

Journal of Visualized Experiments

Isolation and In Vitro Decidualization of Mouse Primary Endometrial Stromal Cells --Manuscript Draft--

| | |
|--|---|
| Manuscript Number: | JoVE55402R2 |
| Full Title: | Isolation and In Vitro Decidualization of Mouse Primary Endometrial Stromal Cells |
| Article Type: | Invited Methods Article - JoVE Produced Video |
| Keywords: | mouse; uterus; implantation; decidualization |
| Manuscript Classifications: | 95.51.1: acceleration effects (biological, animal and plant); 95.51.10: biology (general); 95.51.24: life sciences; 95.51.3: animal biology; 95.51.4: animal models |
| Corresponding Author: | Xiaohuan Liang South China Agricultural University College of Veterinary Medicine Guangzhou, Guangdong CHINA |
| Corresponding Author Secondary Information: | |
| Corresponding Author E-Mail: | xhliang@scau.edu.cn |
| Corresponding Author's Institution: | South China Agricultural University College of Veterinary Medicine |
| Corresponding Author's Secondary Institution: | |
| First Author: | Yuefang Liu |
| First Author Secondary Information: | |
| Other Authors: | Yuefang Liu Zengming Yang |
| Order of Authors Secondary Information: | |
| Abstract: | Successful pregnancy relies on the establishment of decidualization, which involves the morphological and functional reprogramming of the progesterone -primed endometrial stromal cells under the influence of estrogen. In this protocol, we present a method for high purified stromal cell isolation from mouse uterus on day 4 of early pregnancy. Cultured primary stromal cells would subject to further applications, such as RNA interference, overexpression, pharmaceutical treatment, immunoprecipitation, chromatic immunoprecipitation and so on. Additionally, we provide a technique for the in vitro decidualization of cultured stromal cells using estrogen and progesterone. The in vitro decidualization method allows for physical significant study of decidualization-related molecule. Altogether, this protocol provides a reliable and efficient method to facilitate further studies to define molecular mechanism of decidualization during the early pregnancy. |
| Author Comments: | Thank you very much for your comments of our manuscript "Isolation and In Vitro Decidualization of Mouse Primary Endometrial Stromal Cells". We have carefully read through all of the comments from you. In the new version of our manuscript, we have made all the corrections and extensively revised our manuscript. Attached please find our detailed responses for all the comments one by one. Thanks again for your help and comments on our manuscript. These comments have made our manuscript more logical and will be more valuable for our further study. |
| Additional Information: | |
| Question | Response |
| If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter. | |

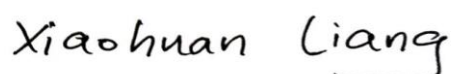
Dear Editor-in-Chief,

Attached please find the manuscript by Liu YF et al., titled “Isolation and in vitro decidualization of mouse primary endometrial stromal cells” for consideration of publication in **JoVE**.

Decidualization is a key step for the establishment of pregnancy. During decidualization, the morphological and functional reprogramming of the progesterone-primed endometrial stromal cells occurs under the influence of estrogen. In this protocol, we present a method for mouse endometrial stromal cell isolation from mouse uterus on day 4 of early pregnancy. Cultured primary stromal cells would subject to further applications, such as RNA interference, overexpression, pharmaceutical treatment, immunoprecipitation, chromatic immunoprecipitation and so on. Additionally, we provide a technique for the in vitro decidualization using estrogen and progesterone. This method allows for further study of decidualization-related molecule. Altogether, this protocol provides a reliable and efficient method to facilitate further studies to define molecular mechanism of decidualization during the early pregnancy. We sincerely appreciate your time and efforts in considering this manuscript for **JoVE**.

Please do not hesitate to contact us if you have any questions.

Very best,

A handwritten signature in black ink that reads "Xiaohuan Liang". The signature is written in a cursive, slightly slanted style. The first name "Xiaohuan" is written in a single line, and the last name "Liang" is written below it, with the 'i' in "Liang" being a small loop.

Xiaohuan Liang

College of Veterinary Medicine, South China Agricultural University,

483 Wushan Road, Tianhe District, Guangzhou, China

TEL: 86-020-85282010

FAX: 86-020-85282010

E-mail: xhliang@scau.edu.cn

TITLE:

Isolation and *In Vitro* Decidualization of Mouse Primary Endometrial Stromal Cells

AUTHORS:

Yuefang Liu, Zengming Yang, Xiaohuan Liang

Yuefang Liu

College of Veterinary Medicine
South China Agricultural University
Guangzhou, China
liuyuef1990@sina.com

Zengming Yang

College of Veterinary Medicine
South China Agricultural University
Guangzhou, China
zmyang@scau.edu.cn

Xiaohuan Liang

College of Veterinary Medicine
South China Agricultural University
Guangzhou, China
xhliang@scau.edu.cn

CORRESPONDING AUTHOR: Xiaohuan Liang, Ph.D

KEYWORDS:

Mouse, uterus, pregnancy, implantation, endometrial stromal cells, decidualization, progesterone, estradiol-17 β

SHORT ABSTRACT:

We aim to provide a method for the isolation and culturing of mouse endometrial stromal cells by enzymatic digestion. The isolated stromal cells are of high purity and can be decidualized by hormones *in vitro*. This protocol represents a feasible and stable method for stromal cell culture and for the further study of decidualization.

