Stem Cell Research (2013) 10, 85–94

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METHODS AND REAGENTS

Efficient cultivation of neural stem cells with controlled delivery of FGF-2

U. Galderisi [b, d, c, 1], G. Peluso [c,*, 1], G. Di Bernardo [b], A. Calarco [c], M. D'Apolito [c], O. Petillo [c], M. Cipollaro [b], F.R. Fusco [e], M.A.B. Melone [a, c]

[a] *Division of Neurology, Department of Clinical and Experimental Medicine and Surgery, Second University of Naples, Naples, Italy*
[b] *Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Second University of Naples, Naples, Italy*
[c] *Institute of Proteins Biochemistry - IBP, C.N.R., Naples, Italy*
[d] *College of Science Technology, Temple University, and Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, PA, USA*
[e] *Laboratory of Neuroanatomy, Santa Lucia Foundation at the European Center for Brain Research, Rome, Italy*

Received 14 March 2012; received in revised form 22 August 2012; accepted 19 September 2012
Available online 26 September 2012

Abstract Neural stem cells (NSCs) raised the hope for cell-based therapies in human neurodevelopmental and neurodegenerative diseases. Current research strategies aim to isolate, enrich, and propagate homogeneous populations of neural stem cells. Unfortunately, several concerns with NSC cultures currently may limit their therapeutic promise. Exhaustion of growth factors and/or their uncontrolled release with burst and fall in their concentration may greatly affect the *in vitro* behavior of NSCs. In this context, we investigate whether a device containing heparan sulfate (HS), which is a co-factor in growth factor-mediated cell proliferation and differentiation, could potentiate and prolong the delivery of fibroblast growth factor-2 (FGF-2) and thus improve *in vitro* NSC cultivation. We demonstrated that NSCs cultivated in media with a controlled release of FGF-2 from a polyelectrolyte polymer showed a higher proliferation rate, and reduced apoptosis and senescence. In these experimental conditions NSCs preserve their stemness properties for a longer period of time compared with controls. Also of interest is that cell fate properties are conserved as well. The controlled release of FGF-2 reduced the level of oxidative stress and this is associated with a lower level of damaged DNA. This result may explain the reduced level of senescence and apoptosis in NSCs cultivated in the presence of hydrogel-releasing FGF-2.

Introduction

Neural stem cells (NSCs) are referred to as self-renewing cells derived from the nervous system that can give rise to cells other than themselves through asymmetric division (Gage, 2000). NSCs have been found not only in the embryonic brain

* Corresponding author at: Institute of Proteins Biochemistry - IBP, C.N.R., Naples, Italy.
E-mail addresses: umberto.galderisi@unina2.it (U. Galderisi), g.peluso@ibp.cnr.it (G. Peluso).
[1] These authors contributed equally to the paper.

1873-5061/$ - see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.scr.2012.09.001

but also in the adult nervous system of all mammalian organisms. These cells are able to proliferate and differentiate into the three major cell types of the nervous system (neurons, astrocytes, and oligodendrocytes) both *in vivo* and *in vitro* (Gritti et al., 1995; Jori et al., 2007; Reynolds and Weiss, 1992; Vescovi et al., 1993).

NSCs hold tremendous promise for fundamental biological studies and cell-based therapies in human neurodevelopmental and neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, for which effective therapies are limited or lacking. Unfortunately, several concerns with NSC cultures currently may limit their therapeutic promise. One major problem is the inability to precisely control stem cell behavior in culture, such as proliferation and specification into different cell types.

NSCs are cultivated in serum-free media supplemented with several hormones and cytokines (Gritti et al., 1995; Jori et al., 2007; Reynolds and Weiss, 1992; Vescovi et al., 1993). It has been established that the adult mouse forebrain contains NSCs that can be cultivated *in vitro* when EGF or FGF-2 or their combination is provided (Gritti et al., 1995). In particular, FGF-2 was shown to promote the growth rate of NSCs *in vitro* thereby maintaining their multilineage differentiation potential (Gritti et al., 1995). However, FGF-2's susceptibility to enzymatic degradation may limit its clinical applications. Several methods have been used to produce a controlled release of FGF-2 both *in vivo* and *in vitro*, namely, encapsulation of heparin-Sepharose bound FGF-2 in alginate beads, impregnation of collagen sponges with heparin-FGF-fibrin mixtures, and FGF-2 incorporation into hyaluronate gels or gelatin hydrogels (DeBlois et al., 1994; Edelman et al., 1991; Tabata et al., 1998; Yamada et al., 1997). However, these delivery systems resulted in complete FGF-2 release either in an initial burst or within 3 days. Differently, the release of FGF lasted weeks or months in poly(d,l-lactide-coglycolide) and acidic gelatin delivery systems, or in heparin conjugated micelle or in other carriers, but it is not known whether these systems can be toxic for *in vitro* cultivation of stem cells (Hile et al., 2000; Ikada and Tabata, 1998; Lee et al., 2008). Furthermore, FGF-2, like other bioactive factors used for regenerative medical applications, usually works in a synchronized manner with other growth factors or co-factors during the cell differentiation process (Chen and Mooney, 2003). Indeed, FGF-2 has an important characteristic: its activity is positively regulated by the addition of exogenous heparan sulfate (HS) (Turnbull et al., 2001). Thanks to their variably sulfated domain structure, HS polysaccharides form charged binding pockets. This process enables many different modes of binding with an individual protein thus endowing the protein with regulatory properties (Bernfield et al., 1999). The binding of FGF-2 to HS protects it from proteolytic degradation and prolongs its half-life. HS also functions as a modulator of FGF-2 activity, not as an anionic binder that stabilizes the receptor complex, but as a specific initiator of FGF signaling (Friedl et al., 1997). In our previous work we demonstrate that a device containing HS, which could potentiate and prolong the delivery of FGF-2, is not toxic for *in vitro* cultivation of various cell types and may even improve their *in vitro* cultivation (Calarco et al., 2010; De Rosa et al., 2004). On this premise, we decided to evaluate this experimental platform for optimizing NSC proliferation and differentiation conditions.

