Video Article

Application of Aorta-gonad-mesonephros Explant Culture System in Developmental Hematopoiesis

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Abstract

The limitation of using mouse embryos for hematopoiesis studies is the added inconvenience in operations, which is largely due to the intrauterine development of the embryo. Although genetic data from knockout (KO) mice are convincing, it is not realistic to generate KO mice for all genes as needed. In addition, performing *in vivo* rescue experiments to consolidate the data obtained from KO mice is not convenient. To overcome these limitations, the Aorta-Gonad-Mesonephros (AGM) explant culture was developed as an appropriate system to study hematopoietic stem cell (HSC) development. Especially for rescue experiments, it can be used to recover the impaired hematopoiesis in KO mice. By adding the appropriate chemicals into the medium, the impaired signaling can be reactivated or up-regulated pathways can be inhibited. With the use of this method, many experiments can be performed to identify the critical regulators of HSC development, including HSC related gene expression at mRNA and protein levels, colony formation ability, and reconstitution capacity. This series of experiments would be helpful in defining the underlying mechanisms essential for HSC development in mammals.

Video Link

The video component of this article can be found at https://www.jove.com/video/56557/

Introduction

Hematopoietic stem cells (HSCs) are tissue-specific adult stem cells that possess multilineage potential including erythroid, myeloid, and lymphoid cells as well as the ability to self-renew. Recent studies have shown that the earliest HSCs arose from a specialized endothelial population, known as hemogenic endothelium (HE), through the endothelial to hematopoietic transition (EHT) at the ventral wall of dorsal aorta[1,2,3,4]. Once formed in the aorta-gonad-mesonephros (AGM) region from embryonic (E) 10.5 to E12.5 in the mouse embryo, HSCs will migrate into the fetal liver for expansion and finally colonize the bone marrow to maintain adult hematopoiesis throughout an individual's life[5,6]. Although this has been studied for many years, the underlying mechanisms of HSC emergence and development remain incompletely understood.

Unlike *in vitro* fertilization and development of zebrafish embryos, intrauterine development of mouse embryos makes the study of definitive hematopoiesis during embryogenesis much more inconvenient. Although genetic experiments using knockout (KO) mice are commonly utilized, the lack of certain KO mice also limits their use in the hematopoietic research field. In addition, *in vivo* rescue experiments are not easily performed in KO mice. Since 1996, the AGM explant culture has been developed for hematopoietic studies by the pioneers in the field[7]. With the help of this culture system, ventral tissues of the AGM region have been identified to promote HSC activity, while dorsal tissues exert an opposite effect[8,9]. The AGM explant culture system has also been applied to determine the roles of Serotonin, Mpl, SCF, BMP, and Hedgehog signaling in HSC development[10,11,12,13,14]. Importantly, it is also a popular method used to rescue hematopoietic defects in mutant embryos[13,15].

Protocol

All the procedures including animal subjects have been approved by the Ethical Review Committee in the Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

1. Material Preparation

1. Sterilize the 0.65 μm filters with ultraviolet rays produced under the ultraviolet ray lamp on the clean bench for 4 h and turn the filters over after the 2 h mark.
   NOTE: When working with UV light, wear appropriate protection.

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2. Sterilize the stainless steel meshes with the autoclave sterilizer at 121 °C for 30 mins.
   NOTE: A certain height of stainless steel mesh is needed to support the filters at the air-liquid interface and this can be realized with the wire on the steel mesh (**Figure 1A**).
3. Dilute Fluoxetine to a final concentration of 10 μM into the M5300 long-term culture medium and mix evenly.
   NOTE: For a six-well plate, 2 mL M5300 long-term culture medium is appropriate for each well. Hydrocortisone at 10^{-6} M can be selectively diluted into the medium.
4. Transfer the medium supplemented with Fluoxetine at 10 μM into six-well plates or dishes and put the sterilized stainless steel meshes into the medium.
5. Wash the 0.65 μm filters with boiling water two times for 3 mins each time in the glass cell culture dish for sterilization. After each washing, put the filters into phosphate buffered saline (PBS) for 2 mins and place the wet filters onto the stainless steel meshes standing at the air-liquid interface as shown in **Figure 1A** [schematic in the center].
   NOTE: Boiling water can be generated with the autoclave sterilizer to achieve the asepsis and equipment should be taken out of autoclave sterilizer in a timely fashion to avoid a decrease in temperature.

2. AGM Explant Culture

1. Carefully separate the AGM regions from the embryos at E10.5 or E11 with forceps.
   1. Strip the uteruses from pregnant mice with the dissecting scissors and separate the embryos from the uteruses.
   2. Wipe off the yolk sac, umbilical cord, and viscera from the embryo body.
   3. Carefully remove the dorsal nerve tissues and surrounding muscular tissues.
   4. Cut off the tissues with the forceps at the site of anterior and hind limbs and separate the AGM region from the embryo body. **Figures 1B-C** show the schematic representation and morphology of dissected AGM.

