Original Article

BMP Inhibition in the Presence of LIF Differentiates Murine Embryonic Stem Cells to Early Neural Stem Cells

(early neural stem cells / mouse/ neurogenesis / embryonic stem cells / guided *in vitro* differentiation)

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Abstract. Early mouse neural stem cells (NSCs) first appear in embryonic day E5.5 and express pluripotency markers *Oct4*, *Sox2*, *Nanog* and early neural marker *Sox1*. Early NSCs are a good model for understanding the role of various pathways that control initial neural commitment. However, a protocol for differentiation of mouse embryonic stem cells (ESCs) into early NSCs by adherent monolayer culture has not yet been established. Hence, in this study, we identified the combination of growth factors and small molecules that differentiated mouse ESCs into early NSCs and supported their proliferation. Leukaemia inhibitory factor (LIF) was the first factor to be tested and it was found that ESCs can differentiate into early neurogenic lineage in the presence of LIF. However, we found that the induction is weaker in the presence of LIF as compared to cells differentiated in its absence. GSK-3 inhibitor, along with BMP and TGF-β pathway inhibitor (dual SMAD inhibition), are commonly used to sequentially direct ESCs towards NSCs. However, when we used this combination, mouse ESCs failed to differentiate into early NSCs. We observed that by adding Wnt inhibitor to the combination of GSK-3 inhibitor, BMP inhibitor, TGF-β inhibitor and LIF, it was possible to differentiate ESCs into early NSCs. qRT-PCR analysis of early NSCs illustrated that they expressed key pluripotency genes *Oct4* and *Nanog*, albeit at levels lower than non-differentiated ESCs, along with early neural markers Sox1 and Pax6.

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Abbreviations: 2i+L – media containing combination of two inhibitors (GSK3 β inhibitor CHIR99021, MEK1/2 inhibitor PD0325901) and LIF, ActB – β-actin, bFGF – basic fibroblast growth factor, BMP – bone morphogenic protein, CHIR99021 – small molecule inhibitor of glycogen synthase kinase (GSK)-3, D3 – mouse embryonic stem cell line derived from blastocysts of a 129S2/SvPas mouse, EGF – epidermal growth factor, ESCs – embryonic stem cells, GSK-3 – glycogen synthase kinase (GSK)-3, LDN193189 – small molecule inhibitor of the bone morphogenetic protein (BMP) pathway, LIF – leukaemia inhibitory factor (LIF/L), NSCs – neural stem cells, PD0325901 – small molecule inhibitor of the MEK/ERK pathway, Pou5f1 – POU domain, class 5, transcription factor 1, also known as Oct4, R1 – mouse embryonic stem cell line established from a 129X1 × 129S1 3.5 day blastocyst, SB431542 – small molecule inhibitor of the TGF-β/activin/NODAL pathway, SMAD – transcriptional comodulators to regulate TGF-β-dependent gene expression, TGF-β – transforming growth factor β, XAV939 – small molecule inhibitor of tankyrase.

Introduction

The adult central nervous system (CNS) has a limited regenerative capacity after injury and in advanced stages of neurodegenerative diseases (Fujimoto et al., 2012, Nakamura and Okano, 2013). Regeneration in the adult brain is mainly carried out by endogenous neural stem cells (NSCs); however, the regeneration capacity is restricted. Transplantation of exogenous NSCs in combination with stimulation of endogenous NSCs would be an important approach to cell-based therapy, as both types of NSCs (exogenous and endogenous) hold the capacity to differentiate into functional neurons and glial cells, which can help to rebuild the damaged neural tissue after nerve injury (Björklund and Lindvall, 2000). Early NSCs express pluripotency factors along with primitive neural marker *Sox1*; as a result, these cells should theoretically have broader regeneration potential compared to definitive NSCs. Definitive NSCs do not express pluripotency genes; however, they express early neural genes *Sox1* and *Pax6* (Tsang et al., 2013).

