RESEARCH ARTICLE

Hydrogel bioink formulation for 3D bioprinting: Sustained delivery of PDGF-BB and VEGF in biomimetic scaffolds for tendon partial rupture repair

Supplementary File

(A) Ink development

Figure S1. Summary of the biological materials selected for the development of the new ink. The first column (Selection) lists the reasons and properties of each material to be selected as component for the development of hydrogel-based scaffolds for partial tendon injuries. The second column (Considerations) includes interesting aspects of the materials to establish the methodology to incorporate them in the ink. Abbreviation: ECM, extracellular matrix.
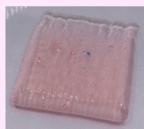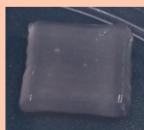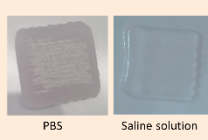
COMPOSITION			PHOTOS	NOTES
Biomaterials	**Concentration**	**Solvent**		
Formulation 1 Glycerol, Gelatin, Hyaluronic acid, Fibrinogen	100 µL mL⁻¹, 35 mg mL⁻¹, 3 mg mL⁻¹, 30 mg mL⁻¹	DMEM, DMEM, DMEM, DMEM		The ink could be easily printed. The obtained scaffolds were difficult to handle. Gelation was achieved enzymatically (fibrinogen-thrombin) and by means of temperature (gelatin).
Formulation 2 Glycerol, Gelatin, Hyaluronic acid, Fibrinogen	120 µL mL⁻¹, 42 mg mL⁻¹, 3.6 mg mL⁻¹, 36 mg mL⁻¹	DMEM, DMEM, DMEM, DMEM (0.9% NaCl)		The handling of the obtained scaffold was somewhat easier than that of formulation 1. The other properties did not seem to be very different between the two formulations. Fibrinogen dissolved more easily using DMEM with 0.9% NaCl.
Formulation 3 Glycerol, Gelatin, Hyaluronic acid, Fibrinogen	140 µL mL⁻¹, 49 mg mL⁻¹, 4.2 mg mL⁻¹, 42 mg mL⁻¹	DMEM, DMEM, DMEM, DMEM (0.9% NaCl)		The strength of the scaffold appeared very similar to formulation 2 despite having a higher concentration of all components.
Formulation 4 Alginate, Gelatin, Hyaluronic acid, Fibrinogen	10 mg mL⁻¹, 42 mg mL⁻¹, 3.6 mg mL⁻¹, 36 mg mL⁻¹	DMEM, DMEM, DMEM, DMEM (0.9% NaCl)		The change from glycerol to alginate meant the inclusion in the formulation of a second crosslinking method. The obtained scaffolds were much easier to handle and appeared more resistant.
Formulation 5 Alginate, Gelatin, Hyaluronic acid, Fibrinogen	10 mg mL⁻¹, 42 mg mL⁻¹, 3.6 mg mL⁻¹, 36 mg mL⁻¹	PBS/ Saline solution, PBS/ Saline solution, PBS/ Saline solution, PBS/ Saline solution	PBS Saline solution	There did not appear to be any differences between formulation 4 and formulation 5 in terms of scaffold handling ability.

Figure S2. Formulations developed using the selected biological materials. Different concentrations and methodologies to dissolve/disperse and to combine the biomaterials were studied. This development process was dynamic and allowed the ink to be gradually defined. Formulations 1–3 had the same composition, but the concentration of the different components varied. The scaffolds obtained using these three formulations were very similar in appearance and handled in a similar manner. Glycerol was replaced by alginate in the formulation 4 (thus adding a second crosslinking method). Through this change, it was possible to improve the rheological properties of the ink. Although the obtained scaffolds had a similar appearance visually, the incorporation of alginate made it possible to make them more manageable. The effect of using other solvents instead of the culture medium (formulation 5) was also studied. No improvements were observed in the obtained scaffolds. Formulation 4 was selected as the final ink formulation.
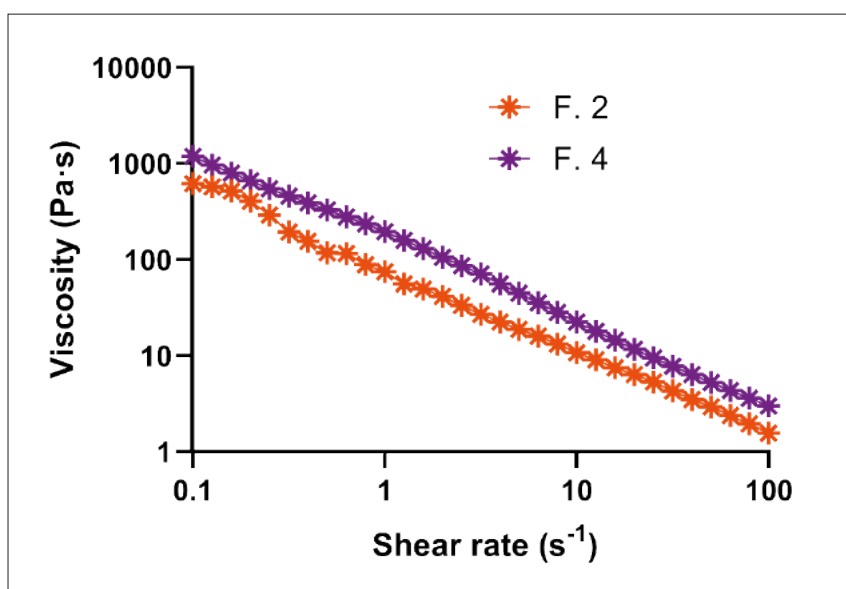
Figure S3. Shear rate sweep assay performed with formulation 2 and formulation 4.

