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

Lotta E. Oikari, Rachel K. Okolicsanyi, Aro Qin, Chieh Yu, Lyn R. Griffiths, Larisa M. Haupt

Genomics Research Centre

Institute of Health and Biomedical Innovation

Queensland University of Technology

Musk Avenue, Kelvin Grove, Brisbane, Queensland 4059, Australia

*Corresponding author:

Dr Larisa M. Haupt, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave, Kelvin Grove QLD 4059 Australia

E-mail: larisa.haupt@qut.edu.au

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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.

Keywords: Neural Stem Cell, Differentiation, Heparan Sulfate Proteoglycan, Syndecan, Glypican

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 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 can also 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 are also shown to be required for NSC proliferation and maintenance (Ligon et al., 2007,

94 Zhou and Anderson, 2002). Thus, the identification of new lineage specification
95 markers and/or defining novel combinations of markers would enable the more
96 efficient utilisation of lineage-specific neural cells.
97
98 The NSC microenvironment, or niche, plays a central role in regulating NSC
99 stemness (self-renewal and differentiation) with local concentrations of signalling
100 molecules mediating NSC maintenance and lineage differentiation (Ramasamy et
101 al., 2013). The distribution and activity of extracellular signalling molecules is
102 mediated by extracellular matrix (ECM) components, including proteoglycans (PGs).
103 PGs consist of a core protein and attached sulfated glycosaminoglycan (GAG)
104 chains that determine their classification and influence local concentrations of growth
105 factors and ligands (Couchman and Pataki, 2012, Dreyfuss et al., 2009). The
106 heparan sulfate proteoglycans (HSPGs) consist of two major families: the type I
107 transmembrane syndecans (SDC1-4), and the globular GPI-anchored glypicans
108 (GPC1-6) (Choi et al., 2011, Filmus et al., 2008).
109
110 HS chains are synthesised post-translationally via a complex temporal process
111 mediated by a number of biosynthesis enzymes to assemble chains to the core
112 proteins. HS chains are first polymerised by exostosin glycosyltransferases 1 and 2
113 (EXT1 and EXT2) (Busse et al., 2007), followed by modifications catalysed by *N*-
114 deacetylase/*N*-sulfotransferases (NDSTs; NDST1-4) and epimerisation catalysed by
115 C5-epimerase (C5-EP) (Grobe et al., 2002). Finally, the HS chains are sulfated by
116 HS 2-*O*-sulfotransferase (HS2ST1) and 6-*O*-sulfotransferases (HS6ST1, HS6ST2
117 and HS6ST3), respectively (Esko and Selleck, 2002). HS chain length along with the
118 *N*- and *O*-sulfation pattern subsequently determine the binding abilities of HSPGs
119 (Esko and Selleck, 2002).
120
121 SDCs and GPCs have been reported to regulate cell adhesion, migration and
122 differentiation and demonstrate specific expression and localisation during CNS
123 development (Choi et al., 2011, Ford-Perriss et al., 2003). The depletion of SDC1,
124 GPC1 and GPC4 *in vitro* in mouse NSC or neural precursor cells alters cell
125 maintenance and proliferation (Abaskharoun et al., 2010, Fico et al., 2012, Wang et
126 al., 2012) and the depletion of EXT1, NDST1, HS2ST1 or HS6ST1 in the mouse
127 CNS results in brain malformations and abnormalities (Grobe et al., 2005, Inatani et

