

TENDON HEALING INDUCED BY CHEMICALLY MODIFIED MRNAS

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Abstract

Tendon disorders are frequent both in human and veterinary medicine with high re-injury rates and unsatisfactory therapeutic treatments. Application of naked, chemically-modified mRNA (cmRNA), encoding for therapeutic proteins, is an innovative approach to address tendon healing. In the current study, we demonstrated that injection of naked cmRNA, diluted in a glucose-containing solution, into tendons resulted in high protein expression in healthy and experimentally-injured tendons. Injection of bone morphogenetic protein 7 (BMP-7)-encoding cmRNA resulted in a significantly higher expression of BMP-7 protein and reduced formation of collagen type III, compared to vehicle control. Moreover, in a large animal model, reporter protein expression was detectable not only in healthy, but also in experimentally-injured, severely inflamed tendons. Summarising, these results demonstrated the potential of cmRNAs encoding for therapeutic proteins as a new class of drugs for the treatment of tendon disorders.

Keywords: mRNA, tendinopathy, tendon rupture, gene therapy, regenerative medicine, sheep.

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Introduction

Tendinopathies are common in animals and humans, especially in athletes (Kvist, 1994). For example, rotator cuff and common calcaneal tendons tendinopathies are frequently diagnosed among humans (Herrmann *et al.*, 2014; Järvinen *et al.*, 2005). Underlying medical reasons are multisided and predisposal factors, such as overloading due to training or joint malposition, age, gender, genetics, body weight or endocrinopathies, are similar in humans and animals (Baird *et al.*, 2014; Ippolito *et al.*, 1975; Magnan *et al.*, 2014; Patterson-Kane and Rich, 2014; Perkins *et al.*, 2005). In numerous cases, subclinical chronic damage, caused by overuse or degenerative processes, leads to

an acute injury or even tendon rupture, accompanied by characteristic clinical symptoms (Järvinen *et al.*, 1997). Besides degenerative processes, failed healing response and inflammation have also been proposed as causative agents for tendinopathies (Del Buono *et al.*, 2011; Maffulli *et al.*, 2010). Millar *et al.* (2015) demonstrated that, in human and experimentally injured tendons, elevated IL-33 expression is a characteristic of early tendinopathy and identified miRNA29a as key regulator of IL-33 function and excessive collagen type III synthesis. Their data provide a molecular mechanism of microRNA-mediated integration of the early pathophysiologic events that facilitate tissue remodelling in human tendon after injury.

The natural healing potential of tendon tissue is low due to hypocellularity, hypovascularity and a low metabolic rate compared to other soft tissues (Bray *et al.*, 1996; Liu *et al.*, 2011; Sharma and Maffulli, 2005a). Therefore, the healing process is slow (James *et al.*, 2008) and takes months or even years. Moreover, the healing process normally results only in tissue repair instead of regeneration and the original tensile strength and elasticity are usually not regained (Sharma and Maffulli, 2005b). As a consequence, recovery is rarely fully functional (Hogan *et al.*, 2011; Sharma and Maffulli, 2005a) and the risk of re-injury or even tendon rupture is greatly increased (Mast, 1997).

Treatment methods can be categorised into conservative and surgical approaches. In cases of acute injury, besides rest and bandaging followed by controlled exercise, conservative therapy methods can also be intra-lesion application of various substances, such as corticosteroids (Hart, 2011; Muto *et al.*, 2014), hyaluronic acid (Foland *et al.*, 1992; Muneta *et al.*, 2012) or glycosaminoglycans (Moraes *et al.*, 2009). Furthermore, physical methods, such as electromagnetic stimulation or shockwave therapy (Bosch *et al.*, 2010; Seeliger *et al.*, 2014) are also used. Also, various approaches using stem cell-based (Martinello *et al.*, 2013; Renzi *et al.*, 2013; Smith, 2008) and growth factor-based therapies have been investigated (Arguelles *et al.*, 2008; Shah *et al.*, 2013; Witte *et al.*, 2011).