Neural induction is one of the earliest events to occur during embryonic lineage specification (Weinstein and Hemmati-Brivanlou, 1999). *In vitro*, neurectoderm cells are commonly observed in embryoid body (EB)-mediated differentiation of ESCs (Watanabe et al., 2005). In addition, monolayer differentiation protocols exist and are

advantageous, as they greatly reduce generation of a mixed population of cells commonly observed in EB-mediated differentiation and ensure emergence of a single population of differentiated cells (Zhang et al., 2001; Ying et al., 2003). Transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP) pathways are known inhibitors of neurogenesis (Hemmati-Brivanlou and Melton, 1994; Bachiller et al., 2000). They supress neural induction by inducing mesodermal or endodermal differentiation (D'Amour et al., 2005; Zhang et al., 2008). Fibroblast growth factor (FGF) is one of the mitogens known to promote self-renewal of NSCs in CNS (Launay et al., 1996; Streit et al., 2000) and can enhance neural differentiation of ESCs (Zhang et al., 2001). In line with these views, application of FGF receptor ligands or antagonists of TGF-β/BMP signalling, in the form of either biological molecules or synthesized chemical inhibitors, are commonly used to direct ESCs to neural fate (Zhang et al., 2001; Kim et al., 2010; Morizane et al., 2011; Zhang et al., 2018). In addition, application of glycogen synthase kinase 3 (GSK-3) inhibitor, in combination with TGF-β/BMP inhibition (dual SMAD inhibition), has been shown to facilitate rapid and robust differentiation of ESCs into early neural stem cells (Bian et al., 2016; Chavali et al., 2020). The above-mentioned research played a crucial role in formulating our combination of factors.

Early NSCs are hierarchically below embryonic stem cells but still express core pluripotency factors (*Oct4*, *Sox2* and *Nanog*). They appear during murine embryonic development from E5.5 to E7.5, and during *in vitro* differentiation of ESCs (Hitoshi et al., 2004; Tsang et al., 2013). Early NSCs form neurospheres when grown in media supplemented with leukaemia inhibitory factor (LIF). On dissociation, LIF cultured cells form secondary neurospheres; however, they require the presence of both LIF and bFGF. Dissociation of secondary neurospheres produces tertiary spheres in the presence of either bFGF or EGF. The change in growth factor requirement is accompanied by differentiation from early NSCs to definitive NSCs (Hitoshi et al., 2004). Definitive NSCs have a different marker expression profile and growth factor requirement compared to early NSCs. ESCs, early NSCs and definitive NSCs can be distinguished from each other based on the expression level of *Oct4*, *Sox1*, *Pax6* and *Nestin* genes (Tsang et al., 2013). For instance, in ESCs, *Oct4* is highly expressed, while expression of *Sox1*, *Pax6* and *Nestin* is low. In early NSCs, expression of *Oct4* and *Sox1* is high, while expression of *Pax6* and *Nestin* is low. Definitive NSCs have negligible expression of *Oct4* and high expression of *Sox1*, *Pax6* and *Nestin* (Tsang et al., 2013).

Based on current understanding, it is not possible to culture early NSCs beyond a single passage. Hence, our aim was to devise the media composition that would help to maintain early NSCs in culture without triggering differentiation or apoptosis.

Material and Methods

Culturing of murine ESCs

ES D3 and R1 cells (ATCC, Teddington, UK) were cultured on a 0.1% gelatin-coated plate in N2B27 media (Life Technologies, Carlsbad, CA). N2B27 media consists of 1 : 1 DMEM/F-12 media (Life Technologies) and Neurobasal media (Life Technologies) with N2 and B27 supplement, LIF (1000 U/ml) (Sigma-Aldrich, Prague, Czech Republic), and 10 ng/ml BMP4 (Peprotech, London, UK) or 2i+L (3 μM CHIR99021 (Tocris Bioscience, Bristol, UK), 1 μM PD0325901 (Tocris Bioscience), and 1000 U/ml LIF).

Differentiation of ESCs

To study the effect of LIF on differentiation, cells were cultured in N2B27 media with or without LIF (L) in the absence of BMP4 for 6 days on a 0.1% gelatin-coated plate. Cells cultured in BMP4+L media were used as a control. To study the effect of BMP inhibition on differentiation, cells were cultured in N2B27 media with 0.2 μM LDN193189 (Tocris Bioscience), 20 μM SB431542 (Tocris Bioscience), 3 μM CHIR99021 (Tocris Bioscience), 2 μM XAV 939 (Tocris Bioscience) in the presence of LIF for 6 days on a 0.1% gelatin-coated plate. Cells cultured in 2i+L media were used as a control.

