# Supporting Information for

# A New Avenue to the Synthesis of GAG-mimicking Polymers Highly Promoting Neural Differentiation of Embryonic Stem Cells**

*Mengmeng Wang, Zhonglin Lyu, Gaojian Chen, Hongwei Wang, Yuqi Yuan, Kaiguo Ding, Qian Yu, Lin Yuan*, Hong Chen**

## Experiment Section

### Materials

Glucosamine hydrochloride (TCI), methacryloyl chloride (Aladdin), sodium 4-vinylbenzenesulfonate (SS) (Sigma), cyanopentanoic acid dithiobenzoate (CTDB) (Sigma) was used as received. 2,2′ -Azobisisobutyronitrile (AIBN) (Sigma) was purified by recrystallization from ethanol and dried under vacuum. Methanol and all the other solvents were purchased from the Shanghai Chemical Reagent Co. and used as received. Hepain (DS 2.60, MW ~3500-8000Da) was bought from Sinopharm Chemical Reagent Co. Ltd. Deionized water with a minimum resistivity of 18.2 MΩ·cm by a Millipore water purification system was used in all experiments.

High glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Hyclone. Fetal bovine serum (FBS), GlutaMAX™ and non-essential Amino-acids were purchased from Gibco. Mitomycin C was purchased from Solarbio. Leukemia inhibitory factor (LIF) was obtained from Chemicon.

### Characterizations

$^1$H NMR spectra in $D_2O$ were acquired using a Varian Mercury-400 spectrometer.

Aqueous gel permeation chromatography (GPC) was conducted on a waters system comprised of a Waters 1515 HPLC pump and a Waters 2414 refractive index detector with a PL aquagel-OH MIXED-M column. The mobile phase consisted 70% 0.2 M NaNO3 and 0.1 M $NaH_2PO_4$ (adjusted to pH 7) and 30% methanol cosolvent at a flow rate of 1.0 mL/ min. Calibration was with near-monodisperse PEO standards.

### Synthesis of 2-methacrylamido glucopyranose (MAG)

MAG was prepared using the method of Zetterlund and Stenzel.[1] Typically, glucosamine hydrochloride ($2.32\times10^{-2}$ mol) and $K_2CO_3$ ($2.32\times10^{-2}$ mol) were vigorously stirred in 125 mL

methanol in a 250 mL single neck round-bottom flask, followed by the dropwise addition of methacryloyl chloride ($2.09\times10^{-2}$ mol) with vigorous stirring under a methanol/ice bath. The mixture was reacted for 4 h before being filtered through a sintered funnel with vacuum suction. The filtrate was then collected and concentrated under reduced pressure. The slurry was purified by being loaded onto a column chromatography with a 4:1 mixture of $CH_2Cl_2$ and methanol as the eluent. 1H NMR ($D_2O$, 400MHz): $H_1(\alpha)$: 5.25 ppm (d, 0.53H), $H_1(\beta)$: 4.70-4.74 ppm (d, 0.53H), $H_2$-$H_6$: 3.45-4.05 ppm (m, 6H), $H_7$: 1.96 ppm (s, 3H), $H_8$: 5.50 ppm (s, 1H), $H_9$: 5.64 ppm (s, 1H) (Figure S1. Supporting Information).

**Polymerization of synthetic polymers**

The polymers were synthesized through reversible additional-fragmentation chain transfer (RAFT) polymerization with CTDB as the chain transfer agent and AIBN as the initiator.[2] Typical polymerization conditions were: $[M]_0/[CTDB]_0/[AIBN]_0=100/1/0.5$. In the homopolymerization, $[M]_0$ represents $[SS]_0$ and $[MAG]_0$ respectively, while in copolymerization, $[M]_0=[SS]_0+[MAG]_0$. Ratios of [SS] unit to [MAG] unit in copolymers were adjusted by changing the initial feeder ratios. Monomers (1.6 mmol), CTDB (0.016 mmol) and AIBN (0.008 mmol) was dissolved in a 25 mL round-bottom flask containing 1:1 mixture of DMF and $H_2O$ as the solvent. After deoxygenation by bubbling with nitrogen for 30 min, the solution was transferred into a glovebox purified with dry nitrogen. The polymerization was carried out at 70 $^oC$. After desired period of time, the polymerization was terminated by quenching with liquid nitrogen followed by exposure in air. The polymer solution was then dialyzed against water for 2 days prior to being isolated by lyophilization.

