Supplementary Materials for

Paracrine signals influence patterns of fibrocartilage differentiation in a lyophilized gelatin hydrogel for applications in rotator cuff repair

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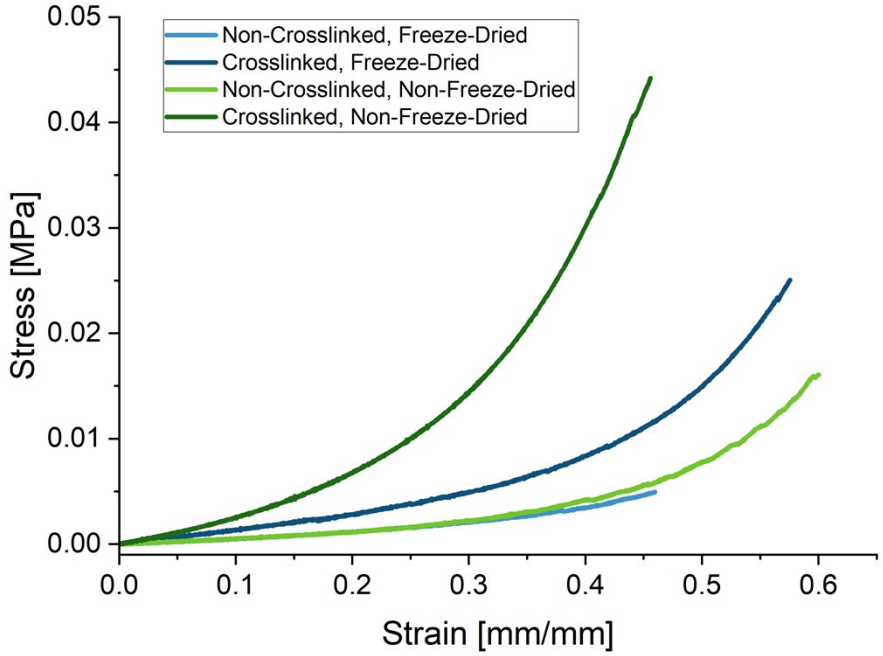
Supplemental Table T1: Taqman Gene Primers for PCR

Gene Name	Taqman Assay ID
GAPDH	Hs02786624_g1
COL2A1	Hs00264051_m1
COL10A1	Hs00166657_m1
ACAN	Hs00153936_m1
SCX	Hs03054634_g1
SOX9	Hs00165814_m1
RUNX2	Hs01047973_m1

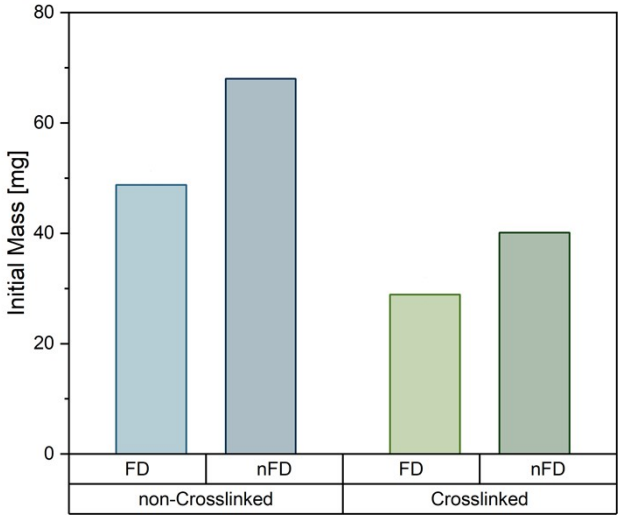
Supplemental Table T2: List of NanoString nCounter Panel gene targets and relevance for entheseal study.