Reverse transcription

Total RNA was isolated using an RNA isolation kit (Zymo Research, Irvine, CA). Two μg of total RNA was reverse transcribed into cDNA using a High Capacity cDNA Synthesis Kit (Life Technologies).

Quantitative real-time PCR

qRT-PCR was performed using a LightCycler 480 SYBR Green I master mix (Roche Life Science, Indianapolis, IN) in the presence of 400 nM final concentration of each primer. The total reaction volume was 15 μl. Expression was normalized to β-actin. Ten ng of cDNA was used for quantification, and data are represented as standard error of mean (SEM) of technical triplicates. qRT-PCR was performed in a BioRad CFX96 Touch instrument (Hercules, CA). Primer sequences are provided in Table 1.

Results

LIF maintains expression of core pluripotency factors and does not inhibit neural differentiation

To study the effect of LIF on neurogenesis, the murine ES D3 cell line was differentiated towards neuroectoderm. Prior to differentiation, cells were cultured in the LIF+BMP4 media. Upon withdrawal of BMP4, cells gradually differentiated towards neural stem cells over six days. In the presence of LIF, differentiated cells mar-

Table 1. Primers used for qRT-PCR

qPCR primers			
Gene	**Sequence (5′-3′)**	**NCBI ID**	**Length**
Pou5f1	CTTTCCCTCTGTTCCCGTCACTG	NM_013633.3	108
	TCTCTTGTCTACCTCCCTTGCCT		
Sox2	CAAGGCAGAGAAGAGAGTGTTTG	NM_011443.4	242
	CTGGCGGAGAATAGTTGGG		
Nanog	ATGCTGCTCCGCTCCATAACTTC	NM_ 028016.3	231
	ACCTGGCTTTGCCCTGACTTTA		
Sox1	GCAGCGTTTCCGTGACTTTATTC	NM_009233.3	239
	AGCACAACCCATCCTCCTTCT		
Pax6	GGGACCACTTCAACAGGACTCAT	NM_001244198.2	218
	GTGCTTCTAACCGCCATTTCTCT		
Nestin	GTCCCTTAGTCTGGAAGTGGCTA	NM_016701.3	154
	CAGGTGCTGGTCCTCTGGTATC		
ActB	CTGTTACTGAGCTGCGTTTTAC	NM_007393.5	88
	GCCATGCCAATGTTGTCTCTTA		

ginally up-regulated expression of core pluripotency factors *Pou5f1* (*Oct4*), *Sox2* and *Nanog* as compared to the control sample, i.e., non-differentiated ESCs cultured in LIF+BMP4 media (Fig. 1). Furthermore, early neurogenic markers *Sox1*, *Nestin* and *Pax6* were also up-regulated (Fig. 1). When the gene expression was compared with cells differentiated in the absence of LIF, it was observed that the core pluripotency factors were down-regulated, while early neural genes had 2-fold higher expression compared to control cells (Fig. 1). In conclusion, LIF maintains expression of core pluripotency factors without obstructing or inhibiting neural differentiation; however, neural induction is weaker compared to cells differentiated in its absence.

Inhibition of BMP and Activin/Nodal pathway induces neuroectoderm fate in ESCs

Malaguti et. al. (2013) had described association of Bmp and E-cadherin and its effect on *Sox1* expression.

