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Methods for derivation of multipotent neural crest cells derived from human pluripotent stem cells

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Summary

Multipotent, neural crest cells (NCCs) produce a wide-range of cell types during embryonic development. This includes melanocytes, peripheral neurons, smooth muscle cells, osteocytes, chondrocytes and adipocytes. The protocol described here allows for highly-efficient differentiation of human pluripotent stem cells to a neural crest fate within 15 days. This is accomplished under feeder-free conditions, using chemically defined medium supplemented with two small molecule inhibitors that block glycogen synthase kinase 3 (GSK3) and bone morphogenic protein (BMP) signaling. This technology is well-suited as a platform to understand in greater detail the pathogenesis of human disease associated with impaired neural crest development/migration.

Keywords

Neural crest cells; human pluripotent stem cells; human embryonic stem cells; human induced pluripotent stem cells

1. Introduction

Neural crest cells (NCCs) arise from the neural plate border during closure of the burgeoning neural tube. NCCs delaminate from the roof plate and migrate to distinct targets

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10Prior to adding the dissociation solution it is critical to evaluate the colonies for spontaneous differentiation. Observe the plate under an inverted scope and identify colonies that show evidence of differentiation or are too large (excessively large colonies are more prone to differentiation). Use an indelible marker to indicate on the plate where the colonies you wish to remove are so that you may remove them in the biological safety cabinet. Appropriate colony morphology is indicated by round, well-defined edged, homogenous colonies that are tightly packed; the cells within retain a characteristic high nuclear to cytoplasmic ratio and display easily identifiable nucleoli. Remove unwanted colonies by first aspirating the culture medium then etching a line around and through the colony(ies) using a 20 μL pipette tip or 20–22G- 1½ needle (this guards against removing the underlying feeder layer in a sheet and prevents removing the colonies you wish to pass). Using a sterile Pasteur pipette, gently slide the pipette along the colony edges and center, being careful to observe and prevent any pulling from the feeder layer. It is critical to work quickly to avoid drying of the plate. Once the colonies are removed, wash with 1x PBS; replace aspirated PBS with fresh hESC maintenance medium. If more than 10% of the colonies in a well/plate are of an inappropriate morphology, the culture is unreliable and should be discarded; you must examine your media preparation as well as your technique to maintain proper hESC identity.

21In order to subsequently differentiate the cells, they should be passaged in hESC maintenance medium (not in a commercial stem cell medium) at least twice prior to switching to neural crest differentiation medium. Differentiation efficiency is dramatically reduced if the cells have not been adapted to the maintenance medium prior to any attempts at differentiation toward NCCs.

throughout the developing embryo where they differentiate to form varied functional tissue [1–4]. Migrating NCCs maintain a characteristic phenotype, however, NCC point of origin and local microenvironments encountered during migration influence cell and ultimately tissue fate [5–8]. NCCs originate from four separate compartments (cranial, cardiac, vagal and trunk), which lie along the rostro-caudal axis of the developing neural tube [7]. As a result of the multipotent nature of NCCs, aberrations that are associated with the development of NCCs from the neural plate border, their migration within the embryo and/or their terminal differentiation lead to a wide variety of diseases or syndromes, known as neurocristopathies [9]. The study of neural crest biology has clear basic science and clinical relevance: NCCs have been dubbed the “fourth germ layer” due to their multipotency. Moreover, the diseases that are a consequence of improper neural crest development or migration span many organ systems and can often have debilitating or lethal outcomes.

The strategy developed by our laboratory was influenced by previous studies focusing on neuroectoderm specification [3, 10]. These approaches lead to low efficiency derivation of NCCs by utilizing concurrent inhibition of transforming growth factor (TGF)-β- and bone morphogenetic protein (BMP)-dependent signaling. The predominant cell type within these culture systems displays a neural progenitor cell (NPC) phenotype with high expression of PAX6. Moreover, these systems were laborious, often relying on feeder layer co-culture and subsequent FACS enrichment steps [3, 10–14]. In an effort to alleviate these limitations, we developed a highly efficient, single-step method for the generation of NCCs from human pluripotent stem cells [15, 16]. The protocol described here is performed under feeder-free conditions, requires no enrichment or selection steps and generates cultures composed of ≥90% cells with neural crest identity. This is accomplished by inhibition of Smad1,5,8 and activation of the canonical Wnt pathway using small-molecule inhibitors of TGF-β signaling (SB431542) and glycogen synthase kinase 3 [GSK3 inhibitor IX (BIO)], respectively. In addition to highly efficient differentiation and ease of culture, the differentiated NCCs are capable of self-renewal for greater than 30 passages. NCCs are then capable of further differentiation into a multitude of additional cell types such as mesenchymal stem cells, peripheral neurons, adipocytes, smooth muscle cells, chondrocytes and osteocytes [15, 16]. Importantly, these cells are also capable of clonal expansion and can be cryopreserved with no loss in potency or self-renewing potential.