Materials and methods

Materials

2-Hydroxyethyl methacrylate (HEMA), 2-methacryloyloxyethyltrimethyl ammonium chloride (METAC) and all chemical reagents were obtained from Sigma-Aldrich, (Milan, Italy). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, trypsin, and Dulbecco's phosphate buffered saline (PBS) were purchased from Hyclone (Milan, Italy). Plastic tissue cultures were from Falcon (Milan, Italy). Surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide) was synthesized and stored in glass containers at −20 °C in the absence of light. Fresh aqueous solutions were prepared from 30 mM stock solutions in dimethyl sulfoxide as needed. Heparan sulfate (HS), and heparinases (I, II, and III) were obtained from Sigma-Aldrich, while human recombinant fibroblast growth factor-2 (FGF-2) was obtained from R&D Systems (Milan, Italy). Stock solutions of 2 mg/ml HS, and 25 mg/ml FGF-2 were prepared.

Scaffold synthesis and FGF-2 release

Polyelectrolyte-modified HEMA hydrogel p[HEMA-co-METAC] (pHM) was obtained by copolymerization of HEMA with the cationic monomer METAC at a ratio of 10:1 mol/mol as previously described (De Rosa et al., 2004; Denizli and Piskin, 1995). The loaded hydrogels were prepared as follows: in the case of pHM dried polymer discs were incubated with an FGF-2 solution, or a heparan sulfate (HS) solution before the addition of FGF-2. The ratios of HS to FGF-2 were 0.5:25 mg/mg. The pHM/HS hydrogel was incubated with FGF-2 (25 μg). To study the kinetics of FGF-2 release, loaded hydrogels were allowed to swell in 2 ml of PBS for 14 days at 37 °C in a humidified atmosphere. Supernatants were removed at different times and stored at −20 °C before assay. The amount of FGF-2 in each sample was determined in triplicate by ELISA. In the presence of HS/FGF-2 complexes, the solution was treated with heparinases before assay. Absorbance was measured at 450 nm on a microplate reader (see also supplemental file 1).

Animal and NSC cultures

Neural stem cells were obtained from the subventricular zone (SVZ) of the brain of adult mice. A thin tissue layer surrounding the ventricles was isolated from coronal slices of the brain of sacrificed animals. After two washes in PBS, the tissue was treated with a dissociation solution, composed of bovine hyaluronidase, type IV-S 660 μg/ml (Sigma-Aldrich Italia, Milano, Italy), DNAse I 40 μg/ml (Sigma-Aldrich Italia), kinurenic acid 0.2 mg/ml (Sigma-Aldrich Italia), trypsin 0.125% (Invitrogen Italia, Milano, Italy) in PBS, for 5 min at 37 °C and triturated with a Pasteur pipette. Then, twenty volumes of DMEM/F12 (1:1) (Invitrogen Italia) were added and the tissues were centrifuged. The pellet was resuspended in DMEM/F12 (1:1) and passed through a 125 μm nylon mesh (BD Biosciences Italia, Milano, Italy). Finally, the cellular suspension was centrifuged again and resuspended in "classical culture medium", composed of DMEM/F12 (1:1), 1× B27, 2.5 μg/ml Fungizone, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Italia) and 20 ng/ml EGF and 20 ng/ml

FGF-2 (Sigma-Aldrich Italia) in bacteriological dishes. After 5–6 days floating neurospheres were evident in the culture. They were pelleted and treated with the dissociation solution to obtain a single cell suspension. Then, cells were plated again in bacteriological dishes at a concentration of 2×10^5/ml in fresh culture medium. Experiments were performed after 10–12 days of cell amplification in the culture. In the control cultures the FGF-2 was added every two days in the medium as above described while in the treated samples the source of FGF-2 was the loaded pHM/HS/FGF-2 polymers. Also in this condition we changed the medium every two days to avoid the accumulation of waste material and cellular debris. Cultures of cells in presence of polymers were carried out as described above with the addition of sterile discs of pHEMA-co-METAC polymers to culture dishes.

NSC differentiation

To induce differentiation, NSCs were plated in DMEM/F12 (1:1), 1× B27, 2.5 μg/ml Fungizone, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Italia) without FGF-2 at a density of 5×10^5 cells/ml onto poly-L-ornithine (Sigma-Aldrich Italia) coated cell culture plates. Cells were incubated for 3–5 days before immunofluorescence analysis (see below).

Cell proliferation and BrdU incorporation

Cell proliferation was evaluated by Quick Cell Proliferation Assay kit II (Biovision, CA, USA). Following silencing, 1000 cells were seeded in 96-well culture plates. At 1, 2, 6, 7, 13, 14, 27, 28 days post-plating, cells were collected and counted. The ratio of the total number of cells at day "n" to the number of cells at day "n-1" was regarded as the cell proliferation rate (Nakamura et al., 2008).

BrdU incorporation was evaluated with Cell proliferation ELISA BrdU colorimetric kit (Roche, Milano, Italy) according to the manufacturer's instructions.

Detection of apoptotic cells

Apoptotic cells were detected by taking advantage of Fluorescein-conjugated Annexin V (Roche, Italy) following the manufacturer's instructions. Apoptotic cells were observed through a fluorescence microscope (Leica Italia, Italy). In every experiment, at least 1000 cells were counted in different fields to calculate the percentage of dead cells in a culture.