Gene Name	HUGO Gene	Probe NSID	Category	Ref.
RUNX2	*RUNX2*	NM_004348.3:1850	Bone, Enthesis Transcription Factor	[1-4]
OSX	*SP7*	NM_001173467.1:1510	Bone Transcription Factor	[5]
OPN	*SPP1*	NM_000582.2:760	Bone Transcription Factor	[6, 7]
BSP	*IBSP*	NM_004967.3:876	Bone, Enthesis Transcription Factor	[7, 8]
ALP	*SLPI*	NM_003064.2:330	Bone Transcription Factor	[3, 9]
SCX	*SCX*	NM_001080514.2:550	Tendon, Enthesis Transcription Factor	[4, 10-13]
TNC	*TNC*	NM_002160.3:1215	Tendon Transcription Factor	[4, 14]
TNMD	*TNMD*	NM_022144.2:462	Tendon Transcription Factor	[2, 15]
IGFBP5	*IGFBP5*	NM_000599.3:3320	Tendon Transcription Factor	[4, 16]
COMP	*COMP*	NM_000095.2:1744	Tendon Transcription Factor	[1, 17]
EGR1	*EGR1*	NM_001964.2:1505	Tendon, Enthesis Transcription Factor	[14]
MKX	*MKX*	NM_173576.2:545	Tendon Transcription Factor	[18]
SOX9	*SOX9*	NM_000346.2:2135	Cartilage, Enthesis Transcription Factor	[2, 4, 10, 12, 18, 19]
Gli1	*GLI1*	NM_005269.1:2885	Enthesis Transcription Factor	[4, 11, 15]
KLF2	*KLF2*	NM_016270.2:1015	Enthesis Transcription Factor	[4]
KLF4	*KLF4*	NM_004235.4:1980	Enthesis Transcription Factor	[4]
Col1A1	*COL1A1*	NM_000088.3:5210	Tendon-to-Bone Matrix Marker	[3, 4, 19-22]
COL2A1	*COL1A2*	NM_001844.4:4745	Tendon-to-Bone Matrix Marker	[1, 2, 20, 23] [3, 4, 19]
Col3A1	*COL3A1*	NM_000090.3:180	Tendon-to-Bone Matrix Marker	[22]
COL5A1	*COL5A1*	NM_000093.3:872	Tendon-to-Bone Matrix Marker	[4]
COL6A1	*COL6A1*	NM_001848.2:3665	Tendon-to-Bone Matrix Marker	[22]
Col9A1	*COL9A1*	NM_001851.4:3198	Tendon-to-Bone Matrix Marker	[2]
Col10A1	*COL10A1*	NM_000493.3:135	Tendon-to-Bone Matrix Marker	[3, 4, 19, 21, 24]
Col11A1	*COL11A1*	NM_001190709.1:2490	Tendon-to-Bone Matrix Marker	[4]
ACAN	*ACAN*	NM_013227.3:335	Tendon-to-Bone Matrix Marker	[1, 10, 20, 21] [4, 19]
DCN	*DCN*	NM_001920.3:420	Tendon-to-Bone Matrix Marker	[25]
BGN	*BGN*	NM_001711.3:1935	Tendon-to-Bone Matrix Marker	[4]

