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TITLE:

Culturing and Manipulation of O9-1 Neural Crest Cells

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SHORT ABSTRACT:

O9-1 is a multipotent mouse neural crest cell line. Here we describe detailed step-by-step protocols for culturing O9-1 cells, differentiating O9-1 cells into specific cell types, and genetically manipulating O9-1 cells by using siRNA-mediated knockdown or CRISPR-Cas9 genome editing.

LONG ABSTRACT:

Neural crest cells (NCCs) are migrating multipotent stem cells that can differentiate into different cell types and give rise to multiple tissues and organs. The O9-1 cell line is derived from the endogenous mouse embryonic NCCs and maintains its multipotency. However, under specific culture conditions, O9-1 cells can differentiate into different cell types and be utilized in a wide range of research applications. Recently, with the combination of mouse studies and O9-1 cell studies, we have shown that the Hippo signaling pathway effectors Yap and Taz play important roles in neural crest-derived craniofacial development. Although the culturing process for O9-1 cells is more complicated than that used for other cell lines, the O9-1 cell line is a powerful model for investigating NCCs *in vitro*. Here, we present a protocol for culturing the O9-1 cell line to maintain its stemness, as well as protocols for differentiating O9-1 cells into different cell types, such as smooth muscle cells and osteoblasts. In addition, protocols are described for performing gene loss-of-function studies in O9-1 cells by using CRISPR-Cas9 deletion and small interfering RNA-mediated knockdown.

INTRODUCTION:

Neural crest cells (NCCs) are multipotent stem-like cells with a remarkable migratory ability and transient existence during embryonic development. NCCs originate between the surface ectoderm and the neural tube and migrate to other parts of the embryo during embryonic development¹. Based on their functional domains, NCCs can be classified into several different types, including cranial, trunk, vagal, sacral, and cardiac NCCs. In addition, NCCs can differentiate into multiple cell lineages, such as smooth muscle cells, bone cells, and neurons, and give rise to various tissues^{2,3}. The development of NCCs is characterized by a complex series of morphogenetic events that are fine-tuned by various molecular signals. Given the complex regulation of NCCs and their important contributions to numerous structures, the dysregulation of NCC development can commonly lead to congenital birth defects, which account for nearly 30% of all human congenital birth defects. Abnormalities during the neural crest development can lead to cleft lip/palate, flawed nose formation, syndromes, defects such as a defective cardiac outflow tract, or even infant mortality^{1,4-7}. Understanding the molecular mechanisms of NCC development is important for developing treatments for diseases caused by defects in NCC development. With the use of various *in vitro* and *in vivo* approaches⁸⁻¹⁵, considerable progress has been made in NCC research. *In vivo*, animal models, including chickens, amphibians, zebrafish, and mice, have been used to investigate NCCs¹. Furthermore, human embryos have been used to study the process of NCC migration in early human embryo development¹⁶. *In vitro*, cell models for NCCs, such as human NCCs that originated from patient subcutaneous fat, have been used to investigate Parkinson's disease¹⁷. The O9-1 NCC line, which was originally derived from mass cultures of endogenous NCCs isolated from E8.5 mouse embryos¹⁸, is a powerful cell model for studying NCCs. Importantly, under non-differentiating culture conditions, O9-1 cells are multipotent stem-like NCCs. However, under varying culture conditions, O9-1 cells can be differentiated into distinguished cell types, such as smooth muscle cells, osteoblasts, chondrocytes, and glial cells¹⁸. Given these properties, O9-1 cells have been broadly used for NCC-related studies, such as investigating the molecular mechanism of cranial-facial defects^{19,20}.

Here, detailed protocols are provided for maintaining O9-1 cells, differentiating O9-1 cells into different cell types, and manipulating O9-1 cells by performing gene loss-of-function studies with CRISPR-Cas9 genome editing and small interfering RNA (siRNA)-mediated knockdown technologies. As a representative example, the use of O9-1 cells to study *Yap* and *Taz* loss-of-function is described. *Yap* and *Taz* are the downstream effectors of the Hippo signaling pathway, which plays a critical role in the cell proliferation, differentiation, and apoptosis. The Hippo pathway has also been shown to be important in the development and homeostasis of several different tissues and organs, as well as in the pathogenesis of different diseases²⁰⁻²⁸. The core components of Hippo signaling include the tumor suppressor sterile 20-like kinases Mst1/2, WW domain-containing Salvador scaffold protein, and the large tumor suppressor homolog (Lats1/2) kinases. Hippo signaling inhibits *Yap* and *Taz* activity and promotes their degradation in the cytoplasm. Without repression from Hippo, *Yap* and *Taz* can translocate into nuclei and function as transcriptional co-activators. We recently showed that specifically inactivating the Hippo signaling effectors *Yap* and *Taz* in mouse NCCs by using the *Wnt1^{cre}* and *Wnt1^{Cre2SOR}* drivers resulted in embryonic lethality at E10.5 with severe craniofacial defects²⁰. We have also performed studies using O9-1 cells to investigate the role of *Yap* and *Taz* in NCCs. To study *Yap*

and *Taz* function in NCC proliferation and differentiation, *Yap* and *Taz* knockdown cells were generated in O9-1 cells by using siRNA, and *Yap* knockout cells were generated by using CRISPR-Cas9 genome editing. The same gene loss-of-function strategies can be applied to different target genes in other pathways. In addition, gain-of-function studies and transfection assays can also be applied to O9-1 cells to study gene function and regulation. The protocols described here are intended to be used by investigators as guides for culturing O9-1 cells to maintain multipotent stemness, for differentiating O9-1 cells into other cell types under different culture conditions, and for studying gene function and the molecular mechanisms of NCCs.

PROTOCOL:

1. Preparation Before O9-1 Cell Culture

Note: Basal media used for O9-1 cell culture must have been conditioned by Sandos inbred mice thioguanine/ouabain-resistant (STO) mouse fibroblast cells; therefore, STO cells need to be obtained and prepared as described below before starting O9-1 cell culture.

1.1. Active STO cell culture

1.1.1. Prepare media for culturing active STO cells by making complete Dulbecco's modified Eagle's media (DMEM) with 7% fetal bovine serum (FBS) (embryonic stem cell culture grade), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (final concentrations are indicated).

Note: STO medium is used for the cultivation of active STO feeder cells. Penicillin, streptomycin, and L-glutamine may form precipitation in storage; dissolve completely by pipetting before use.

1.1.2. Coat a standard 100-mm cell culture plate with 0.1% gelatin for 30 min at room temperature. After the incubation period, aspirate the extra gelatin. Use the plate immediately after coating.

Note: Alternatively, cover the plate with STO media and leave the plate in a humidified incubator to avoid the drying of the plate (this is meant only for short-term storage and not for storing precoated plates).

1.1.3. Thaw the STO cells rapidly in a 37 °C water bath; wipe the cryovial with 70% ethanol before opening, and immediately transfer the whole vial of cells to a centrifuge tube.