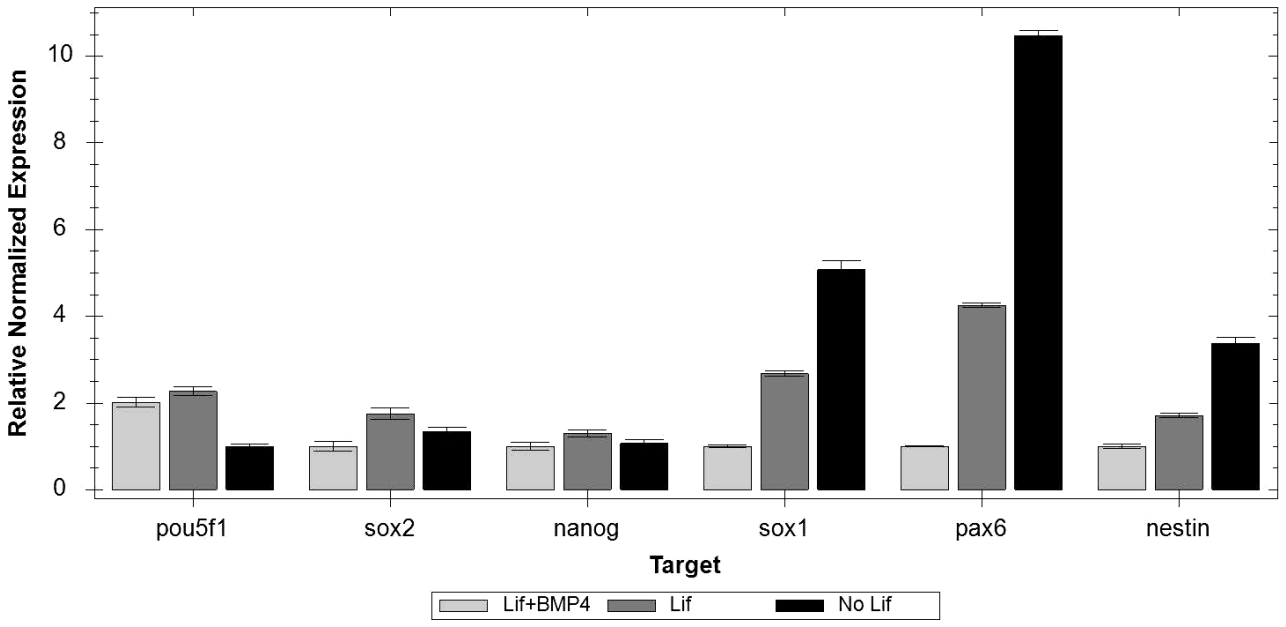

Fig. 1. Effect of LIF on neurogenesis. Murine ES D3 cells were used for analysis and cells cultured in LIF+BMP4 media were used as a control. Neurogenesis was initiated by culturing ESCs in culture media lacking BMP4. The results are presented as +/− SEM of technical replicates.

Furthermore, the authors had characterized Bmp as a negative regulator of Sox1. With this knowledge, the Bmp pathway was inhibited using small molecule LDN 193189 to check its effect on *Sox1* expression. Compared to ESC control, the differentiated cells showed an increase in *Sox1* expression (Figs. 2 and 3). In order to prevent cells from differentiating into mesendoderm, the Activin/Nodal pathway was inhibited using SB431542. Furthermore, LIF was added to the differentiation media to maintain expression of *Oct4*, while CHIR99021 and XAV939 served the purpose of preventing apoptosis and Wnt-mediated differentiation, respectively. Early NSCs formed densely packed colonies resembling ESCs (Fig. 4).

Both the differentiated ESC lines showed an approximate 0.4-fold decrease in expression of pluripotency factors *Oct4* and *Sox2* and a drastic decrease in expression of *Nanog* (Figs. 2 and 3). The ES D3 cell line showed a 4-fold increase, while R1 displayed 2-fold upregulation in *Sox1* expression compared to the control. In summary, under differentiation conditions, BMP inhibition in the presence of LIF directs ESCs towards early NSCs.

Discussion

Most protocols used for differentiation of murine ESCs to neural progenitors highlight activation of the

