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A co-culture model of the hippocampal neurogenic niche reveals differential effects of astrocytes, endothelial cells and pericytes on proliferation and differentiation of adult murine precursor cells

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ABSTRACT

The niche concept of stem cell biology proposes a functional unit between the precursor cells and their local microenvironment, to which several cell types might contribute by cell–cell contacts, extracellular matrix, and humoral factors. We here established three co-culture models (with cell types separated by membrane) for both adherent monolayers and neurospheres to address the potential influence of different niche cell types in the neurogenic zone of the adult hippocampus of mice. Astrocytes and endothelial cells enhanced precursor cell proliferation and neurosphere formation. Endothelial factors also led to a prolonged increase in proliferation after growth factor withdrawal, which otherwise induces differentiation. All niche cell types enhanced cell survival in monolayer cultures, endothelial cells also stimulated neuronal differentiation. A parallel trend elicited by astrocytes did not reach conventional statistical significance. Pericytes had variable effects here. We did not observe changes in differentiation in neurosphere co-cultures. In summary, our data indicate that in precursor cell culture protocols survival could be improved by adding as yet unknown factors physiologically contributed by astrocytes and endothelial cells. Our findings also underscore the complexity of the niche and the differential impact of factors from the different sources on distinct aspects of neuronal development. With the help of the models presented here, identification of these factors and their specific biological activity can now be initiated.

1. Introduction

In the adult mammalian brain, stem and precursor cells localize close to vasculature in two distinct niches, the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Palmer et al., 2000). Although neurogenesis in the SGZ can be increased by systemic interventions like environmental enrichment, physical activity and exploratory behavior (Freund et al., 2013; Kempermann et al., 1997; Kronenberg et al., 2003; Van Praag et al., 1999), and can be down-regulated by stress (Schoenfeld and Gould, 2013), we lack detailed information on how exactly such systemic changes are conveyed to the neurogenic precursor cells in the niche.

The niche concept of stem cell biology proposes that stem cells require local environmental cues to control self-renewal and differentiation (Guilak et al., 2009; Scadden, 2006). In the hippocampus, this mediation will take place at several levels (Kempermann, 2011), presumably in parallel and in an interactive manner, involving direct cell-to-cell signaling via gap-junctions (Kunze et al., 2009), paracrine signals such as Wnt (Lie et al., 2005), neurotransmitters, most notably glutamate (Cameron et al., 1995) and GABA (Ge et al., 2006; Wang et al., 2005), as well as systemic factors, including growth factors and hormones.

A number of studies have addressed the same issue for the SVZ. Lim and Alvarez-Buylla first described the close interaction of astrocytes and neural precursor cells in the SVZ niche (Lim and Alvarez-Buylla, 1999), which paralleled the discovery by the same group that the precursor cells actually have astrocyte-like properties themselves (Doetsch et al., 1999), also in the human brain (Sanai et al., 2004).

That group also highlighted the lineage-relationship between astrocyte-like precursor cells and the generation of oligodendrocytes in the SVZ (Menn et al., 2006). Whereas in the SGZ a small number of new oligodendrocytes is found (Kempermann et al., 2003), these originate from a different set of precursor cells than the neurons and astrocytes. Nevertheless, our precursor cell cultures reveal potency for neuronal, astrocytic and oligodendrocytic lineages (Babu et al., 2011), although extremely few oligodendrocytes are produced. They are thus not routinely assessed in our assays, especially given the fact that they are of different origin in vivo under normal condition. Their production

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can, however, be induced by overexpression of Ascl1 in intermediate progenitor cells (Jessberger et al., 2008).

However, the relative contribution of factors provided by the different niche cells including the precursor cells themselves, endothelial cells, pericytes, neurons and astrocytes to this concert has not yet been systematically explored for the hippocampus. There are no widely usable in vitro systems that would allow further exploration of these questions, although the issue has been discussed for more than a decade (Wurmser et al., 2004). We and others have explored the effects of neuronal activity on precursor cell behavior in co-culture systems, identifying oscillatory excitation and BDNF release as putative mechanisms (Babu et al., 2009; Deisseroth et al., 2004). A few other studies have addressed the contribution of astrocytes (Barkho et al., 2006; Oh et al., 2010; Song et al., 2002). In one of these studies, it was found that astrocyte-dependent Wnt-signaling is a key factor for controlling neuronal differentiation from adult hippocampal precursor cells in vitro (Lie et al., 2005). For further exploration of the factors involved, a parallel examination of different cell types will be useful. Precursor cells from the SVZ have been co-cultured with neurons, endothelial and vascular smooth muscle (vSMC) lines in vitro demonstrating the strength of such an approach (Shen et al., 2004) but without leading to similar follow-up studies for the hippocampus. For the SVZ such studies have already led to a thorough understanding of the vascular niche and the identification of individual factors such as PEGF, SDF1/CXCR4 or VCAM1 (Andreu-Agulló et al., 2009; Kokovay et al., 2010, 2012).

We thus made here use of the advantages of transwell co-culture systems to segregate the cellular cross-talk of the complex niche environment in the SGZ into individual cell type-specific interactions.

2. Results

2.1. The hippocampal niche in vivo

In order to recapitulate and refine interactions in the neurogenic niche of the hippocampus, we first performed 3D reconstructions from images acquired by confocal laser scanning microscopy and visualized the cellular relationships. With one exception (Boström et al., 2014), previous reconstructions had not explicitly included pericytes (Palmer et al., 2000). Nestin-GFP$^+$ hippocampal precursor cells have vascular endfeet (Fig. 1B and B') contacting the endothelial cells (see also (Filippov et al., 2003)). The small blood vessels are built from endothelial cells and are densely covered by pericytes. The neural precursor cells are further surrounded by astrocytes (Fig. 1B and B").

