The expression of tenascin-C in neural stem/progenitor cells is stimulated by the growth factors EGF and FGF-2, but not by TGFβ1

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Abstract
Neural stem/progenitor cells (NSPCs) rely on internal and external cues determining their lineage decisions during brain development. The progenitor cells of the embryonic mammalian forebrain reside in the ventricular and subventricular zones of the lateral ventricles, where they proliferate, generate neurons and glial cells, and respond to external cues like growth factors. The extracellular matrix (ECM) surrounds NSPCs and influences the cell fate by providing mechanical scaffold, trophic support, and instructive signals. The ECM molecule tenascin-C (Tnc) is expressed in the proliferative zones of the developing forebrain and involved in the proliferation and maturation of NSPCs. Here, we analyzed the regulation of the Tnc gene expression by NSPCs cultivated under the influence of different growth factors. We observed that the epidermal growth factor (EGF) and the fibroblast growth factor (FGF)-2 strongly increased the expression of Tnc, whereas the transforming growth factor (TGF)β 1 had no effect on Tnc gene expression, in contrast to previous findings in cell cultures of neural and non-neural origin. The stimulation of the Tnc gene expression induced by EGF or FGF-2 was reversible and seen in constantly treated as well as short term stimulated NSPC cultures. The activation depended on the presence of the respective receptors, which was slightly different in cortical and striatal NSPC cultures. Our results confirm the influence of extracellular stimuli regulating the expression of factors that form a niche for NSPCs during embryonic forebrain development.

Keywords Neural stem cells · Extracellular matrix · Growth factor · Neurospheres · EGF · FGF-2 · TGFβ 1 · Tenascin-C

Introduction
In the developing mammalian forebrain, neural stem/progenitor cells (NSPCs) generate the cell types of the brain in a highly ordered process, which includes the proliferation and differentiation of these cells as reviewed in several publications (e.g., Dimou and Götz 2014; Heide et al. 2017; Taverna et al. 2014). The behavior of the NSPCs and their fate decision in either direction is influenced by intrinsic and extrinsic factors (Faissner et al. 2017; Heide et al. 2017; Martynoga et al. 2012; Pacary et al. 2012; Reinhard et al. 2016; Theocharidis et al. 2014). Two subpopulations of progenitor cells can be distinguished in the proliferative zones of the mammalian forebrain that are characterized by their ability to respond to the epidermal growth factor (EGF) or fibroblast growth factor 2 (FGF-2) (Ciccolini and Svendsen 1998; Martens et al. 2000; Temple 2001; Tropepe et al. 1999). These populations exhibit self-renewal and differentiation capacity, which defines them as stem cells. The stem cell characteristics of these cells can be analyzed in vitro, where they generate free-floating cell aggregates called neurospheres when cultured in defined medium conditions and generate neurons and glia cells under differentiating conditions (Ciccolini and Svendsen 1998; Martens et al. 2000; Reynolds and Weiss 1992). The distinct populations come up in a temporally and spatially ordered fashion during development. At the beginning of neurogenesis, FGF-2-responsive cells can be found in the neurogenic regions of the forebrain, whereas the EGF-responsive populations appear later, when gliogenesis begins (Ciccolini and Svendsen 1998; Martens et al. 2000; Temple 2001; Zhu et al. 1999). The responsiveness of the NSPCs to the growth factors depends on the expression of the corresponding EGF receptor (EGFR) and FGF receptor (FGFR), which are regulated in the respective manner (Burrows et al. 1997; Zhu et al. 1999). These receptor tyrosine kinases activate intracellular signaling cascades, including the mitogen-activated protein kinase (MAPK) pathway, which influences the proliferation
and differentiation of neural progenitors (Boni et al. 1997; Campos et al. 2004; Marshall 1995; Rajan and McKay 1998). The acquisition of the EGFR depends on the activation of the FGFR and therefore succeeds the latter (Ciccioni and Svendsen 1998; Lillien and Raphaël 2000; Tropepe et al. 1999). The maturation from the FGFR-expressing immature towards the more mature FGFR- and EGFR-expressing state is considerably delayed in E12.5 telencephalic and E15 spinal cord-derived NSPCs obtained from tenascin-C (Tnc) knockout tissues (Garcion et al. 2004; Karus et al. 2011). The maturation deficit could be rescued by adding purified Tnc from postnatal CNS tissue to the Tnc−/−-deficient NSPCs in culture (Faissonner et al. 2017; Kazanis and ffrench-Constant 2011; May et al. 2018). These observations clearly suggest that Tnc as a constituent of the niche intervenes in the maturation of NSPCs as an important extrinsic factor (Faissonner et al. 2017; Kazanis and ffrench-Constant 2011; May et al. 2018).

Tenascin-C (Tnc) is a multimodular glycoprotein of the extracellular matrix that is expressed during embryonic and postnatal development in different neural and non-neural tissues (Joester and Faissonner 2001). During brain development, Tnc can be found in the ventricular and subventricular zones of the lateral ventricles, where it is secreted by radial glia cells (Faissonner et al. 2017; Garcion et al. 2001; Gates et al. 1995; Gotz et al. 1997; Temple 2001). During human development, it shows a prominent appearance in the outer SVZ, where basal radial glia serve as neurogenic stem cells (Pollan et al. 2015). In the proliferative zone of the lateral ventricle, the number of actively cycling cells is reduced when Tnc is missing in the homozygous knockout mutant (Garcion et al. 2001). This deficit coincides with the delayed acquisition of the EGFR expression of NSPCs and the correct differentiation of neurons, astrocytes, and oligodendrocytes (Garcion et al. 2001; Garcion et al. 2004; Karus et al. 2011). During later development and in adult mice, Tnc expression can be found in zones of active neurogenesis, whereas it is downregulated in other areas (Faissonner et al. 2017; Gates et al. 1995; Miragall et al. 1990). New neurons are generated in the subventricular zone of the lateral ventricle and in the dentate gyrus of the hippocampus (Bond et al. 2015; Doetsch 2003; Ihrie and Alvarez-Buylla 2011; Kempermann et al. 2004). Tnc is localized in these niches and likely influences the proliferation and fate of the stem cells (Garcion et al. 2001; Garwood et al. 2012; Gates et al. 1995; Nakic et al. 1996).

