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The effect of hypoxia and laminin-rich substrates on the proliferative behavior of human neural stem cells

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Running title: ECM, hypoxia and hNSC proliferation

Abstract

Human neural stem cells (hNSCs) possess an enormous potential to be utilized in novel cell-replacement therapies for neurodegenerative diseases and injuries. The hNSCs are a renewable source of cells with the capacity to generate the major cell types of the central nervous system (CNS). However, the translational potential of cell-based therapy is constrained due to the limited availability of scalable methods to rapidly expand numbers of stem cells *in vitro*. Here, we investigated the possible synergistic effect of oxygen concentration and substrate composition on hNSC growth. The hNSCs were cultured on six different substrates (i.e., collagen I, collagen IV, poly-L-ornithine, fibronectin, laminin, and Matrigel) under normoxic (21% oxygen concentration) and hypoxic (3% oxygen concentration) conditions and then total cell numbers were determined after 2 and 4 d. The percentages of cells undergoing proliferation (EdU^+) and apoptosis ($TUNEL^+$) varied with culture conditions, with a synergistic interaction between Matrigel substrate and hypoxia that resulted in the greatest number of hNSCs after 4 d compared to other conditions. These findings inform new methods to scale up NSC production by identifying potential substrate biomaterial design criteria as well as culture conditions that favor the generation of larger numbers of undifferentiated cells.

Introduction

Neural stem cells (NSCs) are a self-renewing somatic cell type with the capacity to generate the major neural and glial cell types in the brain.[1-3] Due to their ability to self-renew and their multipotency, NSCs are promising candidates to be utilized in cell-replacement therapy in patients with neurological disorders or injury. Preclinical studies have shown that the transplantation of NSCs in animal experimental models such as stroke,[4-6] Parkinson's disease,[7-10] and Alzheimer's disease[11] has resulted in enhanced behavioral recovery. However, the application of stem cells in clinical trials may require as many as 10^{10} cells per patient[12] and therefore a need exists for cost-effective and scalable methods of producing large quantities of hNSCs *in vitro*.

Neural stem cells, *in vivo*, reside within a specialized anatomical compartment known as the NSC niche.[13, 14] The NSC niche provides extrinsic cues that influence cell survival and regulate stem cell proliferation and differentiation.[15, 16] The interaction between NSCs and the extracellular matrix (ECM)[17, 18] as well as the gradient of important cues such as oxygen[19] are among the important regulators of NSC proliferation. For instance, the proliferative behaviors of mouse neuroepithelial cells,[20] as well as mouse and human NSCs [21] have been shown to be influenced by ECM molecules (e.g., laminin). NSC adhesion to ECM is mediated via several cell-surface receptors, of which the major class is integrins.[22] The upregulation of β1 integrin expression, in particular, has been linked to the effect of cell-ECM interaction on NSC proliferation.[23-25] Additionally, the NSC niche is a hypoxic environment with an oxygen concentration between to 4% as opposed to the atmospheric oxygen concentration (normoxia, 21%)[19, 26] conventionally utilized in *in vitro* NSC culture. Further, hypoxia has been shown to enhance the proliferation of both rat and human NSCs. [27, 28] Due to ease of scalability, availability, and no risk of pathogen transmission, manipulation of oxygen concentration in NSC cultures can provide a powerful tool for improving production strategies for NSCs.

In the NSC niche, the ECM varies in composition,[29, 30] where laminin,[25] collagen I,[31, 32] collagen IV,[33] and low levels of fibronectin[34] are among its protein constituents. Yet, the relationship between NSC

proliferation, substrate composition and the concentration of oxygen in culture has not yet been examined. Herein, we test the hypothesis that the synergistic effect of a lowered oxygen concentration across various substrates can enhance the *in vitro* expansion of NSCs.

We assayed the proliferation of human embryonic cortical NSCs cultured on six different substrates and under both normoxic and hypoxic conditions (3% oxygen). Four purified ECM proteins (i.e. laminin, collagen I, collagen IV, and fibronectin), a positively charged non-ECM polymer (i.e. poly-L-ornithine or PLO), and a cell-adhesive matrix for NSCs which is a tissue-derived ECM (Matrigel, reduced-growth factor) were utilized. The proliferation and apoptosis of NSCs when cultured on these substrates and under both normoxia and hypoxia were assessed. In this work, we have shown that the laminin-rich substrates in synergy with low oxygen concentration can significantly contribute to *in vitro* expansion of NSCs.

Materials and Methods

All materials were procured from Sigma Aldrich (St. Louis, MO), Millipore (Billerica, MA), Life Technologies (Grand Island, NY), or Trevigen (Gaithersburg, MD) unless otherwise noted.

Human neural stem cell (hNSC) maintenance and culture

Rencell-CX, an immortalized cell line derived from 14-week gestation human fetal cortex was purchased from Millipore (Billerica, MA) and was used as a model hNSC in these experiments. Cell culture flasks were coated with natural mouse laminin (Life Technologies; 20 μg/ml) and used to maintain cells in serum-free complete growth medium composed of DMEM:

F12 supplemented with 1x B27 supplement, heparin (10 U/ml), L-glutamine (2 mM), gentamicin (30 μg/ml), epidermal growth factor (EGF, 20 ng/ml), and fibroblast growth factor-2 (FGF-2, 20 ng/ml). The cells were passaged every 3 to 4 days using Accutase™ and plated in freshly laminin-coated flasks at a density of ~7×10^3 cell/cm^2. All experiments were carried out using cells between passages 10 to 14.

