Please cite this article as: Shunsuke *et. al.*, (2015). Generation of Mouse Thyroid Calcitonin-producing Cell Tumors from Primary Mouse Tumors, *Bio-protocol* 5 (24): e1681. DOI: 10.21769/BioProtoc.1681.

Generation of Mouse Thyroid Calcitonin-producing Cell Tumors from Primary Mouse Tumors

Shunsuke Kitajima[1, 2], Fengkai Li[1] and Chiaki Takahashi[1*]

[1]Division of Oncology and Molecular Biology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan; [2]Department of Medical Oncology, Dana-Farber Cancer Institute Boston, Boston, USA

[*]For correspondence: chtakaha@staff.kanazawa-u.ac.jp

[Abstract] Medullary thyroid cancers (MTCs) are derived from calcitonin-producing cells (C cells) of neuroendocrine origin. *Rb* heterozygous mice develop low-grade C cell adenocarcinoma following biallelic inactivation of the *Rb* tumor suppressor gene loci. Additional inactivation of another tumor suppressor gene such as *Trp53*, *Arf* or *Cdkn1a* allows Rb-deficient mice to generate more aggressive C cell adenocarcinoma (Takahashi *et al*., 2006; Shamma *et al*., 2009; Kitajima *et al*., 2015). To characterize C cell adenocarcinoma cells derived from Rb-deficient mice of different genetic backgrounds, we attempted to extract C cell adenocarcinoma cells from primary thyroid tumor tissue. Since primary mouse small cell lung cancer (SCLC) cells those originate in neuroendocrine cells that also stems C cells, can be established both as non-adhesive and adhesive cells (Calbo *et al*., 2011), we applied their method to MTCs. Here we describe our isolation technique for non-adhesive and adhesive cell cultures from primary medullary thyroid tumor tissue. We found that the molecular markers of C cell such as Calcitonin and Ascl1 are predominantly enriched in the non-adhesive population (Kitajima *et al*., 2015). This is in line with the fact that one of most commonly distributed human MTC cell line TT is non-adhesive.

Materials and Reagents

1. 12 well cell culture plate (Thermo Fisher Scientific, catalog number: 150628)
2. 100 mm cell culture dish (Thermo Fisher Scientific, catalog number: 172931)
3. 5 ml centrifuge tube (Thermo Fisher Scientific, catalog number: 339650)
4. Phosphate buffered saline (PBS) (pH 7.2) (Life Technologies, catalog number: 20012027)
   Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 20012027".
5. 100x Antibiotic-Antimycotic (Life Technologies, catalog number: 15240062)
   Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 15240062".
6. Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, catalog number: 04330085)
7. 3,000 U/ml Collagenase from *Clostridium histolyticum* (Sigma-Aldrich, catalog number: C5138)

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8. 1,000 U/ml Hyaluronidase (Wako Pure Chemical Industries, catalog number: 08006201)
9. 20 mg/ml Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, catalog number: DN25)
10. Fetal bovine serum (FBS) (Thermo Fisher Scientific, catalog number: SH3091003)
    Note: Currently, it is "GE Healthcare, catalog number: SH3091003".
11. Penicillin-Streptomycin Mixed Solution (Nakarai tesque, catalog number: 2625384)
12. 0.5 g/l-Trypsin/0.53 mmol/l-EDTA Solution, with Phenol Red (Nakarai tesque, catalog number: 3277834)
13. 7-AAD (BD Pharmingen, catalog number: 5168981E)
14. CELLBANKER1 (Nippon Zenyaku Kogyo Co., ZENOAQ, catalog number: CB011)
15. Wash buffer (see Recipes)
16. Digestion solution (see Recipes)
17. Growth medium (see Recipes)

Equipment

1. Surgical scissors and forceps
2. Pipettes
3. Centrifuge
4. Cell culture hood
5. 37 °C, 5% CO_2 cell culture incubator
6. Microscope
7. FACS Aria ll flow cytometer (BD Biosciences)

