Video Article

A Quick and Efficient Method for the Purification of Endoderm Cells Generated from Human Embryonic Stem Cells

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Abstract

The differentiation capabilities of pluripotent stem cells such as embryonic stem cells (ESCs) allow a potential therapeutic application for cell replacement therapies. Terminally differentiated cell types could be used for the treatment of various degenerative diseases. *In vitro* differentiation of these cells towards tissues of the lung, liver and pancreas requires as a first step the generation of definitive endodermal cells. This step is rate-limiting for further differentiation towards terminally matured cell types such as insulin-producing beta cells, hepatocytes or other endoderm-derived cell types. Cells that are committed towards the endoderm lineage highly express a multitude of transcription factors such as FOXA2, SOX17, HNF1B, members of the GATA family, and the surface receptor CXCR4. However, differentiation protocols are rarely 100% efficient. Here, we describe a method for the purification of a CXCR4+ cell population after differentiation into the DE by using magnetic microbeads. This purification additionally removes cells of unwanted lineages. The gentle purification method is quick and reliable and might be used to improve downstream applications and differentiations.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53655/>

Introduction

Pluripotent stem cells such as embryonic stem cells (ESCs) have the capability to differentiate into virtually any cell type of the human body. Thus, *in vitro* differentiation protocols can be used to generate numerous adult cell types such as cardiomyocytes¹, hepatocytes², beta cells³, lung epithelial⁴ or neuronal cells⁵. This makes ESCs a valuable tool for the potential treatment of various degenerative diseases³.

The *in vitro* differentiation of ESCs towards adult tissues of the lung, liver and pancreas requires a pseudo-gastrulation into cells reminiscent of the definitive endoderm (DE)⁶. Since downstream differentiation towards the aforementioned somatic cell types is significantly less efficient, an optimal endoderm differentiation is regarded as rate-limiting⁷. Cells that are committed towards the endoderm lineage undergo characteristic changes in their gene expression profile. Pluripotency master regulator genes are down regulated, whereas the expression of other transcription factors such as FOXA2, SOX17, HNF1B, members of the GATA family and the surface receptor CXCR4 is highly upregulated^{6, 8, 9}. CXCR4 is known to be transactivated by SMAD2/3, downstream of Nodal/TGF- β signaling and SOX17 due to specific binding sites in its promoter region¹⁰. Thus it is a very suitable marker used in a number of reports^{6, 8, 11-13}. These expression changes reflect a pseudo-gastrulation event, in which ESCs first acquire characteristics of a primitive streak-like cell population and subsequently commit into the endoderm germ layer⁶.

However, differentiation protocols are rarely 100% efficient as a few cells may resist the differentiation process or differentiate towards other unintended lineages¹⁴. These cells may negatively influence further differentiation. Furthermore, residual undifferentiated cells harbor great risks for later transplantation experiments and may give rise to teratomas¹⁵⁻¹⁷.

To remove these unwanted cells early-on the surface marker CXCR4 can be used for the purification of cells that are committed towards the DE¹⁸. Here, we describe a method for the positive selection of CXCR4+ cells from DE differentiation cultures. For this, the surface marker CXCR4 is bound by an antibody which then in turn binds to magnetic microbeads. Unlike the harsh conditions during FACS sorting, the magnetically labeled DE-like cells can then easily be purified in a benchtop format using a gentle purification method. This protocol provides a straightforward method for the removal of cell populations that resisted the DE differentiation process.