1.1.4. Add the STO media dropwise to the centrifuge tube; the ratio by volume of the cells to media is 1:10.

1.1.5. Centrifuge the cells at 180 x g for 3 min at RT.

1.1.6. Mix by gentle pipetting and transfer cells to the gelatin-coated plate.

1.1.7. Allow STO cells to grow for 24 h in a standard incubator (37 °C, 5% CO₂).

1.1.8. Change the medium (only after the cells adhere) 24 h after seeding and discard the old medium.

1.1.9. Check STO cells every day under a microscope and passage at 80% confluency.

1.1.9.1. Before starting STO cell passaging, coat plates with 0.1% gelatin (gelatin volume equals half of growth media volume for that vessel) for 30 min at room temperature.

1.1.9.2. To passage STO cells, aspirate growth media and wash cells with phosphate-buffered saline (PBS) twice (add PBS in an equal volume of growth media).

1.1.9.3. Aspirate PBS and add 0.25% trypsin-EDTA (adjust volume according to the vessel size); then, incubate at 37 °C for 5 min.

Note: For a 100-mm plate, use 10 mL of growth media and 3 mL of trypsin solution. For a 150-mm plate, use 20 mL of growth media and 8 mL of trypsin solution. The volume of wash buffer used is equal to the volume of growth media.

1.1.9.4. Inactivate trypsin with an equal volume of STO media. Seed STO cells to new plates with a ratio from 1:3 to 1:10.

Note: A common expanding scheme is to expand from one cryovial into one 100-mm plate, then to one 150-mm plate, then to four 150-mm plates, and finally to 16 150-mm plates.

1.2. Inactivation and freezing of STO cells

1.2.1. For preparing 2x freezing media, add the following to STO media (final concentrations are indicated): 20% FBS, 20% dimethyl sulfoxide (DMSO).

1.2.2. After mixing, filter sterilize the 2x freezing media (pore size 0.22 µm) and store it at 4 °C until use. Make 2x freezing media the same day of use and discard unused freezing media.

1.2.3. Prepare mitomycin C solution in PBS at a concentration of 0.5 mg/mL. To dissolve mitomycin C in PBS, tape the bottle on a vortexer and vortex at a low setting for approximately 45 min to dissolve the particles completely.

Note: Mitomycin C is light sensitive and hard to dissolve.

Caution: Mitomycin C is acutely toxic and may cause cancer. Handle only with proper personal protective equipment.

1.2.4. Inactivate STO cells by adding a final concentration of 0.01 mg/mL mitomycin C to the

existing media. Incubate for 2 h inside a standard incubator (37 °C, 5% CO₂).

1.2.5. Wash plates (150-mm) with 20 mL PBS twice after inactivation with mitomycin C.

1.2.6. Aspirate the PBS solution, dissociate cells by using 8 mL of 0.25% trypsin-EDTA, and incubate for 5 min inside a standard humidified incubator (37 °C, 5% CO₂).

1.2.7. Inactivate trypsin with 8 mL of STO media.

1.2.8. Transfer the cell solution to a centrifuge tube. Combine more than one plate into one centrifuge tube to save time. Centrifuge for 5 min at 180 x g.

1.2.9. Aspirate the supernatant and resuspend the cell pellet in 5 mL PBS.

1.2.10. Use 10 µL of the cell solution to count cells by using a hemocytometer. Count the cells in the central gridded square and multiply the number obtained by 10⁴ to determine the number of cells per 1 mL. Count twice and average the two numbers to obtain the final estimation of the number of cells per milliliter.

1.2.11. Centrifuge cells for 5 min at 180 x g.

1.2.12. Aspirate PBS and add 1:1 (by volume) STO media and 2x freezing media to the cell pellet. Adjust the volume for a final cell concentration of 4 x 10⁶ cell/mL (this amount is good for one 100-mm plate).

Note: For example, from four 150-mm plates yielding a total of 60 x 10⁶ cells, freeze 4 x 10⁶ cells per cryovial, with each cryovial containing 1 mL of cell solution. Four plates yield approximately 15 cryovials (60/4=15). Add 7.5 mL of STO media and 7.5 mL of 2x freezing media to the cell pellet, resuspend, and aliquot 1 mL into each cryovial.

1.2.13. Resuspend by slowly pipetting freezing media with cell pellet. Transfer 1 mL of cell solution to each cryovial and label accordingly.

1.2.14. Place cryovials inside a lidded polystyrene box (this provides a slow cooling rate, which improves cell survival). Transfer the box to a -80 °C freezer for at least 24 h before transferring the cryovial to liquid nitrogen.

Note: For best results, minimize the cell counting time, as well as the time between adding 2x freezing media to cells and transferring vials to -80 °C.

2. O9-1 cell culture

2.1. Prepare basal media for O9-1 cell culture by adding the following in DMEM (final concentrations are indicated): 15% FBS, 0.1 mM minimum essential media (MEM) nonessential

amino acids, 1 mM sodium pyruvate, 55 mM beta-mercaptoethanol, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine, 10^3 units/mL leukemia inhibitory factor (LIF; added immediately before use, do not add to stock bottle), and 25 ng/mL fibroblast growth factor-basic (bFGF; added immediately before use, do not add to stock bottle).

2.2. Filter sterilize the basal media (pore size 0.22 μ m) and wrap in foil to protect from light.

2.3. Collect conditioned basal media from inactivated STO cells.

Note: Proceed only after obtaining inactivated STO cells. O9-1 basal media collected from STO cell plates is hereafter referred to as conditioned basal media.

2.3.1 Thaw inactivated STO cells rapidly as described above (one cryovial containing 4×10^6 cells is good for one 100-mm plate and yields approximately 100 mL conditioned basal media after 10 days of collection). Seed inactivated STO cells to the gelatin-coated plate by using STO media. Allow cells to attach overnight in a standard humidified incubator (37 °C, 5% CO₂).

2.3.2. 24 h after thawing STO cells, discard the existing STO media and replace with the basal media.

Note: Do not add LIF and bFGF to inactivated STO cell culture dishes; only add to O9-1 cell culture dishes.

2.3.3. Every 24 h, change media by using O9-1 basal media and collect the basal media from STO cell plates into a bottle. Wrap the bottle containing conditioned basal media in foil to protect from light. Store all the collected media at 4 °C.

Note: Because the cells were inactivated, they may not appear as healthy as they did after several days of collection; this is to be expected.

2.3.4. Optionally to check for contamination, collect the basal media for each collection day in different tubes (instead of one bottle) wrapped in foil. Using a 24-well plate, place 1 mL of media from each day into each well and incubate overnight in a standard incubator to check for bacterial contamination under the microscope.

Note: The media remains a red color if there is no contamination but changes to yellow if bacterial contamination is present.

2.3.5. After collecting conditioned basal media for 10 days, discard the STO cells. Combine (if applicable) all collected conditional basal media and filter sterilize (pore size 0.22 μ m) into a sterile bottle. Wrap the bottle in foil to protect from light. Keep the filtered conditioned basal media at 4 °C for a maximum of one month from the filter date.

3. Maintaining O9-1 cells

Note: Working O9-1 basal media is filter sterilized conditioned basal media, to which LIF (final concentration 10^3 units/mL) and bFGF (final concentration 25 ng/mL) are added immediately to the cell culture dish before use. This media needs to be protected from light and stored at 4 °C.

3.1. Recovery of O9-1 cells

3.1.1. Slowly thaw the stock bottle of the basement membrane matrix (e.g., Matrigel) overnight at 4 °C to prepare aliquots.

