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Supplementary Materials for

Synthetic presentation of noncanonical Wnt5a motif promotes mechanosensing-dependent differentiation of stem cells and regeneration

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This PDF file includes:

Supplementary Materials

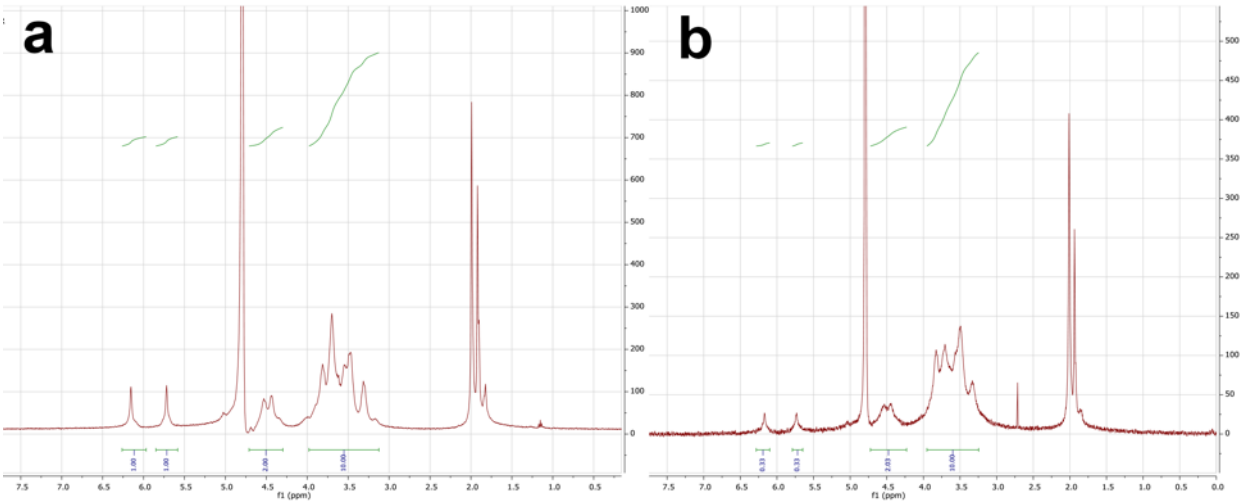

Fig S1. Characterization of the methacrylated hyaluronic acid (MeHA) macromers. ^{1}H nuclear magnetic resonance (^{1}H NMR [400 MHz, D_2O, δ]) results showed that the modification rates of the methacrylated hyaluronic acid (MeHA) polymer are 100% **(A)** and 30% **(B)**, respectively.

