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Supporting Information

Anionic fibroin-derived polypeptides accelerate MSC osteoblastic differentiation in a three-dimensional osteoid-like dense collagen niche

Benedetto Marelli*, Chiara E. Ghezzi, Antonio Alessandrino, Giuliano Freddi and Showan N. Nazhat*

Experimental section

m-MSC culture. m-MSCs, extracted from bone marrow isolated from C57BL/6 mice at < 8 weeks of gestation through mechanical and enzymatic digestion were purchased from Invitrogen (USA). According to manufacturer instructions, C57BL/6 MSCs below passage 10 express a flow-cytometry cell-surface protein profile positive for CD29, CD34, and Sca-1 (>70%), and negative for CD117 (<5%). The cells were expanded and cultured in complete growth medium, prepared from Dulbecco's Modified Eagle Medium (DMEM) F-12 (Gibco®, Invitrogen, USA) with GlutaMAX™-I containing 10% MSC-Qualified Foetal Bovine Serum (Gibco®, Invitrogen, USA) and 5 μg/ml Gentamicin (Gibco®, Invitrogen, USA). m-MSCs were expanded up to passage 8 to prevent dedifferentiation. Osteogenic medium was prepared from complete growth medium supplemented with 0.5% ascorbic acid, 0.5% dexamethasone, and 1% β-glycerophosphate (all from Sigma-Aldrich, Canada). All culture media were replaced at 2-3 day intervals.

Preparation of m-MSC seeded DC-Cs constructs. Cs fibroin derived polypeptides were prepared as previously described.[1] Briefly, Cs polypeptides were isolated by dissolving α-chymotrypsin (an enzyme-to-substrate ratio of 1:100) in an aqueous solution of *Bombyx mori* SF. After 24 hours at 37 °C, a gelatinous precipitate was formed, which was centrifuged and the resulting supernatant was freeze-dried to recover the soluble peptides (Cs fraction).The theoretical

sequences of Cs polypeptides is available using open bioinformatics tools such as "Peptide Cutter" (available at http://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl?P05790) in combination with the reported sequence of the silk fibroin heavy chain (code P5790). Enzymatic cleavage sites of silk fibroin heavy chain by α-chymotrypsin are reported in Table S1. The amino-acidic composition of Cs polypeptides was determined after acid hydrolysis with 6N HCl, at 105 °C for 24 hrs, under vacuum. The free amino acids were determined by high performance liquid chromatography according to the AccQ-Tag Method (Waters). Samples were analysed in duplicate. External standard calibration was used (Amino Acid Standard H, Pierce) (Table S2).

m-MSC seeded DC and DC-Cs rolled gels were prepared using the PC technique as previously described.[2] Cs polypeptides were initially incorporated at 10 dry wt% into 3.2 ml of rat-tail tendon type I collagen (2.05 mg/ml in 0.6% acetic acid, First Link Ltd, UK), 0.8 ml of 10 times concentrated (10x DMEM) (Sigma Aldrich, Canada) and neutralized with 70 μl of 5M NaOH (Fisher Scientific, Canada). m-MSCs were seeded at a density of 2.11×10^5 cells/ml collagen and the solution (4 ml) was cast in a rectangular mould (area 18x43 mm^2) and incubated at 37 °C for 30 minutes for self-assembly. Highly-hydrated gels were removed from the casting chamber and compressed using 1 kPa for 5 minutes in combination with blotting. The resulting rectangular DC sheets (14 wt% in fibrillar density and approximately 150 μm thick) were rolled along their long axis and halved to give cylindrically shaped (roll) specimens; 8 mm in length and 1.5±0.1 mm in diameter. DC rolls were produced as controls.

m-MSC viability. CLSM (LSM 5, Carl Zeiss, Germany) was used to monitor m-MSC viability at days 1, 7, 14 and 21 in culture. DC and DC-Cs scaffolds were incubated for 60 minutes in complete MEM with 1 μM calcein-AM and 2 μM ethidium homodimer-1 (Live/Dead® assay, Invitrogen, USA). Images were acquired using argon laser excitation (488 nm) and HeNe633 laser excitation (543 nm) with a 10X objective. Z-stacks were obtained through the entire

thickness of each roll using 15 μm slices in 5 different regions. Afterwards, z-stacks were assembled into maximum intensity projection images using the "Stacks-MultiD" plug-in from ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2010), and representative images were selected for each time point.