The expression of Tnc is regulated by different extrinsic and intrinsic factors, including different cytokines and growth factors (Giblin and Midwood 2015). FGF-2 and EGF provoke an induction of Tnc expression in different cell culture systems like cortical or hippocampal astrocytes or different tumor cells (DiProspero et al. 1997; Mahler et al. 1997; Meiners et al. 1993; Rettig et al. 1989; Sakai et al. 1995; Smith and Hale 1997; Wirl et al. 1995). After injection of EGF into the adult lateral ventricle, the expression of Tnc by proliferative progenitor cells with glial character is strongly increased (Doetsch et al. 2002). Another factor that shows a strong inductive effect on Tnc expression in diverse cell culture systems is the transforming growth factor (TGF) β1 (Dobbertin et al. 2010; Jinnin et al. 2004; Mackie et al. 1998; Sakai et al. 1994; Smith and Hale 1997; Wirl et al. 1995). TGFβ1 is an important regulator of neural progenitor fate and survival (Falk et al. 2008; Wachs et al. 2006) and induces matrix molecule production in pathogenic situations, which leads to the rearrangement of tissue components in the brain (Dobbertin et al. 2003; Smith and Strunz 2005). Signaling by TGFβ1 involves the activation of the TGFβ receptors 1 and 2 and is conveyed to the nucleus via Smad proteins (Massague 1987; Wrana et al. 1994). Neural stem cells of the adult neural stem cell niche are arrested by TGFβ1 treatment, but their renewal capacity or differentiation potential is not affected (Kandasamy et al. 2014; Wachs et al. 2006).

These examples illustrate that it is not possible to predict the effect of distinct cytokines on the regulation of Tnc. Rather, the cell type and the specific context determine the response that eventually results from the interaction of different signal transduction pathways. In the present study, we therefore investigated the regulation of Tnc expression in NSPC cultures by the growth factors EGF, FGF-2, and TGFβ1. We used an in vitro model to determine the proliferative capacity of different NSPC populations of the mouse embryonic forebrain and analyzed the gene expression pattern of Tnc after long- and short-term stimulation.

Materials and methods

Animals and tissue preparation

Time-mated female NMRI mice were obtained from Charles River Laboratories (Sulzfeld, Germany) or from the in-house animal facility. The present study was carried out in accordance with the European Council Directive of September 22, 2010 (2010/63/EU), for care of laboratory animals and approved by the animal care committee of North Rhine-Westphalia, Germany, based at the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, D-45659 Recklinghausen, Germany). The study was supervised by the animal welfare commissioner of the Ruhr University. Mice were kept according to the German animal protection law and FELASA (Federation for Laboratory Animals Science Association) standards in a 12-h light—12-h dark rhythm with water and food ad libitum. Pregnant females were detected by the appearance of a vaginal plug. They were sacrificed by cervical dislocation, and the embryos of the gestational day
13 (embryonic day (E)13, Theiler stage 21) were removed from the uterus and their brains dissected as described earlier (Sirko et al. 2007; von Holst et al. 2007, 2006). The cerebral cortex (Cor) and the ganglionic eminences (GE) were dissected, and the meninges were removed. The tissue was enzymatically treated with 0.05% trypsin–EDTA in HBSS (Invitrogen) for 10 min at 37 °C before the addition of ovomucoid: 1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich), 50 µg/ml bovine serum albumin (BSA, Sigma-Aldrich), and 40 µg/ml DNaseI (Worthington) in L-15 medium (Sigma-Aldrich). Subsequently the tissue was mechanically triturated to obtain single cell suspensions. These were centrifuged, and the cell pellets were resuspended in neurosphere medium: DMEM/F-12 1:1 (both from Sigma-Aldrich) supplemented with 0.2 mg/ml L-glutamine (Sigma-Aldrich), 2% B27 (Invitrogen), 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen).

**Cell culture**

Dissociated cells from E13 cortical or striatal (GE) embryonic brain tissue were cultured in neurosphere medium in a density of 100,000 cells per ml in the presence of the following growth factors: EGF 20 ng/ml (E; Peprotech #100–15), FGF-2 20 ng/ml (F; Peprotech #100-18B), EGF 20 ng/ml + FGF-2 20 ng/ml (E + F), TGFβ1 10 ng/ml (T; Peprotech #100–21), TGFβ1 10 ng/ml + EGF 20 ng/ml / FGF-2 20 ng/ml (T + E + F). A previous study involving dose–response analyses had revealed robust cellular responses when the cytokines were used in these concentration ranges (Dobbertin et al. 2003). In each FGF-2 containing condition, heparin (Sigma-Aldrich #H3149) was added as co-factor in a concentration of 0.5 U/ml. Control cells were cultured without any additional growth factor (Fig. 1).

**Fig. 1** Scheme of the cell culture protocol. a Dissociated cells from embryonic brain tissue were cultured under defined conditions in medium containing EGF (E), FGF-2 (F), TGFβ1 (T), combinations of these factors (E+F, T+E+F) or without any additional growth factor (wo). Their proliferation capacity was determined after 5 days in vitro (div), whereas RNA and protein isolation were carried out after 3 div. b Cells grown in the presence of EGF and FGF-2 (E+F) generated neurospheres within 1 week. These spheres were dissociated and re-plated for the second passage (c). After withdrawal of the growth factors for 6 h, the neurospheres were treated with the different growth factors for 24 h and subsequently used for RNA isolation and immunocytochemistry. 

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![Diagram](image-url)
The cells were allowed to grow for 3 days in the respective medium before they were used for RNA isolation. To follow the growth over a longer period, some cultures were kept up to 5 days in vitro (div). After that time, neurospheres in 20 randomly chosen visual fields that contained a minimum of 5 assembled cells were counted. The neurosphere index was then calculated by multiplying the mean number of neurospheres (n) with a specific size (larger than 5 cells, larger than 10 (20, 50, or 100) cells) with the respective size (n1 × 5 + n2 × 10 + n3 × 20 + n4 × 50 + n5 × 100). Because under some culture conditions the cells died after the third day (visible as cell debris under the microscope), we used neurospheres grown under optimal conditions with EGF and FGF-2 for further analysis after 1 week. These cultures were transferred to growth factor-free medium for 6 h to remove the bound factors before the different growth factor combinations were added over a period of 24 h, as described above.

