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Controlled Delivery of the Heparan Sulfate/FGF-2 Complex by a Polyelectrolyte Scaffold Promotes Maximal hMSC Proliferation and Differentiation

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ABSTRACT

Growth factors and other regulatory molecules are required to direct differentiation of bone marrow-derived human mesenchymal stem cells (hMSC) along specific lineages. However, the therapeutic use of growth factors is limited by their susceptibility to degradation, and the need to maintain prolonged local release of growth factor at levels sufficient to stimulate hMSC. The aim of this study was to 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 enhance hMSC stimulation. To this aim, we synthesized cationic polyelectrolyte polymers covalently and non-covalently anchored to HS and evaluated their effect on hMSC proliferation. Polymers non-covalently bound to HS resulted in the release of an HS/FGF-2 complex rather than FGF-2 alone. The release of this complex significantly restored hMSC proliferation, which was abolished in serum-free medium and only partially restored by the release of FGF-2 alone as occurred with polymer covalently bound to HS. We also demonstrate that exposure to HS/FGF-2 during early growth but not during post-confluence is essential for hMSC differentiation down the fibroblast lineage, which suggests that both factors are required to establish the correct stem cell commitment that is necessary to support subsequent differentiation. In conclusion, the delivery platform described here is a step towards the development of a new class of biomaterial that enables the prolonged, non-covalent binding and controlled delivery of growth factors and cofactors without altering their potency. J. Cell. Biochem. 110: 903–909, 2010.

KEY WORDS: MESENCHYMAL STEM CELLS; FGF-2; CONTROLLED DELIVERY; POLYELECTROLYTE POLYMER

Surgical treatment for tissue defects and anomalies include autografts, allografts, and artificial implants [Cristino et al., 2005]. However, the limited availability and donor-site morbidity of autografts, and issues with immune responses from allografts and artificial implants are drawbacks to these methods. Autologous stem cells appear to be a promising alternative [Bianco and Robey, 2001; Caplan and Bruder, 2001; Cristino et al., 2005]. To date, mesenchymal stem cells (MSC) stimulated with differentiation-inducing agents have been used to reconstruct fat, cartilage, muscle and bone tissue [Lalan et al., 2001; Otto and Rao, 2004; Lee et al., 2005; Mauney et al., 2005]. Adipogenic differentiation is induced by xantine, dexamethasone, insulin and indometacin; chondrogenic differentiation is induced by culturing a pelleted micromass of MSC with insulin and transforming growth factor-β; and osteogenic differentiation is induced by dexamethasone, ascorbate, and beta-glycerophosphate [Pittenger et al., 1999]. However, exquisite

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control of the cellular microenvironment by the temporally orchestrated release of growth factor(s) is important for tissue regeneration by stem cells [Tsutsumi et al., 2001]. Consequently, the critical issue of the clinical use of growth factors is to learn how to control their chronic supplementation in vivo so that the target stem cells differentiate into the desired cell type.

Fibroblast growth factor (FGF-2) was shown to markedly increase the growth rate of human MCS (hMSC) in vitro thereby maintaining their multilineage differentiation potential [Tsutsumi et al., 2001], to modulate the differentiation of hMSC in chondrogenic, osteogenic, adipogenic and neural cells and to facilitate the healing of tendons and ligaments in vivo [Chan et al., 2000; Neubauer et al., 2004; Watanabe, 2004; Long et al., 2005; Solchaga et al., 2005]. FGF-2 is also useful for tissue engineering of ligament because it enhances the differentiation of MSC into a fibroblast-like cell type [Hankemeier et al., 2005]. However, FGF-2's susceptibility to enzymatic degradation and its short duration of retention at wound sites has limited its clinical applications. Moreover, in a clinical trial, FGF-2 protein infusion in human heart disease was not effective in the long term probably because the recombinant protein infused is rapidly diffused and/or degraded in situ [Laham et al., 2000; Bush et al., 2001].

Various methods have been used to overcome the shortcomings of FGF-2 treatment, namely, encapsulation of heparin-Sepharose-bound FGF-2 in alginate beads [Edelman et al., 1991], impregnation of collagen sponges with heparin-FGF-fibrin mixtures [Deblois et al., 1994], and FGF-2 incorporation into hyaluronate gels [Liu et al., 1998] or gelatin hydrogels [Yamada et al., 1997; Tabata et al., 1998]. 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 3 weeks or longer in poly(d,l-lactide-co-glycolide) and acidic gelatin delivery systems [Hile et al., 2000], but it is not known whether these systems can support mesenchymal cell differentiation.