LONG ABSTRACT:

A successful pregnancy relies on the establishment of decidualization, which involves the morphological and functional reprogramming of the progesterone-primed endometrial stromal cells under the influence of estrogen. In this protocol, we present a method for acquiring highly purified stromal cell isolated from the mouse uterus on day 4 of early pregnancy. Cultured primary stromal cells are then subject to further applications, such as RNA interference, overexpression, pharmaceutical treatment, immunoprecipitation, chromatic immunoprecipitation, and so on. Additionally, we provide a technique for the *in vitro*

decidualization of cultured stromal cells using estrogen and progesterone. The *in vitro* decidualization method allows for the physically significant study of decidualization-related molecules. Altogether, this protocol provides a reliable and efficient method to facilitate further studies to define the molecular mechanism of decidualization.

INTRODUCTION:

Decidualization is a prerequisite for the establishment of pregnancy. In mice, decidualization occurs after the attachment of the embryo to the uterine luminal epithelium during the receptivity stage¹. However, the mechanism underlying decidualization is still not completely understood². Considering the complex compositions of many cell types and the cell-specific expression of different genes in the uterus, there is a high demand for the purification of different cell types to investigate the potential function of each gene in different compartments.

The wall of the uterus consists of three layers: the outer perimetrium; the intermediate myometrium; and, most importantly, the innermost endometrium. The endometrium is a specialized mucosa consisting of simple columnar luminal and glandular epithelia and stroma. Maghsoudlou *et al.* compared three different methods to separate the esophageal epithelial cells and found that intact mucosal sheets yield a single-cell suspension under the influence of a digestive enzyme. The isolated epithelial cells are of high viability and have minimal contamination with other types of cells³. Kalabis *et al.* and Saxena *et al.* also employed trypsin and dispase to collect cells from the rodent esophagus^{4,5}. Accordingly, we use trypsin and dispase as the digestive enzymes to dislodge the epithelial sheets from the integrated uterus. The layer of stroma contains various kinds of matrix metalloproteinases that play a crucial role in the remodeling of connective tissue. Collagenase is widely used in isolating stromal cells from the human endometrium^{6,7}. Taken together, a modified method of isolating mouse stromal cells has come into being.

The mouse appears to be the most commonly used and powerful animal model for scientific research. Estrogen and progesterone are the two main steroid hormones during pregnancy, and they are vital for implantation and decidualization. Therefore, they are employed to mimic the *in vivo* hormone environment in order to induce the cultured stromal cells to differentiate into decidual cells *in vitro*, according to Li Q's method⁸.

PROTOCOL:

All animal procedures were approved by the Animal Care and Use Committee of South China Agricultural University.

1. Preparation

1.1) Animals and treatments

1.1.1) House adult mice (CD-1 strain) in a specific, pathogen-free room with a temperature- and light-controlled environment (12 h light/12 h dark).

1.1.2) Mate mature female mice with fertile males at 16:00 h.

NOTE: The day the vaginal plug is formed is considered day 1 of pregnancy.

1.1.3) Sacrifice the mice by cervical dislocation at 10:00 h on day 4 to collect the uterus for the isolation of mouse stromal cells.

1.2) Laboratory supplies

1.2.1) Soak all glass bottles, pipettes, and 15- and 50-mL centrifuge tubes in chromic acid cleaning solution (1,000 g of potassium dichromate, 1,000 mL of concentrated sulfuric acid, and 9,000 mL of H₂O) overnight and then rinse them with distilled water. Bake all of the glassware at 180 °C for 6 h and sterilize all plastic-ware in an autoclave for 30 min.

1.2.2) Prepare the basal culture medium DMEM/F12 and HBSS according to the manufactures' instructions. Filter them using a 0.22-µm strainer. Store at 4 °C.

1.2.3) Prepare 250 mL of the medium for stromal cell culture: DMEM/F12 supplemented with 10% charcoal-stripped fetal bovine serum (cFBS) and 1% penicillin-streptomycin.

1.2.4) Prepare the digestive enzymes. Dissolve 2 g of trypsin in 40 mL of HBSS for a 5X storage concentration and aliquot 1 mL/tube. Dissolve 1 g of dispase in 16.7 mL of HBSS at the concentration of 60 mg/mL and aliquot 0.5 mL/tube. Dissolve 35 mg of collagenase in 7 mL of HBSS at a concentration of 0.5% and aliquot 180 µL/tube. Store all of the digestive enzymes at -20 °C.

1.2.5) Dissolve estradiol-17β (10 µM) and progesterone (1 mM) in alcohol and store at -20 °C.

2. Isolation and culture of mouse endometrium stromal cells

2.1) Preheat the HBSS at 37 °C in a water bath. Prepare the workbench with ultraviolet light for at least 15 min. Preheat HBSS containing 1% penicillin-streptomycin in 6-cm petri dishes.

2.2) Split the uteri longitudinally to expose the uterine lumen. Wash them 3 times in the prepared HBSS in 6-cm culture dishes to clean out blood, fat, and small pieces of tissue.