To assess secondary neurospheres, EGF- and FGF-2-treated primary neurospheres were collected and dissociated to single cells after 1 week using trypsin–EDTA and re-plated for second passage in the presence of EGF and FGF-2. At the end of the second week, these cells were treated with the different growth factor combinations for 24 h. The different culture and treatment routines are depicted in the schematic representation in Fig. 1.

**RNA isolation and RT-PCR**

Neurosphere cells treated with different growth factors were harvested after a continuous treatment for 3 div or, alternatively, after a 24-h stimulus. The medium was removed from the cells by centrifugation, and their total RNA was isolated using the RNeasy Mini Kit (QIAGEN) following manufacturer’s instructions as described before (von Holst et al. 2007). To remove potentially contaminating genomic DNA we treated the RNA with DNase for 15 min (QIAGEN RNase free DNase Kit).

For cDNA synthesis, 1 µg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas/Thermo Scientific) with random hexamer primers in a total volume of 40 µl. In general, 1 µl of cDNA was used for a PCR reaction in a total volume of 25 µl using 1.5 mM MgCl₂, 5 nmol of each dNTP, 1.25 U Taq polymerase, and 5 pmol of the appropriate forward (for) and reverse (rev) primers (Table 1). The reactions were incubated in a Mastercycler gradient (Eppendorf) with annealing temperatures as displayed in Table 1. The samples were analyzed on 1.5% agarose gels and digitally.

### Table 1: Primers for RT-PCR

| Primer name | Gene (accession number) | Sequence (5´-3´) | Annealing temperature | Cycle number | Product size |
|-------------|-------------------------|------------------|-----------------------|--------------|--------------|
| EGFR for    | egfr NM_207655          | ACCCTCCAAGCAGTGAGTTTA TGTACAATGTGGGCTGCTC | 60 °C | 35 | 554 bp |
| EGFR rev    |                         | CAGGAGGCGGTGTGCTTT CAGCATCATGGTGTCAG | 60 °C | 35 | 356 bp |
| FGFR2 for   | fgfr2 NM_010207         | GGTTGTGCCCATCTTACAT AGAACGCTGACAAATTT | 60 °C | 35 | 315 bp |
| FGFR2 rev   |                         | ATGGACAACTGACACACAC TGAACCGTACCTTGATAT | 60 °C | 35 | 310 bp |
| TGFβR1 for  | tgfbr1 NM_009370        | GGCTTGGCCCATCTTACAT AGAACGCTGACAAATTT | 60 °C | 35 | 312 bp |
| TGFβR1 rev  |                         | CGGAGTACCTTCACACACAA TGGTCACCTGACATGCTT | 60 °C | 30 | 677 bp |
| TGFβR2 for  | tgfbr2 NM_009371        | GCTCTAGAAGGCTGTCCTTG | 370 bp |
| TGFβR2 rev  |                         | ACTCCATCGGCAATTTCC CCTCCACATTGCGCAAGT | 60 °C | 30 | 370 bp |
| TGFβR3 for  | tgfbr3 NM_011578        | GCTCTAGAAGGCTGTCCTTG | 370 bp |
| TGFβR3 rev  |                         | ACTCCATCGGCAATTTCC CCTCCACATTGCGCAAGT | 60 °C | 25 | 247 bp |

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documented. Semi-quantitative endpoint analyses of the resulting band intensities were performed after background subtraction using ImageJ (NIH). Samples were first normalized to their respective GAPDH bands and then to the control conditions without growth factors. The statistical evaluation of three independent experiments (n = 3) was performed as one-way ANOVA with Bonferroni’s multiple comparison post hoc test using GraphPad Prism5 software.

**Immunocytochemical staining**

After a 24-h stimulus with the different growth factor combinations, some neurospheres were dissociated to single cells and plated onto poly-Ornithin (p-Orn, Sigma-Aldrich, 10 µg/ml)-coated cell culture dishes for 3 h. The cells were washed with Krebs–Ringer-HEPES (KRH: 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 2H₂O, 1.2 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 5.6 mM D-Glucose, 25 mM HEPES, pH 7.3) and incubated with a polyclonal anti-Tnc antibody (rabbit, batch KAF14) (Faissner and Kruse 1990), diluted 1:250 in KRH/A (KRH with 1% (w/v) BSA (Carl Roth)), and acetylated and semi-dry blotted to methanol-activated polyvinylidene fluoride (PVDF) membranes. The membranes were blocked and incubated with antibodies for Tnc (polyclonal anti-Tnc, rabbit (batch KAF14), 1:3,000) and α-tubulin (DM1α, 1:10,000, Sigma-Aldrich), probed with appropriate HRP-coupled secondary antibodies (1:10,000, Dianova), and developed with the Clarity Western Blot ECL substrate (Bio-Rad).

**Western blots**

Neurosphere cells cultured with different growth factor combinations for 3 div were harvested by centrifugation and lysed in lysis buffer (50 mM Tris/Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% (v/v) Triton-X100, 0.1% (w/v) deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 40 mM sodium fluoride, 1 mM orthovanadate, pH 10) supplemented with the protease inhibitors PMSF (1 mM, MP Biomedicals), IAA (18.5 µg/ml, Sigma-Aldrich), SBTI (10 µg/ml, Sigma-Aldrich), Aprotinin (10 µg/ml, Sigma-Aldrich), Leupeptin (0.5 µg/ml, Sigma-Aldrich), and Pepstatin (0.1 µg/ml, Sigma-Aldrich) for 30 min on ice before the debris was removed by centrifugation. The conditioned medium was directly used for protein analysis. The lysates and supernatants were fractionated on a 4–10% gradient SDS polyacrylamide gel together with the Precision Plus Protein Dual Color Standard (Bio-Rad) and semi-dry blotted to methanol-activated polyvinylidene fluoride (PVDF) membranes. The membranes were blocked and incubated with antibodies for Tnc (polyclonal anti-Tnc, rabbit (batch KAF14), 1:3,000) and α-tubulin (DM1α, 1:10,000, Sigma-Aldrich), probed with appropriate HRP-coupled secondary antibodies (1:10,000, Dianova), and developed with the Clarity Western Blot ECL substrate (Bio-Rad).