2. Explant the dissected AGMs separately onto the filters at the air-liquid interface (**Figure 1A**) and culture in 5% CO_2 at 37 °C for 2 - 3 days.
   NOTE: Do not place the dissected AGM onto the wire of the steel mesh and make sure the filters are at the air-liquid interface during culture. Besides the AGM, yolk sac and fetal liver can also be cultured with this system[7].

3. Expression Analyses

1. **mRNA level**
   1. Collect the cultured AGMs into 1 mL PBS and centrifuge at 4 °C, 310 x g for 6 min.
   2. After removing the supernatant, total RNA is extracted from the collected AGMs with a monophasic solution of phenol, according to the manufacturer's instructions.
   3. Total RNA (2 μg) is then reverse transcribed using M-MLV reverse transcriptase to obtain cDNA as the template. The quantitative real-time PCR assays are performed with SYBR Green and *Gapdh* is used as the internal control.

2. **Protein level**
   1. Collect the cultured AGM as described above in step 3.1.1.
   2. Prepare the protein from the cultured AGM with the cell lysis buffer (10 mM Tris-HCl, 10 mM NaCl, and 0.5% NP-40) containing protease inhibitor and use for western blotting to detect the protein level as previously described[13].

4. Colony-Forming Units in the Culture (CFU-C) Assay

1. After culturing for 2 - 3 days, collect the AGM regions into 1 mL phosphate buffered saline (PBS) and centrifuge at 4 °C, 310 x g for 6 mins and dissociate with 200 μL collagenase (0.1% in PBS) at 37 °C.
   NOTE: The time needed for collagenase to generate single cell-suspensions is about 20 mins. Shaking the tube containing AGM every 5 mins can accelerate the digestion process. 200 μL 0.1% collagenase is used for each AGM and 200 μL 10% serum is used to stop the reaction.
2. Culture single cell-suspensions in M3434 medium in ultra-low attachment 24-well plates.
   NOTE: To avoid contamination, Penicillin-Streptomycin solution can be selectively added into the medium. Because the M3434 medium is viscous, a 1.0 cc syringe without a needle is used to mix the cells and medium.
3. After 7 - 10 days culturing at 37 °C in 5% CO_2, the number for each type of colonies including Burst forming unit--erythroid (BFU-E), CFU-granulo-monocyte (CFU-GM) and CFU-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) are distinguished based on the morphology and scored with an inverted microscope (**Figure 2C**).

5. *In Vivo* Transplantation Assay

1. **Colony-forming units-spleen (CFU-S) assay**
   1. After 2 - 3 days of explant culturing, collect the AGMs and prepare single cell-suspensions by incubating with 200 μL collagenase (0.1% in PBS) for 20 min.
   2. Perform lethal irradiation (9 Gy of X-rays) of 8- to 10-week old male C57BL/6 mice 4 - 5 h before cell injection.
   3. Put the irradiated mouse into a restrainer to restrict its activity.
   4. Wipe the tail using a cotton swab steeped in 75% alcohol to promote vasodilatation of the vein.
   5. Inject 0.5 embryo equivalent (ee) or 1 ee single cell-suspensions of AGM intravenously into the tail vein of irradiated mouse with a 1.0 cc syringe.

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NOTE: Cells are suspended with PBS and 0.5 mL PBS per recipient is a suitable volume. A needle of 25 gauge or 26 gauge size is used for injection with a 1.0 cc syringe. No air can be introduced into the syringe. Press the injection site with an antiseptic swab to stop bleeding.

6. Eleven days post transplantation, collect and fix the spleens of the recipient mice in Bouin's solution (15 mL 1.22% Picric acid saturated aqueous solution, 2 mL 40% methanol and 1 mL acetic acid) for 1 - 2 days and wash with 80% alcohol for another 1 - 2 days. Count the visible colonies in the spleen macroscopically and determine the number of colonies per ee.
   NOTE: Bouin's fixative, wear appropriate protection.