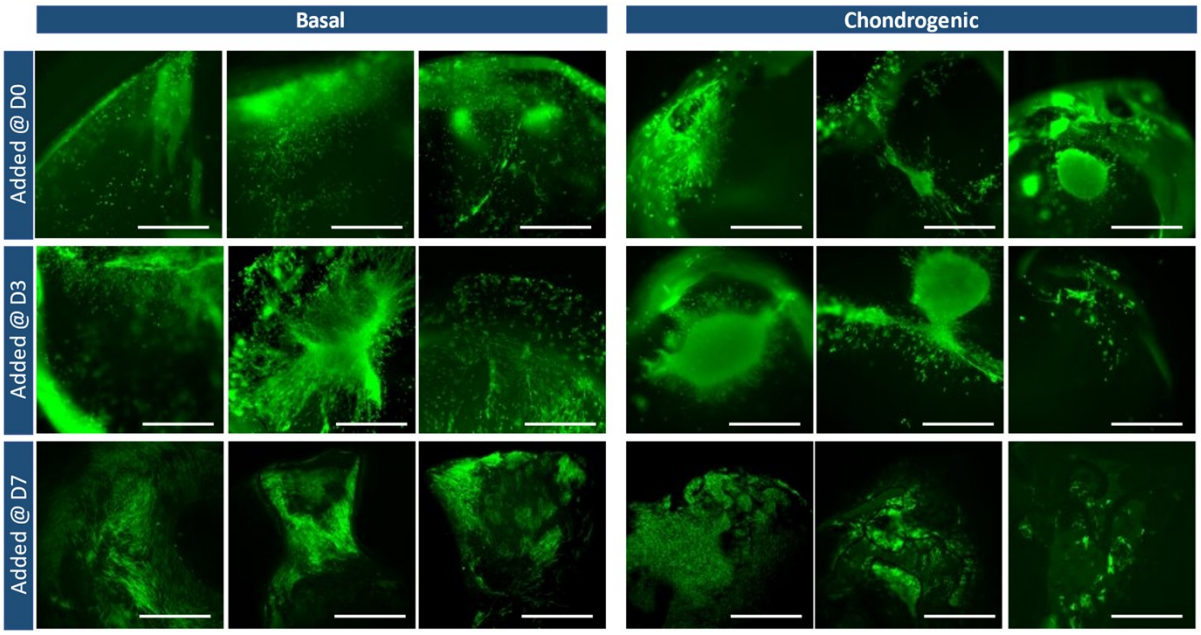
PDGF-BB	*PDGFB*	NM_033016.2:1480	Tendon-to-Bone Growth Factor	[17, 26-28]
TGFβ1	*TGFB1*	NM_000660.3:1260	Tendon-to-Bone Growth Factor	[4, 10, 13, 24, 29]
TGFβ3	*TGFB3*	NM_003239.2:706	Tendon-to-Bone Growth Factor	[4, 10, 24, 29]
BMP2	*BMP2*	NM_001200.2:1515	Tendon-to-Bone Growth Factor	[4, 10, 12, 18, 22, 30]
BMP4	*BMP4*	NM_001202.3:659	Tendon-to-Bone Growth Factor	[4, 10, 18, 30-33]
BMP7	*BMP7*	NM_001719.1:525	Tendon-to-Bone Growth Factor	[1, 4, 10, 18, 30, 34, 35]
BMP12 (GDF7)	*GDF7*	NM_182828.2:954	Tendon-to-Bone Growth Factor	[4, 17, 18, 28, 30, 36]
BMP14 (GDF5)	*GDF5*	NM_000557.2:155	Tendon-to-Bone Growth Factor	[4, 17, 18, 30, 37]
IGF-1	*IGF1*	NM_000618.3:491	Tendon-to-Bone Growth Factor	[28, 36, 38-41]
FGF2 (bFGF)	*FGF2*	NM_002006.4:620	Tendon-to-Bone Growth Factor	[4, 17, 27]
FGF7	*FGF7*	NM_002009.3:190	Tendon-to-Bone Growth Factor	[4, 22]
IHH	*IHH*	NM_002181.2:1693	Tendon-to-Bone Growth Factor	[4, 10, 12, 18]
PTHrP	*PTHLH*	NM_198965.1:605	Tendon-to-Bone Growth Factor	[4, 12, 13, 23]
EGF	*EGF*	NM_001963.4:1022	Tendon-to-Bone Growth Factor	[42]
VEGF	*VEGFA*	NM_001025366.3:1314	Tendon-to-Bone Growth Factor	[43]
SMAD3	*SMAD3*	NM_005902.3:4220	Tendon, Enthesis Relevant Marker	[14]
MMP13	*MMP13*	NM_002427.2:951	Tendon-to-Bone MMP	[1, 30, 44]
MMP9	*MMP9*	NM_004994.2:1530	Tendon-to-Bone MMP	[44, 45]
MCAM	*MCAM*	NM_006500.2:2482	MSC Marker	[22]
MYLK	*MYLK*	NM_053032.2:710	MSC Marker	[22]
PPARγ	*PPARG*	NM_005037.5:345	Adipocyte Marker	[6]
FABP4	*FABP4*	NM_001442.2:415	Adipocyte Marker	[6]
GAPDH	*GAPDH*	NM_001256799.1:386	Housekeeping Gene	[46-49]
ACTB	*ACTB*	NM_001101.2:1010	Housekeeping Gene	[48, 50, 51]
IPO8	*IPO8*	NM_006390.2:860	Housekeeping Gene	[48, 52, 53]
YWHAZ	*YWHAZ*	NM_003406.2:2345	Housekeeping Gene	[49, 50]



