Supplementary Data

TITLE: Protein-engineered microenvironments can promote endothelial differentiation of human mesenchymal stem cells in the absence of exogenous growth factors

AUTHORS: Yeji Kim[a] and Julie C. Liu[a,b,]*

[a] School of Chemical Engineering, Purdue University, West Lafayette, IN 47907

[b] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907

Julie C. Liu (corresponding author), Email: julieliu@purdue.edu, Phone: 1-765-494-1935, Fax: 1-765-494-0805

Western Blot Analysis

Cells were rinsed with PBS twice and treated with radio immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails and phenylmethylsulfonyl fluoride (PMSF). Collected cells were frozen at -20 °C before being assayed. Frozen cells were thawed, vortexed, and centrifuged to remove cell debris, and the supernatant was collected. To measure the total protein concentration of each sample, the QuantiPro BCA assay kit was utilized. Briefly, 25 μL of cell lysate was incubated with an equal amount of the reagent for 1 h at 60 °C, and the absorbance at 562 nm was measured. A standard curve was created using a BSA solution in RIPA buffer to determine the total protein amount of each sample.

A total of 50 μg of proteins were loaded on a 10% SDS-PAGE gel, and the gel was run at 125 V for 1.5 h. The proteins were transferred to a nitrocellulose membrane at 55 V for 10 h at 4 °C. The membrane was blocked with 5 (w/v) % dry milk in Tris-buffered saline with Tween-20 (TBST) buffer at 4 °C overnight. The membrane was incubated with primary antibody (Santa Cruz Biotechnology) in blocking solution for 1.5 h at room temperature and then incubated with horseradish peroxidase (HRP)-tagged secondary antibody (Santa Cruz Biotechnology, sc-2005) in blocking solution for 1 h at room temperature. Details concerning the antibodies used in this study are shown in Table S3. Proteins were detected with a chemiluminescent substrate (Pierce, 32132). The intensity of the desired band was quantified by ImageJ analysis software (NIH) and normalized by that of β-actin. The data are shown relative to the proliferating group at day 0. Three replicates per group were examined.

Table S1. Primer sequences for quantitative reverse transcription-polymerase chain reaction.

Gene	Accession number	Primer sequence (5' → 3')		Efficiency	Product length (bp)	Reference
VE-cad	NM001795.3	FW RV	CCAGGACGCTTTCACCATTGAGACA CCAGAGGCTTCATGGGCTTGATGATG	92%	71	[1]
PECAM	NM000442.3	FW RV	TCTATGACCTCGCCCTCCACAAA GAACGGTGTCTTCAGGTTGGTATTTCA	88%	83	[2]
Ephrin-B1	NM004429.4	FW RV	GTTCTCGACCCCAACGTGTT CAGGCTTCCATTGGATGTTGA	100%	153	[3]
Ephrin-B2	NM004093.3	FW RV	CTCCTCAACTGTGCCAAACCA GGTTATCCAGGCCCTCCAAA	96%	151	[3]
EphB4	NM004444.4	FW RV	CAGCTTTGGAAGAGACCCTGC GTCCACCTGAGGGAATGTACA	107%	77	Designed
COUP-TFII	NM021005.3	FW RV	CTAGGTGGTGATCTGCCCTC GGAAAGAGTCAACTCGCCG	98%	71	Designed
GAPDH	NM002046.3	FW RV	ACAGTCAGCCGCATCTTCTT TTGACTCCGACCTTCACCTT	102%	81	[4]

Table S2. Amino acid analysis of the RZ-QK protein.

	Observed mol%	Calculated mol%
ASX	3.49	3.39
SER	12.37	12.43
GLX	14.59	14.69
GLY	11.40	11.86
HIS	4.90	5.08
ARG	2.26	1.69
THR	7.39	7.34
ALA	12.64	12.43
PRO	11.34	11.30
TYR	1.22	1.13
VAL	0.34	0.00
MET	2.86	3.39
LYS	5.70	5.65
ILE	0.67	0.56
LEU	3.29	3.39
PHE	5.56	5.65
Total	**100.00**	**100.00**

Table S3. Antibodies used for Western blot analysis.