2.3) Prepare the epithelium digestive enzyme solution. Mix 1 mL of trypsin, 0.5 mL of dispase, and 3.5 mL of HBSS in a 15-mL centrifuge tube.

2.4) Digest the uteri in the enzyme solution at 4 °C for 1 h, at room temperature for 1 h, and at 37 °C for 10 min.

2.5) Pour out the digested uteri into a new dish and wipe off the sheets of luminal epithelial cells in the HBSS by swinging each uterine horn about 10 times.

2.6) Prepare the stroma digestive enzyme. Mix 150 µL of collagenase with 5 mL of HBSS.

2.7) Incubate the remaining uterine tissue at 37 °C for 30 min. Vigorously shake the digested

tissue 40 times to disperse the stromal cells. Filter the digested tissue through a 70- μ m filter to eliminate the residual tissue.

2.8) Collect the filterable cell dissociation solution in a new centrifuge tube and centrifuge at 1,500 x g for 5 min. Discard the supernatant, resuspend the cell sediment with HBSS, and centrifuge again.

2.9) Discard the HBSS and resuspend the cells with 4 mL of DMEM/F12 medium containing 2% cFBS. Mix 100 μ L of the resuspended medium with 100 μ L 0.4% trypan blue and then count the live cells under the microscope.

2.10) Dilute the cells with the culture medium to 2×10^5 cells/mL and seed the cells into different culture plates as required. Incubate the cells at 37 °C for 30 min and then change to culture medium with 10% cFBS to eliminate the unattached epithelial cells. Culture the stromal cells overnight for further study.

3. Induction of decidualization *in vitro*

3.1) Preheat the HBSS and DMEM/F12 medium containing 2% cFBS. Wash the cultured stromal cells with HBSS.

3.2) Prepare the decidualization culture medium using DMEM/F12 with 2% cFBS and dilute the stored estradiol-17 β and progesterone to final concentrations of 10 nM and 1 μ M, respectively. Supplement the control group with an appropriate volume of alcohol.

3.3) Add the decidualization culture medium to the culture plates in triplicate and culture the cells for 24 h, 48 h, and 72 h. Replace the working solution every 2 days.

3.4) Confirm the decidualization by observing cell morphology changes and the expression of decidual/trophoblast prolactin-related protein (Dtprp), a reliable decidualization marker.

REPRESENTATIVE RESULTS:

The workflow of procedures are shown in Figure 1.

The purity of isolated mouse endometrium stromal cells

The main morphological feature of the isolated stromal cells was a spindle-like appearance (Figure 2A). To confirm the purity of the isolated stromal cells, immunofluorescence was performed to detect the expression of the stromal cell marker Vimentin. The results showed that a strong Vimentin stain was detected in almost all of the cultured stromal cells (Figure 3). These results indicate that this protocol is efficient in isolating stromal cells from epithelial cells without contamination.

The effect of decidualization *in vitro*

The *in vitro* decidualization method was modified from Li's method⁸. A combination of estrogen and progesterone was applied to induce stromal-cell decidualization. During decidualization,

the morphology of the stromal cells changed from spindle-like to epithelioid (Figure 4A and B). The expression of mesenchymal-epithelial transition (MET)-related markers was detected by Western blot (Figure 5A). The expression level of the mesenchymal marker Snail was downregulated, while the expression of the epithelial marker E-cadherin was upregulated. To further confirm the process of decidualization, the decidualization marker Dtprp was detected by real-time PCR. The results showed that Dtprp was remarkably upregulated in mouse endometrial stromal cells during *in vitro* decidualization in a time-dependent manner (Figure 5B).

FIGURE LEGENDS:

Figure 1: Schematic representation of workflow.

Figure 2: Light microscopy image of cultured mouse endometrial stromal cells at 24 h after seeding. Mouse primary endometrial stromal cells cultured for 24 h displayed a typical spindle shape. This microphotograph was taken before the induction of decidualization. Scale bar = 100 μ M.

Figure 3: Immunofluorescence images of cultured endometrial stromal cells. The immunofluorescence of primary mouse endometrial stromal cells demonstrated the expression of the stromal cell marker, Vimentin (in green). PI was used as a control. Scale bar = 100 μ M.

Figure 4: Microphotograph of decidualized mouse endometrial stromal cells induced with estrogen and progesterone for 24 h. The control stromal cells were spindle-shaped, while the decidualized stromal cells displayed epithelioid morphology. Scale bar = 100 μ M.

Figure 5: Verification of the *in vitro* decidualization of stromal cells. (A) Western blot of a MET-related molecule. (B) Real-time PCR results showed that the expression of Dtprp was upregulated significantly at different time points of decidualization induction.

DISCUSSION:

The uterus, the residence of the conceptus, is of great importance for a successful pregnancy. A tight interaction between the conceptus and the maternal uterine endometrium initiates implantation and then the proliferation and differentiation of the stromal cells around the implantation sites, which will turn into specialized decidual cells under the influence of ovarian hormones. As a result of differentiation, decidual cells acquire various new functions during this transformation, such as providing a nutritional supply to the developing embryos, regulating trophoblast invasion, and modulating maternal immunity. Any aberration in this process will cause decidualization failure and adverse pregnancy outcomes⁹. Even though this is such a significant process, the molecular mechanism of decidualization is still not completely understood.

