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Cell surface heparan sulfate proteoglycans as novel markers of human neural stem cell fate determination

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ABSTRACT

Multipotent neural stem cells (NSCs) provide a model to investigate neurogenesis and develop mechanisms of cell transplantation. In order to define improved markers of stemness and lineage specificity, we examined self-renewal and multi-lineage markers during long-term expansion and under neuronal and astrocyte differentiating conditions in human ESC-derived NSCs (hNSC H9 cells). In addition, with proteoglycans ubiquitous to the neural niche, we also examined heparan sulfate proteoglycans (HSPGs) and their regulatory enzymes. Our results demonstrate that hNSC H9 cells maintain self-renewal and multipotent capacity during extended culture and express HS biosynthesis enzymes and several HSPG core protein syndecans (SDCs) and glypicans (GPCs) at a high level. In addition, hNSC H9 cells exhibit high neuronal and a restricted glial differentiative potential with lineage differentiation significantly increasing expression of many HS biosynthesis enzymes. Furthermore, neuronal differentiation of the cells upregulated SDC4, GPC1, GPC2, GPC3 and GPC6 expression with increased GPC4 expression observed under astrocyte culture conditions. Finally, downregulation of selected HSPG core proteins altered hNSC H9 cell lineage potential. These findings demonstrate an involvement for HSPGs in mediating hNSC maintenance and lineage commitment and their potential use as novel markers of hNSC and neural cell lineage specification.

1. Introduction

Neural stem cells (NSCs) are retained in the adult brain in discrete locations and maintain the ability to self-renew and differentiate into neural cell lineages — neurons, astrocytes and oligodendrocytes (Gage, 2000). Isolated NSCs not only can be propagated in vitro in the presence of fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) as free-floating neurospheres (Reynolds and Weiss, 1992) but also can be derived from embryonic stem cells (ESCs), which can be expanded as an adherent monolayer circumventing the challenges associated with long-term neurosphere culture (Conti et al., 2005, Zhang et al., 2001). NSCs provide a model of nervous system development and they have great therapeutic potential for the treatment of CNS injuries and disease. A better understanding of factors regulating their behaviour is required to fully exploit the capacity of these cells.

NSCs with self-renewal and multipotentiality express the intermediate filament nestin, transcription factors SOX1 and SOX2 and the RNA-binding protein Musashi 1 (MSI1), all shown to play a role in NSC self-renewal and thus in the maintenance of the NSC pool (Christie et al., 2013, Okano et al., 2005). In addition, the expression of telomerase (TERT) is considered a marker of true stem cell self-renewal (Thomson et al., 1998). Neuronal differentiation is indicated by increased expression of neuron-specific markers including βIII-tubulin (TUBB3), microtubule-associated protein 2 (MAP2), neurofilaments (NEFs) and doublecortin (DCX) (Brown et al., 2003, Laser-Azogui et al., 2015, Song et al., 2002). Markers denoting the astrocyte lineage include glial fibrillary acidic protein (GFAP), surface marker CD44 and S100B calcium binding protein (Donato, 2001, Reeves et al., 1989, Sosunov et al., 2014) and finally, oligodendrocyte lineage markers include galactosylceramidase (GalC), transcription factors Olig1 and Olig2 and surface markers O1 and O4 (Barateiro and Fernandes, 2014, Tracy et al., 2011).

However, there is an overlap in expression of these markers between lineages. For example nestin, MSI1 and MAP2, expressed by immature NSCs and neuronal cells are also expressed by reactive astrocytes (Duggal et al., 1997, Geisert et al., 1990, Oki et al., 2010) and Olig1 and Olig2 expressed by motor neurons with Olig2 also shown to be required for NSC proliferation and maintenance (Ligon et al., 2007, Zhou and Anderson, 2002). Thus, the identification of new lineage specification markers and/or defining novel combinations of markers would enable the more efficient utilisation of lineage-specific neural cells.

