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Protocol Article

Generating primary murine vaginal fibroblast cell lines

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A B S T R A C T

Primary human vulvovaginal fibroblast cell lines are useful for studying biological mechanisms underlying genital pain, pelvic organ prolapse, and the spread of sexually transmitted infections. However, the vaginal biopsies necessary for establishing these cell lines are invasive and relatively difficult to obtain. Primary mouse fibroblast cell lines derived from pre-clinical animal models of these conditions can be used for better controlled experiments that can help us dissect disease mechanisms. To our knowledge, there are no published protocols for establishing primary murine vaginal fibroblast cell lines to date. Here, we describe a protocol for the establishment of murine vaginal fibroblast cell lines via enzymatic digestion of vaginal canal tissue. Cell lines generated using this method can be used for *in vitro* studies of these important structural cells in a variety of pre-clinical mouse models; such studies are required to identify and characterize relevant regulatory and therapeutic targets in a wide array of diseases of interest. As shown in our representative data, this protocol yields viable cell lines from ND4 Swiss outbred mice. These cells bear surface markers characteristic of fibroblasts and are capable of producing inflammatory cytokines in response to treatment with bacterial and yeast antigens *in vitro*.

A R T I C L E I N F O

Method name: Generating primary murine vaginal fibroblast cell lines
Keywords: Contact hypersensitivity, Vulvodynia, Sexually transmitted infections, Pelvic organ prolapse
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Specifications table

Subject Area:	Immunology and Microbiology
More specific subject area:	Allergy, contact hypersensitivity, chronic pain
Protocol name:	Isolation of Infiltrating Leukocytes from Mouse Skin Using Enzymatic Digest and Gradient Separation [1] Establishing primary adult fibroblast cultures from rodents [2]
Reagents/tools:	Female ND4 Swiss mice (Harlan Laboratories, Indianapolis, IN, USA) Electric Shaver (Wahl) 1 mL syringes (BD) Sterile 0.9% saline 2-Methyl-1,2-thiazol-3(2H)-one (Sigma) Surgical scissors and forceps Hank's Balanced Salt Solution (-Ca-Mg) (HyClone Laboratories) Heat-inactivated Fetal Bovine Serum (VWR) EDTA (VWR) HEPES (VWR) Collagenase D (Roche) 40 µm strainers (Fisher Scientific) Dulbecco's Modified Eagle's Medium (VWR) Penicillin/Streptomycin/L-glutamine (Gibco) 6 cm and 10 cm tissue culture treated dishes (Corning) 1X PBS (Corning) 0.25% Trypsin-EDTA (Gibco) Lipopolysaccharides from *E. coli* (Sigma) Zymosan from *S. cerevisiae* (Alfa Aesar) Anti-Mouse CD45 violetFluor450 (Tonbo) Anti-Mouse CD90.2 PE-Cyanine7 (Tonbo) Mouse IL-6 DuoSet ELISA (R&D Systems) PowerWave XZ microplate spectrophotometer (Biotek Instruments) CytoFLEX Flow Cytometer (Beckman Coulter)
Experimental design:	Establishment of primary vaginal fibroblast cell lines via enzymatic digest of vaginal tissue from previously sensitized mice repeatedly exposed to an irritant allergen in our model of allergy-driven genital pain
Trial registration:	N/A
Ethics:	15-week old female ND4 Swiss mice, conventionally housed with free access to food and water, were used for these studies. The experimental protocol used was approved by Macalester College's IACUC (B19F02).
Value of the Protocol:	• Novel protocol for establishing murine vaginal fibroblast primary cell lines • Potential for elucidating vaginal fibroblast function in murine models • Pre-clinical tool to supplement human vaginal fibroblast cell lines

Rationale

Human vulvovaginal fibroblast cell lines derived from tissue biopsies have been a valuable tool for understanding pelvic organ prolapse, chronic vulvar pain, and transmission of STIs [3–7]. However, there are several challenges associated with using these cell lines in a research setting. Biopsies used to generate control cell lines are often derived from patients with underlying conditions other than the condition being studied, rather than from healthy patients. Furthermore, biopsies are painful, and expose patients to risk of infection. Primary mouse vaginal fibroblasts present an attractive alternative to human cell lines, can be acquired more easily, and allow detailed investigation of disease mechanisms.