Protocol

1. Differentiation of Human ESC towards the Definitive Endoderm

1. Cultivate human embryonic stem cells (ESCs) in an incubator at 37 °C and 5% CO₂.

2. Coat a new 6-well cell culture plate with 1 ml of a basement membrane matrix and incubate the culture-ware for at least 30 min at RT. For specific details please turn to the respective manufacturer's instructions.
3. Confirm that the cultured human ESCs have reached 80%-90% confluency under the microscope using a low magnification (e.g., 4X). Aspirate the medium from the cavities by sucking off the medium with a sterile glass Pasteur pipet. Wash the cells once with phosphate buffered saline (PBS) solution. For this, add 2 ml PBS to each well softly shake the plate and suck off the solution to remove dead cells and cell debris.
4. Add 1 ml of enzyme-free passaging solution reagent for gentle dissociation of cell clusters. Incubate the cells at 37 °C and 5% CO₂ until the cells show clear signs of disruption into small clusters.
NOTE: The incubation time depends on the reagent used. For the enzyme-free passaging solution mentioned in the materials section, incubation time is roughly 7 min.
5. Add 1 ml DMEM/F-12 medium and disrupt the remaining cell aggregates into single cells by pipetting up and down using a 1 ml pipette tip. Use this to flush the cells from the surface and transfer the cells to a centrifugation tube. To retrieve all cells, wash each well with 1 ml of DMEM/F-12 medium and add the medium to the centrifugation tube.
6. Centrifuge the cells for 5 min at 300 x g. Aspirate the supernatant and resuspend the cells in 5 ml ES cell culture medium containing 10 µM Rho-Kinase (ROCK) inhibitor.
7. Count the cells under the microscope using a hemocytometer and seed 150,000 - 400,000 cells per 6-well or in another plate layout, depending on the ES cell line used. Use culture medium containing 10 µM ROCK inhibitor to avoid apoptosis and culture the cells in an incubator at 37 °C and 5% CO₂.
8. Approximately 24 hr after seeding aspirate the medium with a sterile glass Pasteur pipet and add 2 ml of primitive streak induction medium.
NOTE: This medium contains final concentrations of 1% glutamine, 0.2% FCS, 5 µM CHIR-99021 and 50 ng/ml activin A in Advanced RPMI-1640 medium. Commonly, use 2 ml of medium for cultivation in 6-well plates.
9. 48 hr after seeding, replace the medium to endoderm induction medium. Cultivate the cells in this medium for another 48 hr with daily medium change.
NOTE: This medium contains 1% glutamine, 0.2% FCS and 50 ng/ml activin A in Advanced RPMI-1640 medium. Cells that are committed towards the definitive endoderm express the surface marker CXCR4. The staining of CXCR4 can be used to quantify the number of DE-committed cells.

2. Staining of CXCR4+ Definitive Endoderm Cells for Flow Cytometry Analysis

1. For the final 24 hr but at least 1 hr before harvesting the cells add 10 µM ROCK inhibitor to the culture medium.
2. Coat the cell culture-ware that will be used for re-seeding with a basement membrane matrix, i.e., 12-well plates for qPCR analysis or chamber slides for immunofluorescence staining. Incubate the plates or slides for at least 30 min at RT.
3. Aspirate the medium with a sterile glass Pasteur pipet from the wells of the differentiated cells used for staining and/or sorting.
4. Add 1 ml of enzyme-free passaging solution reagent for the gentle dissociation of cell clusters. Incubate the cells at 37 °C and 5% CO₂ until the cells show clear signs of disruption into small clusters.
NOTE: The incubation time depends on the reagent used. For the enzyme-free passaging solution mentioned in the materials section, incubation time is roughly 7 min.
5. Add 1 ml DMEM/F-12 medium and disrupt remaining cell aggregates into single cells by pipetting up and down using a 1 ml tip. Use this to flush the cells from the surface and transfer the cells to a centrifugation tube. To retrieve all cells, wash each well with 1 ml of DMEM/F-12 medium w/o FCS and add the medium to the centrifugation tube.
6. Count the cells under the microscope using a hemocytometer. Cells from three wells of a 6-well plate should result in 10-15 x 10⁶ cells after three days of differentiation. Centrifuge the cells for 5 min at 300 x g.
NOTE: The exact number of obtained cells will depend on the pluripotent cell line used for the differentiation.
7. Aspirate the supernatant and resuspend the cells in PEB buffer containing 10 µM ROCK inhibitor. Use 100 µl of this buffer for up to 10⁷ cells. Add the 10 µM ROCK inhibitor on the day of staining. Use this buffer for all downstream applications (referred to as PEB buffer + RI).
NOTE: PEB buffer contains 0.5% BSA and 2 mM EDTA in PBS. If more cells are to be stained, adjust the buffer volume accordingly.
8. Add 10 µl of a CXCR4-APC antibody per 10⁷ cells in 100 µl, this roughly represents a dilution of 1:10.
NOTE: The usage of an APC-linked antibody is not mandatory. Instead it can be substituted depending on the microbeads used. If more cells are to be stained adjust the antibody volume accordingly.
9. Mix by gently flipping the tube with the fingers and incubate at 4 °C in a refrigerator for 15 min. Resuspend the cells with 1-2 ml PEB buffer. Centrifuge the cells for 5 min at 300 x g.
10. Aspirate the supernatant with a sterile glass Pasteur pipet and resuspend the cell pellet in 80 µl PEB per 10⁷ cells and add 20 µl anti-APC microbeads.
NOTE: If more cells are to be stained, adjust the micro-bead volume accordingly.
11. Mix by gently flipping the tube with the fingers and incubate at 4 °C in a refrigerator for 15 min. Resuspend the cells with 1-2 ml PEB buffer + RI. Centrifuge the cells for 5 min at 300 x g. Aspirate the buffer. Resuspend in 500 µl PEB buffer + RI.