	Catalog number	Primary antibody dilution	Secondary antibody dilution
VEGF receptor 2 (VEGF R2)	sc-6251	1:100	1:1000
β-actin	sc-47778	1:200	1:2000

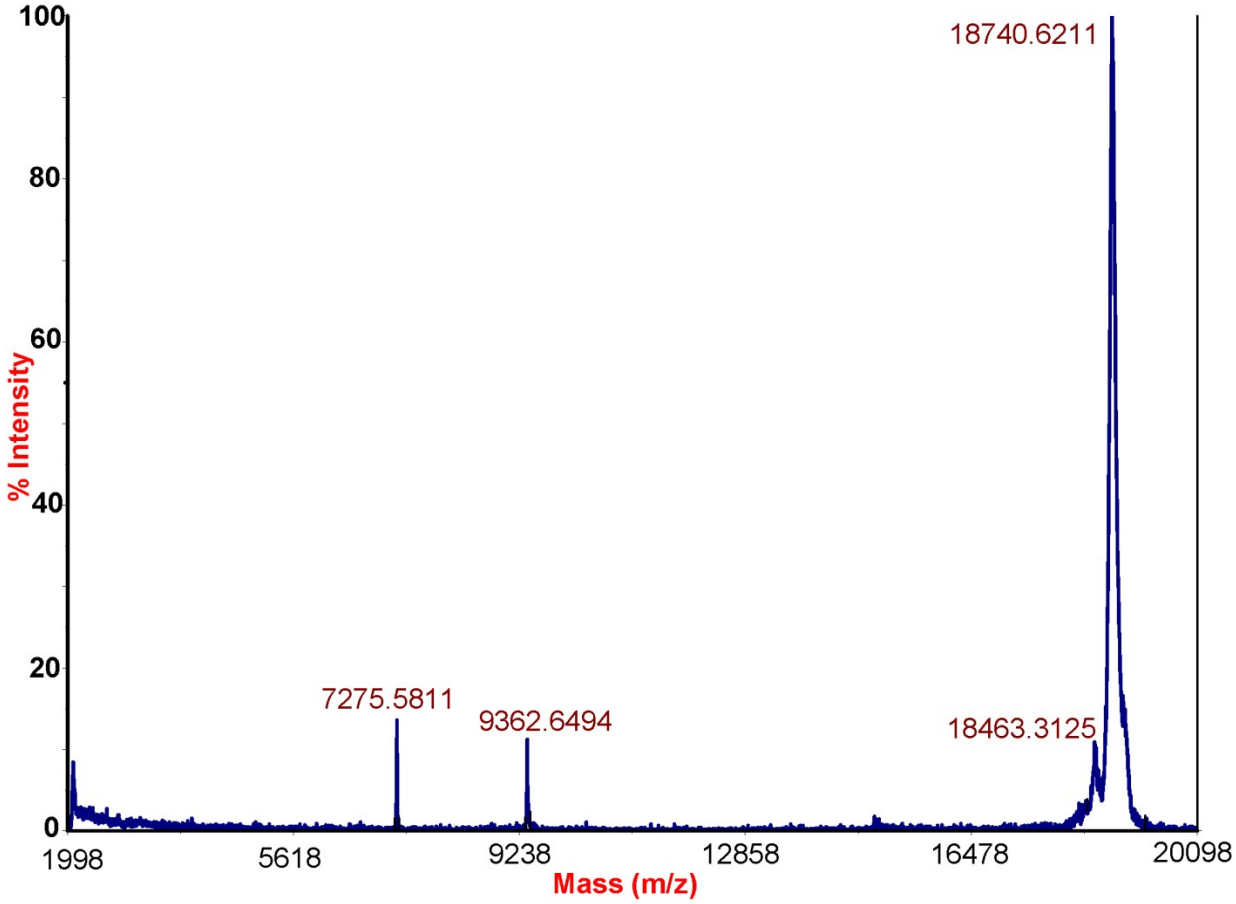

Figure S1. MALDI-TOF mass spectroscopy of the RZ-QK protein. The measured molecular weight of RZ-QK (18740 Da) was within 29 Da (0.15 %) of the expected molecular weight (18711 Da).