We here focused on astrocytes, pericytes and endothelial cells, presumably forming the vascular niche proper. Besides these cells, the niche also contains nestin-negative progenitor cells (type-3), microglia and granule cell neurons. There are only capillaries in the SGZ, so no vascular smooth muscle cells are found (Ehret et al., 2015) but current isolation protocols for pericytes do not yet distinguish between these populations.

2.2. Establishing a selective hippocampal niche model

For further analysis of humoral cell-to-cell interaction in the SGZ we next established primary cultures of the three niche cell types found in close proximity to the precursor cells. Primary culture of astrocytes, endothelial cells and pericytes/vSMCs were set up to bring these niche models as close as possible to the in vivo situation (Fig. 2A). Pericyte/vSMC and endothelial cell enriched cultures were established from adult cerebral cortex tissue, because isolation from the dentate gyrus did not yield sufficient numbers of proliferating cells. Vessel cells only stay proliferative for 1 to 2 passages (P), thus fresh primary cultures had to be established 2 weeks prior to each co-culture. For this, cortical tissue from 6 to 8 adult mice was centrifuged through sucrose cushions to specifically isolate vessels. By using different sieves, large vessel could be separated from smaller vessel and digested separately. The larger vessels (>100 μm) were used for pericytes/vSMC preparations and the smaller vessels (50–100 μm) were used for endothelial cell culture; selective media was used to enrich these primary cultures. For comparison, an established brain endothelial cell line (bEnd3, purchased from ATCC) was used. Hippocampal astrocyte culture was established from adolescent mice (4 weeks old). Cells were cultured on poly-D-lysin coated surfaces. As an alternative source of astrocytes, neuronal precursor cells (P8) were differentiated by the addition of 2% fetal bovin serum (FBS). All primary cultures reached a purity of 75–90% depending on the preparation as evaluated by immunocytochemistry (Fig. 2B–P). Primary astrocytes isolated from the dentate gyrus showed 3–5% contamination by microglia (CD11b$^+$ cells; Fig. 2M), 3–6% endothelial cells (VE-Cadherin$^+$ or vWF$^+$ cells) and 8–10% vSMC cells (smooth muscle cell actin, SMCA$^+$ cells). Astrocytes differentiated from neural precursor cells did not show any contamination with neurons, microglia or oligodendrocytes (Fig. 2G, L). Pericyte/vSMC culture showed 4–7% contamination with endothelial cells (VE-cadherin$^+$ or vWF$^+$ cells) and 2–5% astrocytes (GFAP$^+$ cells) but no neuronal contamination (Fig. 2I and N). Primary endothelial cells showed 3–6% contamination by smooth muscle cells (SMCA$^+$

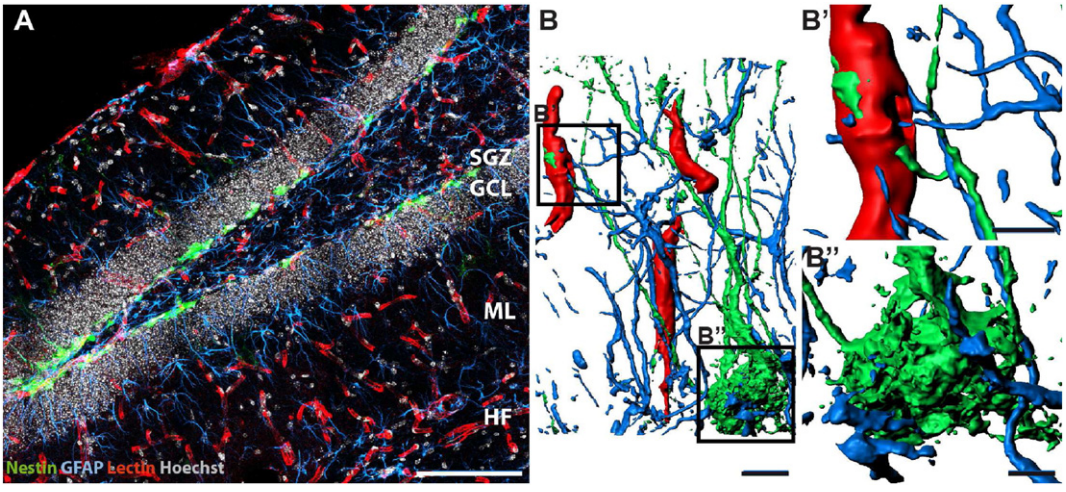


Fig. 1. The neurogenic niche of the hippocampus. Nestin$^+$ stem and precursor cells of the subgranular zone (SGZ) dentate gyrus are in contact with small capillaries and surrounding astrocytes. (A) Microscopic image of a dentate gyrus tissue section depicts the vascular network and the distribution of astrocytes in the dentate gyrus. (B) 3D reconstruction of confocal images of the neurovascular niche stretching from SGZ to granular cell layer (GCL). (B') Spatial proximity of Nestin$^+$ precursor cells and astrocyte projections close to blood vessel. (B") Astrocytic projection in contact with soma of Nestin$^+$ cells. ML, molecular layer; HF, hippocampal fissure. Scale bar represents 100 μm (A), 10 μm (B) and 5 μm (B' and B").