3. Magnetic Separation of CXCR4+ Cells

1. Place a medium sized magnetic column in a magnetic field as per manufacture instructions. Pre-rinse the column with 500 µl PEB buffer + RI. Apply the entire cell suspension to the column. Collect the flow through as not all cells will bind to the column. Make sure not to disturb the columns for optimal retrieval of CXCR4+ cells.
2. Wash the column three times with 500 µl PEB buffer + RI. Collect the first flow through and combine it with the collected cells from Step 3.1. Remove the magnetic column from the magnetic field and place it in a suitable collection tube. Add 1 ml PEB buffer + RI onto the column. To elute the cells firmly press down the plunger into the column.
3. Optional: Collect all flow through samples separately and use 20 µl each to analyze the number of CXCR4+ cells using flow cytometry. Their number should decline with every washing step.
NOTE: At least 2 x 10⁴ viable, gated cells should be counted for reliable results.

4. Repeat Steps 3.1-3.3 with the collected-flow through sample from Step 3.1 and the first flow through sample from step 3.2 using a new column. Do not re-use the previous column. By using the plunger air is pressed into the column, which blocks it.
5. Count the cells under the microscope using a hemocytometer. Depending on the efficiency of the differentiation up to 6×10^6 cells can be sorted initially and another 1×10^6 cells by using a second column when using 10^7 cells for the procedure.
6. Centrifuge the cells at $300 \times g$ for 5 min. Aspirate the supernatant and with a sterile glass Pasteur pipet and resuspend the cells in 1 ml endoderm induction medium from Step 1.9 with additional 10 μ M ROCK inhibitor.
7. Count the cells under the microscope using a hemocytometer. Seed the cells at an appropriate density, *i.e.*, $\sim 4 \times 10^5$ cells per well of a 12-well plate (app. 3.6 cm^2 surface) or $\sim 1.5 \times 10^5$ cells per well of an 8-well chamber-slide.

4. Optional: Analysis of Purified Definitive Endoderm Population

1. For immunofluorescence staining fix the purified cells from Step 3.7 roughly 24 hr after seeding with 4% paraformaldehyde and stain for definitive endoderm (DE) and/or pluripotency marker proteins.
NOTE: Commonly used DE markers include FOXA2 and SOX17, commonly used pluripotency markers include OCT3/4, NANOG and SOX2^{6,8}.
2. For RT-qPCR analysis, harvest the purified cells directly or 24 hr after seeding, extract total-RNA and reverse transcribe cDNA from the extracted total-RNA samples. Use 10 ng cDNA as template per RT-qPCR reaction (triplicates) to analyze the expression of DE and pluripotency marker genes^{6,8}.
NOTE: Commonly used marker genes are mentioned in Step 4.1. Cycle conditions are 5 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C, followed by melting curve analysis.

Representative Results

Upon differentiation ESCs undergo drastic changes in gene and protein expression. **Figure 1** depicts typical marker genes that can be used to verify a successful endoderm differentiation. Prime targets for a gene expression analysis are *GSC*, *FOXA2*, and *SOX17*. In a relative gene expression analysis especially *FOXA2* and *SOX17* are increased by $> 2,000$ fold when compared to undifferentiated ESCs. *GSC* is already expressed very early within 24 hr during primitive streak formation but it is nonetheless induced > 100 fold on day four. The expression of these genes is consequently increased upon sorting using the CXCR4 surface marker⁶. Pluripotency master regulators such as *OCT3/4* (*POU5F1*) and *NANOG* are significantly down regulated although the expression of these genes is still detectable after four days. This indicates that some cells do not adequately respond to the treatment regimen with CHIR-99021 and activin A. *SOX7* gene expression is typically addressed to discriminate extra-embryonic endoderm formation against DE. In the first case *SOX17* is co-expressed with *SOX7* and in the latter case *SOX17* is co-expressed with *FOXA2*. The results in **Figure 1** indicate that along with DE some extra-embryonic cells may have formed during differentiation, although we have not yet been able to stain *SOX7*⁺ cells.

