (40 cages) will be used. This would amount to approximately 100 cages of mice a year. These are projected estimates.

3. Breeding scheme to generate mice with uterine deletion of *Msx1 and Msx2*

$PR^{Cre/+}$ ♂ x $Msx1^{loxP/loxP}/Msx2^{loxP/loxP}$ ♀ (P)

↓

$Msx1^{loxP/+}/Msx2^{loxP/+}/PR^{Cre/+}$ ♂ (F1) **x** $Msx1^{loxP/loxP}/Msx2^{loxP/loxP}$ ♀ (P)

↓

$Msx1/Msx2^{d/d}$ ($Msx1^{loxP/loxP}/Msx2^{loxP/loxP}/PR^{Cre/+}$) ♂ (F2) **x** $Msx1/Msx2^{f/f}$ ($Msx1^{loxP/loxP}/Msx2^{loxP/loxP}/PR^{+/+}$) ♀ (F2)

↓

$Msx1/Msx2^{f/f}$ ♀ (F3) and $Msx1/Msx2^{d/d}$ ♀ (F3) **for experiments**

We have successfully generated $Msx1/Msx2^{f/f}$ and $Msx1/Msx2^{d/d}$ mice as evident from our preliminary data. We predict that we will use about 20 males (20 cages) per year for breeding purposes. We will set up one male with 3 females to generate a maximum number of litters. This will require 60 females (15 cages). Pregnant dams will be separated before delivery, requiring an estimated total of about 25 cages for breeding purposes. Every 6-8 months, males are exchanged to ensure optimal fertility. Litters will be weaned at 19-21 days and genotyped, with $Msx1/Msx2^{f/f}$ and $Msx1/Msx2^{d/d}$ females then being separated. Genotyping is conducted within 5 days of weaning. For various experiments, 20 cages of $Msx1/Msx2^{f/f}$ and $Msx1/Msx2^{d/d}$ females each (40 cages) will be used. This would amount to approximately 100 cages of mice a year. These are projected estimates.

Veterinary care of animals. The animal facility at the [redacted] is fully accredited by the [redacted]. All mice will be housed in barrier facilities according to the National Institutes of Health and institutional guidelines on the care and use of laboratory animals under the surveillance of an Institutional Animal Care Committee. Furthermore, an assurance is on file with the Office for Laboratory Animal Welfare. All animals are housed in filtered ventilated racks, and a mixed bedding program is used to monitor potential mouse pathogens. The [redacted] also has a Veterinary Medicine service. Mice will be observed on a routine basis and all veterinary care will be under the supervision of [redacted]

Prevention of discomfort, distress and pain to animals. Most treatments will involve subcutaneous or intraperitoneal injections with little discomfort to the animals. All surgical procedures including vasectomy in males and ovariectomy and embryo transfer in females will be performed inside a disinfected hood and will follow aseptic procedures, which include the use of sterile surgical gloves as well as sterile instruments re-sterilized via heat in between procedures. Mice will be anesthetized with avertin before surgeries. They will be euthanized at the end of each study under deep anesthesia in an Isoflurane-saturated jar followed by cervical dislocation. The details on the anesthesia methods and surgical procedures are described below. All personnel conducting experiments will be appropriately trained. No procedures will be conducted in the absence of anesthesia that will cause more than momentary distress.

***Anesthetizing mice using Isoflurane in a drop jar*:** Place gauze in the bottom of the jar and cover with paper towels. Add 300µl isoflurane to the gauze. Place one mouse in the jar and quickly close the lid; watch closely. When the mouse is lying on its side and its breathing has slowed, remove the mouse from the jar. The mouse will be at a surgical plane of anesthesia for 30 seconds to 1 minute; Add another 300 µl of isoflurane to the jar after every third or fourth mouse. All procedures will be carried in a running fume hood.

Anesthetizing mice using Avertin: Avertin is made by mixing equal amounts of tribromyl ethyl alcohol and tertiary amyl alcohol, and then diluting to 2.5% in saline. During storage, the Avertin solution is protected from light at 4˚C to prevent decomposition. Mice will receive an intraperitoneal injection of Avertin solution at a concentration of 10 mg/0.4 ml/mouse, which will result in the rapid induction of short-term surgical anesthesia (approximately 1 hour).