Here, we describe a novel method for the establishment of murine vaginal fibroblast cell lines, which provide a cell type-specific *in vitro* experimental system for investigating fibroblast behavior in pre-clinical rodent models of disease. We validate this methodology in two ways: 1) we evaluate surface marker expression and visually examine morphology of cells and 2) we demonstrate that

primary fibroblasts derived from vaginal canals of previously sensitized ND4 mice repeatedly exposed to an allergic irritant in our model of chronic allergy-provoked genital pain replicate the heightened cytokine response to bacterial and yeast antigens observed in fibroblast cell lines derived from vulvar biopsies taken from vulvodynia patients [5–6,8].

Method details

15-week old outbred female ND4 Swiss mice, conventionally housed with free access to food and water, were used for these studies. The experimental protocol used was approved by Macalester College's IACUC.

Vaginal Canal Extraction

Extract vaginal canal tissue in a BSL1 hood to minimize any likelihood of contamination. Grip the clitoris of a euthanized mouse with forceps and lift it upwards to expose the introitus or the opening of the vaginal canal. Insert curved forceps into the vaginal canal and grip the cervix. Maintaining a firm grip on the cervix, invert the vaginal canal by pulling the cervix out through the introitus. Use surgical scissors to remove the vaginal canal tissue by cutting at the base of the vaginal opening; the vaginal canal is now inside out. Grasp the vaginal canal with a pair of tweezers held in one hand and re-invert it over the end of a pair of tweezers held it the other hand. Remove the cervix using a clean, sharp surgical blade. See Yano, et al. for a video of vaginal canal extraction [9].

Pre-digestion Tissue Softening

Place two vaginal canals together into a 15 mL tube containing 1 mL of HBSS Media (Hank's Balanced Salt Solution (-Ca, -Mg) with 10% heat-inactivated FBS, 5 mM EDTA, and 2.383 g/L HEPES), which should be prepared the same day as the collection. Tightly close the tube, seal with parafilm, and secure it to a shaker inside a warming oven set to 37^0C. Incubate the vaginal canals for 30 min with shaking set to 800 rpm to soften the tissue. Vortex vigorously for 10 s at the end of the incubation.

Conduct all further steps involving manipulation of the tissue pieces in a BSL2 hood. Open the tube containing the vaginal canals and use sterile, autoclaved tweezers to transfer the tissue to a new 15 mL tube containing 1 mL of fresh HBSS media. Clean tweezers with ethanol in between samples and let dry. Mince the vaginal canal tissue into ~1 mm^2 pieces using autoclaved sharp-tipped scissors inside the tube; the consistency of minced tissue should resemble finely minced garlic. Clean scissors with ethanol in between samples. Tightly close the tube and seal with parafilm. Remove it from the hood and repeat the 30 min softening step at 37^0C described above to further soften the tissue in preparation for enzymatic digestion. Vortex vigorously for 10 s at the end of the incubation. These softening steps and the later digestion step can be scaled up to use larger amounts of tissue, i.e. from more than two vaginal canals.

Collagenase Digestion of Vaginal Canals

Unseal and open the tube and pipette the contents up and down to break up any clumps. Drain the medium from the tissue fragments by pipetting the contents of the tube over a 40 µm strainer into a 50 mL waste collection tube. Rinse out the tube with an additional 4 mL of HBSS media to ensure that all tissue fragments are collected. Use sterile tweezers to transfer the tissue pieces in the strainer to a new 15 mL tube containing 2 mL of digestion media consisting of 6 parts HBSS media and 1 part Collagenase D stock solution (5 mg/mL in Hank's Balanced Salt Solution (-Ca, -Mg)). The final concentration of Collagenase D during digestion is 0.11 U/mL in Wunsch units. Tightly

close and seal the tube with parafilm. Remove it from the hood and secure it to a shaker in a 37^0C incubator. Incubate the tissue pieces in the digestion media for 30 min with shaking at 800 rpm. Vortex vigorously for 10 s at the end of the incubation.