**Culture of embryonic stem cells (ESCs)**

Mouse ESCs (R1/E, Stem Cell Bank, Chinese Academy of Sciences) were cultured on feeder cells (mitomycin C-inactivated mouse embryonic fibroblasts, mEFs) in DMEM containing 10 % FBS, 1 % penicillin/streptomycin, 2.0 mM GlutaMAX™, 0.1 mM non-essential amino-acids, 0.1 mM β-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor (LIF; Chemicon). Medium was changed daily. ESCs were passaged every 2 days.

**Differentiation of ESCs**

Before differentiation experiments, ESCs grown on feeder cells were harvested by trypsinization and further directly cultured in a gelatin-coated cell culture flask for two passages to remove mEFs. For differentiation experiments, ESCs at a density of $3\times10^5$ cells/$cm^2$ were grown without feeder cells on new gelatin-coated plates in growth medium (DMEM containing 10 % FBS, 1 % penicillin/streptomycin, 2.0 mM GlutaMAX™, 0.1 mM non-essential amino-acids and 0.1 mM 2-mercaptoethanol) or neural differentiation medium (growth medium supplemented with 1.0 μM RA). Medium was changed every 2 days.

## Cell proliferation assay

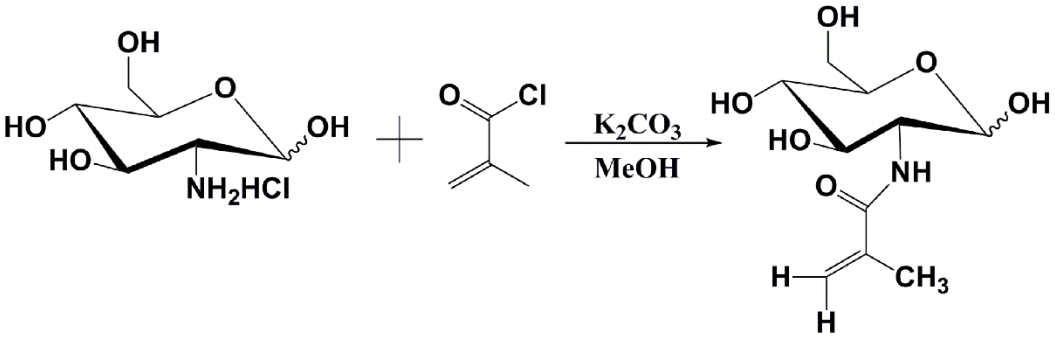
L929 is fibroblast from the connective tissue of mouse. They were grown directly on cell culture plate. ESCs were grown without feeder cells as for differentiation study. Proliferation of L929 cells and ESCs was assessed using an MTT test. After desired incubation time, cells treated with different synthetic polymers were further cultured in 200 μL fresh medium containing 20 μL MTT (5 mg/mL) for 4 h. Then the medium was replaced by 220 μL DMSO to dissolve the formazan crystals generated by living cells and the absorbance of the resulting solution was measured at 490 nm by a microplate reader (Varioskan Flash, Thermo Scientific).

## Immunofluorescent staining

Differentiated cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100/PBS for 5 min. The cells were then blocked with 3% bovine serum albumin (BSA) in PBS for 30 min before being incubated with the primary antibody (Rabbit anti-mouse anti-β3-tubulin antibody, Cell Signaling) in 1% BSA/PBS overnight at 4°C. The corresponding secondary antibody (FITC-labeled goat anti-rabbit antibody, Invitrogen) was added and the cells were incubated at room temperature for 1 h, followed by a 5 min incubation with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) stain. Washing of the wells with PBS was done after each abovementioned step. Immunofluorescent staining images were obtained using an Olympus IX 71 fluorescence microscope..

## Reverse transcription polymerase chain reaction (RT-PCR) and Real-time quantitative polymerase chain reaction (Real-time qPCR)

A total RNA extraction kit (Tiangen Biotech Co., Ltd.) was used to extract the total RNA of differentiated cells according to the manufacturer's instructions. The extracted total RNA samples were then reverse transcribed for first-strand cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) using oligo (dT) as the reverse transcription primer. qPCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) utilizing SYBR Green master mixed with a ROX reference dye (ABI). The reaction solution contained 200 nM of each primer in a 20 μL total volume. β-actin was used as the housekeeping gene. Reaction conditions were as follows: 95 °C for 20 s, followed by 45 cycles at 95°C for 15 s and 60 °C for 30 s. Each sample was analyzed in triplicate. Relative expression of β3-tubulin in mESCs of each experimental group was calculated compared to that in mESCs of the control group on condition that the expression of housekeeping gene was the same for cells in all groups.