**Tissue preparation for cryosections**

Embryonic whole mounts were fixed in 4% (w/v) PFA overnight and subsequently cryo-protected in 20% (w/v) sucrose in PBS treated with DEPC (diethyl pyrocarbonate, 1:1,000, autoclaving after overnight incubation). After settlement the tissue was frozen in tissue freezing medium (Leica) on dry ice before the sectioning of 14-µm slices in a Leica cryostat. Sections were immediately dry-mounted and stored at −20 °C until use.

**In situ hybridization**

The protocol was adapted from Akita et al. (2008). Tissue sections were dried at room temperature and primed in 0.1 M TEA (triethanolamine hydrochloride, pH 8.0) before acetylation (0.25% (w/v) acetic anhydrate in TEA). After washing with 50 mM phosphate buffer (PB), slices were incubated for 2 h at 60 °C with hybridization buffer (50% (v/v) formamide, 10% (w/v) dextran sulfate, 1× Denhardt’s reagent (Sigma-Aldrich), 100 µg/ml yeast RNA (Roche), 250 µg/ml salmon sperm DNA (Roche), 2× SSC (standard saline citrate, prepared as 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 50 mM sodium phosphate, pH 7.0, 0.2% (w/v) SDS). Subsequently, the sections were hybridized with a Tnc riboprobe (Czopka et al. 2009) 1:500 in hybridization buffer), which was denatured at 80 °C for 5 min before application. After overnight incubation at 60 °C, the sections were washed stringently at 60 °C in these buffers: 4× SSC for 10 min, 2× SSC containing 50% formamide for 20 min twice, 2× SSC for 10 min, 0.2× SSC for 20 min twice. The last two washing steps in Tris/NaCl buffer (0.15 M NaCl, 0.1 M Tris–HCl, pH 7.5) for 10 min were performed at room temperature, before the sections were blocked for 30 min with 1% (w/v) skimmed milk powder in Tris/NaCl buffer. Afterwards, the alkaline phosphatase-coupled anti-Dig Fab fragments (Roche) were applied (1:2,000 in blocking buffer) overnight at 4 °C. After washing with Tris/NaCl buffer thrice, the alkaline phosphatase substrates nitroblue tetrazolium (NBT, 0.34 mg/ml, Roche) and
Neural stem/progenitor cells proliferated in the presence of EGF and/or FGF-2. NSPCs from the cerebral cortex (Cor, a–c) or the ganglionic eminence (GE, d–f) of embryonic mice grown in defined medium generated neurospheres when treated with EGF (E), FGF-2 (F), or the combination of both (E+F). The additional treatment with TGFβ1 (T+E+F) reduced the proliferation capacity of E+F-treated cells. When no growth factor was added to the medium (w/o) or only TGFβ1 (T), the cells started with an initial proliferation but died between the 3rd and 5th day under these conditions (#). The graphical representation (g,h) shows the neurosphere index representing the number and size of neurospheres in the individual cultures. Thin lines represent the median, error bars show the standard deviation (SD), and symbols represent the results of each individual experiment (n=2).

5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.18 mg/ml, Roche) were applied in detection buffer containing 5% (w/v) polyvinyl alcohol, 0.1 M NaCl, 50 mM MgCl2, 0.1 M Tris–HCl, pH 9.5. The development of the color reaction was carried out at 37 °C and stopped with 1 mM EDTA, 10 mM Tris–HCl, pH 7.5 when clear signals were visible under microscopic control.

Immunohistochemistry
Cryosections were dried at room temperature and blocked with 5% (v/v) goat serum (Jackson Immuno Research) in PBS containing 1.7% (w/v) NaCl for 1 h, before the polyclonal anti-Tnc antibody (rabbit, batch KAF14) was applied 1:300 in PBT-1 (PBS with 1% (w/v) BSA (Carl Roth) and 0.1% (v/v) Triton X-100 (Sigma-Aldrich)). After overnight incubation and washing with PBS/A, the Cy3-coupled secondary antibody (anti-rabbit Cy3, 1:500, Dianova) was applied 1:300 in PBT-1 (PBS with 1% (w/v) BSA (Carl Roth) and 0.1% (v/v) Triton X-100 (Sigma-Aldrich)). After overnight incubation and washing with PBS/A, the Cy3-coupled secondary antibody (anti-rabbit Cy3, 1:500, Dianova) was added in combination with Hoechst 33258 nuclear marker (1:105) in PBS/A for 2 h at room temperature. After washing with PBS, the sections were mounted with Immu-Mount (Shandon/Thermo Scientific) and documented with the Zeiss Axiophot 2.

Results
Proliferation of neural stem/progenitor cells of the forebrain is dependent on the presence of growth factors. Epidermal growth factor (EGF) and fibroblast growth factor (FGF-2) stimulate the proliferation of neural progenitors in vitro, which leads to the formation of freely floating cell aggregates, the so-called neurospheres (Ciccolini and Svendsen 1998; Reynolds et al. 1992). Neural stem/progenitor cells (NSPCs) isolated at embryonic day (E)13 from the dorsal (Cor) or ventral (GE) embryonic mouse forebrain generated neurospheres in culture (Fig. 2). The resulting spheres were counted and quantified using the neurosphere index, which combines the number of neurospheres and their respective size. Depending on the culture conditions, we observed considerable variability of sphere diameters. We decided not to exclude outliers in order to avoid introducing an observer’s bias. This resulted in large error bars in some cases (Fig. 2). Control cells without growth factor support yielded few and little spheres, but most of the cells died after about 3 days in the absence of trophic support, visible as cell debris. When EGF was added to the culture medium, viable neurospheres grew to a proper size, but their numbers remained low. More neurospheres arose when FGF-2 was supplied in the culture medium. However, when both growth factors were combined, the number and size of neurospheres were larger, resulting in higher indices. This observation strongly suggests that EGF and FGF-2 synergized to drive the expansion of neurospheres. This indicates that both cytokines acted independently, possibly stimulating different fractions of progenitor cells. This may explain why the combination of both factors led to the generation of more and larger neurospheres than either of the factors alone. As remarked previously, transforming growth factor (TGFβ1) had no stimulative effect on the proliferation of neural precursors (Falk et al. 2008; Siegenthaler and Miller 2005; Wachs et al. 2006). In contrast, TGFβ1 rather reduced the proliferation rate of the NSPCs when it was added to the promotive factors FGF-2 and EGF. This was visible for cortical cells and for cells from the GE (Fig. 2). Initially, TGFβ1-treated cells started proliferation during the first 3 days, but then suddenly stopped dividing and died between the 3rd and 5th day. In this they behaved like the control cultures in the absence of growth factors, indicative of missing trophic support (Fig. 2).