Procedure

1. Euthanize the mouse when the thyroid tumor has reached appropriate size, approximately 50-500 mm^3 (refer to Video 1 and Figure 1).
2. Expose and remove the thyroid tumor from trachea using sterile scissors. Remove the surrounding tissues as carefully as possible.
3. Place the thyroid tumor onto 12 well type plate, and wash it several times with 2 ml of cold wash buffer.
4. Mince the thyroid tumor into small pieces as small as possible using sterile scissors on ice.
5. Collect all of the tumor pieces into 15 ml centrifuge tube.
6. Wash with 5 ml wash buffer by centrifugation at 300 rcf for 5 min at 4 °C.
7. Resuspend the tumor pieces in 5 ml digestion solution.
8. Incubate at 37 °C for 60 min. Meanwhile mix gently every 15 min.
9. Centrifuge at 300 rcf for 5 min at room temperature, and remove the supernatant.

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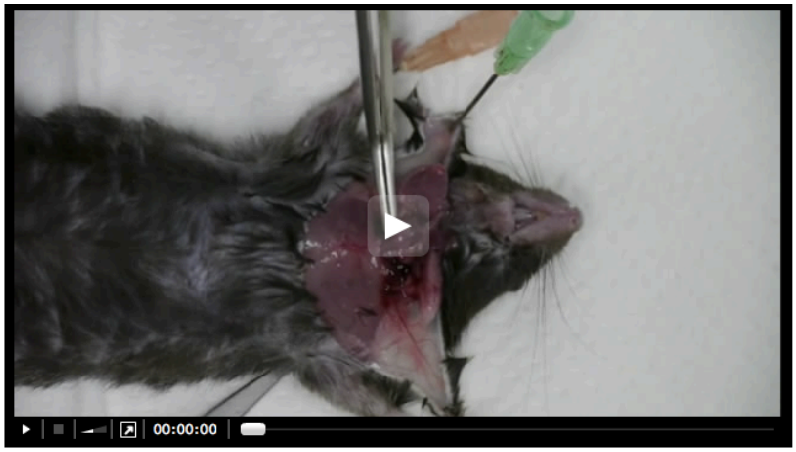
10. Wash 2 times with 5 ml wash buffer by centrifugation at 300 rcf for 5 min at room temperature.
11. Resuspend the tumor pieces in 10 ml growth medium, and plate them onto a 100 mm cell culture dish.
12. Incubate at 37 °C in 5% CO_2 for 4~5 days.
13. You will see some cells attach to the dish and grow, while the major population of cells is floating.
14. Collect all culture medium containing floating cells into 15 ml centrifuge tube.
15. Add 1 ml growth medium to the 100 mm cell culture dish, wash and recollect it into 15 ml centrifuge tube. Repeat this step at least twice in order to recover all of the floating cells.
16. Centrifuge at 300 rcf for 5 min at room temperature, discard the supernatant, resuspend the cells in 10 ml growth medium, and plate them onto a new 100 mm cell culture dish.
17. Add 10 ml growth medium to the used 100 mm cell culture dish, and maintain the attached cells in the cell culture incubator as adhesive population.
18. Repeat steps 13-17 after 3-4 day culture at least 3-4 times until attached cells never appear from floating cells in order to completely separate the non-adhesive population from the adhesive population.
19. You will detect the markers of C cells such as Calcitonin and Ascl1 positive cells are highly enriched in the non-adhesive population.
20. Dead cells should be removed by cell sorter using staining reagents such as 7-AAD for some applications.
21. Non-adhesive and adhesive populations can be stored in liquid nitrogen tank with cell stock solution such as CELLBANKER1.

