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Patterning factors during neural progenitor induction determine regional identity and differentiation potential *in vitro*

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ABSTRACT

The neural tube consists of neural progenitors (NPs) that acquire different characteristics during gestation due to patterning factors. However, the influence of such patterning factors on human pluripotent stem cells (hPSCs) during *in vitro* neural differentiation is often unclear. This study compared neural induction protocols involving *in vitro* patterning with single SMAD inhibition (SSI), retinoic acid (RA) administration and dual SMAD inhibition (DSI). While the derived NP cells expressed known NP markers, they differed in their NP expression profile and differentiation potential. Cortical neuronal cells generated from 1) SSI NPs exhibited less mature neuronal phenotypes, 2) RA NPs exhibited an increased GABAergic phenotype, and 3) DSI NPs exhibited greater expression of glutamatergic lineage markers. Further, although all NPs generated astrocytes, astrocytes derived from the RA-induced NPs had the highest GFAP expression. Differences between NP populations included differential expression of regional identity markers *HOXB4*, *LBX1*, *OTX1* and *GSX2*, which persisted into mature neural cell stages. This study suggests that patterning factors regulate how potential NPs may differentiate into specific neuronal and glial cell types *in vitro*. This challenges the utility of generic neural induction procedures, while highlighting the importance of carefully selecting specific NP protocols.

1. Introduction

Human pluripotent stem cells (hPSCs) can generate different neural cell types *in vitro*. To generate intermediate neural progenitors (NPs), multiple *in vitro* neural induction protocols have been developed (Shi et al., 2012; Falk et al., 2012; Koch et al., 2009; Nat et al., 2007), and often involve dual SMAD inhibition. Downstream activation of transforming growth factor β (TGFβ) and bone morphogenetic proteins (BMP) signaling is mediated by SMAD proteins, which transduce extracellular signals to the nucleus and activate downstream gene transcription. However, single SMAD inhibition has also been shown capable of generating NP populations (Kerr et al., 2010). Additionally, neural induction procedures lacking SMAD inhibition, such as administration of retinoic acid (RA) may also generate NP populations (Izrael et al., 2007). Despite reports on methods producing NP populations, the actual regional identity and differentiation potential of NPs generated *via* current *in vitro* protocols is often unclear. Given the labor-intensive nature of establishing neural differentiation protocols for hPSCs, a comparative study of existing protocols would be valuable.

At different time points, regions and concentration gradients, various signaling molecules, also called patterning factors, combine to determine the regional identity and differentiation potential of NP populations in the developing neural tube of the central nervous system (CNS) (Temple, 2001; Wurst and Bally-Cuif, 2001; Wessely and De Robertis, 2002; Sirbu and Duester, 2006; Briscoe and Novitch, 2008; Rowitch and Kriegstein, 2010; Groves and Labonne, 2014; Tao and Zhang, 2016). Patterning factors include wingless-related integration side proteins (WNTs) (Steventon and Mayor, 2012; Heeg-Truesdell and Labonne, 2006), BMPs (Londin et al., 2005; Marchal et al., 2009), fibroblast growth factors (FGFs) (Streit et al., 2000; Bertocchini et al., 2004; Cohen et al., 2010; Huang et al., 2010; Feng et al., 2014; Grabiec et al., 2016), sonic hedgehog (SHH) (Patten and Placzek, 2000) and RA (Sasai et al., 1996; Streit and Stern, 1999). FGF signaling is known to be an important inducer of posterior regionalization (Rentzsch et al., 2004;

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Deimling and Drysdale, 2011). Gradual expression of WNT (Nordstrom et al., 2002; Kim et al., 2000) and BMP (Koshida et al., 2002; Gonzalez et al., 2000), both members of the TGFβ superfamily, are required for anterior-posterior patterning. Furthermore, RA is an important inducer of hindbrain and spinal cord development (Glover et al., 2006; Maves and Kimmel, 2005). Along the length of the neural tube, floor plate-expressed SHH stimulates ventral structures (Ribes et al., 2010), while roof plate-expressed WNTs and BMPs are crucial for dorsal development (Ulloa and Marti, 2010; Chizhikov and Millen, 2005). In conclusion, similar to neural patterning in the CNS, it might be expected that administration of patterning factors *in vitro* may similarly affect the characteristics and potency of hPSC-derived NPs.

Here we performed a comparative study of neural induction protocols, involving single SMAD inhibition (SSI), RA administration and dual SMAD inhibition (DSI) as well as different plating conditions such as adherent and non-adherent (*i.e.* embryoid body (EB)) cultures. We demonstrate that all neural induction protocols resulted in NP populations that expressed known NP cell markers. However, the NP populations showed variations in differentiation potential towards neuronal and glial cell types. Additional analysis, including embryonic regional marker expression, showed differences in characteristics of the generated NP populations. The results of this study indicate that neural induction protocols should be chosen carefully to obtain NP populations with appropriate potency for research goals.

2. Material and methods

Extra information, including cell culture techniques, and used antibodies and primers, can be found in supplementary materials and methods.

2.1. Pluripotent stem cell culturing

Human embryonic stem cells (hESCs, H1 and H9; WiCell) and control human induced pluripotent stem cells (hVS-88, 74 day old male) (Holmes and Heine, 2017) were maintained in E8 media (Life Technologies) on GelTrex- (Life Technologies) coated plates, and passaged with 0.5 mM EDTA in PBS (Life Technologies). The medium was supplemented with 10 μM ROCK inhibitor (RI) (SelleckChem) after passaging.