To assess hNSC proliferation, the cells were seeded at a density of 3×10^4 cells/cm^2 onto 96-well tissue culture plates coated with the various substrates; cultures were carried out for 2 and 4 d and the medium was changed every 2 d. All the proliferation experiments were carried out using complete growth medium containing growth factors.

The normoxic cultures were carried out in a humidified, 5% CO_2, 37 C incubator. The hypoxic cultures were carried out in a portable, humidified, and isolated hypoxic chamber (COY Laboratory, Grass Lake, MI) with 3% O_2, 5% CO_2 and 92% N_2. The hypoxic chamber was housed in a 37 C incubator. The concentration of oxygen in air was measured using O_2-sensitive electrode system (Dwyer Instruments, Michigan City, IN).

Substrates

Solutions of human fibronectin, laminin, reduced-growth factor Matrigel, and poly-L-ornithine (30000-70000 MW) in DMEM: F12 as well as collagen I (Corning, Corning, NY) in 0.02 N acetic acid and collagen IV (Santa Cruz Biotechnology, Dallas, TX) in 0.05 N hydrochloric acid were prepared for coating 96-well tissue plates. Fibronectin, laminin and poly-L-ornithine solutions were incubated in the 96-well plates overnight in a 37 C, 5% CO_2, humidified incubator and then the wells were rinsed with phosphate-buffered saline (PBS) before

plating the cells. Matrigel was incubated in the wells at room temperature for 2 h and subsequently the wells were rinsed and incubated in DMEM: F12 overnight in a humidified 37 C, 5% CO_2 incubator. Collagen I and collagen IV solutions were incubated in the wells at room temperature for 2 h and then the wells were rinsed with sterile water, PBS, and DMEM: F12 before plating the cells. The final concentration of all protein substrates and PLO in the wells was assumed to be 3 $\mu g/cm^2$.

Proliferation assay

To detect hNSC proliferation during culture, the Click-iT EdU proliferation assay (Life Technologies) was performed. The hNSCs were incubated with EdU for 2 and 4 d with EdU being replenished every 2 d to prevent depletion. The cells were then fixed in 4% buffered formaldehyde (Thermo Fisher Scientific, Pittsburgh, PA) for 20 min, permeabilized with Tris-buffered saline (TBS) containing 0.1% Triton-X-100, and incubated with EdU reaction cocktail for 30 min followed by 4',6-diamidino-2-phenylindole (DAPI, 300 nM) nuclear stain. The samples were then imaged under fluorescence microscopy (Olympus, Center Valley, PA) to measure the number of cells that proliferated during EdU incubation. The nuclei of EdU^+ cells fluoresced green because of Alexa Fluor 488 azide fluorescent dye detection. DAPI (blue) stained all nuclei. The numbers of EdU^+ and $DAPI^+$ cells were counted.

Apoptosis assay

To detect cell apoptosis, the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Life Technologies) was performed. After 4 d in culture, hNSCs were fixed in

4% buffered formaldehyde, permeabilized, and incubated with terminal deoxynucleotidyl transferase (TdT) buffer for 10 min. The cells were then incubated for 1 h at 37 C with TdT reaction cocktail (according to the manufacturer's instructions) and were subsequently washed two times with 3% goat serum in PBS for 5 min followed by incubation with TUNEL Click-iT reaction cocktail for 30 min. The samples were then stained with DAPI and imaged under fluorescence microscopy. All nuclei fluoresced blue because of the DAPI nuclear stain; also, the nuclei of $TUNEL^+$ cells fluoresced green due to Alexa Fluor 488 dye detection. The numbers of $TUNEL^+$ and $DAPI^+$ cells were counted.

Immunofluorescence imaging

The hNSCs cultured onto 96-well tissue culture plates after 4 d were fixed, washed with TBS, and incubated with 10% goat serum in PBS for 30 min in room temperature. The hNSCs were incubated overnight with primary antibodies SOX-2 (1:1000, Millipore), nestin (1:2000, Millipore), GFAP (1:1000, Abcam, Cambridge, MA), and TUJ-1 (1:2000, Covance, Princeton, NJ), washed with TBS, and then incubated with appropriate fluorescently-conjugated secondary antibodies (Abcam). The incubations of both primary and secondary antibodies were performed at 4 C. The samples were then washed with TBS, stained with DAPI and imaged using fluorescence microscopy. The washing steps consisted of 2 washes with TBS buffer, permeabilization with TBS containing 0.1% Triton-X-100, and then 3 more washes with TBS. The duration of each washing step was 10 min.

Statistical Analysis

Experimental results are represented as mean ± SD (n≥3). Analysis of variance (ANOVA) followed by post-hoc tests were performed to analyze statistical significance of variances among groups.

Results

To test the effect of substrate type and oxygen concentration on hNSC proliferation, we first determined the total number of cells in culture after 2 and 4 d. Identical numbers of cells were seeded on 96-well plates coated with PLO, collagen I, collagen IV, fibronectin, laminin, and reduced-growth factor Matrigel, and cultured under both normoxic and hypoxic conditions. The hNSCs were subsequently fixed, stained with DAPI nuclear stain, and imaged using fluorescent microscopy. After 2 d and 4 d, we did not detect any $DAPI^+$ hNSCs on collagen I and PLO coated wells (results not shown), suggesting that the hNSCs did not adhere to these two substrates and were washed away during medium replacement or fixation.