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].

HS has been widely used for growth factor delivery because of its electrostatic nature; for example, it interacts weakly with positively charged polymers and strongly with FGF-2 [Thompson et al., 1994]. Moreover, in addition to its role as cofactor for FGF-2-mediated cell proliferation, HS per se has peculiar biological properties. It can increase the expression of differentiating genes in preosteoblast cells, which suggests that exogenous HS has the potential to shift cells from proliferative to differentiative phenotypes [Jackson et al., 2007]. Various approaches have been tried to covalently immobilize HS on biomaterials in order to promote the binding of growth factor to the polymer [Nilasaroya et al., 2008]. However, the decoration of biomaterials with HS to enhance the affinity of a growth factor for biomaterial may improve the loading efficiency of the growth factor but compromise the release of HS and/or the formation of the HS/FGF-2 complex.

Therefore, new implantable delivery system(s) able to control the local and temporal delivery of pro-differentiating factors and co-factors are needed to stimulate the proliferation of tissue progenitor cells, whether endogenous or transplanted. The development of biomaterials that mimic the physiological binding of growth factors to the extracellular matrix (ECM) is a promising strategy for the development of growth factor delivery systems [Young et al., 2005]. In vivo, FGF-2 binds stably to HS, which is a major component of the ECM. Only proteolytic degradation of extracellular matrix (ECM) proteins results in the release of HS and the activation of stored growth factors, which, in turn, leads to the rapid generation of highly localized signals.

In this context, we speculated that FGF-2 significantly stimulates the proliferation of hMSC when associated with HS. Therefore, we investigated the contribution of HS to the regulation of hMSC proliferation and differentiation by FGF-2 using a new delivery platform that was able to release FGF-2 alone or in a complex with HS (see Fig. 1).

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

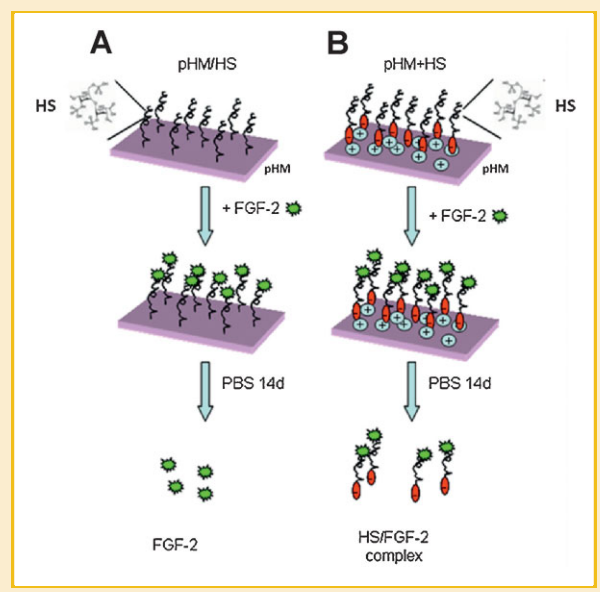

Fig. 1. FGF-2 platform delivery. Adsorption and release of FGF-2 from HS covalently (A) and non-covalently (B) bound polyelectrolyte polymers.

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) and anti-FGF-2 antibodies were obtained from R&D Systems (Milan, Italy). Stock solutions of 2 μg/ml HS, and 25 μg/ml FGF-2 were prepared. The amount of anti-FGF-2 antibody added to the culture medium was calculated on the basis of FGF-2 amount present in the supernatant and in the polymer at the moment of the addition, considering that 50 ng/ml of antibody was able to inhibit the growth of hMSC induced by 1 ng/ml of FGF-2.