2.2. Neural induction protocols

To test different NP induction procedures, we chose 3 different culture conditions based on commonly used growth factors and medium supplements, together with different culturing techniques (adherent and non-adherent) (Shi et al., 2012; Kerr et al., 2010; Izrael et al., 2007). Our study design compares the following NP cell induction protocols (Fig. 1):

Condition 1 (Single SMAD inhibition; non-adherent; C1-SSI): To create embryoid bodies (EBs), hESC colonies were fragmented using 0.5 mM EDTA in PBS and plated in the ratio of 2:1 wells on anti-adhesive (AA) poly-2-hydroxyethyl methacrylate (Sigma) coated plates. The cells were cultured in N2B27 medium supplemented with FGF2 (20 ng/ml; Peprotech), FGF4 (20 ng/ml; R&D), Noggin (200 ng/ml; Peprotech) and RI (10 μM) and 2/3 of the medium was changed every other day. RI was omitted from the medium after 3 days in all EB protocols. On day 10, the EBs were plated on GelTrex-coated 6WP. Initial plating of EBs was considered passage 0 (P0) and NPs were maintained in the same induction medium until used for neuronal/ glial differentiations. The plated EBs formed rosette-like structures. These cells were used for immunocytochemical (ICC) analysis, and passaged to P1 at day 14 using Accutase (Sigma-Aldrich). At day 18, RNA samples were collected, and the cells were frozen to use for further neuronal and glial differentiations.

Condition 2 (RA administration; non-adherent; C2-RA): EBs were created and handled as described for Condition 1, with the exception that the cells were cultured in N2B27 medium supplemented with T3 (40 ng/ml; Sigma), FGF2 (4 ng/ml) and EGF (20 ng/ml; Peprotech). On day 3, the medium was switched to N2B27 supplemented with T3 (40 ng/ml), FGF2 (4 ng/ml), EGF (20 ng/ml) and RA (10uM; Sigma). The EBs were plated on day 10, passaged to P1 at day 14, and frozen at day 18. From day 10 onwards, RA and FGF2 were omitted from the medium.

Condition 3 (Dual SMAD inhibition; adherent; C3-DSI): hESC and hiPSC colonies were fragmented with 0.5 mM EDTA in PBS and plated in the ratio of 3:2 on GelTrex-coated 12WP in 1.5 ml E8 medium supplemented with RI (10 μM; considered as day −3). From day −3 to 0, half of the media was refreshed daily. At day 0, medium was switched to N2B27 medium supplemented with Dorsomorphin (1 μM; Tocris bioscience), SB431542 (10 μM; SelleckChem) and refreshed entirely every day for 12 days. When rosette-like structures formed, NP cells were passaged using dispase (Sigma) or manually picked and transferred into a PLO/Laminin- (20 g/ml) coated 6WP. NP cells were maintained the same induction medium and passaged using TrypLE and defined trypsin inhibitor (DTI; both Life Technologies). The day rosettes were plated was treated as P0.

2.3. Mixed neuronal differentiation

NP cells (P2) from all the 3 protocols were differentiated towards neurons. An earlier described mixed cortical neuronal differentiation protocol was used (Nadadhur et al., 2017) with the modification of neuronal density at 125 K cells/ well of 12WP.

2.4. Astrocyte differentiation

NP cells from all the 3 protocols were differentiated towards astrocytes, using a glial differentiation protocol described previously with small adaptations (Izrael et al., 2007; Tcw et al., 2017). In short, P2 NP cells were thawed to begin astrocyte differentiation protocol and cultured in N2B27 medium without vitamin A (N2B27-vitA) supplemented with T3 (40 ng/ml) and EGF (20 ng/ml) on GelTrex-coated plates. The medium was refreshed completely every other day. When confluent, the cells were passaged using Accutase and plated at density of 1000 K cells/ well of 6WP. After 4 passages (~20 days), the medium was switched to N2B27-vitA supplemented with T3 (40 ng/ml), EGF (5 ng/ml), FGF2 (5 ng/ml), Noggin (50 ng/ml Peprotech), Vitamin C (50 μg/ml, Sigma) and Laminin (1 μg/ml, Sigma). After 5 days, EGF and FGF2 were omitted from the medium. After 3 passages, the medium was switched to astrocyte medium (ScienCell, Sanbio b.v.) for another 2 passages. At the end of the protocol (~55 days), the cells were collected for ICC and RNA analysis.

Human primary astrocytes isolated from cerebral cortex (ScienCell) were used as a positive control in QPCR experiments, and cultured similarly to hESC-derived astrocytes, *i.e.* on Geltrex-coated plates in astrocyte medium (ScienCell, Sanbio b.v.).

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA, Electron microscopy sciences) in PBS for 15 min at room temperature (RT). Fixed cells were washed (3–6 times with PBS over 30 min), blocked with blocking buffer (PBS, 0.1% BSA, 5% NGS, 0.3% Triton) at RT for 1 h, and then incubated with primary antibodies in the blocking buffer overnight at 4 °C. After washing (3–6 times with PBS over 30 min), secondary antibodies were added (in blocking buffer) and incubated for 1–2 h at RT. Then the cells were washed (3–6 times in PBS over 30 min) and incubated with DAPI (in PBS) for 2–3 min at RT. Finally, the cells were washed (2 times with PBS) and slides were mounted with Fluormount G solution (Southern Biotech). Fluorescent images were taken using a Carl Zeiss 510Meta confocal with 40× (1.2 Numerical Aperture) oil