MACS&more

Excerpt from MACS&more Vol 14 – 2/2012

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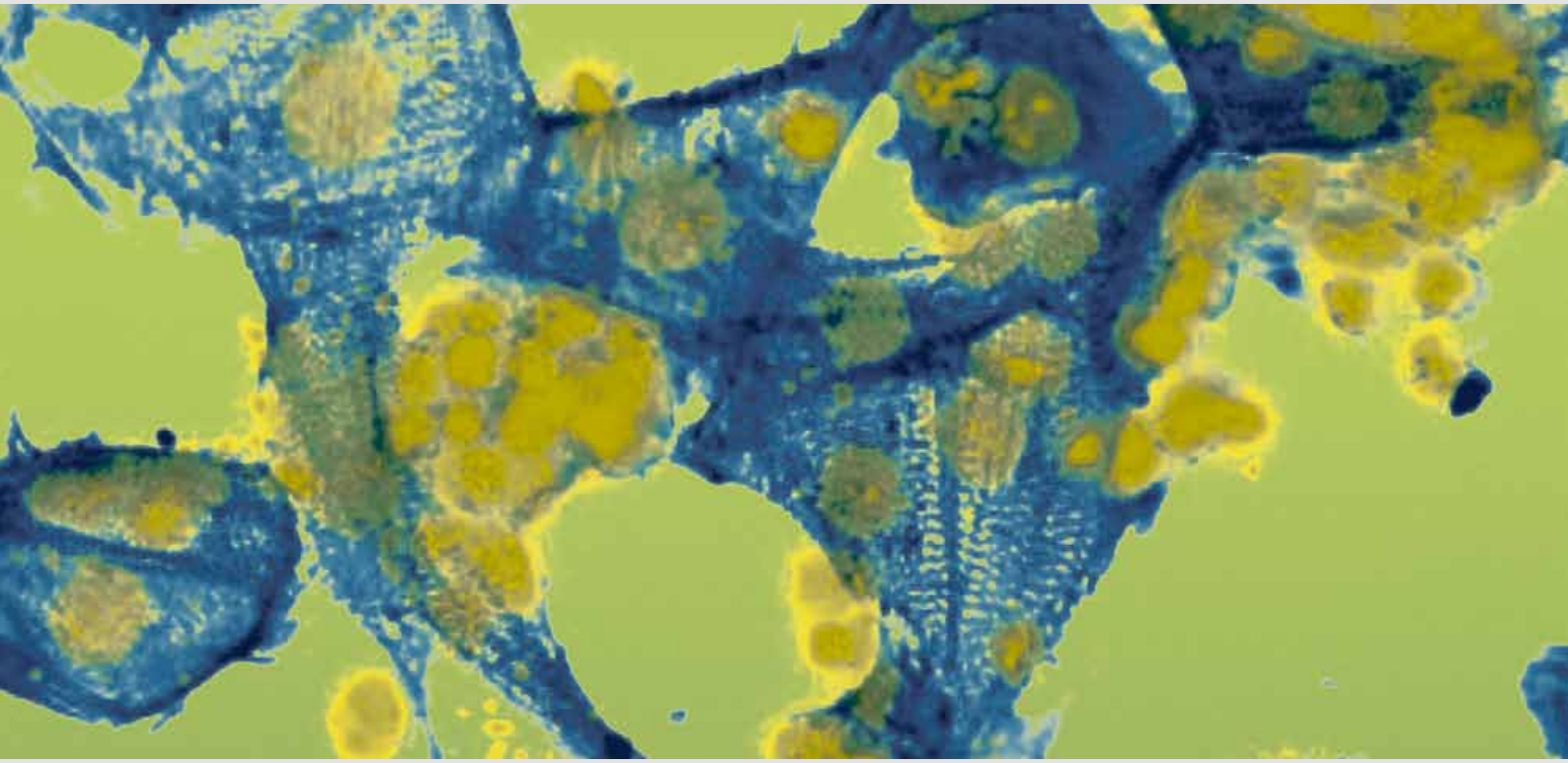

Sequential magnetic enrichment of TRA-1-60$^+$ PSCs and CD271 (p75)$^+$ NCSCs enhances peripheral neuron differentiation