128 al., 2003, Pratt et al., 2006). Currently the role of HSPGs in human NSC (hNSC)
129 lineage specification is limited and reliant upon rodent models: despite the
130 acknowledged differences in development, structure and regulation between human
131 and rodent nervous systems (reviewed in (Oikari et al., 2014)). To elucidate key
132 HSPGs in hNSC regulation we expanded hESC-derived NSCs (hNSC H9 cells) and
133 examined the expression of NSC self-renewal and neural cell lineage markers along
134 with HS biosynthesis enzymes and SDC and GPC core proteins in basal and lineage
135 specific differentiation (neuronal and glial) cultures. Our results identify HSPGs as
136 potential regulators of hNSC lineage potential and support their use as additional
137 markers of neural cell specification.
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160 Materials and Methods
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162 hNSC H9 cell expansion
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164 Two populations of human neural stem cells derived from the NIH approved H9
165 (WA09) human embryonic stem cells (hNSC H9 cells) were purchased from Life
166 Technologies and expanded until passage 31 (P31) corresponding to approximately
167 100 days in culture. Basal culture conditions included expanding cells as a
168 monolayer on Geltrex® coated culture vessels in neural stem cell serum-free
169 medium (NSC SFM) containing Knockout™ DMEM/F-12, 2% StemPro® Neural
170 Supplement, 20 ng/mL of FGF-2 and EGF, and 2 mM GlutaMAX™-I all obtained
171 from GIBCO®, Life Technologies. Cells were maintained at 37°C in 5% CO_2 in a
172 humidified atmosphere and passaged every 3-5 days using TrypLE (Life
173 Technologies) and re-plated at a density of $5x10^4$ cells/cm^2. The viability of the cells
174 was monitored using Trypan Blue and an automated cell counter (Bio-Rad) and via
175 hemacytometer.
176
177 hNSC H9 neuron and astrocyte differentiation cultures
178
179 hNSC H9 cells were cultured under lineage-specific differentiation conditions
180 according to protocols provided by Life Technologies. For neuronal lineage
181 differentiation, hNSC H9 cells were plated on poly-L-ornithine-laminin coated culture
182 vessels at a seeding density of $2.5x10^4$ cells/cm^2; for astrocyte lineage
183 differentiation, hNSC H9 cells were plated on Geltrex® coated culture vessels at a
184 seeding density of $2x10^4$ cells/cm^2. Cells were allowed to attach in NSC SFM for two
185 days. Neuronal differentiation was induced by maintaining cells in Neurobasal®
186 Medium (Life Technologies) supplemented with 2% B-27® Serum-Free Supplement
187 and 2 mM GlutaMAX™-I (Life Technologies) with the astrocyte differentiation
188 conditions consisting of DMEM supplemented with 1% N-2 supplement (Life
189 Technologies), 2 mM GlutaMAX™-I and 1% FBS. Cells were maintained in
190 differentiating conditions for 15 to 18 days with the medium changed every 3-4 days.
191
192 RNA-interference
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194 hNSC H9 P5 cells were plated in NSC SFM at a density of $5x10^4$ cells/cm^2 and
195 allowed to attach for 48h prior to treatment with Accell^{TM} Smartpool Human siRNAs
196 (Dharmacon), a pool of four siRNA transcripts specific to GPC1 (E-004303-00-0010)
197 or GPC4 (E-011271-00-0010). For siRNA delivery, the growth medium was changed
198 to Accell^{TM} siRNA Delivery Media containing 1 µM of siRNA supplemented with 2%
199 StemPro® Neural Supplement and 20 ng/mL of FGF-2 and EGF. Untreated and
200 non-targeting (scramble) siRNA (D-001910-10-20) treated cells were used as a
201 control. Cells were incubated with siRNAs for 72h after which cell number and
202 viability was assessed and cells harvested for RNA extraction.
203
204 Total RNA extraction, cDNA synthesis and Q-PCR
205
206 RNA was harvested using TRIzol® reagent (Invitrogen) with the Direct-zol^{TM} RNA
207 miniprep kit (Zymo Research) according to the manufacturer's instructions. During
208 extraction samples were treated in-column with DNase I (Zymo Research) for 15 min
209 to eliminate DNA contamination. cDNA synthesis was performed using Roche
210 Transcriptor Reverse Transcriptase. Briefly, 150 ng of RNA was incubated with 3 µg
211 of Random Primer (Invitrogen) at 65°C for 10 min in a reaction volume of 19.5 µl.
212 Samples were then incubated with 10 U of RT enzyme in 1x RT reaction buffer, with
213 1 mM dNTPs (New England Biolabs), 20 U of RNaseOUT (Invitrogen) in a total
214 reaction volume of 30 µl for 10 min at 25°C, followed by 30 min at 55°C and a final
215 step of 5 min at 85°C.
216
217 Q-PCR reactions were performed in quadruplicate per sample for each gene studied
218 in a 384-well microtiter plate. Each reaction contained 120 ng of cDNA template, 5 µl
219 of SYBR®-Green PCR Master Mix (Promega), 200 ng of forward and reverse primer
220 and 0.1 µl of CXR reference dye (Promega). Amplification was monitored using a
221 Life Technologies QuantStudio^{TM}-7 with an enzyme activation of 2 min at 50°C and 3
222 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Cycle
223 threshold (Ct) values were normalised against the endogenous control gene 18S Ct
224 values (ΔCt) included in each run. Relative gene expression was determined by
225 calculating the $^{\Delta\Delta}$Ct value ($2^{(-\Delta Ct)}$) and relative expression values presented on bar
226 graphs are $^{\Delta\Delta}Ctx10^6$. All primer sequences for the genes studied can be found in
227 tables 1 and 2 in (Oikari et al., 2015).

228

229 Immunofluorescence

230

231 For immunofluorescent (IF) detection of target proteins, cells in basal growth
232 conditions were plated on CC2-coated chamber slides (Lab-Tek) at $5x10^4$
233 cells/chamber and allowed to attach for 2 days. Differentiating cells were plated in
234 48-well culture dishes (Corning) and stained between D14-D18. Prior to staining,
235 cells were washed with 1X PBS with Ca^{2+} and Mg^{2+} and fixed with 4%
236 paraformaldehyde then blocked with 1% BSA and 5% donkey serum in 1X PBS with
237 Ca^{2+} and Mg^{2+}. For intracellular proteins 0.1% of Triton-X was included in the
238 blocking solution to allow permeabilisation. After blocking, primary antibodies diluted
239 in blocking solution were applied to cultures and incubated overnight at 4°C. The
240 following primary antibodies and dilutions were used: anti-Nestin (ab22035, Abcam,
241 1:200), anti-SOX2 (AB5603, Millipore, 1:1000), anti-TUBB3 (ab18207, Abcam,
242 1:1000), anti-S100B (ab868, Abcam, 1:200), anti-O1 (MAB344, Millipore, 1:500),
243 anti-syndecan 4 (ab24511, Abcam, 1:1000), anti-glypican 1 (ab137604, Abcam,
244 1:1000), anti-glypican 4 (ab100843, Abcam, 1:500) and 10E4 HS antibody (370255-
245 S, amsbio 1:500). After overnight incubation cells were washed with 1X PBS with
246 Ca^{2+} and Mg^{2+} secondary antibodies applied and cells incubated at room
247 temperature for 2h. Finally, cells were rinsed with 1X PBS with Ca^{2+} and Mg^{2+}, and
248 counterstained with fluoroshield mounting medium with DAPI (ab104139, Abcam).
249 Images were taken on an Olympus IX81 inverted phase-contrast fluorescent
250 microscope using Volocity software (Perkin Elmer) on a Hamamatsu Orca camera.
251 Isotype controls and secondary antibodies used can be found in table 3 in (Oikari et
252 al., 2015).