After the initial growth phase of 3 days in culture, we isolated the RNA of the precursor cells to determine the gene expression levels of genes of interest under the influence of the different growth factors. The cell cultures could be analyzed using this approach because they provided sufficient amounts of sample, as documented with the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was equally detectable in the different settings. NSPCs of the dorsal forebrain (Cor) did not express detectable amounts of EGF receptor (EGFR) yet (Fig. 3). In contrast, the FGF receptor (FGFR) was indeed present in each culture analyzed (Fig. 3) and presumably mediated the proliferative response. The neural development in the ventral forebrain appeared slightly faster than that of the dorsal forebrain, which was mirrored by the expression of the EGFR on the precursor cells (Ciccolini and Svendsen 1998; Martens et al. 2000). The receptor was already detectable in neurospheres from the GE. Therefore
a stimulus with EGF could lead to a cellular response in this particular case. It has been demonstrated before that the presence of FGF-2 induces the gene expression of the EGFR (Lillien and Raphael 2000). This is in agreement with our results, because the EGFR was seen in all cultures where FGF-2 had been added (Fig. 3).

The expression of the TGFβR2 required the active signaling via EGF/EGFR, which had previously been documented...
in other studies (Yamane et al. 2007, 2003), e.g., for corneal epithelial cells (Shu et al. 2019). When EGFR activation was combined with the addition of FGF-2, the TGFβR2 expression was further enhanced, beyond a level obtained with FGF-2 treatment alone. This speaks in favor of a synergism of FGF-2 and EGF with regard to TGFβR2 expression that was maintained in the presence of TGFβ1, whereas TGFβ1 by itself completely repressed TGFβR2. The genes of the two other known TGFβ receptors TGFβR1 and TGFβR3 were detectable under all analyzed conditions and did not appear regulated by any of the growth factors used in this study (Fig. 3).

The strong regulation of the Tnc expression appeared most intriguing in our setting on mRNA level (Fig. 3) and on protein level (Fig. 4). Thus, the precursor cells from the cortex expressed only very weak levels of Tnc mRNA when no growth factor was present in the cultures (Fig. 3). This was expected because the expression of Tnc in situ was not yet prominent in this region at E13 (compare to Fig. 5). As soon as FGF-2 or EGF were added, the expression of the ECM molecule was strongly enhanced. FGF-2 stimulated the expression of Tnc in cortical cells, as well as in ventrally derived cell cultures — where the EGFR could already be detected — the stimulation by EGF resulted in a strong increase of Tnc expression on mRNA level. Both growth factors combined resulted in
a much stronger expression of \( Tnc \) in NSPCs that proved statistically significant. Also in this situation the addition of FGF-2 to EGF-responsive cells caused an augmented \( Tnc \) expression that exceeded notably what each factor could achieve by itself. The expression pattern of \( Tnc \) observed in vivo in E13 mouse embryos (Fig. 5) might be explained by the emergence of EGFR-positive neural progenitor cells and the subsequent activation of the intracellular signaling pathways leading to the increased transcription of the \( Tnc \) gene in neural progenitors of the glial lineage (Burrows et al. 1997; Temple 2001). Two days later, when gliogenesis begins at E15 and most of the progenitors in the lateral ventricular zones are responsive to EGF, the expression of \( Tnc \) expanded in the ventral as well as in the dorsal part along the whole ventricle (data not shown, and see Faissner et al. 2017).

TGFβ1 strongly promotes the expression of \( Tnc \) in numerous cell culture systems (Mackie et al. 1998; Sakai et al. 1994; Schnadelbach et al. 1998; Smith and Hale 1997; Wirl et al. 1995). As \( Tnc \) is released by NSPCs, we examined whether TGFβ1 also induces its upregulation in this context. In order to be activated by TGFβ1, the responsive cells need to express the type 2 receptor (Wrana et al. 1994). Cultures treated with a combination of EGF, FGF-2, and TGFβ1 expressed the \( TGFβR2 \), as detailed above. In the presence of TGFβ1,
the neurosphere-forming capacity was clearly reduced. In contrast, the expression of Tnc was barely affected (Fig. 3). Message levels are not necessarily translated into different protein concentrations. In order to examine the consequences of factor exposure for downstream Tnc release, Western blots using the polyclonal Tnc antibody were performed (Fig. 4). The band intensities mirrored the outcome of the PCR analysis. The NSPCs secreted the ECM molecule into the medium, where it could weakly be detected in control and TGFβ1-treated cultures. EGF and FGF-2 strongly increased the amount of detectable Tnc, and the joint treatment with both growth factors exerted the most stimulatory effect on Tnc expression. The fact that EGF treatment had a slight, positive effect on neurosphere survival (Fig. 2) and on the Tnc expression, although the EGFR could not be detected by RT-PCR, leads to the question how EGF can exert these effects. Very low amounts of the EGF receptor, below the RT-PCR detection limit, might be expressed that mediate EGF signaling to a certain degree. As Tnc comprises several alternatively spliced domains (Joester and Faissner 1999; von Holst et al. 2007) and is highly glycosylated (Giblin and Midwood 2015), not one clearly circumscribed band was visible in the Western blot. Rather, Tnc glycoproteins distributed as rather diffuse bands covering a broad molecular weight range, in particular when the expression was substantial (Fig. 4).