2. Materials

2.1 Medium Preparation

Special care should be made to prepare all medium using freshly thawed factors from frozen aliquots. Bulk medium preparation (greater than 500 mL) should be avoided. Unless otherwise stated, all medium should be utilized within seven days of preparation. For the most consistent and reliable results, maintenance and differentiation medium should be prepared, as needed, every one to two days. When not in use, medium must be refrigerated at +4°C.

1. Collagenase IV Dissociation Solution:

Combine enough collagenase IV powder and DMEM/F12 (1:1) to reach a final concentration of 400 units/mL (*see* Note 1). Filter-sterilize the solution and store at 4°C for a week. Alternatively, the solution may be placed in aliquots and stored at −20°C for up to 2 months.

2. StemPro® hESC Culture Medium: StemPro® should be mixed according to the manufacturer's instructions.

3. Basal Defined Medium: For each 500mL of basal defined medium, combine 50 mL of a 20% (vol/vol) stock solution of Probumin®, 5 mL of penicillin (10,000 IU) and Streptomycin (10,000 μg/ml) 100x, 5 mL of L-alanyl-l-glutamine (43.44 mg/mL) 100x, 5 mL of MEM non-essential amino acids, 0.5 mL of trace elements A 1,000x, 0.5 mL of trace elements B 1,000x, 0.5 mL of trace elements C 1,000x (Cellgro), 0.9 mL of 2-mercaptoethanol, transferrin (10 μg/mL), (+)-sodium L-ascorbate (50 μg/mL), and reach final volume of 500 mL by adding 432 mL of DMEM/F12 (1:1). Filter sterilize (0.22 μm pore) and store at 4°C for up to two weeks for use with maintenance and differentiation media preparation (*see* Note 2).

4. Human Embryonic Stem Cell (hESC) Maintenance Medium. Add the following factors to the basal defined medium with the specified final concentrations: Heregulin β-1 (10 ng/mL), Activin A (10 ng/mL), LONG® R3 IGF-I (200 ng/mL) and Fgf2 (8 ng/mL) (*see* Notes 2 & [3]).

5. Neural crest differentiation medium. Add the following factors and inhibitors to the basal defined medium with the specified final concentrations: Heregulin β-1 (10 ng/mL), LONG® R3 IGF-I (200 ng/mL), and FGF2 (8 ng/mL), GSK3 inhibitor IX (BIO) (2 μM), and SB431542 (20 μM) (*see* Notes 2–[4]).

6. Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix -coated plates: Prepare 1 mL aliquots of Geltrex™ according to manufacturer's protocol (*see* Note 5). Thaw a 1 mL aliquot of Geltrex ™ and dilute it to 1:30 or 1:200 (*see* Notes 6 & [7]) in DMEM/F12 before plating. Add 1.5 mL of the Geltrex™ dilution to a 35-mm tissue culture plate or 3 mL of the final dilution

