Sequential magnetic enrichment of TRA-1-60$^+$ PSC and CD271 (p75)$^+$ NCSC enhances peripheral neuron differentiation

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Introduction

Human pluripotent stem cells (PSC) constitute an invaluable source for the generation of defined cell types, which may be used for basic research, drug compound screening, and ultimately for regenerative medicine. The specification of neural cell lineages has become relatively convenient through the development of 2D differentiation protocols, replacing the traditional embryoid body/neurosphere induction protocols. The new protocols are based on the inhibition of SMAD signaling[1,2] and give rise to Pax6$^+$Sox1$^+$ neural stem cells (NSC) as well as CD271 (p75/LNGFR)$^+$ AP2$^+$ neural crest stem cells (NCSC) within 5–10 days after induction. As an intermediate step, PSC that are cultivated on mouse embryonic fibroblast (mEF) feeder cells are usually transfered to Matrigel and expanded in conditioned medium (CM) before neural differentiation is initiated. Quality and density of the cells in the Matrigel culture are critical parameters that bias the differentiation fate towards NSC or NCSC.

In order to optimize neural differentiation towards either cell type, we have developed a magnetic cell separation protocol to standardize quality and number of true PSC before inducing neural differentiation. Our newly developed method is based on magnetic enrichment of TRA-1-60$^+$ PSC that are immediately transferred to neural induction conditions. At the same time, defined cell densities can be adjusted in order to control the differentiation fate towards NSC or NCSC. NCSC that arise 10 days after neural induction can in turn be isolated based on CD271 (p75) expression by magnetic cell sorting. Whereas unsorted cells gave rise to only few peripheral neurons, magnetically enriched cells produced nearly homogenous peripheral neuron cultures after another 3 weeks of differentiation.

Results

1 Homogenous populations of pluripotent TRA-1-60$^+$ stem cells can be generated by magnetic cell sorting

Human PSC are characterized by their expression of the stem cell surface antigen TRA-1-60. TRA-1-60 is dynamically regulated in PSC cultures and loss of TRA-1-60 is an early sign of differentiation. Using MACS Technology, TRA-1-60$^+$ cells can be isolated from heterogenous cell populations (fig. 1). The resulting positive fraction is enriched in TRA-1-60^{bright} cells, whereas some dimly expressing cells as well as feeder cells are removed. Consequently, magnetic enrichment of stem cell populations based on TRA-1-60 expression results in reduced culture heterogeneity and provides a homogenous starting population for downstream applications. As the procedure requires the generation of single-cell suspensions, it is mandatory to add a small molecule to the medium, which supports the isolated PSC. The best results are obtained when sorted cells are seeded in media containing either 2 µM thiazovivin (Rho kinase inhibitor), or alternatively 2.5 µM (-)-blebbistatin (non-muscle myosin II inhibitor). Cells attach efficiently within 24 hours, after which the medium is routinely replaced with standard culture medium.