m-MSC metabolic activity. AlamarBlue™ reagent (Invitrogen, USA) was used to investigate m-MSC metabolic activity within DC and DC-Cs rolled gels at days 1, 7, 14, and 21 in culture. Specimens (n=3) were incubated at 37 °C with 10% AlamarBlue™ reagent for 4 hours. Post incubation, 100 μl aliquots of medium were collected in triplicate. Fluorescence intensity was measured at 585 nm using 550 nm excitation with a LB940 Multimode Microplate Reader (Berthold Technologies, Germany), as an expression of cellular reduction according to the resazurin indicator. Acellular DC and DC-Cs were used as background reference.

m-MSC Differentiation. Expression levels of ALP, Runx2 and OPN genes were assessed by RT-qPCR to investigate seeded m-MSC osteogenic differentiation at days 14 and 21 in culture. Total RNA was extracted from m-MSCs seeded scaffolds with TRIzol® (Invitrogen, USA) following manufacturer instructions. After RNA separation, nucleic acid concentration and integrity were determined with an Eppendorf BioPhotometer Plus (Eppendorf, Hamburg, Germany). Total RNA (250 ng) was mixed with 0.25 ng of random hexamers (Invitrogen, USA) and reverse-transcribed into complementary DNA (cDNA) with 200 U of Superscript Reverse Transcriptase II (Invitrogen, USA) and RNasin (Promega). RT-qPCR was performed with an ABI Prism 7900 HT 139 (Applied Biosystems). Each PCR reaction contained 9 μl of cDNA, 0.5 μl of both forward and reverse primers (10 μM), and 10 μl of SYBR Green (Applied Biosystems). The cycling conditions were: 50 °C for 2 minutes, initial denaturation at 95 °C for 10 minutes, and 45 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Relative quantification of target gene expression

was achieved first by normalizing against an endogenous reference gene (housekeeping gene GAPDH) to correct different amounts of input RNA, and then relating the expression of the target genes to a reference sample (MSCs extracted from DC) at day 1, using the -2ΔΔCt method.[3]

Histological analysis. Histological analysis was used to evaluate m-MSC distribution, construct morphology and mineralization at days 14 and 21 in culture. Specimens were washed in phosphate buffered saline and fixed in 10% neutral buffered formalin (Protocol, Fisher Scientific) overnight. Constructs were then dehydrated through a series of graded ethanol, embedded in paraffin and cut in transverse sections of 5 μm thickness. Histological sections were then deparaffinized, rehydrated through a series of graded ethanol, and stained with Von Kossa's method. Histological sections were analyzed with a light microscope (Leica DM500) using 4X and 10x objectives.

SEM. SEM was used to characterize seeded m-MSCs morphology and the presence of a mineral phase in DC and DC-Cs constructs at day 14 in osteogenic medium. Specimens were fixed in 4% paraformaldehyde – 0.1 M sodium cacodylate solution at 4 °C overnight, washed with deionized water, and dehydrated through sequential immersion in a graded series of increasing ethanol concentrations. Afterwards, specimens were processed with a critical point dryer (Ladd Research Industries, USA), sputter coated with Au/Pd (Hummer VI Sputter Coater, Ladd Research Industries, USA) and analyzed with a S-4700 Field Emission-STEM (Hitachi, Japan) at 2 kV and 10 μA.

ATR-FTIR. ATR-FTIR (Spectrum 400, Perkin-Elmer, USA) was used to characterize structural changes of DC and DC-Cs constructs and to evaluate mineralization within the collagenous matrices as a function of time in culture. Analysis was carried out using a resolution of 2 cm^{-1},

128 scans, and a spectrum range of 4000-650 cm^{-1}. Spectra were tuned up with an *ad hoc* baseline correction.

XRD. XRD was used to characterise m-MSCs-seeded DC and DC-Cs rolls as made, and at day 21 of culture in osteogenic medium. XRD patterns were recorded with a Bruker D8 Discover from 6 to 60° 2 theta at 40 kV and 20 mA. Two frames of 25° were recorded for 10 minutes and then merged during data post processing. Phase composition was determined by comparing the acquired spectra with peaks identified in the International Centre for Diffraction Data (ICDD) database.

*Statistical Analysis.*_Data were analyzed for statistical significance using a two-way ANOVA with a significance level=0.05 and Tukey-Kramer and Holm-Bonferroni methods for means comparison (Origin Pro v.8 software, OriginLab, USA).