The cell lysates (Fig. 4b, b’) also provided evidence for the regulation of Tnc protein by the growth factors, but the signals obtained were weaker. The protein fraction that was associated with the cell membranes or cell cytoplasm was less abundant than the secreted fraction. The cell lysates offered the opportunity to compare the protein amounts between the different culture conditions by comparison with the reference protein α-tubulin. The tubulin bands were similar for all cell cultures, confirming that comparable cell numbers were available in the samples. This underlined the regulatory impact of the growth factors with regard to Tnc in the samples under study (Fig. 4c, c’).

Because NSPC cultures in media devoid of growth factors or replenished solely with TGFβ1 displayed very limited growth after the third day in vitro and failed to express TGFβR2, an alternative approach was used to assess their responsiveness to a short-term exposure to different growth factors. In a first step, neurospheres were generated in the presence of EGF and FGF-2 and thereafter freed of potentially bound growth factors by incubation in growth factor-free medium for 6 h. Subsequently, NSPCs were treated with different cytokine combinations over a period of 24 h (see methods scheme in Fig. 1b). This procedure tempered any effect that could result from poor cell survival or proliferation and created equal starting conditions. This method also rendered possible the generation of secondary derived from primary neurospheres maintained in the presence of EGF and FGF-2 (Fig. 1c). The number of NSPCs in secondary was higher than in primary neurospheres, which could potentially lead to different expression patterns (Reynolds and Rietze 2005; Reynolds and Weiss 1996). Cells from the cortex and the GE treated with EGF and FGF-2 for 1 week expressed the TGFβ receptors TGFβR1, TGFβR2, and TGFβR3 (Fig. S1), which was not achieved after the shorter period of 3 days (Fig. 3). In this situation, therefore TGFβ1 could recruit TGFβR1 and TGFβR2. After 24 h we observed only faint expression of Tnc in control and TGFβ1-treated cultures, consistent with immunofluorescence analysis of dissociated cells and RT-PCR analysis performed with 1st and 2nd passage GE cultures (Fig. 6). Although the expression of the relevant receptors warranted the responsiveness of NSPCs, the exposure to TGFβ1 did not elevate Tnc expression in the first or the second passage. We conclude that TGFβ1 does not induce the expression of Tnc in neurosphere cells, different from what has been reported for diverse cell types of neural and non-neural origin (Mackie et al. 1998; Sakai et al. 1994; Smith and Hale 1997; Wirl et al. 1995).

In contrast, EGF or FGF-2, or both factors in combination strongly augmented Tnc expression in NSPCs. The protein was secreted by NSPCs that deposited a pericellular ECM in the respective environment around them, as visualized by immunostaining (Fig. 6a–f). Using RT-PCR, we detected significantly stronger bands when the NSPCs were treated with EGF in cultures derived from the GE and also a similar tendency was seen in cells from the cortex (Fig. 6g, h–k). Because the EGF receptor was upregulated by FGF-2 in the antecedent culture condition, the difference between cortical and striatal cultures was waived. Consequently, EGF fostered a strong upregulation of the Tnc gene expression. FGF-2 also increased the transcription rate of Tnc, as expected for continuously treated cultures. Both factors led to a high expression level that reached saturation and could not be increased further, neither by the combination of both factors nor by the addition of TGFβ1 (Fig. 6). First and second passage NSPCs yielded similar results, which underlined that the stimulation of Tnc expression by EGF and FGF-2, but not by TGFβ1 proved a recurrent theme for these slightly different cultures. It is of interest to note that the stimulatory effect by the classical growth factors of neurosphere cultures proved reversible, because cells initially treated with EGF and FGF-2 lost high Tnc expression when these factors were removed from the culture (Fig. 6).
Stem cells of the developing brain undergo symmetric and asymmetric divisions to enlange the stem cell pool and generate differentiated cells of neural and glial lineages (Temple 2001). Along this pathway, NSPCs depend on the activity of growth factor signaling by EGF and FGF family members. When we analyzed the proliferation capacity of NSPCs in the presence of the growth factors EGF, FGF-2, and TGFβ1, we found that the progenitor cells divided frequently upon EGF and FGF-2 treatment, whereas TGFβ1 tempered their proliferation rate. The stimulation of NSPCs with EGF or FGF-2 resulted in the generation of neurospheres from embroyonic forebrain tissues, in agreement with previous reports (Ciccolini and Svendsen 1998; Tropepe et al. 1999). As it had been reported for adult NSPCs, the addition of TGFβ1 led to a decreased proliferation rate of embryonic NSPCs, and the factor by itself was not able to prevent poor growth and regression of neurospheres. We assume that TGFβ1 rather arrested the stem cells in a non-proliferative, non-differentiating state, analogous to the adult neural stem cell response to TGFβ1 (Wachs et al. 2006).

Tnc is a constitutive compound of the stem cell niche in various organs (Chiquet-Ehrismann et al. 2014). Genetic transcriptome screens have revealed that Tnc is conspicuously enriched in neural stem cells in comparison with embryonic or hematopoietic stem cells (Ramalho-Santos et al. 2002). In vivo the localization of the ECM glycoprotein Tnc in the CNS coincides with the upregulation of the EGFR in the developing forebrain, which suggests that Tnc is expressed by a resident subpopulation of progenitor cells. Indeed, cells with glial characteristics produce Tnc, which has especially been shown for radial glia cells serving as glial progenitors in this case (Faissner et al. 2017). Independently of EGF, also the cultivation of NSPCs in the presence of FGF-2 led to an upregulation of Tnc expression, but the EGF-dependent regulation resulted in a much more prominent increase. The neurosphere cultures reproduced the correct timing of forebrain development, shown by the EGFR and FGFR expression that could be detected in a developmental stage and region-dependent manner. The pattern of expression underpinned the basis for the stimulation of Tnc expression by cytokines. Both receptor systems involve the MAPK pathway that fosters an increase of proliferation (Campos et al. 2004). The MAPK signaling eventually impacts gene regulation in the nucleus, where it might induce an increase of Tnc expression. The maturation of the stem cells reflected by the acquisition of the EGFR is promoted by Tnc in the ECM environment, as evidenced by the observation that in Tnc-deficient mice the upregulation of the EGFR is delayed (Garcion et al. 2004; Karus et al. 2011).