Supplementary Scheme S1. Synthesis of MAG.

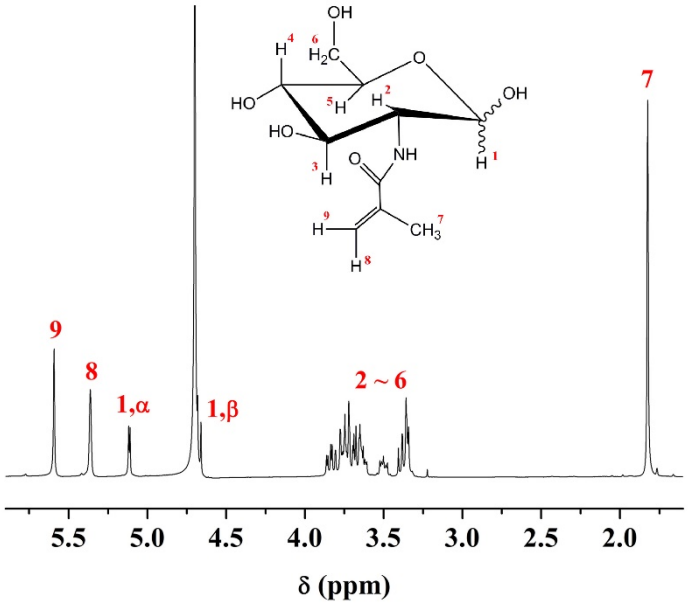



**Figure S1**. $^{1}$H NMR spectra of MAG.

**Table S1**. Characterization of synthetic polymers.

| Polymers | Ratios of SS to MAG ($^{1}$H-NMR) | Mn (GPC) | PDI |
|---|---|---|---|
| $pS_2G_1$ | 1:0.56 | $8.4\times10^3$ | 1.19 |
| $pS_1G_1$ | 1:0.93 | $8.9\times10^3$ | 1.19 |
| $pS_1G_2$ | 1:1.83 | $8.8\times10^3$ | 1.20 |
| | | | |

