

Supplementary Materials

Differential effects of heparin and hyaluronic acid on neural patterning of human induced pluripotent stem cells

Julie Bejoy¹, Zhe Wang², Brent Bijonowski¹, Mo Yang², Teng Ma¹,

Qing-Xiang Sang^{2,3}, Yan Li^{*,1,3}

¹Department of Chemical and Biomedical Engineering; FAMU-FSU College of Engineering; Florida State University; Tallahassee, FL USA

²Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida, USA

³Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida, USA

Number of pages: 16

Number of figures: 12

Number of tables: 3

Table of contents

Supplementary Figure S1. ¹H NMR histogram confirming the methylation of HA.

Supplementary Figure S2. Synthesis of Thiolated-Heparin (Hep-SH).

Supplementary Figure S3. Pluripotent marker expression for hiPSCs grown with mTeSR + ECM medium.

Supplementary Figure S4. Illustration of the cortical neural spheroid differentiation protocols from hiPSCs.

Supplementary Figure S5. Effect of ECMs on the viability of neural spheroid outgrowth.

Supplementary Figure S6. Effects of ECMs on neural lineage commitment of hiPSCs.

Supplementary Figure S7. The influence of ECM treatment on expression of glutamate and GABA.

Supplementary Figure S8. Effects of ECM treatment on the expression of F-actin stress fiber and outgrowth proliferation.

Supplementary Figure S9. Enlarged confocal images of YAP from the represented groups to show cytoplasmic and nuclear YAP (supporting data for Figure 4Bi).

Supplementary Figure S10. Supporting data (another two repeats) for Western blot of active β -catenin.

Supplementary Figure S11. Active beta-catenin expression under IWP4 treatment.

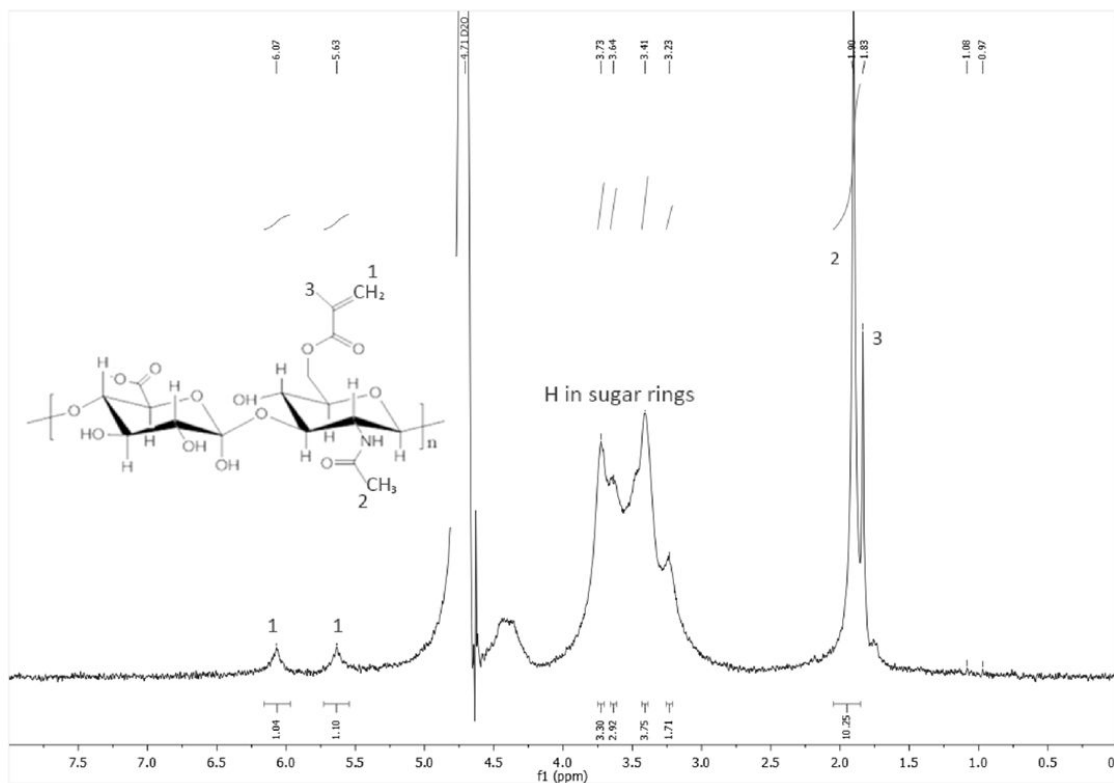
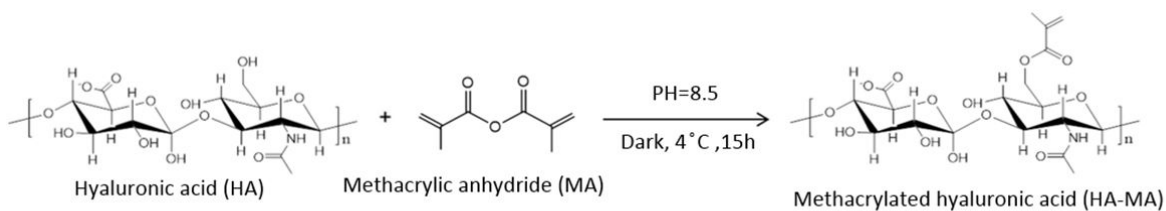
Supplementary Figure S12. BRN2 and PROX1 expression for cortical spheroids outgrowth cultured within different Hep-HA hydrogels.