References

1. B. Marelli, C. E. Ghezzi, A. Alessandrino, J. E. Barralet, G. Freddi and S. N. Nazhat, *Biomaterials*, 2012, 33, 102-108.
2. R. A. Brown, M. Wiseman, C. B. Chuo, U. Cheema and S. N. Nazhat, *Advanced Functional Material*, 2005, 15, 1762-1770.
3. K. J. Livak and T. D. Schmittgen, *Methods*, 2001, 25, 402-408.
4. C. Drouet, *BioMed Research International*, 2013, Article ID 490946, 12 pages

Figure S1

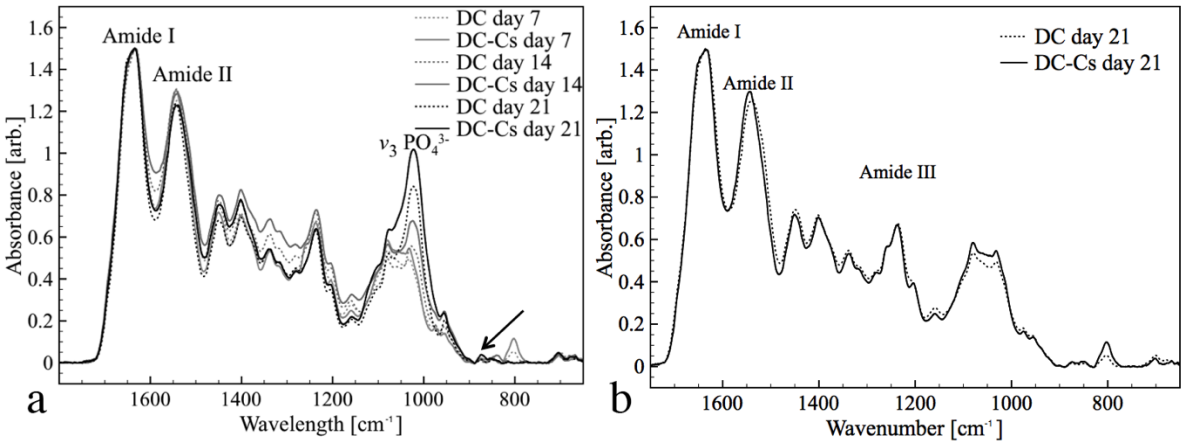

Figure S1: ATR-FTIR spectra of DC and DC-Cs rolled gels at days 7, 14 and 21 in culture in osteogenic medium. a) There was an increase in the absorbance of v_3 PO_4^{3-} at 1018 cm^{-1} and of v_2 CO_3^{2-} at 872 cm^{-1} (black arrow) in m-MSC seeded gels, suggesting the formation of carbonated hydroxyapatite. The higher absorbance of the v_3 PO_4^{3-} vibration in DC-Cs relative to DC indicated accelerated mineralization. b) Acellular DC and DC-Cs did not indicate mineralization at day 21.