Video 1. The video for how to remove the thyroid tumor



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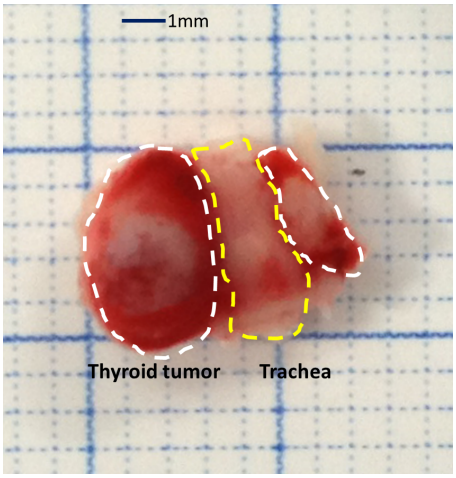

Figure 1. The picture of mouse thyroid tumor

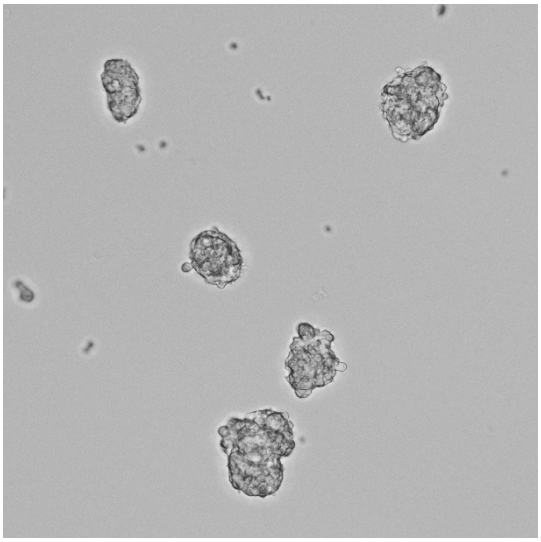

Figure 2. Phase-contrast image of the non-adhesive cell population

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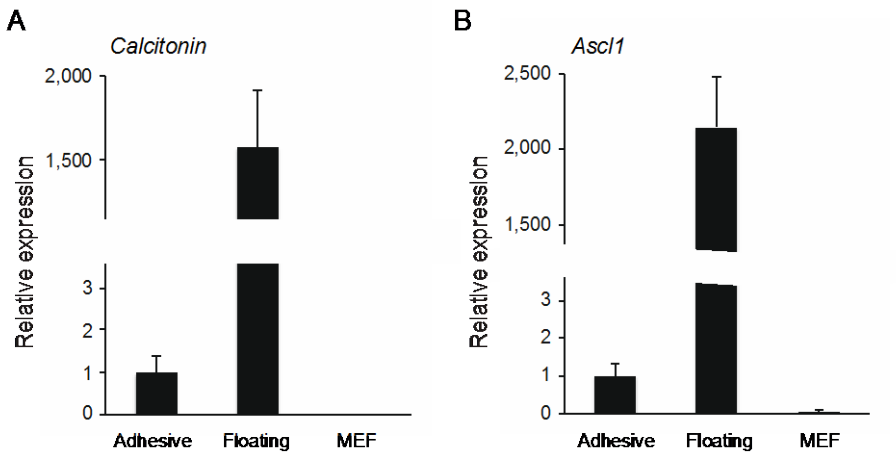

Figure 3. The non-adhesive population highly expressed C cell markers (A. Calcitonin, B. Ascl1) as compared to adhesive population and mouse embryonic fibroblast

Recipes

1. Wash buffer
   2x antibiotic
   Antimycotic in PBS (pH 7.2)
2. Digestion solution
   DMEM containing 300 units/ml collagenase, 100 units/ml hyaluronidase and 0.1 mg/ml DNase I
3. Growth medium
   DMEM supplemented 10 % FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin

Acknowledgments

We thank S. Kohno, H. Muranaka and Y. Nishimoto for helping preparation of this manuscript. Also, we thank A. Berns's group for stimulating us to create this protocol. This work was supported by Funding Program for Next Generation World-Leading Researchers (NEXT), Grant-in-Aid for Scientific Research (MEXT), Astellas Foundation for Research on Metabolic Disorders, Takeda Science Foundation, Naito Foundation, Daiichi-Sankyo Foundation for Life Science, NOVARTIS Foundation (Japan) for Promotion of Science and Hokkoku Foundation for Cancer Research.

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