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Introduction

Human pluripotent stem cells (PSCs) 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, PSCs that are cultivated on mouse embryonic fibroblast (mEF) feeder cells are usually transferred 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 NSCs 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 PSCs before inducing neural differentiation. Our newly developed method is based on magnetic enrichment of TRA-1-60$^+$ PSCs 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 NSCs or NCSC. NCSCs that arise ten days after neural induction can in turn be magnetically isolated based on CD271 (p75) expression. Whereas unsorted cells gave rise to only few peripheral neurons, magnetically enriched cells produced highly enriched peripheral neuron cultures after another three weeks of differentiation.

Materials and methods

Enrichment of TRA-1-60$^+$ PSCs

TRA-1-60$^+$ PSCs were isolated from standard mEF cocultures[3] by using the Anti-TRA-1-60 MicroBead Kit. To check the separation efficiency, unseparated cells and enriched TRA-1-60$^+$ cells were additionally labeled with Anti-Feeder antibodies and analyzed by flow cytometry using the MACSQuant® Analyzer.

Neural induction of enriched TRA-1-60$^+$ PSCs

The enriched TRA-1-60$^+$ cells were subjected to neural induction immediately after the enrichment procedure. Cells were plated on Matrigel at 25,000, 50,000, or 100,000 cells/cm^2 in NI medium (80% DMEM/F12, 20% KOSR, 1×NEA, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 µM dorsomorphin (compound C)). Thiazovivin (2 µM) was added for the first 24 hours to support attachment of the cells to the plate. Ten days after induction, cells were analyzed by fluorescence microscopy using antibodies against Pax6 and CD271 (p75), detecting NSCs and NCSCs, respectively. Cell nuclei were visualized by DAPI staining.

For comparison we processed cells according to the methods by Chambers *et al.*[1] and Zhou *et al.*[2].

Enrichment of CD271 (p75)$^+$ NCSCs

CD271 (p75)$^+$ NCSCs were obtained through neural induction of enriched TRA-1-60$^+$ PSCs for ten days at an initial seeding density of 25,000 cells/cm^2. NCSCs were then enriched using Neural Crest Stem Cell MicroBeads, human.

Differentiation of NCSCs into peripheral neurons

Enriched CD271 (p75)$^+$ NCSCs or unseparated progenitors were cultured in PND medium (N2 medium/MACS® Neuro Medium (1:1), 1% MACS Supplement B27 PLUS, 100 ng/mL dcAMP, 10 ng/mL BDNF, 20 ng/mL NGF, 100 µM ascorbic acid) for 21 days. Formation of peripheral neurons was monitored by fluorescence microscopy using an antibody against peripherin.

Results

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

Chambers *et al.*[1] and Zhou *et al.*[2] 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 by using the TGF-β superfamily receptor inhibitor dorsomorphin alone. Both protocols include an intermediate cultivation step, during which the stem cell culture is transferred to feeder-free conditions using Matrigel-coated plates and conditioned medium (fig. 1). This step eliminates the inhibitory effect of feeder cells on differentiation, but at the same time it increases the risk for spontaneous differentiation.

In order to circumvent this step we isolated TRA-1-60$^+$ PSCs by MACS Technology. TRA-1-60 is dynamically regulated in PSC cultures and loss of TRA-1-60 is an early sign of differentiation. The positive fraction contained the TRA-1-60^{bright} cell population, whereas some TRA-1-60^{dim} cells as well as feeder cells were removed. Consequently, magnetic enrichment of stem cell populations based on TRA-1-60 expression provided a homogeneous starting population (fig. 1). As magnetic sorting requires working with single-cell suspensions, it was mandatory to add a small molecule to the separation buffer and culture medium to support attachment of the PSCs to the culture vessel. The best results were obtained when the cells were seeded in media containing 2 µM thiazovivin (Rho kinase inhibitor, active component of the hES Cell Cloning & Recovery Supplement). Cells attached efficiently within 24 hours, after which the medium was routinely replaced with standard culture medium.