The comparison of the 2 d and 4 d results of cultures on fibronectin and collagen IV substrates show that the total numbers of hNSCs decrease over time (**Fig. 1**). The hNSCs cultured on fibronectin and collagen IV, under both normoxic and hypoxic conditions, exhibited ~65% and ~20% decreases in cell density, respectively. To determine whether the decrease in the total cell number is due to cell death or a lack of proliferation, we used EdU pulse-labeling[35] to label the cells in the S-phase of division. After 4 d, the EdU^+ cells constituted 16±1% and 16±0.9% of hNSCs cultured on collagen IV under normoxic and hypoxic conditions, respectively (**Fig. 2A**). The hNSCs cultured on fibronectin, after 4 d, consisted of 31±1% and

32±1.1% of EdU^+ cells under normoxia and hypoxia, respectively (**Fig. 2A**). The presence of actively proliferating cells in both cultures suggests that the decrease in cell density over time is likely due to cell death. A one-way ANOVA analysis also showed no statistical difference in cell density or the percentage of EdU^+ cells between normoxia and hypoxia for both fibronectin and collagen IV substrates. This suggests that oxygen concentration did not influence the proliferation of hNSCs cultured on fibronectin and collagen IV.

The total number of hNSCs cultured on laminin and laminin-rich Matrigel substrates increased over time (**Fig. 1**). The hNSCs cultured on laminin, after 4 d, exhibited ~18% and ~25% increases in number under normoxia and hypoxia, respectively. The hNSCs cultured on reduced-growth factor Matrigel represent ~160% and ~179% increases in number, after 4 d in normoxic and hypoxic conditions. A two-way ANOVA analysis indicates significant effects of oxygen ($p<0.05$) and substrate ($p<0.0001$) on hNSC total number after 4 d of culture on laminin and Matrigel. The EdU proliferation assay for laminin, after 4 d, determined that 11±1% and 14±1% of cells were EdU^+ under normoxia and hypoxia, respectively (**Fig. 2B**). For reduced-growth factor Matrigel, 24±1.1% and 31±0.9% of stem cells under normoxia and hypoxia, respectively, were actively proliferating after 4d (**Fig. 2B**). Similar to cell density, the two-way ANOVA analysis indicates significant effect of oxygen ($p<0.01$) and substrate ($p<0.0001$) on hNSC proliferation.

To clarify whether the temporal decrease in cell numbers in collagen IV and fibronectin cultures is due to cell death, we performed a TUNEL assay to detect the apoptotic hNSCs in culture.[36] After 4 d, in collagen IV cultures, 32±0.6% (normoxia) and 33±0.87% (hypoxia) of hNSCs were apoptotic $TUNEL^+$ cells (**Fig. 3A**). The hNSCs cultured on fibronectin consisted of 19±0.99% and 20±0.64% of $TUNEL^+$ cells under normoxia and hypoxia, respectively (**Fig. 3A**).

The hNSCs cultured on laminin-rich substrates (i.e., laminin and reduced-growth factor Matrigel) were associated with less apoptosis vs fibronectin and collagen IV substrates. The hNSCs cultured on laminin had 8±0.24% (normoxia) and 7±0.95% (hypoxia) apoptotic cells (**Fig. 3B**). Similarly, in reduced-growth factor Matrigel cultures, 8±0.71% and 8±0.44% of hNSCs were apoptotic under normoxia and hypoxia, respectively (**Fig. 3B**). A two-way ANOVA indicates a significant effect of substrate ($p<0.001$) on hNSC apoptosis and no effect of oxygen concentration. Therefore, we concluded that the relatively lower cell density in collagen IV and fibronectin cultures may be due to increased apoptosis vs cells cultured on laminin-rich substrates.

The molecular markers nestin and SOX-2 were utilized to detect the hNSCs that maintained stemness. To detect possible differentiation, cells were stained by neuronal (TUJ-1) and astrocytic (GFAP) markers. The majority of cells (>95%) cultured on all substrates under both normoxic and hypoxic cultures were both nestin and SOX-2 positive (**Fig. 4A to 4D**). No neuronal differentiation (TUJ-1^+ cells) was detected and limited (<5%) astrocytic differentiation was observed (**Fig. 4E and 4F**). The EdU proliferation, TUNEL apoptosis, and immunofluorescence results suggest that hypoxic culture on Matrigel supports the expansion of undifferentiated hNSCs *in vitro*.

Discussion

Our results demonstrate a synergistic effect of laminin-rich substrates (i.e., reduced-growth factor Matrigel) and oxygen concentration on the expansion of hNSCs. These results are consistent with published works[20, 21, 37] showing that laminin-rich substrates promote the

proliferation of mouse and human NSCs. We observed that the stem cells exhibit a temporal increase in density when cultured on laminin and laminin-rich Matrigel substrates (**Fig. 1**). However, the numbers of hNSCs cultured on fibronectin and collagen IV decreased over time (**Fig. 1**). To start to understand this distinction, we considered the three possible scenarios for the fate of a population of hNSCs: self-renewal, differentiation, or death.[1] The immunofluorescence analysis using stemness markers (i.e., nestin and SOX-2) showed that the majority (>95%) of cells in culture maintained their stemness and did not differentiate into neural or glial progenies (**Fig. 4A to 4F**). In the absence of differentiation, the total cell number in culture is regulated via two cellular behaviors (i.e., proliferation and death). The EdU assay confirmed that hNSC proliferate regardless of substrate type and oxygen concentration. Therefore, the relative differences in total cell numbers under different conditions could be explained by differing extents of cell death.