Post-digestion Tissue Washing and Plating

For the first wash, transfer the digestion media and tissue pieces to a 50 mL tube, and rinse the digestion tube 3 times with 10 mL of HBSS media, adding the rinsing media to the new tube containing the tissue pieces. Parafilm the tube and spin it at 500 × g for 5 min at room temperature. Being careful not to disturb the pelleted tissue pieces, remove and discard the supernatant. Complete two more washes by resuspending the pellet in 30 mL HBSS media, centrifuging, and discarding the supernatant.

Resuspend the tissue pieces in 5 mL of culture media (Dulbecco's Modified Eagle's Medium with 15% heat-inactivated FBS, 2 mM L-glutamine, 1 U/mL penicillin, and 1 µg/mL streptomycin). Pipette up and down several times to ensure that tissue pieces are not clumping together, and plate the tissue pieces and media in a sterile 6 cm tissue culture-treated plate. Place the plates in a 37^0C tissue culture incubator with 5% CO_2 and 95% humidity.

Fibroblast Migration

Monitor the color of the culture media daily for the first 7 days of culture. Media should be pink in color, but if the media turn yellow, check cultures for contamination. Fibroblasts will begin to migrate out of the tissue pieces and adhere to the plate around the fourth day of culture. This is a benchmark that can be used to assess whether the culture is progressing normally; only a few cells will migrate out onto the plate during the first week of culture.

After 7 days of culture, transfer the tissue pieces and media to a 50 mL tube and spin them at 500 x g for 5 min at room temperature to pellet the tissue pieces. Discard the supernatant and resuspend the tissue pieces in 5 mL of fresh culture media, transferring them to a new 6 cm plate. Allow the cells another 7 days to migrate out of the tissue pieces and adhere to the plate. Since most of the cells emerge from the tissue pieces during the second week of incubation, the plate used to grow the cells during their first week can be discarded. In the event that a substantial number of cells migrated out of the tissue pieces during the first week of culture, replace the media on the plate from the first week of culture with fresh media and allow cells to grow for another week.

Passages and Assays

Two weeks after the digestion, passage the cells to a new plate via the following steps. Remove media from plates and wash twice with 1 mL of 1X PBS. Add 0.5 mL of 0.25% trypsin-EDTA and place plates in a 37^0C incubator for 5–7 min. At the end of the incubation, visually inspect the plates under the microscope to confirm that the cells have detached from the plate surface. Dilute the trypsin by adding 4.5 mL of media to each plate, and pipette the cell suspension over the surface of the plate to detach any stubborn adherent cells. Transfer cell suspension to a 50 mL tube and spin for 5 min at 500 x g to pellet cells. If plates from both the first and second week of culture were saved, their cell suspensions can be combined and spun together in the previous step. After spinning, remove the supernatant, resuspend the cells in 5 mL fresh media, and dispense the cell suspension into a new 6 cm plate.

One week after passage 1, passage cells to 10 cm plates with a total media volume of 10 mL per plate, and allow them to grow to confluence to maximize the number of cells available for experiments. If possible, conduct experiments on passage 3 and standardize passage number across all cell lines in an experiment for the most robust and consistent cytokine response to treatment. In our experience, cell lines derived from allergen-exposed tissue tend to be slower growing, which may increase the passage number at which there are enough cells available for an experiment. In this case, experiments can be conducted between passages 3–5, but should not be conducted after passage 6 due to substantial declines in functional performance.