3.1.2. Add 5 mL of DMEM with 10% FBS to 5 mL of the stock basement membrane matrix membrane. Mix well by pipetting with cold pipette tips stored at -20 °C. Keep the original bottle and aliquot tubes on ice. Freeze aliquots in different volumes (0.5 mL or 1 mL aliquots) depending on the experiment needs. Avoid freeze-thawing basement membrane matrix multiple times.

Note: Basement membrane matrix polymerizes rapidly at room temperature; use cold pipette tips and keep tubes cold when working to avoid unintentional polymerization.

3.1.3. Thaw an aliquot of the basement membrane matrix at 4 °C or on ice 2 h before starting the experiment. Do not store the matrix at 4 °C for an extended time.

3.1.4. Use filter-sterilized DMEM with 10% FBS to dilute the basement membrane matrix to a final concentration of 0.5 mg/mL to coat the plates. Coat plates with the basement membrane matrix (0.5 mg/mL) at room temperature for 1 h. Ensure that it fully covers the plate (as shown in **Figure 1**).

3.1.5. Tilt the plate when aspirating the basement membrane matrix to avoid touching the coated surface; dry plates at 37 °C for 30 min. Do not over-dry coated plates.

Note: Proceed only when all media and reagents are ready to use (conditioned basal media, sterile-filtered 10% FBS in DMEM, LIF, and bFGF).

3.1.6. Recover O9-1 cells rapidly by placing the cryovial in a 37 °C water bath; slowly swirl the vial until the solution completely turns to liquid and proceed immediately to the next step.

3.1.7. Transfer all the cell solution from the cryovial (approximately 1 mL) to a 15-mL centrifuge tube. Add 5 volumes of DMEM with 10% FBS (filter sterilized with a filter pore size of 0.22 µm) and resuspend.

3.1.8 Centrifuge for 3 min at 180 x g. Aspirate the supernatant without disturbing the cell pellet. Resuspend the cell pellet (by tapping) in conditioned basal media supplemented with LIF and bFGF. Count the cells by using a hemocytometer.

Note: Conditioned basal media supplemented with LIF and bFGF is critical to maintain the

309 multipotency of the O9-1 cell line. Use care to add the correct media to avoid unintentional cell
310 differentiation.

311
312 3.1.9 Seed cells from 10,000 to 15,000 cell/cm² to a 6-well plate. Allow cells to attach and grow
313 in a standard cell culture incubator (37 °C, 5% CO₂) overnight before changing media.

314
315 3.1.10 Ensure that the cells are attached before proceeding to change media (healthy attached
316 cells look like those shown in **Figure 2**).

317
318 3.1.11. Always change culture media the next day after the recovery of O9-1 cells (use
319 conditioned basal media supplemented with LIF and bFGF).

320 321 **3.1. Passage of O9-1 cells**

322
323 3.2.1. When O9-1 cells reach 80% confluency, thaw the basement membrane matrix and
324 prepare a basement membrane matrix-coated plate as described above. Add conditioned basal
325 media supplemented with LIF and bFGF to the vessel. Alternatively, add DMEM without FBS to
326 keep the basement membrane matrix from drying and store inside a standard humidified
327 incubator for no more than 3 days.

328
329 3.2.2. Rinse the O9-1-containing wells (6-well plate) with 2 mL of Dulbecco's phosphate-
330 buffered saline (DPBS) twice and gently pipette to avoid losing cells.

331
332 3.2.3. Dissociate cells with 1 mL of 0.05% trypsin-EDTA at 37 °C for 3 min. Neutralize trypsin with
333 an equal amount of DMEM with 10% FBS. Repeatedly pipette the liquid over the whole surface
334 of the well to dissociate as many cells from the plate as possible.

335
336 **Note:** Trypsin concentration is 0.05% instead of 0.25%. Use DPBS to dilute from the stock bottle.
337 Avoid generating bubbles when dissociating cells from the plate.

338
339 3.2.4. Transfer all cell solution to a centrifuge tube and centrifuge for 3 min at 180 x g. Aspirate
340 the supernatant without disturbing the cell pellet. Resuspend the cell pellet in the centrifuge tube
341 by gentle pipetting in conditioned basal media supplemented with LIF and bFGF.

342
343 3.2.5. Use a hemocytometer to count the total cell number as described above. Seed cells
344 (10,000 to 15,000 cells/cm²) to the coated plate prepared as described in steps 3.1.4 to 3.1.8.
345 One well of a 6-well plate would require 100,000 cells to seed.

346
347 3.2.6. Allow cells to attach and grow in a standard cell culture incubator (37 °C, 5% CO₂). Always
348 change culture media the next day after passaging O9-1 cells.

349 350 **3.3. Freezing of O9-1 cells**

351
352 3.3.1. To prepare 2x freezing media for O9-1 cells, dilute the following in DMEM (final

concentrations are indicated): 40% FBS and 20% DMSO.

3.3.2. Filter sterilize 2x freezing media and keep it at 4 °C. The 2x freezing media must be made on the day it is used. Discard all unused freezing media.

Note: Freeze O9-1 cells at 80% confluency.

3.3.3. Rinse wells with DPBS twice; pipette gently to avoid losing cells.

3.3.4. Dissociate cells with 0.05% trypsin-EDTA at 37 °C for 3 min, then neutralize trypsin with an equal amount of 10% FBS in DMEM. Repeatedly pipette the liquid over the whole surface of the well to dissociate as many cells from the plate as possible.

3.3.5. Transfer all plate contents to a centrifuge tube and centrifuge for 3 min at 180 x g. Aspirate the supernatant and add 2 mL of PBS and resuspend by tapping.

3.3.6. Use 10 µL of cell solution to count cells by using a hemocytometer as described above.

3.3.7. Centrifuge cell solution for 3 min at 180 x g. Aspirate the supernatant and adjust the amount of basal media needed; then, add an equal amount of 2x freezing media.

Note: Cells are frozen at a concentration of 1×10^6 cells/mL (1 mL per cryovial).

3.3.8. Transfer cells to cryovials and label accordingly. Place cryovials inside a lidded polystyrene box for a slow cooling rate at -80 °C for at least 24 h before transferring to liquid nitrogen.

Note: For best results, minimize the cell counting time and the time between adding 2X freezing media and transferring vials to -80 °C.

4. Manipulation of O9-1 cells

4.1. Performing siRNA knockdown in O9-1 cells

4.1.1. Thaw and coat a 24-well plate with basement membrane matrix as described above (steps 3.1.4-3.1.8) before starting the experiment. Recover and seed O9-1 cells, allowing them to grow to 60% to 80% confluency.

4.1.2. Dilute liposomes in serum-free media according to the appropriate well volume. Dilute siRNA in serum-free media to the final. Add diluted siRNA to diluted liposomes in a ratio recommended by the manufacturer. Mix well by pipetting and incubate.

Note: Follow the manufacturer's guide on the volume used, time and temperature of incubation.

4.1.7. Add an appropriate volume of siRNA-lipid complex to cells according to the

manufacturer's recommendations.

4.1.8. Incubate cells for 24 h in a standard cell culture incubator (37 °C, 5% CO₂). Perform downstream steps as appropriate (extract RNA, extract proteins, staining, etc.)

Note: siRNA knockdown times and concentrations can vary depending on the individual experiment.