We thus investigated hNSC apoptosis when cultured under all conditions by performing the TUNEL apoptosis assay (**Fig. 3A and 3B**). Our results indicate that the substrate composition had a significant effect ($p<0.001$) on the survival of hNSCs with no significant difference between normoxia and hypoxia. Percentages of apoptotic ($TUNEL^+$) cells were greatest for collagen IV (~32%) and fibronectin (20%) whereas in laminin and Matrigel cultures the percentages of apoptotic cells were lower (~8%). Therefore, the higher percentage of apoptotic cells in collagen IV and fibronectin cultures may explain the lower density of hNSCs on these two substrates.

The binding of cells to ECM is mediated via cell-surface receptors such as integrins.[38] The hNSCs express high levels of $\alpha_6\beta_1$ integrin,[39] which binds to laminin. However, hNSCs express only low levels of fibronectin-binding receptors ($\alpha_5\beta_1$ and $\alpha_V\beta_1$) and collagen IV

receptors ($\alpha_1\beta_1$ and $\alpha_2\beta_1$)[22, 39, 40]. For instance, it has been shown that ≤2% of hNSCs express α_1 and α_2 integrin subunits[21] which are required for adhesion to collagen I and collagen IV. For a variety of cells, proper integrin-mediated binding to ECM plays a crucial role in cell survival.[41, 42] Thus, the lower survival of hNSCs cultured on fibronectin and collagen IV may be due to low expression of appropriate integrin receptors and consequently insufficient cell-ECM adhesion.

The hNSCs cultured for 4 d on Matrigel had a ~2 fold greater increase in total cell numbers in comparison with the cells cultured on laminin regardless of oxygen concentration. The number of actively proliferating cells, as determined by an EdU assay, is also ~2 fold higher on Matrigel versus laminin at 4 d regardless of oxygen concentration. Matrigel is a secreted ECM extracted from Engelbreth-Holm-Swarm mouse tumor and is a complex mixture of laminin, collagen IV, entactin, and heparin sulfate proteoglycans.[43] Since laminin is one of many constituents of Matrigel, the concentration of laminin in Matrigel-coated substrates is expected to be lower than on laminin-coated substrates. Therefore, other components of Matrigel are likely responsible for the enhanced cell numbers vs laminin alone. Growth factor signaling in synergy with β_1 integrin signaling play a regulatory role in hNSC maintenance.[44] Therefore, the enhanced proliferation of hNSCs on Matrigel may be due to the presence of heparin sulfate proteoglycans, which stabilize heparin-binding growth factors such as FGF, providing protection from degradation,[45-47] and allowing upregulation of growth factor signaling processes involved in adhesion and survival.

For the hNSCs cultured on laminin and Matrigel, hypoxic conditions are associated with significant increases both the stem cell density (**Fig. 1**) and the percentage of actively proliferating (EdU^+) cells over time (**Fig. 2B**). However, for hNSCs cultured on fibronectin and collagen IV, the lowered oxygen concentration does not affect hNSC density and proliferation.

Thus in summary, we report that the synergistic effect of the laminin-rich substrate (i.e. Matrigel) and lowered oxygen concentration increases the rate of hNSC *in vitro* expansion. Manipulation of oxygen concentration in culture provides a scalable and a xeno-free alternative to enhance *in vitro* hNSC growth. We acknowledge that Matrigel and laminin are both animal-derived and hence xenogeneic. However, this result may inform the development of new xeno-free biomaterial substrates with the capability of binding to $\alpha_6\beta_1$ integrins, stabilizing growth factors, and maintaining high levels of proliferation.

References

1. F. H. Gage, Science, 2000, 287, 1433-1438.
2. S. Temple, Nature, 2001, 414, 112-117.
3. I. L. Weissman, D. J. Anderson and F. Gage, Annual review of cell and developmental biology, 2001, 17, 387-403.
4. M. M. Daadi, A.-L. Maag and G. K. Steinberg, PLoS One, 2008, 3, e1644.
5. Y. Mine, J. Tatarishvili, K. Oki, E. Monni, Z. Kokaia and O. Lindvall, Neurobiology of disease, 2013, 52, 191-203.
6. E. J. Smith, R. P. Stroemer, N. Gorenkova, M. Nakajima, W. R. Crum, E. Tang, L. Stevanato, J. D. Sinden and M. Modo, Stem cells, 2012, 30, 785-796.
7. T. Ben-Hur, M. Idelson, H. Khaner, M. Pera, E. Reinhartz, A. Itzik and B. E. Reubinoff, Stem Cells, 2004, 22, 1246-1255.
8. K. B. Bjugstad, Y. D. Teng, D. E. Redmond, J. D. Elsworth, R. H. Roth, S. K. Cornelius, E. Y. Snyder and J. R. Sladek, Experimental neurology, 2008, 211, 362-369.
9. A. D. Ebert, A. J. Beres, A. E. Barber and C. N. Svendsen, Experimental neurology, 2008, 209, 213-223.