For extended storage of cell lines, freeze cells at passage 3 or 4. Proceed through the passaging protocol until cells have been pelleted. Remove supernatant and resuspend the pellet in 1.5 mL of DMEM with 30% FBS and 10% DMSO and store at -80^{0}C. Cells stored in a -80^{0}C freezer remain viable for about 5 weeks.

Method validation

Murine vaginal canal-derived primary fibroblasts have characteristic fibroblast morphology and surface antigen presentation

To validate fibroblast surface markers and confirm cellular identity, we stained cells with fluorescently-conjugated monoclonal antibodies and analyzed using flow cytometry. We removed media from plates and washed twice with 1X PBS before detaching cells from plates by trypsinizing with 0.25% Trypsin-EDTA. We quenched the trypsin with culture media and spun cells at 500 x g for 5 min at 4^{0}C. After spinning, we discarded the supernatant and resuspend the pellet in staining buffer (1X PBS with 2% FBS). We spun the cells again to remove any residual trypsin, and resuspended the pellet in the appropriate volume of staining buffer to reach a concentration of 10^7 cells/mL. At this concentration, we stained cells with fluorophore-conjugated antibodies against CD45 and CD90.2 diluted 1:100 for 30 min in the dark at 4^{0}C. We spun stained cells and resuspended in 100 µL of staining buffer before evaluating the expression of surface markers via flow cytometry. We compensated fluorescence intensity readouts using single-stained controls.

On passage 3, ~99% of the cells in the culture did not display surface expression of the hematopoietic lineage marker CD45, confirming that the vaginal cell lines we generated were non-hematopoietic (Fig. 1). We also evaluated surface expression of CD90.2, which is present in mouse thymocytes, peripheral T cells, hematopoietic stem cells, neurons, and fibroblasts [10]. Almost ~100% of the cells were CD90.2+, which in combination with the lack of CD45 expression and the tissue of origin, suggests fibroblast cellular identity. All cells in the culture also showed the characteristic spindle-like morphology of fibroblasts. Based on this cell morphology and the observed surface marker expression, we are confident that our method produces fibroblast cell lines.

Murine primary vaginal fibroblasts show exacerbated cytokine responses to yeast and bacterial antigens in vitro

We have developed a murine model of allergy-driven genital pain that recapitulates clinical characteristics of the chronic pain condition localized provoked vulvodynia, including mast cell accumulation in vaginal tissue and long-lasting tactile sensitivity to stimulation of the anogenital ridge, using repeated exposures to the allergenic cosmetic preservative methylisothiazolinone (MI) [8,11–12]. Primary fibroblast cell lines derived from vulvar biopsies of vulvodynia patients have been shown to exhibit a heightened cytokine response when treated with yeast and bacterial antigens [5,6]. To explore whether our model replicates this altered functional response, we administered *in vitro* treatments of lipopolysaccharide and Zymosan (a mixture of yeast antigens) to vaginal fibroblasts derived from a MI-sensitized mouse challenged daily for 10 days with MI in the vaginal canal and a MI-sensitized mouse challenged daily for 10 days with saline, respectively. We then evaluated production of the pro-inflammatory cytokine INTERLEUKIN-6 (IL-6) by Enzyme-Linked Immunosorbent Assay (ELISA). Our experimental methods are described below.

For the treatments, we seeded fibroblasts in triplicate in 6-well plates at a concentration of 9×10^5 cells/well with a total media volume of 2 mL per well and allowed cells to grow to confluency (3–4 days). We replaced the culture medium with low serum containing DMEM (0.05% FBS, 2 mM L-glutamine, 1 U/mL penicillin, and 1 µg/mL streptomycin), and serum starved cells for 48 h before treatment. We then treated cells with 500 ng/mL LPS or Zymosan. After 24 h of treatment, we collected the supernatant, spun it at 500 × g for 10 min at 4^{0}C to pellet debris, and stored at -80^{0}C.