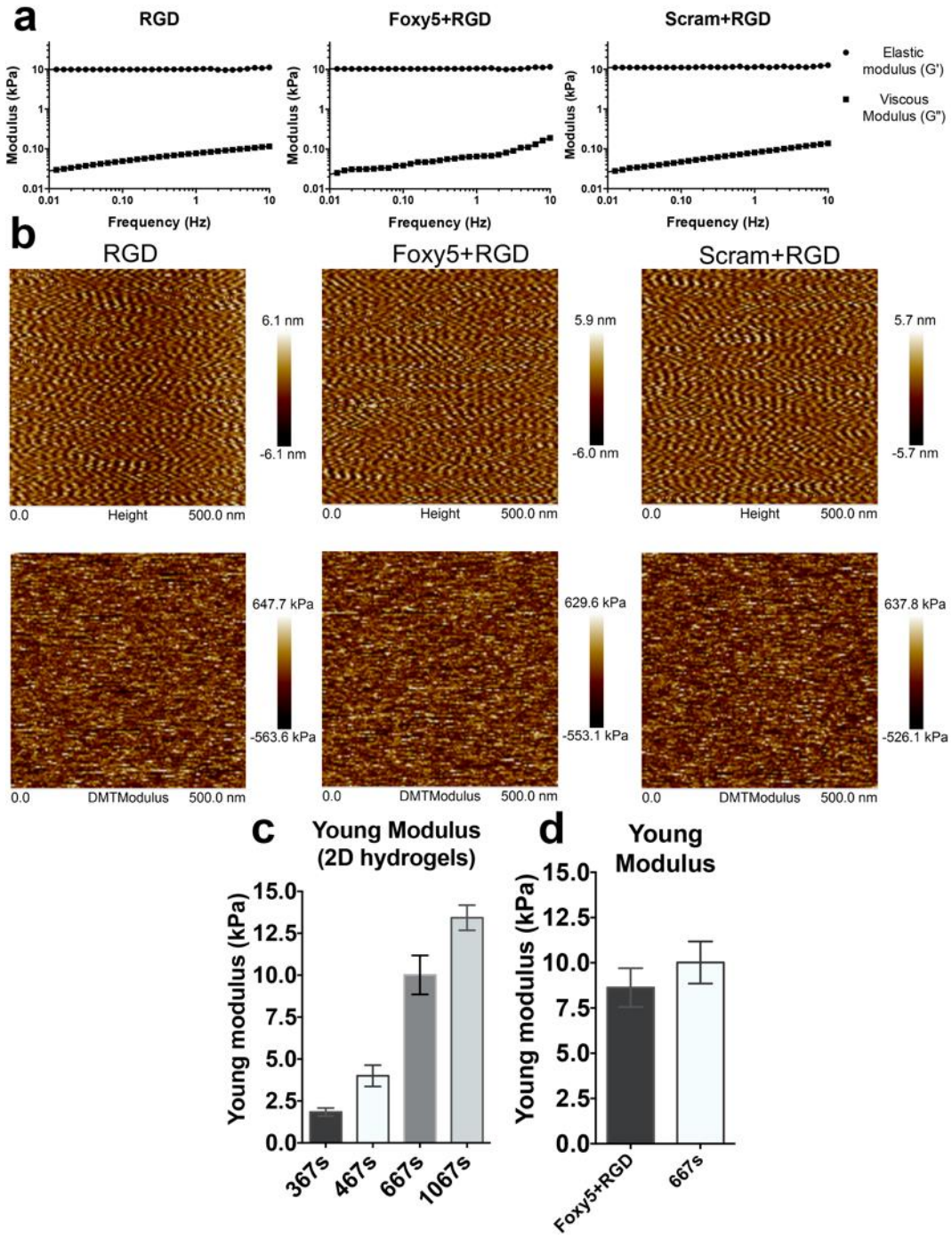

Fig. S2. The mechanical characterizations of the MeHA hydrogels conjugated with various groups of peptide. (A) The storage moduli and loss moduli acquired from frequency sweep analysis were not significantly different among different hydrogels in the RGD, Foxy5+RGD, and Scram+RGD groups. **(B)** Characterization of the 2D MeHA hydrogels, conjugated with RGD peptides, Foxy5 and RGD peptides, or scrambled Foxy5 and RGD peptides. AFM images showed that the surface roughness and the average moduli of the 2D hydrogels are not significantly different among groups (n=3). **(C)** The Young moduli of the UV-crosslinked 2D MeHA (modification rate = 30%) hydrogels were verified using the Mach-1 mechanical tester. **(D)** The average young modulus of the UV-crosslinked Foxy5+RGD hydrogels with 667 seconds of radiation is not significantly different from the DTT-crosslinked Foxy5+RGD MeHA (modification rate=100%) hydrogel (n=3).