Supplementary Table S1. A list of antibodies.

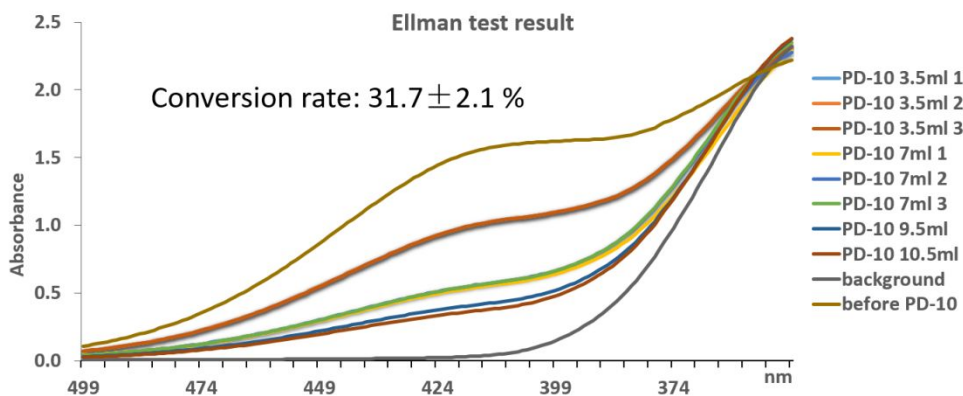
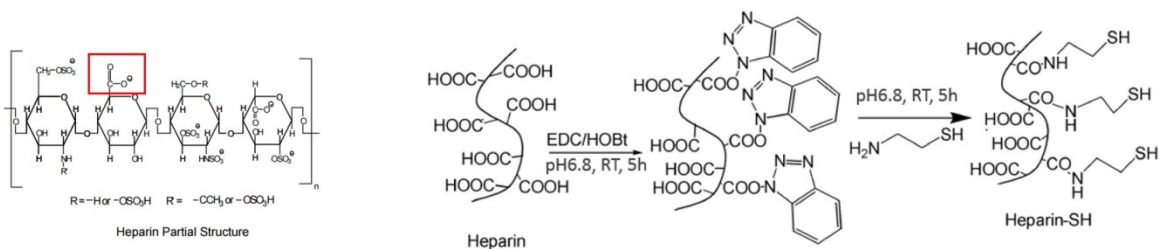
Supplementary Table S2. Primer sequence for target genes.

Supplementary Table S3. Two-way ANOVA analysis for Figure 3, 5, and 6.

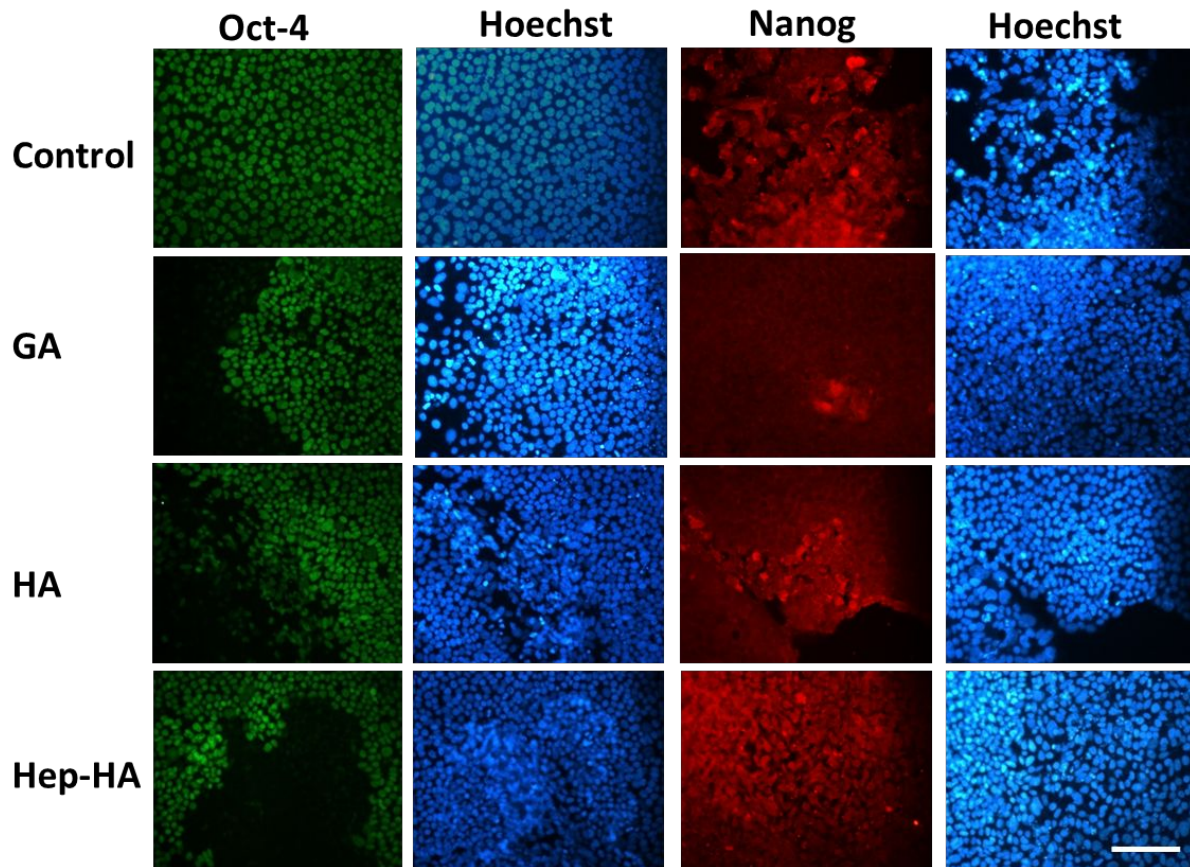
Supplementary Figure S1. ^1H NMR histogram confirming the methylation of HA. Peaks at 5.63 and 6.07 ppm were attributed to the methacrylate protons ($\text{C}=\text{CH}_2$). The peak at 1.9 ppm belonged to the protons of methyl (CH_3). According to the integral area of protons, HA-MA has 29.5% methacrylation degree. HA and MA reaction was modified from [1].