[1]If unit concentrations of collagenase IV are not given, use 1 mg/mL.
[2]To ensure proper concentration of growth factors, it is best to follow strict aseptic technique with no need to filter the medium; however, if factors or other reagents are shared or their handling/aliquoting can not be accounted for, the medium must be filter-sterilized using a 0.22 μm pore.
[3]Media should be pre-equilibrated to 37°C prior to use.
[4]The use of commercially available stem cell media, such as StemPro® or mTesR®, is not recommended for this protocol, as the presence of Activin A and/or TGF-β inhibits efficient NCC differentiation. Additionally, the use of serum-rich or KSR media is also not recommended due to the undefined nature of their components and poor efficiency in NCC yield.
[5]In our lab, we initially aliquot 1 mL containing a 1:1 solution of Geltrex™: DMEM/F12 by adding 5 mL of ice cold DMEM/F12 to 5 mL of frozen Geltrex™ and allow the mix to completely thaw on ice before thoroughly mixing by pipetting. It is important to work quickly as Geltrex™ will gel in 5–10 minutes at temperatures above 15°C. To avoid the solution reaching this temperature, we keep the aliquoted tubes on ice until we finish portioning out the solution. These aliquots are immediately frozen (−20°C) for later use.
[6]When adapting cells to feeder free conditions, we utilize a 1:30 dilution of Geltrex™ to DMEM/F12. This is met by diluting a 1mL aliquot of 1:1 Geltrex™: DMEM/F12 as in Note 5 into a further 14 mL of DMEM/F12 for a final volume of 15 mL. The cellular stress upon change from the feeder layer to Geltrex™ appears to be lessened by using this higher concentration, as cell survival is enhanced. After 2–3 passages, the cells may be transitioned further to a Geltrex™:DMEM/F12 dilution of 1:200. Cell survival and spontaneous differentiation are unaffected, while considerable cost savings can be attained by this increased dilution.
[7]For best results, coated plates may be kept for five days at 37°C in a 5% CO_2 incubator, provided the plates are not allowed to dry out. Take care to monitor coated plates and add additional DMEM/F12 if needed after solidification to prevent drying. Alternatively, the plates may be wrapped with Parafilm™ and stored at 4°C for no greater than two weeks.

to a 60-mm tissue culture plate. Incubate coated plates at 37°C for at least 1 hour prior to use.

3. Methods

This neural crest differentiation protocol works equally well for hESCs or hIPSCs, however, cultures must be adapted to feeder-free and single-cell growth conditions.

3.1 Adaptation of hESC and hiPSC cultures to feeder-free conditions

Skip to step 3.2 (Adaptation of hESC/hiPSC cultures to single-cell growth conditions) if hESC/hiPSC cultures are already maintained in feeder-free conditions.

It is advisable to only passage one well/plate at a time and to work as quickly as possible through each step.

1. Once colonies reach proper confluence, aspirate the culture medium and wash with 1x PBS (*see* Note 8); aspirate the PBS and add enough collagenase IV dissociation solution to fully cover one well/plate (*see* Notes 9–[11]).
2. Manual passage of the colonies yields the most uniform results and leads to improved survival upon passage. Many methods can be used for manual passage, however, the most facile method utilizes the StemPro® EZ™ Passage Tool (*see* Note 12).
3. Upon addition of the collagenase IV solution, immediately begin manual passage. Once colonies have been cut into pieces, incubate them in the collagenase solution at 37°C for 5–30 min or until the colony fragments begin to lift up from the plate; observe the colonies under the microscope periodically to detect this change (*see* Note 13).
4. Gently collect the colonies using a 5 mL pipette and place in a 15 mL conical tube. If the colonies are difficult to remove by gentle pipetting, you may use a cell scraper to gently detach them from the plate (*see* Note 14). Once the cells have been collected in the conical tube, wash the plate with 2–5 mL of 1 x PBS (depending on well/plate size) to collect remaining colonies in the dish and to dilute

[8]You may use PBS with or without Ca^{2+}/Mg^{2+}, as they do not affect collagenase activity. The wash step is included to rid the plate of components that may inhibit or reduce collagenase IV activity, such as Fe^{2+}.
[9]Prior to beginning any plating or dissociation work with cells, ensure that you have Geltrex-coated plates prepared and that they are ready to be utilized.
[11]When working with multiple wells/plates of cells, it is critical to add the cell dissociation solution one well/plate at a time. For example, if using a 6 well plate, add the solution to one well and proceed to the next step with that well before moving on to the next well. This is time consuming but yields the most reliable results and protects cells from over exposure to the dissociation solution.
[12]The StemPro® EZ™ Passage Tool separates the colonies into optimally sized, uniform pieces, which survive passage with greater frequency and yield "goldilocks" colonies that are not too large or too small- leading to decreased spontaneous differentiation and more reliable culture. When attempting to pass several wells/plates the StemPro® EZ™ Passage Tool allows the greatest time-savings and therefore the least amount of exposure of the culture to contaminants. Use 1 Tool per well/plate.
[13]Once you have scraped the colonies in one well containing the collagenase solution, you may begin the process for other wells/plates now- **do not** exceed 6 wells/plates at one time, as time will become a limiting factor for good technique and ultimately culture survival/reliability.
[14]Excessive, vigorous pipetting will damage colonies or reduce their size to suboptimal dimensions. Gentle pipetting 2–3x should be enough to remove the colonies from the plate. If this is not enough, you may not have incubated the cells in collagenase solution long enough. You may use gentle scraping with a sterile cell scraper, but make sure to add PBS or hESC medium to the plate first.