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]. As a control, HEMA covalently coupled with HS was copolymerized with METAC to obtain the HS-grafted p(HEMA-co-METAC) copolymer (pHM/HS) as described elsewhere [Denizli, 1999]. The loaded hydrogels were prepared as follows: in the case of pHM, dried polymer discs (Ø = 1.5 cm) were incubated with an FGF-2 solution, or an HS solution before the addition of FGF-2. The ratios of HS to FGF-2 were 0.5:25, and 2:25 (μg/μg), respectively. The pHM/HS hydrogel was incubated with FGF-2 (25 μg). In summary, six samples were prepared: neat pHM, pHM loaded with FGF-2 (pHM + FGF-2), pHM loaded with 0.5 μg HS and 25 μg FGF-2 (pHM + HS/FGF-2 0.5:25), pHM loaded with 2 μg HS and 25 μg FGF-2 (pHM + HS/FGF-2 2:25), pHM modified with grafted HS (pHM/HS), and HS-grafted pHM loaded with FGF-2 (pHM/HS + FGF-2).

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.

CELL CULTURE

Human MSC were obtained from Cambrex (Milan, Italy) and were cultured in Mesenchymal Stem Cell Basal Medium (Cambrex) in a humidified incubator at 37°C with 5% CO_2 and subcultured before the cells became confluent in the presence or absence of 10% SingleQuot™ stimulatory supplements and antibiotics (Cambrex). The media without SingleQuot™ (basal media) were used for all the experiments in which the bioactivity of FGF-2 was tested.

hMSC PROLIFERATION ASSAY

Cells ($6 \times 10^3/cm^2$) were serum-deprived for 24 h, seeded on the polymer disks placed into individual wells of 24-well plates, and allowed to proliferate for 22 h in medium containing 0.2% FCS. The proliferation of cells was determined by DNA assay using a fluorimetric dsDNA quantification kit (PicoGreen, Molecular Probes). Briefly, cells were collected on days 1, 3, 5, 7, and 9, and washed twice with a sterile PBS solution and transferred into 1.5-ml microtubes containing 1 ml of ultrapure water. Samples were incubated for 1 h at 37°C in a water bath and then stored at −80°C until testing. Samples were thawed and sonicated for 15 min before DNA quantification. Samples and standards (ranging between 0 and 2 μg/ml) were prepared for each well of an opaque 96-well plate. To each well, 28.7 μl of sample or standard, plus 71.3 μl of PicoGreen solution and 100 μl of Tris-EDTA buffer were added. Triplicates were made for each sample or standard. The plate was incubated for 10 min in the dark and fluorescence was measured on a microplate reader (Fluostar Optima, bMG Labtechnologies) using an excitation wavelength of 490 nm and an emission of 520 nm. A standard curve was created and sample DNA values were read off from the standard graph. Cells were also cultured on pHM + HS/FGF-2 (low and high HS concentration) polymers in the presence or absence of 10 μM surfen for 7 days.

RT-PCR

Human MSC cultured on the scaffolds for 14 days were harvested by trypsinization and washed twice with PBS. Total RNA was extracted from cells using TRizol reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions at 1 and 2 weeks. RNA concentration was determined using nanodrop (NanoDrop Technologies) and 200 ng RNA was used to synthesize cDNA with SuperScript First-Strand Synthesis System and an oligo dT according to the manufacturer's protocol (Invitrogen). Primer sequences are shown in Table I. After cDNA synthesis, the PCR reaction consisted of 35 cycles of denaturing at 95°C for 30 s, annealing at different temperature as reported in Table I for 30 s, extension at 72°C for 1 min, and a further 6 min at 72°C in the last cycle. The PCR products were analyzed on 2% agarose gels and visualized with ethidium bromide. The relative expression was

TABLE I. Specific Primers Used in RT-PCR

Genes	Forward primers (5′–3′)	Reverse primers (5′–3′)	Annealing	
Collagen type I (col α1 (I)	CTGGCAAAGAAGGCGGCAAA	CTCACCACGATCACCACTCT	63°C	30 s
Collagen type II (col α1 (II)	CACACTCAAGTCCCTCAACAAC	GGTATGTTTCGTGCAGCCATC	64°C	30 s
Collagen type III (col α1 (III)	TTGACCCTAACCAAGGATGC	CACCTTCATTTGACCCCATC	64°C	30 s
Osteocalcin	CCACCGAGACACCATGAGAG	CCATAGGGCTGGGAGGTCAG	66°C	30 s
Tenascin C	GAGATTTAGCCGTGTCTGAGGTTTG	GCCATCCAGGAGAGATTGAAGC	68°C	30 s
Ppar-γ2	CATTCTGGCCCACCAACTT	CCTTGCATCCTTCACAAGCA	66°C	30 s
Scleraxis	CCTGAACATCTGGGAAATTTAATTTTAC	CGCCAAGGCACCTCCTT	60°C	30 s
Gapdh	ACATGTTCCAATATGATTCCA	GGACTCCACGAGCTACTCAG	72°C	30 s