Quantitative senescence-associated beta-galactosidase assay

A substrate of beta-galactosidase, 4-methylumbelliferyl-β-D-galactopyranoside (4-MUG) does not fluoresce until cleaved by the enzyme to generate the fluorophore 4-methylumbelliferone. Assay was carried out on lysates obtained from cells that were grown in 96 well plates as reported (Gary and Kindell, 2005). The production of the fluorophore was monitored at an emission/excitation wavelength of 365/460 nm.

TRAP assay

TRAP assay was carried out according to Kim and co-workers (Kim and Wu, 1997). In the TRAP PCR reactions the primers ACX and M2(TS) amplified the telomerase products, while the primers M2(TS) and NT amplified the TRAP internal control. PCR products were resolved on polyacrylamide gels stained with Gelstar (Cambrex Bioscience, Denmark).

NSC differentiation and immunofluorescence analysis

We determined the cell type of NSCs by performing immonofluorescence double stainings on cells grown on glass coverslips. Three days after the induction of differentiation, cells were fixed with 4% paraformaldehyde for 15 min followed by three washes in PBS. Slides were then treated with 0.3% Triton X-100 in PBS for 15 min, washed three times in PBS and incubated for 30 min in a blocking solution containing 5% nonfat dry milk in PBS. At this point, cells were incubated with two primary antibodies at the same time. Antibodies were used for 90 min at room temperature at the following dilutions in 1% nonfat dry milk in PBS: monoclonal anti-Map2 (Sigma-Aldrich Italia) 1:20, polyclonal anti-GFAP (Sigma-Aldrich Italia) 1:1000 and monoclonal anti-Rip (DSHB, Iowa City, IA) 1:500. Afterwards, slides were washed three times with PBS and incubated for 45 min at room temperature with two secondary antibodies simultaneously (FITC-conjugated anti-mouse Ig and TRITC-conjugated anti-rabbit Ig, both from Jackson Immunoresearch, West Grove, PA), diluted 1:100 in 5% nonfat dry milk in PBS. Finally, after further washes in PBS, glass coverslips were counterstained with Hoechst 33342 (Sigma-Aldrich Italia) 100 μg/ml for 5 min, washed with water, mounted on glass slides with Mowiol (Calbiochem, San Diego, CA) containing 2.5% DABCO (Sigma-Aldrich Italia) and observed through a fluorescence microscope (Leica Mycrosystems Italia, Milano, Italy). In every experiment, at least 2000 cells were counted in 15–20 different fields to calculate the percentage of neurons, astrocytes, and oligodendrocytes in culture.

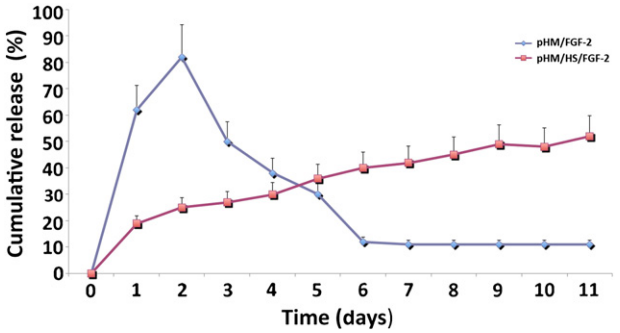
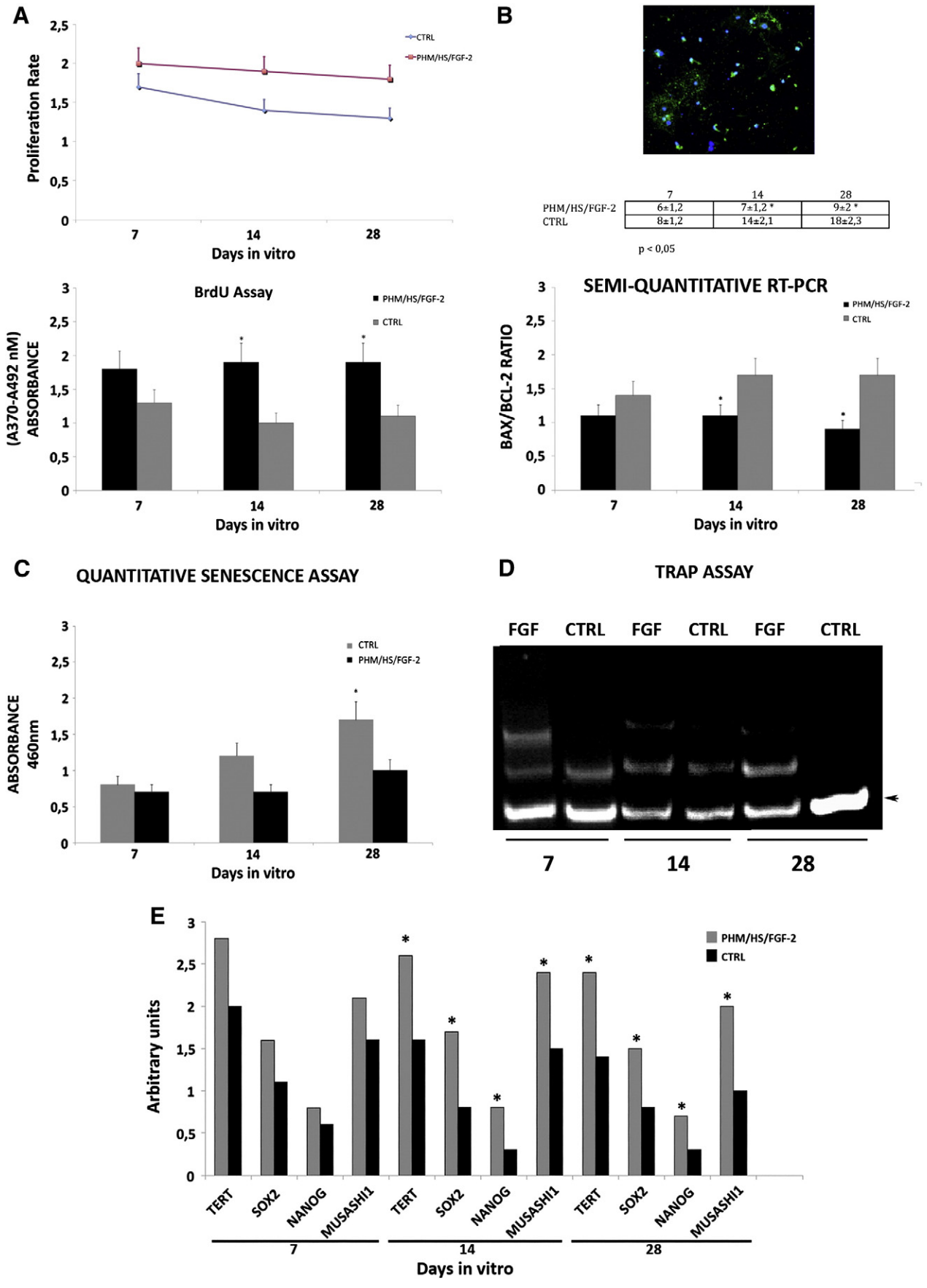


