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Supplementary Materials for

Combinatorial screening of biochemical and physical signals for phenotypic regulation of stem cell–based cartilage tissue engineering

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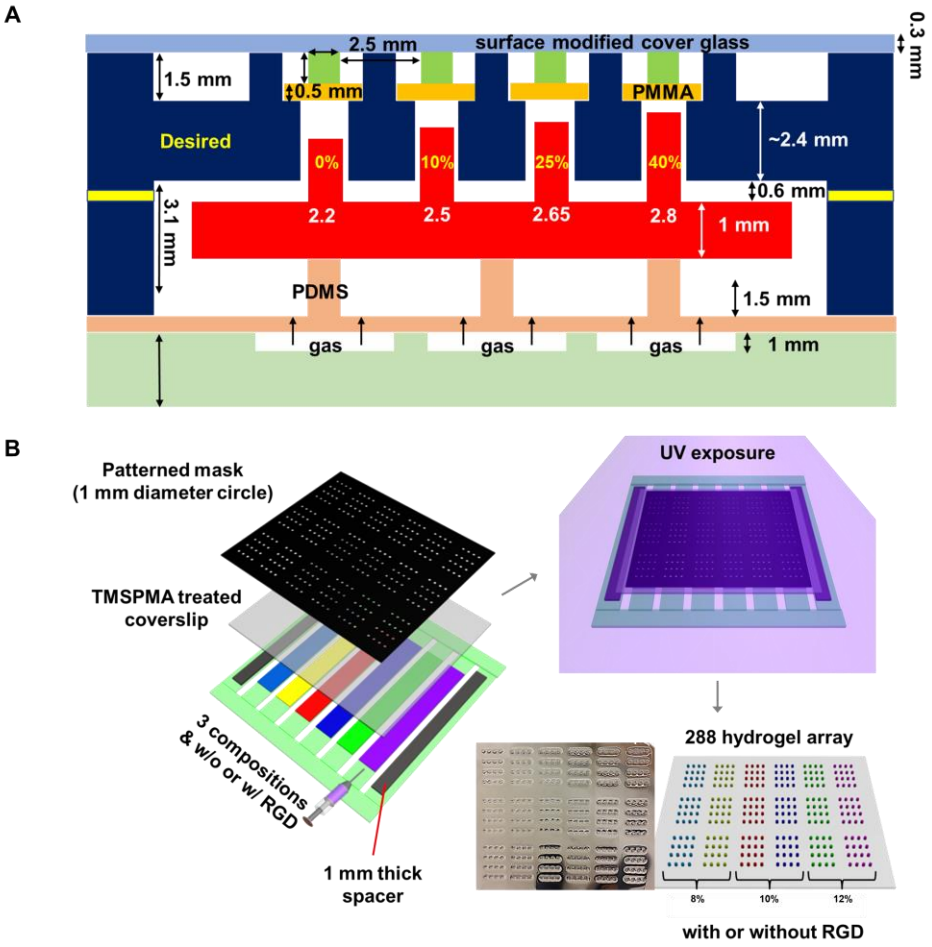
A
2.5 mm
surface modified cover glass
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PMMA
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2.8
1 mm
PDMS
1.5 mm
gas
gas
gas
1 mm
B
Patterned mask
(1 mm diameter circle)
TMSPMA treated
coverslip
3 compositions
& w/o or w/ RGD
1 mm thick
spacer
UV exposure
288 hydrogel array
8%
10%
12%
with or without RGD