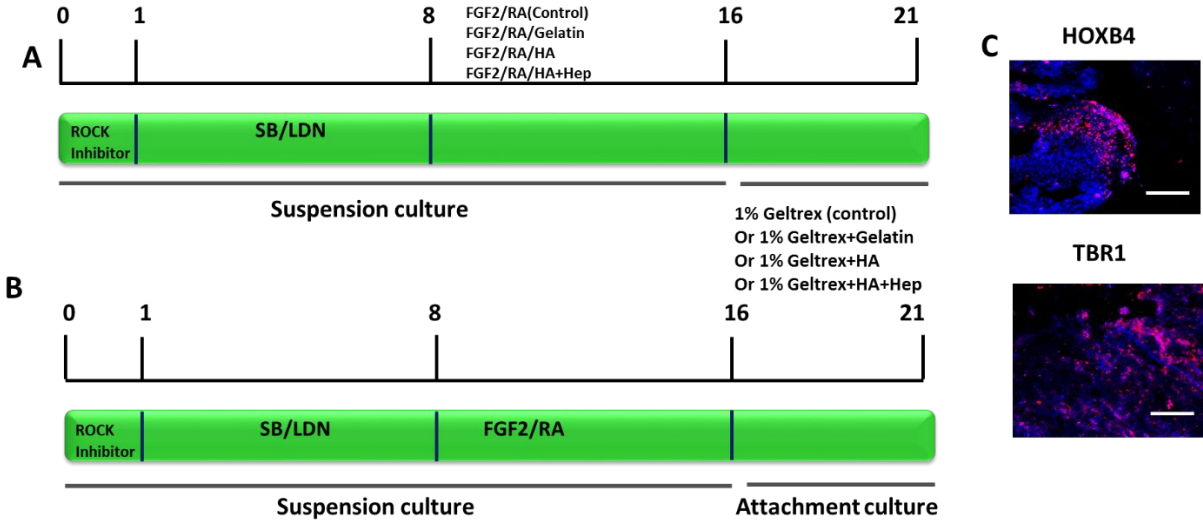
Supplementary Figure S2. Synthesis of Thiolated-Heparin (Hep-SH). Ellman tests showed that the conversion rate was $31.7 \pm 2.1\%$. The illustration of Heparin modification reaction was modified from [2].



Supplementary Figure S3. Pluripotent marker expression for hiPSCs grown with mTeSR + ECM medium. The cells grown with mTeSR + ECM media were analyzed for pluripotency markers. Fluorescent images of pluripotency markers Oct-4 and Nanog at day 10. Scale bar: 100 μm .