TABLE II. Sequence of Primers Used in Real Time-Polymerase Chain Reaction

Genes	Forward primers (5′–3′)	Reverse primers (5′–3′)
Collagen type I	AGGGCCAAGACGAAGACATC	AGATCACGTCATCGCACAACA
Collagen type III	GACCCTAACCAAGGATGCAA	GGAAGTTCAGGATTGCCGTA
TenascinC	GGAAGTCTTCTGTGACATGAC	CATATGCCTTCCAGTTTTGGTA
Scleraxis	CCTGAACATCTGGGAAATTTAATTTTAC	CGCCAAGGCACCTCCTT
Gapdh	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

quantified densitometrically using the GelDoc System (BioRad, Milan, Italy).

REAL-TIME POLYMERASE CHAIN REACTIONS

Real-time PCRs were performed on BioRad iCycler using SYBR green detection (BioRad). MacVector was used to design primers for Collagen type I, Collagen type III, Tenascin C, Scleraxis (Table II). For each experimental condition, mRNA levels were normalized to those of GAPDH for each time point and are reported as fold changes versus basal values (day 0). All runs were performed according to the default PCR protocol (50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min). For each sample, real-time PCR reactions were performed in triplicate and the average threshold cycle (Ct) was calculated. A standard curve was generated to convert the Ct to copy numbers. We used the delta/delta calculation method described by Winer et al. [1999] for quantification.

STATISTICAL ANALYSIS

All data are reported as mean ± SD unless specified otherwise. Statistical significance calculated by Student's *t*-test was used for pairwise comparisons, and one-way ANOVA was used for multiple comparisons. Statistical significance was analyzed on data from at least three independent experiments on three different polymer samples performed in triplicate. P values <0.05 were defined as significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

RESULTS

EFFECT OF HS ON THE KINETICS OF FGF-2 RELEASE FROM POLYMERS

To determine the effect of HS on FGF-2 release from the cationic polymers, we first examined FGF-2 release (Fig. 2). The delivery of FGF-2 from p[HEMA-co-METAC] (pHM) in the absence of HS was very fast. Almost all the FGF-2 was released within 3 days. In contrast, the release of FGF-2 after the addition of HS to pHM was slower and depended on the concentration of HS in the loading solution. Indeed, the FGF-2 release rate decreased as the concentration of HS in the loading solution increased. Almost all the FGF-2 was released within 7 days from pHM + HS/FGF-2 polymers that contained the lowest HS concentration (HS to FGF-2 ratio, 0.5:25) (Fig. 2). In contrast, pHM + HS/FGF-2 polymers with the highest HS concentration (HS to FGF-2 ratio, 2:25) were characterized by an initial burst followed by a continuous slow release such that 23% of the FGF-2 loaded was in the polymer 14 days after loading (Fig. 2). Interestingly, the presence of HS covalently coupled to the cationic polymer (pHM/HS) profoundly changed the kinetics of FGF-2 release. When pHM/HS was loaded with FGF-2 and incubated with PBS at 37°C, about 47% of the incorporated FGF-2 molecules was released from the hydrogels within 3 days with no substantial release thereafter (Fig. 2).