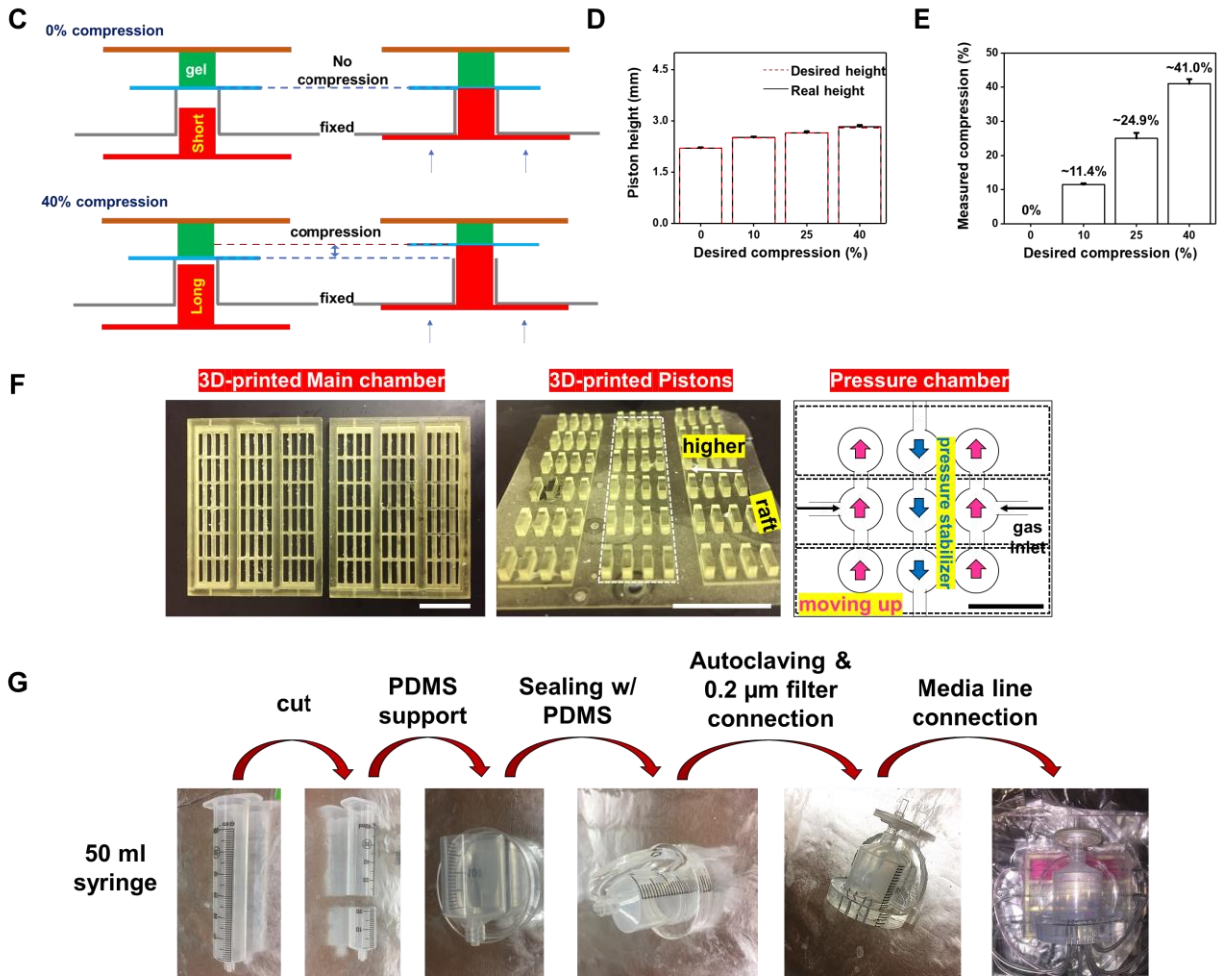


Fig. S1. Design of combinatorial system for screening of biochemical and physical signals for chondrogenesis of hMSCs. (**A**) Schematic showing cross-sectional view of combinatorial system (i.e., TMSPA-treated glass slides with hydrogel arrays, caps to maintain gel height, main chamber, pistons with different height, membrane with pillars, pressure chamber). (**B**) Illustrations showing the process to separate six different biomaterial conditions (three different PEG:OMA compositions: 8%, 10%, 12% and with or without RGD) and fabricate 288 hydrogel arrays on a TMSPA-treated glass slide (photo credit: Junmin Lee, UCLA). (**C**) Schematic illustrations of the principle of compression. (**D**) Measured height of pistons with desired height of 2.2, 2.5, 2.65, 2.8 mm using Auto CAD drawing and 3D printing (N = 4). (**E**) Measured compression of hydrogels in combinatorial system with desired strain of 0, 10, 25, 40% (N = 4). (**F**) Representative images showing 3D-printed main chambers and pistons and design of pressure chamber (photo credit: Junmin Lee, UCLA). Scale bar is 20 mm. (**G**) Representative images showing the procedure to make a device be open system preventing pressure accumulation which leads to a leaking issue (photo credit: Junmin Lee, UCLA).

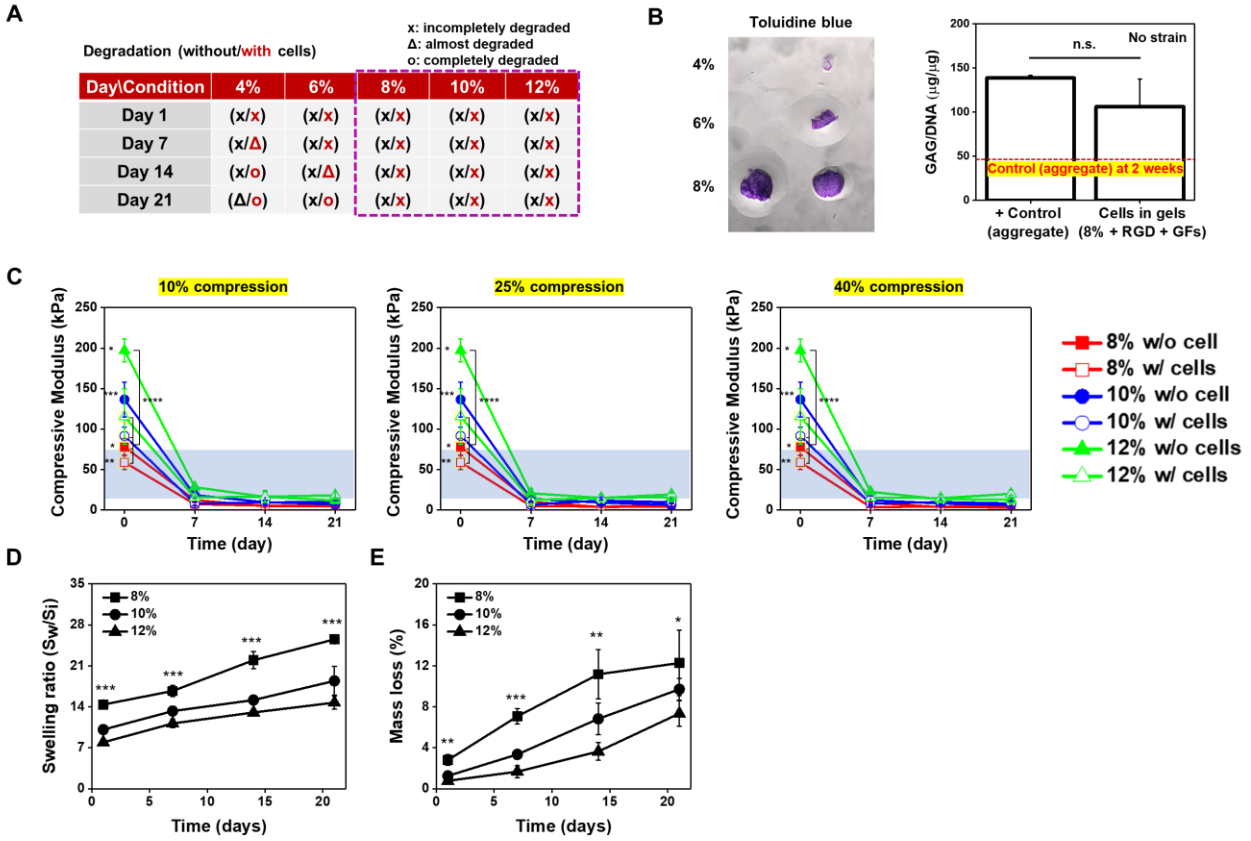


Fig. S2. Selection of PEG/OMA hybrid biomaterial compositions and their characteristics. (**A**) Selection of OMA/PEG hybrid biomaterial compositions after 21 days of degradation (N = 3). (**B**) Representative photograph of Toluidine Blue staining after 21 days of chondrogenic differentiation (4%, 6%, 8%) and quantitative GAG deposition analysis of hMSCs cultured in 8% PEG/OMA hydrogels for 21 days. Positive control: aggregation culture of hMSCs for 14 or 21 days (N = 3). (One-way ANOVA with Tukey post hoc testing) (**C**) Time profile of PEG/OMA hydrogel (with or without cells) degradation with compression (10, 25, and 40%) for 21 days (N = 5). The same samples were used for the day 0 measurements in all 3 plots. (One-way ANOVA with Tukey post hoc test; *p<0.05 compared with 10% w/o cell group, **p<0.05 compared with 12% w/ cells group, ***p<0.005 compared with 8% w/ cells and ****p<0.005 compared with 12% w/o cell group at day 0). (**D**) Swelling ratio and (**E**) mass loss of PEG/OMA hydrogels without compression for 21 days (N = 3). (*p<0.05, **p<0.005, ***p<0.0005 based on one-way ANOVA with Tukey post hoc testing).

