Video Article

Fabrication of Tongue Extracellular Matrix and Reconstitution of Tongue Squamous Cell Carcinoma *In Vitro*

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Abstract

In order to construct an effective and realistic model for tongue squamous cell carcinoma (TSCC) *in vitro*, the methods were created to produce decellularized tongue extracellular matrix (TEM) which provides functional scaffolds for TSCC construction. TEM provides an *in vitro* niche for cell growth, differentiation, and cell migration. The microstructures of native extracellular matrix (ECM) and biochemical compositions retained in the decellularized matrix provide tissue-specific niches for anchoring cells. The fabrication of TEM can be realized by deoxyribonuclease (DNase) digestion accompanied with a serious of organic or inorganic pretreatment. This protocol is easy to operate and ensures high efficiency for the decellularization. The TEM showed favorable cytocompatibility for TSCC cells under static or stirred culture conditions, which enables the construction of the TSCC model. A self-made bioreactor was also used for the persistent stirred condition for cell culture. Reconstructed TSCC using TEM showed the characteristics and properties resembling clinical TSCC histopathology, suggesting the potential in TSCC research.

Video Link

The video component of this article can be found at https://www.jove.com/video/57235/

Introduction

The tongue has various important functions, such as deglutition, articulation, and tasting. Thus, the impairment of tongue function has great impact on patients' quality of life[1]. The most common malignancy in the oral cavity is tongue squamous cell carcinoma (TSCC), which usually occurs in people who drink alcohol or smoke tobacco[2].

In recent years, little progress has been achieved in fundamental research on TSCC. The lack of efficient *in vitro* research models remains to be one of the biggest problems. Thus, the extracellular matrix (ECM) turns out to be a potential solution. Since ECM is a complex network frame composed of highly organized matrix components, scaffold materials having an ECM-like structure and composition would be competent for cancer research. Decellularized ECM can perfectly provide the niche for the cells from the same origin *in vitro*, which turns out to be the most significant advantage of ECM.

ECM can be retained with cellular components being removed from the tissues through the decellularization using detergents and enzymes. Various ECM components, including collagen, fibronectin, and laminin in decellularized matrix provide a native-tissue-like microenvironment for cultured cells, promoting the survival, proliferation, and differentiation of the cells[3]. Moreover, the immunogenicity for transplantation can be reduced to a minimal level with the absence of cellular components in ECM.

So far, fabrication methods for decellularized ECM have been tried in different tissues and organs, such as heart[4,5,6,7], liver[8,9,10,11], lung[12,13,14,15,16,17], and kidney[18,19,20]. However, no relevant research has be found on similar work in the tongue to the best of our knowledge.

In this study, decellularized tongue extracellular matrix (TEM) was fabricated both efficiently and cheaply by a series of physical, chemical, and enzymatic treatment. Then the TEM was used to recapitulate TSCC *in vitro*, showing an appropriate simulation for TSCC behavior and development. TEM has good biocompatibility as well as the ability to guide the cells to the tissue-specific niche, which indicates that TEM may have great potential in TSCC research[3]. The protocol shown here provides a choice for researchers studying on either pathogenesis or clinical therapies of TSCC.

Protocol

All animal work was performed in accordance with animal welfare act, institutional guidelines and approved by Institutional Animal Care and Use Committee, Sun Yat-sen University.