4.2. Performing gene knockout in O9-1 cells by using CRISPR-Cas9 genome editing

Note: Refer to the previously published protocol for using CRISPR-Cas9 in mammalian cell lines²⁹. Provided here is a simplified description of CRISPR-Cas9 genome editing and related steps.

4.2.1. Obtain sgRNA sequences by using a sgRNA design tool.

4.2.2. Ligate the sgRNA to pSpCas9(bb)-2a-GFP vector³⁰.

4.2.3. Transfect O9-1 cells with the vector by using a standard lipofection protocol according to the manufacturer's recommendations.

4.2.4. Incubate cells in a standard cell culture incubator at 37 °C with 5% CO₂ for 24 h.

4.2.5. Perform fluorescence-activated cell sorting (FACS) based on GFP/7-AAD. Seed GFP+ single cells into 96-well plates.

4.2.6. Grow the cells in the incubator for an additional 4 days. Discard all wells that have more than one colony.

4.2.7. Perform PCR with primers designed for detecting the deletion. After PCR, run PCR products on an agarose gel to evaluate the band size. Validate the knockout efficiency achieved by using CRISPR-Cas9 editing by performing Western blotting (an example of *Yap* knockout results is shown in **Figure 3**).

5. O9-1 cell differentiation

5.1. Differentiation of O9-1 cells into osteoblasts

5.1.1. To prepare osteogenic differentiation media, dilute the following in alpha-MEM (final concentrations are indicated): 0.1 mM dexamethasone, 100 ng/mL bone morphogenetic protein 2 (BMP2), 50 µg/mL ascorbic acid, 10 mM β-glycerophosphate, 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin.

5.1.2 Detect the osteoblast marker, osteocalcin, in differentiated osteoblasts by immunostaining as shown in **Figure 4**.

Note: Osteogenic differentiation can also be evaluated by using other markers of osteoblasts or with Alizarin red staining or alkaline phosphatase staining.

5.2. Differentiation of O9-1 cells into chondrocytes

5.2.1. To prepare chondrocyte differentiation media, dilute the following in alpha-MEM (final concentrations are indicated): 5% fetal calf serum (FCS), 1% insulin-transferrin-selenium (ITS), 100 U/mL penicillin, 100 mg/mL streptomycin, 10 ng/mL transforming growth factor beta (TGF- β 3), 50 mg/mL ascorbic acid, 10 ng/mL BMP2, 0.1 mM dexamethasone, and 1 mM sodium pyruvate.

5.2.2. First culture a monolayer of O9-1 cells with osteogenic media for 3 days and then trypsinize and culture as a micromass format in chondrocyte differentiation media for an additional 7 days.

5.2.3. Assess chondrogenic differentiation with Alcian blue staining or markers of chondrocytes.

5.3. Differentiation of O9-1 cells into smooth muscle cells

5.3.1. To prepare smooth muscle cell differentiation media, dilute the following in DMEM (final concentrations are indicated): 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% FBS.

5.3.2. Assess smooth muscle cell differentiation with immunofluorescence staining by using antibodies against markers of smooth muscle cells, such as smooth muscle actin (SMA), shown as an example in **Figure 5**.

5.4. Differentiation of O9-1 cells into glial cells

5.4.1. To prepare glial cell differentiation media, dilute the following in DMEM/F12 (final concentrations are indicated): 1x B-27 supplement, 2 mM L-glutamine, 50 ng/mL BMP2, 100 U/mL penicillin, 100 mg/mL streptomycin, 50 ng/mL LIF, and 1% heat-inactivated FBS.

5.4.2. Evaluate glial cell differentiation by performing immunofluorescence staining with antibodies against glial cell markers such as fatty acid binding protein 7 (FABP7).

REPRESENTATIVE RESULTS:

The goal of our knockdown and knockout experiments was to study the effects of *Yap* and *Taz* loss-of-function in O9-1 cells. Before the knockdown and knockout experiments, we have to make sure that prepare for basal media and culture O9-1 cells as described above (for example, basement membrane matrix needs to cover the whole plate as shown in **Figure 1**, and O9-1 cells recovered from liquid nitrogen as shown in **Figure 2**). We performed knockdown experiments as described above in which *Yap* and *Taz* were simultaneously knocked down. Equal amounts of *Yap*

siRNA and *Taz* siRNA were added to the final volume recommended by the manufacturer. To the control plate, an equal volume of nontargeting siRNA was added.

A *Yap*-null O9-1 cell line was generated by deleting exon 3 of *Yap* by using CRISPR/Cas9 genome editing, as described by Wang *et al*²⁰. We performed knockout experiments by using CRISPR-Cas9 genome editing as described above and in Wang *et al*²⁰. We used the following sgRNA sequences, which flanked exon 3 of *Yap*: 5'-caccgtggattacgtgggtatgtt-3' (sgRNA1-forward), 5'-aaacaacatacccacgtaatccac-3' (sgRNA1-reverse), 5'-caccgagatgggtctaattgtagtga-3' (sgRNA2-forward), 5'-aaactcactacattagaccatctc-3' (sgRNA2-reverse)

CACC was added to the forward strand, and AAAC was added to the reverse strand. G was added at the 5' end of the forward strand because the oligo did not begin with G, and C was added to the 3' end of the reverse strand. During the CRISPR-Cas9 genome editing steps described above and in Wang *et al*²⁰, cells were in conditioned basal media for O9-1 cells supplemented with LIF and bFGF to avoid unwanted differentiation. The following PCR primers were used for detecting the *Yap* deletion: 5'-AAAACAGTCTCCACTACCCCTT-3' (forward) and 5'-GGCCATCATAGATCCTGGACG-3' (reverse). The position of the sgRNAs is from base #7973399 to #7974433 on mouse chromosome 9. The PCR primer position on the chromosome is from base #7973306 to #7974478. After PCR, the PCR band for the *Yap* knockout with exon 3 deleted appeared as a 138-bp band on an agarose gel; wild-type *Yap* appeared as an 1173-bp band. The efficiency of *Yap* knockout by using CRISPR-Cas9 genome editing was validated with Western blotting, as shown in **Figure 3**.

As described above, O9-1 cells can be cultured in specific differentiation-inducing media to promote differentiation into particular cell types. Evaluation of differentiation can be done by using various approaches, such as quantitative PCR or immunostaining of specific cell markers. As an example, **Figure 4** shows O9-1 cells that were cultured in osteoblast-inducing media and differentiated into osteoblasts, which were evaluated by immunostaining cells with the antibody against osteocalcin (Ocn, an osteoblast marker). Combined with the gene knockdown and knockout experiments described above, O9-1 cells can be broadly used for gene function studies and phenotypic analyses. As an example, both wild-type and *Yap*-null O9-1 cells were cultured in smooth muscle cell differentiation media and evaluated by immunostaining cells with the antibody against smooth muscle actin (SMA, a smooth muscle cell marker). Most wild-type O9-1 cells gave rise to SMA-positive smooth muscle cells (**Figure 5A**), whereas, *Yap*-null O9-1 cells failed to differentiate into SMA-positive smooth muscle cells (**Figure 5B**), which indicated that *Yap* plays a critical role in the differentiation of NCCs into smooth muscle cells.