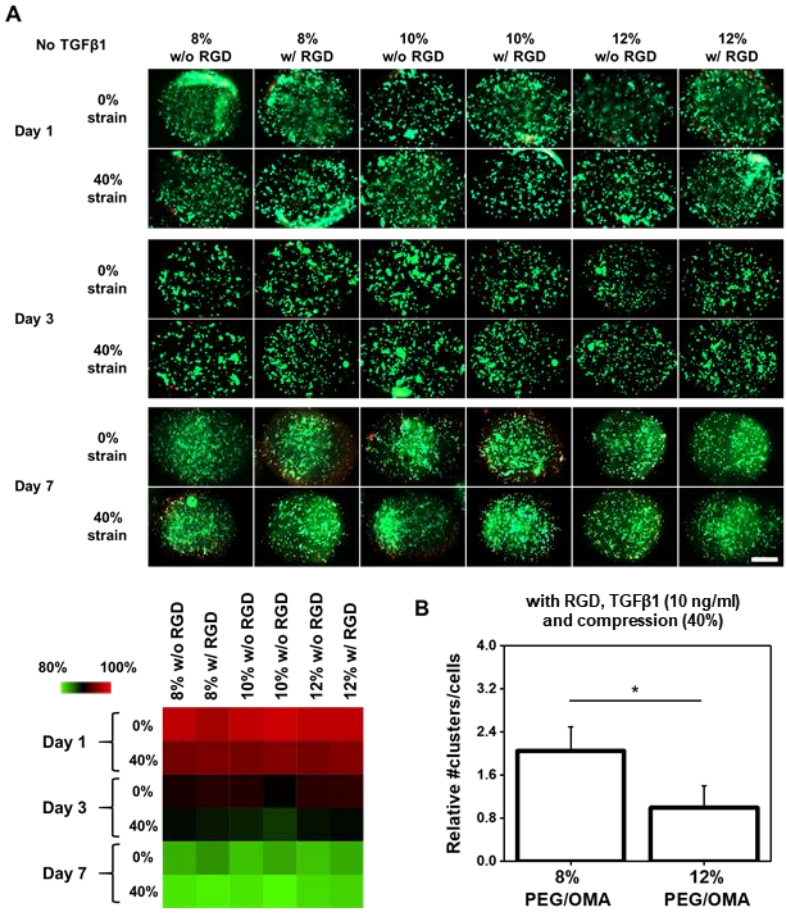
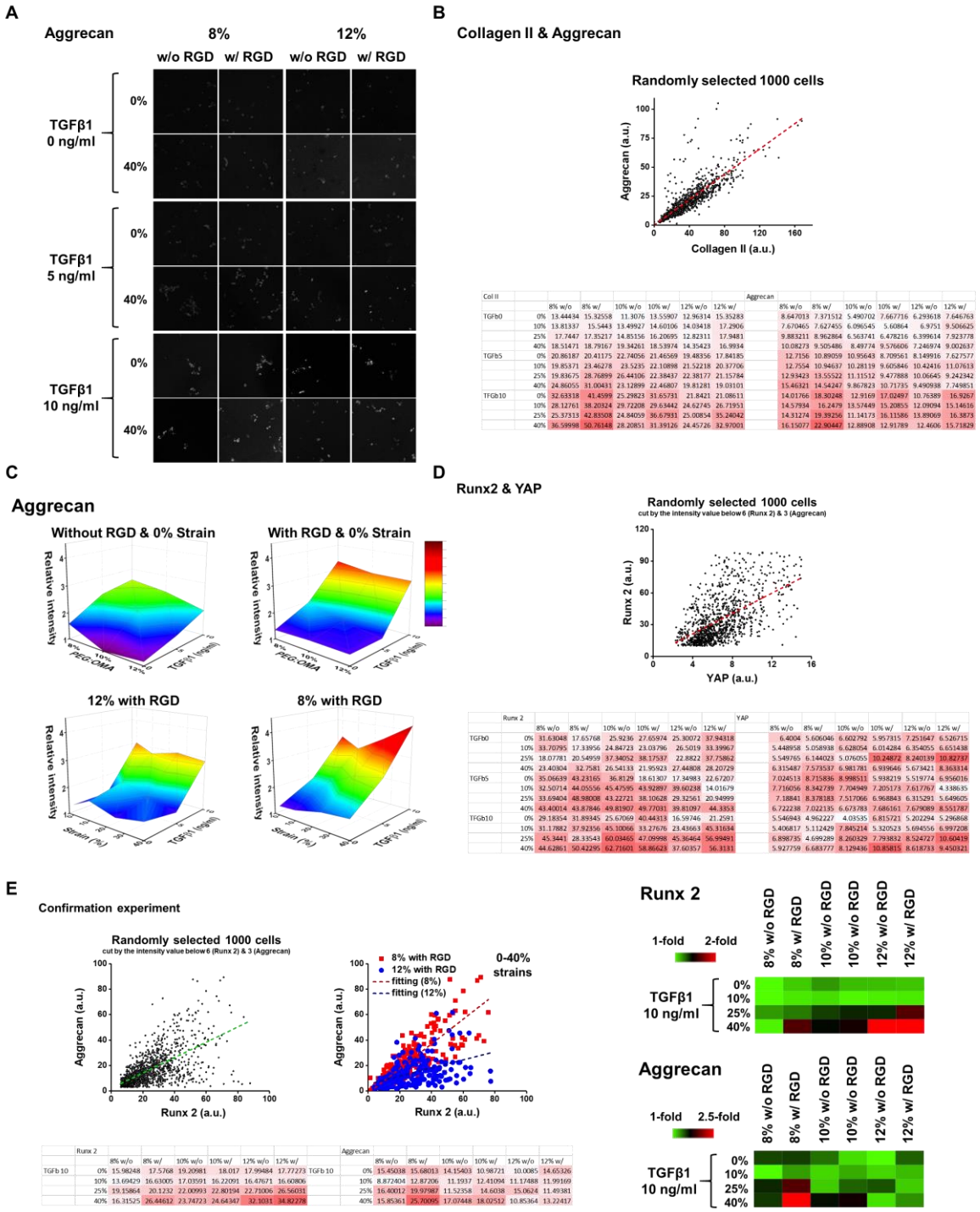


Fig. S3. Viability and relative cluster numbers of hMSCs cultured in PEG/OMA hybrid hydrogels. (**A**) Representative Live/Dead assay images of hMSCs and quantification of cell viability in different conditions at day 1, 3, and 7 days (N = 4). Scale bar, 300 μm. (**B**) Quantitative cluster number of hMSCs after 21 days of chondrogenic differentiation (8% and 12% PEG/OMA hydrogels) with the presence of other parameters (N = 5). Scale bar, 30 μm.