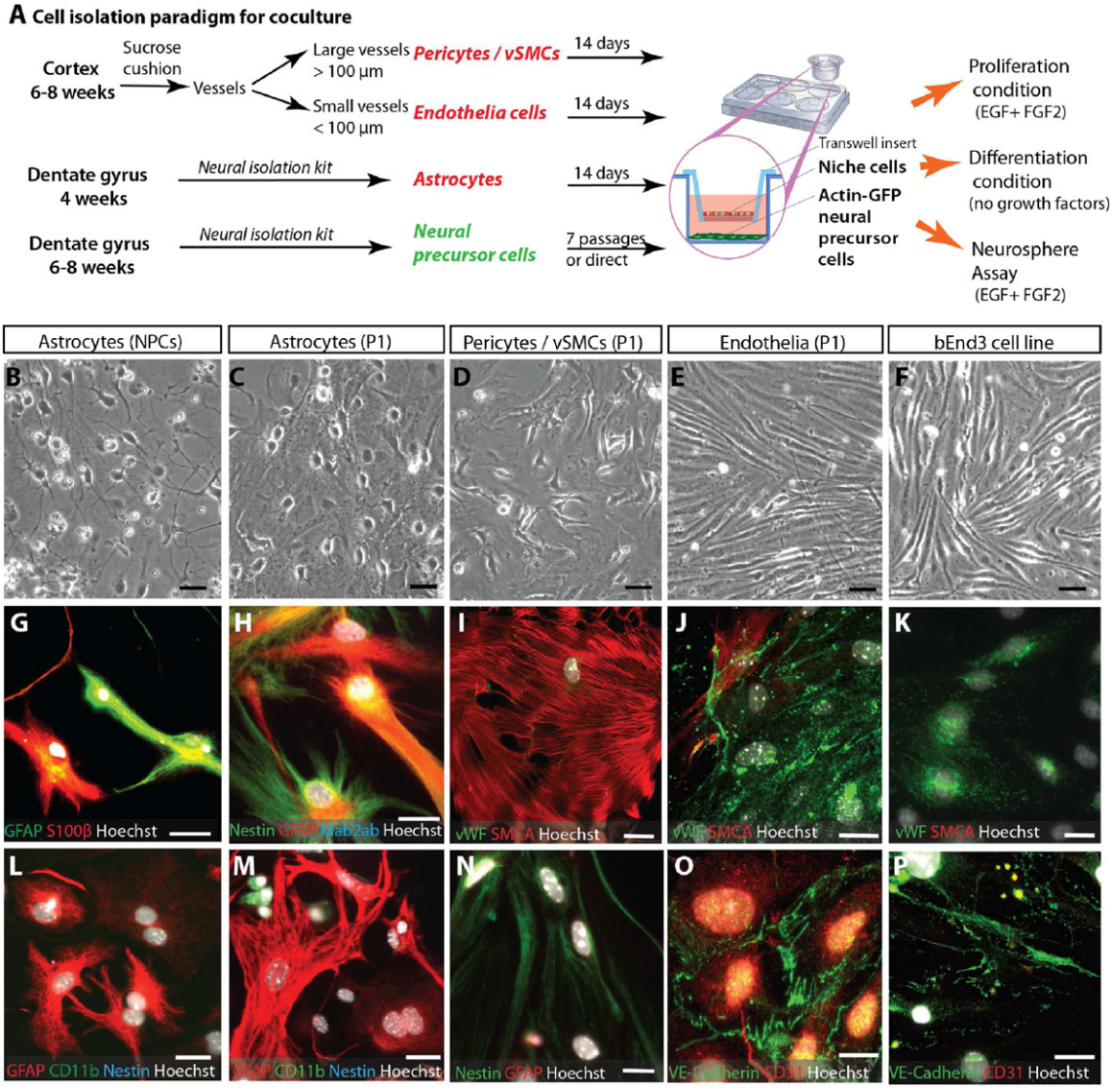


Fig. 2. Isolation and characterization of the different niche cell populations. (A) Illustration of cell isolation paradigm for primary cells used in this co-culture system. (B–F) Phase contrast images of the different cell population used in the niche co-culture. (G, L) Neural precursor cells differentiated into astrocytes by fetal calf serum express GFAP, S100β but not CD11b or Nestin. (H, M) Primary astrocytes express Nestin, GFAP and some CD11b$^+$ cells but no Mab2ab$^+$ cells were found. (I, N) Pericyte/vascular smooth muscle cell (vSMC) cultures express SMC actin (SMCA) and low levels of Nestin and only a view positive cells that are GFAP$^+$ or vWF$^+$ were found. (J, O) Primary endothelial cells express Von-Willebrand-factor (vWF), VE-Cadherin and CD31 and show only little contamination through SMCA. (K, P) brain endothelial cell line (bEnd3) showed no expression of SMCA but also only a weak expression of vWF, VE-Cadherin, CD31. Scale bar 10 µm (B–O).