Figure 2 illustrates the different cell populations that are present after the differentiation of ESCs into the DE. In Step 1 ESCs are seeded as single cells and then differentiated by treatment with CHIR-99021 and activin A for four days. The IF staining of the DE markers *SOX17* and *FOXA2* (**Figure 2A**) shows that both markers are uniformly co-expressed within the nuclei. This is regarded as a hallmark of DE commitment. However, some cells resist the differentiation process and express neither of the two DE marker proteins (**Figure 2A**). In fact, two distinct cell populations are observed after the four day differentiation. While many cells express the DE marker *FOXA2* there are still cells remaining that express the pluripotency marker *SOX2* (**Figure 2B**). These two proteins are expressed in two separate populations and no co-expression can be observed (**Figure 2B**).

On day three or four of differentiation the surface protein CXCR4 is used to sort the DE-committed CXCR4⁺ population and thereby remove the remaining pluripotent cells and the other unwanted lineages. Depending on the differentiation efficiency of the used cell line $> 80\%$ CXCR4⁺ cells can be obtained using the differentiation protocol outlined in Step 1⁸. The HES3 line used here yielded $66\% \pm 3\%$ CXCR4⁺ cells before sorting and $92\% \pm 4\%$ after sorting ($n=3$). **Figure 3** depicts flow cytometry data after staining and MACS purification of CXCR4⁺ cells after differentiation towards DE and shows the expression of common DE and pluripotency marker proteins using immunofluorescence (IF) staining before and after MACS purification. On day three of differentiation roughly 60% CXCR4⁺ cells using the immunofluorescence staining for CXCR4 described in Step 2 were obtained (**Figure 3A**, middle panel). CXCR4⁺ cells move from Q4 to Q1 upon staining with an APC conjugated anti-CXCR4 antibody. After the MACS purification described in Step 3 the CXCR4⁺ population is enriched to 85%. The purification process, however, does not eliminate all unwanted lineages from the cultures (**Figure 3A**, right panel).

After the MACS purification, the CXCR4⁺ cells can be seeded for further analysis or optionally a second round of sorting may be performed to yield higher purities but reduced viability. Prior to differentiation the wide-spread expression of the pluripotency marker *SOX2* (stained in green) is detected by IF staining. In contrast, the DE marker *FOXA2* (stained in red) cannot be detected (**Figure 3B**). In **Figure 3C** CXCR4⁺ cells are stained with antibodies for *FOXA2* (green) and are co-stained for the pluripotency marker *SOX2* (red). After seeding of the purified CXCR4⁺ cells only few *SOX2*⁺ cells are detected. The majority of the seeded cells are positive for the DE marker *FOXA2*.

Figure 1. Representative changes in gene expression of different pluripotency and endoderm marker genes during a four day endoderm differentiation. (A) Illustration of the protocols being used for endoderm differentiation followed by gene expression analysis. Random denotes to culture conditions without any growth factors, RP denotes to the differentiation using a reference protocol⁷, A a treatment with activin A alone, and CA-A (CHIR-99021 and activin A) the protocol, which is used for MACS sorting (see details in⁸). The media used in the CA-A protocol are here referred as primitive streak induction medium and endoderm induction medium. (B) Depicted is the gene expression measured by RT-qPCR of GSC, SOX17 and FOXA2, which are expressed upon definitive endoderm commitment without further MACS sorting. POU5F1 (OCT3/4) and NANOG are pluripotency regulators and typically down regulated upon differentiation, whereas SOX7 is expressed in extra-embryonic endoderm. The gene expression was normalized against three stably expressed housekeeping genes (*TBP*, *TUBA1A*, *G6PD*) resulting in CNRQ values. The expression of the above mentioned genes in undifferentiated cells was set to 1 and changes are plotted as fold change of gene expression. Values are means $\pm$ SEM, n = 4-5. ANOVA plus *Bonferroni's* post hoc test. **P $\leq$ 0.01 compared with Random and #P $\leq$ 0.05, ##P $\leq$ 0.01 compared with RP (all data within this figure have recently been published in⁸). [Please click here to view a larger version of this figure.](#)