Col II		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb0	0%	13.44434	15.32558	11.3076	13.55907	12.96314	15.35283
	10%	13.81337	15.5443	13.49927	14.60106	14.03418	17.2906
	25%	17.7447	17.35217	14.85156	16.20695	12.82311	17.9481
	40%	18.51471	18.79167	19.34261	18.53974	14.35423	16.9934
TGFb5	0%	20.86187	20.41175	22.74056	21.46569	19.48356	17.84185
	10%	19.85371	23.46278	23.5235	22.10898	21.52218	20.37706
	25%	19.83675	28.76899	26.44106	22.38437	22.38177	21.15784
	40%	24.86055	31.00431	23.12899	22.46807	19.81281	19.03101
TFGb10	0%	32.63318	41.4599	25.29823	31.65731	21.8421	21.08611
	10%	28.12761	38.20324	29.72208	29.63442	24.62745	26.71951
	25%	25.37313	42.83508	24.84059	36.67931	25.00854	35.24042
	40%	36.59998	50.76148	28.20851	31.39126	24.45726	32.97001

Aggrecan		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb0	0%	8.647013	7.371512	5.490702	7.667716	6.293618	7.646763
	10%	7.670465	7.627455	6.096545	5.60864	6.9751	9.506625
	25%	9.883211	8.962864	6.563741	6.478216	6.399614	7.923778
	40%	10.08273	9.505486	8.49774	9.576606	7.246974	9.002637
TGFb5	0%	12.7156	10.89059	10.95643	8.709561	8.148916	7.627577
	10%	12.7554	10.94637	10.28119	9.605846	10.42416	11.07613
	25%	12.93423	13.55522	11.11512	9.477888	10.06645	9.242342
	40%	15.46321	14.54247	9.867823	10.71735	9.490938	7.749851
TFGb10	0%	14.01766	18.30248	12.9169	17.02497	10.76389	16.9267
	10%	14.57934	16.2479	13.57449	15.20855	12.09094	15.14616
	25%	14.31274	19.39256	11.14173	16.11586	13.89069	16.3873
	40%	16.15077	22.90447	12.88908	12.91789	12.4606	15.71829

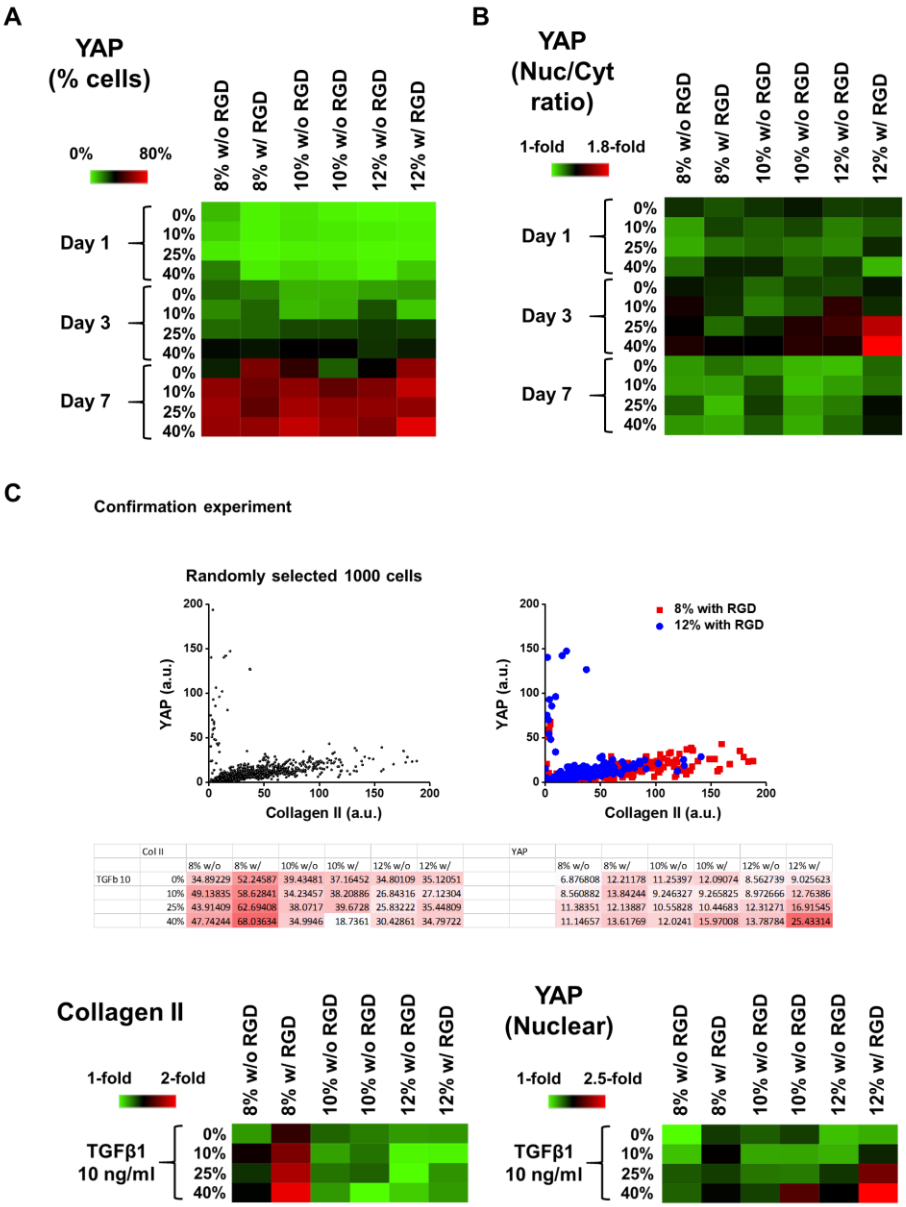
Runx 2		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb0	0%	31.63048	17.65768	25.9236	27.65974	25.30072	37.94318
	10%	33.70795	17.33956	24.84723	23.03796	26.5019	33.39967
	25%	18.07781	20.54959	37.34052	38.17537	22.8822	37.75862
	40%	23.40304	32.7581	26.54133	21.95923	27.44808	28.20729
TGFb5	0%	35.06639	43.23165	36.8129	18.61307	17.34983	22.67207
	10%	32.50714	44.05556	45.47595	43.92897	39.60238	14.01679
	25%	33.69404	48.98008	43.22721	38.10628	29.32561	20.94999
	40%	43.40014	43.87846	49.81907	49.77031	39.81097	44.3353
TFGb10	0%	29.18354	31.89345	25.67069	40.44313	16.59746	21.2591
	10%	31.17882	37.92356	45.10066	33.27676	23.43663	45.31634
	25%	45.3441	28.33543	60.03465	47.09998	45.36464	56.99491
	40%	44.62861	50.42295	62.71601	58.86628	37.60357	56.3131