Figure 1 FGF-2 release kinetic. The graphic shows the FGF-2 release from the pHM/FGF-2 and pHM/HS/FGF-2. The values represent the mean ± standard deviation. Experiments were repeated three times. Supernatants were removed at different times and the amount of FGF-2 in each sample was determined in triplicate by ELISA. At day 2, 4, 6, 8, 10, 12 the amount of FGF-2 was determined before medium change.


A
Proliferation Rate
CTRL
PHM/HS/FGF-2
Days in vitro
B
7 14 28
PHM/HS/FGF-2 6±1,2 7±1,2 * 9±2 *
CTRL 8±1,2 14±2,1 18±2,3
p < 0,05
BrdU Assay
(A370-A492 nM) ABSORBANCE
SEMI-QUANTITATIVE RT-PCR
BAX/BCL-2 RATIO
C
QUANTITATIVE SENESCENCE ASSAY
ABSORBANCE 460nm
D
TRAP ASSAY
FGF CTRL FGF CTRL FGF CTRL
E
Arbitrary units
TERT SOX2 NANOG MUSASHI1
Days in vitro

Detection of reactive oxygen species (ROS)

The generation of ROS in NSCs was monitored by the conversion of fluorogenic 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to highly fluorescent dichlorofluorescein diacetate (DCF) within cells by ROS. In brief, NSC cultures were incubated for 1 h at 37 °C with 20 μM DCFH-DA in PBS. Cells were washed with PBS and then incubated for 30 min with 300 μM for 1 h. Cells were then lysed in a buffer containing 0.1% Triton X-100 for 10 min. Fluorescence was measured with a fluorometric plate reader at 480 nm/530 nm.

8-Oxoguanine detection

8oxodG within DNA was detected with Oxoguanine Glycosylase 1 (OGG1) ELISA Kit (antibodies-online.com, Germany) The kit is a sandwich enzyme immunoassay for *in vitro* quantitative measurement of 8oxodG levels in DNA. NSC homogenates were treated according to the manufacturer's instruction. In brief, cell lysates were added to the microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for OGG1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells containing OGG1, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color that was read at 450 nm. The concentration of OGG1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

RNA extraction, RT real-time PCR

Total RNA was extracted from cell cultures using OMNIZOL (Euroclone, Italy), according to the manufacturer's protocol. Levels of mRNA were measured by RT-PCR amplification, as previously reported (Galderisi et al., 1999). Real-time PCR assays were run on an Opticon-4 machine (Biorad, CA, USA). The reactions were performed according to the manufacturer's instructions using SYBRGreen PCR Master mix. The mRNA levels were normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times. The variations in gene expression are given as arbitrary units.

Western blotting

Cells were lysed in a buffer containing 0.1% Triton for 30 min at 4 °C. The lysates were then centrifuged for 10 min at 10,000 g at 4 °C. After centrifugation, 10–40 μg of each sample was loaded, electrophoresed in a polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. All the primary antibodies were used according to the manufacturers' instructions. Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA) and reacted with ECL plus reagent (GE Healthcare, Italy). Each reaction was repeated at least three times. A semi-quantitative analysis of protein levels was carried out by "Quantity One" software associated with "Gel Doc 2000 System" (Biorad Company, CA, USA).

Statistical analysis

Statistical significance was evaluated using ANOVA analysis, followed by Student's t and Bonferroni's tests.

Results

The supplemental file 2 shows a schematic time-line of study design.

Kinetics of FGF-2 release

Polyelectrolyte-modified HEMA hydrogel p[HEMA-co-METAC] (pHM) were used to load FGF-2 both in presence and in absence of heparan sulfate (HS) this to evaluate the effect of