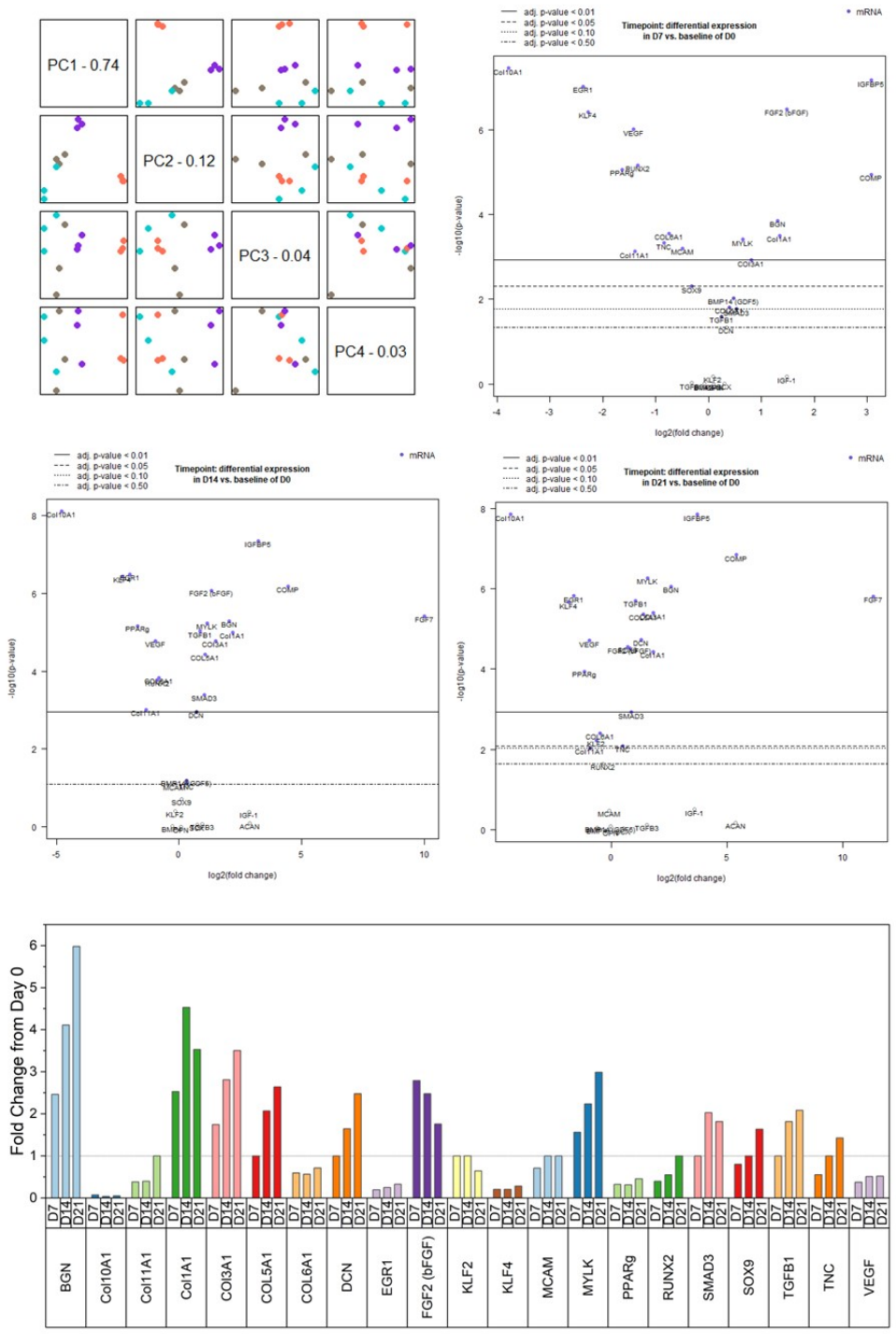
Supplemental Figure S1: Enthesis hydrogel mechanical properties. Representative stress-strain diagrams for compression tests of Gel-SH hydrogels as a function of fabrication processing (non-Freeze Dried vs. Freeze Dried) and post-fabrication crosslinking (Non-Crosslinked vs. Crosslinked). Constructs used for cell activity studies are Freeze Dried and Crosslinked.