FIGURE AND TABLE LEGENDS:

Figure 1. Matrigel fully covering a 35-mm plate.

Figure 2. O9-1 cells 24 h after recovery from liquid nitrogen.

Figure 3. Western blot data showing the efficient knockout (ko) of *Yap* in O9-1 cells by using

CRISPR-Cas9 genome editing. wt, wild-type.

Figure 4. Immunofluorescence staining of osteoblast marker osteocalcin (Ocn) indicating that O9-1 cells gave rise to osteoblast cells under osteogenic differentiation conditions. Cells were stained with osteoblast marker Ocn antibody (green), and nuclei were stained with DAPI (blue). Arrows indicate Ocn-positive cells. Osteocalcin (Ocn, an osteoblast marker); DAPI (',6-diamidino-2-phenylindole).

Figure 5. Immunofluorescence staining of smooth muscle actin (SMA) indicating that, under smooth muscle cell differentiation conditions, most wild-type O9-1 cells gave rise to smooth muscle cells (A), whereas *Yap*-null O9-1 cells failed to differentiate into smooth muscle cells (B). Cells were stained with SMA antibody (red), and nuclei were stained with DAPI (blue). Arrows indicate SMA-positive cells.

DISCUSSION:

The NCC is a versatile and key contributor to different tissues and organs during embryonic morphogenesis. The O9-1 cell line maintains its potential to differentiate into many different cell types and mimics the *in vivo* characteristics of NCCs, making it a useful *in vitro* tool for studying gene function and molecular regulation in NCCs. The different status of O9-1 cells may correspond to different neural crest progeny *in vivo*, depending on the culture conditions of O9-1 cells. O9-1 cells can be maintained in the multipotent state when cultured under non-differentiating culture conditions, similar to regular stem cell culture. The non-differentiating O9-1 cells may correspond to pre-migratory and migratory multipotent stem-like NCCs *in vivo*. Furthermore, O9-1 cells can differentiate into many different cell types under specific differentiation culture conditions, which is an important advantage for studying gene function and regulation during the differentiation of NCCs from multipotent stem cells into a specific differentiated cell type. The differentiating O9-1 cells may correspond to post-migratory neural crest progeny *in vivo*, which have a different cell fate to differentiate into various cell types. Depending on the research interest, O9-1 cells can be manipulated and cultured differently to complement *in vivo* NCC studies with animal models.

There are various ways to manipulate O9-1 cells to study NCC events, including gene loss-of-function and gain-of-function. Here, examples are presented in which siRNA knockdown and CRISPR-Cas 9 gene editing experiments were performed in O9-1 cells for Hippo pathway effector *Yap* loss-of-function studies²⁰. With the combined use of a mouse model and O9-1 cells, our study indicated that *Yap* plays a critical role in regulating NCC proliferation and differentiation into smooth muscle cells²⁰. Other studies in different models have also indicated an important role for *Yap* in NCCs. *Yap* was shown to be required for mouse neural crest smooth muscle differentiation³¹ and to enhance human NCC fate and migration³². Furthermore, *Yap* is expressed during early *Xenopus* development³³. The efficiency of gene knockdown and knockout in O9-1 cells, combined with the convenience of performing other downstream molecular techniques such as quantitative PCR (qPCR), Western blotting, and immunostaining supports that the O9-1 cell line is an excellent NCC model that can be easily manipulated *in vitro*. In addition to the loss-

of-function studies we have described above and previously²⁰, various other applications of O9-1 cells have been described for studying NCCs. O9-1 cells can be used as an efficient alternative for experiments that require a relatively large amount of tissue, such as chromatin immunoprecipitation sequencing (ChIP-seq). However, many *in vivo* cell events such as NCC migration rely on complex tissue-to-tissue interactions, so it remains unclear how well O9-1 cells can accurately mimic *in vivo* NCC migration. Because of the limitations of *in vitro* studies with O9-1 cells, it is recommended to validate observations made with O9-1 cells *in vivo* by using animal models.

When using the O9-1 cell line to study NCCs *in vitro*, maintaining the multipotency of the cells during routine culture is critical. The multipotency of O9-1 cells can be tested at the beginning of culture by evaluating the expression of NCC marker genes such as AP-2a and Sox9. Similarly, the differentiation ability of O9-1 cells can be tested by differentiating them into specific cell types. To avoid unwanted differentiation during the routine culturing of O9-1 cells, the experimental procedures must be carried out in a manner consistent with the established protocol described here. Careful preparation of the conditioned basal media and the fresh addition of the correct concentrations of LIF and bFGF are critical for maintaining the multipotency of O9-1 cells. In addition, the experimental timeline needs to be carefully planned, given that culturing O9-1 cells requires first preparing inactive feeder STO cells and collecting conditioned basal media, which is good for only about one month. Previous studies have shown that Basement membrane matrix is important for maintaining the differentiation potential of muscle and neural precursor cells, as well as mesenchymal stem cells³⁴⁻³⁶. In the context of the protocol described here, Basement membrane matrix provides a platform for O9-1 cells to attach and helps maintain differentiation potential. Therefore, Basement membrane matrix is an important factor that may affect experimental results and their consistency when repeated. We recommend preparing Basement membrane matrix strictly according to the protocol described above and avoiding multiple freeze-thaw cycles of the Basement membrane matrix. Also, when recovering O9-1 cells from the frozen state to culture, the process of moving the cells from liquid nitrogen to a 37 °C water bath needs to be done quickly, avoiding any vortexing during the entire process. In addition, the overgrowth of O9-1 cells also needs to be avoided to maintain the multipotency of O9-1 cells. When passaging O9-1 cells, ensure that the trypsin concentration is diluted to 0.05% instead of 0.25% and that cells appear properly dissociated. Repeatedly pipetting very gently to achieve a single-cell suspension is necessary for avoiding the formation of aggregated cells. In general, the entire process of handling the O9-1 cell line needs to be done gently and carefully.

In summary, the O9-1 cell line is a very useful tool for studying NCCs *in vitro*, especially when used as a method complementary to *in vivo* studies of NCCs. O9-1 cells have obvious advantages such as the ease with which they can be accessed and the ease with which sufficient cell numbers can be obtained for experiments. Given the differentiation capabilities of O9-1 cells, they have great potential for use in a wide range of applications in various fields of research. O9-1 cells can be conveniently used for applications such as quick drug testing and screening, ChIP-seq, transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) and RNA sequencing (RNA-Seq), gene gain-of-function and loss-of-function studies, as well as signaling regulation studies. The use of a standardized and complete protocol for the handling of O9-1 cells