Vasectomy: Male mice will be anesthetized with Avertin. Their abdomen will be shaved (clipped) and washed with betadine and 70% ethanol. To open the abdomen, a cut (1.5 cm) will be made along the midline with a pair of small sterile dissection scissors. Both testes can be reached through one incision. Using the blunt forceps, the fat pad on the left side will be pulled out. The left testis, vas deferens, and epididymis will come out with the fat pad. The vas deferens lies underneath the testis and can be recognized by a blood vessel running along one side. With a pair of sharp forceps, a hole will be poked in the membrane beneath the vas deferens and pulled through a loop of silk suture. The loop will be cut to yield two pieces of suture. A double knot will be tied in each thread, with knots placed 4-5 mm apart. The vas deferens will be cut between the knots. The fat pad will be picked up with a pair of blunt forceps and the testes will be carefully placed back inside the wall. The procedure will be repeated on the right side. The body wall will be sewn up with two to three stitches. The skin will be held together with autoclips and mice will be allowed to recover in a warm cage. Wound clips will be removed in 7-10 days.

***Ovariectomy*:** Female mice are brought to the surgery and anesthetized with Avertin. The dorsal skin is shaved and washed with skin disinfectant solution of betadine, followed by 70% ethanol; this will be repeated 3 times. An incision (1cm) will be made with a pair of scissors. The dorsal skin is pulled to one side and a small incision made in the body wall to expose the ovary. The ovary is isolated with silk suture to prevent bleeding and removed with an iridectomy scissors; the procedure is repeated on the contralateral side. The skin incision is closed with 7mm wound clips and mice are allowed to recover in a warm cage. Wound clips are removed in 7-10 days, if mice are not killed within this time period.

***Embryo transfer*:** For embryo transfer experiments, mice are anesthetized with Avertin as described above. The dorsal skin is shaved and washed with a skin disinfectant solution of betadine, followed by 70% ethanol. This will be repeated 3 times and a 1cm incision made with a pair of scissors. The skin is pulled to one side and a small incision made in the body wall to expose the utero-oviductal end and the embryos are transferred to the uterine lumen from this end and the uterus is put back to peritoneal cavity. This procedure is repeated on the contralateral horn. The skin is closed with 7mm wound clips and the animal is allowed to recover in a warm cage.

***Experimental decidualization*:** For induction of decidualization, pseudopregnant female mice are anesthetized with Avertin as described above. The dorsal skin is shaved and washed with skin disinfectant solution of betadine, followed by 70% ethanol; these steps are repeated 3 times. An incision (1cm) is made with a pair of scissors. The skin is pulled to one side and a small incision made in the body wall to expose the utero-oviductal junction, and 10 µl of sesame seed oil is delivered into one uterine lumen by a 27-gauge needle fitted to a microsyringe. The uterus is put back into the peritoneal cavity. The non-infused contralateral horn will serve as a control. The skin is closed with 7mm wound clips and the animal is allowed to recover in a warm cage.

Methods of euthanasia of animals. All mice will be euthanized by cervical dislocation under deep anesthesia or in a CO_2 gas chamber. These methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Literature Cited

1. [redacted]

5. [redacted]

6. [redacted]

13. [redacted]

21. [redacted]

30. [redacted]

41. [redacted]

43. [redacted]

[redacted]

[redacted]

February 2, [redacted]

[redacted]

Dear [redacted]:

[redacted]

[redacted].

Sincerely,

[redacted]

An Equal Opportunity Employer

Resource Sharing Plan. We have a long track record of sharing research resources, including mouse models, cell lines, antibodies, DNA clones and protocols with numerous scientists, postdoctoral and graduate students all over the world. We affirm again that such resources and reagents developed with NIH funds will be made readily available for research purposes to qualified individuals within the scientific community for pursuing investigations in biomedical fields to improve the quality of life.

PHS 398 Checklist

OMB Number: [redacted]

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: [redacted]

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)

5. * Disclosure Permission Statement

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

☐ Yes ☒ No