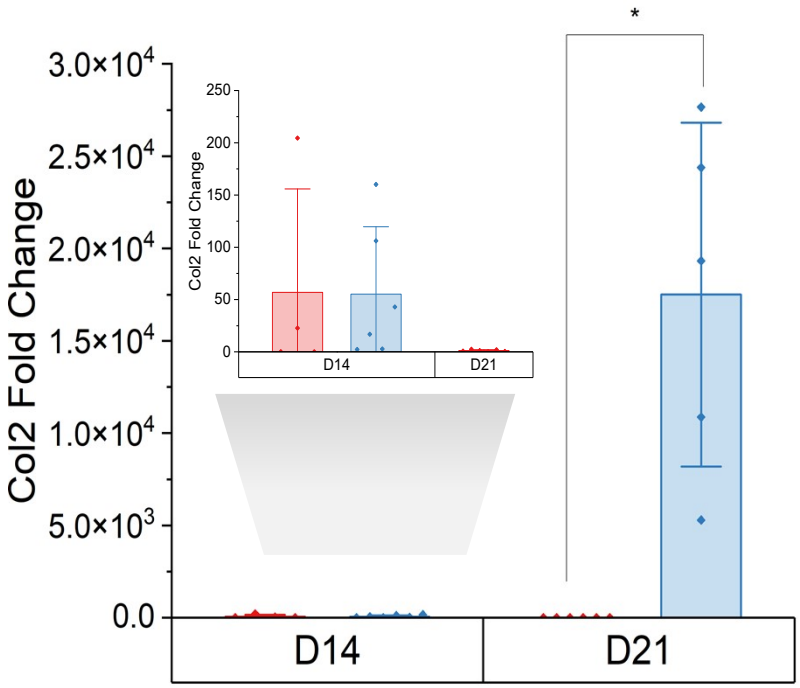
Supplemental Figure S2: Initial mean mass for Gel-SH hydrogel degradation testing. Average initial mass (reported as average weight from a single measurement of the aggregate mass of n=10+ individual hydrogel specimens) as a function of fabrication processing (non-Freeze Dried, “nFD” vs. Freeze Dried, “FD”) and post-fabrication crosslinking (Non-Crosslinked vs. Crosslinked). Constructs used for cell activity studies are Freeze Dried and Crosslinked.



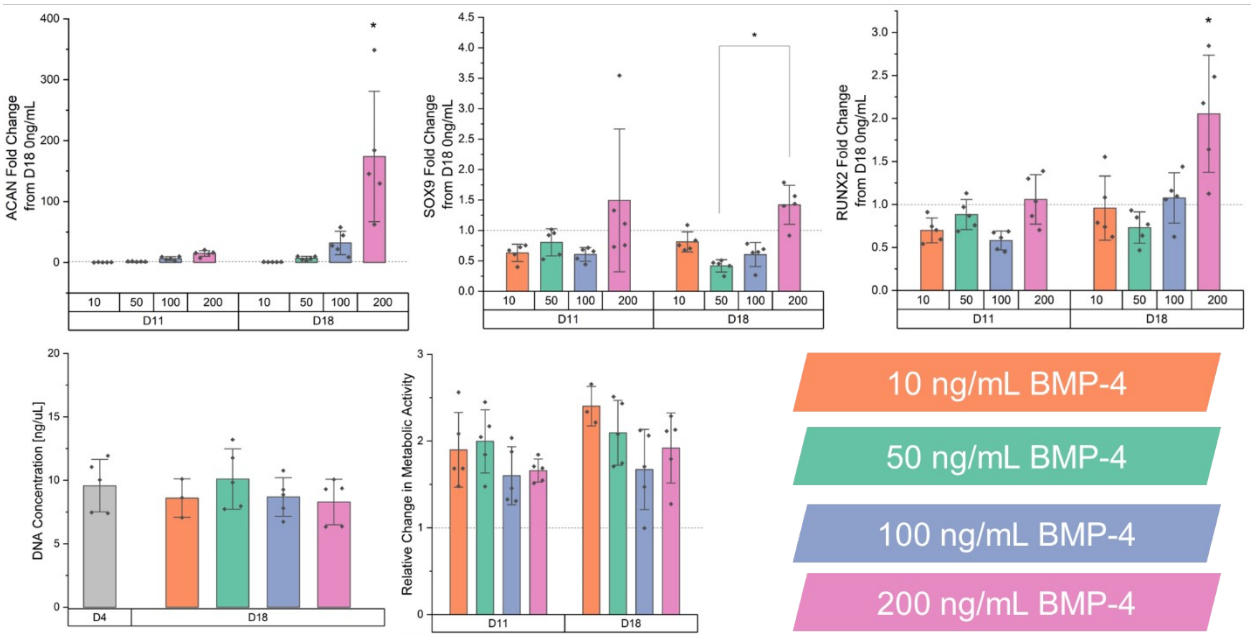
Supplemental Figure S3: hMSC proliferation and invasion analysis within Gel-SH constructs. Images display calcein-stained live hMSCs, comparing basal and chondrogenic conditions if chondrogenic media is added at various times. Scale bar: 1mm