The endometrium is mainly composed of fibroblastic stromal and epithelial cells. The ovarian hormones, estrogen and progesterone, are the main steroid hormones for embryo implantation, decidualization, and maintenance of early pregnancy. On day 1 of pregnancy, the

pre-ovulatory estrogen promotes epithelial cell proliferation. On day 3 of pregnancy, the newly-developed corpora luteum starts secreting progesterone, which stimulates stromal cell proliferation. The proliferative effect of the stromal cells is further consolidated by the preimplantation of ovarian estrogen on day 4 of pregnancy¹⁰. Taken together, after day 4 of pregnancy, the uterus is at the appropriate stage for isolating stromal cells.

Trypsin, dispase, and collagenase are the common digestive enzymes that are widely used in separating epithelial cells from other tissues³. The isolated stromal cells showed non-contamination of epithelial cells and high proliferative potential, as shown in Figure 2. The cultured stromal cells can be used for further studies, such as RNA interference, overexpression, plasmid transfection, and drug treatment.

After being cultured overnight in the medium containing 10% cFBS, the stromal cells are decidualized in culture medium with 2% cFBS containing estrogen and progesterone. Our previous result suggested that MET is a potential marker of decidualization¹¹. In our results, the expression of Snail was downregulated and the expression of E-cadherin was upregulated. Furthermore, Dtprp, the most widely-used and reliable marker of decidualization, was highly expressed during *in vitro* decidualization in a time-dependent manner. Furthermore, decidual polyploidy has been reported to be important for successful pregnancies, and molecules such as amiloride binding protein 1 (Abp1) and Tdo2 have been used as markers of decidual polyploidy¹². In our previous study, Abp1 was up-regulated during *in vitro* decidualization performed according to the method mentioned above¹³. These results suggest that this method is suitable for maintaining the polyploidy characteristics observed *in vivo*.

The maternal uterus undergoes dynamic changes and developmental processes post-implantation, including the proliferation and differentiation of stromal cells, the formation of vessels, the migration of immune cells, and the remodeling of the endometrial extracellular matrix—all of which are of great importance to the establishment of a pregnancy. Our protocol for endometrial decidualization will open a new avenue for the functional study of the genes highly expressed in the decidua after implantation. It is difficult to study the physiological significance of a gene during pregnancy if genetically-modified mice are not available. Whole-body drug injections are also challenged by indirect effects and toxicity. Studies of purified stromal cells can estimate the function and molecular mechanism of a gene. As there is reciprocal crosstalk between epithelial and stromal cells, the results from isolated stromal cells will fall short of the interplay functions demonstrated by these two cell types. Meanwhile, there are two different decidualization zones at the implantation site: the primary decidualization zone (PDZ) and the secondary decidualization zone (SDZ). This *in vitro* decidualization method cannot reliably mimic decidual cells in these two regions. Altogether, although we need to pay more attention to the discrepancy between the decidualization processes *in vivo* and *in vitro*, the method we provide here is still currently the most suitable model for functional studies.

ACKNOWLEDGMENTS:

This work was funded by the National Basic Research of China (2013CB910803) and the National Natural Science Foundation of China (31471397, 31272263 and 31501206).