Figure 2. Different cell populations after endoderm differentiation. (A) Staining of the DE markers SOX17 and FOXA2 with respective antibodies on d4 of differentiation reveals their homogenous co-localization. However, some cells resisted the differentiation process and do not express these markers (blue nucleus staining only). The merge of green and red staining is shown in yellow. (B) After DE differentiation there are two distinct cell populations. DE-like cells express FOXA2 whereas cells that resisted the differentiation process still express the pluripotency marker SOX2. (A-B) Nuclei were counterstained with DAPI (blue). Scale bar in panel (A) is 50 μ m and in panel (B) is 100 μ m. Imaging was carried out at 670 nm (red, Cy5), 520 nm (green, FITC) and 433 nm (blue, DAPI), respectively. [Please click here to view a larger version of this figure.](#)

Figure 3. MACS sorting of differentiated DE cell populations and representative staining after sorting. (A) CXCR4⁺ stained cells (Q1) are enriched after MACS sorting. The number of CXCR4⁻ cells (Q4) is decreased. Depending on the cell line being used the differentiation protocol yields >80% CXCR4⁺ cells⁸ and MACS may be used to further enrich them. (B) Undifferentiated cells express the pluripotency marker SOX2 (green) but not the DE marker FOXA2 (red). (C) After the MACS sorting only very few undifferentiated SOX2⁺ cells remain (red), whereas the sorted population almost uniformly expresses the DE marker FOXA2 (green). (B-C) Nuclei were counterstained with DAPI (blue). Scale bar in panel (C) is 100 µm and applies to all panels. Imaging was carried out at 670 nm (red, Cy5), 520 nm (green, FITC) and 433 nm (blue, DAPI), respectively. [Please click here to view a larger version of this figure.](#)

Discussion

Currently used differentiation protocols rarely result in 100% differentiated cells. For reasons that still have to be addressed some cells resist the differentiation process. Depending on the efficiency of the used differentiation protocol and the propensity of the ESC line a certain number of residual pluripotent cells are commonly observed even after differentiation into the definitive endoderm. These residual cells may impair downstream differentiations or further analysis such as transcriptomics, proteomics, and miRNA expression analysis. Residual pluripotent cells or other unwanted lineages may also exhibit paracrine effects that may interfere with the differentiation goals. Consequently, the removal of these cells may result in improved reproducibility.

The purification of CXCR4⁺ expressing endoderm cells after differentiation can be used to enrich these populations and to remove cells that resisted the differentiation process. The surface DE marker protein CXCR4 can be used for the specific purification of endoderm cells. The MACS purification protocol at hand can be completed within less than 2 hr and can be executed in a simple bench top format in every cell culture lab without expensive laboratory equipment and devices. FACS purification is a commonly used technique but employs harsh conditions. During FACS purifications cells are usually kept in suspension for a prolonged period of time and the cells are subject to other challenging factors, e.g., high pressure in the nozzle of the FACS device. In comparison the MACS procedure is fast and gentle. This improves the cells' ability to reattach during reseeding after the purification process. To further ease this reattachment a ROCK inhibitor is added to the culture media after and prior to the purification to prevent apoptosis. Another important factor that influences reattachment is the density at which cells are reseeded. This strongly depends on the used cell line. The cell numbers used for seeding in this study are representative cell numbers that work well in our hands. However, there may be need to adjust these numbers for different cell lines. Both measures, the addition of a ROCK inhibitor and the evaluation of the cell numbers for reseeding, are critical to the success of the further cell culture after the sorting.

With the used DE differentiation protocol typically >70% CXCR4⁺ cells can be obtained without sorting⁸. The performance of the protocol used for DE generation consequently influences the efficiency of the subsequent MACS purification protocol. In general, efficient differentiation (> 80% CXCR4⁺ cells) yields a higher purity after MACS sorting (up to 95%). Thus, the usage of this purification method reduces the number of undifferentiated cells substantially but 100% purity cannot be achieved. In the future, lineage specific surface markers may be defined that will permit the purification of more terminally differentiated cells. The MACS sorting procedure is the prime selection for this purpose.

Disclosures

The authors declare that they have no competing financial interests.

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