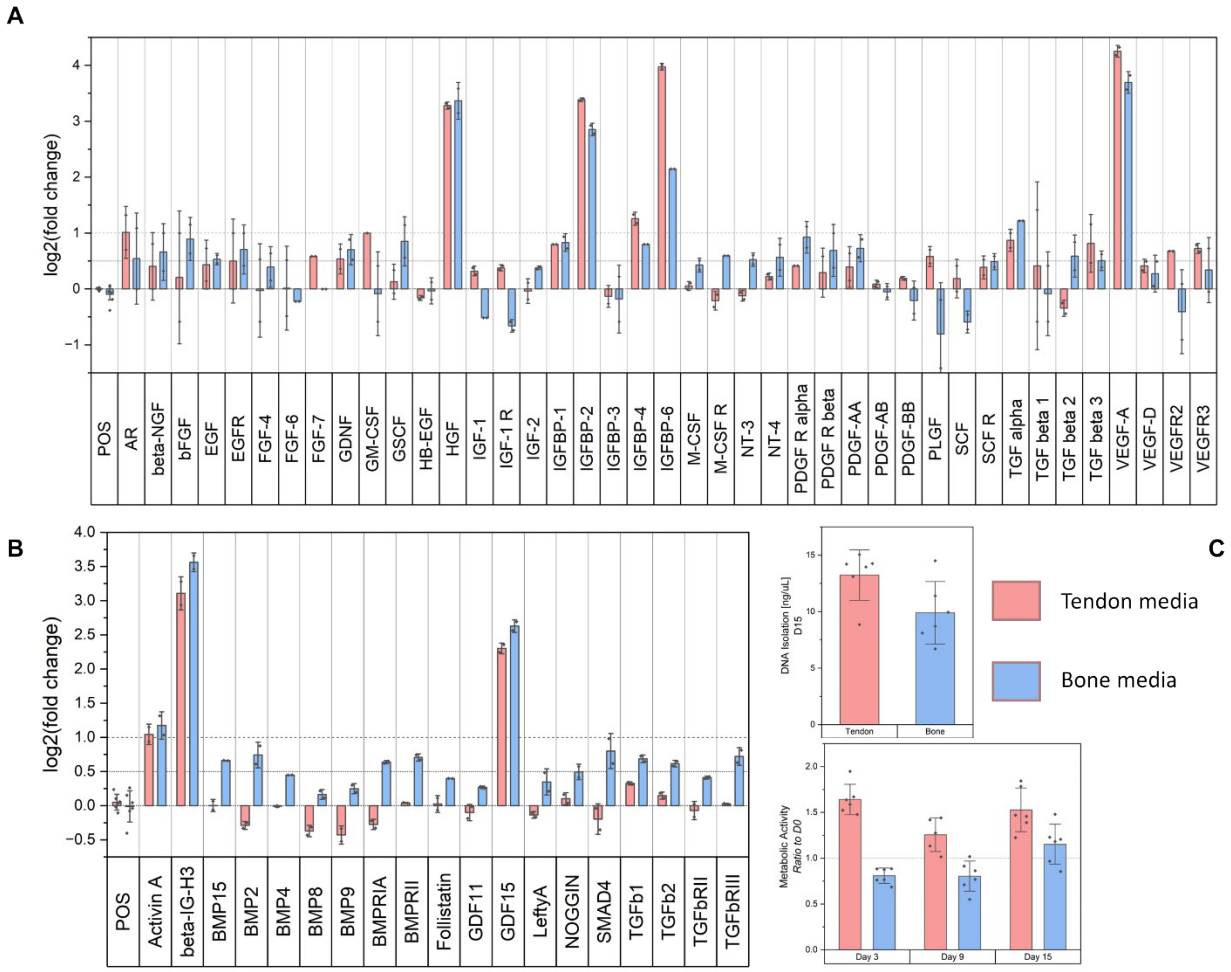
Supplemental Figure S4: Individual differentiation patterns of hMSCs in crosslinked, lyophilized Gel-SH hydrogels in basal growth media. Expression patterns (n=3) via nCounter mRNA expression panel, shown with principal component analysis, differential expression analysis, and summary bar graph. All bars deviating from the baseline denote a significant ($p<0.05$) change in expression. IGFBP5, COMP, and FGF7 have been omitted for scaling purposes; all are upregulated by a minimum of 10-fold by Day 21, with FGF7 reaching over 2500-fold by Day 21.