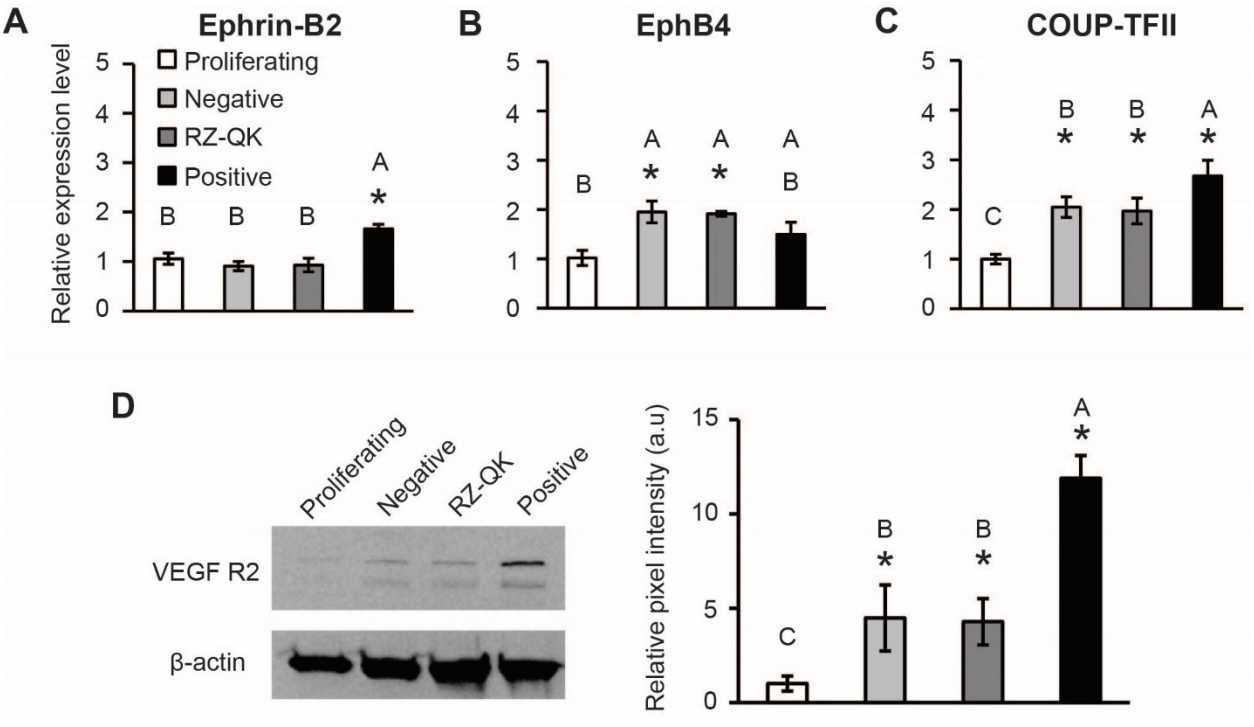

Figure S2. Expression levels of endothelial markers. Gene and protein expression levels were measured by (A-C) qPCR after 4 days of differentiation and (D) Western blot analysis after 12 days of differentiation. Arterial and venous gene expression levels for (A) Ephrin-B2 (arterial), (B) EphB4 (venous), and (C) COUP-TFII (venous) were normalized by GAPDH levels and are shown relative to the proliferating group at day 0. (D) VEGF R2 protein expression was examined and shown relative to the proliferating group (day 0). Data are represented as the average ± standard deviation of three to five replicates. Letters indicate statistically different Tukey groups. * represents a statistical difference compared to the proliferating group at day 0 ($p < 0.05$) as assessed by Dunnett's test.

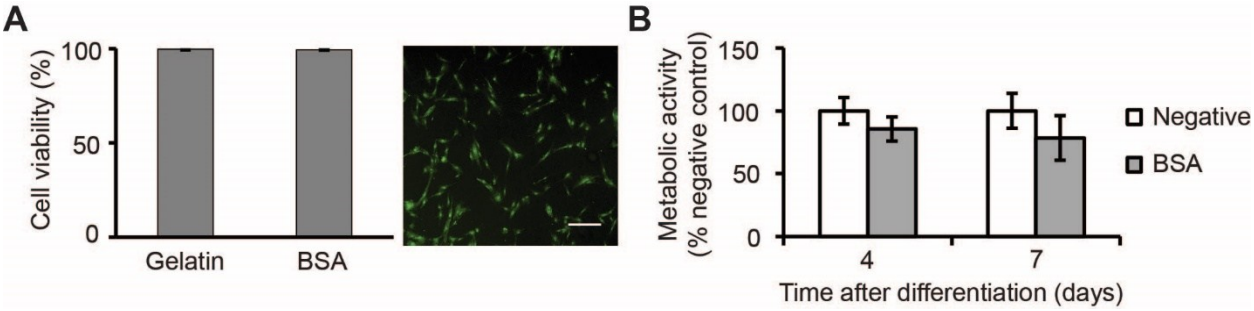

Figure S3. Cell viability and metabolic activity on BSA surfaces. (A) Cell viability on BSA surfaces (99.5%) was statistically equivalent to that on gelatin surfaces (99.8%) (left). The cell viability data on gelatin surfaces is from the same experiment as shown in Figure 2A. An image of the LIVE/DEAD assay for cells grown on BSA surfaces is shown (right). Scale bar represents 250 μm. (B) There was no difference in metabolic activity, as confirmed by one-way ANOVA, between cells on BSA surfaces and negative control cells at two time points. The negative control data is from the same experiment as shown in Figure 2B. Data are represented as the average ± standard deviation of (A) four or (B) five replicates.