1. Preparation of TEM

1. Execute mice by cervical dislocation and remove the tongues using sterile surgical scissors and tweezers.
2. Immerse the tongues in 75% ethanol for 3 min, then put each tongue into a 1.5 mL Eppendorf (EP) tube with 1 mL of 10 mM sterile phosphate buffered solution (PBS).
   NOTE: The concentration of PBS in all the following steps is same as the concentration in this step.
3. Cell lysis by freeze thaw: Freeze the tongues in EP tubes at -80 °C for 1 h, and then thaw the tongues at room temperature for 45 min for 3 cycles.
4. Load each tongue onto a piece of surgical suture using a surgical needle and wrap the end of the suture with a small piece of sterile tinfoil. Perform the operation in a 3.5 cm or 6 cm culture dish containing 75% ethanol in sterile conditions.
   NOTE: The appropriate length of each piece of surgical suture is about 20 cm, and the appropriate size of each piece of tinfoil is about 0.3 cm^2 (1 cm × 0.3 cm). The tongue should be loaded near the tinfoil.
5. Rinse each tongue with 3 mL of sterile PBS in a 3.5 cm or 6 cm culture dish for 30 s. Perform this operation in sterile conditions.
6. Wash the tongues with ultrapure water: Add ampicilin into a wide-mouth bottle with 250 mL of sterile ultrapure water to a final concentration of 90 μg/mL. Put the tongues into the bottle containing a stir bar. Tighten the bottle cap with part of the suture remaining outside the bottle. Perform this operation in sterile conditions.
   NOTE: Up to 5 tongues can be put into the same bottle in consideration of twining of the suture. The tinfoil is at the end of the suture in the bottle to prevent the tongue from slipping off. The tongues should be placed 2 cm high from the bottom of the bottle by adjusting the length of the suture remaining inside the bottle. This note is also for steps 1.8, 1.10, 1.12, and 1.16.
7. Put the bottle on a magnetic stirrer for 12 h.
8. Wash the tongues with NaCl: Add ampicilin into a wide-mouth bottle with 250 mL of sterile 1 M NaCl to a final concentration of 90 μg/mL. Move the tongues and the stir bar into the bottle. Tighten the bottle cap with part of the suture remaining outside the bottle. Perform this operation in sterile conditions.
9. Put the bottle on a magnetic stirrer for 24 h.
10. Cell lysis by Triton X-100: Add ampicilin to a final concentration of 90 μg/mL into a wide-mouth bottle with 250 mL of sterile 2% Triton X-100 in PBS. Move the tongues and the stir bar into the bottle. Tighten the bottle cap with part of the suture remaining outside the bottle. Perform this operation in sterile conditions.
11. Put the bottle on a magnetic stirrer for 48 h.
12. Wash tongues with $CaCl_2$/$MgCl_2$: Add ampicilin into a wide-mouth bottle with 250 mL of sterile 5 mM $CaCl_2$/$MgCl_2$ to a final concentration of 90 μg/mL. Move the tongues and the stir bar into the bottle. Tighten the bottle cap with part of the suture remaining outside the bottle. Perform this operation in sterile conditions.
13. Put the bottle on a magnetic stirrer for 24 h.
14. Digestion by DNase: Add 1 mL of Hank's balanced salt solution (HBSS) to each EP tube. Add DNase into HBSS respectively to a final concentration of 300 μM. Move each tongue into each EP tube, with part of the suture outside the tube. Perform this operation in sterile conditions.
    NOTE: Make sure that the part of suture which remains inside the bottles in previous steps also remains inside the EP tube in this step, and make sure that the part of suture which remains outside the bottles in previous steps also remains outside the EP tube in this step.
15. Incubate the tongues in EP tubes at 37 °C for 24 h.
16. Wash the tongues with PBS: Add ampicilin into a wide-mouth bottle with 250 mL of sterile PBS to a final concentration of 90 μg/mL. Move the tongues and the stir bar into the bottle. Tighten the bottle cap with part of the suture remaining outside the bottle. Perform this operation in sterile conditions.
17. Put the bottle on a magnetic stirrer for 24 h.
18. Store the prepared TEM in sterile PBS at 4 °C until use.

2. Three-dimensional (3D) Reconstitution of TSCC

1. **Static TSCC model construction**
   1. Seed 1.0 x 10^6 single TSCC cells (Cal27) into a 3.5 cm culture dish. Add 3 mL of Dulbecco's modified Eagle's medium/F12 (DF12) containing 10% fetal bovine serum (FBS), 90 μg/mL ampicilin, and 90 μg/mL kanamycin.
   2. Culture the Cal27 cells at 37 °C for 2 to 3 days. Make sure that the cells cover at least 60% area of the dish bottom.
   3. Load TEM onto the Cal27 monolayer in the culture dish.
   4. Put the dish into a CO_2 incubator at 37 °C for 28 days.
   5. Refresh the culture medium every day during the cell culture process. The CO_2 concentration in the incubator is 5%.