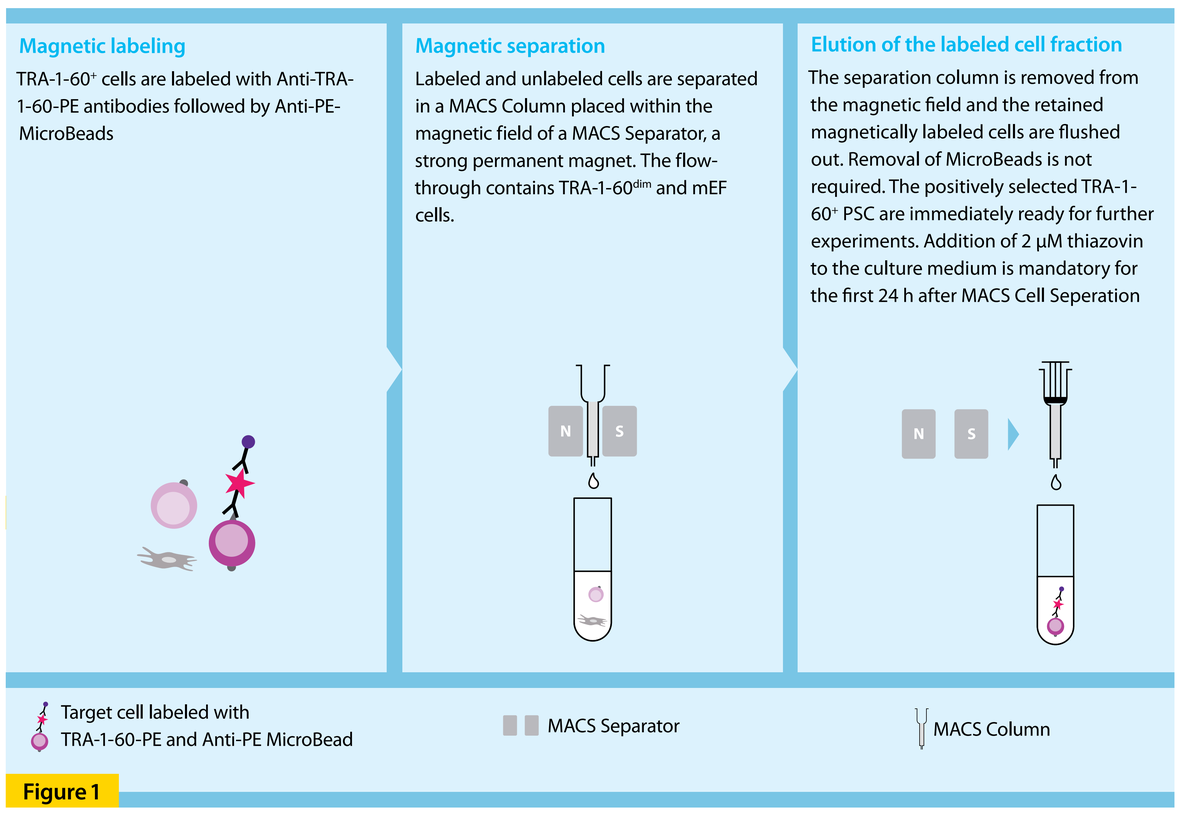

Figure 1

2 Magnetic enrichment of TRA-1-60$^+$ cells shortens 2D neural induction protocols by 3 days

Chambers *et al.*[1] and Zhou *et al.*[2] have introduced highly efficient 2D neural induction protocols that rely on the abrogation of SMAD signaling either by combining Noggin and the TGF-β inhibitor SB431542 or the TGF-β superfamily receptor inhibitor dorsomorphin. Both protocols comprise an intermediate cultivation step, during which the stem cell culture is transferred to feeder-free conditions using Matrigel-coated plates and conditioned medium (fig. 2). Differentiation is initiated after a certain degree of confluency has been reached, typically three days after seeding. The initial seeding density was found to be critical for influencing the cell fate during the subsequent neural induction phase[1]. Therefore, we sought to circumvent this cultivation step in order to standardize the culture conditions. Our shortened protocol led to the following results (fig. 2):

- Magnetic enrichment reproducibly generated a homogenous and undifferentiated TRA-1-60^{bright} cell population.
- In parallel, feeder cells were discriminated efficiently, which eliminated the need for intermediate re-expansion of PSC in conditioned medium, which in turn prevented spontaneous differentiation.
- Seeding density of PSC could be controlled immediately before shifting to neural induction conditions.
- Use of thiazovivin or (-)-blebbistatin allowed efficient plating of isolated PSC as single-cell suspensions.
- TRA-1-60$^+$ cells seeded directly into dorsomorphin-containing induction medium gave rise to NSC as well as NCSC with the same efficiency as the traditional protocols.