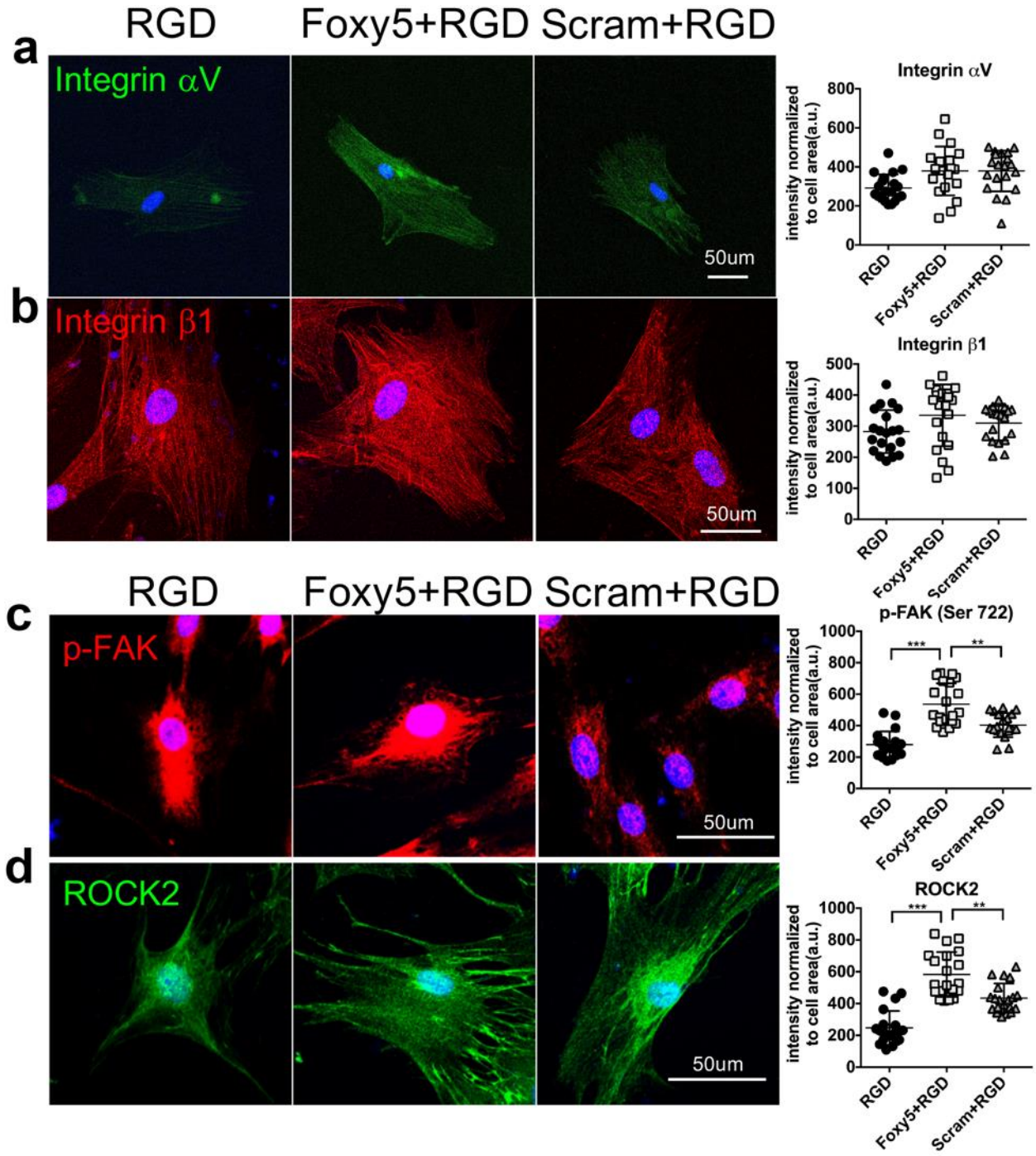

Fig. S3. Foxy5 peptide-conjugated hydrogels upregulate the expression of mechanotransduction signaling molecules in the seeded hMSCs. (A) Fluorescence micrographs and analysis of hMSCs stained for integrin αV (green), **(B)** integrin β1 (red), **(C)** p-FAK(red), **(D)** ROCK2 (green), and nuclei (blue), of the hMSCs cultured on the RGD, Foxy5+RGD, and Scram+RGD hydrogels. Analysis of the cytoplasmic fluorescence intensity of integrin αVβ1 of representative cells cultured on 2D hydrogels in the different experimental groups showed that the staining intensity of the integrin aVβ1 in the Foxy5+RGD group is not significantly different with the intensities in the RGD group and Scram+RGD group. Scale bars represent 50 μm. Data are shown as the mean ± standard deviation. (n=20) Statistical significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significant difference.