Figure 2 *Panel A* Top: The graphic shows the proliferation rate of NSCs grown in classical medium (CTRL) and in a medium with controlled release of FGF-2 (pHM/HS/FGF-2). The values represent the mean ± standard deviation. Experiments were repeated three times. Bottom: BrdU assay on NSCs grown in control medium and with controlled release of FGF-2. The values represent the mean ± standard deviation. Experiments were repeated three times. * $p<0.05$. *Panel B* Top: Detection of apoptotic cells by Annexin V staining. The fluorescence photomicrograph is a representative picture of NSCs stained with Annexin V (green), which binds to phosphatidylserine residues exposed on the outer layer of the cell membrane during the early stages of apoptosis. Nuclei were counterstained with Hoechst 33342 (blue). The table shows the mean expression values (±SD, n = 3) in control (CTRL) and in cultures with controlled FGF-2 release (pHM/HS/FGF-2). * $p<0.05$. Bottom: Semi-quantitative RT-PCR analysis of BAX/BCL2 ratio. * $p<0.05$. *Panel C* Quantitative senescence-associated beta-galactosidase assay carried out on lysates obtained from control NSCs and from cultures with controlled FGF-2 release (pHM/HS/FGF-2). Data are expressed in arbitrary units. Experiments were repeated three times. Statistically significant variations: *$p<0.05$. *Panel D* Polyacrylamide gel electrophoresis of TRAP assay products obtained from NSCs grown in different conditions. The picture shows the PCR-amplified products indicating telomerase activity. Black arrow: TRAP internal control. The assay measures the enzymatic activity of telomerase. In the first step of the reaction, active telomerase in cell extracts adds a varying number of telomeric repeats (TTAGGG) onto the 3′ end of the substrate oligonucleotide M2 (TS). Next, PCR is used to amplify the extended products. The M2 primer serves as a forward primer, while ACX is the reverse primer for PCR. The products are then electrophoresed on a polyacrylamide gel. The internal control of the reaction (arrow) demonstrates that the PCR reactions were properly carried out. *Panel E* Semi-quantitative RT-PCR analysis of "stemness" genes. HPRT was used as the internal control and data are expressed in arbitrary units. Experiments were repeated three times. Statistically significant variations: *$p<0.05$.

HS on FGF-2 release. To study the kinetics of FGF-2 release, loaded hydrogels were incubated in 2 ml of PBS for 14 days at 37 °C in a humidified atmosphere. To mimic the NSC culture conditions the PBS was changed every two days. Supernatants were removed at different times and the amount of FGF-2 in each sample was determined in triplicate by ELISA (Fig. 1). At days 2, 4, 6, 8, 10, and 12 the amount of FGF-2 was determined before medium change. The release of FGF-2 from p[HEMA-co-METAC] (pHM/FGF-2) in the absence of HS was fast. Almost all the FGF-2 was released within 6 days. In contrast, the release of FGF-2 after the addition of HS to pHM was slower (Fig. 1). Concentration of FGF-2 in medium containing pHM/FGF-2 was about 22 ng/ml at day 1; it reached a peak of 30 ng/ml at day 2 and then declined (Fig. 1). The level of FGF-2 in medium with pHM/HS/FGF-2 was about 7 ng/ml at day 1. It reached 18 ng/ml at day 6 and then remained stable for at least 6 more days (Fig. 1).

This result suggested that pHM/HS/FGF-2 polymer produced a better controlled-release of FGF-2. We then grew NSCs in classical medium with FGF-2 supplementation every two days (control cultures) or in a medium with pHM/HS/FGF-2 polymer. In detail, pHM/HS/FGF-2 polymers were pre-incubated for 5 days in PBS at 37 °C and then were added to NSC cultures. Every 7 days, the polymer was changed. We adopted this procedure to have a stable FGF-2 concentration in culture media.

Effect of FGF-2 controlled release on cell proliferation, apoptosis, and senescence

The cell proliferation curves showed a significant increase ($p<0.05$) in cell growth of cultures incubated with pHM/HS/FGF-2 compared with the control. Differences are particularly evident at 14 and 28 days *in vitro* (Fig. 2A). This data is in agreement with BrdU incorporation assay carried out at the same time as points of cell proliferation analysis (Fig 2A). In several experiments, we observed an increase of S-phase cells in cultures treated with pHM/HS/FGF-2 compared with the control. Of note, at the beginning of treatment (day 1 and day 3), we did not detect any significant difference in proliferation rate between cells grown in pHM/HS/FGF-2 and those grown in classical medium.

Annexin assays evidenced a reduced percentage of apoptotic cells in cultures from pHM/HS/FGF-2 treated samples as compared to the controls (Fig. 2B). Results are in agreement with expression analysis of apoptosis-related genes. In NSCs grown in pHM/HS/FGF-2, we detected a decrease of Bax/Bcl-2 ratio compared to controls (Fig. 2B). Also in this case, at the beginning of treatment (day 1 and day 3), we did not detect any significant difference in apoptosis rate in cells grown in pHM/HS/FGF-2 and those grown in classical medium.

We observed progressive signs of senescence in cells grown in classical medium, as detected by quantitative acid-beta-galactosidase assay. Senescence increased also in pHM/HS/FGF-2 treated cultures but it was lower than in the control (Fig. 2C). To extend this finding, we measured telomerase activity by a primer extension assay in which telomerase reverse transcriptase (TERT) synthesizes telomeric repeats onto oligonucleotide primers (TRAP assay). Extracts obtained from NSCs grown in control medium were telomerase-positive, as expected in stem cell samples. However, the telomerase activity decreased *during in vitro* cultivation due to progressive replicative senescence phenomena (Fig. 2D). Of note, reduction in telomerase activity occurred more slowly in NSC cultures with controlled delivery of FGF-2 (Fig. 2D).

Studies on transcriptional profiling of stem cells allowed a preliminary identification of "stemness" genes participating in the control of stem cell properties, such as self-renewal ability and retention of an uncommitted state (Mikkers and Frisen, 2005; Squillaro et al., 2010). We analyzed a panel of embryonic stemness genes (*Sox2*, *Nanog*, *Musashi-1* and *Tert*) to evaluate the effects of FGF-2 controlled delivery on their expression (Ferron et al., 2009; Leonard et al., 2009; Sundberg et al., 2011). All the genes showed a progressive decline of expression during *in vitro* cultivation (Fig. 2E). Also in this case reduction in gene expression occurred more slowly in NSC cultures with controlled delivery of FGF-2 compared with the control (Fig. 2E).

Altogether these results strongly suggest that controlled delivery of FGF-2 decelerated the apoptosis and senescence phenomena, which occur during *in vitro* cultivation of NSCs.