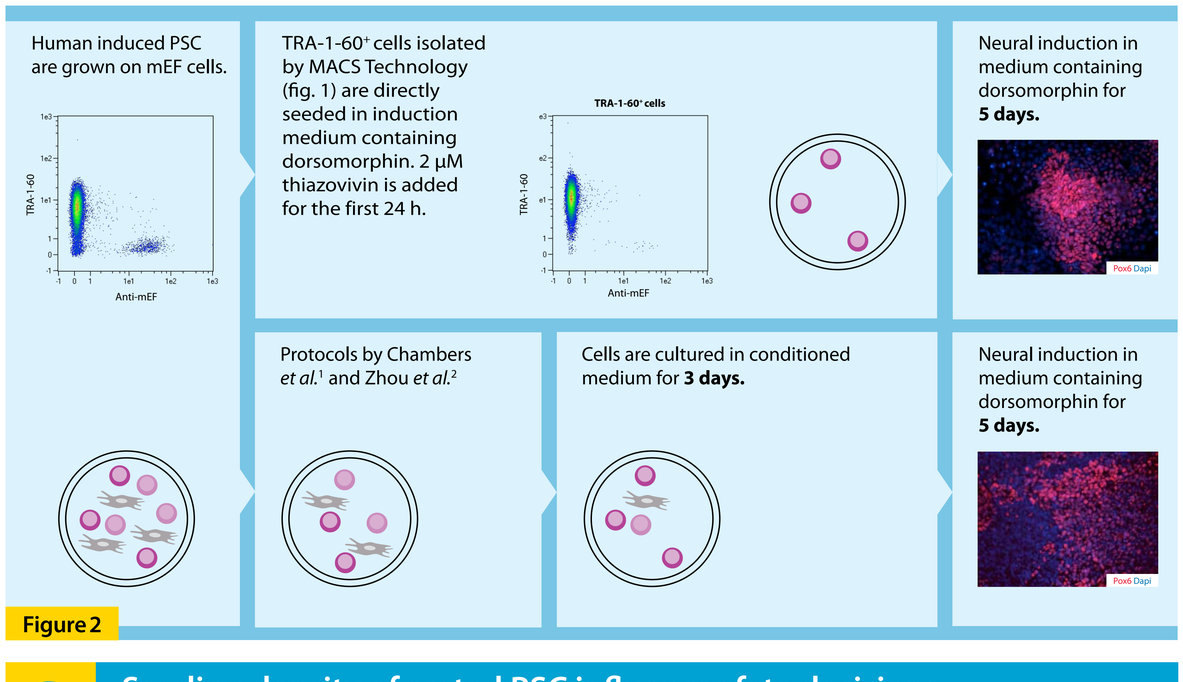

Figure 2

3 Seeding density of sorted PSC influences fate decisions during neural induction

One of the major drawbacks of the existing protocols is the rather ill-defined intermediate expansion step of PSC in conditioned medium (fig. 2), where initial feeding densities have to be adjusted three days before induction of differentiation. We were able to omit this intermediate cultivation period by using isolated TRA-1-60$^+$ iPS cells for differentiation. Consequently, the cell density could be adjusted immediately at the time point of neural induction. Cell densities between 25,000 and 100,000 cells/cm^2 were compared. The seeding density positively correlated with a fate decision towards the neural over the NCSC lineage at day 10 of differentiation (fig. 3): At high densities differentiating iPS cells mainly gave rise to Pax6$^+$Sox1$^+$ NSC, whereas lower seeding densities, promoted differentiation towards CD271$^+$ NCSC.