will facilitate the reproducibility of studies in which they are used.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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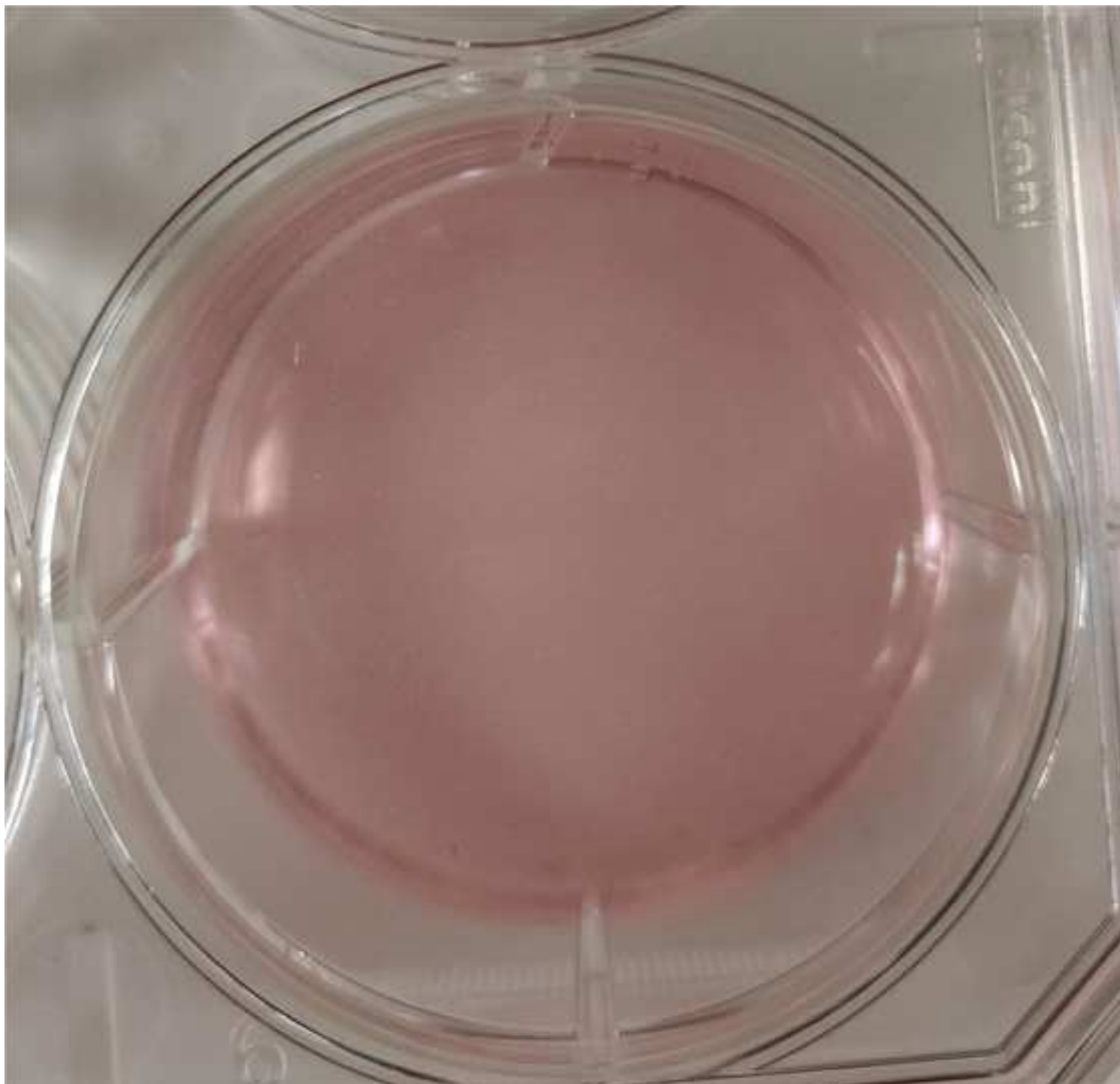
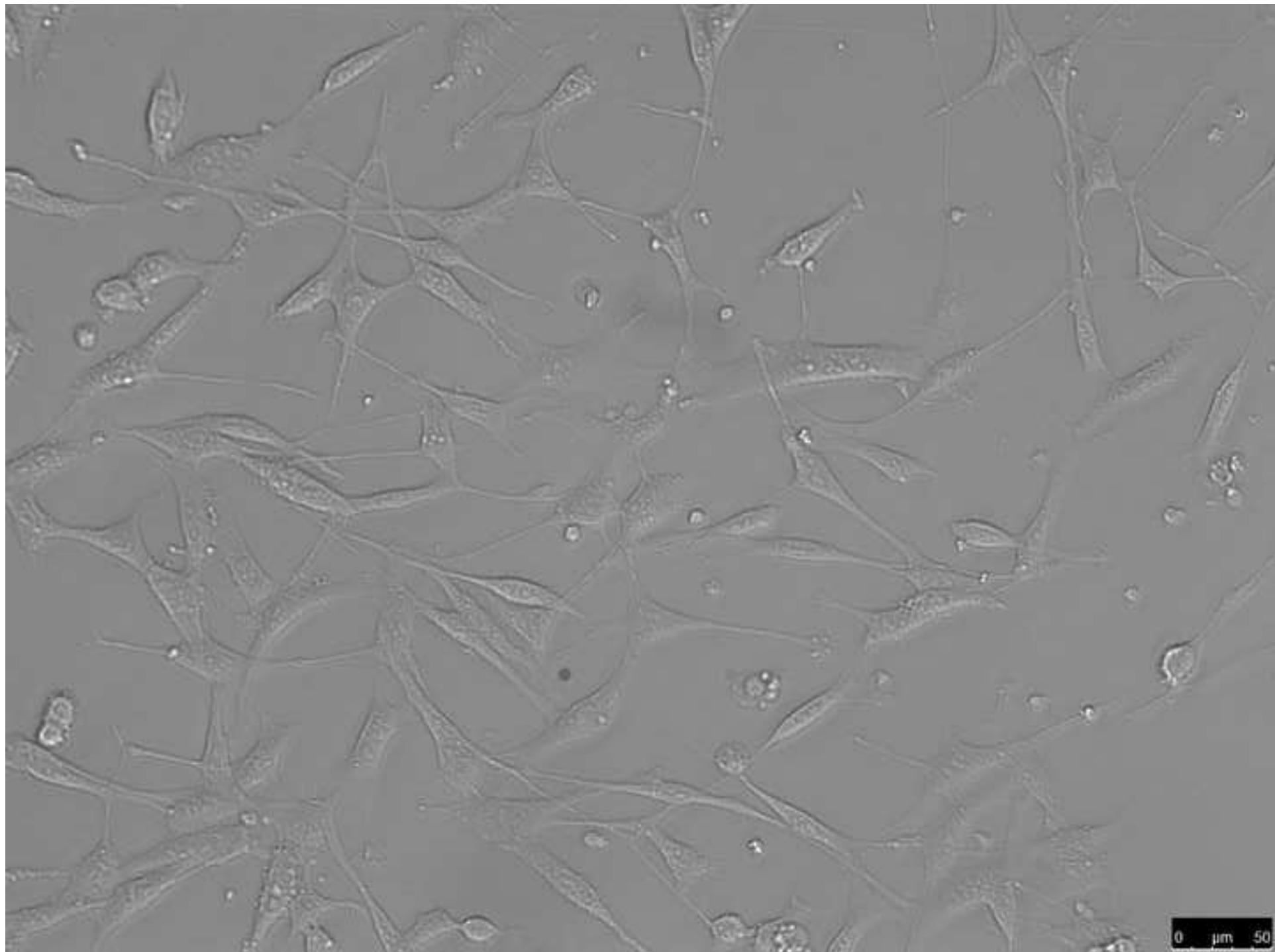