the collagenase in the conical tube (See note 15). Centrifuge the cells for 4 min at 200g at RT.

5. Carefully aspirate the collagenase/PBS supernatant and resuspend the cell pellet in 4 mL of pre-equilibrated StemPro™ medium.

6. Plate the cells using a 1:4 (vol/vol) ratio (*see* Note 16) onto freshly prepared Geltrex-coated plates. Add pre-equilibrated StemPro® medium for a total volume of 2mL for a 35-mm dish and a total volume of 4 mL for a 60 mm dish.

7. Maintain the cells at 37°C in a 5% CO_2 incubator and replenish spent medium with fresh pre-equilibrated StemPro® medium every day. Repeat steps 1–7 as necessary until colonies begin to need passaging every 4–5 days (*see* Notes 17 & [18]). The colonies may be kept in this manner indefinitely, however, for neural crest differentiation, they must be adapted to Accutase passage for single-cell culture as described below.

3.2 Accutase® and single-cell culture adaptation

1. When hESC/hiPSC colonies have become adapted to Geltrex™, they must be further adapted to Accutase® dissociation and single-cell culture before they may be differentiated to neural crest stem cells. Aspirate the StemPro® medium and add enough Accutase® cell dissociation reagent to cover the plate (~2 mL/60 mm dish). Leave the dish at room temperature (RT) for 5–10 minutes, checking frequently to ensure that the colonies begin to conform to a rounded shape and the colony edges begin to detach from the plate but are still loosely adherent (*see* Note 19).

2. Gently collect the colonies using a 5 mL pipette and place in a 15-mL conical tube and centrifuge the cells for 4 min at 200*g* at RT.

3. Carefully aspirate the Accutase® supernatant and resuspend the cells in 4–5 mL of StemPro® medium, if the cells are still quite sensitive to Accutase® treatment or hESC maintenance medium, if they have become well tolerant of Accustase®. While adapting the cells to Accutase®, it is best to pass at a ratio of 1:2, until passaging is required every 4–5 days (*see* Notes 20–[22]).

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[15]Adding PBS to the collagenase solution accomplishes two goals: 1) it makes aspirating the supernatant from the conical tube after centrifugation easier, as an undiluted viscous collagenase solution can easily pull the entire pellet with it, and 2) it further dilutes the collagenase to be removed- if collagenase is not removed, the colonies will not adhere well, thus leading to reduced plating efficiency and spontaneous differentiation.

[16]It is advisable to use two Geltrex™ plates and two MEF plates and place 1 mL of the 1:4 (vol/vol) colony/StemPro® dilution onto each plate; that way, the two MEFs plates serve as reserves in the event the cells do not survive well on the new Geltrex™ plates. If the colonies are successful upon transitioning to Geltrex™, then the two reserve MEF plates make great frozen stocks should you need to return to MEF plates in the future.

[17]Reaching a point at which colonies need to be passed every 4–5 days may take 2–3 weeks for reliable passaging and survival on Geltrex™.

[18]As with culturing hESC colonies on feeders, passage the colonies when any of the following criteria are observed: 1) colonies are becoming too large, 2) colonies are becoming too dense (proximity to neighboring colonies decreases or colonies begin touching), 3) spontaneous differentiation begins, or 4) 10 days pass between passages and the colonies have not violated the 3 preceding criteria.

[19]Overly confluent cells require longer Accutase® incubation; it is not optimal for the cells to fully detach, and not to pipette too much, as this decreases cell health [viability??].

[20]The cells may take up to 3 weeks to become adapted to Accutase treatment.