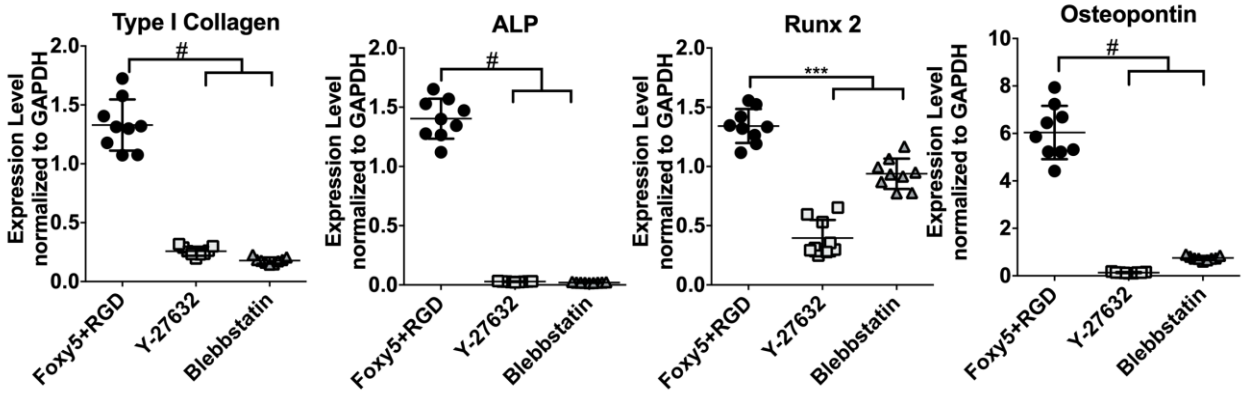

Fig. S4. The promoting effect of conjugated Foxy5 peptide on the osteogenesis of hMSCs is dependent on ROCK and non-muscle myosin II activities. To evaluate the influence of RhoA activation by Foxy5 presentation, pharmacological inhibitors of ROCK2 (Y-27632) and non-muscle myosin II (blebbistatin) were separately added to the osteogenic media in the Foxy5+RGD group for 7 days of culture before the analysis of quantitative gene expression of osteogenic markers (type I collagen, RUNX2, ALP, and OPN) in hMSCs seeded in porous hydrogels conjugated with Foxy5 and RGD peptides (Foxy5+RGD). Data are shown as the mean ± standard deviation. (n=9) Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and # $p<0.0001$ significant difference.