Figure 2

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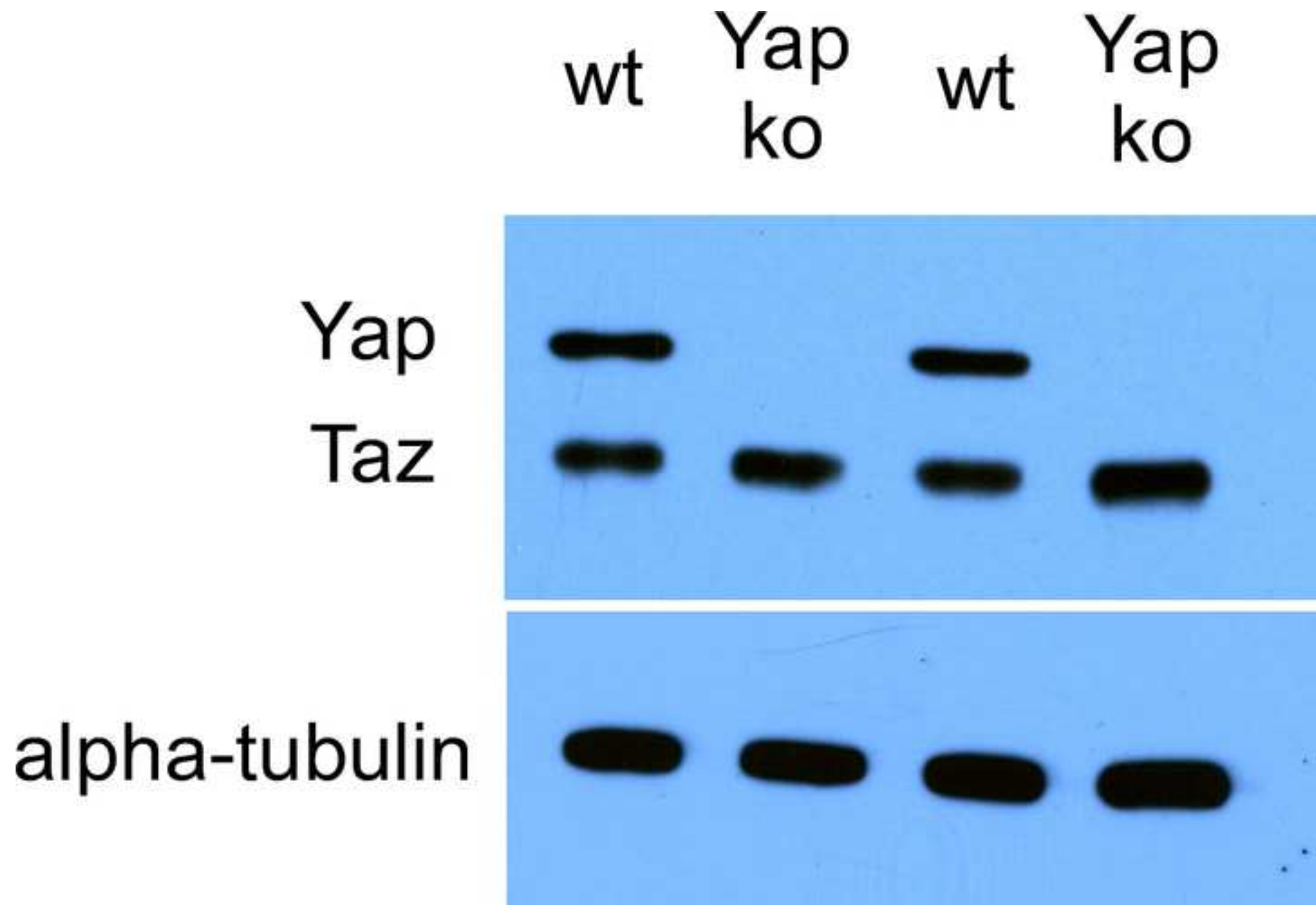
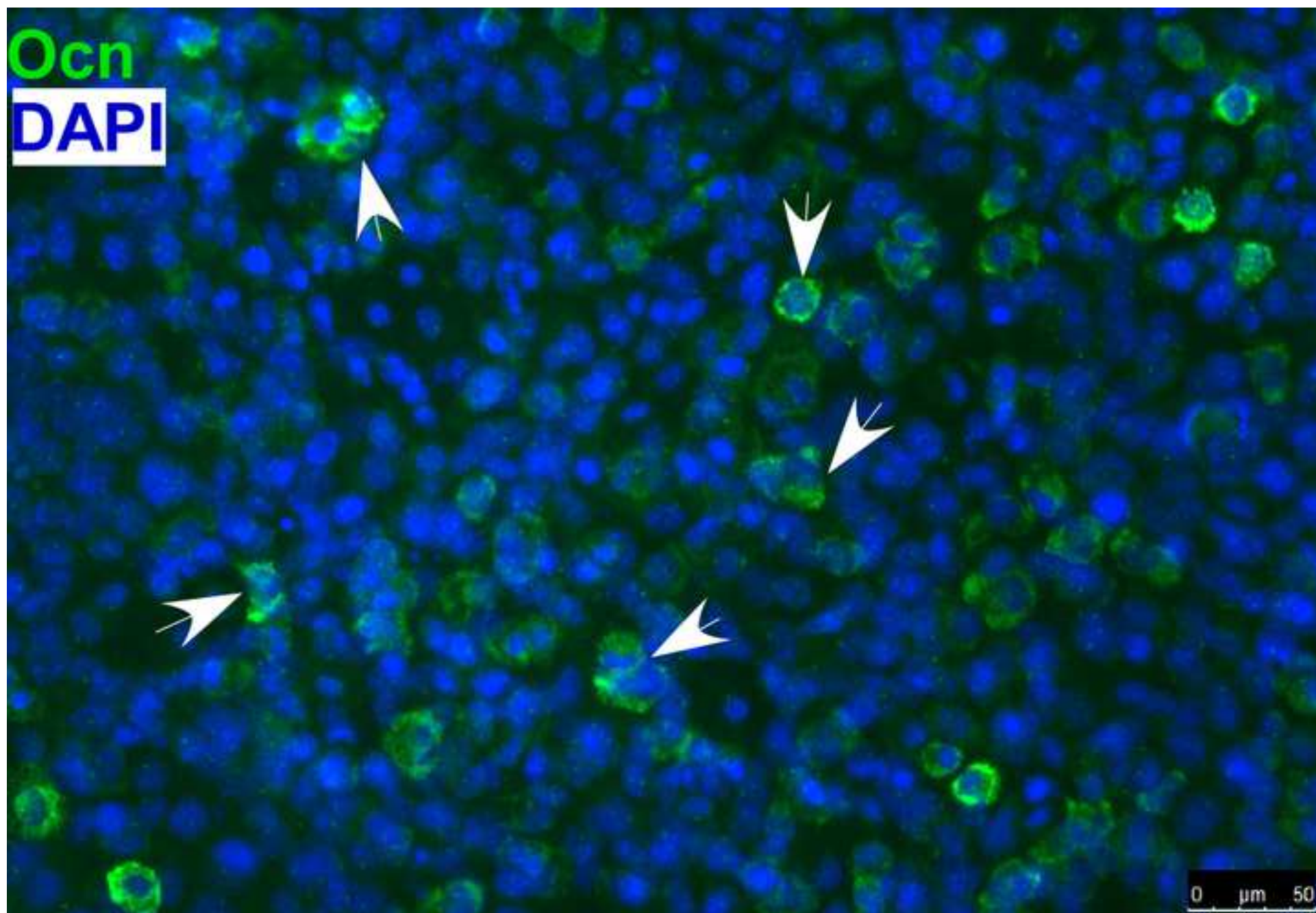
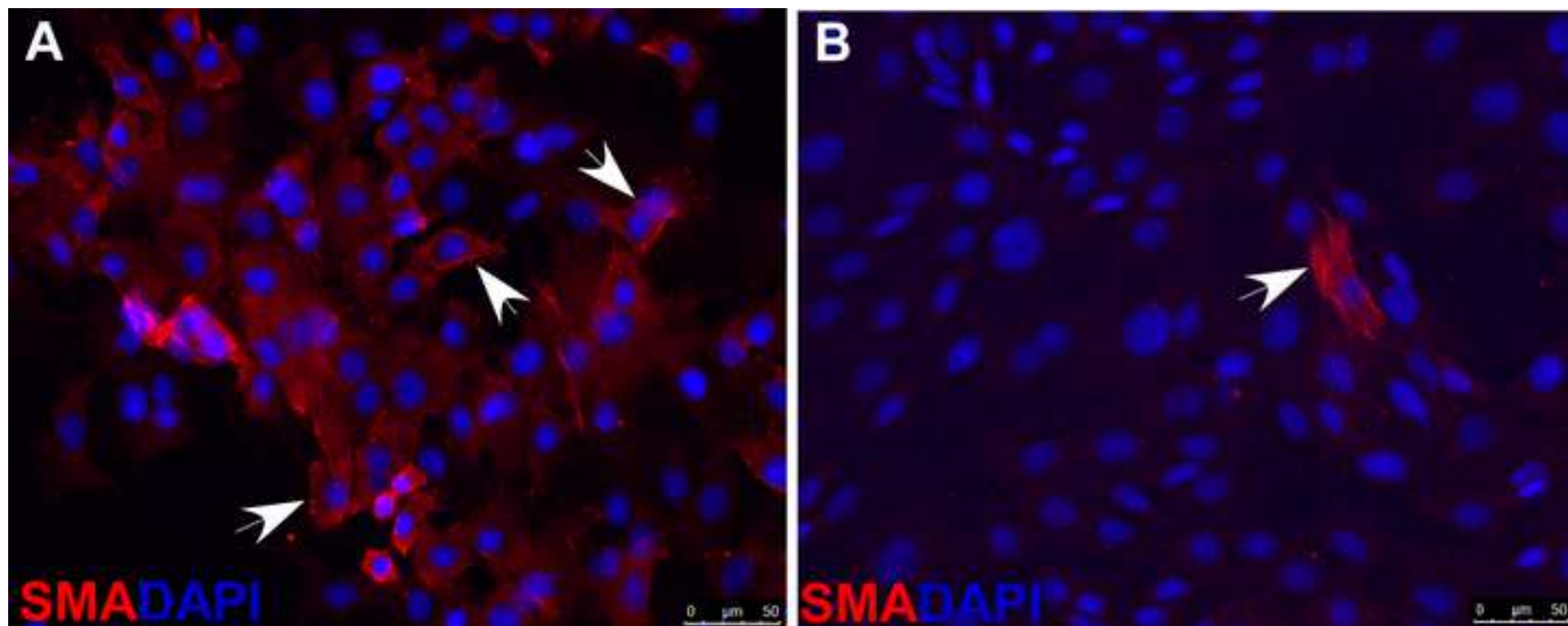