In the adult mouse subventricular zone, slowly dividing stem cells generate transient amplifying type C cells that are EGF-responsive (Doetsch et al. 2002). Upon exposure to EGF, the type C cells downregulate Dlx2 and start to proliferate. The cells obtained from mature mouse brain displayed pronounced Tnc expression when cultured with EGF, which suggests stimulation of Tnc expression by this growth factor. Cells from this postnatal stage do express the EGFR, which renders them independent of FGF-2 dependent growth. Consequently, the injection of EGF into the lateral ventricle of adult mice leads to the upregulation of Tnc expression by the NSPC pool, which is localized there (Doetsch et al. 2002). In our present study, we provide further evidence that the response to EGF is not confined to adult, but can also be seen in embryonic NSPCs as soon as these have acquired EGFR expression.

TGFβ1 reduced the proliferation capacity of NSPCs from the embryonic forebrain, similar to what has been reported for cultures of adult neural stem cells and astrocytes (Vergeli et al. 1995; Wachs et al. 2006). In this respect the response to this external stimulus seems comparable between the different cell culture systems. Remarkably, we observed opposing effects regarding the stimulation of Tnc gene expression by TGFβ1. The cytokine strongly upregulates Tnc in cultures of primary astrocytes (Dobbertin et al. 2010), which did not occur in our NSPC cultures. Reactive astrocytes that produce ECM components contribute a pivotal cellular component of glial scars involved in the inhibition of axonal regeneration (Fitch and Silver 2008; Vogelaar et al. 2015). TGFβ1 is an inducer of glial scar formation both in vivo and in vitro (Howe et al. 2019; Song et al. 2019). Thus, reactive glia in lesion situations reacts differently to the TGFβ1 stimulus compared to NSPCs that represent actively cycling cells involved in tissue development and remodeling. Interestingly, also the response to EGF and FGF-2 differed between NSPCs and reactive astrocytes, because the latter do not increase Tnc production upon stimulation with either growth factor (Dobbertin et al. 2010). By comparison, EGF and FGF-2 promoted a strong upregulation of Tnc in NSPCs. This might indicate that the release of Tnc into the micromilieu of the neural NSPC niche is required to maintain the stemness and proliferative capacity of this cell.
population. The enrichment of Tnc in stem cell niches of the CNS and other organs supports this interpretation (Chiquet-Ehrismann et al. 2014; Faissner et al. 2017). It would be interesting to examine in which way adult neural stem cells respond to TGFβ1, because they share characteristics both with embryonic NSPC as well as with astrocytes. Considering regional differences, it is conceivable that TGFβ1 leads to distinct effects in cells from the midbrain, which showed stronger responsiveness to the factor than forebrain cells, in agreement with the report that these regions also display differential responses to the factor in vivo (Falk et al. 2008).

In conclusion, Tnc is regulated by growth factors in different and occasionally even opposed ways in selected tissues, depending on the cell type and the mode of presentation. Several studies reported an increased Tnc expression when cells are treated with TGFβ1, some of them distinguishing between the distinct splice variants of the molecule (Dobbertin et al. 2010). This is clearly not the case for NSPCs. FGF-2 stimulates Tnc expression both in neural and non-neural cell cultures (Chen et al. 2009; Meiners et al. 1993). Our findings clearly demonstrate an increased expression in FGF-2-treated NSPCs. This might generate a conducive microenvironment for proliferation and differentiation of the neural stem cell pool (Faissner et al. 2017).

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**Declarations**

**Ethics approval** The present study was carried out in accordance with the European Council Directive of September 22, 2010 (2010/63/EU) for care of laboratory animals and approved by the animal care committee of North Rhine-Westphalia, Germany, based at the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, D-45659 Recklinghausen, Germany). The study was supervised by the animal welfare commissioner of Ruhr University. Male and female mice were housed individually with a constant 12-h light–dark cycle and access to food and water ad libitum. All efforts were made to reduce the number of animals in the experiments. Embryos of both sexes were used.

**Conflict of interest** The authors declare no competing interests.

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**References**

Akit a K, von Holst A, Furukawa Y, Mikami T, Sugahara K, Faissner A (2008) Expression of multiple chondroitin/dermatan sulphatransferases in the neurogenic regions of the embryonic and adult central nervous system implies that complex chondroitin sulfates have a role in neural stem cell maintenance. Stem Cells 26:798–809

Bond AM, Ming GL, Song H (2015) Adult mammalian neural stem cells and neurogenesis: five decades later. Cell Stem Cell 17:385–395

Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky L, Stahl N, Yancopoulos GD, Greenberg ME (1997) Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 278:477–483

Burrows RC, Wancio D, Levitt P, Lillien L (1997) Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron 19:251–267

Campos LS, Leone DP, Relvas JB, Brakebusch C, Fässler R, Suter U, Ffrench-Constant C, (2004) Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contribute to their maintenance. Development 131:3433–3444

Chen J, Guerraiero E, Sado Y, SundarRaj N (2009) Rho-mediated regulation of TGF-beta1- and FGF-2-induced activation of corneal stromal keratocytes. Invest Ophthalmol vis Sci 50:3662–3670

Chiquet-Ehrismann R, Orend G, Chiquet M, Tucker RP, Midwood KS (2014) Tenascins in stem cell niches. Matrix Biology : Journal of the International Society for Matrix Biology 37:112–123

Ciccolini F, Svendsen CN (1998) Fibroblast growth factor 2 (FGF-2) promotes acquisition of epidermal growth factor (EGF) responsiveness in mouse striatal precursor cells: identification of neural precursors responding to both EGF and FGF-2. J Neurosci 18:7869–7880