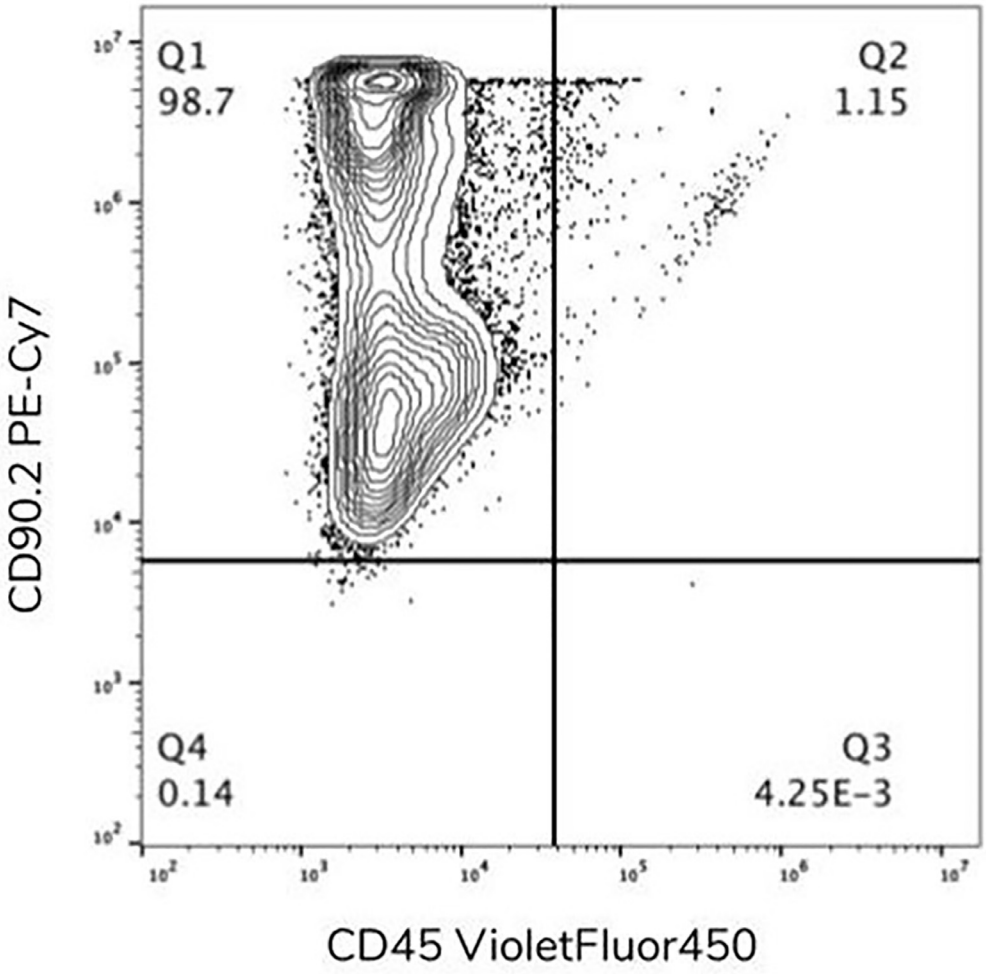

Fig. 1. Primary murine vaginal cell lines are predominantly CD45- and CD90.2+

We determined the concentration of INTERLEUKIN-6 (IL-6) in the stored supernatant samples using an ELISA (R&D Systems) according to the manufacturer's instructions.

Fibroblasts derived from MI-challenged vaginal canals (MI/MI (10) 1D) produced higher levels of IL-6 compared to those derived from saline-challenged tissue (MI/Sal (10) 1D) when challenged with LPS and Zymosan *in vitro*, mirroring the behavior of vulvar fibroblasts derived from biopsies from vulvodynia patients (Fig. 2). Therefore, the fibroblasts derived using this protocol are able to be used for functional assays in our pre-clinical model of allergy-driven pain-associated tissue changes, and allow us to dissect the contribution of fibroblasts to pain responses. It is likely that fibroblasts derived from experimental subjects in other pre-clinical mouse models will be useful for functional assays that are important and informative in those contexts as well.