Seeding density of sorted PSCs influences fate decisions during neural induction

Another major drawback of the expansion in conditioned medium is that seeding densities have to be adjusted three days prior to neural induction (fig. 1). We were able to omit this intermediate cultivation period by using isolated TRA-1-60$^+$ iPS cells directly 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 NSC over the NCSC lineage at day 10 of differentiation (fig. 2). At high densities, differentiating iPS cells mainly gave rise to Pax6$^+$Sox1$^+$ NSCs, whereas lower seeding densities, promoted differentiation towards CD271 (p75)$^+$ NCSCs.

Magnetic enrichment of NCSCs allows highly efficient differentiation of peripheral neurons

In a next step, we enriched CD271 (p75)$^+$ NCSCs to facilitate peripheral neuron differentiation. Using the most permissive conditions for NCSC differentiation, i.e., an initial seeding density of 25,000 cells/cm^2 (fig. 2), 17.74% (±3.53%, n=11) NCSCs were obtained ten days after neural induction (fig. 3, stage 1). This cell population could then be induced to differentiate into peripheral neurons (fig. 3, stage 2). Unsorted stage-1 neural progenitor populations gave rise to only few peripheral neurons (fig. 3, top right). In contrast, NCSCs that were magnetically enriched based on CD271 (p75) expression gave rise to virtually homogeneous peripheral neuron cultures after three weeks (fig. 3, bottom right).