YAP		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb0	0%	6.4004	5.666046	6.602792	5.957315	7.251647	6.526715
	10%	5.448958	5.058938	6.628054	6.014284	6.354055	6.651438
	25%	5.549765	6.144023	5.076055	10.24872	8.240139	10.82737
	40%	6.315487	7.573537	6.981781	6.939646	5.673421	8.363314
TGFb5	0%	7.024513	8.715836	8.998511	5.938219	5.519774	6.956016
	10%	7.716056	8.342739	7.704949	7.205173	7.617767	4.338635
	25%	7.18841	8.378183	7.517066	6.968843	6.315291	5.649605
	40%	6.722238	7.022135	6.673783	7.686161	7.679089	8.551787
TFGb10	0%	5.546943	4.962227	4.03535	6.815721	5.202294	5.296868
	10%	5.406817	5.112429	7.845214	5.320523	5.694556	6.997208
	25%	6.898735	4.699289	8.260329	7.793832	8.524727	10.60419
	40%	5.927759	6.683777	8.129436	10.85815	8.618733	9.450321

Runx 2		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb 10	0%	15.98248	17.5768	19.20981	18.017	17.99484	17.77273
	10%	13.69429	16.63005	17.03591	16.22091	16.47671	16.60806
	25%	19.15864	20.1232	22.00993	22.80194	22.71006	26.56031
	40%	16.31525	26.44612	23.74723	24.64347	32.1031	34.82778

Aggrecan		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb 10	0%	15.45038	15.68013	14.15403	10.98721	10.0085	14.65326
	10%	8.872404	12.87206	11.1937	12.41094	11.17488	11.99169
	25%	16.40012	19.97987	11.52358	14.6038	15.0624	11.49381
	40%	15.85361	25.70695	17.07448	18.02512	10.85364	13.22417

Fig. S4. Expression levels of articular and hypertrophic markers for hMSCs guided by different levels of both degradation and stiffness. (**A**) Representative 3D confocal images of hMSCs stained with Aggrecan in different combinations of parameters. (**B**) Plot of measured immunofluorescence intensity data for 1000 randomly selected cells cultured for 21 days and co-stained with Collagen II and Aggrecan. Averaged intensity of cells cultured in different conditions for 21 days (N = 4). (**C**) Surface plot displaying the effect of the two variables on chondrogenic marker expression (Aggrecan) of hMSCs when other two factors are fixed. (**D**) Plot of measured immunofluorescence intensity data for randomly selected 1000 cells cultured for 21 days and co-stained with Runx2 and YAP. Averaged intensity of cells cultured in different conditions for 21 days (N = 4). (**E**) Plot of measured immunofluorescence intensity data for 1000 randomly selected cells cultured for 21 days and co-stained with Runx2 and Aggrecan. Intensity of co-stained markers for the group of cells cultured in 8% or 12% PEG/OMA hydrogels with RGD presence for 21 days. Quantified heatmaps of Runx2 and YAP for cells encapsulated in the hydrogel micro-arrays cultured with the combinations of some selected factors for 21 days (N = 4).



Col II									YAP							
		8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/			8% w/o	8% w/	10% w/o	10% w/	12% w/o	12% w/
TGFb 10	0%	34.89229	52.24587	39.43481	37.16452	34.80109	35.12051			6.876808	12.21178	11.25397	12.09074	8.562739	9.025623
	10%	49.13835	58.62841	34.23457	38.20886	26.84316	27.12304			8.560882	13.84244	9.246327	9.265825	8.972666	12.76386
	25%	43.91409	62.69408	38.0717	39.6728	25.83222	35.44809			11.38351	12.13887	10.55828	10.44683	12.31271	16.91545
	40%	47.74244	68.03634	34.9946	18.7361	30.42861	34.79722			11.14657	13.61769	12.0241	15.97008	13.78784	25.43314

Fig. 5. YAP quantification and staining results revealing hypertrophic chondrogenesis of hMSCs through YAP-dependent mechanotransduction. Quantified heatmaps of (**A**) % cells over the threshold and (**B**) nuclear/cytoplasm intensity ratio at day 1, 3, 7 to define the role of YAP in lineage specification of hMSCs (N = 4). (**C**) Plot of measured immunofluorescence intensity data for 1000 randomly selected cells cultured for 21 days and co-stained with Collagen II and YAP. Intensity of co-stained markers for the group of cells cultured in 8% or 12% PEG/OMA hydrogels with RGD presence for 21 days. Quantified heatmaps of Collagen II and YAP for cells encapsulated in the hydrogel micro-arrays cultured with the combinations of some selected factors for 21 days (N = 4).

Rank	PEG:OMA	RGD	Strain	TGF-β	Col II	Aggrecan	Runx2	YAP	COl II scaled	Aggrecan scaled	Runx2 scaled	YAP scaled	score
1	8%	w RGD	25	10	46.039439	20.252768	27.088213	4.638592	0.961518	0.962677	0.368590	0.064861	0.372686
2	8%	w RGD	0	10	39.300062	17.019819	21.266216	4.489886	0.775980	0.757281	0.223295	0.041950	0.317004
3	8%	w RGD	40	10	47.437241	20.840235	42.584921	6.079284	1.000000	1.000000	0.755327	0.286827	0.239461
4	10%	w RGD	0	10	34.058895	19.343055	27.317505	6.328301	0.631689	0.904881	0.374312	0.325193	0.209266
5	8%	w/o RGD	0	10	31.245410	13.449179	19.752073	5.315387	0.554233	0.530432	0.185508	0.169134	0.182506
6	8%	w RGD	10	10	35.651932	14.427535	34.322415	4.717333	0.675546	0.592588	0.549127	0.076993	0.160504
7	12%	w RGD	0	10	20.830888	14.543805	18.795868	5.022307	0.267517	0.599975	0.161645	0.123980	0.145467
8	10%	w RGD	10	10	30.723548	15.413718	32.725678	5.153268	0.539866	0.655243	0.509279	0.144157	0.135418
9	12%	w RGD	10	5	19.084896	10.061776	12.318693	4.217604	0.219450	0.315223	0.000000	0.000000	0.133668
10	10%	w RGD	25	10	42.477124	18.773962	41.535888	7.283922	0.863446	0.868726	0.729147	0.472425	0.132650

Metric to compare different combinations:

$$score = 0.25 \times Col_{scaled} + 0.25 \times Aggrecan_{scaled} - 0.25 \times Runx2_{scaled} - 0.25 \times YAP_{scaled}$$