Table S1. Parameter values of the Carreau–Yasuda model for both formulation 2 and formulation 4

Property	Meaning for the 3D printing process	Value for formulation 2	Value for formulation 4
Power-law exponent (n)	It characterizes the shear-thinning behavior. For 3D printing, a shear-thinning material is often desirable as it facilitates extrusion through the nozzle during printing and promotes layer adhesion. $0 < n < 1$: shear thinning $n > 1$: shear thickening $n = 1$: Newtonian fluid	0.14	0.15
Shape parameter (a)	It describes the transition between low and high shear rate behaviors. An appropriate value contributes to a smooth transition in viscosity, avoiding sudden changes during printing.	0.10	0.13
Relaxation time (λ)	It influences how quickly the material responds to changes in shear rate. An optimal λ ensures that the material adapts swiftly to variations in flow conditions during printing.	17.73 s	10.12 s
Zero-rate viscosity (η_0)	It represents the material's viscosity at low shear rates. For 3D printing, a moderate value is often desirable (to ensure that it flows through the printing nozzle but also that it maintains its position once extruded).	1.12×10^{-5} Pa·s	0.18 Pa·s
Infinite-rate viscosity (η_∞)	It represents the viscosity at high shear rates. A lower value is generally preferable for 3D printing, enabling the material to flow easily during the printing process.	1100.62 Pa·s	4039.11 Pa·s
Adjustment of the model (R^2)		0.999833	0.999629

(B) Rheological characterization: parameters

Table S2. Rheological parameters of the developed ink

Property/Parameter [a]	Value	Determined from
Non-Newtonian fluid	Non-linear shear rate/shear stress behavior	Shear rate sweep
Shear thickening	Decrease in viscosity with increasing shear rate	Shear rate sweep
Herschel–Bulkley fluid	Exhibits a non-linear strain rate/shear stress behavior after the yield stress	Shear rate sweep
Zero-rate viscosity	4039.11 Pa·s	Shear rate sweep
Infinite-rate viscosity	0.18 Pa·s	Shear rate sweep
Yield stress (σ_y)	107.48 Pa	Shear rate sweep
Thixotropic behavior	Reversible, time-dependent decrease in viscosity in response to shear rate. 1st cycle: 36.12% recovery after 2 min; 2nd cycle: 24.38% recovery after 2 min	Recovery test
Values in LVER G' G'' G* Tan δ	323.18 Pa 114.27 Pa 342.79 Pa 0.35	Strain sweep
Linearity limit (γ_L)	67.18%	Strain sweep
Critical strain	345.23% (G' = G'' = 107.21 Pa)	Strain sweep
Flow transition index (τf/τy)	2.28	Oscillation stress
Yield point (τ_y)	232.35 Pa	Stress sweep
Flow point or flow stress (τ_f)	528.83 Pa	Stress sweep
Beginning of gel formation (t_{CR})	3 min 30 s	Time sweep
Gel point (t_{SG})	10 min 10 s	Time sweep
G', G'' stabilization	55 min	Time sweep
Viscoelastic liquid and solid-like gel behavior	G' is higher than G'' for all the frequency range; with increasing frequency both modulus increase	Frequency sweep
Melting temperature (T_m)	23.56°C	Temperature sweep
Gelation temperature (T_g)	28.14°C	Temperature sweep

[a] The conditions under which the ink showed these rheological properties are described in the materials and methods section.