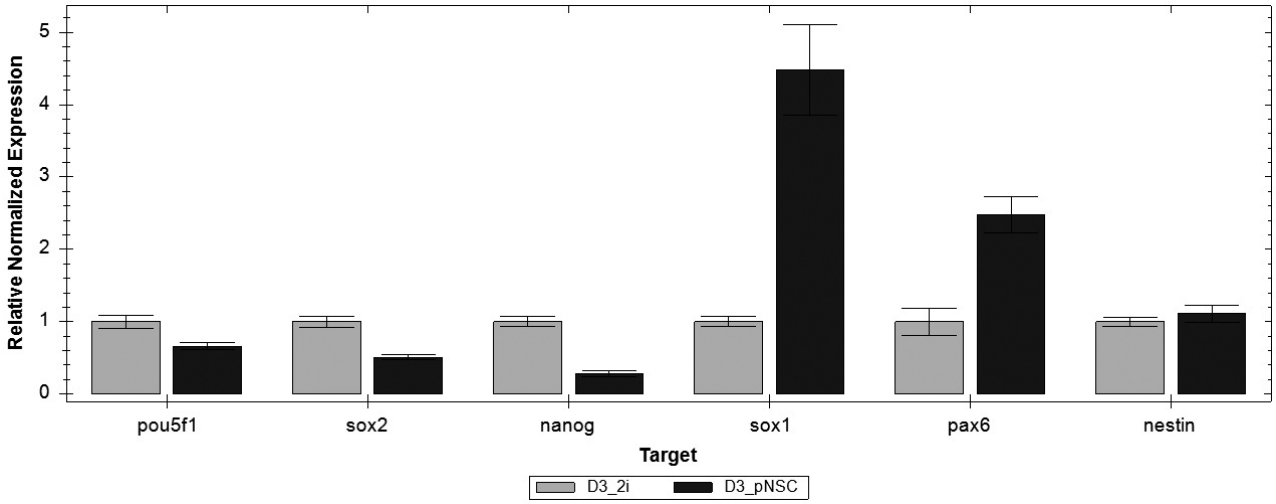

Fig 2. Gene expression analysis of differentiated ES D3 cell line. Results of differentiated ES D3 cells are presented relative to non-differentiated ES D3 cell line. *β-actin* was used as a reference gene and the results represent gene expression after 6 days of cell differentiation. Data are expressed as average of biological replicates, N = 2 (mean ± SEM).

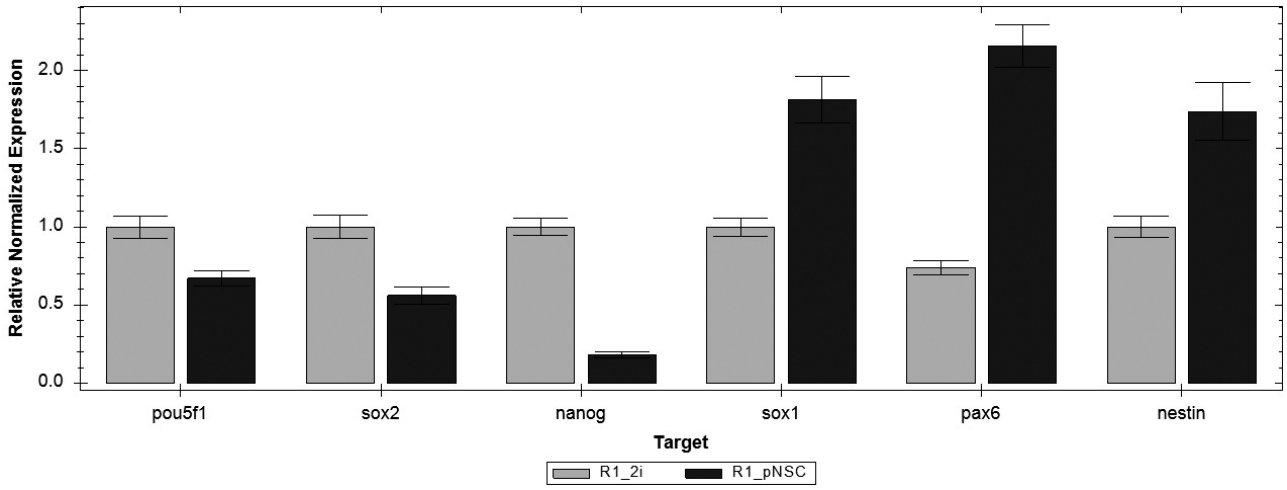

Fig 3. Gene expression analysis of differentiated ES R1 cell line. Results of differentiated R1 cells are presented relative to non-differentiated ES R1 cells. *β-actin* was used as a reference gene and the results represent gene expression after 6 days of differentiation. Data are expressed as average of biological replicates, N = 2 (mean ± SEM).