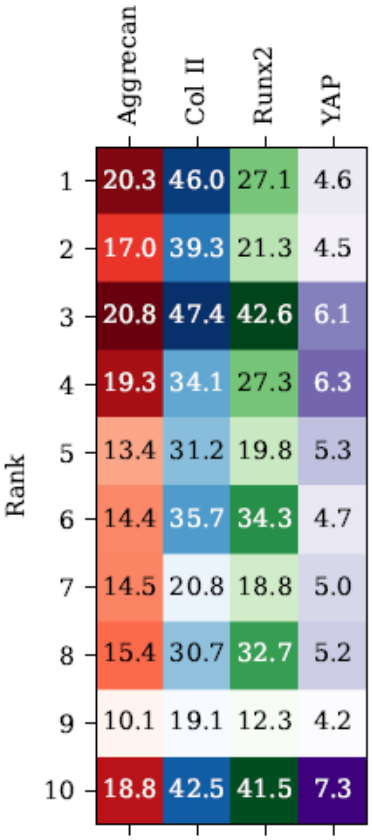
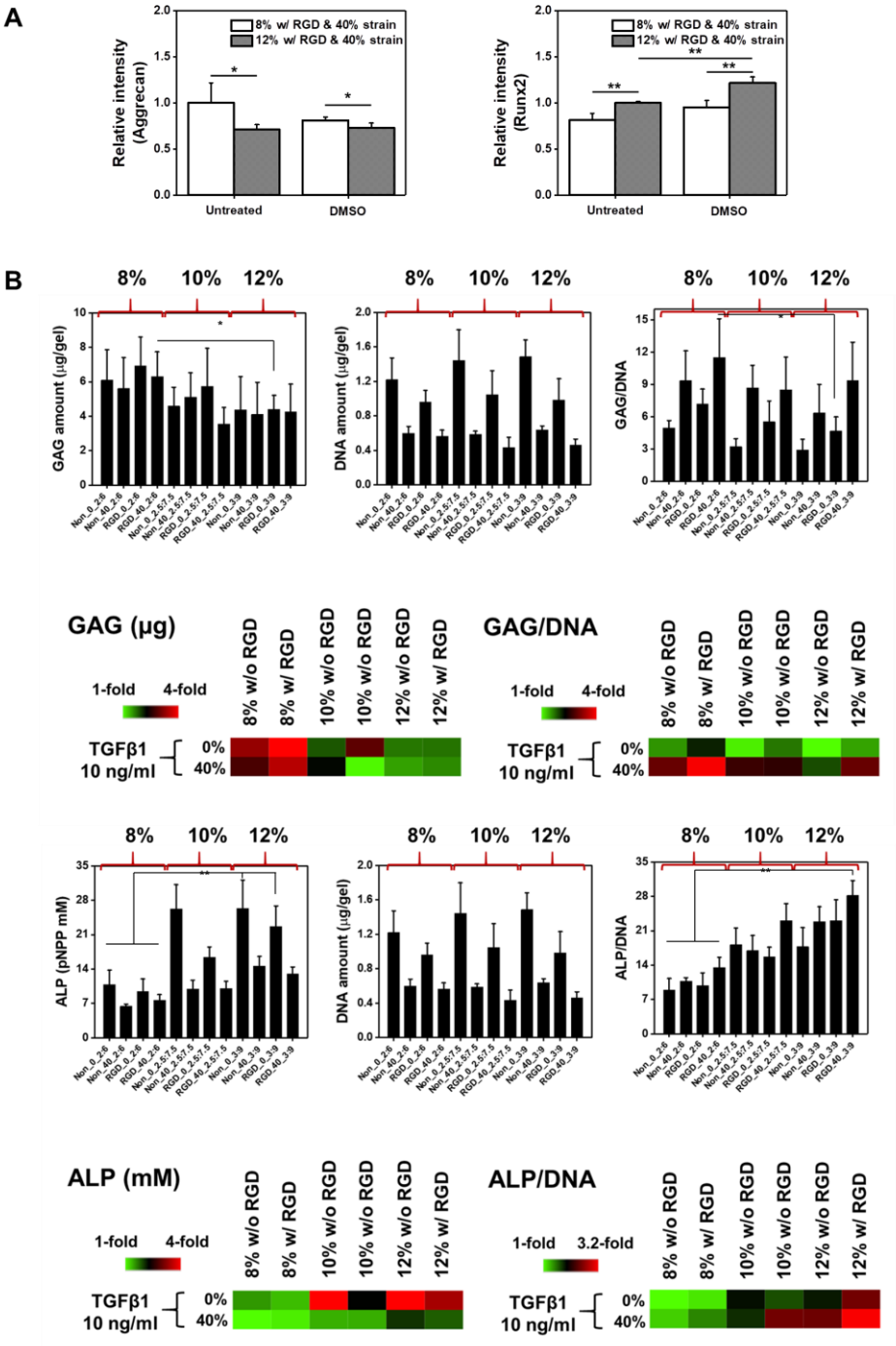


Fig. S6. Top-10 combinations for the best chondrogenic metrics. Light colors represent low values and dark colors represent high values. The opposite trend in the figure 3d and 3e is for hypertrophic chondrogenesis.


A
8% w/ RGD & 40% strain
12% w/ RGD & 40% strain
Relative intensity (Aggrecan)
Untreated
DMSO
Relative intensity (Runx2)
B
8%
10%
12%
GAG amount (μg/gel)
DNA amount (μg/gel)
GAG/DNA
GAG (μg)
1-fold
4-fold
8% w/o RGD
8% w/ RGD
10% w/o RGD
12% w/o RGD
12% w/ RGD
TGFβ1
10 ng/ml
0%
40%
ALP (pNPP mM)
ALP/DNA
ALP (mM)
3.2-fold