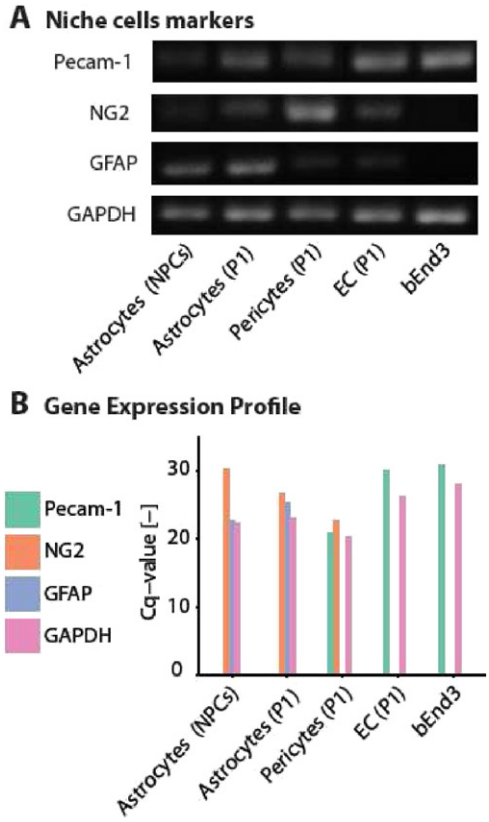
cells) and 2–3% astrocytes (GFAP$^+$ cells) but no neuronal contamination (Fig. 2J and O). The bEnd3 cell line was free of astrocytes, pericytes or neurons but showed also low expression levels of VE-Cadherin, CD31 or vWF (Fig. 2K, P). It can be concluded that none of the primary cultures was 100% pure but that a sufficient enrichment of the desired cell population had been obtained. Messenger RNA from the different cell populations was isolated to confirm the enrichment of the different cell populations. Gene expression for endothelia marker Pecam-1, pericyte marker NG2 and astrocyte marker GFAP were analyzed by PCR and confirmed sufficient enrichment of the targeted cell population (Fig. 3A).

These enriched niche cells were co-cultured with neuronal precursor cells isolated from the dentate gyrus. For this, neural precursor cell cultures from actin-GFP expressing mice were created (Babu et al., 2011). GFP expression of neural precursor cell was stable over several passages and used to directly monitor cell growth. Cells were used at passage 8 and showed 100% purity, as evaluated by the expression of Nestin and Sox2 (Fig. 3B–C). These cultures showed multipotency in vitro and could be differentiated into astrocytes and neurons.

2.3. Co-cultures revealed critical interaction of endothelial cells and astrocytes with hippocampal precursor cells

Niche cells were seeded into transwell net inserts 2 days prior to co-culture in their respective growth medium to allow attachment. On the day of co-culture, the niche cells were transferred to DMEM with or without growth factors depending on the intended analysis. Adherent actin-GFP neural precursor cells (P8) were seeded into the wells. For comparison, primary neuronal precursor cells from actin-GFP mice (6–8 weeks old) were also cultured as neurospheres, which represent self-assembling agglomerates with certain intrinsic niche properties.

After 5 days in proliferation medium the number of neural precursor cells without co-cultured niche cells had increased 4.3-fold, whereas precursor cells co-cultured with primary endothelial cells increased 7.4-fold and with primary astrocytes 6.1-fold ($F_{(5,59)} = 2.94$, $p = 0.019$; comparison endothelial cells vs. no niche cells: $p = 0.007$ [Dunnett's test]; comparison astrocytes vs. no niche cells: $p = 0.095$).



Cell survival was assessed under differentiation condition (graded growth factor withdrawal) over a period of 7 days. All three co-culture conditions resulted in a significant enhancement of cell survival compared to precursor cells without niche cells (repeated measures ANOVA $F_{(5,39)} = 7.663$, $p < 0.001$; endothelial cells vs. no niche cells $p < 0.001$ [Dunnett's test]; bEnd3 vs. no niche cells: $p < 0.001$; astrocytes vs. no niche cells: $p = 0.019$; astrocytes (NPC) vs. no niche cells: $p = 0.039$; pericytes vs. no niche cells: $p = 0.0265$). In addition, we found a significant enhancement of neuronal differentiation in co-cultures with primary endothelial cells (10.96 ± 6.89%) and bEnd3 cell line (11.55 ± 6.94%) in comparison to precursor differentiation without co-culture (1.13 ± 1.13%; one-way ANOVA $F_{(5,30)} = 4.51$, $p = 0.003$; endothelial cells vs. no niche cells: $p = 0.013$; bEnd3 vs. no niche cells: $p = 0.008$; Fig. 4G). Primary astrocytes promoted neuronal differentiation at $p = 0.101$. On average, differentiating neurons in co-cultures developed 1–3 neurites over the observation period, which did not obviously differ from baseline conditions (Fig. 4G).

Taken together, analysis of proliferation, survival and differentiation of adherent neural precursor cells in co-culture with niche cells in a transwell inset above revealed that endothelial cells enhanced proliferation of precursor cells and differentiation into neuronal linage. Pericytes and astrocytes showed partial effects.

Analysis of the differentiation potential of the neural precursor cells into astrocytes, in contrast did not reveal any co-culture dependent influences (one-way ANOVA, $F_{(5,30)} = 0.16$; $p = 0.97$).

2.4. Astrocytes enhance neurosphere formation

In contrast to this observation we found that co-culture with primary astrocytes increased the number of neurospheres 2.4-fold, whereas co-cultures with astrocytes differentiated from neural precursor cells led to a 1.9-fold increase. Co-cultures with primary endothelial cells or pericytes did not significantly increase sphere formation, but co-cultures with the bEnd3 cell line increased neurosphere formation by 1.7-fold (one-way ANOVA, $F_{(5,50)} = 7.91$, $p < 0.001$ M; astrocytes (NPCs) vs. no niche cells $p < 0.001$; astrocytes vs. no niche cells $p = 0.021$; bEnd3 vs. no niche cells $p = 0.031$). When only the number of large spheres (≥100 μm) was taken into consideration, only astrocytes derived from neural precursor cells lead to a significant 3-fold induction ($p = 0.002$). A similar trend was also found for primary astrocytes (one-way ANOVA $F_{(5,45)} = 8.75$, $p < 0.001$; astrocytes vs. no niche cells: $p = 0.127$). Thus, astrocytes played a major role in providing soluble trophic support to enhance formation and growth of neurospheres, but had fewer effects on neural precursor cells under monolayer conditions.