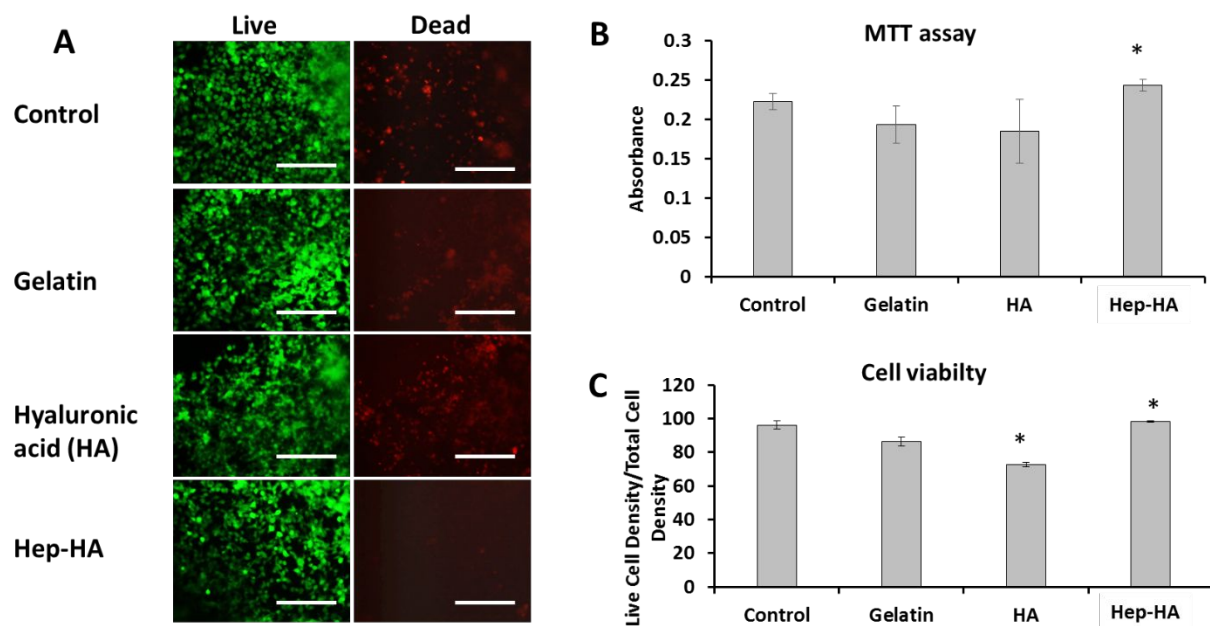


Supplementary Figure S4. Illustration of the cortical neural spheroid differentiation protocols from hiPSCs. The cortical differentiation protocols for ECMs treatment at (A) Day 8 and (B) Day 16. (C) Representative confocal images of HOXB4 (a hindbrain marker) and TBR1 (a deep cortical layer IV marker) expression in the cortical spheroids (day 24). Scale bar: 100 μ m.

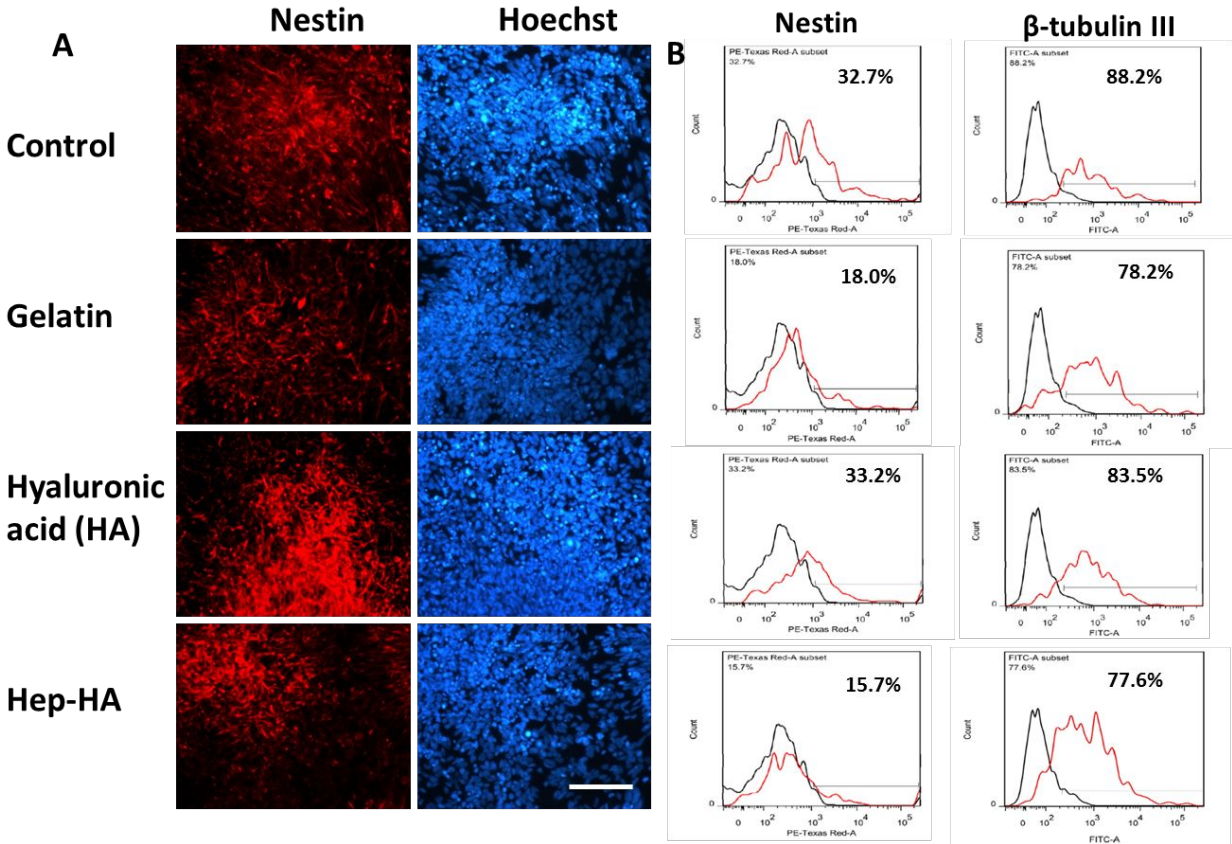


Supplementary Figure S5. Effect of ECMs on the viability of neural spheroid outgrowth. (A)