Abbreviations: G', storage modulus; G'', loss modulus; G*, complex modulus; LVER, linear viscoelastic region.

(C) Printability and printing fidelity

In this work, four parameters were selected to characterize the printability of the developed ink: the formation of filaments, the level of gelation (under-gelation, optimal gelation, and over-gelation), the shape fidelity, and the strand printability. The four tests and the methodology used to determine the values of the mentioned parameters are schematically shown in Figure S4.

The formation of filaments in the 3D extrusion printing process is presented in Figure S4 (panel 1). When the ink is too gelated, the filaments are more curved and have a "clumpy" appearance due to breakage in the printing process of the gel previously created in the syringe (hyper-gelated filament). When the ink has the proper gelation, the filaments do not present discontinuities or gel fragments and are continuous and uniform (continuous filament). When the ink is under-gelated, no filaments are obtained (no filament-drops). The ink comes out of the syringe needle in the shape of droplets. The second mentioned parameter is the level of gelation (Figure S4, panel 2). The filaments and printed structures can be differentiated in three states: under-gelation, proper gelation, and over-gelation. The third analyzed parameter is the strand printability, and, for that, single filaments are evaluated (Figure S4, panel 3). The diameter of the filaments is measured at different points, and then the media are done. The resulting value is compared with the theoretical diameter of the filament in the CAD design. The parameter is represented as percentage. Finally, the last parameter is the shape fidelity of the printed structures (Figure S4, panel 4). It has different names in literature (ex. filament fusion test or deflection test). A structure

with increasing distances between filaments is printed at different printing parameters. The comparison between the obtained areas and the areas in the design is analyzed. The result of this assay gives information about the filaments as well as the resolution that can be obtained with the ink at the printing conditions.
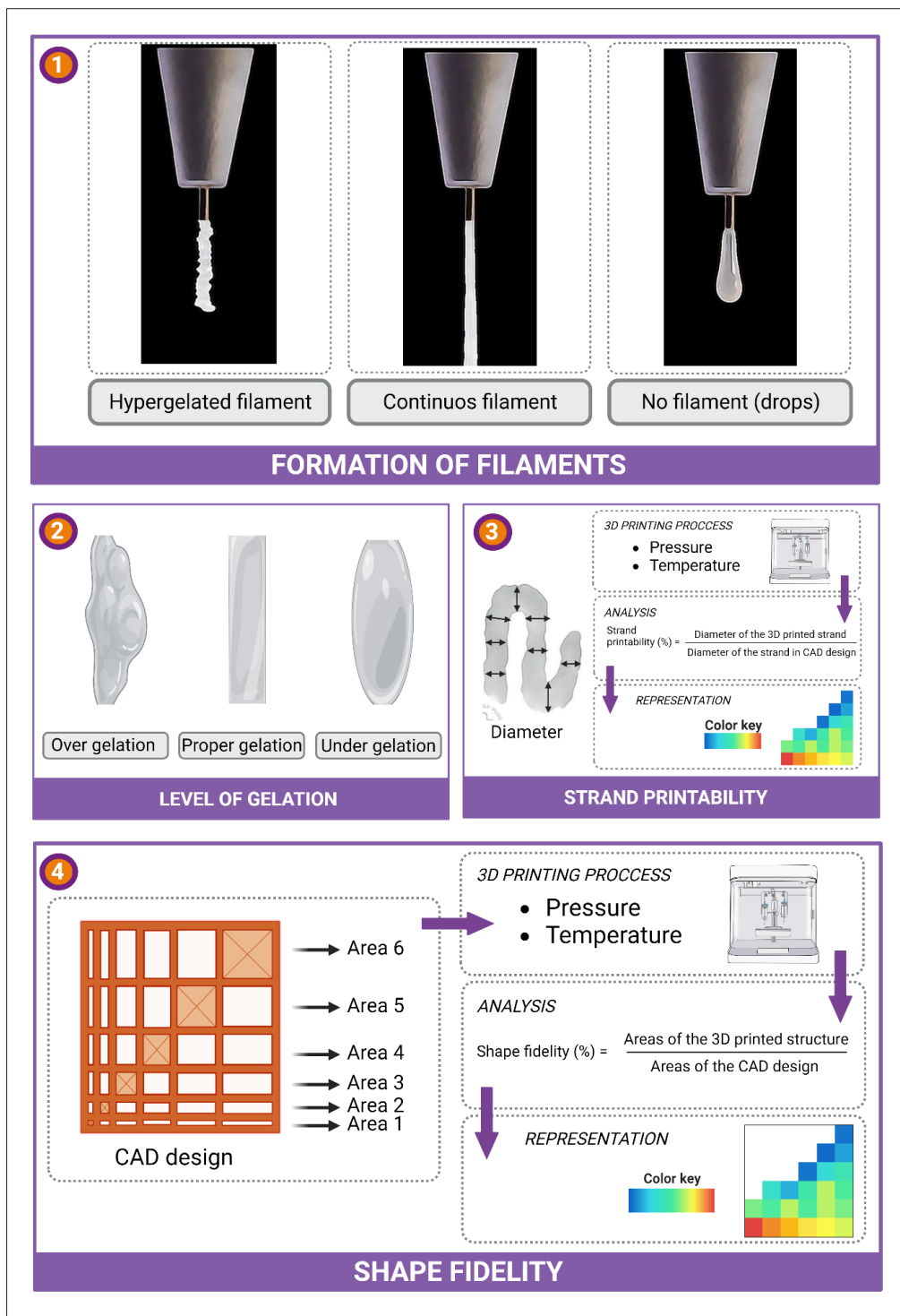