EFFECT OF POLYMER LOADING ON hMSC PROLIFERATION

To assess whether HS affected the proliferation of hMSC induced by FGF-2, we seeded growth-arrested cells (in FCS-deprived basal medium for 24 h) on the various loaded polymers in 0.2% FCS and then measured cell proliferation. Because the HS/FGF-2 ratio did not affect the cell proliferation rate, we report only the results obtained at a low HS/FGF-2 ratio (0.5:25) (Fig. 3). The growth of hMSC on pHM was barely discernable although the cells remained viable. After 5 and 7 days of culture, the growth of hMSC on FGF-loaded polymers (pHM + FGF) was significantly higher than on polymers without FGF-2 ($P<0.05$), which demonstrates that FGF-2 stimulated hMSC proliferation. Cell proliferation did not increase further after 7 days of culture (Fig. 3). Besides improving hMSC growth versus unstimulated cells, the pHM + HS/FGF-2 polymers significantly improved hMSC growth versus FGF-loaded polymers (Fig. 3). On the contrary, the release of FGF-2 from polymer covalently coupled to HS (pHM/HS + FGF-2) did not induce a significant rate of hMSC proliferation (Fig. 3). Taken together, these results show that HS is an important cofactor for the signaling of exogenous FGF-2, and suggest that soluble HS/FGF-2 complexes are crucial for promoting and regulating hMSC proliferation.

To verify that cell growth stimulation of pHM + HS/FGF-2 was due to the sustained and controlled release of HS/FGF-2 complexes, we carried out experiments with surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide), a small molecule antagonist of HS. A previous study showed that surfen, at a concentration below 20 μM, interacts with HS and antagonizes HS-dependent biological

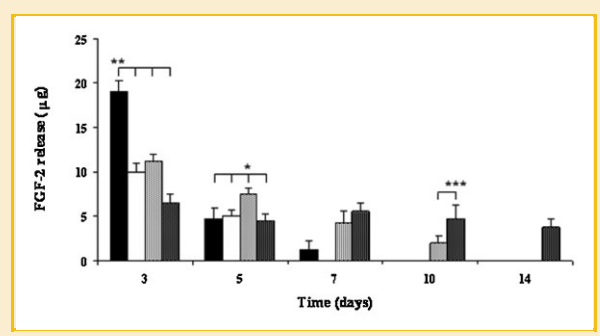

Fig. 2. FGF-2 release kinetics of different cured polymers. FGF-2 release from the pHM + FGF-2 (■), pHM/HS + FGF-2 (□) and pHM + HS/FGF-2 (▨ 0.5:25, and ▩ 2:25 HS/FGF-2 ratio) polymers was monitored for 14 days. The amount of FGF-2 loaded in the polymers was 25 μg. The values represent the mean ± SD (n = 5). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

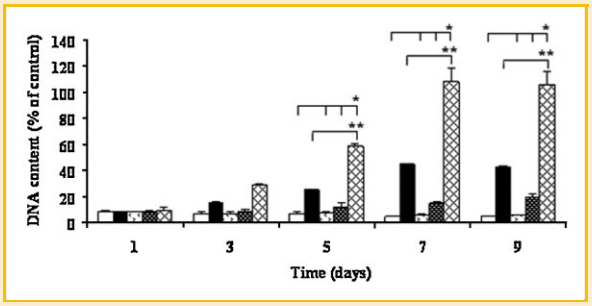

Fig. 3. Proliferation of hMSC on different polymers. Proliferation of serum-deprived hMSC seeded in 24-well plates at an initial density of $6 \times 10^3/cm^2$ on different cured polymers. Cells were cultured for 9 days on pHM (□), pHM + FGF-2 (■), pHM/HS (▨), pHM/HS + FGF-2 (▩), and pHM + HS/FGF-2 (▨, HS/FGF-2 ratio 0.5:25). The proliferation of cells was evaluated at various time points by DNA assay using a fluorimetric dsDNA quantification kit. The values represent the mean ± SD (n = 5). $^*P < 0.05$ and $^{**}P < 0.01$.

processes such as the formation of ternary complexes with FGF-2 and with FGF-2 receptors [Schuksz et al., 2008]. In our study, the addition of surfen to hMSC cultured on pHM + HS/FGF-2 significantly decreased cell proliferation (Fig. 4).

