

SUPPLEMENTARY INFORMATION

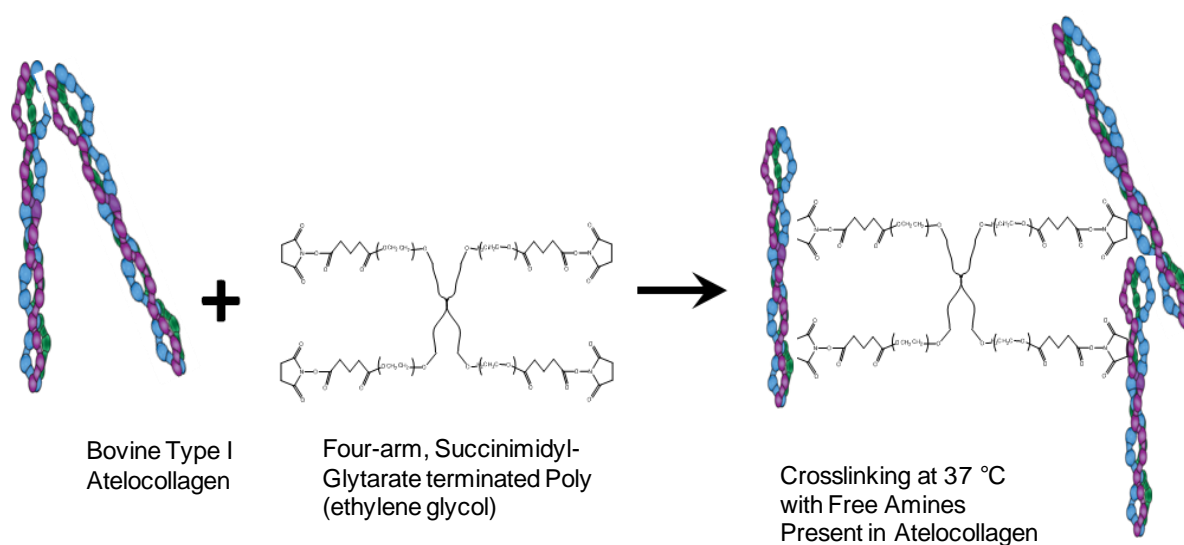


Figure S1. Type I atelocollagen and 4S-StarPEG reaction. Succinimidyl glutarate is an NHS-ester which binds with amine groups and therefore the succinimidyl groups react with the amine groups present on the molecules of type I atelocollagen at 37 °C.

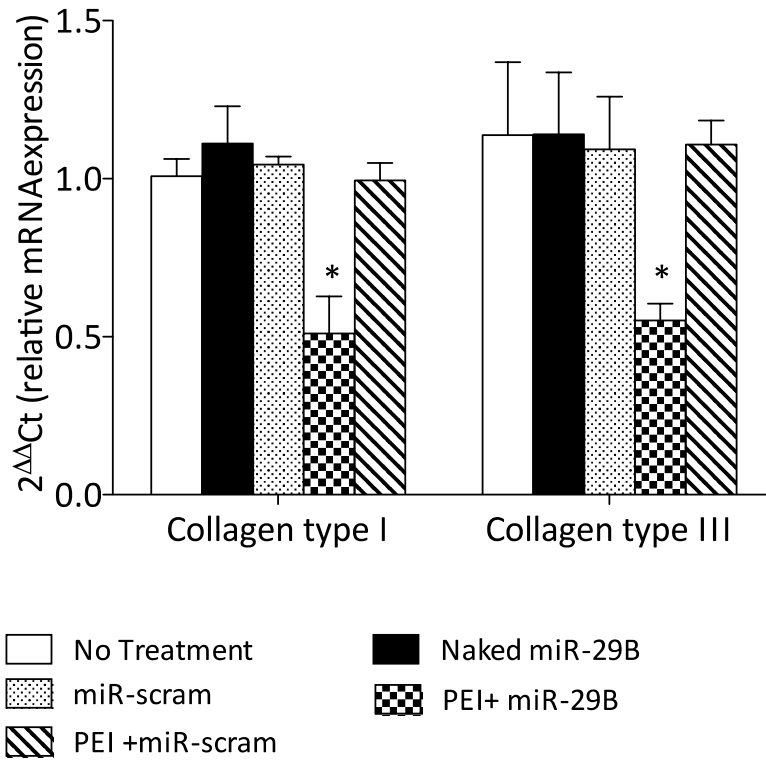
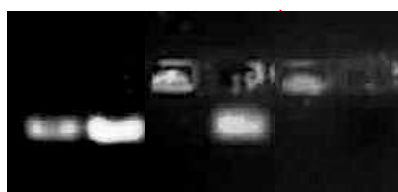