10. D. E. Redmond, K. B. Bjugstad, Y. D. Teng, V. Ourednik, J. Ourednik, D. R. Wakeman, X. H. Parsons, R. Gonzalez, B. C. Blanchard and S. U. Kim, Proceedings of the National Academy of Sciences, 2007, 104, 12175-12180.
11. D. Park, H. J. Lee, S. S. Joo, D.-K. Bae, G. Yang, Y.-H. Yang, I. Lim, A. Matsuo, I. Tooyama and Y.-B. Kim, Experimental neurology, 2012, 234, 521-526.
12. V. F. M. Segers and R. T. Lee, Nature, 2008, 451, 937-942.
13. F. Doetsch, Current opinion in genetics & development, 2003, 13, 543-550.
14. A. Spradling, D. Drummond-Barbosa and T. Kai, Nature, 2001, 414, 98-104.
15. E. Fuchs, T. Tumbar and G. Guasch, Cell, 2004, 116, 769-778.
16. S. J. Morrison and A. C. Spradling, Cell, 2008, 132, 598-611.
17. D. E. Discher, D. J. Mooney and P. W. Zandstra, Science, 2009, 324, 1673-1677.
18. R. Peerani and P. W. Zandstra, The Journal of clinical investigation, 2010, 120, 60.
19. A. Mohyeldin, T. Garzón-Muvdi and A. Quiñones-Hinojosa, Cell stem cell, 2010, 7, 150-161.
20. J. Drago, V. Nurcombe and P. F. Bartlett, Experimental cell research, 1991, 192, 256-265.
21. L. A. Flanagan, L. M. Rebaza, S. Derzic, P. H. Schwartz and E. S. Monuki, Journal of neuroscience research, 2006, 83, 845-856.
22. R. O. Hynes, Cell, 2002, 110, 673-687.
23. T. S. Jacques, J. B. Relvas, S. Nishimura, R. Pytela, G. M. Edwards and C. H. Streuli, Development, 1998, 125, 3167-3177.
24. I. Kazanis, J. D. Lathia, T. J. Vadakkan, E. Raborn, R. Wan, M. R. Mughal, D. M. Eckley, T. Sasaki, B. Patton and M. P. Mattson, The Journal of Neuroscience, 2010, 30, 9771-9781.
25. J. D. Lathia, B. Patton, D. M. Eckley, T. Magnus, M. R. Mughal, T. Sasaki, M. A. Caldwell, M. S. Rao and M. P. Mattson, Journal of Comparative Neurology, 2007, 505, 630-643.
26. J. Dings, J. Meixensberger, A. Jäger and K. Roosen, Neurosurgery, 1998, 43, 1082-1094.
27. G. Santilli, G. Lamorte, L. Carlessi, D. Ferrari, L. Rota Nodari, E. Binda, D. Delia, A. L. Vescovi, L. De Filippis and J. Najbauer, PloS one, 2010, 5, e8575.

28. L. Studer, M. Csete, S.-H. Lee, N. Kabbani, J. Walikonis, B. Wold and R. McKay, The Journal of Neuroscience, 2000, 20, 7377-7383.

29. E. D. Hay, The Journal of cell biology, 1981, 91, 205s-223s.

30. R. O. Hynes, Science, 2009, 326, 1216-1219.

31. A. Alvarez-Buylla and D. A. Lim, Neuron, 2004, 41, 683-686.

32. F. Mercier, J. T. Kitasako and G. I. Hatton, Journal of Comparative Neurology, 2002, 451, 170-188.

33. S. A. Ali, I. S. Pappas and J. G. Parnavelas, Developmental brain research, 1998, 110, 31-38.

34. A. L. Pearlman and A. M. Sheppard, Progress in brain research, 1995, 108, 117-134.

35. A. Krishan and R. M. Hamelik, in Current Protocols in Cytometry, John Wiley & Sons, Inc., 2001, DOI: 10.1002/0471142956.cy0736s52.

36. Y. Gavrieli, Y. Sherman and S. A. Ben-Sasson, The Journal of cell biology, 1992, 119, 493-501.

37. P. E. Hall, J. D. Lathia and M. A. Caldwell, BMC neuroscience, 2008, 9, 71.

38. K. R. Legate, S. A. Wickström and R. Fässler, Genes & development, 2009, 23, 397-418.

39. P. E. Hall, J. D. Lathia, N. G. A. Miller, M. A. Caldwell and C. Ffrench-Constant, Stem Cells, 2006, 24, 2078-2084.

40. M. Barczyk, S. Carracedo and D. Gullberg, Cell and tissue research, 2010, 339, 269-280.

41. S. M. Frisch and H. Francis, The Journal of cell biology, 1994, 124, 619-626.

42. S. M. Frisch and E. Ruoslahti, Current opinion in cell biology, 1997, 9, 701-706.

43. H. K. Kleinman, M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason and G. R. Martin, Biochemistry, 1982, 21, 6188-6193.

44. D. P. Leone, J. B. Relvas, L. S. Campos, S. Hemmi, C. Brakebusch, R. Fässler and U. Suter, Journal of cell science, 2005, 118, 2589-2599.

45. D. Gospodarowicz and J. Cheng, Journal of cellular physiology, 1986, 128, 475-484.

46. G. F. Whalen, Y. Shing and J. Folkman, Growth Factors, 1989, 1, 157-164.

47. T. H. Nguyen, S.-H. Kim, C. G. Decker, D. Y. Wong, J. A. Loo and H. D. Maynard, Nature chemistry, 2013, 5, 221-227.

Figure Captions

Fig.1. Number of total cells ($DAPI^+$) at days 2 and 4 cultured on fibronectin, collagen IV, laminin, and Matrigel substrates. After 2 and 4 d, there were no statistical differences in cell number between normoxia and hypoxia for the cells cultured on fibronectin and collagen IV. The total number of cells was significantly higher under hypoxia in comparison with normoxia at day 4 for the cells cultured on laminin and Matrigel ($p<0.05$). The total number of cells cultured on Matrigel are significantly higher than laminin under both normoxic and hypoxic conditions ($p<0.0001$). Symbol (*) denotes significant differences ($p<0.05$) between normoxic and hypoxic groups. n>9 samples; one representative set of experimental data is shown. n>50 hNSCs/sample.