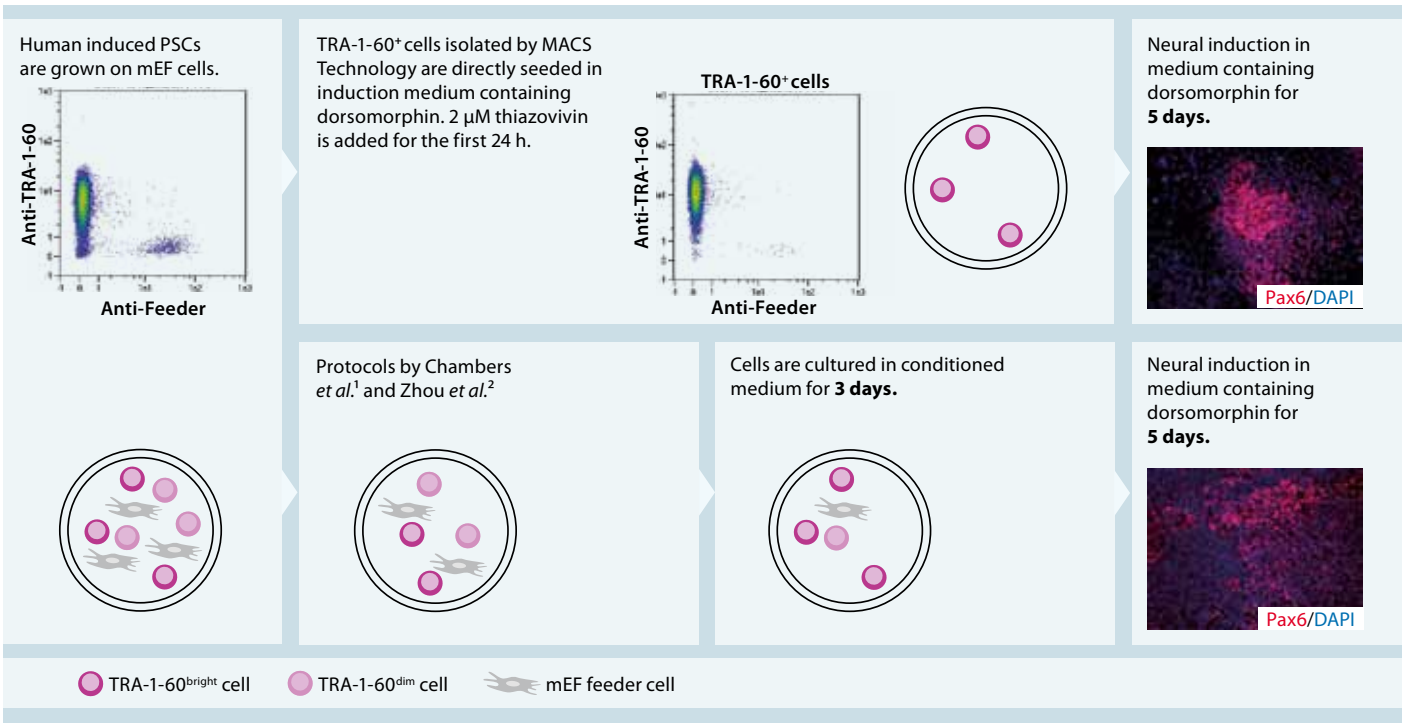

Figure 1 **Newly developed, short protocol for neural induction compared to protocols by Chambers *et al.*[1] and Zhou *et al.*[2]** To illustrate the results of magnetic cell separation, unseparated cells or isolated TRA-1-60$^+$ cells were analyzed by flow cytometry. Neural induction was monitored by fluorescence microscopy using Pax6 antibodies to visualize NSCs (red). Cell nuclei were visualized by DAPI staining (blue).

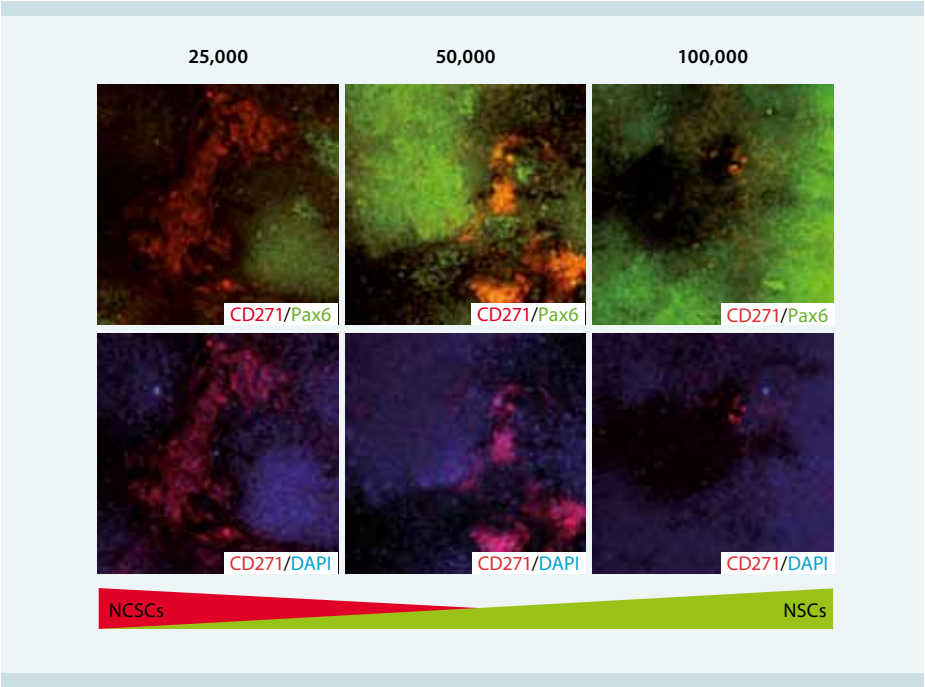

Figure 2 **Neural induction of TRA-1-60$^+$ PSCs at various cell densities.** Enriched TRA-1-60$^+$ cells were seeded in NI medium at the indicated numbers of cells per cm^2. After ten days cells were analyzed by fluorescence microscopy using antibodies against Pax6 and CD271 (p75), detecting NSCs (green) and NCSCs (red), respectively. Cell nuclei were visualized by DAPI staining (blue).

Conclusion

- Magnetic enrichment of TRA-1-60$^+$ PSCs reproducibly generated homogeneous starting populations of undifferentiated TRA-1-60^{bright} cells.
- Use of thiazovivin allowed efficient handling and plating of isolated PSCs in single-cell suspension.
- The use of magnetically enriched TRA-1-60$^+$ cells shortened the neural induction protocol by three days by omitting pre-expansion in conditioned media.
- The seeding densitiy of isolated TRA-1-60^{bright} cells could be adjusted immediately prior to neural induction, thus allowing tighter control over the decisions for NCSC or NSC fate.
- Magnetic enrichment of PSC-derived CD271 (p75)$^+$ NCSCs enables more efficient, nearly homogeneous differentiation into peripheral neurons.