2. **Stirred TSCC model construction**
   1. **Preparation of a self-made stirred minibioreactor**
      1. Take out the plunger from a 10 mL syringe.
      2. Dig a hole (diameter of 1 cm) near the lower terminal of the rod and load a stir bar in the hole.
      3. Dig a hole (diameter of 0.5 cm) at the center of the bottle cap of a plastic wide-mouth bottle and put the piston rod through the cap.
      4. Cut half of a 50 mL centrifuge tube and weld it on the outer side of the bottle cap.
      5. Attach fishhooks to the rod by wrapping the rod with fishing lines which are tied to the fishhooks.
         NOTE: Up to 4 fishhooks can be attached to a rod.
      6. Autoclave the self-made complex before use.
         NOTE: Do not autoclave the plastic wide-mouth bottle. Use a new sterile plastic wide-mouth bottle while culturing cells.

3. **Dynamic cell culture**

1. Seed 1.0 x 10^6 single Cal27 cells in the self-made minibioreactor. Add 150 mL of DF12 medium which contains 10% FBS, 90 µg/mL ampicilin, and 90 µg/mL kanamycin.
2. Load TEM onto the minibioreactor using the fishhooks attached to the rod.
3. Tighten the bottle cap and put the minibioreactor on a magnetic stirrer. Activate the minibioreactor at 200 rpm in a CO_2 incubator at 37 °C for 7 to 14 days.
   NOTE: The concentration of CO_2 in the CO_2 incubator is 5%.

Representative Results

This protocol for the preparation of TEM turns out to be efficient and appropriate. The TEM showed perfect decellularization compared with native tongue tissues. The efficacy of decellularization was confirmed by hematoxylin-eosin (HE) staining (**Figure 1A-B**). The HE staining results revealed complete disappearance of nuclear staining in TEM (**Figure 1B**). Moreover, DNA content quantification from previous work showed that DNA was almost completely removed from TEM[3]. This protocol also showed rare damage to the tissue integrity while removing cell components (**Figure 1B**).

3D reconstitution of TSCC using TEM and a self-made minibioreactor (**Figure 2A-B**) achieved satisfying results. HE staining showed that Cal27 cells in the TEM presented typical TSCC pathological characteristics (**Figure 2C-D**). The cells in stirred culture conditions presented single-cell migration (**Figure 2C**) or collective migration (**Figure 2D**) in different lesion areas. In static culture conditions, Cal27 cells also formed invasive structures in the TEM, but it took a longer time (**Figure 2E**). Furthermore, a human osteosarcoma cell line U2OS was also introduced to the same stirred culture system. Though U2OS cells could live in the culture medium, they were not found in the TEM (**Figure 2F**). The TEM showed different biocompatibility for different types of cancer cells, suggesting that different tumor cells may need different microenvironments to flourish.

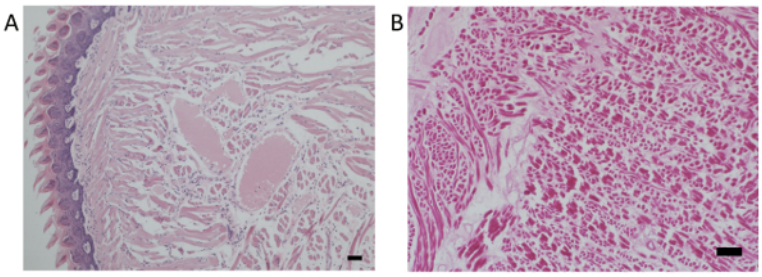

Figure 1: Preparation of TEM. (A) HE staining of native tongues from mice. Scale bar = 100 µm. (B) HE staining of decellularized TEM from mice. Scale bar = 100 µm. Please click here to view a larger version of this figure.