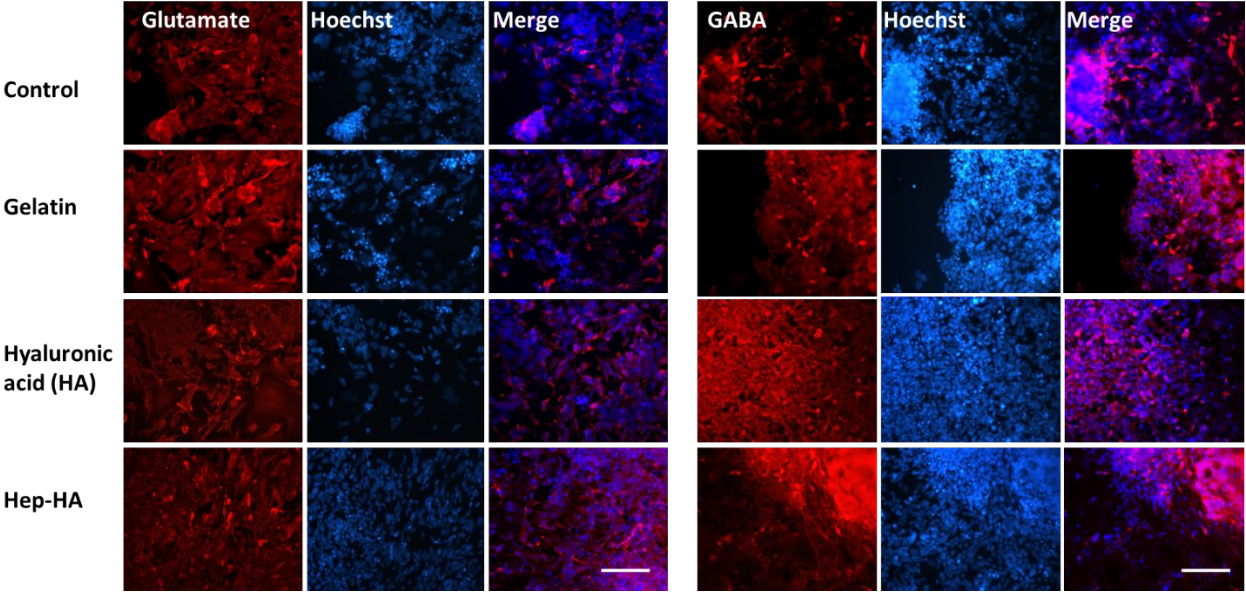
Live/Dead images of neural spheroids treated with ECMs at day 20. Scale bar: 100 μm . After 72 hours, the cells were incubated in DMEM-F12 containing 1 μM calcein-AM (green and for live cells) and 2 μM ethidium homodimer I (red and for dead cells) for 30 min. The samples were imaged under a fluorescent microscope. (B) MTT activity. (C) Quantification of the cell viability based on Live/Dead assay. *indicates $p < 0.05$.



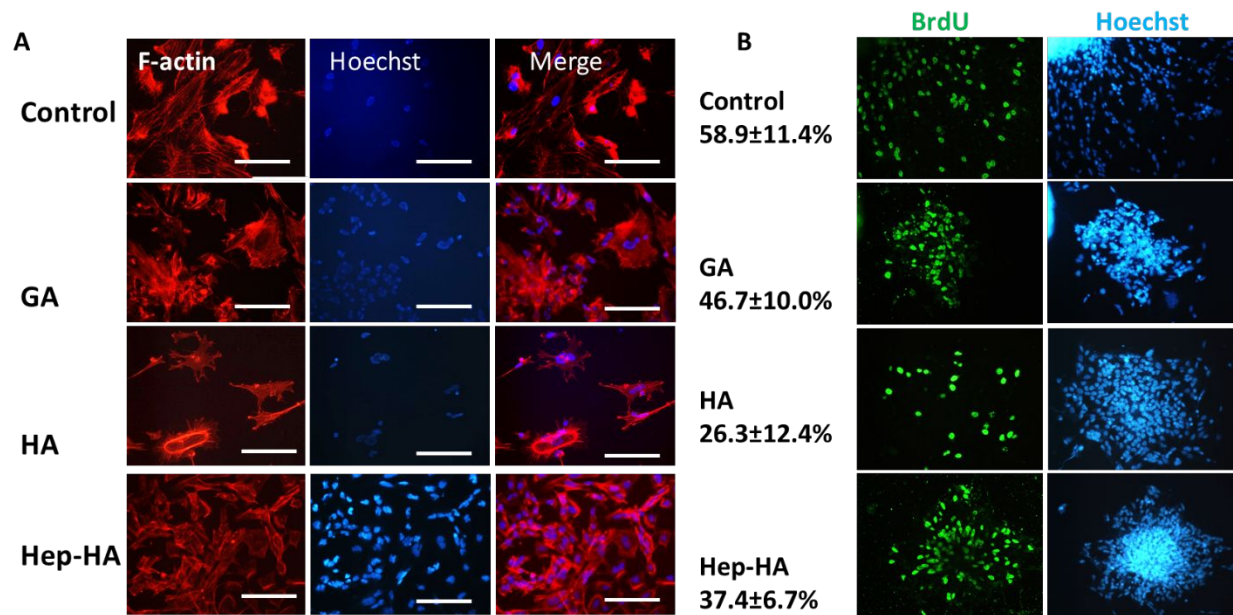
Supplementary Figure S6. Effects of ECMs on neural lineage commitment of hiPSCs. The cells treated with ECMs at Day 16 were analyzed for neural markers. (A) Fluorescent images of neural progenitor marker Nestin. Scale bar: 100 μ m. (B) Flow cytometry analysis of Nestin and β -tubulin III expression. Black line: negative control; Red line: marker of interest.