Figure S4. Schematic representation of the printability assays. (1) Analysis of the formation of filaments in the 3D extrusion printing process. (2) Level of gelation observed in the filaments and printed structures. (3) Strand printability. (4) Shape fidelity of the printed structures. Created with Biorender.com

(D) Mechanical properties of the acellular scaffolds and tissue constructs: compressive test

Parameters based on the stress vs. strain curve were analyzed and discussed in the main text. Nevertheless, there are other important parameters that can also be determined from the compressive test. These parameters are the maximum force and the stiffness of the scaffolds and tissue constructs. In this case, instead of using the stress vs. strain curves, the force vs. displacement curves need to be analyzed (Figure S5). On day 1, the maximum force was observed on the structures: 7.67 N for acellular scaffolds and 6.46 N for tissue constructs. The stiffness value was also higher on day 1: 14.36 N mm^{-1} for acellular scaffolds and 9.93 N mm^{-1} for tissue constructs. In the following time points, the structures presented lower mechanical properties. No significant differences were observed between the maximum force of the tissue constructs and the acellular scaffolds. However, statistical differences between tissue constructs and acellular scaffolds were observed in the case of stiffness.
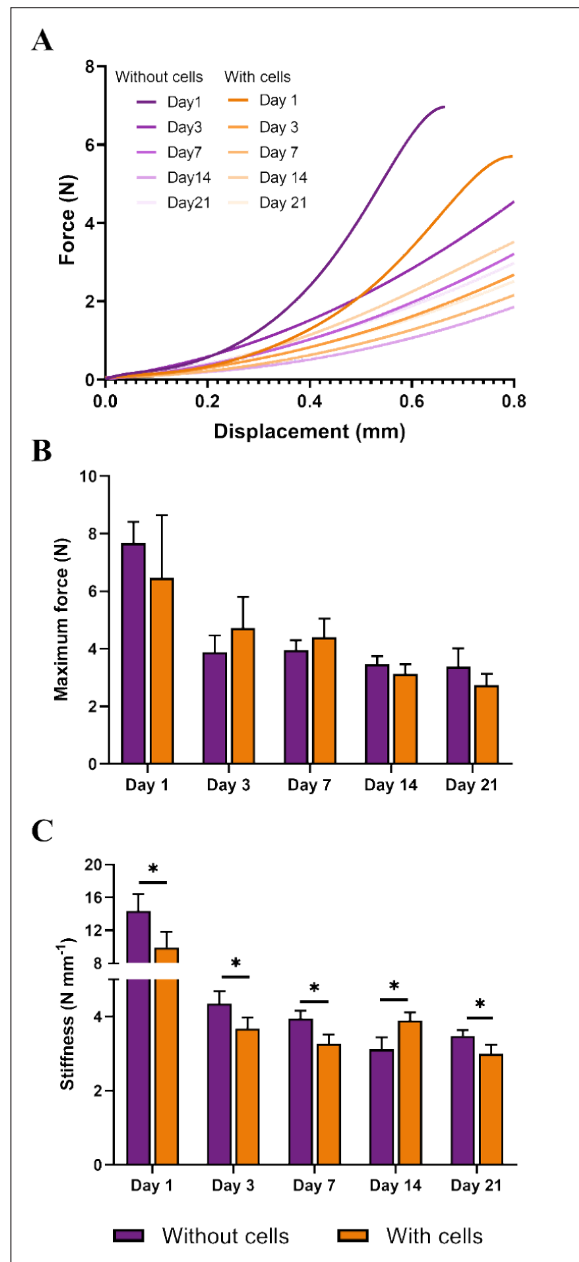