The differentiation of neurospheres after niche cell co-culture revealed no clear statistically significant effect. While the one-way ANOVA ($F_{(5,30)} = 2.16$) resulted in a p-value of $p = 0.085$, the individual post hoc comparisons were not statistically significant at $p < 0.05$. Although co-cultures of primary endothelial cells had a small impact on to differentiation into neuronal linage, only a weak interaction was detected (18.3 ± 5.8% in EC co-culture vs. in co-cultures with no niche cells; $p = 0.580$). On the other hand, pericyte co-culture might have a slight negative influence on neuronal differentiation, which was not statistically significant under the present conditions, however (2.6% ± 1.4% neurons vs. 11.5 ± 3.2% with no niche cells, $p = 0.324$).

Fig. 3. Marker expression of the different niche cells and hippocampal precursor cells. (A) Pecam-1 an endothelia cell marker is expressed at high levels in primary endothelial cells and in the endothelial cell line bEnd3. NG2, a gene expressed in pericytes shows high levels of expression in primary pericyte culture. GFAP an astrocyte marker is expressed at high levels in primary astrocytes and astrocytes differentiated from neural precursor cells (NPCs). Quantification of GAPDH was used as a loading control. (B) qPCR analysis of the different niche cell preparations. (C) Phase contrast image of the proliferative hippocampal precursor cells. (D) Expression of Nestin and Sox-2 in all hippocampal precursor cells. Scale bar, 10 μm (C–D).

Neurite outgrowth from primary spheres was not noticeably affected by co-culture conditions. Analysis of astrocytic differentiation revealed that in all preparations 43–75% of the sphere cells were GFAP$^+$ astrocytes and no significant impact of co-culture conditions could be found (one-way ANOVA, $F_{(5,30)} = 1.81$, $p = 0.143$). Taken together, the formation of neurospheres can be enhanced by astrocytic co-culture but