[22]Avoid exceeding densities over 85–90% confluence in single cell culture conditions, as this dramatically increase spontaneous differentiation and decreases reliable differentiation to lineage specific cell types later on. Also, it is best not to plate at densities, which lead to excessive passage, i.e. cultures should not need passing more than every 4 days.

4. Count the cells with a hemocytometer and replate them at a density of ~5–8 × 10^4 cells/cm^2. Once the cells have become well adapted to Accutase® passage they may be passed without cell number determination at a 1:4 to 1:5 (vol/vol) ratio on a Geltrex™-coated plate.

5. Maintain the cells at 37°C in a 5% CO_2 incubator and replenish spent medium with fresh pre-equilibrated medium every day. Human ESCs maintained in these conditions should remain positive for pluripotent markers such as SOX2, OCT4 and NANOG.

3.3 Differentiation of hESC to Neural Crest Stem Cells (NCCs)

1. When hESC plates have reach 75–85% confluence, they are ready to be passaged and replated for differentiation into NCCs. Aspirate hESC maintenance medium from culture dish and add enough Accutase® cell dissociation reagent to cover the plate (~2 mL/60 mm dish). Leave the dish at room temperature (RT) for 5–10 minutes, checking frequently to ensure that the cells conform to a rounded shape, but are still loosely adherent (*see* Note 19).

2. Collect the cells in a 15 mL tube and centrifuge for 4 min at 200*g* at room temperature.

3. Aspirate the supernatant and resuspend the cells in 4–5 mL of pre-equilibrated hESC maintenance medium.

4. Count the cells with a hemocytometer and replate them at a seeding density of ~9 × 10^4 cells/cm^2 onto Geltrex™-coated plates in hESC pre-equilibrated maintenance medium.

5. After 24 hours, aspirate the hESC maintenance medium, wash the cells with 1xPBS, (*see* Note 23) and replace with neural crest differentiation medium.

6. Replenish spent medium with fresh neural crest differentiation medium every day.

7. Differentiating cells will reach 75–85% confluence within 3–4 days and density/morphology should be monitored daily. Morphological changes should become apparent around days 4–5 (*see* Figure 1A) after exposure to neural crest differentiation medium, and subsequent neural crest morphology should become apparent between 7–12 days of differentiation in neural crest differentiation medium (*see* Figure 1A).

8. Upon reaching proper confluence (75–85%), typically every 3–4 days, the differentiating cells should be passed using Accutase® according to the method described above and continued to be reseeded in neural crest differentiation medium at the same density.

9. NCC identity can be analyzed as early as 15 days post initial exposure to neural crest differentiation medium However, it may take up to 21 days to reach full

[23]It is critical to wash the cells with PBS in order to remove any remaining Activin A in the medium. The presence of any Activin A will reduce the NCC differentiation.

maturity (*See* Figure 1). Analyses include immunocytochemistry, flow cytometry and/or RT-PCR (Figure 1B–D). If you are using immunocytochemistry, NCCs should be positive for markers such as p75, Hnk1, AP2. Flow cytometric analysis of NCCs should yield $p75^+$ and $HNK1^+$ cell populations. If you carry out RT-PCR, NCCs should express genes such as PAX3, AP2, ZIC1, SOX9 and SOX10, among others. (*See* Figure 1)