Molecular analysis of controlled FGF-2 treatment

After analyzing the biological consequences of controlled release of FGF-2 in NSCs, we attempted to elucidate the molecular pathways involved. We analyzed the expression of the P53-P21-P16-RB pathways, which are known to control cell cycle arrest, differentiation, apoptosis, and/or senescence processes (Campisi and d'Adda di Fagagna, 2007; Galderisi et al., 2006).

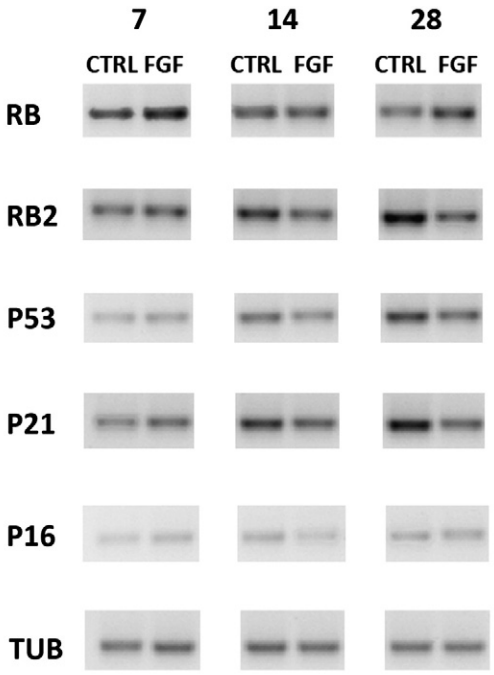
In vitro cultivation of NSCs induced a significant upregulation of P53 and P21 proteins (Fig. 3). This finding is in agreement with progressive decrease of proliferative potential and onset of senescence (Campisi and d'Adda di Fagagna, 2007; Galderisi et al., 2006). Upregulation of these proteins was lower in pHM/HS/FGF-2 treated cultures (Fig. 3).

In the control cultures, we detected a gradual down-regulation of RB1 and an increase of RB2/P130 during *in vitro* cultivation. This is in agreement with the role of RB2/P130 in senescence and regulation of definitive cell cycle exit (Helmbold et al., 2011). Accordingly, cultures with controlled FGF-2 release showed lower expression of RB2/P130 since in these samples we detected higher proliferation and less senescence compared with the control (Fig. 3).

Surprisingly, P16 protein levels did not change significantly in NSC control cultures as expected in cells undergoing replicative senescence. It should be pointed out that senescence triggered by different stimuli might occur in a P16-independent way (Haferkamp et al., 2009; Kim et al., 2009; Zanichelli et al., 2012). Also in cultures with controlled release of FGF-2 we did not detect a significant increase during *in vitro* cultivation (Fig. 3).

FGF-2 release from loaded hydrogel prevents cell fate skewing

Both control neurosphere cultures and those grown in dishes with hydrogels were induced to differentiate by incubation



	7 DAYS		14 DAYS		28 DAYS	
	CTRL	FGF	CTRL	FGF	CTRL	FGF
RB	10.0±1.5	9.0±1.6	7.0±1.1	7.0±0.9	5.5±0.4 #	5.0±0.6 #
RB2	6.0±0.5	6.0±0.7	8.0±1.2	7.0±0.8	12.0±1.5#	7.5±1.0 *
P53	3.8±0.5	4.1±0.5	6.0±0.8	5.0±0.7	8.5±1.0 #	6.0±1.0
P21	8.0±1.1	10.5±1.1	12.0±1.8	11.5±1.5	16.0±1.8#	11.0±1.3*
P16	6.0±0.6	7.5±0.9	7.5±1.1	6.0±0.8	7.1±1.1	7.5±1.2

Figure 3 A representative gene expression analysis of RB- and P53-related pathways of NSCs grown in classical medium (CTRL) and in a medium with controlled release of FGF-2 (pHM/HS/FGF-2). Protein levels were normalized with alpha-tubulin, chosen as the loading control. The table shows protein mean expression values (±SD, n=3). Data are expressed in arbitrary units. For every treatment (classical medium and controlled FGF-2 release, respectively) we carried out a statistical comparison of protein expression at 7 days *in vitro* versus 14 and 28 days *in vitro* # $p<0.05$. Moreover, at each time point we carried out a statistical comparison between CTRL and pHM/HS/FGF-2 samples. * $p<0.05$.

on poly-L-ornithine coated cell culture plates in absence of FGF-2. Differentiation experiments were carried out on NSCs that had been grown in proliferating media for 7, 14, and 28 days.

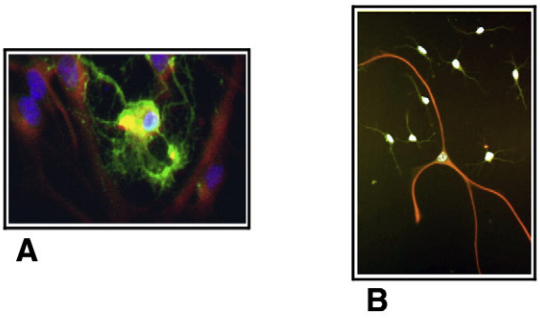
After three–five days of differentiation, we performed immunofluorescent stainings to ascertain whether the proliferation of NSCs in medium with controlled release of FGF-2 might modify cell fate specification. In addition, we detected the expression levels of cell type-specific molecular markers by semi-quantitative RT-PCR. We used primers specific for MAP-2 and GFAP for neurons and astrocytes and primers specific for CGT for oligodendrocytes (Jori et al., 2007).