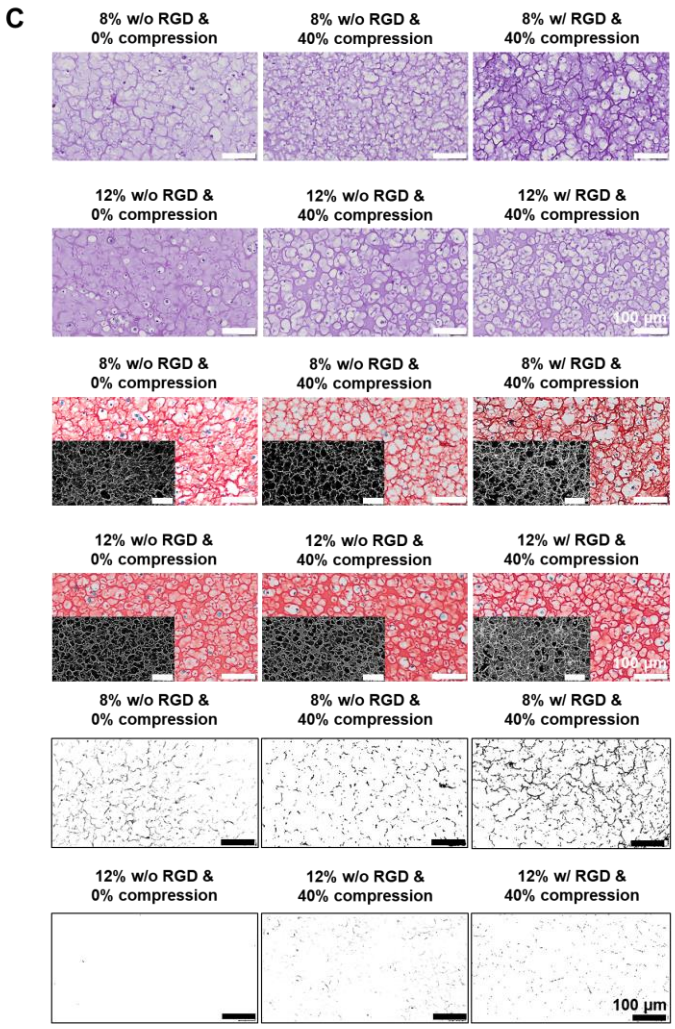


Fig. S7. Crosslinking density of PEG/OMA regulating articular or hypertrophic chondrogenesis of hMSCs. (**A**) Relative Aggrecan or Runx2 intensity of hMSCs when supplemented with DMSO for 21 days (N = 4). (**B**) Quantification of DNA, GAG (GAG/DNA), and ALP (ALP/DNA) for 21 days in culture of hMSCs in with or without RGD-conjugated PEG/OMA gels with the supplement of TGF-β1 under 40% compression compared to those cultured without compression (N = 6). (another experiment set, different from Fig. 4A) (One-way ANOVA with Tukey post hoc testing across the all conditions, *p<0.05, **p<0.005) (**C**) Hematoxylin and eosin and Safranin O staining of cartilage tissues where cells are cultured in different compositions (8% or 12%) with or without RGD or compression. Images of Safranin O staining were thresholded for imaging analysis. Scale bar is 100 μm.

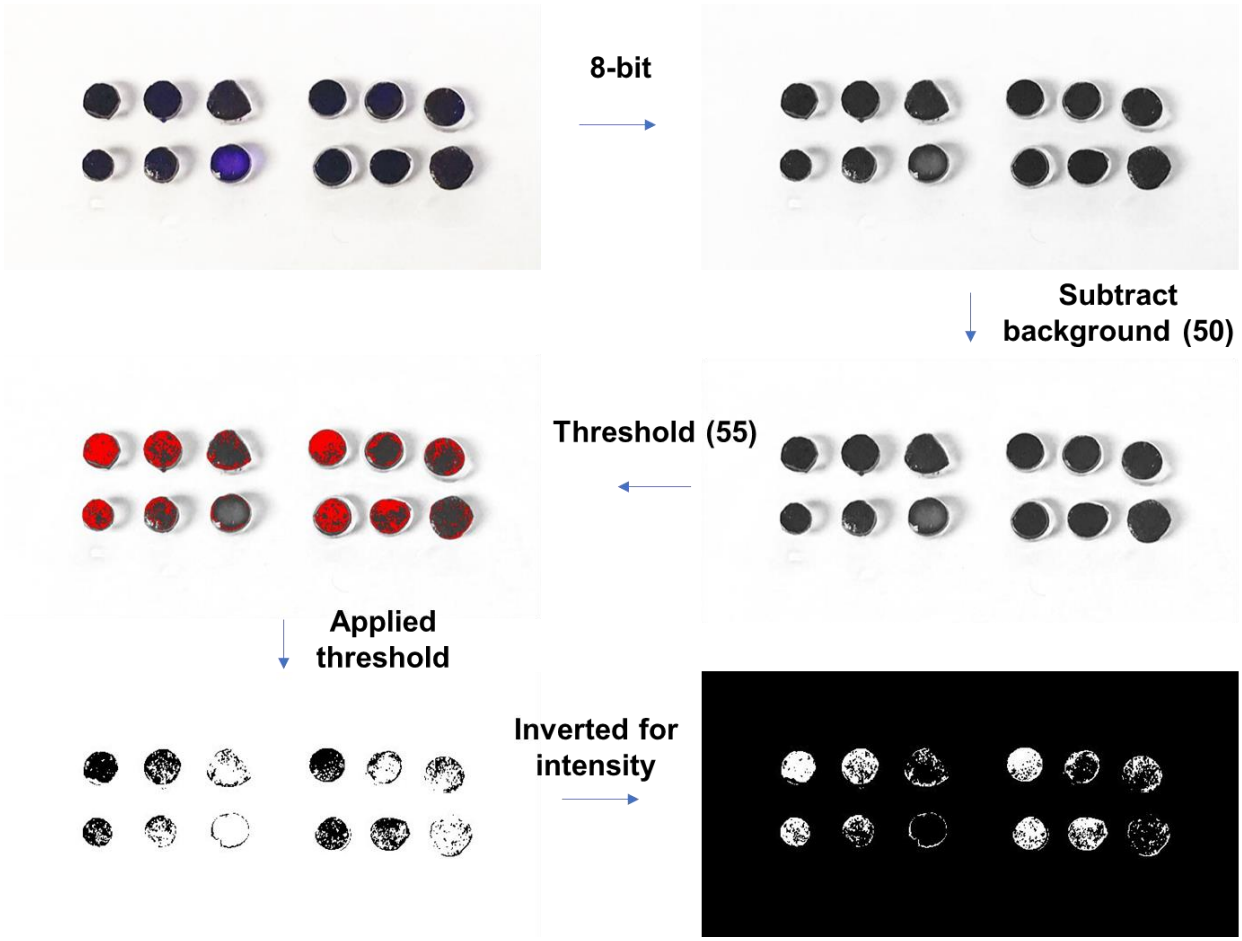
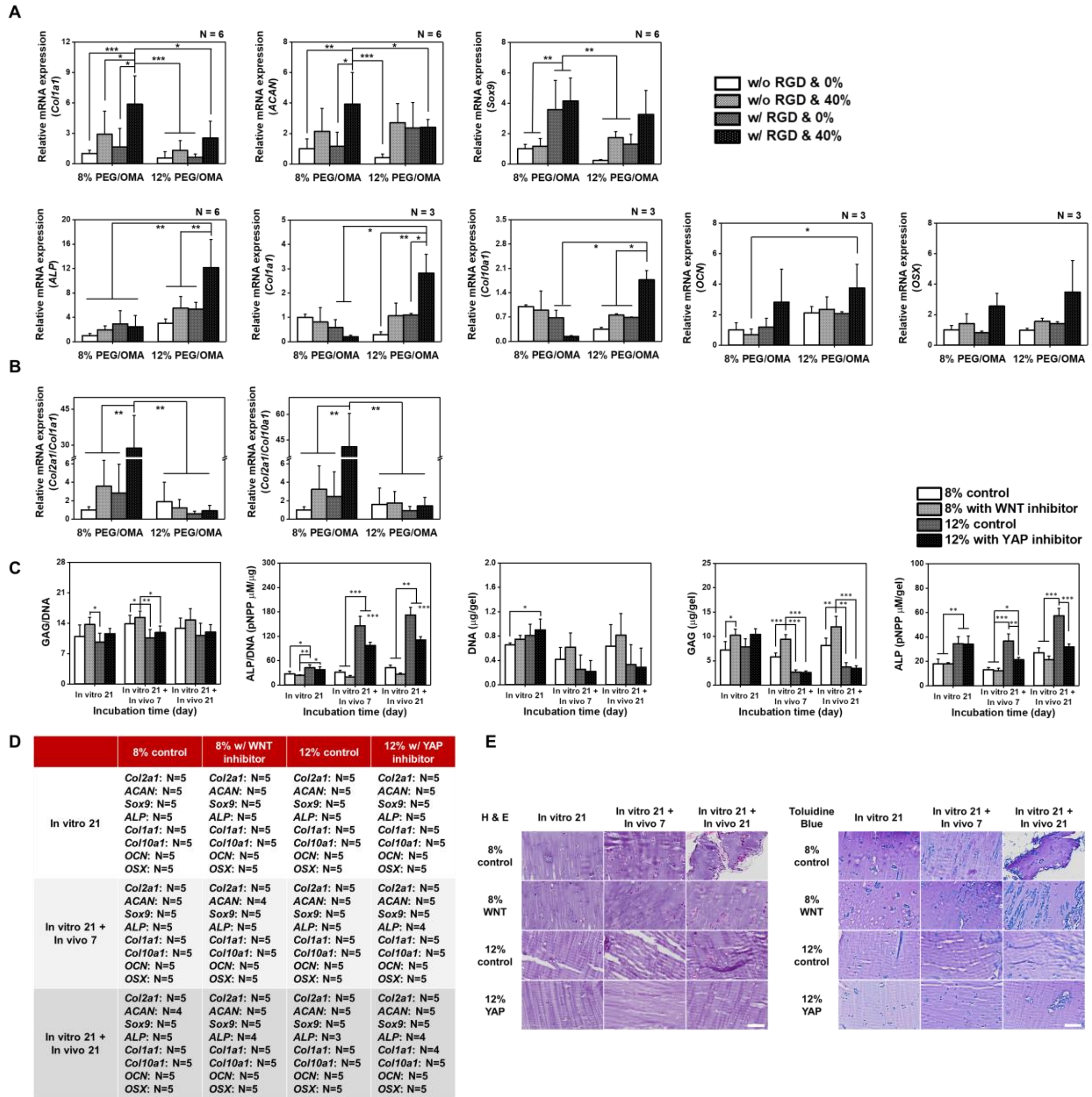