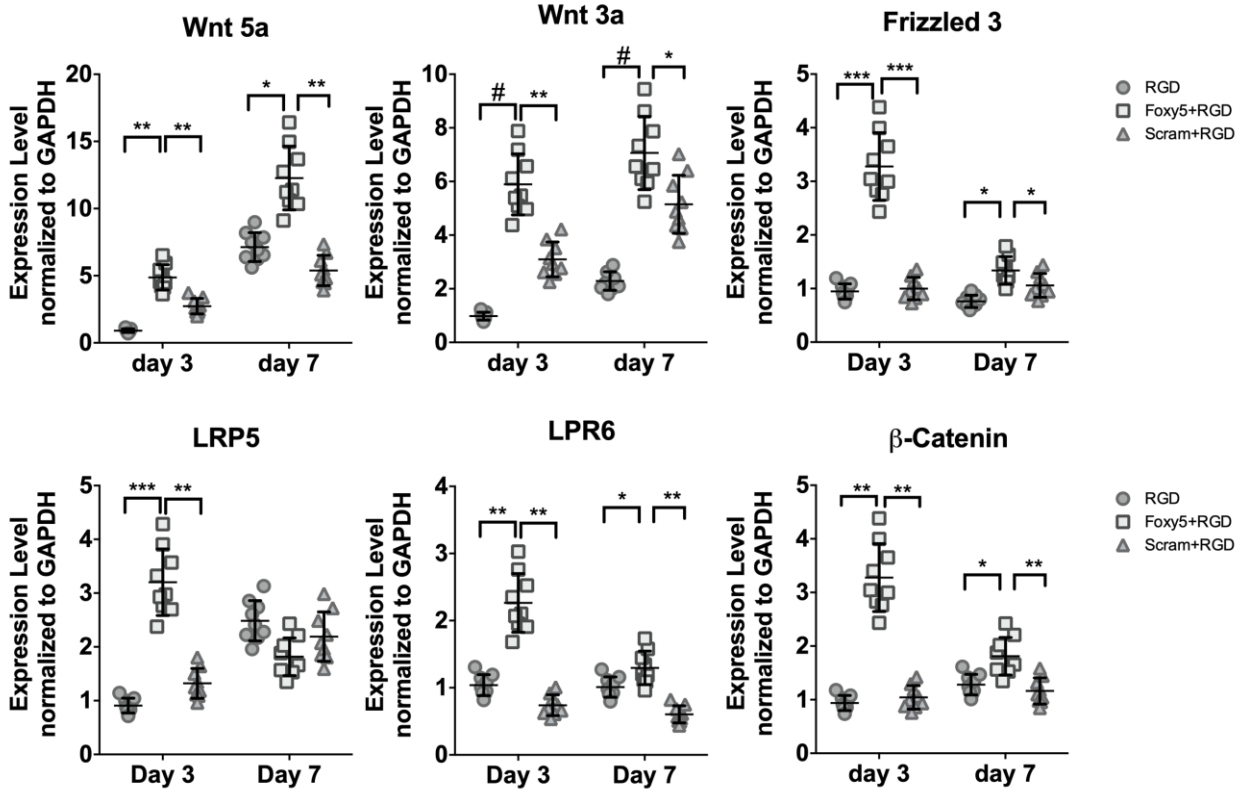

Fig. S5. Conjugated Foxy5 peptides enhance the expression of canonical Wnt-related genes of hMSCs seeded in the 3D hydrogel. Quantitative gene expression of Wnt-related markers (Wnt5a, Wnt3a, Frizzled 3, LRP5, LRP6, and β-Catenin) in hMSCs seeded in porous hydrogels conjugated with RGD peptide alone (RGD), Foxy5 and RGD peptides (Foxy5+RGD), or Scram and RGD peptides (Scram+RGD) in osteogenic culture for 7 days before analysis. Data are shown as the mean ± standard deviation (n=9). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and # $p<0.0001$ significant difference.