The NSC microenvironment, or niche, plays a central role in regulating NSC stemness (self-renewal and differentiation) with local

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concentrations of signalling molecules mediating NSC maintenance and lineage differentiation (Ramasamy et al., 2013). The distribution and activity of extracellular signalling molecules are mediated by extracellular matrix (ECM) components, including proteoglycans (PGs). PGs consist of a core protein and attached sulfated glycosaminoglycan (GAG) chains that determine their classification and influence local concentrations of growth factors and ligands (Couchman and Pataki, 2012, Dreyfuss et al., 2009). The heparan sulfate proteoglycans (HSPGs) consist of two major families: the type I transmembrane syndecans (SDC1-4), and the globular GPI-anchored glypicans (GPC1-6) (Choi et al., 2011, Filmus et al., 2008).

HS chains are synthesised post-translationally via a complex temporal process mediated by a number of biosynthesis enzymes to assemble chains to the core proteins. HS chains are first polymerised by exostosin glycosyltransferases 1 and 2 (EXT1 and EXT2) (Busse et al., 2007), followed by modifications catalysed by *N*-deacetylase/*N*-sulfotransferases (NDSTs; NDST1-4) and epimerisation catalysed by C5-epimerase (C5-EP) (Grobe et al., 2002). Finally, the HS chains are sulfated by HS 2-*O*-sulfotransferase (HS2ST1) and 6-*O*-sulfotransferases (HS6ST1, HS6ST2 and HS6ST3), respectively (Esko and Selleck, 2002). HS chain length along with the *N*- and *O*-sulfation pattern subsequently determines the binding abilities of HSPGs (Esko and Selleck, 2002).

SDCs and GPCs have been reported to regulate cell adhesion, migration and differentiation and demonstrate specific expression and localisation during CNS development (Choi et al., 2011, Ford-Perriss et al., 2003). The depletion of SDC1, GPC1 and GPC4 in vitro in mouse NSC or neural precursor cells alters cell maintenance and proliferation (Abaskharoun et al., 2010, Fico et al., 2012, Wang et al., 2012) and the depletion of EXT1, NDST1, HS2ST1 or HS6ST1 in the mouse CNS results in brain malformations and abnormalities (Grobe et al., 2005, Inatani et al., 2003, Pratt et al., 2006). Currently the role of HSPGs in human NSC (hNSC) lineage specification is limited and reliant upon rodent models: despite the acknowledged differences in development, structure and regulation between human and rodent nervous systems (reviewed in Oikari et al., 2014). To elucidate key HSPGs in hNSC regulation we expanded hESC-derived NSCs (hNSC H9 cells) and examined the expression of NSC self-renewal and neural cell lineage markers along with HS biosynthesis enzymes and SDC and GPC core proteins in basal and lineage specific differentiation (neuronal and glial) cultures. Our results identify HSPGs as potential regulators of hNSC lineage potential and support their use as additional markers of neural cell specification.

2. Materials and methods

2.1. hNSC H9 cell expansion

Two populations of human neural stem cells derived from the NIH approved H9 (WA09) human embryonic stem cells (hNSC H9 cells) were purchased from Life Technologies and expanded until passage 31 (P31) corresponding to approximately 100 days in culture. Basal culture conditions included expanding cells as a monolayer on Geltrex® coated culture vessels in neural stem cell serum-free medium (NSC SFM) containing Knockout™ DMEM/F-12, 2% StemPro® Neural Supplement, 20 ng/mL of FGF-2 and EGF, and 2 mM GlutaMAX™-I all obtained from GIBCO®, Life Technologies. Cells were maintained at 37 °C in 5% CO_2 in a humidified atmosphere and passaged every 3–5 days using TrypLE (Life Technologies) and re-plated at a density of 5×10^4 cells/cm^2. The viability of the cells was monitored using Trypan Blue and an automated cell counter (Bio-Rad) and via hemacytometer.