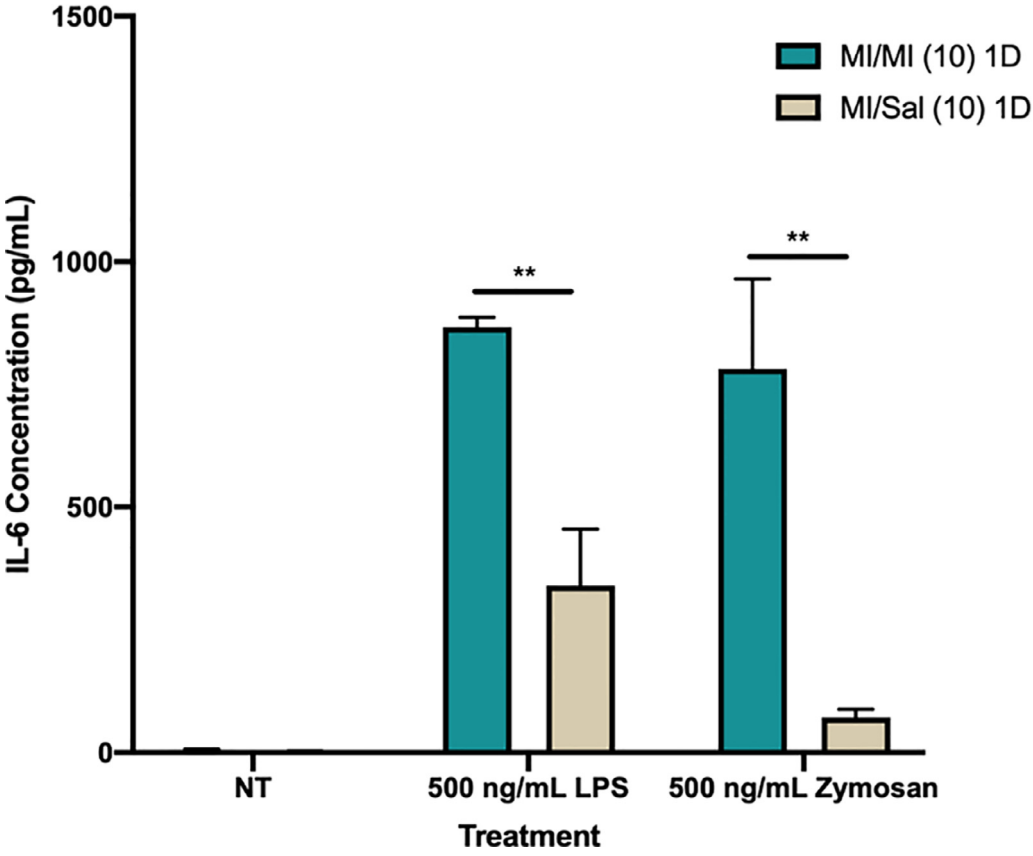

Fig. 2. Murine vaginal fibroblasts derived from allergen-exposed tissue exhibit enhanced production of IL-6 following treatment with bacterial and yeast antigens

Conclusion

Vaginal fibroblasts play a key role in tissue maintenance, remodeling, and repair, and have been implicated in a variety of disease processes [3–7]. While primary human vulvovaginal fibroblast cell lines are a valuable tool for elucidating fibroblast function in homeostatic tissue and in disease states, murine vaginal fibroblast cell lines offer the same opportunities, with the added benefit that they can be established from mouse tissue which is easier to acquire. The method described above generates primary vaginal cell lines which adhere to the characteristic morphology and surface expression profile of vaginal fibroblasts, and which can be used to conduct functional assays in order to assess the fidelity of a murine model to the disease interest and untangle disease pathways. The availability of this new *in vitro* system creates the potential for more nuanced therapeutic development that modulates the behavior of vaginal fibroblasts and their interactions with other cell types.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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