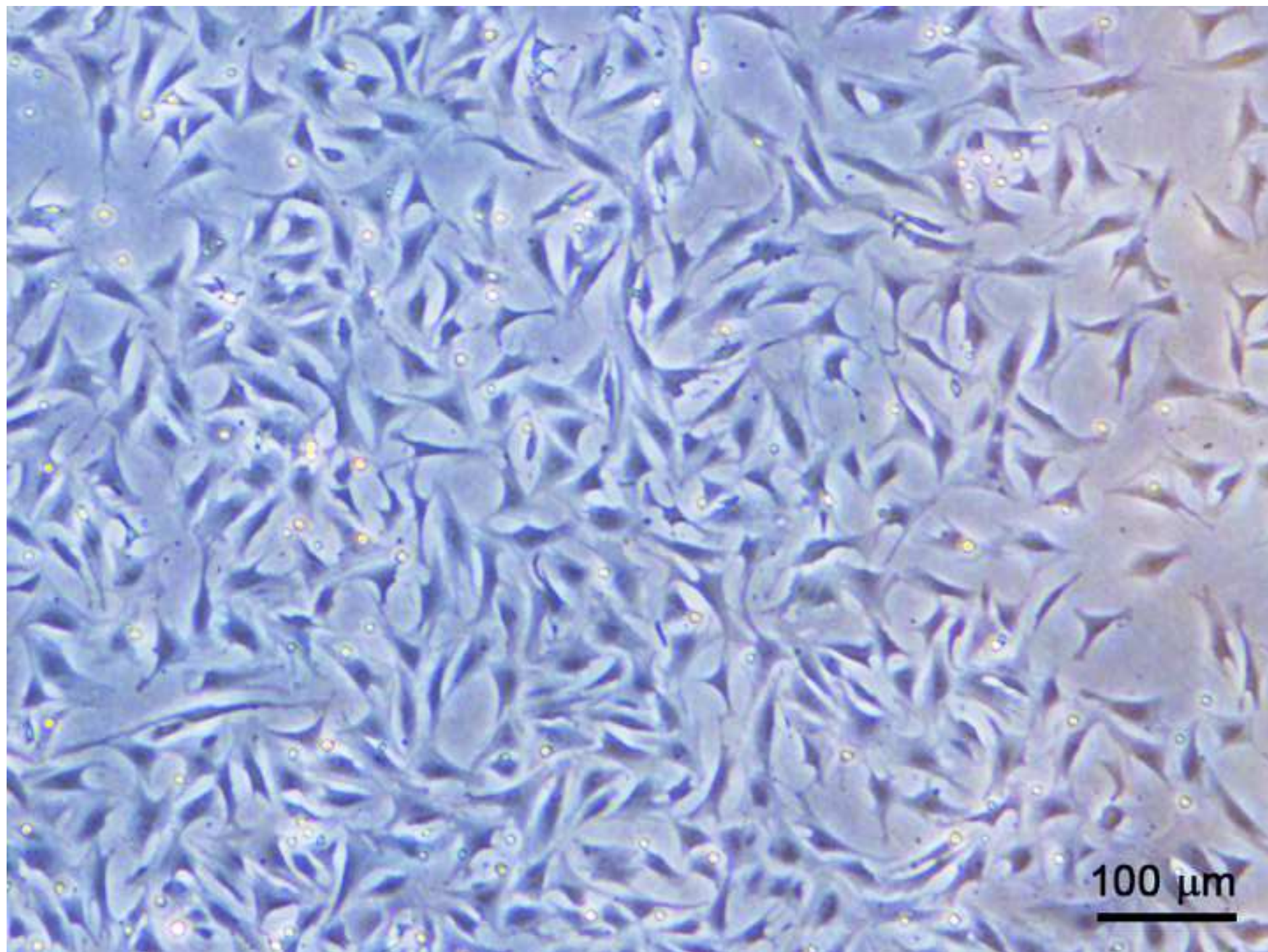
DISCLOSURES:

The authors have nothing to disclose.

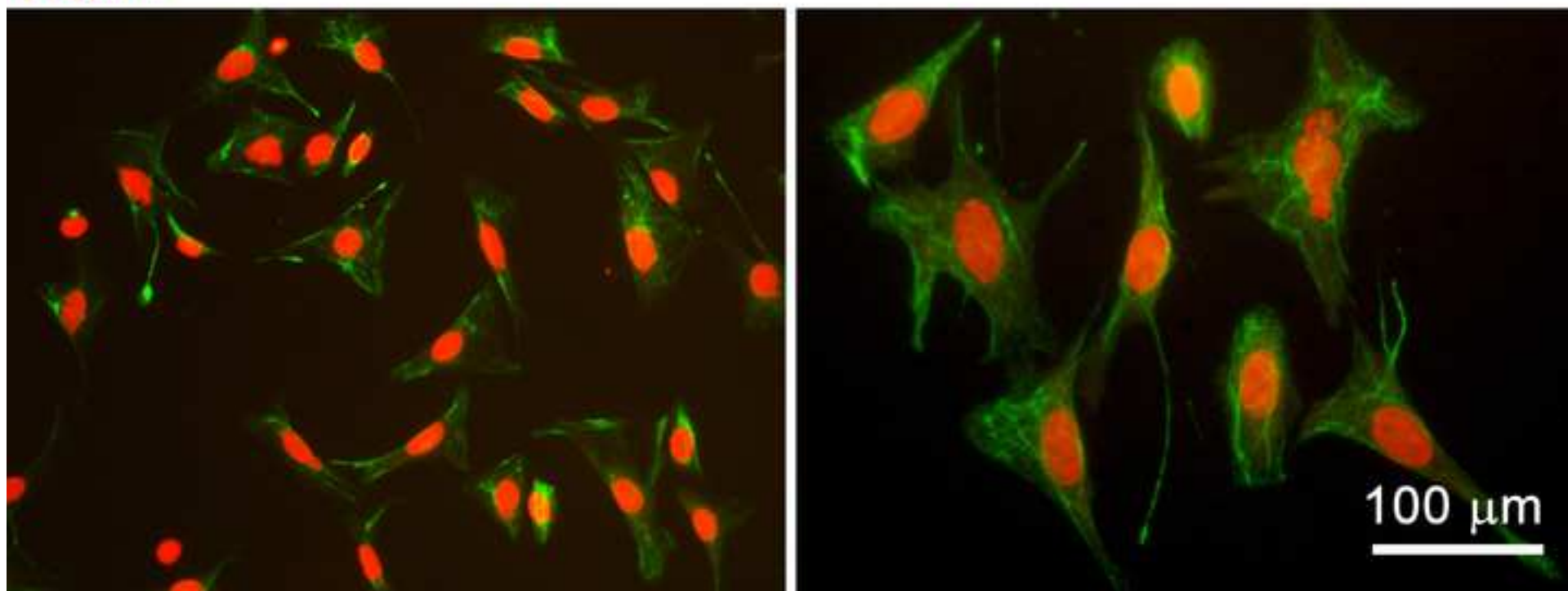
REFERENCES:

- 1 Ramathal, C. Y., Bagchi, I. C., Taylor, R. N. & Bagchi, M. K. in *Seminars in reproductive medicine*. 017-026 (© Thieme Medical Publishers).
- 2 Van Mourik, M. S., Macklon, N. S. & Heijnen, C. J. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *J Leukoc Biol.* **85** (1), 4-19 (2009).
- 3 Maghsoudlou, P. *et al.* Isolation of esophageal stem cells with potential for therapy. *Pediatr Surg Int.* **30** (12), 1249-1256 (2014).
- 4 Kalabis, J. *et al.* A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. *J Clin Invest.* **118** (12), 3860-3869 (2008).
- 5 Saxena, A. K., Ainoedhofer, H. & Höllwarth, M. E. Esophagus tissue engineering: in vitro generation of esophageal epithelial cell sheets and viability on scaffold. *J Pediatr Surg.* **44** (5), 896-901 (2009).
- 6 Martelli, M., Campana, A. & Bischof, P. Secretion of matrix metalloproteinases by human endometrial cells in vitro. *J Reprod Fertil.* **98** (1), 67-76 (1993).
- 7 Masuda, A. *et al.* An improved method for isolation of epithelial and stromal cells from the human endometrium. *J Reprod Dev.* **62** (2), 213 (2016).
- 8 Li, Q. *et al.* Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human. *J Biol Chem.* **282** (43), 31725-31732, doi:10.1074/jbc.M704723200, (2007).
- 9 Cha, J. M. & Dey, S. K. in *Regulation of Implantation and Establishment of Pregnancy in Mammals* 69-85 (Springer, 2015).
- 10 Carson, D. D. *et al.* Embryo implantation. *Dev. bio.* **223** (2), 217-237 (2000).
- 11 Zhang, X. H. *et al.* The mesenchymal-epithelial transition during in vitro decidualization. *Reprod Sci.* **20** (4), 354-360, doi:10.1177/1933719112472738, (2013).
- 12 Bian, F. *et al.* Polycomb repressive complex 1 controls uterine decidualization. *Sci Rep.* **6** 26061, doi:10.1038/srep26061, (2016).
- 13 Liang, X. H. *et al.* Estrogen regulates amiloride-binding protein 1 through CCAAT/enhancer-binding protein-beta in mouse uterus during embryo implantation and decidualization. *Endocrinology.* **151** (10), 5007-5016, doi:10.1210/en.2010-0170, (2010).

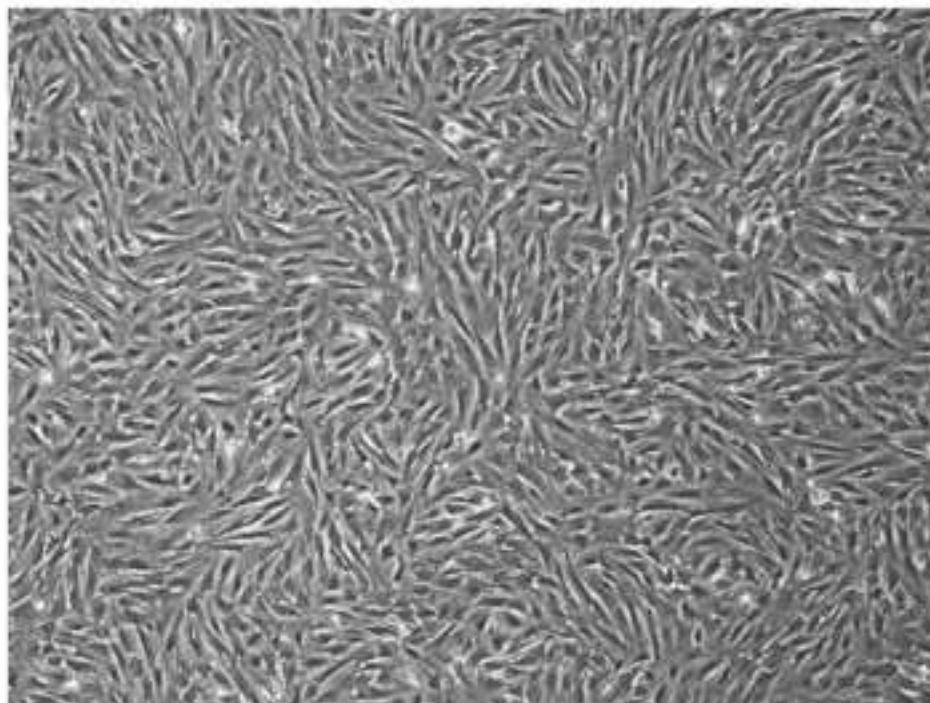
1. Mouse uteri collection
2. Split the uteri longitudinally
3. Wash with HBSS for 3 times
4. Digest with enzymes tripsin and dispase
5. Wash with HBSS for 3 times
6. Digest in collagenase
7. Shake by hand for 40 times
8. Filter and contrifuge
9. Wash with HBSS and resuspend with culture medium
10. Count and seed
11. Change culture medium 30 minutes later
12. Cells are cultured overnight
13. Was with HBSS
14. Treat with estrogen and progesterone in culture medium with 2% CFBS
15. Cells are collected at different time points after decidualization induction