Figure S2. qRT-PCR data demonstrating the effect of miR-29B in silencing collagen type I and type III mRNA *in vitro* in rat cardiac fibroblasts compared to scrambled control 48 hours after delivery of miR mimics. * indicates a statistically significant difference compared to the other treatments, $p < 0.05$. Data is presented as the mean \pm SD, $n = 3$.

Table S1: Rheological evaluation of crosslinked scaffolds.

	0.5 mM	1 mM	2 mM	GTA
Gelation Time	18 ± 9	7 ± 1.1	8 ± 1.3	Instant*
Storage Modulus	201 ± 165.1	260 ± 81.7	192 ± 27.8	4934 ± 518*
Storage Modulus (15 Days)	45 ± 28.6#	307 ± 68.4	402 ± 257.7	4014 ± 223.2*

Data presented is n = 3, mean ± standard deviation. * indicates a statistically significant difference when using GTA compared to crosslinking with 4S-StarPEG, p < 0.05. # indicates a statistically significant difference in storage modulus at 15 days when using 0.5 mM of 4S-StarPEG when compared to the other groups.



RNA	+	+	+	+	-	-
Collagen	-	-	+	+	+	+
Crosslinked	-	-	-	+	-	+

Figure S3. Agarose gel electrophoresis to indicate the binding of RNA complexes to the collagen 4S-StarPEG scaffold. The components in each lane are indicated by the grid below the picture of the gel. Bands towards the bottom of a lane indicates that the RNA has a net negative charge and is migrating towards the anode (-) whereas bands still within the loading lane or migrating upwards have a net positive charge and are migrating towards the cathode (+). Naked RNA migrates towards the anode. A non-crosslinked scaffold offers the naked RNA a positive charge whereas a crosslinked scaffold minimises the interaction of the scaffold with naked RNA.