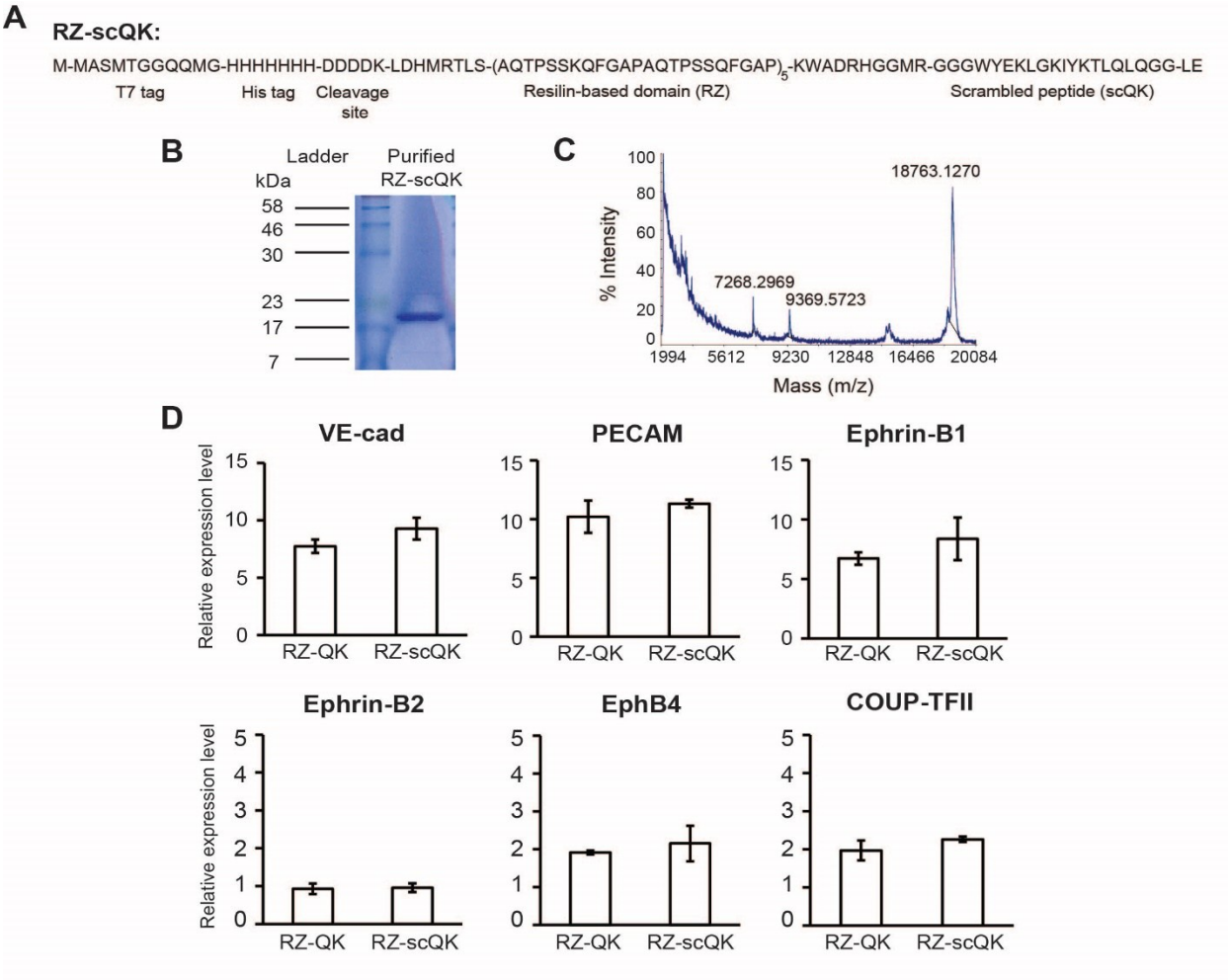

Figure S4. Examination of specificity of the QK domain on cell differentiation. (A-C) The RZ-scQK protein was manufactured and analyzed and (D) gene expression levels of cells on the RZ-scQK protein were measured. (A) The full amino acid sequence of the RZ-scQK protein is shown. (B) The purity of the protein was confirmed by SDS-PAGE gel. The RZ-scQK protein appeared at the expected molecular weight of 18.7 kDa. The purity was 96.1% as determined by densitometry analysis. (C) MALDI-TOF mass spectroscopy of the RZ-scQK protein. The measured molecular weight of RZ-QK (18763 Da) was within 52 Da (0.27 %) of the expected molecular weight (18711 Da). (D) Endothelial gene expression levels of cells cultured on the RZ-scQK protein for 4 days were assessed by qPCR. For all markers, there was no statistical

difference in expression levels between cells on RZ-QK and RZ-scQK proteins as evaluated by a one-way ANOVA. The RZ-QK data are from the same experiments shown in Figures 5A-C and S2A-C. Data are normalized to GAPDH, which was used as the housekeeping gene, and expression levels were determined relative to proliferating cells at day 0. Data are represented as the average ± standard deviation of three to five replicates.

References

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[4] Pinhu L, Park JES, Yao WX, Griffiths MJD. Reference gene selection for real-time polymerase chain reaction in human lung cells subjected to cyclic mechanical strain. Respirology 2008;13:990-999.