GENE EXPRESSION MARKERS OF hMSC CULTURED ON THE DIFFERENT CATIONIC POLYMERS

We next determined whether HS and FGF-2 exerted cooperative effects on hMSC differentiation. To this aim, we first confirmed by RT-PCR that under our experimental conditions hMSC did not express any detectable levels of mRNAs specific for osteogenic (osteocalcin), chondrogenic (collagen type II) or adipogenic (PPARγ) differentiation markers (data not shown). Then, again using RT-PCR, we measured the gene expression markers of fibroblast differentiation, namely, collagen I, collagen III, tenascin C and scleraxis mRNA, in hMSC cultured on pHM, pHM/HS + FGF-2 and pHM + HS/FGF-2, after 14 days of culture (Table I). Under these conditions, the transcription level of the four genes examined was not significantly upregulated in hMSC cultured on polymers without HS or with covalently bound HS (data not shown). A different result was obtained with the pHM + HS/FGF-2 polymers. In this case, although pHM + HS/FGF-2 polymers promoted hMSC proliferation to the same extent irrespective of the HS/FGF-2 ratio, only the polymer with a low content of HS promoted hMSC differentiation. On the other hand, HS without FGF-2 neither increased nor inhibited hMSC differentiation. The only difference between the pHM + HS/FGF-2 polymers loaded with a low or high amount of HS was the kinetics of FGF release. At the highest HS concentration, the FGF-2 release profile was characterized by an initial relatively fast delivery followed by a prolonged slow release phase that continued into the post-confluence period.

To evaluate the inhibitory or the stimulatory effect of HS or FGF-2 on cell differentiation, we cultured hMSC on pHM + HS/FGF-2 at a high HS concentration for 7 days, and continued to culture the cells on the same polymer for a further 7 days with the addition of anti-FGF-2 blocking antibodies in the presence or absence of surfen. Only in the presence of anti-FGF-2 blocking antibodies, did hMSC resume differentiation (Fig. 5). The contemporary presence of surfen did not induce any additive effect (data not shown). These results suggest that, upon confluence, even a low amount of FGF-2 is sufficient to inhibit hMSC differentiation, and may explain why continuous stimulation with exogenous FGF-2 is sufficient to prevent the differentiation of hMSC in other experimental models [Jacob et al., 2006; Woei et al., 2007].

It has been demonstrated that it is critical for hMSC differentiation to maintain a high density of hMSC over several weeks [Dombrowski et al., 2009]. Consequently, it is possible that the pro-differentiating effect of HS that we identified after 7 days of culture is due only to its capacity to induce greater proliferation of FGF-2-stimulated hMSC. Therefore, to determine whether the effect of HS on differentiation was due only to its ability to promote the effect exerted by FGF-2-induced cell proliferation, we cultured hMSC on

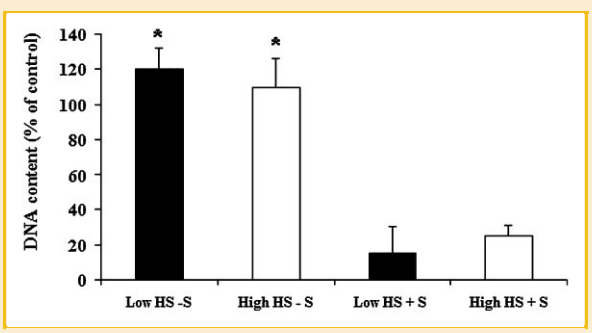

Fig. 4. Role of HS in hMSC proliferation. Serum-deprived hMSC were seeded in 24-well plates at an initial density of $6 \times 10^3/cm^2$ on low and high HS-polymers, and cultured for 7 days in the presence or absence of 10 μM surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide). Cell proliferation was measured by DNA assay using a fluorimetric dsDNA quantification kit. The values represent the mean ± SD (n = 5). $^*P < 0.05$ HS-surfen versus HS + surfen.

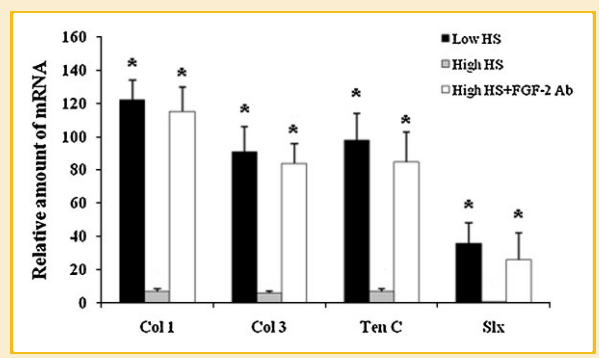

Fig. 5. Modulation of the expression of selected genes in relation to HS content in pHM + HS/FGF-2 polymer. The mRNA levels of collagen I (Col1), collagen III (Col 3), tenascin C (Ten C) and scleraxis (Scx) in hMSC cultured on pHM+HS/FGF-2 polymer with low (■, 0.5:25 HS/FGF-2 ratio) or high (□, 2:25 HS/FGF-2 ratio) HS content in the presence of anti-FGF-2 antibody were determined by quantitative real-time PCR. The relative expression levels of each gene were calculated with the expression level of day 0 set at 1. Each value is the average ± SD of three independent experiments. $^*P < 0.05$ low HS and high HS + FGF-2 Ab versus high HS.