Fig. S8. A flow chart for threshold-based imaging analysis of cartilage tissues stained with toluidine blue O where cells are cultured in different compositions (8% or 12%) with or without RGD or compression.



	8% control	8% w/ WNT inhibitor	12% control	12% w/ YAP inhibitor
In vitro 21	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5
In vitro 21 + In vivo 7	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=4 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=4 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5
In vitro 21 + In vivo 21	*Col2a1*: N=5 *ACAN*: N=4 *Sox9*: N=5 *ALP*: N=5 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=4 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=3 *Col1a1*: N=5 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5	*Col2a1*: N=5 *ACAN*: N=5 *Sox9*: N=5 *ALP*: N=4 *Col1a1*: N=4 *Col10a1*: N=5 *OCN*: N=5 *OSX*: N=5

Fig. S9. Inhibition of YAP-dependent mechanotransduction and WNT signaling pathway enabling the prevention of hypertrophic transition of prechondrogenic hMSCs. (**A**) Quantification of real-time PCR to measure the gene expression associated with chondrogenic or osteogenic differentiation for cells cultured for 21 days with or without RGD or 40% compression. (**B**) Ratio of relative mRNA expression (collagen II/Collagen I and Collagen II/Collagen X) for cells cultured for 21 days. (**C**) Quantification of DNA, GAG, and ALP for hMSCs precultured (in 8% PEG/OMA with or without WNT inhibitor or 12% PEG/OMA with or without YAP inhibitor) for 21 days in vitro and implanted in vivo for 7 or 21 days (N = 6). (**D**) Number of replicates for different markers and conditions. (**E**) Hematoxylin and eosin and toluidine blue staining of cartilage tissues after encapsulated cells were cultured for 21 days with or without inhibitors and transplanted in vivo for 7 or 21 days after the in vitro pre-treatment. Scale bar is 100 µm. (*p<0.05, **p<0.005, ***p<0.0005 based on One-way ANOVA with Tukey post hoc testing)

A

Antibody	Company	Catalog #	Dilution/ Application
DAPI	SIGMA	D9564	1:5000/IF
Actin (594-phalloidin)	INVITROGEN	A12381	1:200/IF
Goat 488-anti-rabbit	ABCAM	AB150077	1:200/IF
Goat 647-anti-mouse	ABCAM	AB60316	1:200/IF
Collagen II	ABCAM	AB34712	1:500/IF
Aggrecan	ABCAM	AB3778	1:250/IF
Runx2	ABCAM	AB23981	1:500/IF
YAP	SANTA CRUZ BIOTECHNOLOGY	SC-271134	1:250/IF
a5b1	MILLIPORE	MAB1969	1 μg/ml, blocking
Toluidine blue O	Fisher chemical	BP107-10	Vendor's protocol
Safranin O	Fisher chemical	S670-25	Vendor's protocol
Hematoxylin	Fisher chemical	H345-25	Vendor's protocol
Eosin	Thermo Scientific Richard-Allan Scientific	71-11L	Vendor's protocol

B

Primary	Forward	Reverse
Col2a1	GGAAACTTTGCTGCCCAGATG	TCACCAGGTTCACCAGGATTGC
ACAN	TGCGGGTCAACAGTGCCTATC	CACGATGCCTTTCACCACGAC
Sox9	CACACAGCTCACTCGACCTTG	TTCGGTTATTTTTAGGATCATCTCG
ALP	CCACGTCTTCACATTTGGTG	GCAGTGAAGGGCTTCTTGTC
Col1a1	GATGGATTCCAGTTCGAGTATG	GTTTGGGTTGCTTGTCTGTTTG
Col10a1	AAAGGCCCACTACCCAACAC	CTTCCGTAGCCTGGTTTTCC
OCN	ATGAGAGCCCTCACACTCCTC	CGTAGAAGCGCCGATAGGC
OSX	TGGCTAGGTGGTGGGCAGGG	TGGGCAGCTGGGGGTTCAGT
GAPDH	GGGGCTGGCATTGCCCTCAA	GGCTGGTGGTCCAGGGGTCT

Table S1. (**A**) Antibody information for immunostaining, flow cytometry analysis, and integrin blocking. (**B**) qRT-PCR primer sequence information.