2. **Long-term transplantation assay**
   1. After 2 - 3 days explant culturing, collect the AGMs and prepare single cell-suspensions by incubating with 200 µL collagenase (0.1% in PBS) for 20 min.
   2. Perform lethal irradiation (9 Gy of X-ray) of 8- to 10-week old male C57BL/6 mice 4 - 5 hrs before cell injection.
   3. Put the irradiated mouse into a restrainer to restrict its activity.
   4. Wipe the tail using a cotton swab steeped in 75% alcohol to promote vasodilatation of the vein.
   5. Inject 0.5 embryo equivalent (ee) or 1 ee single cell-suspensions of AGM intravenously into the tail vein of an irradiated mouse with a 1.0 cc syringe. AGM are injected at a dose of 1 ee per recipient with 2×10^5 bone marrow cells (CD45.1 background).
      NOTE: No air can be introduced into the syringe. Press the injection site with an antiseptic swab to stop bleeding.
   6. Four months post-transplantation, collect the bone marrow of the recipient and use it for the reconstitution assay by FACS. Only the recipients with ≥10% donor-derived chimerism are considered to exhibit successful reconstitution.

Representative Results

A recent publication reported endothelial cell-derived serotonin promotes the survival of HSCs by inhibiting the pro-apoptotic pathway in the AGM[13]. To confirm the promoting effect of serotonin on HSC development, Fluoxetine was included. As a selective serotonin re-uptake inhibitor (SSRI), Fluoxetine has been demonstrated to inhibit serotonin re-absorption in peripheral tissues[16,17,18]. Using the AGM explant culture system, the effect of Fluoxetine on HSC development was examined by following the procedure presented above. The expression analyses showed increased expression of Runx1, which is defined as a pivotal gene for HSC development[19], in Fluoxetine-treated AGMs at mRNA and protein levels, respectively (**Figure 2A** and **2B**). Fluoxetine can also significantly increase the colony formation ability of HSCs in the AGM, including BFU-E, CFU-GM, and CFU-GEMM (**Figure 2C** and **Figure 2D**). In addition, *in vivo* transplantation results showed that HSCs in the AGM treated with Fluoxetine can increase the number of spleen colonies in irradiated adult recipients (**Figure 2E**).

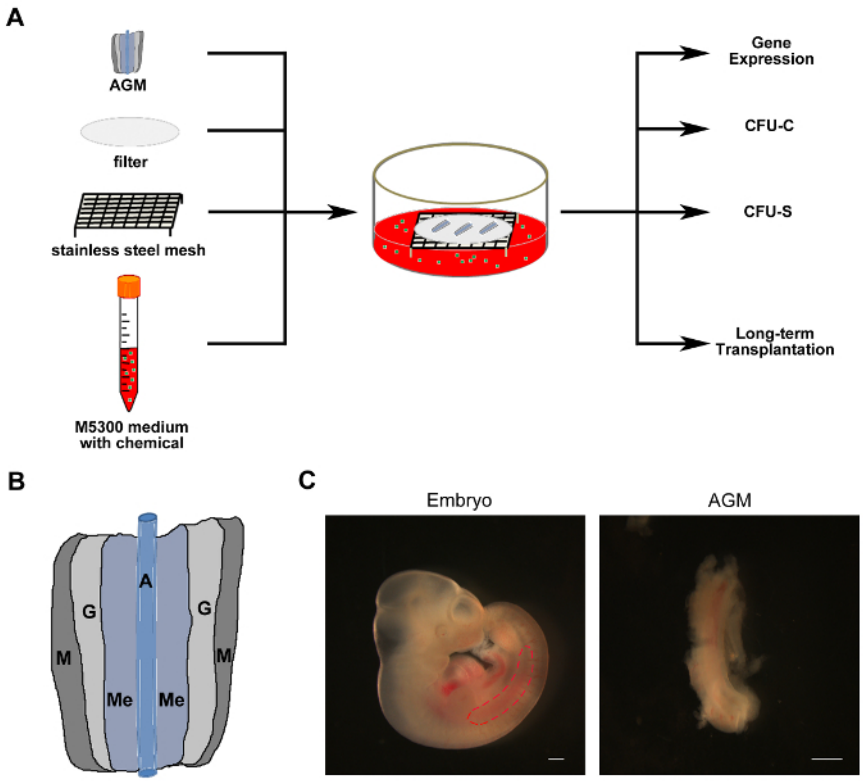

Figure 1. Schematic representation of AGM structure and explant culture system.
(A) The flow chart for using the AGM explant culture systemto examine the effect of chemicals on HSCs development. Briefly, AGMs are dissected from embryos and cultured on filters at the air-liquid interface with chemicals diluted in M5300 long-term culture medium. 2 - 3 days later, the cultured AGMs are collected and used to examine the expression of hematopoietic related genes, colony formation ability, and repopulation capacity. AGM, aorta-gonad-mesonephros; CFU-C, colony-forming units in the culture; CFU-S, colony-forming units-spleen. **(B)** The structure of AGM is shown in this diagram. A, dorsal aorta; Me, mesenchyme; G, gonad; M, mesonephros. **(C)** The morphology of AGM. The AGM is separated from the caudal half of the embryonic body with the surrounding mesenchymal cells at E10.5. Scale bars = 1 mm. Please click here to view a larger version of this figure.