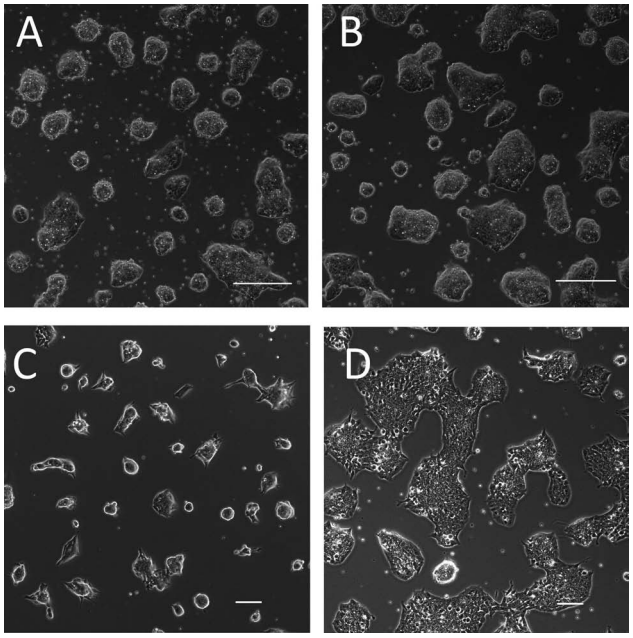

Fig. 4. Differentiation of murine ESCs into early NSCs. Phase-contrast images of mouse early NSCs derived from ES D3 (**A**) and R1 ESCs (**B**). For comparison, phase-contrast images of D3 ESCs (**C**) and R1 ESCs (**D**). Scale bar, 100 μm.

Wnt pathway (Li et al., 2011; Bian et al., 2016). When we tried to use these protocols for obtaining early NSCs, it was noticed that ESCs bypass the initial step of neurogenesis, i.e., occurrence of early NSCs, and instead they directly differentiate into definitive NSCs. This observation was based on gene expression analysis. Cells differentiated using previous published protocols (Li et al., 2011; Bian et al., 2016) failed to express *Sox1*, which is the most important gene expressed by early NSCs. Moreover, they directly up-regulated *Pax6* and *Nestin* genes, which are identification markers of definitive NSCs (Tsang et al., 2013).

In this study, we developed an adherent monolayer system and defined conditions for differentiation of ESCs into early NSCs. Morphologically, early NSCs appear like ESCs; however, a difference exists in the expression of genes *Oct4*, *Sox2*, *Sox1*, *Pax6*, and *Nestin*. Early NSCs express *Oct4*, *Sox2* and *Nanog*, albeit at low levels compared to ESCs, with simultaneous up-regulation of *Sox1* and *Pax6* genes. In the absence of LIF and serum, ESCs differentiate into the neural lineage (Ying et al., 2003), and the presence of LIF does not inhibit neurogenesis but delays the process. Moreover, LIF alone can maintain expression of *Oct4*, *Sox2* and *Nanog* under differentiation conditions; however, it is unable to prevent differentiation (Ying et al., 2003).

Guided stem cell differentiation using small molecules is a promising approach to generating clinically relevant cell types for regenerative medicine and drug discovery. Zhang et al. (2016) showed that fibroblasts can be transdifferentiated into NSCs using a combination of nine small molecules, CHIR99021, LDN193189, A83-01, retinoic acid, Hh-Ag1.5, RG, Parnate, SMER28, and bFGF. Furthermore, human adipose-derived stem cells can be transdifferentiated into NSCs by inhibition of SMAD signalling (Park et al., 2017).

As it is known, early NSCs are difficult to propagate because of the unclear mechanism governing early neurogenesis. Based on our results, it is seen that LIF is essential for maintaining pluripotency, and it can drive neural differentiation on withdrawal of either BMP or serum. However, apoptosis and cell cycle arrest are seen in differentiating cells. Bian et al. (2016) and Pachenari et al. (2017) have shown that GSK-3 inhibitor prevents apoptosis and promotes proliferation. Moreover, several protocols have shown a positive effect of Wnt activation on neural induction (Avery and Dalton, 2016; Leung et al., 2016; Gomez et al., 2019). However, activation of the canonical Wnt pathway directs ESCs towards the mesoderm lineage (Lindsley et al., 2006; Funa et al., 2015) and inhibition of GSK-3 activates the canonical Wnt pathway. Hence, in order to prevent apoptosis and mesodermal differentiation of ESCs, we used a combination of GSK-3 inhibitor and tankyrase inhibitor XAV939. This dual inhibitor combination allows GSK-3 inhibition without activation of the canonical Wnt pathway. The combination of molecules used by us is unique and has not been previously used. Moreover, we are the first group to publish a protocol for differentiating ESCs to early NSCs in adherent monolayer culture.

Our results have cleared a few missing links governing the early neural fate acquisition. However, further work needs to be done to check the differentiation capacity of early NSCs into various neurogenic lineages, as well as their regenerative capacity in injury models.

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