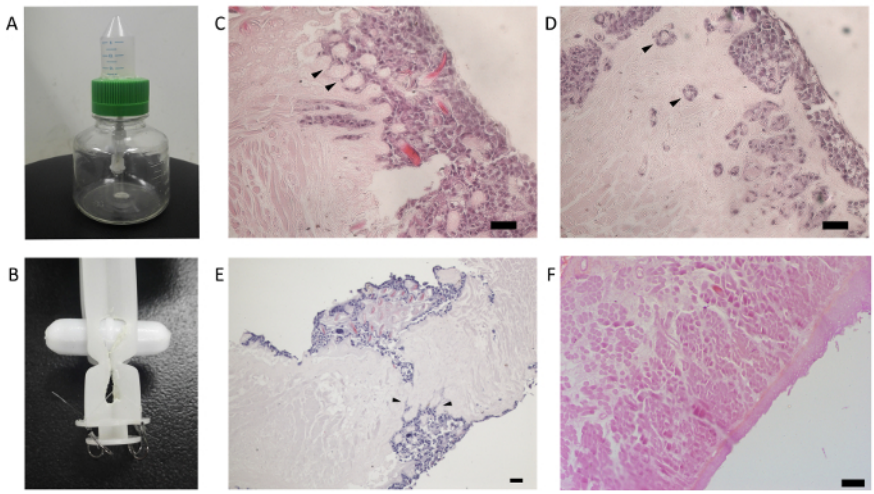

Figure 2: Reconstitution of TSCC by TEM. (A) The overview of a self-made minibioreactor. (B) The view of the TEM-loading position of a self-made minibioreactor. (C) HE staining of 14-day stirred cultured TSCC with TEM. Single cell invasion phenomena are indicated by black arrows. Scale bar = 50 µm. (D) HE staining of 14-day stirred cultured TSCC with TEM. Collective invasion phenomena are indicated by black arrows. Scale bar = 50 µm. (E) HE staining of 28-day static cultured TSCC with TEM. Single cell invasion phenomena are indicated by black arrows. Scale bar = 100 µm. (F) HE staining of 14-day stirred cultured U2OS cells with TEM. Scale bar = 50 µm. Please click here to view a larger version of this figure.

Discussion

A well-established protocol for decellularized ECM fabrication should retain the native ECM composition while removing cellular components in tissues nearly completely[21]. Despite currently reported decellularization protocols which require perfusion through the vasculature to remove cellular materials by convective transport, mechanical agitation was adopted here, known as a traditional simple and cheap method[22,23,24,25,26]. Moreover, since the tongue is rich in lingualis and has few bulky vascular vessels, this protocol is more suitable for tongue tissues than other protocols described above.

Furthermore, this protocol for TEM production carries out an appropriate moderate-strength decellularization, avoiding the destruction or dissolution of the base membrane which may be caused by high-strength decellularization such as sodium dodecyl sulfate (SDS) treatment[3]. In addition, the protocol also worked effectively in the tongue of rat and pig (data not shown), suggesting that the method could be commonly used upon tongues from various species[3].

It's worth noting that there are some critical steps or details in this protocol, which could directly influence the results. One important step is the digestion of tongue cells by DNase. If the digestion time is not enough or the DNase doesn't work efficiently, the decellularization of the tongue would hardly be achieved. Another thing which should be noticed is that the rotary speed for the bioreactor shouldn't be too fast, considering the damage to TEM. Moreover, a sterile environment for this operation is very important in the protocol.

In spite of the strict rules above, the protocol for TEM preparation can be adjusted to some extent. Washing the tongues with ultrapure water or PBS for a few more hours than the time which the protocol suggests would not obviously affect the fabrication of TEM. However, since the protocol needs almost one week to prepare the TEM, it cannot meet immediate demands for TEM.

The TEM shows great value in TSCC model construction. Together with a self-made minibioreactor, suspended Cal27 cells can attach in TEM and form similar infiltrative structure resembling human TSCC histopathology[3]. This could be an ideal model for monitoring and investigating the invasion and metastasis of TSCC *in vitro*. The model could also benefit the work on drug tests on TSCC. In consideration of all these, the protocol presented here may have great potential in TSCC research.

Disclosures

The authors have nothing to disclose.

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