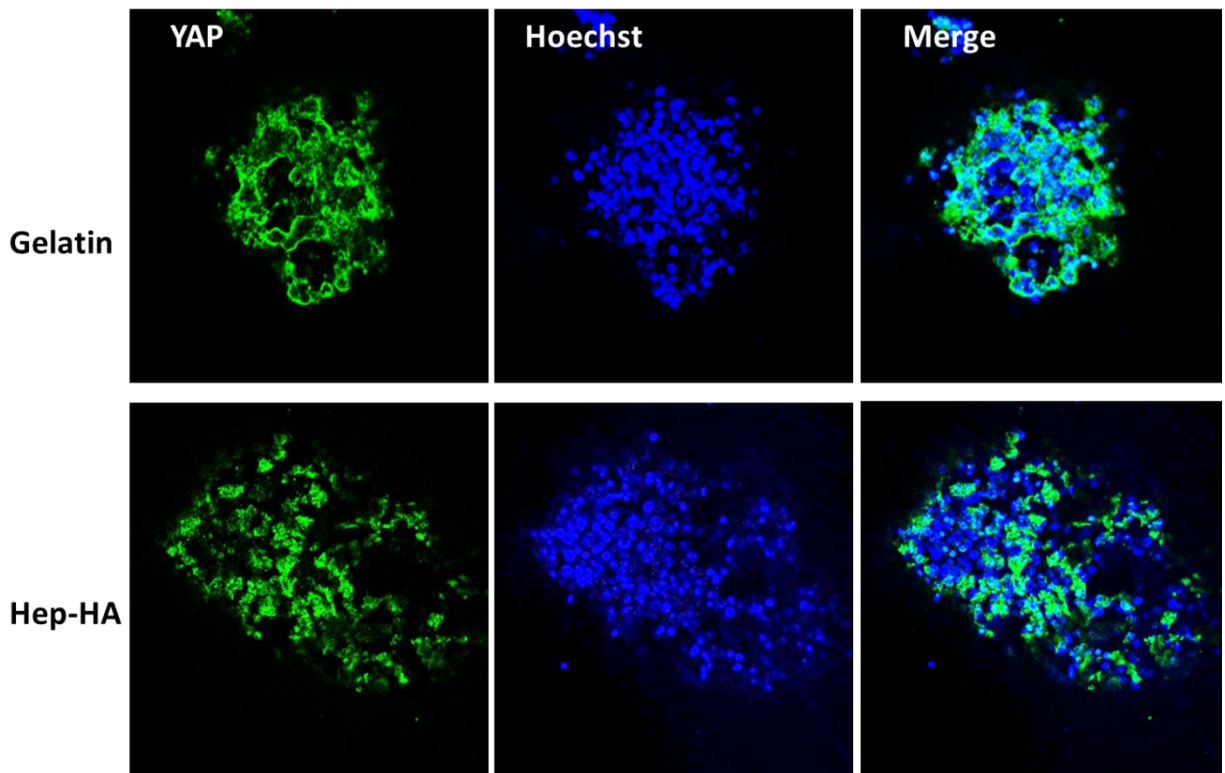
Supplementary Figure S7. The influence of ECM treatment on expression of glutamate and GABA. Fluorescent images of neural markers Glutamate (red)/Hoechst (blue) (for excitatory neurons) and GABA (red)/Hoechst (blue) (for inhibitory neurons). Scale bar: 100 μ m.



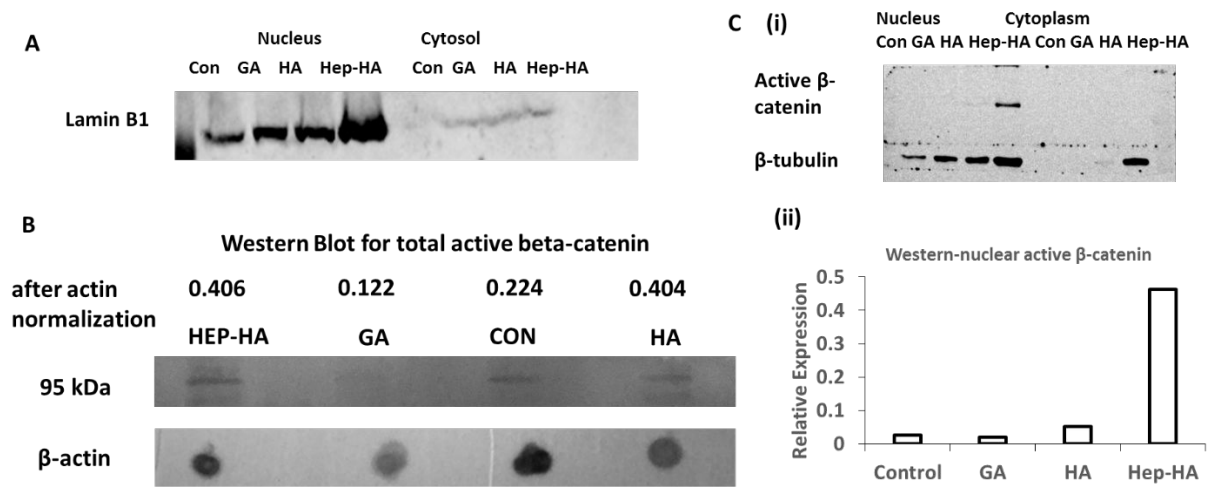
Supplementary Figure S8. Effects of ECM treatment on the expression of F-actin stress fiber and outgrowth proliferation. (A) Fluorescent images of F-actin stress fiber after treatment of iPSCs with ECMs (day 20). Scale bar: 100 μ m. (B) Images of BrdU positive cells for different ECM groups (day 20). Scale bar: 100 μ m.