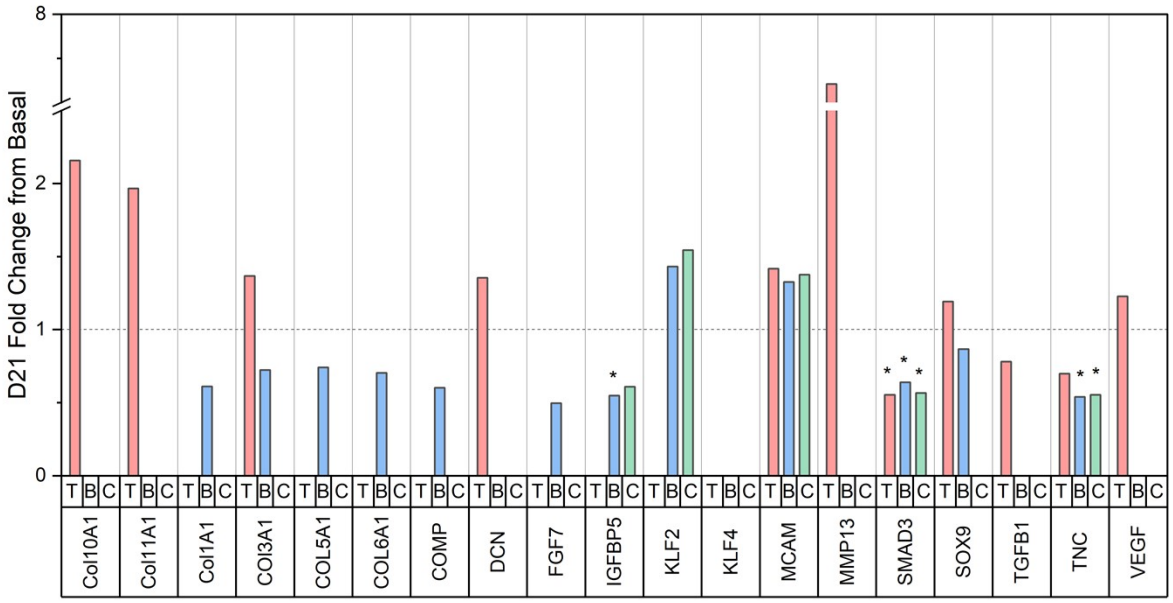
Supplemental Figure S5: *COL2* expression of hMSCs in crosslinked, lyophilized Gel-SH hydrogels. Expression patterns (n=5) for cartilage matrix protein (COL2) after 7 (Day 14) or 14 (Day 21) days of exposure to chondrogenic (blue) vs basal (red) media