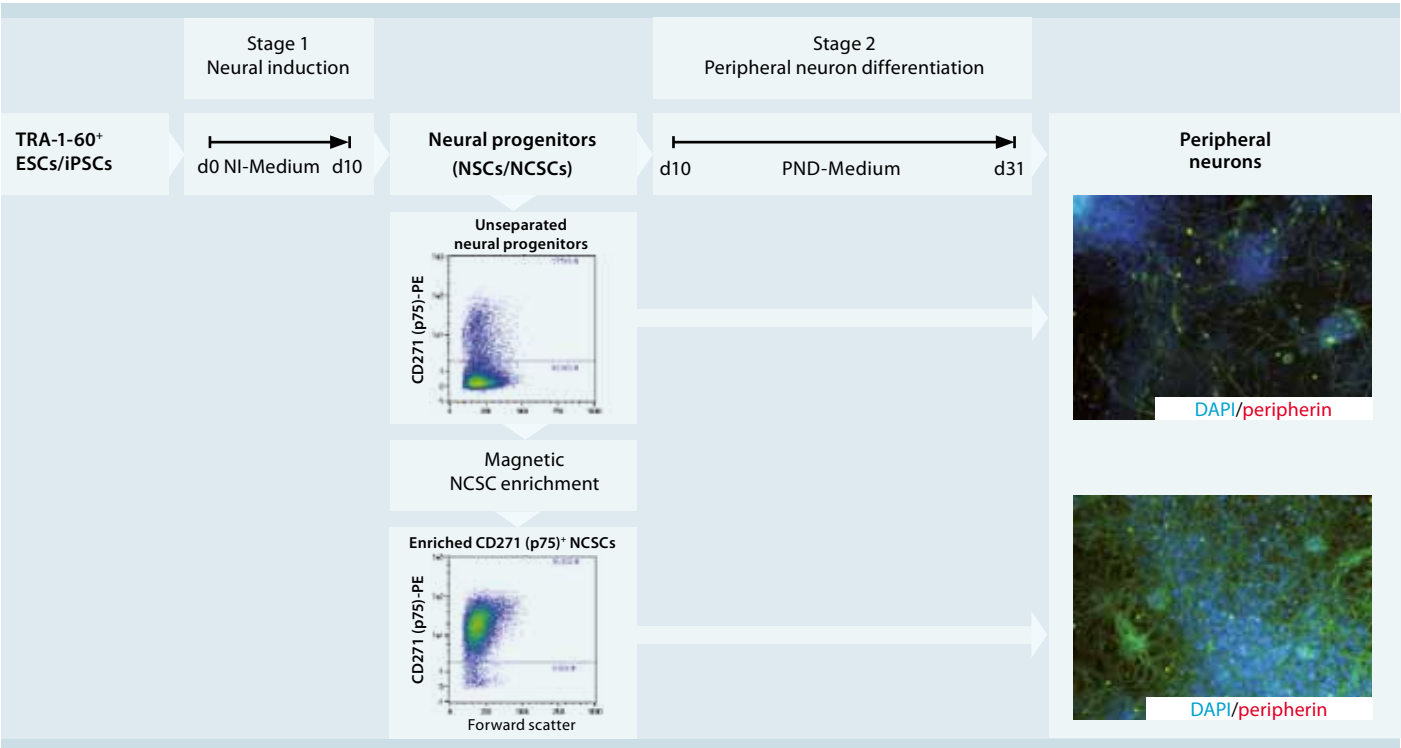

Figure 3 **Newly developed protocol for the differentiation of peripheral neurons.** Enriched TRA-1-60$^+$ cells were subjected to neural induction in NI medium for ten days (stage 1), giving rise to neural progenitors (NSCs and NCSCs). Subsequently, CD271 (p75)$^+$ NCSCs were magnetically enriched. Both unseparated neural progenitors and enriched CD271 (p75)$^+$ NCSCs were subjected to peripheral neuron differentiation in PND medium (stage 2). After 21 days cells were analyzed by fluorescence microscopy using an antibody against peripherin (green). Cell nuclei were visualized by DAPI staining (blue).

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.
3. Haase, A. *et al.* (2009) Cell Stem Cell 5: 434–441.

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