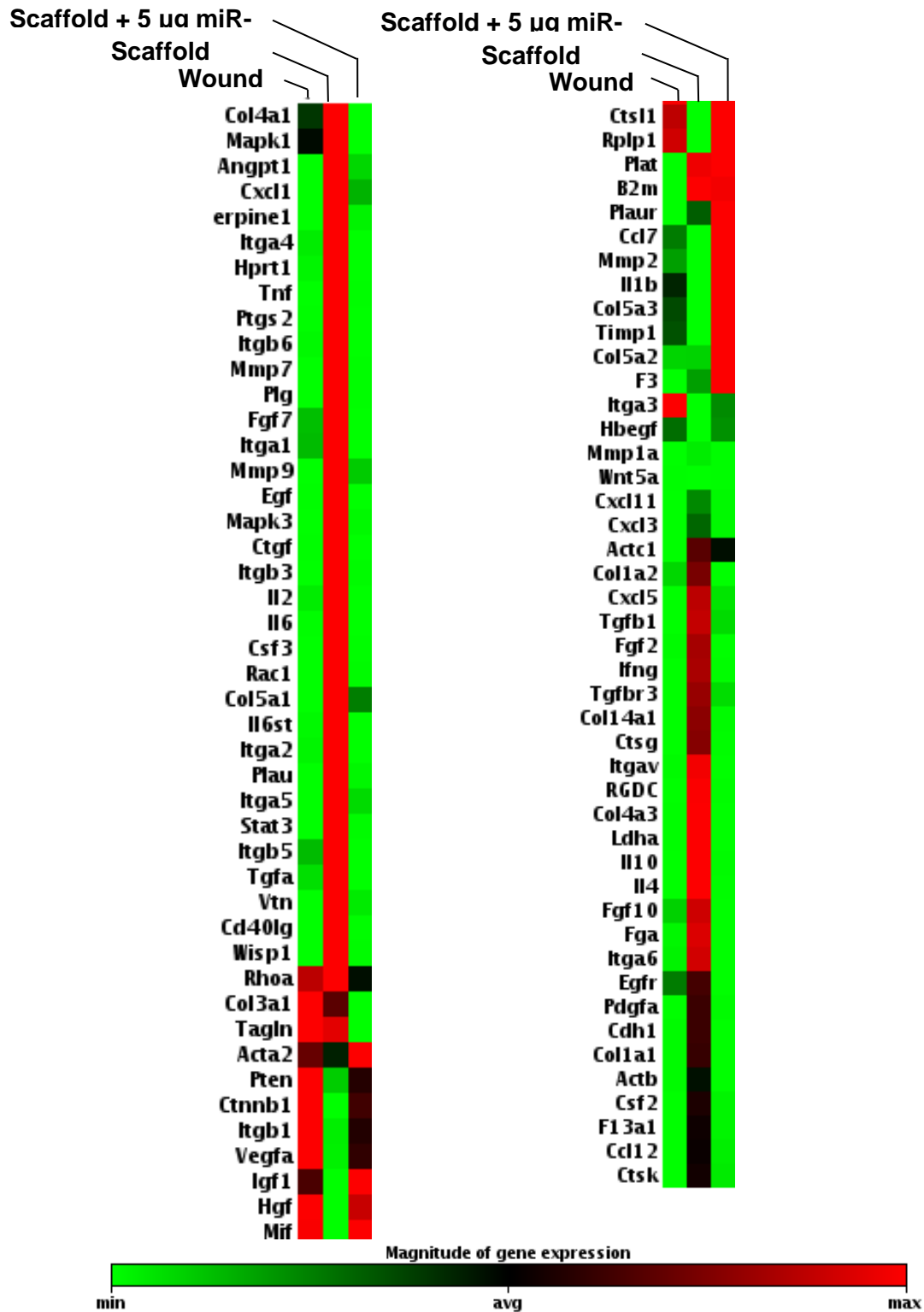


Figure S4: An example heat map of gene expression data obtained from wound healing RT-PCR array. Data presented is the upregulated (red) and downregulated (green) genes after 28 days when compared with healthy skin. Treatments analysed include No Treatment (wound only) and treatment of wounds with a 1 mm 4S-StarPEG crosslinked collagen scaffold alone, or a 1 mm 4S-StarPEG crosslinked collagen scaffold with 5 µg of miR-29B.