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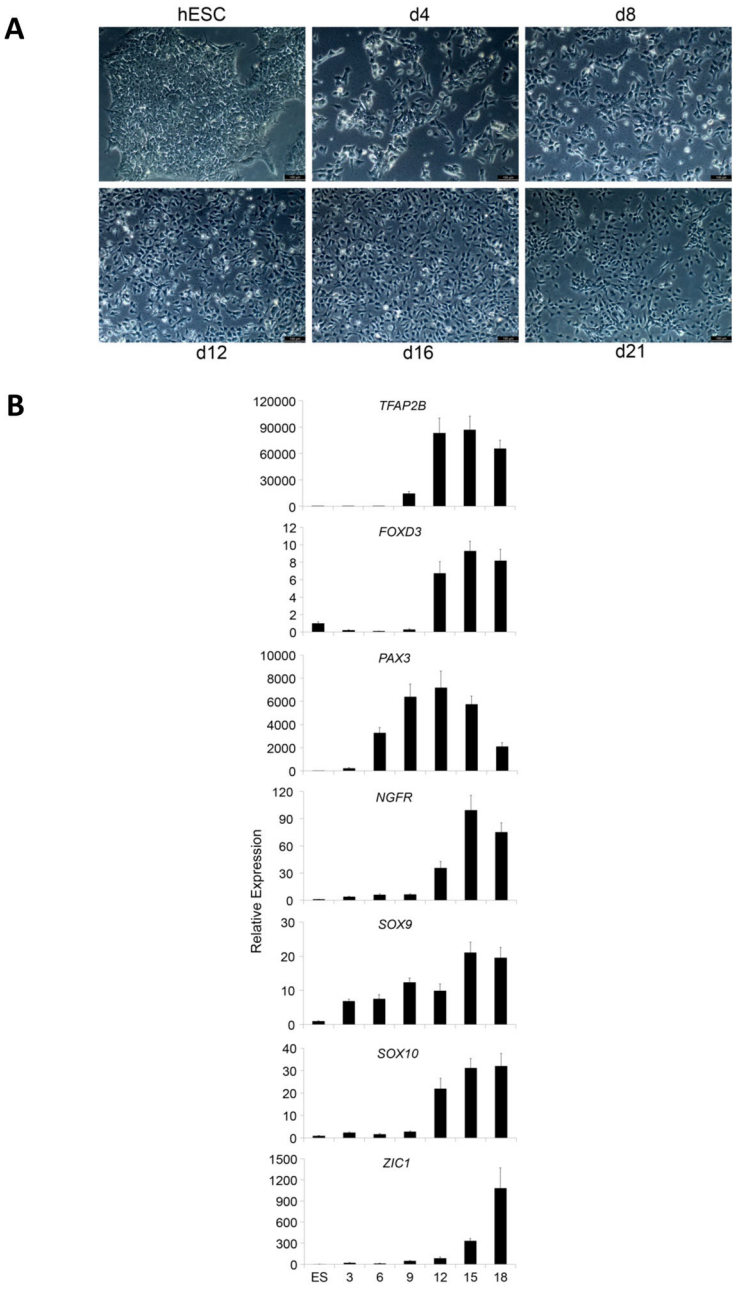

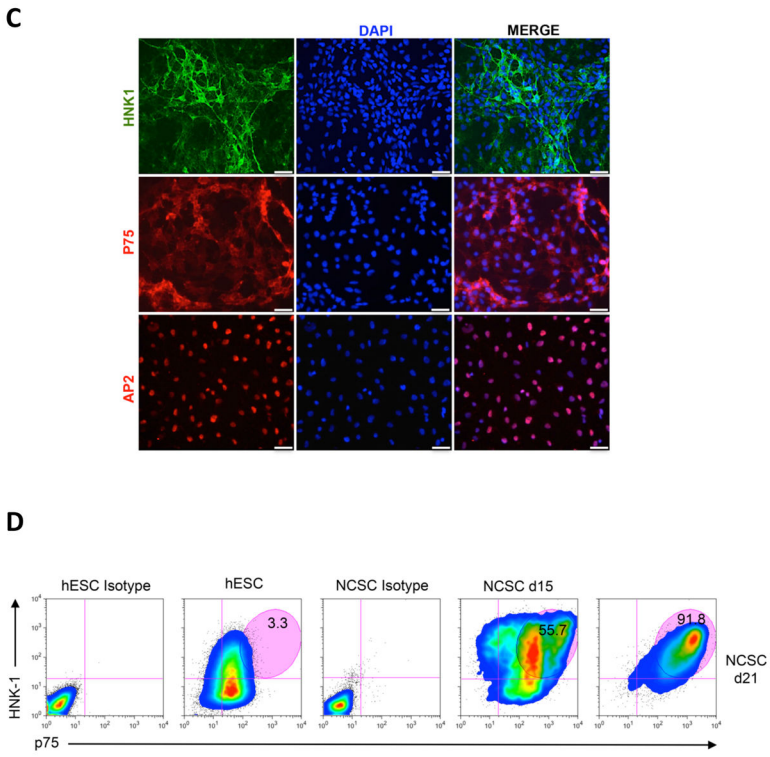

Figure 1.