Fig.2. The proliferation of hNSCs on fibronectin and collagen IV (**A**) and laminin and Matrigel (**B**) substrates. The cells cultured on fibronectin and collagen IV did not exhibit a significant difference in number of actively proliferating cells (EdU^+) between normoxia and hypoxia. The cells cultured on laminin and Matrigel had higher proliferation rates under hypoxia in comparison with normoxia ($p<0.01$). The percentage of EdU^+ cells was significantly higher for the cells cultured on Matrigel compared to laminin under both normoxic and hypoxic conditions. Symbols denote significant differences (*, $p<0.01$ and **, $p<0.0001$). A two-way ANOVA analysis indicated significant effects of substrate type and oxygen concentration on hNSC proliferation. Bars represent mean ± SEM. n>9 independent experiments; n>50 NSCs/sample.

Fig.3. The apoptosis of hNSCs on cultured for 4 days on fibronectin and collagen IV (**A**) and on laminin and Matrigel (**B**) substrates. The cells cultured on collagen IV and fibronectin exhibited ~4 fold and ~2.5 fold higher percentages of apoptotic cells ($TUNEL^+$) compared to laminin-rich substrates, respectively. A two-way ANOVA analysis indicated a significant effect of substrate ($p<0.001$) on hNSC apoptosis with no significant effect of oxygen concentration. Bars represent mean ± SEM. n>9 independent experiments; n>50 NSCs/sample.

Fig.4. Expression of stemness markers in hNSCs culture. Representative fluorescent images of hNSCs cultured on laminin-coated 96-well plates. The hNSCs were labeled for SOX-2 (**A and B**), nestin (**C and D**), and GFAP (**E and F**). No neuronal ($TUJ\text{-}1^+$) cells were detected. Scale bar, 50 µm.