pHM + FGF-2 without HS having first seeded the cells at high density. This did not induce significant differentiation of hMSC although the number of cells on pHM + FGF-2 at day 7 overlapped that of cells cultured on pHM + HS/FGF-2 (data not shown). Therefore, it appears that exposure to FGF-2 and HS during the growth phase may be essential for hMSC differentiation down the fibroblast lineage, which suggests that both factors play an important role in establishing the correct stem cell commitment that is necessary to support subsequent differentiation.

DISCUSSION

Here we report a new growth factor delivery system in which HS is used for the non-covalent linkage of FGF-2 to the pHM polymer thereby resulting in the controlled release of the HS/FGF-2 complex. In turn, the controlled delivery of HS/FGF-2 enhanced the growth of hMSC and improved their differentiation. Much of the potential of our pHM polymer as a biomaterial stems from its cationic nature and high-charge density in solution [De Rosa et al., 2004]. The charge density allows the polymer to form insoluble complexes with anionic molecules such as HS. In addition, HS, which forms ionic complexes with the cationic polymer, maintains the ability to bind FGF-2.

According to the polyelectrolyte interaction theory [Maccarana and Lindahl, 1993], which has been used to analyze the contribution of electrostatics in reactions involving polycations and heparin, HS elutes from cationic polymers at a relatively low ionic strength of the medium because electrostatic forces significantly contribute to its binding. On the other hand, in the case of the HS/FGF-2 complex, which dissociates in the presence of more than 1 M NaCl, only approximately 30% of the binding energy results from pure electrostatic forces [Thompson et al., 1994]. This demonstrates that the FGF-2 released from pHM + HS/FGF-2 polymers is linked to HS.

The finding that HS covalently anchored to pHM does not induce a significant biological response in terms of hMSC proliferation has important implications. Indeed, it confirms that only soluble HS, once linked to FGF-2, promotes receptor-mediated intracellular protein kinase cascades that ultimately lead to cell proliferation.

Another important finding to emerge from this study is the ambivalent role played by HS and FGF-2 in the differentiation of hMSC. FGF-2 exerts a variety of effects on tissue progenitor cells. hMSC stimulated with FGF-2 differentiate preferentially towards the fibroblastic lineage as demonstrated at transcriptional level by the detection of mRNA coding for ligament or tendon extracellular matrix proteins [Arakawa et al., 1994; Altman et al., 2002; Moreau et al., 2005]. In particular, scleraxis, a basic helix–loop–helix transcription factor, is highly expressed in tissues that generate abundant extracellular matrix consisting of collagen I and III such as tendons [Cserjesi et al., 1995]. Scleraxis is an important transcription factor during tendon development as shown by the finding that scleraxis-null mutants exhibit severe defects in the force transmitting tendons [Murchison et al., 2007]. Moreover, scleraxis seems to be required to direct condensations of tendon progenitor cells to form the force transmitting tendons [Murchison et al., 2007]. In this study, we demonstrate that FGF-2 and HS are required for the commitment of hMSC towards the fibroblastic lineage, but FGF-2 must disappear during the post-confluence phase when hMSC cease proliferation, because continuous release of FGF-2 perturbates the differentiation process. Indeed, blocking antibodies resulted in resumption of normal differentiation of hMSC. Therefore, the timing of growth factor administration is clearly crucial for regenerative medicine.

In conclusion, we demonstrate that HS is important in regulating the effect of FGF-2 on hMSC. Modulation of the release of FGF-2 and HS by the delivery platform we report will provide the opportunity to engineer stem cells to follow specific differentiation programs that may be useful for the development of cell replacement therapies.

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