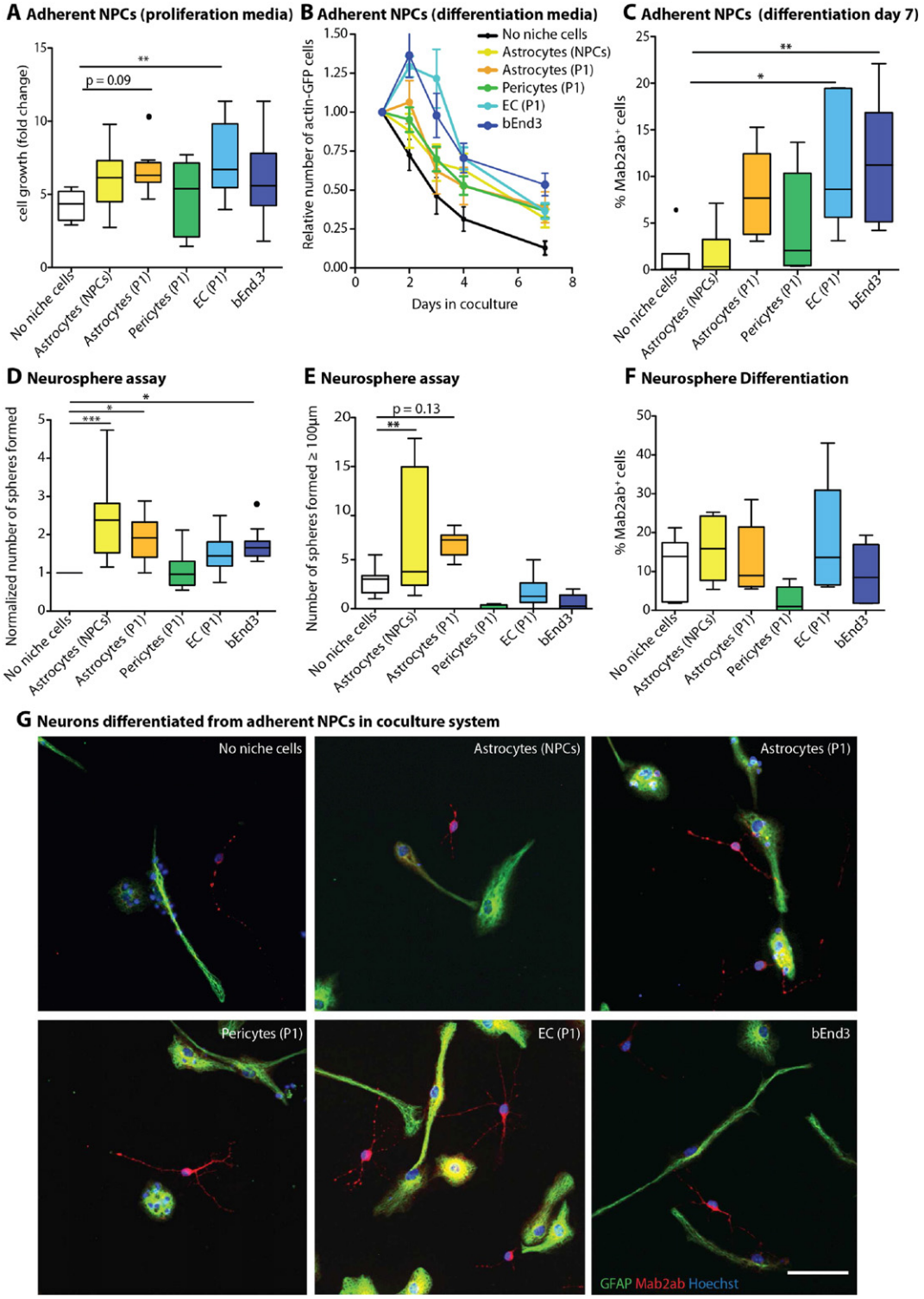


Fig. 4. Effects of niche co-culture on to cell proliferation, survival and neuronal differentiation of hippocampal precursor cells. The influence of niche cell co-culture on neural precursor cells (NPCs) proliferation differentiation and neurosphere formation. (A) Adherent NPCs under proliferative conditions increased cell growth significantly in co-culture with primary endothelial cells (EC) ($n = 9$–11). (B) Adherent NPCs under differentiation conditions survived significantly better in all niche co-cultures over a time-course of 7 days ($n = 11$). (C) The differentiation of NPCs into neuronal linage was increased in co-culture with primary EC and the endothelial cell line bEnd3 ($n = 6$). (D) Primary neurosphere formation after 10 days in co-culture was increased in preparations with astrocytes from primary culture or differentiated from NPCS as well as in co-cultures with EC ($n = 10$). (E) Particularly astrocyte co-culture enhanced the formation of large spheres over 100 µm ($n = 8$). (F) Co-culture does not affect the differentiation of neurospheres into neuronal and astrocytic linage ($n = 6$). (G) New neurons differentiated from adherent NPCs in co-culture with EC, pericytes and bEnd3 cells showed increased dendritic complexity. Data are represented as Box Plot with whiskers to $\pm 1.5 \times$ interquartile range. Scale bar 50 µm (G).

neither astrocyte nor endothelial cell co-culture significantly influenced the differentiation of the precursor cells.

3. Discussion

We here evaluated the humoral influences of three different niche cell populations on proliferation and differentiation of adult hippocampal precursor cells in two culture models. We found that astrocytes provide soluble factors enhancing cell growth of neural precursor cells; whereas endothelial cells rather support their differentiation into the neuronal linage. Pericytes did not secrete soluble factors influencing cell growth or differentiation.

The essential role of astrocytes in the adult stem cell niche has been rarely investigated in the adult hippocampus. Only one study by Song et al. had shown that astrocytes from the adult hippocampus of rats increased the rate of proliferation and neuronal fate commitment (Song et al., 2002). A similar relationship was observed in SVZ precursor cells co-cultured with murine astrocytes of the early postnatal period (Lim and Alvarez-Buylla, 1999). Thus, we could reproducibly show that astrocytes secrete factors enhancing cell growth. This is also in line with the finding that astrocytic adenosine 5′-triphosphate release induces precursor cell proliferation in the adult hippocampus (Cao et al., 2013). On the other hand, we did not find here that astrocytes enhanced neuronal fate as proposed by Lie et al. (Lie et al., 2005). The key difference between their study and ours is, however, that they used neural precursor cells in direct contact with a feeder layer of astrocytes, which might point to the plausible idea that secreted and direct cell-to-cell mediators must go hand in hand. On one side this underscores the limitations of reductionistic assays, on the other side it confirms that single experiments will not deliver the full and complex picture.

Second, we showed that endothelial cells can enhance neuronal differentiation. The influence of endothelial cells on supporting neuronal differentiation in murine precursor cells from adult SVZ had been observed previously (Shen et al., 2004). Although the study by Shen et al. was also performed in a transwell system with no direct cell contact of endothelial cells and precursor cells, an increased activation of Notch signaling target Hes1 was detected and seen as a mechanism for the continuous proliferation. Another study by Leventhal et al. showed that rat neural precursor cells from SVZ grown on top of endothelial cells had increased cell survival and generated more neurons, whereas co-cultures with fibroblasts and astrocytes did not (Leventhal et al., 1999). Taken together, our observed effects are in line with the reports by Shen et al. and Leventhal et al. but provide an additional perspective on the regulation in the hippocampal niche.

One of the factors that have been identified as mediator between endothelial cells and neural stem cells of the SVZ is Pigment epithelium-derived factor (PEDF) (Ramírez-Castillejo et al., 2006). PEGF had a distinct effect on self-renewal (see, for example discussion in Ref. (Pumiglia and Temple, 2006)) and enhances Notch signaling (Andreu-Agulló et al., 2009). Our culture system will now allow similar investigations for adult hippocampal precursor cells.

