**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-17β

**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 esophagus4,5. 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 endometrium6,7. 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 °Cfor 30 min. Vigorously shake the digested tissue 40 times to disperse the stromal cells. Filter the digested tissue through a 70-μm 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 μL of the resuspended medium with 100 μL 0.4% trypan blue and then count the live cells under the microscope.

2.10) Dilute the cells with the culture medium to 2x105 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-17β and progesterone to final concentrations of 10 nM and 1 μM, 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 µM.

**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 µM.

**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 µM.

**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 methodcannot 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).