2.2. hNSC H9 neuron and astrocyte differentiation cultures

hNSC H9 cells were cultured under lineage-specific differentiation conditions according to protocols provided by Life Technologies. For neuronal lineage differentiation, hNSC H9 cells were plated on poly-L-ornithine-laminin coated culture vessels at a seeding density of 2.5×10^4 cells/cm^2; for astrocyte lineage differentiation, hNSC H9 cells were plated on Geltrex® coated culture vessels at a seeding density of 2×10^4 cells/cm^2. Cells were allowed to attach in NSC SFM for two days. Neuronal differentiation was induced by maintaining cells in Neurobasal® Medium (Life Technologies) supplemented with 2% B-27® Serum-Free Supplement and 2 mM GlutaMAX™-I (Life Technologies) with the astrocyte differentiation conditions consisting of DMEM supplemented with 1% N-2 supplement (Life Technologies), 2 mM GlutaMAX™-I and 1% FBS. Cells were maintained in differentiating conditions for 15 to 18 days with the medium changed every 3–4 days.

2.3. RNA-interference

hNSC H9 P5 cells were plated in NSC SFM at a density of 5×10^4 cells/cm^2 and allowed to attach for 48 h prior to treatment with Accell™ Smartpool Human siRNAs (Dharmacon), a pool of four siRNA transcripts specific to GPC1 (E-004303-00-0010) or GPC4 (E-011271-00-0010). For siRNA delivery, the growth medium was changed to Accell™ siRNA Delivery Media containing 1 μM of siRNA supplemented with 2% StemPro® Neural Supplement and 20 ng/mL of FGF-2 and EGF. Untreated and non-targeting (scramble) siRNA (D-001,910-10-20) treated cells were used as a control. Cells were incubated with siRNAs for 72 h after which cell number and viability were assessed and cells harvested for RNA extraction.

2.4. Total RNA extraction, cDNA synthesis and Q-PCR

RNA was harvested using TRIzol® reagent (Invitrogen) with the Direct-zol™ RNA miniprep kit (Zymo Research) according to the manufacturer's instructions. During extraction samples were treated in-column with DNase I (Zymo Research) for 15 min to eliminate DNA contamination. cDNA synthesis was performed using Roche Transcriptor Reverse Transcriptase. Briefly, 150 ng of RNA was incubated with 3 μg of Random Primer (Invitrogen) at 65 °C for 10 min in a reaction volume of 19.5 μl. Samples were then incubated with 10 U of RT enzyme in 1× RT reaction buffer, with 1 mM dNTPs (New England Biolabs), 20 U of RNaseOUT (Invitrogen) in a total reaction volume of 30 μl for 10 min at 25 °C, followed by 30 min at 55 °C and a final step of 5 min at 85 °C.

Q-PCR reactions were performed in quadruplicate per sample for each gene studied in a 384-well microtiter plate. Each reaction contained 120 ng of cDNA template, 5 μl of SYBR®-Green PCR Master Mix (Promega), 200 ng of forward and reverse primer and 0.1 μl of CXR reference dye (Promega). Amplification was monitored using a Life Technologies QuantStudio™-7 with an enzyme activation of 2 min at 50 °C and 3 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Cycle threshold (Ct) values were normalised against the endogenous control gene 18S Ct values (ΔCt) included in each run. Relative gene expression was determined by calculating the $^{\Delta\Delta}$Ct value ($2^{(-\Delta Ct)}$) and relative expression values presented on bar graphs are $^{\Delta\Delta}Ct \times 10^6$. All primer sequences for the genes studied can be found in Tables 1 and 2 in Oikari et al. (2015).

2.5. Immunofluorescence

For immunofluorescent (IF) detection of target proteins, cells in basal growth conditions were plated on CC2-coated chamber slides (Lab-Tek) at 5×10^4 cells/chamber and allowed to attach for 2 days. Differentiating cells were plated in 48-well culture dishes (Corning) and stained between D14 and D18. Prior to staining, cells were washed with 1× PBS with Ca^{2+} and Mg^{2+} and fixed with 4% paraformaldehyde then blocked with 1% BSA and 5% donkey serum in 1× PBS with Ca^{2+} and Mg^{2+}. For intracellular proteins 0.1% of Triton-X was included in the blocking solution to allow permeabilisation. After blocking, primary