While we showed that endothelial cells and astrocytes contribute to the regulation in the neurogenic niche of the hippocampus, the role of pericytes is far less conclusive. For most time pericytes have received relatively little attention and only more recently their role in the context of regeneration and plasticity has been highlighted by demonstrating their activity in scarring after spinal cord injury (Göritz et al., 2011). Additional studies investigated the specific function of these vSMC-like cells in the regulation of blood flow and vascular integrity in health and disease as well as during aging (Bell et al., 2010; Hall et al., 2015). But pericytes had not been tested in a neurogenic niche model. Shen et al. had used a smooth muscle cell line in a transwell co-culture system only in the context of SVZ precursor cells (Shen et al., 2004). They found that vSMC indeed increased proliferation but clones were less cohesive and spontaneously differentiated into glial like progeny. We similarly observed no effect in neuronal cell fate choice and found that most cells differentiated into glial like progeny (75.68 ± 5.99%). Pericyte co-cultures also increased the adhesion of clones to the plate and thus rather inhibited sphere formation. Thus, pericytes appear to reduce cohesive properties in the hippocampus and in their presence precursor cells rather started to grow on the uncoated plate surface.

The presented transwell assays allow taking into account three major niche cell populations in parallel to compare their influences on neural precursor cell proliferation and differentiation. With this model we might begin to understand the interaction of the concert of factors secreted in the neurogenic niche, which will complement the physical interaction of the cells, including the biophysical properties of the tissue itself.

Direct co-cultures of hippocampal precursor cells with the three different niche cells are as yet impossible because of their different growth requirements. This, by itself, is an interesting observation, indicating that in vivo, the cells must manage to listen to only the signal relevant to them or have found cell-autonomous mechanisms that actually reduce niche impact.

Independent of this, our study provides evidence that endothelial cells, astrocytes and pericytes might have distinct, even though presumably overlapping functions in the neurogenic niche of the adult hippocampus. We hypothesize that pericytes mediate cell adhesion, astrocytes primarily support proliferation and endothelial cells might predominantly control neuronal commitment. The exact nature of the secreted molecules remains to be established. And the goal has to remain to create three-dimensional models representing the impact of both humoral factors and the direct cellular interactions in the niche.

4. Material and methods

4.1. Animals

C57BL/6 mice (Charles River), actin-GFP mice (Okabe et al., 1997) and Nestin-GFP mice (Yamaguchi et al., 2000) were maintained at Medizinisch Theoretisches Zentrum, Technische Universität Dresden, Germany. All experiments were conducted in accordance with the applicable European and national regulations (Tierschutzgesetz) and approved by the responsible authority (Regierungspräsidium Dresden). Mice were maintained on a 12 h light/dark cycle with food and water provided ad libitum.

4.2. Tissue preparation and immunohistochemistry

Mice were killed by an overdose of ketamine and transcardial perfusion with 0.9% NaCl and 4% paraformaldehyde (PFA). 40 μm thick serial coronal brain sections were cut on a freezing microtome (Microm HM430, Thermo Scientific) and stored at −20 °C in cryoprotectant solution (25% ethylene glycol, 25% glycerol in 0.1 M phosphate buffer, pH 7.4). Immunohistochemistry of the brain section was performed as described previously (Kempermann et al., 2003). In brief, after 1 h blocking in 10% donkey serum with 0.3% Triton-X100, the sections were incubated in primary antibody solution for 24 h at 4 °C in 3% donkey serum. The following antibodies were used: chicken anti-GFAP (1:500; ab50738, Abcam), biotinylated *Lycopersicon Esculentum* lectin (1:4000; B1175, Covance). After primary incubation, sections were washed in TBS, followed by secondary antibody incubation over night at 4 °C. All secondary antibodies were purchased from Dianova, streptavidin Dye Light 647, anti-chicken Cy3. Sections were washed again and mounted onto glass slides using Vectorshield mounting media (Vector laboratories). The images were obtained using a confocal microscope (LSM 780 NLO, Zeiss).

4.3. Immunocytochemistry

Cells in differentiation condition were fixed at day 7 for 10 min with 4% PFA and stained for analysis of differentiation. Fix cells were permeabilised with 0.2% Triton-X100 for 10 min when PFA fixation was used otherwise no permeabilisation was needed. Followed by blocking for 1 h with 10% donkey serum and incubated overnight at 4 °C with primary antibody solution in 3% donkey serum. The following antibodies were used: rat anti Cd11b (1:300; MAB1124, R&D), mouse anti-CD31 (1:200; sc13537, Santa Cruz), rabbit anti-GFAP (1:500, Z0334, Dako), mouse anti-Mab2ab (1:500; M1406, Sigma), rabbit anti-NG2 (1:200; sc20162, Santa Cruz), mouse anti-SMC-actin (1:2000; A2547, Sigma), S100β, rabbit anti-vWF (1:200; F3520, Sigma). After primary incubation, the sections were washed in TBS, followed by secondary antibody incubation for 2 h at room temperature. All secondary antibodies were purchased from Dianova: anti-goat Alexa 488, anti-mouse Alexa 488, anti-mouse Dye Light 549, anti-rabbit Cy3, anti-rabbit Alexa 647, anti-rat Alexa 488. After a final wash step, the sections were mounted in fluorescence mounting medium (Dako).

4.4. Image analysis

For 3D reconstruction Imaris software V7.4.2 (Bitplane, USA) was used. For automated counting of GFP+ cells the images were pre-processed (stitching, z-projection, contrast enhancement, gamma correction, edge enhancement). Segmentation and automatic counting was performed with object counter plugin in Fiji (objects between 50 and 200 μm). Correct object recognition was confirmed by validation through Hoechst labeling.

4.5. mRNA analysis

Messenger RNA was isolated with TRI Reagent (Sigma). All samples were digested with DNAse I (Invitrogen), transcribed into cDNA with SensiFAST cDNASynthesis Kit (Bioline #BIO-65,053) and amplified with Sensifast SYBR Mix (Bioline) in a real time PCR system (CFX96, Biorad).