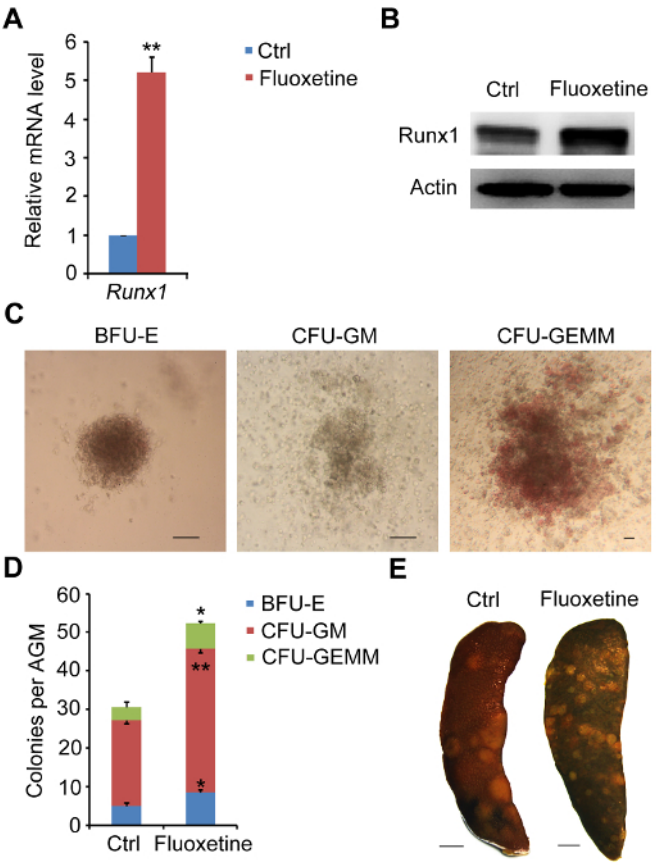

Figure 2. Application of the AGM explant culture system to detect the effect of Fluoxetine on HSC development. (A) The mRNA level of *Runx1* in the AGMs treated with Fluoxetine at 10 μM was detected by quantitative real-time PCR. Student's *t* test: **, P<0.01. The results are presented as mean ± SEM. **(B)** Western blotting analysis further confirmed the increased expression of Runx1 at the protein level in the AGMs treated with Fluoxetine. **(C)** The representative images show the morphology of BFU-E, CFU-GM and CFU-GEMM. Scale Bars = 100 μm. **(D)** CFU-C assay showed that Fluoxetine treatment can increase the number of each type of colony. One embryo equivalent (ee) was used. Student's *t* test: *, P<0.05; **, P<0.01.The results are presented as mean ± SEM. **(E)** CFU-S assay with AGMs treated with Fluoxetine showed that Fluoxetine can increase the number of colonies in the spleen of irradiated adult recipients. Scale bars = 1.5 mm. 1 ee was used. Please click here to view a larger version of this figure.

Discussion

It is well known that mature HSCs in the bone marrow can repopulate the blood system of irradiated recipients. Unlike these functional HSCs, the emerging HSCs in the AGM of mouse embryos are immature. Direct transplantation results showed that type I (VE-cad$^+$ CD45$^-$ CD41$^+$) and type II (VE-cad$^+$ CD45$^+$) pre-HSCs possess no reconstitution ability[20]. Taking advantage of the AGM explant culture system, the nascent HSCs in the AGM region can reconstitute the recipients in the transplantation assay[4,20]. Furthermore, due to the intrauterine development of the mouse embryo, *in vivo* experiments to rescue the impaired hematopoiesis in KO mice are inconvenient to perform. The AGM explant culture is an alternative system, which is well suited for rescue experiments, simply by adding the chemicals (growth factors and cytokines) into the medium[13,15]. In addition to the AGM, the yolk sac and fetal liver can also be cultured with this system[7].

At the beginning of this experiment, the strict sterilization of filters and stainless steel meshes is quite important to avoid contamination. More importantly, the critical step in the culture is to make the dissected AGM cultured on the filters at the air-liquid interface. Too much medium will cause the AGM to be cultured in the liquid and it may be decayed, whereas too little medium will make the AGM dry. During culturing, maintaining the proper humidity of the cell incubator is also important to prevent the evaporation of the medium.

Further improvement of this explant culture system to truly mimic the development of HSCs *in vivo*, together with recent advancements in organoid culture techniques[21], will greatly expand its application in hematopoiesis studies, from HSC self-renewal, multi-lineage differentiation, and HSC-niche interaction to disease modeling and drug discovery.

Disclosures

The authors have no conflicting financial interests.

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