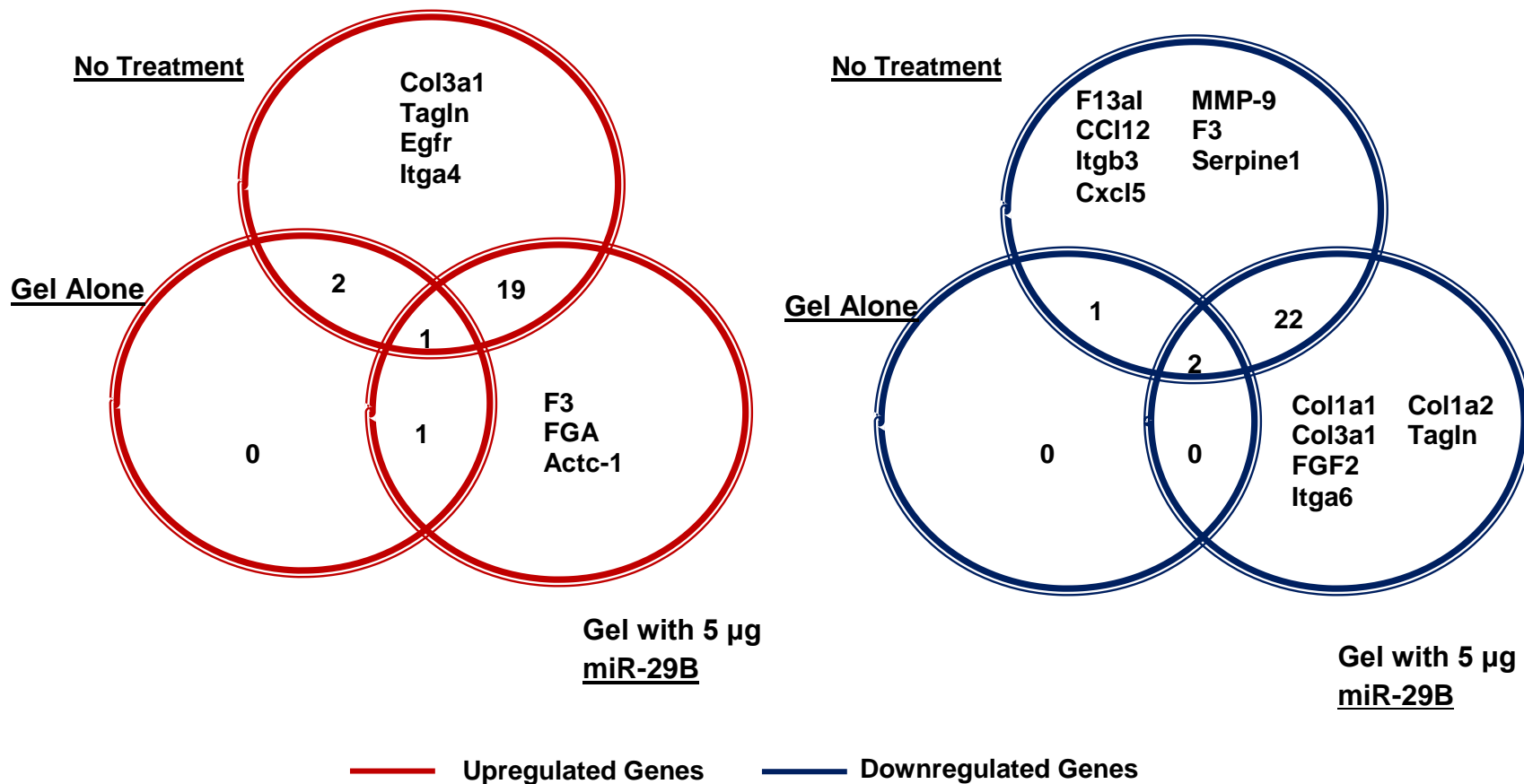


Figure S5: Overview of number of genes altered in the different comparisons studied in the PCR array analysis with fold change cut off being five. Data presented is the upregulated (red) and downregulated (blue) genes after 28 days when compared with healthy skin. Treatments analysed include No Treatment (wound only) and treatment of wounds with a 1 mm 4S-StarPEG crosslinked collagen scaffold alone, or a 1 mm 4S-StarPEG crosslinked collagen scaffold with 5 µg of miR-29B.

Supplementary Discussion

Rheological studies were carried out to estimate the gel time of the scaffolds and was determined to be the time when G' equaled G'' . No statistical difference in gelation time ($p < 0.05$) was detected between samples after varying crosslinker concentrations (**Table S1**). The optimal crosslinking concentration (1 mM) had a mean gelation time of 6.67 minutes. Similarly, no statistically significant difference between the storage moduli of the gels was detected and the average storage modulus of the 1 mM crosslinked scaffold was 192 Pa.

To remove any ambiguity regarding the specificity of miR-29B to silence collagen type I and collagen type III, RT-PCR was performed on cardiac fibroblasts treated with miR-29B and non-targeting, scrambled controls (**Fig. S2**). As expected, the scrambled control did not produce any significant silencing of collagen type I and collagen type III mRNA which eliminates the possibility of global, non-specific silencing due to simply using double stranded RNA. While it is true there are perhaps targets of miR-29B that are not validated here and have yet to be discovered, there are no noticeable effects on cellular viability or proliferation detected in response to miR-29B specifically.