Figure S5. Mechanical characterization of the acellular scaffolds (purple) and tissue constructs (orange). This characterization was performed with a compression test. Results are represented as mean ± SD ($n = 4$). *$p < 0.05$.
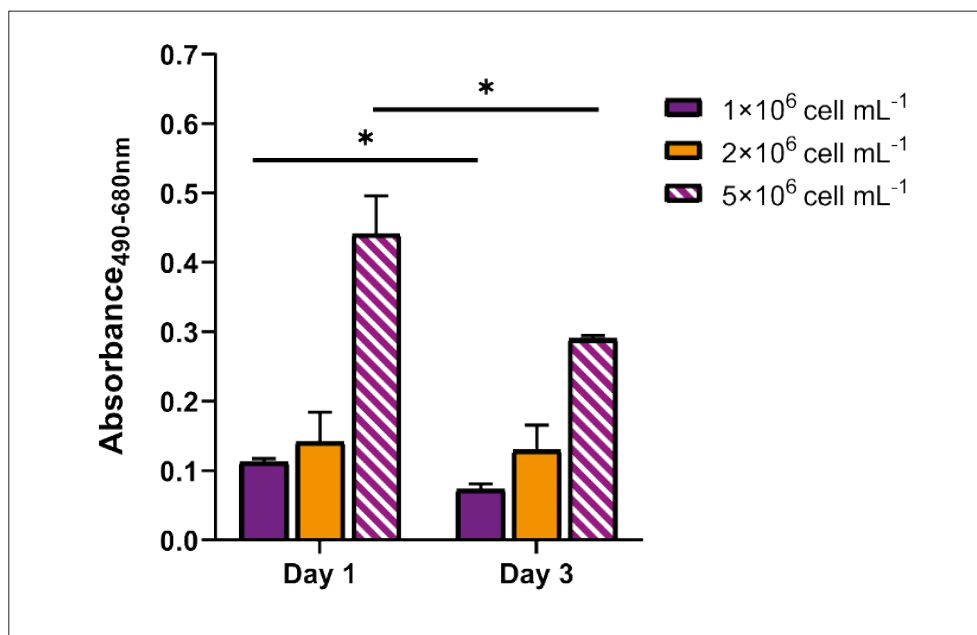
(E) Printing effect on cells (stress)

Figure S6. Lactate dehydrogenase assay. Results are represented as mean ± SD (n = 3). Statistical differences between days are shown. *p < 0.05.

(F) Correlation between the expressions of tendon-related genes

As mentioned in the main text, the studied genes include scleraxis BHLH transcription factor (*Scx*), tenascin C (*Tnc*), collagen type I alpha 1 chain (*Col1a1*), collagen type III alpha 1 chain (*Col3a1*), Fos proto-oncogene (*c-Fos*), and fibronectin 1 (*Fn1*). Although it was not the main focus of this study, analyzing the correlation between the expressions of different genes is a fundamental aspect of molecular biology and genetics research. Spearman statistics were used to analyze the obtained results (Table S2). The analysis revealed that several of the studied genes showed direct correlation in their expression levels (Figure S7): *Col1a1* with *Fn1*, *Tnc* with *c-Fos*, *c-Fos* and *Tnc* with *Col3a1*, and *Scx* with *Col1a1*. Despite not being statistically significant, there also seems to be a certain direct correlation between *Scx* and *Fn1*. It becomes apparent that there are two clusters of genes with related expressions. In the first cluster, the correlation between *Col1a1* and *Fn1* may be related to the participation of fibronectins in the regulation of type I collagen deposition. Both are molecules of the ECM, thus justifying a similar expression profile. Furthermore, the expression of both genes could be positively regulated by the expression of the *Scx* gene, as described in the literature.[1,2] In the second cluster, the relationship between *Tnc* and *c-Fos* may be due to

the fact that the expression of both genes increases in response to mechanical stimuli (in this case, it could be due to the compression they suffer within the scaffold).[3] No information has been found in the literature to offer an explanation on why the expression of *c-Fos* and *Col3a1* are related.