Czopka T, Von Holst A, Schmidt G, ffrench-Constant C, Faissner A (2009) Tenascin C and tenasin R similarly prevent the formation of myelin membranes in a RhoA-dependent manner, but antagonistically regulate the expression of myelin basic protein via a separate pathway. Glia 57:1790–1801

Dimou L, Götz M (2014) Glial cells as progenitors and stem cells: new roles in the healthy and diseased brain. Physiol Rev 94:709–737

DiPorepero NA, Meiners S, Geller HM (1997) Inflammatory cytokines interact to modulate extracellular matrix and astrocytic support of neurite outgrowth. Exp Neurol 148:628–639

Dobbertin A, Czvitkovich S, Theocharidis U, Garwood J, Andrews MR, Properzi F, Lin R, Fawcett JW, Faissner A (2010) Analysis of combinatorial variability reveals selective accumulation of the fibronectin type III domains B and D of tenasin-C in injured brain. Exp Neurol 225:60–73

Dobbertin A, Rhodes KE, Garwood J, Properzi F, Heck N, Rogers JH, Fawcett JW, Faissner A (2003) Regulation of RTPbeta/phosphatase expression and glycosaminoglycan epitopes in injured brain and cytokine-treated glia. Mol Cell Neurosci 24:951–971

Doetsch F (2003) A niche for adult neural stem cells. Curr Opin Genet Dev 13:543–550
Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710

Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1–13

Sakai T, Kawakatsu H, Ohta M, Saito M (1994) Tenasin induction in tenasin nonproducing carcinoma cell lines in vivo and by TGF-beta 1 in vitro. J Cell Physiol 159:561–572

Sakai T, Ohta M, Furukawa Y, Saga Y, Aizawa S, Kawakatsu H, Saito M (1995) Tenasin-C induction by the diffusible factor epidermal growth factor in stromal-epithelial interactions. J Cell Physiol 165:18–29

Schnädelbach O, Mandl C, Faissner A (1998) Expression of DSD-1-PG in primary neural and glial-derived cell line cultures, upregulation by TGF-beta, and implications for cell-substrate interactions of the glial cell line Oli-neu. Glia 23:99–119

Shu DY, Hutcheon AEK, Zieske JD, Guo X (2019) Epidermal growth factor stimulates transforming growth factor-beta receptor type II expression in corneal epithelial cells. Sci Rep 9:8079

Siegenthaler JA, Miller MW (2005) Transforming growth factor beta 1 promotes cell cycle exit through the cyclin-dependent kinase inhibitor p21 in the developing cerebral cortex. J Neurosci 25:8627–8636

Sirkó S, von Holst A, Wizenmann A, Götz M, Faissner A (2007) Chondroitin sulfate glycosaminoglycans control proliferation, radial glia cell differentiation and neurogenesis in neural stem/progenitor cells. Development 134:2727–2738

Smith GM, Hale IH (1997) Macrophage/Microglia regulation of astrocytic tenasin: synergistic action of transforming growth factor-beta and basic fibroblast growth factor. J Neurosci 17:9624–9633

Smith GM, Strunz C (2005) Growth factor and cytokine regulation of chondroitin sulfate proteoglycans by astrocytes. Glia 52:209–218

Song G, Yang R, Zhang Q, Chen L, Huang D, Zeng J, Yang C, Zhang T (2019) TGF-beta secretion by M2 macrophages induces glial scar formation by activating astrocytes in vitro. Journal of Molecular Neuroscience: MN 69:324–332

Taverna E, Götz M, Huttner WB (2014) The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. Annu Rev Cell Dev Biol 30:465–502

Temple S (2001) The development of neural stem cells. Nature 414:112–117

Theocharidis U, Long K, Ffrench-Constant C, Faissner A (2014) Regulation of the neural stem cell compartment by extracellular matrix constituents. Prog Brain Res 214:3–28

Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208:166–188

Vergeli M, mazzanti B, Ballerini C, Gran B, Amaducci L, Massacesi L (1995) Transforming growth factor-beta 1 inhibits the proliferation of rat astrocytes induced by serum and growth factors. J Neurosci Res 40:127–133

Vogelaar CF, König B, Krafft S, Estrada V, Brazda N, Ziegler B, Faissner A, Müller HW (2015) Pharmacological suppression of CNS scarring by deferoxamine reduces lesion volume and increases regeneration in an in vitro model for astroglial-fibrotic scarring and in rat spinal cord injury in vivo. PLoS One 10:e0134371

von Holst A, Egbers U, Prochiantz A, Faissner A (2007) Neural stem/progenitor cells express 20 tenasin C isoforms that are differentially regulated by Pax6. J Biol Chem 282:9172–9181

von Holst A, Sirkó S, Faissner A (2006) The unique 473HD-chondroitin sulphate epitope is expressed by radial glia and involved in neural precursor cell proliferation. J Neurosci 26:4082–4094

Wachs FP, Winner B, Couillard-Despres S, Schiller T, Aigner R, Winkler J, Bogdahn U, Aigner L (2006) Transforming growth factor-beta 1 is a negative modulator of adult neurogenesis. J Neuroupathol Exp Neurol 65:358–370

Wirl G, Hermann M, Ekblom P, Fassler R (1995) Mammary epithelial cell differentiation in vitro is regulated by an interplay of EGF action and tenasin-C downregulation. J Cell Sci 108(Pt 6):2445–2456

Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J (1994) Mechanism of activation of the TGF-beta receptor. Nature 370:341–347

Yamane K, Asano Y, Tamaki K, Ihn H (2007) Epidermal growth factor up-regulates transforming growth factor-beta receptor type II in human dermal fibroblasts via p38 mitogen-activated protein kinase pathway. Biochem Biophys Res Commun 352:69–77

Yamane K, Ihn H, Tamaki K (2003) Epidermal growth factor up-regulates expression of transforming growth factor receptor type II in human dermal fibroblasts by phosphoinositide 3-kinase/Akt signaling pathway: resistance to epidermal growth factor stimulation in scleroderma fibroblasts. Arthritis Rheum 48:1652–1666

Zhu G, Mehler MF, Mabie PC, Kessler JA (1999) Developmental changes in progenitor cell responsiveness to cytokines. J Neurosci Res 56:131–145

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