253

254 Western blotting

255

256 Total protein was extracted using protein-lysis buffer (20 mM HEPES, 25% Glycerol,
257 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5% Igepal CA-630, 0.2
258 mM Na_3VO_4, 1 mM PMSF and dH_2O containing protease and phosphatase
259 inhibitors). Protein concentration was determined using the BCA protein quantitation
260 assay (Pierce) with ~50 µg of total protein separated by SDS-PAGE using 12% pre-
261 cast gels (Mini-PROTEAN®TGX™, Bio-Rad). For the detection of HSPG core

262 proteins using the monoclonal antibody HS Δ3G10, protein samples were treated
263 with 1.5 mU Heparitinase (Seikagaku Biobusiness) for 2.5h at 37°C before
264 separation. After separation, protein was transferred to a PVDF membrane, the
265 membrane blocked with 5% milk and primary antibodies diluted in 5% BSA
266 incubated on the membrane overnight. Primary antibodies used were: anti-SOX2
267 (#AB5603, Millipore), anti-TUBB3 (ab18207, Abcam), anti-CD44 (ab6124, Abcam),
268 anti-Δ3G10 (H1890-75, US Biological) and anti-GAPDH (#2118, Cell Signaling) as
269 loading control. The membrane was then washed with TBST and incubated with
270 HRP-conjugated secondary antibodies (anti-Rabbit IgG, #7074 and anti-Mouse IgG,
271 #7076, both from Cell Signaling) diluted in 5% BSA for 2h at room temperature.
272 Detection of target proteins was performed with ECL (Clarity™ ECL, Bio-Rad) using
273 the Fusion FX Spectra chemiluminescence system (Vilber Lourmat, Fisher Biotec)
274 with optical quantitation performed using Bio-1D software.
275
276 Statistical analysis
277
278 Expansion, differentiation and RNA-interference (RNAi) experiments consisted of
279 two biological replicates (hNSC H9 POP1 and POP2) with RNA and protein samples
280 of differentiated and RNAi cultures consisting of 2-4 pooled wells. For IF images the
281 average signal intensity was calculated by normalising the mean signal intensity to
282 exposure time and dividing it by the number of nuclei. The signal intensities and
283 nuclei count were obtained using Volocity software and values presented are an
284 average between 2-4 images. Statistical significance was determined using a paired
285 t-test and defined as * $p<0.5$, ** $p<0.01$ and *** $p<0.001$.
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291 Results
292
293 *hNSC H9 expansion and lineage characterisation*
294
295 Commercially available hNSC H9 cells (POP1 and POP2) were independently
296 established and expanded as a monolayer on Geltrex® until passage 31 (P31)
297 corresponding to approximately 100 days in culture (Fig. 1A). Cells were passaged
298 at 90-100% confluence approximately every 3-4 days and re-plated at a seeding
299 density of $5x10^4$ cells/cm^2. Cell number and viability was assessed using Trypan
300 Blue on an automated cell counter as well as via hemacytometer with morphology of
301 the cells monitored under a phase contrast microscope and images taken at each
302 passage at D1. For gene expression analysis, RNA was harvested from both
303 populations at passages 2, 5, 7, 11, 13, 17, 21, 23, 27/28 and 31 and gene
304 expression results presented as the average for both populations at passages P2-
305 P5, P7-P11, P13-P17, P21-23 and P27-P31.
306
307 Cells exhibited linear growth and a high level of homogeneity throughout expansion
308 (Fig. 1A-B). A similar growth rate was observed for both populations until P6, after
309 which POP2 grew faster with P31 reached at D87 with POP1 reaching P31 after 105
310 days in culture (Fig. 1A). The averaged viability for both populations remained
311 between 60-84% with decreased viability observed between P2 (84%) and P31
312 (60%) (Fig. 1A). Cells acquired minimal morphological changes between P2, P5,
313 P17 and P31 (corresponding to averaged 2, 12, 52 and 96 days in culture) (Fig. 1B)
314 and maintained human TERT (hTERT) expression, with no significant change
315 observed in expression between P27-P31 and P2-P5 (Fig. 1C).
316
317 Expanded hNSC H9 cultures were examined for expression of NSC self-renewal as
318 well as neural specific lineage markers at passages P2-P5, P7-P11, P13-P17, P21-
319 P23 and P27-P31. NSC markers nestin, SOX1, SOX2 and MSI1 gene expression
320 was observed throughout expansion (Fig. 2A). SOX2 was expressed at the highest
321 level and its expression significantly upregulated (p=0.008) in P27-P31 cultures
322 when compared to P2-P5. Neuronal markers TUBB3, MAP2, medium molecular
323 weight NEF (NEFM) and DCX were expressed during expansion with TUBB3
324 demonstrating the highest expression level, followed by MAP2, NEFM and DCX,