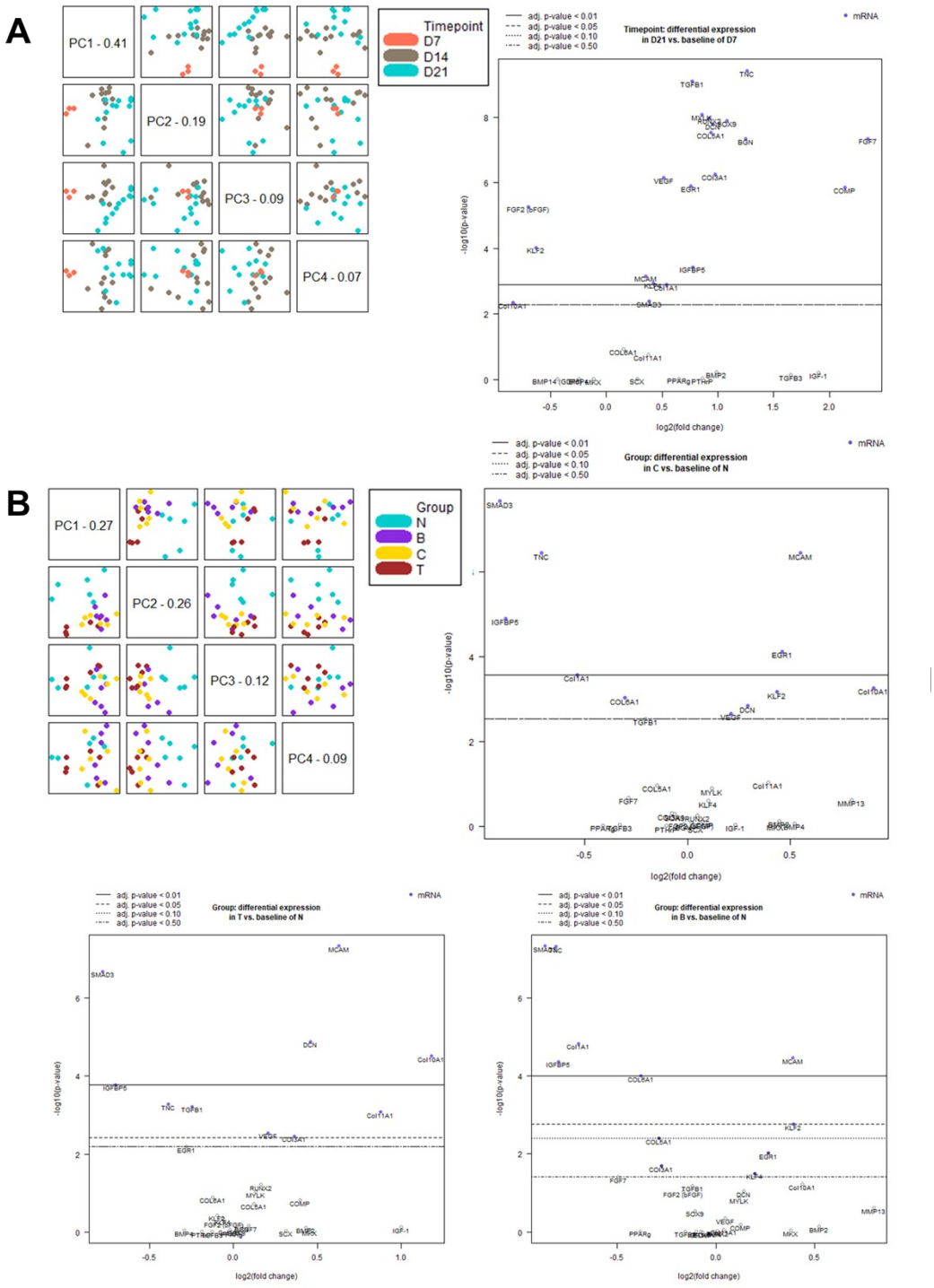
Supplemental Figure S6: hMSC response to differential dosage of BMP-4. In a truncated study, hMSCs were exposed to 2 weeks of BMP-4 supplementation after 4 days of culture; all other methods were conducted identically to those for chondrogenic and single biomolecule assessment



Supplemental Figure S7. ***Conditioned media analysis from hMSCs seeded onto tendon- or bone-mimetic collagen scaffolds.*** (A-B) Relative cytokine levels (n=2) present in conditioned media relative to a basal media control along with (C) overall metabolic activity (n=6) and DNA concentration (n=6) of hMSCs within their constructs over the isolation period.



Supplemental Figure S8*:* Individual differentiation patterns of hMSCs in crosslinked, lyophilized Gel-SH hydrogels at Day 21 of culture in response to conditioned media, relative to basal D21 control. Expression patterns (n=3) via nCounter mRNA expression panel. All bars denote trends with a p-value < 0.20. Absence of a bar indicates no trend with p-value <0.20. An asterisk indicates a significant ($p<0.05$) change in expression.



Supplemental Figure S9: Complete conditioned media PCA and gene fold-change data. (A) Principal component analysis (left) and differential expression analysis (right) on hMSC gene expression across all conditioned media treatments as a function of time (D7-D21, D7 baseline). (B) Principal component analysis (left) and differential expression analysis (right) on hMSC gene expression between conditioned media treatment groups ("N" baseline) across relevant time points (D14, D21). PCA plots denote the top four principal components plotted against one another, with each label denoting the corresponding row (x-axis plot) or column (y-axis plot) it appears in.

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