The use of miR-29B in a naked unprotected form did not exhibit effective silencing of collagen type I or collagen type III mRNA (1.11 and 1.14 relative expression, $2\Delta\Delta Ct$) which suggests the requirement for a complexing agent when performing miR delivery to cells in 2D culture. However, complexation of miR-29B with PEI did show effective silencing of collagen type I and type III mRNA expression (0.51 and 0.55 relative expression, $2\Delta\Delta Ct$ respectively), However, although PEI is an effective carrier of nucleic acid to cells, it poses many drawbacks among which are that it is toxic to cells and impairs cellular viability, important considerations if any therapy is to be clinically translatable.

miR-29B has been previously reported to silence collagen type I expression *in vitro* and in all studies reported the employment of a complexing agent (in the majority of cases commercial agents such as Lipofectamine™ 2000 have enabled this silencing) as it is highly unlikely that the administration of suspended miRs towards will cells be effective *in vitro* at the small doses employed [12-14]. Furthermore, commercial agents are primarily intended for diagnostics and research purposes since their compromised toxicity limits their therapeutic potential.

Supplementary Materials and Methods

Materials

All solvents were of analytical or HPLC grade and were obtained from Sigma Aldrich Chemical Company (Ireland) unless otherwise stated. All oligonucleotides and primers were purchased from Eurofins MWG GmbH Ebersberg, Germany. Four- star poly (ethylene glycol) succinimidyl glutarate, $M_w = 10,000$ (4S-StarPEG) was purchased from JenKem Technology Co. Ltd., Texas, USA.

Collagen Gel Preparation

Atelocollagen was isolated as described elsewhere [16]. Nine parts of collagen solution (3.5 mg/ml w/v) was gently and thoroughly mixed with one part 10 x phosphate buffered saline (PBS). The solution was neutralized by the drop-wise addition of 2 M sodium hydroxide (NaOH) until a final pH of 7–7.5 was reached and kept in an ice bath to delay gel formation. 4S-StarPEG was then added to final concentrations of 0.125 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM but always in a volume of 50 μ l. 0.625% glutaraldehyde (GTA) was used as a positive control. The solutions were incubated for 1 hour at 37 °C in a humidified atmosphere to induce gelation.

Rheological Evaluation

In order to identify the gel time of the hydrogel as a function of cross-linking, rheological measurements were performed at 37 °C using a Haake Modular Advanced Rheometer

System™ (MARS) rheometer (Thermo Haakes, Germany) as described before [20]. Briefly, type I atelocollagen, 10 x PBS and 1 M NaOH alone, with 0.625% GTA or with different concentrations of 4S-StarPEG (0.5 mM, 1 mM and 2 mM), were added to the plate at 37 °C. The rheometer was equipped with a circulating water bath to accurately control the temperature. To minimize the influence of water loss on mechanical behavior, samples were coated with paraffin oil. Dynamic frequency sweep experiments were carried out to determine the storage (G') and loss (G'') *moduli* as a function of time at 37 °C. The measurements of the storage (G') and loss (G'') *moduli* during the gelation were recorded as a function of time for five different frequencies (a, b, c, d and e rad/s) using multi-wave facilities. The gel point was defined as the time that G' equaled G'' .

Evaluating Matrix Binding of RNA

To further understand the mechanism of how RNA is held within the scaffold, the electrophoretic kinetics of RNA complexes within the scaffold were evaluated using agarose gel electrophoresis. 1 % (w/v) agarose was heated in tris-acetic acid-ethylenediaminetetraacetic acid (EDTA) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA respectively) and a 10,000 fold dilution of Syber® Safe dye was added prior to gel casting. Non-crosslinked atelocollagen scaffolds or scaffolds crosslinked with 4S-StarPEG, to a volume of 10 μ l, were deposited into the running wells of this gel with 330 ng of naked RNA or PEI-complexed RNA. Naked RNA and scaffolds containing no RNA were applied as controls. Additionally, naked RNA mixed with an indicative loading buffer was run to monitor the migration of the RNA on the gel. However, this was not applied to the other samples to avoid any interference with the kinetics of the scaffolds. A voltage of 50 V was applied to the agarose gel and the current was set to 'auto'. After 20 minutes, the loading buffer indicated sufficient migration to observe the electrophoretic mobility of the RNA under UV light.