325 respectively (Fig. 2B). TUBB3 (p=0.002) and MAP2 (p=0.02) were significantly
326 upregulated at late passages (P27-P31) when compared to early passage (P2-P5)
327 cells.
328
329 hNSC H9 cells also expressed glial markers, denoting astrocyte (S100B and CD44)
330 and oligodendrocyte (GalC, Olig1 and Olig2) lineages (Fig. 2C-D). The observed
331 expression levels of glial markers remained markedly lower than neuronal markers
332 with the neuronal marker TUBB3 (>$50x10^6$) expressed approximately at a 50 times
333 higher level than the glial markers (~$1x10^6$) at P2-P5. Significant upregulation of the
334 astrocyte marker S100B (p=0.0008) and oligodendrocyte markers Olig1 (p=0.04)
335 and Olig2 (p=0.007) in late passages (P27-P31) was observed (Fig. 2C-D) with
336 GFAP expression absent or only intermittently present in a few passages (data not
337 shown).
338
339 We then examined the cultures for expression of a panel of NSC and neural cell
340 lineage markers via IF. NSC markers nestin and SOX2 as well as neuronal (TUBB3),
341 astrocyte (S100B) and oligodendrocyte (O1) markers were observed at early (P5),
342 mid (P17) and late (P31) passages (Fig. 2E). Nestin, SOX2 and TUBB3
343 demonstrated a homogeneous expression pattern with S100B showing high
344 expression only in a few cells and the average signal intensity for O1 was the lowest
345 when compared to other markers studied (Fig. 2F). Increased SOX2 expression was
346 demonstrated via Western analysis indicative of self-renewal during extended culture
347 (Fig. 2G). The combined expression profile of neuronal, astrocyte and
348 oligodendrocyte lineage markers examined demonstrate hNSC H9 cells maintain
349 multipotentiality during extended *in vitro* expansion.
350
351 *HSPGs during hNSC H9 expansion*
352
353 Following confirmation of maintenance of self-renewal and expression of multi-
354 lineage markers during long-term expansion we then studied the expression of HS
355 regulatory enzymes and SDCs and GPCs in hNSC H9 cells from early (P2-P5) to
356 late stages (P27-P31) of expansion. HS biosynthesis genes were expressed in
357 hNSC H9 cells including elongation (EXT1 and EXT2), modification (NDST1-4 and
358 C5-EP) and sulfation (H2ST1, HS6ST1-3) enzymes with clear differences in

359 expression levels observed between several of the enzymes (Fig. 3A-D). This
360 included higher relative expression of EXT1 than EXT2 with EXT1 expression
361 significantly downregulated (p=0.02) in late passage cultures (P27-P31) (Fig. 3A).
362 NDST1 demonstrated the highest expression level followed by NDST2 with NDST3
363 and NDST4 expressed at a low level (Fig. 3B). Of the sulfotransferases HS2ST1 was
364 most highly expressed, followed by HS6ST1, HS6ST2 and HS6ST3, respectively
365 (Fig. 3D). In addition, HS2ST1 expression was significantly downregulated (p=0.01)
366 and HS6ST2 significantly upregulated (p=0.03) in late passage cultures.
367
368 HSPG core protein SDC2 demonstrated the highest expression level followed by
369 SDC3, SDC1 and SDC4, respectively (Fig. 3E) in hNSC cultures. Both SDC2
370 (p=0.03) and SDC3 (p=0.02) significantly increased expression at late passages
371 (P27-P31) when compared to early passages (P2-P5) (Fig. 3E). GPC1, GPC2,
372 GPC3, GPC4 and GPC6 were expressed in hNSC H9 cultures, but no GPC5
373 expression was detected (Fig. 3F). GPC4 was most abundantly expressed followed
374 by GPC1, GPC2, GPC3 and GPC6, respectively (Fig. 3F). The expression of GPC2
375 (p=0.0003) and GPC4 (p=4.3e-5) was highly significantly increased in later passage
376 (P27-P31) cultures. Detection of the HS 10E4-epitope via IF demonstrated localised
377 HS providing further confirmation that hNSC H9 cells maintain HS expression
378 throughout expansion (P5 to P31) (Fig. 3G).
379
380 *Markers of hNSC H9 neuronal and astrocyte lineage differentiation*
381
382 In order to confirm hNSC multipotency and to identify any associated changes in the
383 HSPG profile following lineage commitment, cells were examined during neuronal
384 and astrocyte differentiating conditions. Due to low differentiation efficiency and poor
385 viability of the cells following initiation of oligodendrocyte differentiation, this lineage
386 was not further examined. Neuronal and astrocyte differentiation was induced for 14-
387 18 days in both populations in P5 cells and during differentiation, cells were
388 monitored for morphological changes as well as expression changes in lineage and
389 HSPG genes.
390
391 Cells cultured in neuronal differentiating conditions acquired thin, elongated
392 protrusions, while astrocyte lineage cultures exhibited a large cell body when

393 compared to basal and neuronal cultures (Fig. 4A). Gene expression levels of the
394 NSC markers nestin, SOX1 and SOX2 were not altered following differentiation while
395 MSI1, also a marker of reactive astrocytes, was highly significantly upregulated in
396 astrocyte cultures when compared to basal (p=7.2e-6) and neuronal (p=0.0001)
397 cultures (Fig. 4B). All neuronal markers examined were significantly upregulated in
398 neuronal cultures when compared to basal cultures including: TUBB3 (p=0.03),
399 MAP2 (p=0.02), NEFM (p=0.0006) and DCX (p=0.02) (Fig. 4C). Furthermore, NEFM
400 (p=0.002) and DCX (p=0.03) were significantly upregulated in neuronal cultures
401 when compared to astrocyte cultures (Fig. 4C). Interestingly, TUBB3 (p=0.009),
402 MAP2 (p=5.8e-8), NEFM (p=0.01) and DCX (p=0.03) were also upregulated in the
403 astrocyte cultures when compared to basal cultures, however, the expression level
404 of NEFM and DCX in astrocyte cultures remained lower than in neuronal cultures
405 (Fig. 4C). The astrocyte marker S100B was undetectable by Q-PCR in neuronal
406 cultures, while it was highly significantly upregulated (p=6e-5) in astrocyte cultures
407 when compared to basal cultures (Fig. 4D). In addition, the glial marker CD44 was
408 significantly upregulated in both neuronal (p=0.008) and astrocyte (p=0.0003)
409 cultures when compared to basal hNSC H9 cells, with higher relative expression
410 observed in neuronal cultured cells (Fig. 4D). Western analysis confirmed
411 upregulation of TUBB3, SOX2 and CD44 in neuronal cultures when compared to
412 both basal and astrocyte cultures (Fig. 4E). No protein level expression of the
413 astrocyte marker S100B was observed under any culture conditions.
414
415 IF demonstrated that the neuronal cultures exhibited significantly decreased average
416 signal intensity of nestin (vs. hNSC H9 p=0.04, vs. astrocyte p=0.005) and SOX2 (vs.
417 hNSC H9 p=0.048, vs. astrocyte p=0.029) compared to other culture conditions with
418 astrocyte cultures demonstrating increased nestin intensity compare to basal
419 cultures (p=0.02) (Fig. 4F-G). Furthermore, although not significant, neuronal
420 cultures demonstrated the highest TUBB3 signal intensity compared to other
421 conditions and a significantly decreased signal intensity of S100B compared to basal
422 (p=0.008) and astrocyte (p=0.03) cultures. The average signal intensity of S100B in
423 astrocyte cultures remained lower than in basal cultures (p=0.008) (Fig. 4G),
424 however, a high number of cells demonstrated strong S100B staining in these
425 cultures (Fig. 4F).
426