Control NSCs cultivated for 7 days in proliferating medium and then induced to differentiate displayed 50% of astrocytes, 22% of neurons and 6% of oligodendrocytes (Fig. 4). This is in agreement with our previous results (Jori et al., 2007). The percentage of differentiated cells from NSCs grown in proliferating medium with controlled release of FGF-2 did not change compared to control cultures, suggesting that hydrogel do not alter differentiation potential (Fig. 4).

It is well known that *in vitro* cultivation alters differentiation potential of NSCs (Moses et al., 2006; Ostenfeld et al., 2002; Pagani et al., 2006). Also in our experimental system we detected a bias toward astrocyte differentiation of NSCs as the days *in vitro* increased (Fig. 4, compare 7 days versus 14 and 28 days). Nevertheless, the cultivation of NSCs in medium with controlled

release of FGF-2 slowed down the alteration of NSC differentiation potential (Fig. 4). RT-PCR analysis of differentiation markers confirmed the immunocytochemistry data (Fig. 4).

Overall this finding is in agreement with results showing that cultivation in presence of hydrogel-releasing FGF-2 improved NSC proliferation and reduced senescence and apoptosis phenomena.



	7		14		28	
	PHM/HS/FGF-2	CTRL	PHM/HS/FGF-2	CTRL	PHM/HS/FGF-2	CTRL
Astrocytes	48±6	50±7	51±2	60±7	54±6*	74±6
Neurons	21±4	22±4	23±3	15±2	19±3	10±2
Oligodendrocytes	7±1	6±1	4±1	3±1	4±1*	ND
Undifferentiated	24±4	22±5	22±3	22±4	23±2	16±2

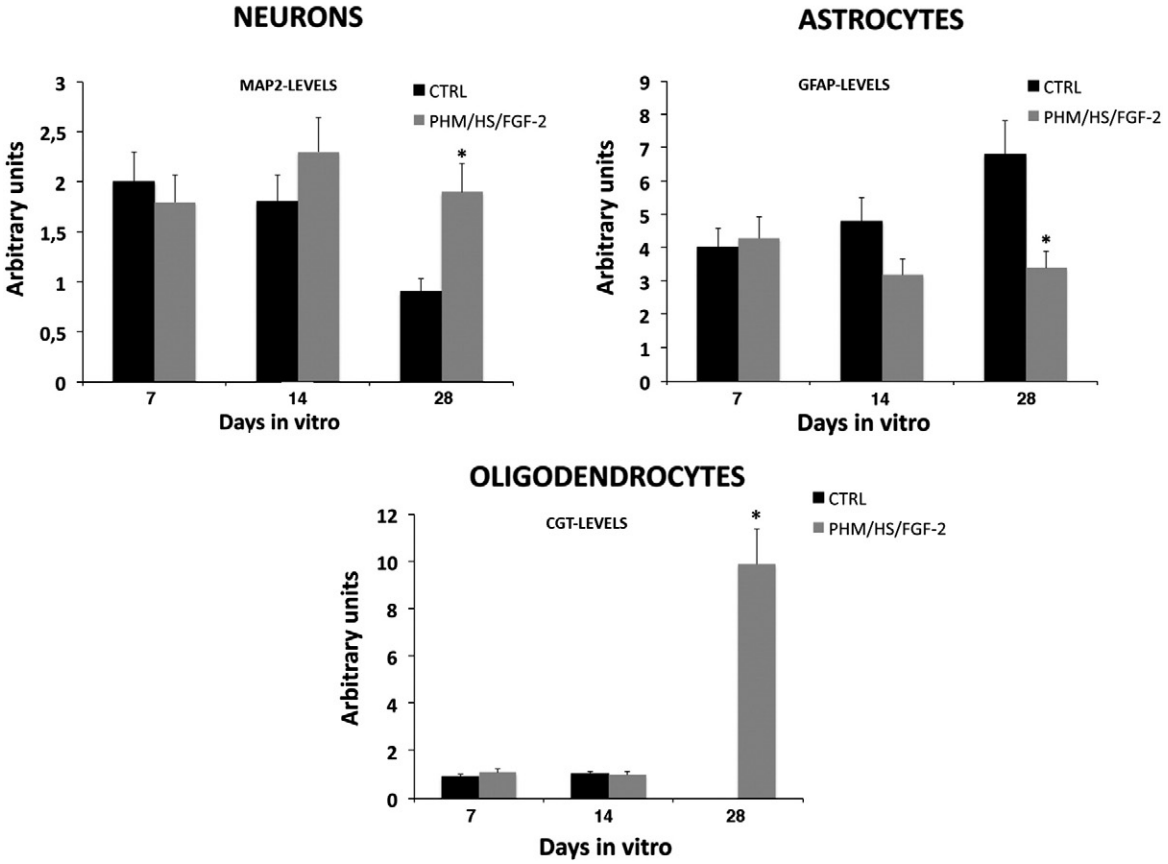


Figure 4 Effects of controlled delivery of FGF-2 on the cell-fate of NSCs grown *in vitro* for 7, 14, and 28 days and then differentiated for 3 days onto poly-L-ornithine-coated plates in medium without FGF-2. Top: A representative fluorescence photomicrograph of differentiated NSCs. Cells were stained for MAP-2 (green) and GFAP (red) (upper panel) or for Rip (green) and GFAP (red) (lower panel). Nuclei were counterstained with Hoechst 33342. The table shows the percentages of NSCs differentiated into neurons, astrocytes, and oligodendrocytes obtained with different experimental conditions (*$p<0.05$). Bottom: semiquantitative RT-PCR analysis of mRNA expression levels of molecular markers of differentiation: MAP-2 (neurons) and GFAP (astrocytes) and CGT (oligodendrocytes). The densitometric values, given as arbitrary units, were normalized with respect to HPRT, chosen as internal control (*$p<0.05$).