Cell Extraction

Primary rat cardiac fibroblasts were isolated from neonatal pups (day 3-5) as previously described [21]. Briefly, neonatal rat ventricle myocytes were isolated from the cardiac ventricles of 3–5 days old Sprague-Dawley pups. Hearts were removed from the thoracic cavity and placed in a tube containing cold (4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid) HEPES solution (20 mM HEPES, pH 7.4). Ventricles were separated from other tissue using scissors and minced into several pieces. Subsequently, cardiomyocytes and fibroblasts were detached from the extracellular matrix by repeated incubation in collagenase, supplemented with 2 mg/ml trypsin and 20 µg/ml DNase. Cells were collected by centrifugation and tissue clumps were removed by filtration. Following this, cells were pre-plated in cell culture dishes in 50 ml Dulbecco's Modified Eagle Medium (DMEM) /F12 (50:50) medium with 5% fetal bovine serum (FBS) for 45 minutes. During this period, most non-cardiomyocyte cells (mainly fibroblasts) attached to the dish, whereas cardiomyocytes remained in solution. Fibroblasts were subsequently cultured in DMEM/F12 medium containing 10% FBS.

In Vitro Silencing

Primary rat cardiac fibroblasts were seeded on a 6-well plate at a density of 1×10^6 cells per well. After one day incubation at 37 °C, 5% CO₂ to ensure adherence and acclimatization, miR-29B or miR-scram, uncomplexed or complexed with PEI, was added to each well in a total volume of 250 µl with a concentration of 0.5 µg of miR. After ten minutes, the total volume of supernatant in the wells was brought to 1 ml with the addition of DMEM/F12 containing FBS at a concentration of 5%. The experiment was maintained for 48 hours following the addition of this media.

RNA Extraction

One mL of TRI Reagent® (Applera Ireland, Dublin, Ireland) was added to each well and incubated for five minutes at room temperature. Phase separation was performed by adding chloroform (Sigma-Aldrich), and total RNA was purified using an RNeasy™ kit (Qiagen), according to the supplier's recommended procedure.

Real-time Reverse Transcription Polymerase Chain Reaction

Total RNA quantity and purity were determined using spectrophotometry at 260 and 280 nm using an ultraviolet spectrophotometer (NanoDrop™ ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was checked electrophoretically using the RNA 6000 Nano LabChip™ kit with an Agilent Bioanalyser 2100

(Agilent Technologies, Cork, Ireland). Reverse transcription (RT) was performed using the ImProm-II™ RT system according to the manufacturer’s protocol (Promega, Southampton, UK). Gene transcription was examined using real-time RT polymerase chain reaction (PCR). Reactions were performed and monitored using an ABI 7000® sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan® Real-time Gene Expression Mastermix (TaqMan, Applied Biosystems) and specific primers which are detailed in Table S2. The primers were designed and their specificity checked using primer-BLAST (www.ncbi.com) and their efficiency determined by RT-PCR on ten-fold serial dilutions of template cDNA. Gene transcription was inferred from calibration samples and normalized in relation to transcription of the housekeeping gene; glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The $2^{\Delta\Delta-Ct}$ method was used to calculate relative gene expression for each target gene.

Table S2. Sequences of primers used in RT-PCR

Primer	Forward (5`→3`)	Reverse (5`→3`)
<i>Collagen 1A1</i>	AACAAATCCCCACACACAC	ACACACAAAGACAAGAACGAG
<i>Collagen 3A1</i>	GCCTCCCAGAACATTACATACC	ACTGTCTTGCTCCATTACC
<i>GAPDH</i>	AAGAAGGTGGTGAAGCAGG	CAAAGGTGGAAGAATGGGAG

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