427 *Lineage specific changes in HSPG expression*

428

429 Multiple HS biosynthesis enzymes exhibited a significant increase in expression
430 when examined by Q-PCR following neuronal and astrocyte lineage differentiation.
431 The HS polymerising enzyme EXT2 was highly significantly upregulated in neuronal
432 cultures when compared to both basal (p=4.2e-5) and astrocyte (p=0.0001) cultures
433 and the HS modifying enzyme C5-EP was significantly upregulated in neuronal
434 cultures when compared to astrocyte cultures (p=0.02) (Fig. 5A). Following lineage
435 commitment NDST2 was significantly upregulated (p=0.04) in neuronal cultures
436 compared to basal and astrocyte cultures; NDST3 was significantly upregulated in
437 both neuronal and astrocyte (p=0.003) cultures compared to basal cultures, with a
438 significant difference also observed between neuronal and astrocyte cultures
439 (p=0.04); and finally, NDST4 was highly significantly upregulated in neuronal
440 cultures when compared to basal (p=4.1e-5) and astrocyte (p=0.0006) cultures (Fig.
441 5B). The expression level of HS2ST1 was not altered following hNSC H9 cell
442 differentiation, however, HS6ST2 (p=0.006) and HS6ST3 (p=0.02) were significantly
443 upregulated in neuronal cultures when compared to basal cultures with HS6ST2 also
444 significantly upregulated (p=0.03) in neuronal cultures when compared to astrocyte
445 cultures (Fig. 5C). Finally, HS6ST1 (p=0.049) and HS6ST2 (p=0.013) were
446 significantly upregulated following astrocyte lineage differentiation when compared to
447 basal hNSC H9 cultures (Fig. 5C).

448

449 Gene expression of SDC1, SDC2 and SDC3 were not altered following neuronal
450 differentiation, interestingly, however, SDC4 expression was significantly
451 upregulated (P=0.03) in neuronal cultures when compared to basal and astrocyte
452 cultures (Fig. 5D). In addition, SDC2 was significantly upregulated (p=0.011) in
453 astrocyte cultures when compared to neuronal cultures (Fig. 5D). Significant
454 increases in expression were observed for several GPCs in neuronal cultures when
455 compared to basal and astrocyte cultures as follows: GPC1 neuronal vs. basal
456 (p=0.049), neuronal vs. astrocyte (p=0.048); GPC2 neuronal vs. basal (p=0.009) and
457 neuronal vs. astrocyte (p=0.011); GPC3 neuronal vs. basal (p=0.014) and neuronal
458 vs. astrocyte (p=0.013); and GPC6 neuronal vs. basal (p=0.009) and neuronal vs.
459 astrocyte (p=0.02) (Fig. 5E). GPC4 was highly significantly upregulated in astrocyte
460 differentiating cultures when compared to basal (p=0.0001) and neuronal (p=1.8e-5)

461 cultures with GPC6 also showing significant upregulation (p=0.02) in the astrocyte
462 cultures when compared to basal cultures (Fig. 5E). Finally, the HS Δ3G10 epitope
463 was detected in basal and lineage differentiated cultures via a Western analysis
464 following heparitinase digest with three major bands representing multiple HSPGs
465 associated with the cell membrane of approximately 70 kDa (glypicans), 35 kDa and
466 15 kDa (syndecans) (Haupt et al., 2009). Relative quantitation normalised to GAPDH
467 demonstrated higher intensity for the 70 kDa band in neuronal and astrocyte cultures
468 with the 35 kDa and 15 kDa bands demonstrating higher intensity in the basal
469 cultures suggesting HSPG modifications during lineage commitment.
470
471 SDC4, GPC1 and GPC4 were further examined via IF under all culture conditions
472 demonstrating cellular localisation. Neuronal differentiating cultures demonstrated
473 strong SDC4 and GPC1 staining. Although the average signal intensity of these
474 markers was not significantly higher to basal cells, GPC1 demonstrated significantly
475 stronger staining in neuronal cells when compared to astrocyte cultures (p=0.03).
476 (Fig. 6A). While IF of GPC4 did not demonstrate significantly higher signal intensity
477 in the astrocyte cultures, high levels of expression were localised to areas of dense
478 cell-cell contact reflective of the observed phenotypic changes when compared to
479 the basal and neuronal cells (Fig. 6A). Finally, to examine the potential influence of
480 GPC1 and GPC4 on hNSC H9 lineage potential, we downregulated the expression
481 of these core proteins in undifferentiated hNSC H9 P5 cells at the mRNA level using
482 gene specific RNAi pools and studied changes in marker genes in the knockdown
483 (KD) cultures compared to control conditions (untreated and scramble). GPC1kd and
484 GPC4kd cultures expressed significantly reduced levels of GPC1 (p=1.6e-10, 58%
485 reduction to control) and GPC4 (p=9.3e-8, 73% reduction to control), respectively,
486 with no changes observed in cell number or viability when compared to control
487 cultures (Fig. 6B). KD of GPC1 significantly reduced the gene expression levels of
488 nestin (p=0.005), MSI1 (p=0.004), TUBB3 (p=0.004) and NEFM (p=0.003) with the
489 KD of GPC4 significantly reducing NEFM (p=0.03) and S100B (p=0.004) gene
490 expression (Fig. 6C-D).
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494