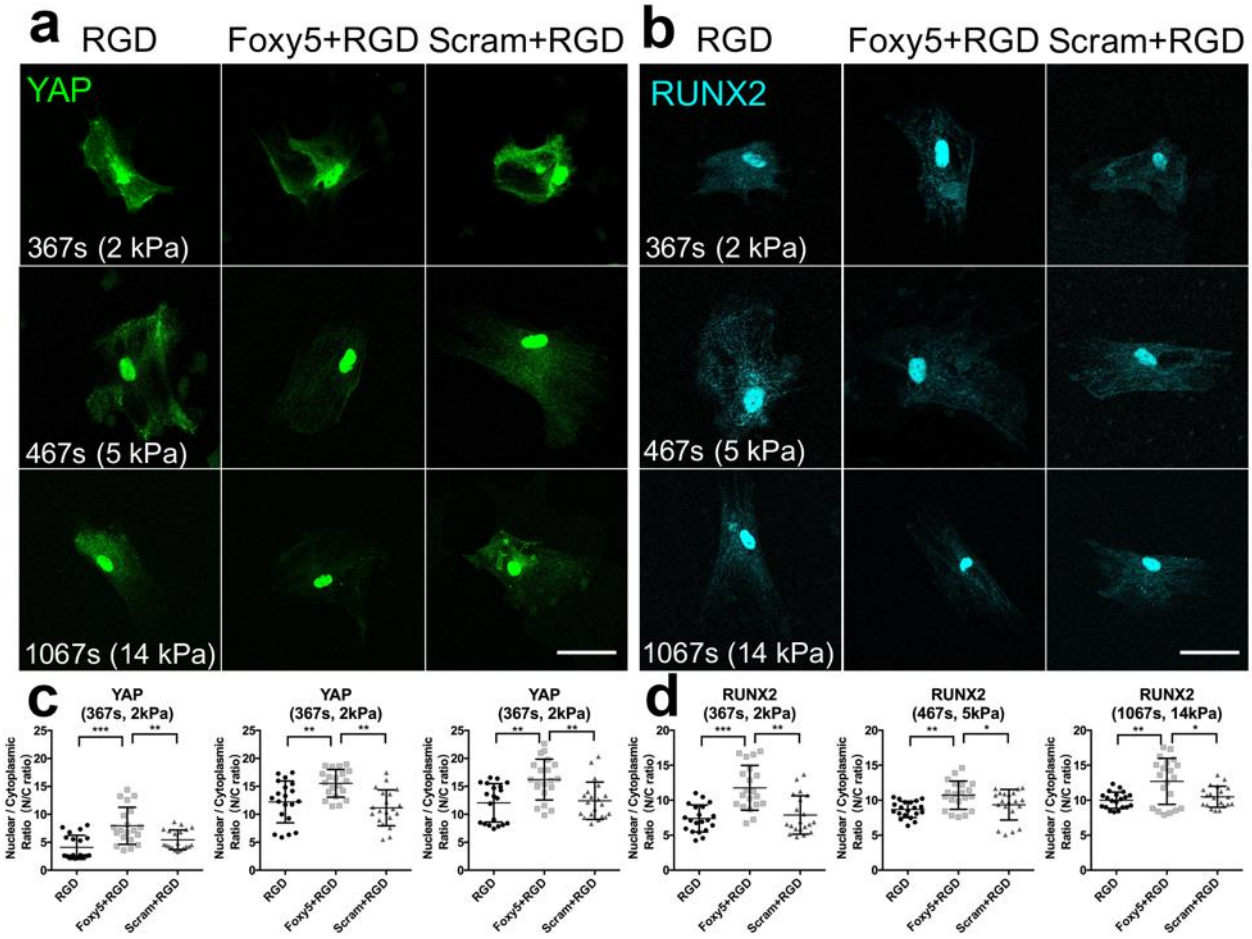

Fig. S6. The hydrogel-conjugated Foxy5 peptide promotes the osteogenesis in a wide range of hydrogel substrate stiffness. (A) Fluorescence micrographs of hMSCs stained for F-actin (red), nuclei (blue), and the mechanosensing marker YAP (green) or the osteogenic marker RUNX2 (cyan) **(B)** of the hMSCs cultured on the RGD, Foxy5+RGD, and Scram+RGD hydrogels with Young moduli of 2 kPa, 5 kPa and 14 kPa, respectively. **(C)** Analysis of the nuclear localization of YAP determined by the nuclear-to-cytoplasmic fluorescence intensity ratio (N/C ratio) and **(D)** RUNX2 nuclear localization of representative cells cultured on 2D hydrogels in the different experimental groups. Scale bars represent 50 μm in the fluorescence micrographs. Data are shown as the mean ± standard deviation. (n=20) Statistical significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significant difference.