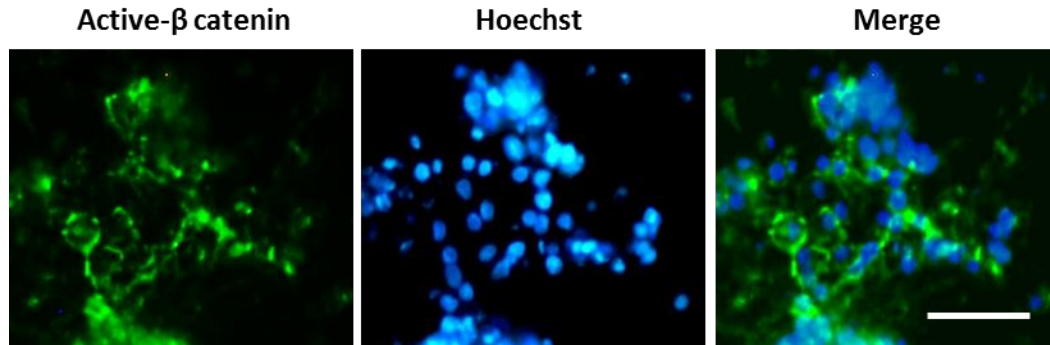
Supplementary Figure S9. Enlarged confocal images of YAP from the represented groups to show cytoplasmic and nuclear YAP (supporting data for Figure 4Bi). The images show the more cytoplasmic YAP in Gelatin (GA) group and the more nuclear YAP in Hep-HA group.



Supplementary Figure S10. Supporting data (another two repeats) for Western blot of active β -catenin. (A) Western blot bands of lamin B1 to confirm the extraction method for obtaining nuclear or cytoplasmic proteins. (B) Western blot for total active β -catenin protein. (C) (i) Western repeat for nuclear active β -catenin protein; (ii) Quantification of nuclear active β -catenin protein (Supporting data for Figure 4C).

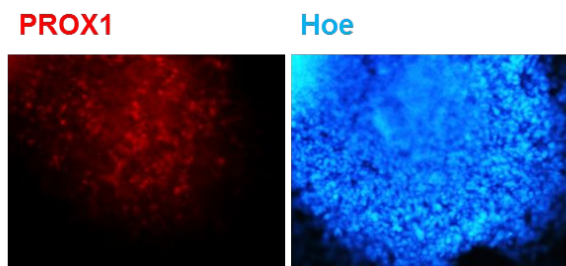
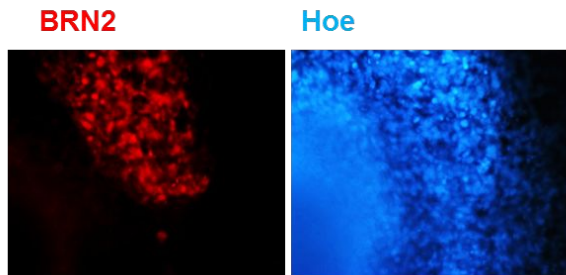


Supplementary Figure S11. Active beta-catenin expression under IWP4 treatment. This result is to confirm the effect of IWP4 on canonical Wnt signaling inhibition. Scale bar: 50 μm .

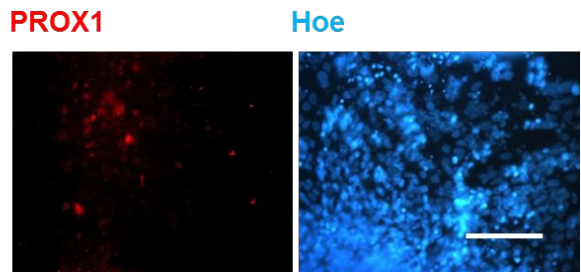
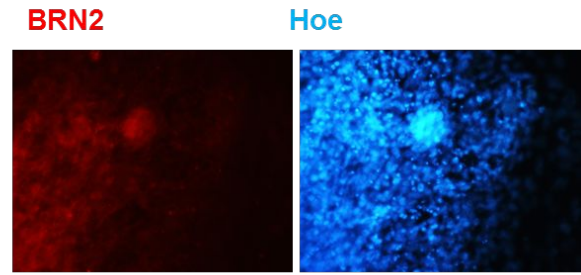


Supplementary Figure S12. BRN2 and PROX1 expression for cortical spheroids outgrowth cultured within different Hep-HA hydrogels. Representative fluorescent images of forebrain markers BRN2 (red) and PROX1 (red) for 2 wt% and 4 wt% hydrogel cultures (day 24). Hoechst: blue. Scale bar: 100 μ m.

2 wt%



4 wt%



Supplementary Table S1. A list of antibodies.