In the context of the correlation between *Tnc* and *Col3a1*, prior studies in the literature have reported that incubating vascular smooth muscle cells[4] and human dermal fibroblasts[5] with Tenascin-C (TNC), a glycoprotein, resulted in the upregulation of *Col3a1* expression. Therefore, in the case of tenocytes embedded in the hydrogel, there could be an upregulation of the expression of type III collagen caused by an increase in the expression of *Tnc*.

References

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Table S2. Values of the Spearman correlation coefficient for the analyzed tendon-related genes

	Scx	*c-Fos*	*Tnc*	*Col1a1*	*Col3a1*	*Fn1*
Scx	-	-0.286	-0.024	0.738	-0.071	0.69
c-Fos	-	-	0.621	0.095	0.721	0.048
Tnc	-	-	-	0.429	0.700	0.476
Col1a1	-	-	-	-	0.333	0.976
Col3a1	-	-	-	-	-	0.286
Fn1	-	-	-	-	-	-

Abbreviations: *Scx,* scleraxis BHLH transcription factor; *c-Fos*, Fos proto-oncogene; *Tnc*, tenascin C; *Col1a1*, collagen type I alpha 1 chain; *Col3a1*, collagen type III alpha 1 chain; *Fn1*, fibronectin 1.
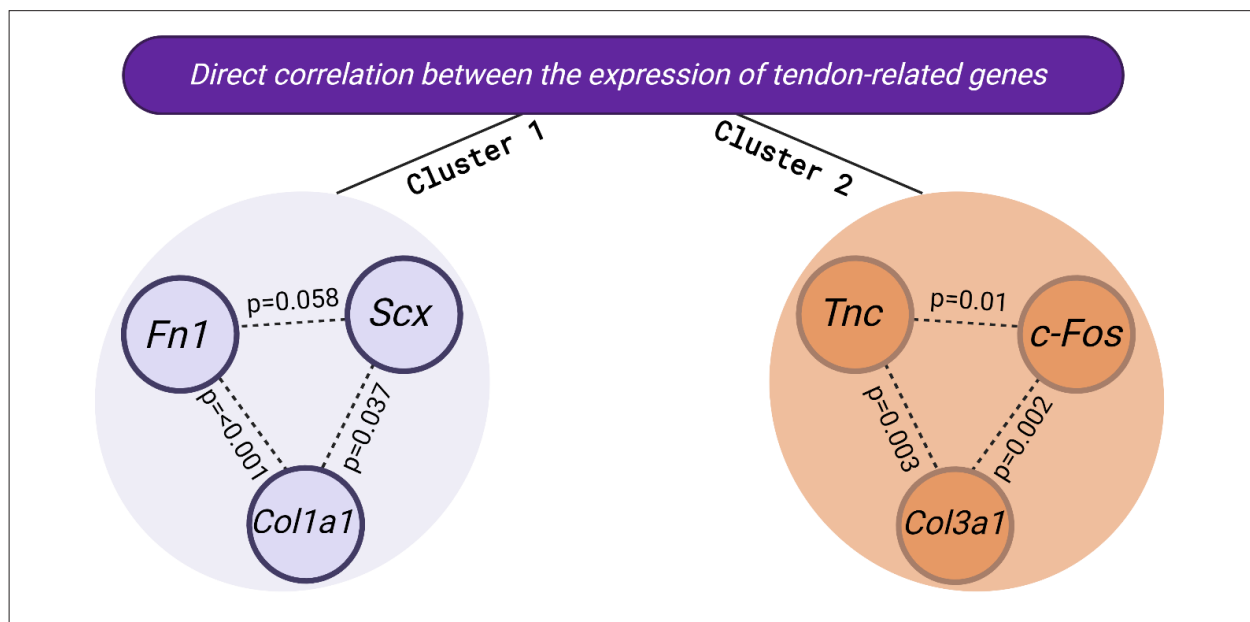

Figure S7. Direct correlation between the expressions of the analyzed tendon-related genes. Spearman statistics were used to analyze the relationship. The *p*-value of the analysis is determined for each pair of genes that showed a statistically significant correlation. Created with Biorender.com. Abbreviations: *Scx*, scleraxis BHLH transcription factor; *c-Fos*, Fos proto-oncogene; *Tnc*, tenascin C; *Col1a1*, collagen type I alpha 1 chain; *Col3a1*, collagen type III alpha 1 chain; *Fn1*, fibronectin 1.