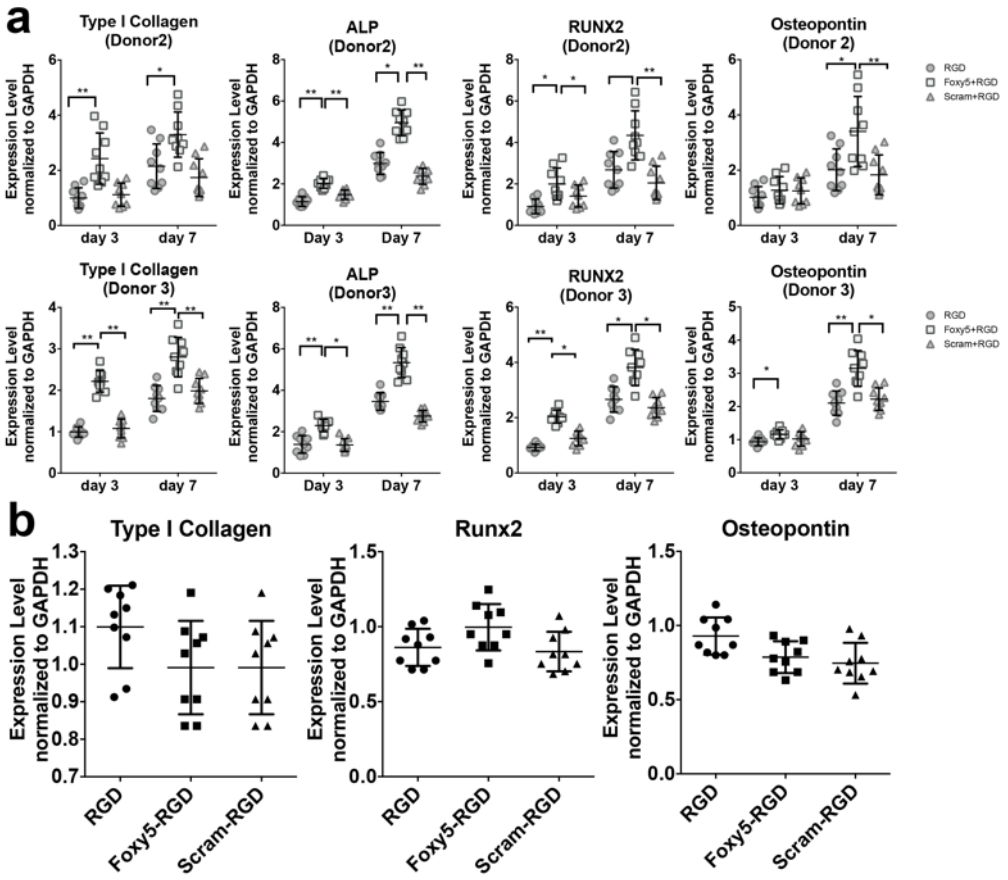

Fig. S7. The conjugated Foxy5 peptide promotes the osteogenesis of the hMSCs from multiple donors. (A) Quantitative gene expression of osteogenic markers (type I collagen, ALP, RUNX2, and osteopontin) in hMSCs from different donors seeded in porous hydrogels conjugated with RGD peptide alone (RGD), Foxy5 and RGD peptides (Foxy5+RGD), or Scram and RGD peptides (Scram+RGD) in osteogenic culture for 3 and 7 days before analysis. Data are shown as the mean ± standard deviation (n=9). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and # $p<0.0001$ significant difference. **(B)** Quantitative gene expression of osteogenic markers (type I collagen, RUNX2, and osteopontin) in hMSCs seeded in porous hydrogels conjugated with RGD peptide alone (RGD), Foxy5 and RGD peptides (Foxy5+RGD), or Scram and RGD peptides (Scram+RGD) in osteogenic culture for 14 days before analysis. Data are shown as the mean ± standard deviation (n=9). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and # $p<0.0001$ significant difference.