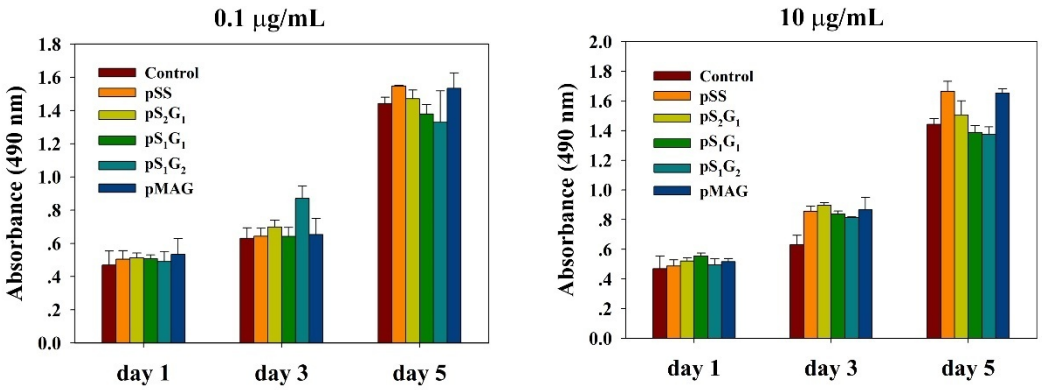



**Figure S2**. Influences of synthetic polymers on the proliferations of L929 cells.

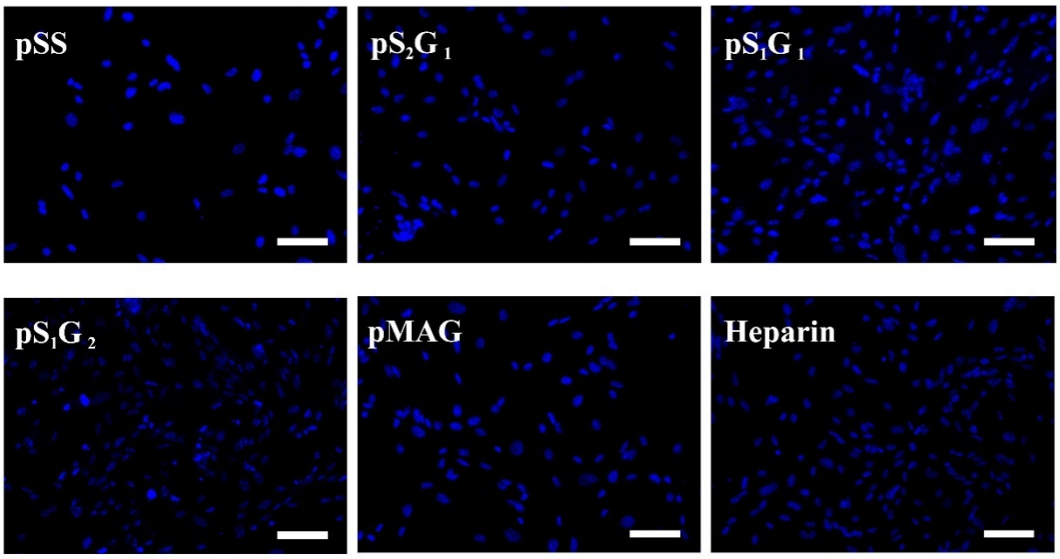



**Figure S3**. Fluorescence images of mESCs after the treatment with synthetic polymers and heparin respectively for 20 days (blue: nuclei/DAPI) (scale bar: 100 μm).

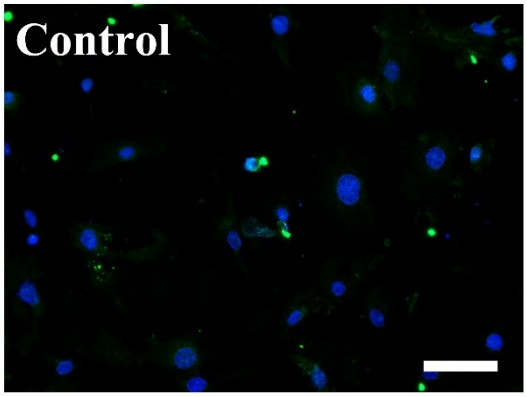



**Figure S4**. Immunofluorescence image (green: β3-tubulin; blue: DAPI) of mESCs after 20 days culture without any synthetic polymers or heparin (scale bar: 100 μm).

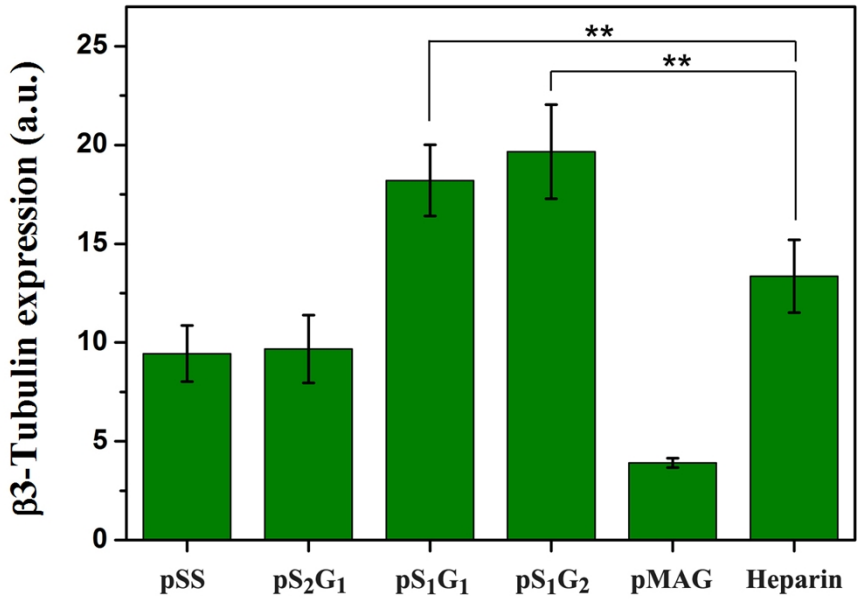



**Figure S5**. The expression of β3-tubulin in mESCs treated by the synthetic polymers. ** represents the significant difference between mESCs treated by heparin and the copolymers ($pS_1G_1$ and $pS_1G_2$) ($p < 0.01$).

**References**

[1] S. S. Ting, E. H. Min, P. B. Zetterlund and M. H. Stenzel, *Macromolecules,* 2010, **43**, 5211-5221.

[2] A. B. Tekinay and M. O. Guler, *Biomacromolecules,* 2011, **12**, 3508-3519.