495 Discussion

496

497 Self-renewing and multipotent hNSCs provide an *in vitro* model to study human
498 neurogenesis and have potential in the regenerative treatment of CNS injuries.
499 Understanding the mechanisms regulating expansion, 'stemness' and lineage
500 commitment of hNSCs is critical to our improved understanding of the cells for these
501 applications. With the extracellular microenvironment contributing to the regulation of
502 stem cell fate, cell-surface HSPGs associated with hNSCs and localised within the
503 neural niche may provide novel markers for the characterisation and isolation of
504 hNSCs and their progeny and with which to control hNSC lineage specification. The
505 central findings of this study are summarised in figure 7, with several HSPGs
506 proposed as novel markers of hNSCs and lineage specificity.

507

508 *hNSC expansion, stemness, multi-lineage capacity and HSPGs*

509

510 With limitations associated with the long-term culture of brain-derived neurospheres
511 (Anderson et al., 2007, Ostenfeld et al., 2000, Wright et al., 2006, Zhang et al.,
512 2001), we utilised adherent hESC-derived NSCs (hNSC H9 cells) as a model of NSC
513 self-renewal and neurogenesis. During extended culture limited morphological
514 changes were observed in the cells with cultures continuing to express hTERT as
515 well as increased NSC self-renewal (SOX2) and neural lineage marker expression,
516 (TUBB3, MAP2, S100B, Olig1 and Olig2) (Fig. 7) suggesting the cells maintain
517 stemness and multipotency. Previous studies utilising human neural progenitor cells
518 (NPCs) isolated from the cortical tissue have demonstrated the loss of neurogenic
519 potential during extended culture as indicated by downregulation of neuronal
520 markers, such as TUBB3 and upregulation of glial markers, such as S100B
521 (Anderson et al., 2007, Wright et al., 2006), While glial markers were also
522 upregulated in hNSC H9 cells during long-term expansion, these cells appear to also
523 retain their self-renewal and neuronal differentiative potential (SOX2, TUBB3 and
524 MAP2) highlighting the advantage of utilising hESC-derived NSCs as a self-renewing
525 NSC model.

526

527 HSPG ECM proteins including biosynthesis enzymes and cell-anchored SDCs and
528 GPCs regulate multiple cellular functions and are potential targets for the control of

529 hNSC fate. The expression of central HS synthesising and modifying EXT1, NDST1
530 and HS2ST1 (Busse et al., 2007, Grobe et al., 2002, Kreuger and Kjellen, 2012) in
531 basal hNSC H9 cultures suggests active production of HS chains. The observed
532 decrease in expression of EXT1 and HS2ST1 during extended culture likely reflects
533 reduced synthesis of new HS chains with the increased 6-*O*-sulfation suggesting
534 continued modification of the HS chains (Fig. 7). HS chain modifications are
535 associated with a shift in growth factor binding abilities (e.g. FGF-2 vs. FGF-1)
536 (Brickman et al., 1998, Johnson et al., 2007), indicating altered requirements in
537 hNSC H9 cells during expansion.
538
539 Of the SDC and GPC core proteins, SDC1, SDC2, SDC3, GPC1 and GPC4
540 demonstrated high expression in basal hNSC H9 cells with extended culture
541 upregulating SDC2, SDC3, GPC2 and GPC4 expression (Fig. 7). In addition, KD of
542 GPC1 downregulated the expression of nestin in basal hNSC H9 cells. Previous
543 reports have shown SDC1, GPC1 and GPC4 to be required for mouse NPC and
544 ESC maintenance and SDC2 and SDC3 have been shown to localise to rodent
545 neurons and NSCs (Abaskharoun et al., 2010, Fico et al., 2012, Ford-Perriss et al.,
546 2003, Hienola et al., 2006, Inatani et al., 2001, Wang et al., 2012) suggesting these
547 HSPGs are likely key contributors to the hNSC niche. Continued dissection of the
548 function of these HSPGs within the neural niche will allow us to more fully
549 understand their contribution to both stemness and lineage specification.
550
551 *Markers and HSPGs of neuronal differentiation of hNSCs*
552
553 Understanding markers of neurogenesis is crucial for more efficient neuronal lineage
554 differentiation of hNSCs. High expression levels of neuronal markers in basal hNSC
555 H9 cells indicated the high inherent neuronal potential of these cells, confirmed by
556 upregulation of the neuronal markers examined in neuronal lineage differentiative
557 culture conditions. Specific neuronal markers upregulated in neuronal lineage cells
558 when compared to both basal and astrocyte lineage cells, included TUBB3 (protein
559 level), NEFM (transcript) and DCX (transcript) (Fig. 7). Interestingly, MAP2 which is
560 commonly used to characterise neuronal lineage cells (Song et al., 2002), was highly
561 upregulated in astrocyte cultures and showed no significant difference in expression
562 between neuronal and astrocyte cultures. This supports previous reports of MAP2