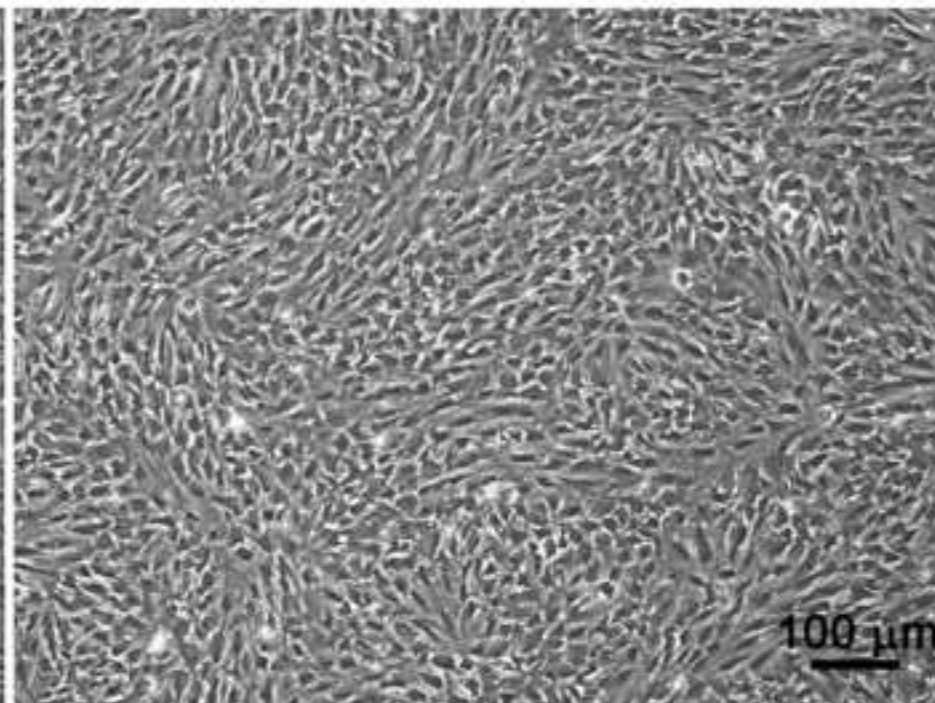
Green: Vimentin
Red: PI

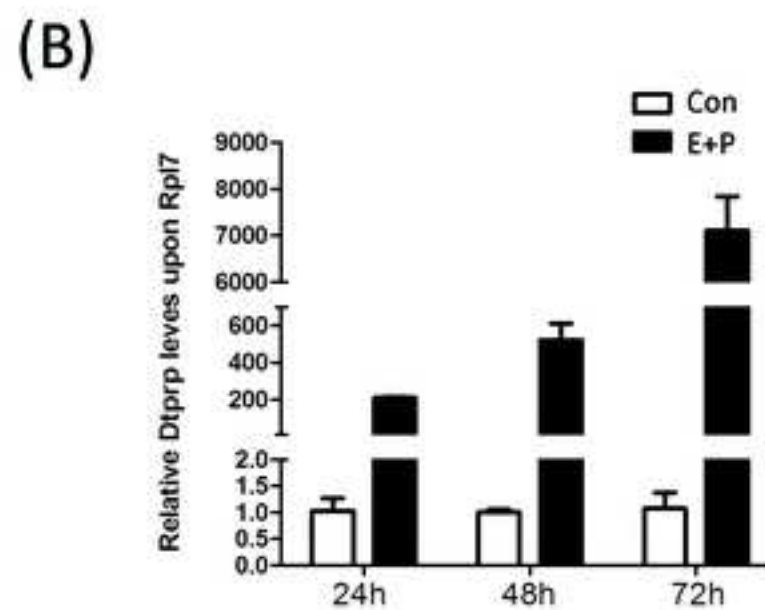
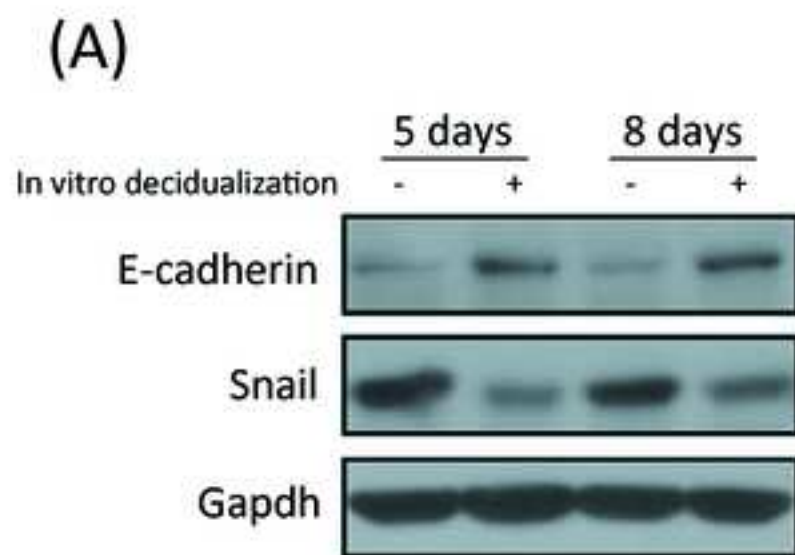