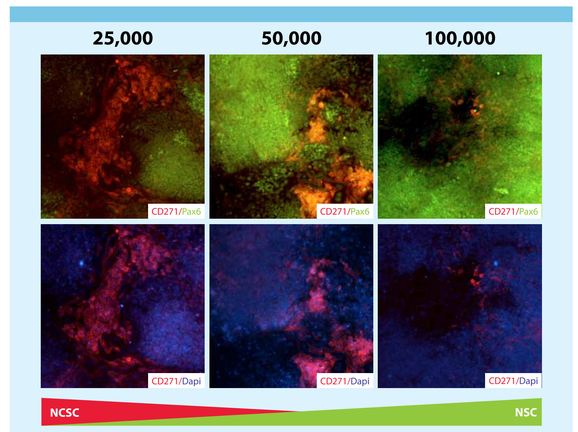

Figure 3

4 Magnetic enrichment of neural crest stem cells allows highly efficient differentiation of peripheral neurons

In a next step, magnetic enrichment of NCSC was optimized in order to facilitate peripheral neuron differentiation. Using the most permissive conditions for NCSC differentiation, i.e., initial seeding density of 25,000 cells/cm^2 (fig. 3), 17.74% (± 3.53%, n=11) NCSC were obtained 10 days after neural induction (fig. 4, stage 1). This cell population could then be induced to differentiate into peripheral neurons (fig. 4, stage 2). Unsorted stage-1 neural progenitor populations gave rise to only few peripheral neurons. In contrast, NCSC that were magnetically enriched based on CD271 (p75) expression gave rise to virtually homogenous peripheral neuron cultures 3 weeks later (fig. 4).

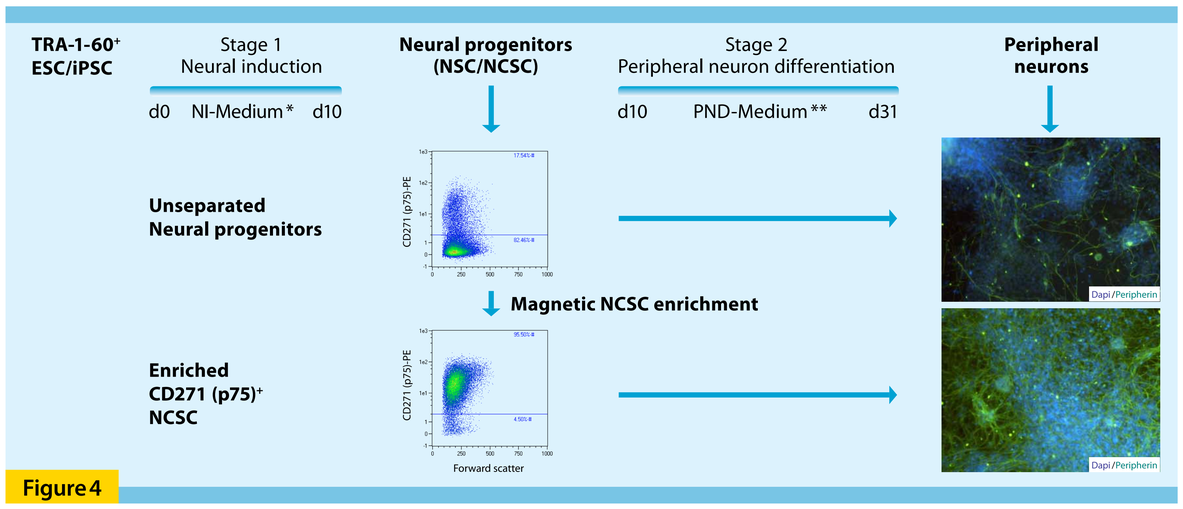

Figure 4

* NI-Medium: 80% DMEM/F12, 20% KOSR, 1× NEA , 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol, 1 µM dorsomorphin (Compound C), ± 2 µM thiazovivin ** PND-Medium: N2-Medium/MACS Neuro Medium (1:1), 1% B27-Plus, 100 ng/mL dcAMP, 10 ng/mL BDNF, 20 ng/mL NGF, 100 µM ascorbic acid

Conclusion

- 2D differentiation of PSC into neural and NCSC lineages can be optimized by using magnetically enriched TRA-1-60$^+$ cells.
- The protocol is shortened by three days by omitting pre-expansion of PSC in ill-defined conditioned media.
- Magnetic separation reproducibly generates homogenous undifferentiated starting populations.
- Fate decisions during neural induction may be influenced by adjusting the seeding density of sorted PSC, allowing predictable differentiation results.
- Magnetic enrichment of PSC-derived CD271 (p75)$^+$ NCSC enables homogenous differentiation into peripheral neurons.

References

1. Chambers, S.M. *et al.* (2009) [published erratum in Nat. Biotechnol. 2009; 27: 485] Nat. Biotechnol. 27: 275–280.
2. Zhou, J. *et al.* (2010) Stem Cells 28: 1741–1750.