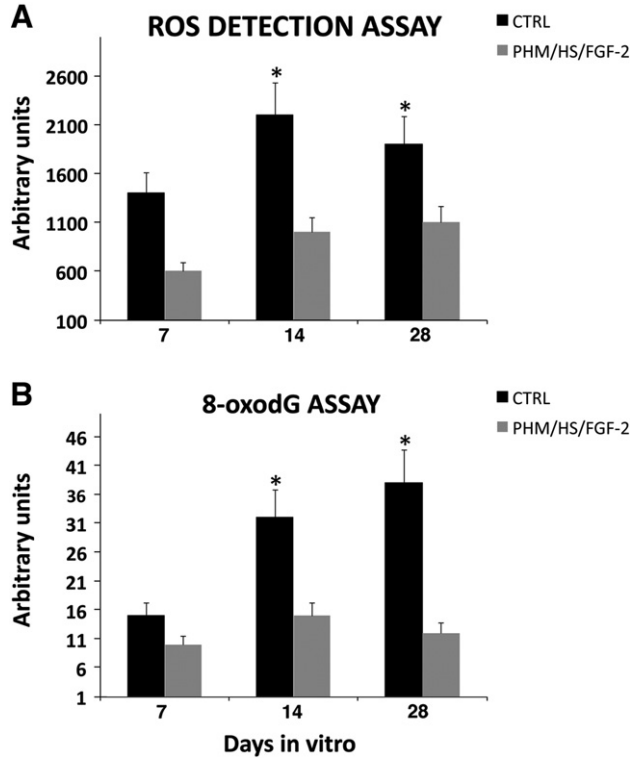


Figure 5 *Panel A* Fluorimetric analysis of ROS production in NSC cultures. The conversion of fluorogenic 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to highly fluorescent dichlorofluorescein diacetate (DCF) within cells by ROS was measured with a fluorometric plate reader at 480 nm/530 nm. The histogram shows the mean expression values (±SD, n = 3) in control (CTRL) and in cultures with controlled FGF-2 release (pHM/HS/FGF-2). * $p < 0.05$. Data are expressed as arbitrary units. *Panel B* Elisa assay to detect 8oxodG level in NSC cultures. The histogram shows the mean expression values (±SD, n = 3) in control (CTRL) and in cultures with controlled FGF-2 release (pHM/HS/FGF-2). * $p < 0.05$. Data are expressed as arbitrary units.

Controlled release of FGF-2 reduced culture oxidative stress

Oxidative stress occurs in all cell culture media. It is especially important in media with low or no serum concentration because many of the anti-oxidation properties of serum are missing. Oxidative stress can modify both cells and cell products. It can lead to cell apoptosis, and/or senescence.

Many formulation strategies aim to reduce oxidative stress associated with serum-free and protein-free media. We evaluated if controlled release of FGF-2 avoiding burst and fall in hormone concentration may reduce cell stress. The detection of ROS by Elisa assay evidenced that the production of reactive oxygen species was significantly reduced in cultures with controlled release of FGF-2 compared with the control (Fig. 5A). Of note, this decrease in oxidative stress produced less DNA damage, as detected with 8-oxodG assay (Fig. 5B). This is in good agreement with reduced apoptosis and senescence phenomena observed in NSCs cultivated with hydrogel.

Discussion

NSCs are stem cells in the nervous system that can self-renew and give rise to differentiated progenitor cells to generate lineages of neurons as well as glia, such as astrocytes and oligodendrocytes. To facilitate the use of NSCs in clinical scenarios, we need to explore the biology of these cells in greater detail. One clear goal is to be able to definitively identify, purify, and expand *in vitro* NSCs. Neural stem and progenitor cells are maintained and expanded as neurospheres in chemically defined media, which are optimized for the culture of human, mouse, and mammal neural stem cells isolated from normal and tumor tissues. Exhaustion of growth factors and an uncontrolled release with burst and fall in their concentration may greatly affect the *in vitro* behavior of NSCs. We evaluated the effect of a controlled release of FGF-2 in presence of HS on the expansion of NSCs in culture while maintaining self-renewal, proliferation, and differentiation potential.

Our results clearly evidenced that controlled release of FGF-2 from polyelectrolyte hydrogel improved *in vitro* cultivation of NSCs. Cultures in presence of pHM/HS/FGF-2 showed a higher proliferation rate, reduced apoptosis, and senescence and preserve their stemness properties for a longer time compared with the controls (Figs. 2, 3). Also of interest is that cell fate properties are conserved as well (Fig. 4).

We demonstrated that controlled release of FGF-2 reduced the level of oxidative stress and this is associated with a lower level of damaged DNA (Fig. 5). This result may explain the reduced level of senescence and apoptosis in NSCs cultivated in presence of hydrogel-releasing FGF-2.

Further studies could dissect the molecular mechanism that associates controlled release of FGF-2 with reduction in ROS production. It should be considered that this may be an innovative research issue and few other reports have described a connection between FGF-2 and oxidative stress. In detail some findings provided evidences that FGF-2 can protect cultured neurons from ROS accumulation (Mark et al., 1997; Zhang et al., 1993). According to other scientists this antioxidant property of FGF-2 may be related to its ability to induce the activity of antioxidant enzymes (Mark et al., 1997; Zhang et al., 1993). It remains to be determined why uncontrolled release of FGF-2 as it occurs in classical NSC cultures produces more ROS than its controlled release.

Collectively, our study supports the conclusion that cultivation of NSCs in medium with controlled release of FGF-2 optimizes proliferation and differentiation conditions for NSCs. This result further proves the versatility of our delivery platform, since in previous works we demonstrated that pHM/HS/FGF-2 hydrogels promote maximal proliferation and differentiation of other stem cells (Calarco et al., 2010; De Rosa et al., 2004). Our delivery platform may contribute to the development of a new class of biomaterial that enables the prolonged controlled delivery of growth factors.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2012.09.001.

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