con



Deci





| Name of Reagent/ Equipment | Company | Catalog Number | Comments/Description |
|--|-----------------------|----------------|------------------------------------|
| Hanks’ Balanced Salts | Sigma | H4891 | For washing the tissues or cells |
| Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham | Sigma | D2906 | For mESC culture |
| Trypsin | Amresco | 0 458 | For epithelial sheets dissociation |
| | Roche Applied | | |
| Dispase | Science | 0 4942078001 | For epithelial sheets dissociation |
| Collagenase I | Invitrogen | 17100-017 | For stromal cells dissociation |
| Charcoal Stripped Foetal Bovine Serum | Biological Industries | 04-201-1A | For the culture of mESC |
| Progesterone | Sigma | 850454 | For in vitro decidualization |
| Estradiol-17 | Sigma | E1024 | For in vitro decidualization |
| | Cell Signaling | | |
| Vimentin | Technology | 3932s | Marker of stromal cells |
| 0.22 µm Sterile Filter | Millipore | SLGP033RB | For filtration medium |
| CO ₂ Incubator | Thermo | 3111 | For the culture of mESC |



15 Avenue of the Sciences #2010
 Cambridge, MA 02142-1101
 Tel: 617 552 9800
 www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Isolation and In vitro Differentiation of Mouse Primary Endometrial Stromal Cells
 Author(s): Yuxiang Liu, Zengming Yang, Xiaohuan Liang

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JOVE**” means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JOVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JOVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JOVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JOVE agreeing to publish the Article, the Author hereby grants to JOVE, subject to Sections 4 and 7 below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in Item 1 above, JOVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:

Xiaohuan Liang

Department:

College of Veterinary Medicine

Institution:

South China Agricultural University

Article Title:

Isolation and In Vitro Differentiation of Mouse Primary Endometrial Stromal Cells

Signature:

Xiaohuan Liang

Date:

2016.8.26

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Jaydev,

Thanking you for reviewing our manuscript entitled “**Isolation and *In Vitro* Decidualization of Mouse Primary Endometrial Stromal Cells**”. We have revised the manuscript to address each of the comments followed by our responses highlighted in *blue*. Below is a detailed discussion of our amendments:

Reviewer #1:

1. Whether the author examined the polyploidization of uterine stromal cells by immunofluorescence or flow cytometry after treatment with estrogen and progesterone. Please provide the data in the results if any or at least be mentioned in the discussion.

Response: Thanks. We have added relevant content in the Discussion part.

2. Line 41, "isolation" should be changed to "isolated".

Response: Thanks for the suggestion. It has been corrected.

3. Please double check line 48, "during the early pregnancy" looks like the redundancy.

Response: Thanks for your reminding. It has been deleted.

4. Line 58, "consists" should be changed to "consists of"

Response: Thanks for your reminding. It has been corrected by the editor.

5. Line 249, please substitute "uterine/endometrial decidualization" for "endometrial stromal cells and decidualization".

Response: Thanks. We have corrected these in Line 244.

Reviewer #2:

None.

Reviewer #3:

Major Concerns:

This is an important technique however more data is required to show these cells undergo a true decidual reaction. The only true evidence in the morphology (which is not convincing) and the expression of Snail and Dtprrp. This manuscript should show the expression of Pgr, Bmp2 and Wnt 4. The cells should be stained for alkaline phosphatase. To be truly valuable resource a more expensive characterization is required.

*Response: This is a very good point, we appreciate the thoughtful and encouraging comments of this reviewer. The stromal cells experience both morphologic and functional changes after embryo implantation *in vivo*. The decidualized stromal cells around embryo which named primary stromal cells process epithelial characteristics*

with highly expressed E-cadherin and decreased Snail. While the outer stromal cells are mainly composed by polyploidy stromal cells with secreting nature. It's hard to imitate all kinds of decidualized stromal cells just by the simple combination of estrogen and progesterone *in vitro*. We failure to detect the increase of Pgr, Bmp2 and Wnt4 in current model, which consistent with the complexity of decidualization. Although our current model will improve in vitro studies, more efforts will be taken to optimize this model in our further study.

Minor Concerns:

Finally, the writing style in the manuscript needs improvement.

Response: Thanks for your suggestion. The manuscript has been modified by a native English speaker.

We hope that our responses are now satisfactory for consideration of publication in **JOVE**

Again, we appreciate the effort and time of the reviewers in reviewing this manuscript and to you and your staff for handling this task.

Sincerely,

Xiaohuan Liang

Xiaohuan Liang

College of Veterinary Medicine, South China Agricultural University,

483 Wushan Road, Tianhe District, Guangzhou, China

TEL: 86-020-85282010

FAX: 86-020-85282010

E-mail: xhliang@scau.edu.cn