The following primers were used: GFAP (forward: ACCCAGAAGACT GTGGATGG; reverse: CACATTGGGGGTAG GAACAC), NG2 (forward: GCGACTTGCGACTCGGTGCT; reverse: CCCGAAGAAGGAGGCAGGAGC), Pecam-1 (forward: CCGCGGTGTTCAGCGAGATCC; reverse: ACGTGATT GGGCTCGTCCCCT), GAPDH (forward: ACCCAGAAGACTGTGGATGG, reward: CACATTGGGGGTAGGAACAC).

PCR Products were load onto 1.5% agarose gel run in an electrophoresis chamber (Biorad) and analyzed in the darkhood (Biostep).

4.6. Cell culture

For all cell isolations mice were anesthetized, quickly decapitated and tissue of interest was microdissected. For isolation of hippocampal precursor cells the dentate gyri were microdissected (Walker and Kempermann, 2014) from actin-GFP mice. The tissue was enzymatically digested using the Neural Tissue Dissociation Kit (Miltenyi) according to the manufacturer's instructions. Cells were washed with HBSS (GE Healthcare) and filtered through a 40 μm cell sieve followed by centrifugation at 500 × g for 5 min. For adherent cultures, the pellet was resuspended in Neurobasal medium (Gibco) with B27 supplement (Gibco), 10 ng/ml human-EGF (Peprotec), 10 ng/ml human FGF2 (Peprotec) and heparin (MP Biomedicals) and seeded into laminin-coated wells. Cells were passaged five times before stock was frozen down and analyzed for the expression of Nestin and Sox-2. For neurosphere assay the same neuronal tissue dissociation kit (Miltenyi) was used, but after final centrifugation the cell pellets were resuspended in DMEM medium with 20 ng/ml EGF, 20 ng/ml FGF2 and heparin seeded into a 24 cell plate with niche cells in co-culture. The neurosphere assay was analyzed 10 days after plating.

For astrocyte-enriched culture, dentate gyri from 4-week-old mice were microdissected. Tissue was enzymatically digested with neural tissue dissociation kit (Miltenyi) and seeded onto PDL coated plates for one passage before co-culture with neural precursor cells.

For primary pericyte and endothelial cell culture, cortex tissue from 7 to 8 mice was dissected and homogenized with B-type douncer (Wheaton) in HBSS with 2% FBS. Homogenate was mixed with dextran solution (final concentration of 15%, Sigma Aldrich) centrifuged at 6000 ×*g* for 20 min. The vessel pellet was resuspended in 2% FBS in HBSS and filtered through a 100 μm nylon cell strainer filter (BD). Vessels trapped on top of the filter were collected for pericyte/vSMC culture and flow through was filtered through a 40 μm nylon cell strainer. Captured vessels were collected for endothelia cell culture. Vessels of the two different sizes were digested with 1 mg/ml collagenase A (Roche) for 5 h in DMEM F12 medium (Invitrogen). Afterwards pericytes were grown in DMEM F12 containing 10% FBS, 0.1 mM non-essential amino acids (Sigma-Aldrich); endothelial cells were grown in MCDB131 (PAA Laboratories) with 5% human plasma derived platelet poor serum (Sigma-Aldrich), 5 μg/ml insulin–transferrin–selenium (BD) and 15 U/ml heparin (MP Biomedicals). Cells were cultured in humidified incubator at 37 °C for 14 days prior to co-culture assay. Some cells from the different cultures were always seeded onto laminin coated glass slides for evaluation of the preparation by immunocytochemistry.

4.7. Co-culture assay

Two days prior to the co-culture endothelial cells, pericyte/vSMC and astrocytes were seeded into 24 well Millicell hanging inserts (PET transparent, 1 μm pore size, Millipore) in representative growth medium. At the day of co-culture, the niche cells were washed and transferred to differentiation medium (DMEM medium with B27 without growth factors) or proliferation medium (DMEM with B27, 10 ng/ml EGF and 10 ng/ml bFGF). Adherent actin-GFP neural precursor cells P8 or freshly isolated actin-GFP neural precursor cells were seeded into glass bottom 24 well plates and transwell inserts containing the different niche cells were placed on top. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2. GFP^+ neural precursor cells were imaged daily with spinning disk microscope (Axio observer Z1, Zeiss) with AxioCam MRm Rev.3 (Zeiss) using 488 nm laser excitation.

Co-cultured neurospheres were counted and measured 10 days after seeding using a 10× objective with a scale plate inside on a CKX41 microscope (Olympus). Then neurosphere were plated onto laminin coated glass slides for 7 days in DMEM medium with B27 for differentiation analysis. Cells from neurosphere culture and adherent culture were fixed with 4% PFA and stained against Mab2ab and GFAP. Eight frames per condition and preparation were analyzed manually on a fluorescence microscope (ApoTome.2, Zeiss).

4.8. Statistical analysis

Statistical analysis was performed using JMP Pro 10 (SAS). All data were analyzed by one-way-ANOVA using Turkeys least significant difference as a post hoc test. A p value of less than 0.05 was considered to be statistically significant.

Author Contributions

F.E. established performed histological analysis and established the primary cultures of EC, vSMC and pericytes; S.V. established astrocyte primary culture; F.E. and S.V. collected and analyzed the data; G. K. supervised the entire project and provides financial support; F.E. and G. K. wrote the manuscript.

Conflicts of interest

The authors declare that no competing interests exist.

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