Cells	Primary Antibody	Origin/ Isotype	Supplier/ Cat#	Dilution
Neural cells	Nestin	Rabbit IgG	Sigma, N5413	1:100
	PAX6	Mouse IgG1	Santa Cruz, sc-81649	1:100
	β -tubulin III	Mouse IgG1	Millipore, MAB1637	1:200
	MAP-2	Rabbit IgG	ABCAM, ab32454	1:200
	tau	Mouse IgG ₁	Sigma, T9450	1:100
	TBR1 (layer VI)	Rabbit IgG	ABCAM, ab31940	1:200
	BRN2 (layer III)	Goat IgG	Santa Cruz, sc-6029	1:200
	HOXB4	Rabbit IgG	ABCAM, ab76093	1:200
	PROX1 (hippocampal)	Rabbit IgG	ABCAM, ab101851	1:200
	Islet-1	Rabbit IgG	Millipore, AB4326	1:300
	Glutamate	Rabbit IgG	Sigma, G6642	1:1000
	GABA	Rabbit IgG	Sigma, A2052	1:1000
Pathway	Active β -catenin	Mouse IgG1	Millipore, 05-665	1:100
	YAP	Rabbit IgG	Santa Cruz, sc-15407	1:100
	F-actin	Alexa Fluor® 594 Phalloidin	Life Technologies, A12381	1:200
Mesoderm	Nkx 2.5	Rabbit IgG	Santa Cruz, SC-14033	1:200
Endoderm	FOXA2	Rabbit IgG	Millipore, AB4125	1:200
Pluripotency	Oct4	Mouse IgG1	Millipore, MAB4419	1:200
	Nanog	Rabbit IgG	Millipore, AB9220	1:200
Proliferation	BrdU	Mouse IgG1	Life Technologies, 03-3900	1:200
Secondary	Alexa 488, goat anti-mouse IgG1	-	Life Technologies, A-21121	1:200
	Alexa 488, goat anti-rabbit IgG	-	Life Technologies, A-11034	1:200
	Alexa 594, goat anti-rabbit IgG	-	Life Technologies, A-11012	1:400
	Alexa 594, donkey anti-goat IgG	-	Life Technologies, A-11058	1:400

Supplementary Table S2. Primer sequence for target genes.

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
TBR1	CCCCCTCGTCTTTCTCTTACC	TAATGTGGAGGCCGAGACTTG
HOXB4	AATTCCTTCTCCAGCTCCAAGA	CCTGGATGCGCAAAGTTCA
PROX1	GACTTTGAGGTTCCAGAGAGA	TGTAGGCAGTTCGGGGATTG
Beta-actin	GTACTCCGTGTGGATCGGCG	AAGCATTGCGGTGGACGATGG

Supplementary Table S3. Two-way ANOVA analysis for Figure 3, 5, and 6.

Figure 3. Two-way ANOVA F-value and P-value

Gene	Method	Group		Day		Group*Day	
		F-value	P-value	F-value	P-value	F-value	P-value
HOXB4	ICC	9.7001	0.0031**	22.3643	0.0005**	2.0960	0.1657
	Flow	13.2872	0.0062**	129.8439	<0.0001**	14.9904	0.0046**
TBR1	ICC	3.3725	0.0688	96.6488	<0.0001**	16.5043	0.0004**
	Flow	11.0053	0.0098**	8.4233	0.0273*	1.5497	0.2867
PROX1	ICC	10.2960	0.0025**	47.8427	<0.0001**	28.7838	<0.0001**
	Flow	0.7357	0.5179	0.2710	0.6213	0.4098	0.6811
ISL1	ICC	8.8506	0.0043**	0.3402	0.5705	16.8447	0.0003**
	Flow	7.8851	0.0209*	14.2662	0.0092**	0.6392	0.5602

Figure 5. IWP4 treatment two-way ANOVA F-value and P-value

Gene	Group		Treatment		Group*Treatment	
	F-value	P-value	F-value	P-value	F-value	P-value
HOXB4	2.6118	0.1528	3.6389	0.1051	0.1064	0.9007
TBR1	1.0848	0.3961	23.9717	0.0027**	3.3757	0.1042
PROX1	1.1085	0.3893	0.6894	0.4382	0.4687	0.6469
ISL1	0.1621	0.8540	17.9479	0.0055**	0.7289	0.5207

Figure 6. Cyto D treatment two-way ANOVA F-value and P-value

Gene	Group		Treatment		Group*Treatment	
	F-value	P-value	F-value	P-value	F-value	P-value
HOXB4	1.4878	0.2898	0.0013	0.9722	4.1964	0.0465*
PROX1	3.9126	0.0545	1.4087	0.2693	0.3931	0.7615

* indicates $p < 0.05$ and ** indicates $p < 0.01$

References:

- [1] K. Gwon, E. Kim, G. Tae, Heparin-hyaluronic acid hydrogel in support of cellular activities of 3D encapsulated adipose derived stem cells, *Acta Biomater* 49 (2017) 284-295.
 [2] G. Tae, Y.J. Kim, W.I. Choi, M. Kim, P.S. Stayton, A.S. Hoffman, Formation of a novel heparin-based hydrogel in the presence of heparin-binding biomolecules, *Biomacromolecules* 8(6) (2007) 1979-86.