Figure 1

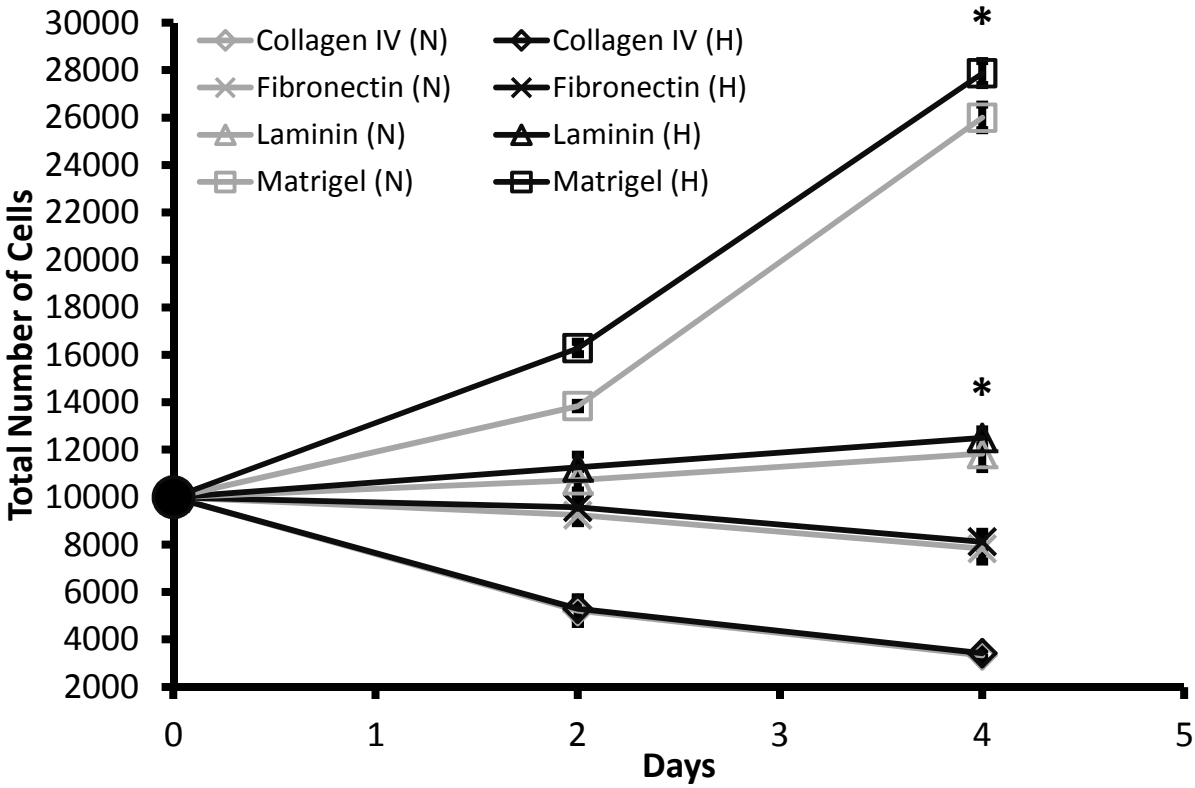


Figure 2

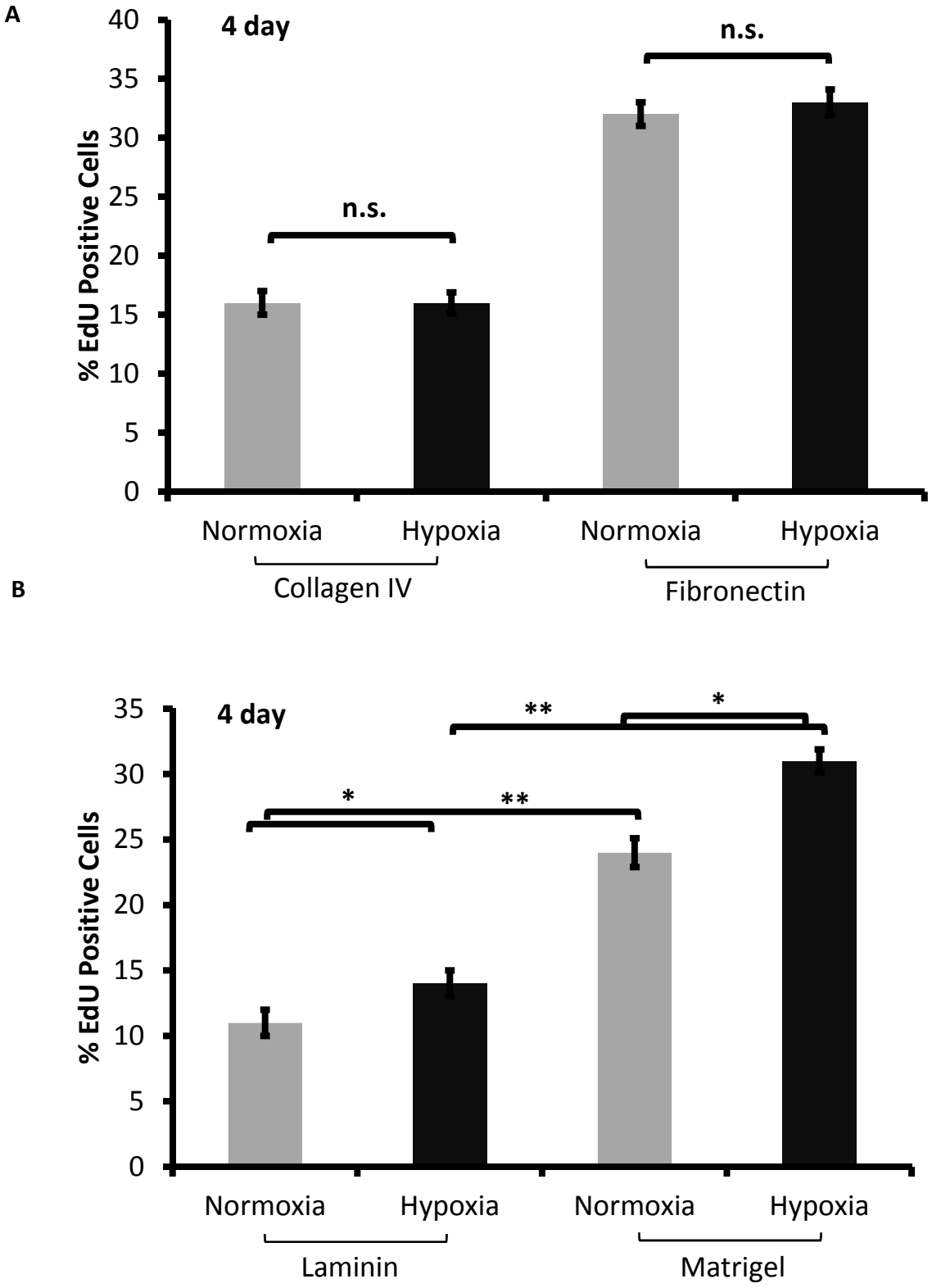


Figure 3

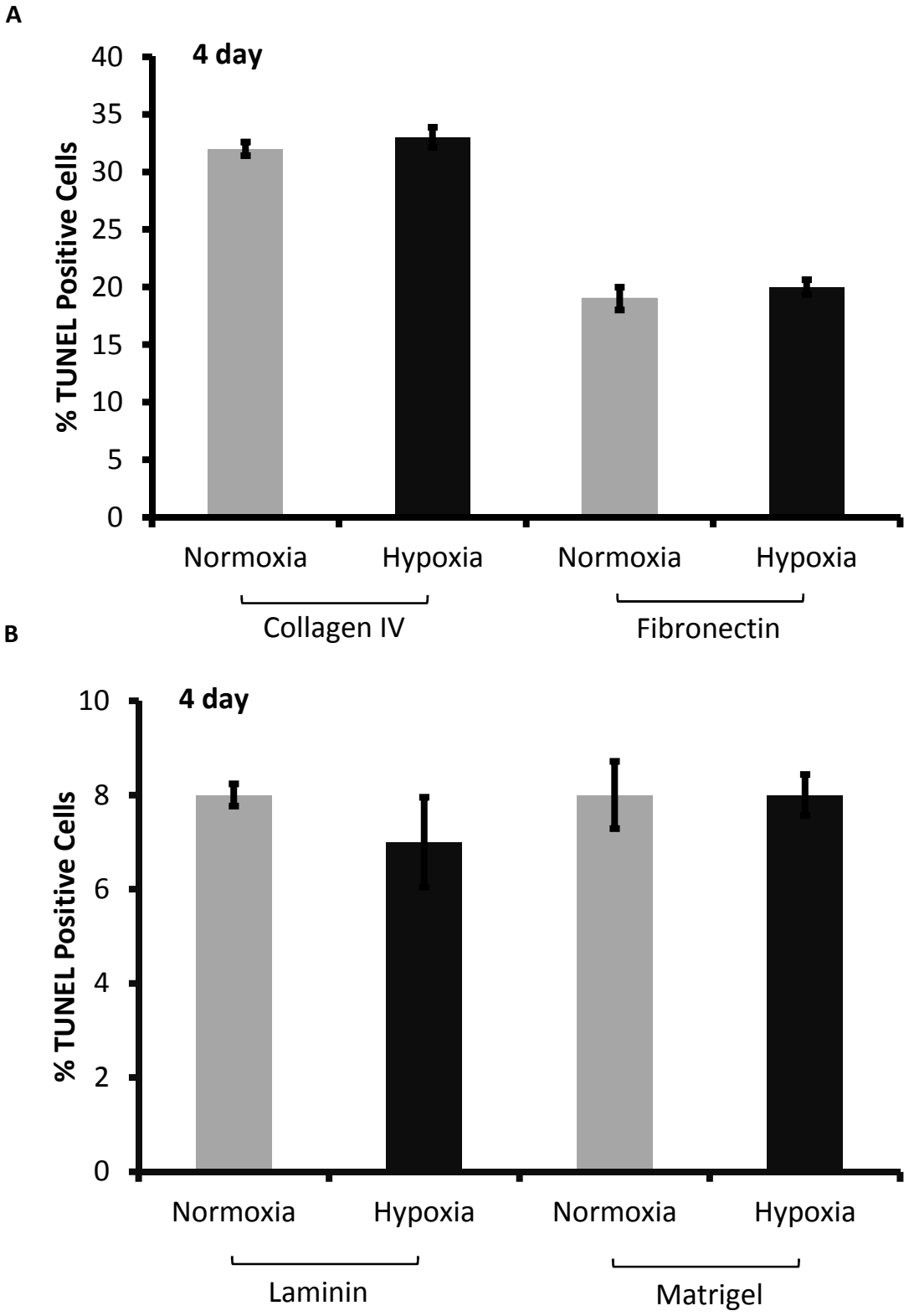
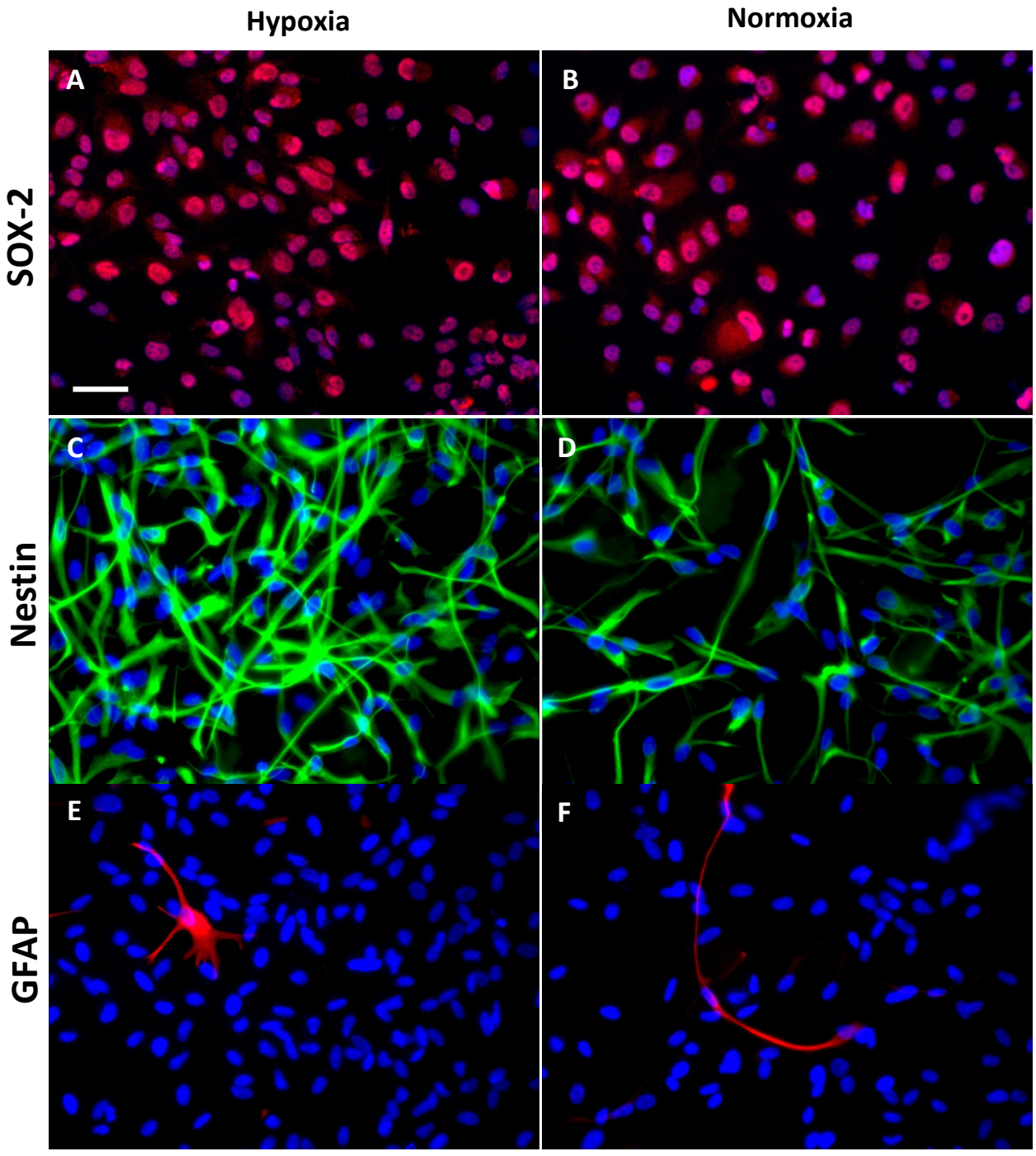


Figure 4



Graphical Abstract for Ghourichaee and Leach

Human neural stem cells cultured on laminin and Matrigel under hypoxia significantly increases both the stem cell density and the percentage of actively proliferating cells.