Figure 4





Name of Material/ Equipment

Active STO feeder cells

O9-1 mouse cranial neural crest cell line

DMEM, high glucose, no glutamine

DMEM, high glucose

FBS (fetal bovine serum)

Penicillin - streptomycin

L-glutamine 200mM (100X)

Gelatin from porcine skin

Trypsin-EDTA 0.25% in HBSS

DPBS (Dulbecco's phosphate buffered saline) without calcium or magnesium

MEM non-essential amino acids (MEM NEAA) 100X

Sodium pyruvate (100mM)

2-Mercaptoethanol

ESGRO leukemia inhibitory factor (LIF) 10^6 unit/ml

Recombinant human fibroblast growth factor-basic (rhFGF-basic)

Mitomycin C

Matrigel matrix

DMSO (dimethylsulfoxide)

Lipofectamine RNAiMAX

Opti-MEM I (1X)

Minimum essential medium, alpha 1X with Earle's salts, ribonucleosides, deoxyribonucleosides, & L-glutamine

ON-TARGETplus Wwtr1 siRNA

ON-TARGETplus Non-targeting Pool

ON-TARGETplus Yap1 siRNA

FCS (fetal calf serum)

ITS (insulin-transferrin-selenium)

TGF- β 3

Ascorbic acid

BMP2 (bone morphogenetic protein 2)

Dexamethasone

B-27 supplement

Company	Catalog Number
ATCC	ATCC CRL-1503
Millipore Sigma	SCC049
Gibco	11960-044
Hyclone	SH30243.01
Millipore Sigma	ES-009-B
Gibco	15140-122
Gibco	25030-081
Sigma	G1890
Genesee Scientific	25-510
Lonza	17-512F
Gibco	11140-050
Gibco	11360-070
Sigma	M-7522
Millipore Sigma	ESG1106
R&D Systems	233-FB-025
Roche	10107409001
Corning	356234
Millipore Sigma	MX1458-6
Thermo Fisher Scientific	13778-075
Gibco	31985-070
Corning	10-022-CV

Dharmacon	L-041057
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Dharmacon	D-001810
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Dharmacon	L-046247
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Also available in mitomycin C-inactivated form, catalog # ATCC 56-X



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July 23, 2018

Vineeta Bajaj, Ph.D.
Review Editor
JoVE
617.674.1888

Re: *JoVE* submission JoVE58346

Dear Dr. Bajaj,

We highly appreciate your evaluation of our manuscript and the valuable feedback that you have provided. To address your concerns, we have revised the manuscript accordingly and have included a point-by-point response below. All of our changes are shown as tracked. We believe that the revisions have improved our manuscript and hope that you will now find it suitable for publication in *JoVE*.

Sincerely,

A handwritten signature in dark ink, appearing to be "Jun Wang", with a long horizontal stroke extending to the right.

Jun Wang, Ph.D.

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1. *The editor has formatted the manuscript to match the journal's style. Please retain the same.*

Response: We retained the same format.

2. *Please address specific comments marked in the manuscript.*

Response: We have addressed the specific comments marked in the manuscript.

3. *For the protocol section please write exactly how you did your experiment with all specific details. Please avoid redundancy and use complete sentences in the numbered step.*

Response: We have revised our manuscript accordingly.

4. *Please number all sentences/steps and do not have paragraphs of text. So 1 should be followed by 1.1. which should be followed by 1.1.1. and so on.*

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5. *Please use imperative tense throughout directing someone how to do your experiment.*

Response: We have revised our manuscript accordingly.

6. *Also please show some figures for section 2 of culturing and growing the cells as well.*

Response: We have added two figures in section 2 related to the culturing and growing of the cells.

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Response: We have proofread the manuscript thoroughly.