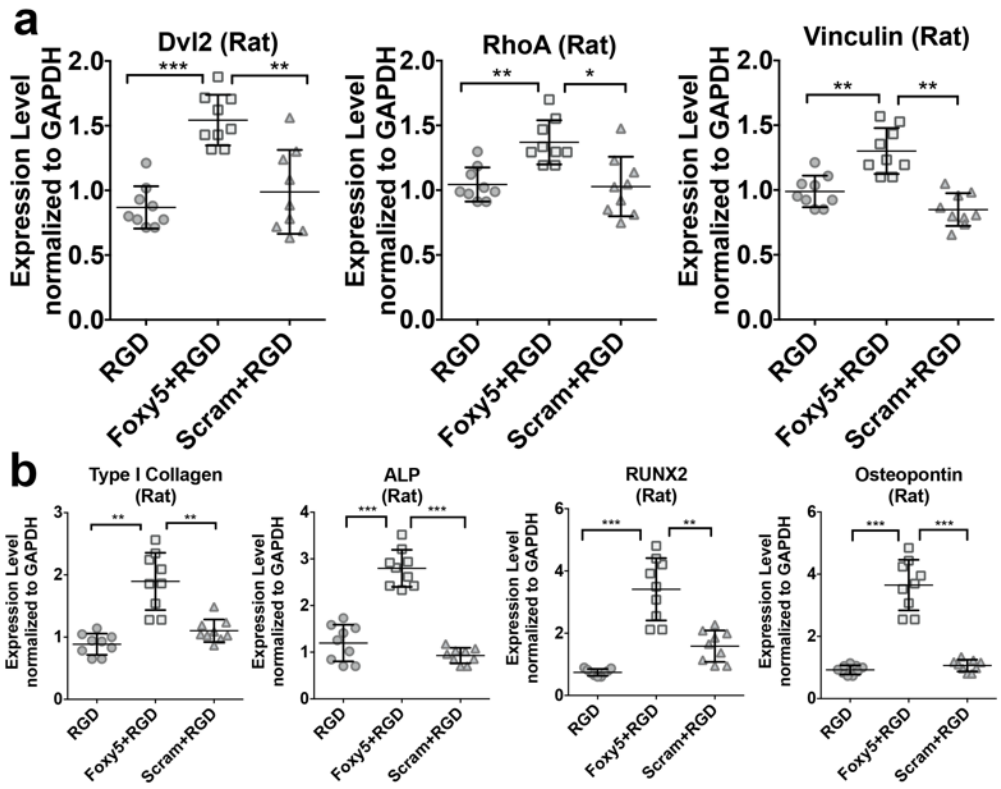

Fig. S8. The conjugated Foxy5 peptide promotes the mechanotransduction and osteogenesis of the seeded rMSCs. Quantitative gene expression of mechanotransduction-related markers **(A)** (Dvl2, RhoA, and Vinculin) and osteogenic markers **(B)** (type I collagen, RUNX2, and osteopontin) in rMSCs seeded in porous hydrogels conjugated with RGD peptide alone (RGD), Foxy5 and RGD peptides (Foxy5+RGD), or Scram and RGD peptides (Scram+RGD) in osteogenic culture for 7 days before analysis. Data are shown as the mean ± standard deviation (n=9). Statistical significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significant difference.

Table S1. The sequence of the primers and probes used for real-time PCR are listed.

Gene	Forward primer	Reverse primer	Probe
GAPDH	AGGGCTGCTTTTAACTCTGGTAAA	GAATTTGCCATGGGTGGAAT	CCTCAACTACATGGTTTAC
Collagen type I	CGGAACTCCTGACCCTTGAC	TGTTCAGCTCGTACTGCATGTC	TCGAAGAGACCCAATAGGT
ALP	AGGACAAGAGGCATGTCTGGTT	GGACATCAGGCGCAGGAA	TTCCAGTTCGAGTATGGC
RUNX2	GGAGGCAAAAAGGCAGAGGTT	CCCAAACTCCTGTAAGGTTAAGCAT	TTCCTTTCTACTACCCGCTC

Table S2. The donor information of the hMSCs used for the in vitro experiments is listed.

Donor	Brand	Batch Number	Age	Gender	Race
Donor 1	Lonza	PT-2501 0000494678	21	M	H
Donor 2	Lonza	PT-2501 0000372262	39	M	B
Donor 3	Stem Cell Technology	70022	33	F	W