563 functioning as a reactive astrocyte marker (Geisert et al., 1990) indicating its lack of
564 specificity to the neuronal lineage.
565
566 Neuronal differentiation of hNSC H9 cells induced the expression of multiple HS
567 biosynthesis enzymes, including EXT2, NDST2, NDST4, HS6ST2 and HS6ST3
568 indicating altered HS biosynthesis activity following neuronal commitment (Fig. 7).
569 The upregulation of these enzymes has been reported during embryoid body (EB)
570 formation, the process used as an intermediate step in the derivation of NSCs from
571 ESCs (Nairn et al., 2007). In addition, the upregulation of NDST4 and 6-*O*-
572 sulfotransferase gene expression has been shown to be highly upregulated following
573 neural differentiation of mouse ESCs indicating altered HS sulfation and subsequent
574 changes in growth factor binding (Grobe et al., 2002, Johnson et al., 2007). With the
575 increased expression of HS modifying enzymes correlating with the upregulation of
576 neuronal markers, an importance for HS modifications during neuronal lineage
577 commitment is highlighted (Oikari et al., 2015).
578
579 Consistent with increased HS biosynthesis enzyme transcription, the increased
580 expression of multiple cell surface HSPG core proteins, including SDC4, GPC1,
581 GPC2, GPC3 and GPC6 were also observed following neuronal differentiation (Fig.
582 7). Interestingly, SDC4 has previously been identified as glial-specific (Avalos et al.,
583 2009, Hsueh et al., 1998) with its upregulation in neuronal cultures suggesting it may
584 also have a role in neuronal lineage specification. The importance of, in particular,
585 GPC proteins in the neuronal lineage is supported by their coordinated expression
586 with neuronal lineage markers (Oikari et al., 2015) and previous studies reporting the
587 localisation of GPC1 and GPC2 to rodent neurons (Ford-Perriss et al., 2003, Ivins et
588 al., 1997, Jen et al., 2009, Luxardi et al., 2007) as well as the reported upregulated
589 expression of GPC2, -3 and -6 following mouse EB differentiation (Nairn et al., 2007,
590 Zhang et al., 2001). Furthermore, in our study the downregulation of GPC1 in basal
591 hNSC H9 cells resulted in reduced expression of TUBB3 and NEFM, further
592 highlighting the importance of GPCs in neuronal lineage regulation.
593
594 *Markers and HSPGs of astrocyte differentiation*
595

596 The observed low expression levels of glial markers (astrocyte and oligodendrocyte)
597 in basal hNSC H9 cultures indicated low inherent glial differentiative potential of the
598 cells. The oligodendrocyte cultures of hNSC H9 cells were not viable and the
599 efficiency of astrocyte differentiation was difficult to determine, due to low expression
600 levels of astrocyte lineage markers. However, the significantly increased expression
601 of S100B following astrocyte differentiation compared to basal and neuronal cultures
602 indicated astrocyte lineage commitment of the cells (Fig. 7). The NSC marker MSI1,
603 previously reportedly expressed in reactive astrocytes (Oki et al., 2010) was also
604 highly significantly upregulated in astrocyte differentiation conditions, potentially
605 providing a new astrocyte specific marker (Fig. 7). With the glial marker CD44
606 showing greater expression in the neuronal lineage cells, higher neuronal specificity
607 for this marker is indicated (Fig. 7) reinforcing the requirement to define more
608 accurate human astrocyte lineage markers.
609
610 Expression of the HS biosynthesis enzymes showed few changes following astrocyte
611 differentiation likely reflective of the lower efficiency of astrocyte differentiation.
612 However, upregulation of NDST3 and HS6ST1 following astrocyte commitment
613 again suggests the importance of HS modifications and the incorporation of 6-*O*-
614 sulfation sites during hNSC differentiation (Fig. 7). NDST3 has a more restricted
615 expression profile than NDST1/2 but is expressed during embryonic development
616 (Nairn et al., 2007). Low levels of NDST3 were observed in basal hNSC H9 cells and
617 was upregulated following hNSC H9 (neuronal and astrocyte) lineage commitment
618 suggesting NDST3 may play a role within the neural niche during lineage
619 specification but not during stem cell maintenance.
620
621 Our observed changes in the hNSC H9 HSPG profile following astrocyte
622 differentiation suggest GPC4 as an astrocyte lineage marker (Fig. 7). GPC4
623 expression at the gene expression levels was highly upregulated in astrocyte
624 cultures with its IF localisation demonstrating a distinct “clustering” pattern that
625 differed from basal and neuronal cultures. Additionally, KD of GPC4 in basal hNSC
626 H9 cultures resulted in the downregulation of S100B further supporting its
627 importance within astrocytes. Finally, the combination of GPC4 and GPC6 may be of
628 importance in the astrocyte niche, as increased transcription of GPC6 was also
629 observed in the astrocyte cultures compared to basal cultures, with previous studies