Figure S2

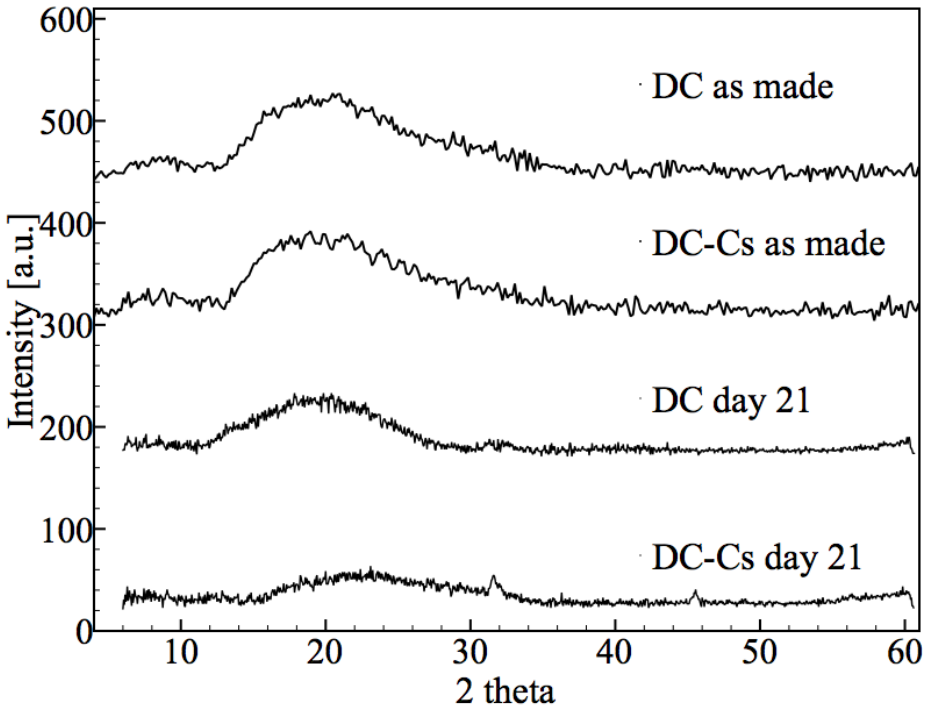

Figure S2: X-ray diffraction of DC and DC-Cs rolled gels, as made, and at day 21 in culture in osteogenic medium. XRD diffractographs of as made gels indicated the amorphous nature of DC and DC-Cs gels with a broad peak around 20°, distinctive of the random orientation of the collagen nanofibrils. X-ray diffractographs of cellular DC and DC-Cs gels at day 21 in osteogenic medium displayed the formation of an apatitic phase via a peak around 31°.[4] The peak was more evident for DC-Cs gels as a consequence of the anionic peptides-driven accelerated mineralization of the collagenous constructs.

Table S1. Enzymatic cleavage sites of silk fibroin heavy chain by α-chymotrypsin.

Enzyme	**No. of cleavages**	**Position of cleavage sites**
α-Chymotrypsin	317	6 15 18 26 30 31 84 115 123 129 135 141 145 171 189 201 213 225 237 249 261 273 311 323 333 341 363 369 385 393 397 405 409 483 491 537 545 553 561 631 639 643 651 678 681 687 693 695 697 701 741 749 753 761 851 859 929 937 941 949 1031 1039 1043 1051 1115 1123 1127 1135 1193 1197 1205 1232 1235 1241 1244 1246 1248 1254 1286 1294 1298 1306 1370 1374 1382 1440 1448 1452 1460 1518 1522 1530 1588 1596 1600 1608 1635 1638 1644 1647 1649 1651 1657 1691 1695 1703 1779 1787 1791 1799 1826 1829 1835 1838 1840 1842 1848 1894 1902 1906 1914 1990 1998 2062 2070 2074 2082 2104 2112 2116 2124 2212 2220 2224 2232 2266 2270 2297 2300 2306 2309 2311 2313 2319 2347 2355 2359 2367 2389 2397 2407 2447 2451 2459 2505 2513 2517 2525 2589 2616 2619 2625 2628 2630 2632 2638 2672 2680 2684 2692 2744 2796 2804 2808 2816 2888 2892 2900 2956 2964 2968 2976 3084 3092 3096 3104 3164 3168 3176 3184 3192 3228 3255 3258 3264 3267 3269 3271 3277 3311 3319 3359 3411 3415 3423 3427 3435 3487 3495 3499 3507 3579 3583 3591 3647 3655 3659 3667 3747 3755 3759 3767 3794 3797 3803 3806 3808 3810 3816 3838 3842 3850 3858 3866 3908 3912 3920 3928 3936 3988 3992 4000 4008 4016 4094 4102 4106 4113 4159 4186 4189 4194 4197 4199 4201 4207 4229 4237 4241 4249 4323 4331 4335 4343 4413 4417 4425 4433 4441 4501 4505 4532 4535 4541 4544 4546 4548 4554 4576 4584 4588 4596 4654 4658 4666 4674 4682 4754 4758 4766 4774 4782 4852 4860 4928 4932 4940 4946 4950 4958 5008 5016 5024 5072 5114 5139 5142 5148 5154 5156 5158 5164 5208 5214 5233 5235 5255

Table S2: Amino acidic composition of silk fibroin yarn and of the soluble α-chymotryptic fraction (Cs polypeptides) Standard deviation for each measurement is ≤1% of the average value.

Amino acid	Silk fibroin yarn (mol%)	Cs fraction (mol%)
Asp	1.6	4.5
Ser	11.3	9.2
Glu	1.2	3.1
Gly	45.9	36.9
His	0.3	0.5
Arg	0.5	1.3
Thr	0.9	1.9
Ala	28.6	24.0
Pro	0.4	1.0
Cys	0.0	0.0
Tyr	5.1	7.5
Val	2.1	4.2
Met	0.1	0.3
Lys	0.3	0.9
Ile	0.6	1.6
Leu	0.5	1.7
Phe	0.7	1.5