630 in rodents further supporting this hypothesis (Allen et al., 2012), Identification of
631 regulators of the astrocyte lineage would greatly enhance the more efficient
632 derivation of astrocytes from hNSCs.
633
634 Conclusions
635
636 Our data demonstrates hNSC H9 cells provide a good model to study human NSC
637 self-renewal and neurogenesis and suggest HSPGs as important proteins of lineage
638 specificity for these cells. The HSPG SDCs were highly expressed in basal hNSC H9
639 cultures and lineage differentiation resulted in an altered HSPG profile with multiple
640 GPC proteins likely playing a role in neuronal differentiation and GPC4 identified as
641 a potential mediator of astrocyte differentiation. Overall, our results support the
642 importance of HS modifications during lineage commitment and the requirement for
643 continued reorganisation of the localised niche during lineage specification.
644 Importantly, in combination with identified lineage markers, HSPGs provide
645 additional markers of hNSC and neural cell lineage characterisation, and as cell
646 surface proteins, key HSPGs may provide targets to enable the more efficient
647 isolation, enrichment and potentially differentiation of hNSCs and neural cell
648 lineages.
649
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841 Figure legends:
842
843 Figure 1. Expansion of hNSC H9 cells. A) Growth curve of hNSC H9 populations
844 (POP1 and POP2) expanded to passage 31 (P31). Cells exhibit a linear growth
845 pattern and maintain 60-80% viability. B) Phase contrast images (20X magnification,
846 scale bar 130 μM) of hNSC H9 cells at P2, P5, P17 and P31 corresponding to an
847 average of 2 days, 12 days, 52 days and 96 days in culture. C) Relative expression
848 of hTERT in hNSC H9 cells at passages 2-5, 7-11 13-17, 21-23 and 27-31 (error
849 bars = SEM).
850
851 Figure 2. hNSC H9 cells express NSC self-renewal, neuronal, astrocyte and
852 oligodendrocyte lineage markers throughout expansion. Averaged (POP1 and
853 POP2) relative expression in hNSC H9 cultures of A) NSC markers nestin, SOX1,
854 SOX2 and MSI1, B) neuronal markers TUBB3, MAP2, NEFM and DCX, C) astrocyte
855 markers S100B and CD44 and D) oligodendrocyte markers GalC, Olig1 and Olig2 at
856 passages 2-5, 7-11 13-17, 21-23 and 27-31 (error bars = SEM, * $p<0.05$, ** $p<0.01$,
857 *** $p<0.001$). Immunofluorescence of E) NSC markers nestin and SOX2, neuronal
858 marker TUBB3, astrocyte marker S100B and oligodendrocyte marker O1 in hNSC
859 H9 cells at passages 5, 17 and 31, counterstained with DAPI (40X magnification,
860 scale bar 130 μM). F) Average signal intensity of IF (error bars = SD). G) Western
861 blot analysis of SOX2 at passage P3-P5, P7-P11, P13-P17, P21-P23 and P27-P31
862 with GAPDH loading control.
863
864 Figure 3. hNSC H9 cells express HSPGs and HS biosynthesis enzymes. Averaged
865 (POP1 and POP2) relative expression in hNSC H9 cultures of A) EXT1 and EXT2,
866 B) *N*-deacetylase/*N*-sulfotransferases (NDST1-4), C) C5-Epimerase, D) 2-*O*/6-*O*
867 sulfotransfereases, E) SDC 1-4 and F) GPC 1-4 and 6 at passages 2-5, 7-11, 13-17,
868 21-23 and 27-31 (error bars = SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). G)
869 Immunofluorescence of pan HS 10E4 epitope in hNSC H9 cells at P5, P17 and P31
870 counterstained with DAPI (40X magnification, scale bar 130 μM).
871

872 Figure 4. Neuronal and astrocyte differentiation of hNSC H9 cells alters cell
873 morphology and marker expression. A) Neuronal and astrocyte lineage phase
874 images of hNSC H9 P5 cells (20X and 40X magnification, scale bar 130 μM).
875 Averaged (POP1 and POP2) relative expression of B) NSC C) neuronal and D)
876 astrocyte markers and E) Western analysis of TUBB3, SOX2 and CD44 (GAPDH
877 loading control) following neuronal and astrocyte differentiation. F)
878 Immunofluorescence of nestin and SOX2, TUBB3 and S100B in hNSC H9, neuronal
879 and astrocyte lineage cells (20X magnification, scale bar 130 μM) with average
880 signal intensity graph (G). (Error bar = SD for CD44 relative expression astrocyte
881 condition and average signal intensity graph, other error bars = SEM, * $p<0.05$, **
882 $p<0.01$, *** $p<0.001$.)
883
884 Figure 5. HS biosynthesis enzymes and HSPG core protein (SDC and GPC)
885 expression in hNSC H9 cells following neuronal and astrocyte lineage differentiation.
886 Averaged (POP1 and POP2) relative expression of A) exostoses EXT1 and EXT2
887 and C5-Epimerase, B) *N*-deacetylase/*N*-sulfotransferases and C) HS 2-*O*/6-*O*-
888 sulfotransferases D) SDC1-4 and E) GPC1-4, 6 following hNSC H9 neuronal and
889 astrocyte differentiation (error bar for NDST3 basal hNSC H9 condition = SD, other
890 error bars = SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). F-G) Western analysis and
891 optical quantitation of the HS Δ3G10 epitope in basal and lineage differentiated cells.
892
893 Figure 6. SDC4, GPC1 and GPC4 core proteins in hNSC H9 cell lineage
894 specification. A) Immunofluorescence of SDC4, GPC1 and GPC4 in hNSC H9,
895 neuronal and astrocyte lineage cells (20X magnification, scale bar 130 μM) with
896 representative average signal intensity (error bars = SD). B) Relative expression of
897 GPC1 and GPC4 and cell number and viability in control hNSC H9 P5 cells and in
898 GPC1 and GPC4 knockdown (KD) cultures. Relative expression of C) NSC and D)
899 neural lineage markers following GPC1 and GPC4 kd (error bars = SEM, * $p<0.05$, **
900 $p<0.01$, *** $p<0.001$).
901
902 Figure 7. A schematic summary of changes in NSC and lineage markers (neuronal
903 and glial), HS biosynthesis enzymes and HSPG core proteins in hNSC H9 cells. Key
904 differences were identified between early (P2-P5) and late (P27-P31) passage
905 cultures and following neuronal and astrocyte lineage differentiation.