The assumption that cytokines and growth factors play a key role during the healing process – namely that they stimulate cell proliferation as well as cell differentiation and formation of extracellular matrix components (Evans, 2014; Grotendorst, 1988; Molloy *et al.*, 2003) – generated considerable interest in growth-factor based gene therapy. Various *ex vivo* and *in vivo* experiments provided promising results, such as improved tendon healing in a rat Achilles tendon model after bone morphogenetic protein (BMP)-12 gene transfer (Majewski *et al.*, 2008) or a positive influence

of platelet-rich plasma on core lesions of superficial digital flexor tendon (SDFT) in horses (Bosch *et al.*, 2010). In recent years, in the field of growth factor-based therapies, several studies have been focused on DNA gene transfer (Evans, 2014; Lou *et al.*, 2001; Majewski *et al.*, 2008; Nakamura *et al.*, 1998). However, the results are still not highly efficient, due to limitations of nuclear entry in non-dividing cells (Tong *et al.*, 2009), and in addition gene transfer carries the potential risk of mutagenesis. On the other hand, messenger RNA has been successfully applied in several mouse models. Kormann *et al.* (2011) reported increased haematocrit levels after single intramuscular injection of modified murine erythropoietin-encoding mRNA. In addition, in a congenital mouse disease model of surfactant protein B deficiency, aerosol application of surfactant protein B-encoding mRNA reconditioned large parts of the lung. In the present study, we focused on application of chemically modified naked mRNA (cmRNA) in intact, as well as, surgically and chemically injured tendons.

Materials and Methods

All mRNAs used in the current study were produced by Ethris GmbH (Planegg, Germany), using proprietary SNIM® RNA (Stabilised Non-Immunogenic mRNA) technology.

Ex vivo transfection, cultivation and bioluminescence imaging

Deep digital flexor tendons (DDFT), derived from horses, cattle, sheep or pigs, and common calcaneal tendons, derived from rats, were harvested within 1 h after animals were euthanised (horses, sheep and rats) or slaughtered (pigs and cattle) and rinsed twice in ice cold Dulbecco's phosphate-buffered saline (PBS) (Life Technologies GmbH, Darmstadt, Germany) containing 1 % penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). Modified naked mRNAs, encoding for luciferase (cmRNA^{LUC}), β -galactosidase (cmRNA^{LacZ}) and human bone morphogenetic protein 7 (cmRNA^{hBMP-7}), were diluted in different solvents and at different concentrations. Injection of cmRNA was performed in parallel to tendon fibre orientation using 23-G needles (Sterican, B. Braun Melsungen AG, Melsungen, Germany) and 1 mL Injekt®-F syringes (B. Braun Melsungen AG) for major tendons and 30-G insulin syringes (BD Micro-Fine, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for rat tendons. cmRNA^{LUC} was diluted in different solvents and prepared using commercially available solutions and standard laboratory chemicals (Table 1). HEPES-buffered glucose (HBG) (Sigma-Aldrich Chemicals, Schnelldorf, Germany) was adjusted to pH 7.4 and prepared in four different concentrations, namely 2.5 %, 3.75 %, 5 % and 10 %. cmRNA^{LacZ} and cmRNA^{BMP-7} were prepared in saline solution only. Complexed cmRNA^{LUC} was prepared using DreamFect^{FM} Gold (OZ Biosciences, Marseille, France) as recommended by the manufacturer (10 μ g cmRNA^{LUC}/40 μ L DreamFect^{FM} Gold) and using branched polyethyleneimine (brPEI) 25 kDa (Sigma-Aldrich,

Schnelldorf, Germany) at an N/P ratio of 10 using 25 μ g cmRNA^{LUC}.

Ex vivo transfected tendons were incubated at 37 °C and 5 % CO₂ in 50 mL DMEM (1 \times) + GlutaMAX medium (Life Technologies, Darmstadt, Germany) containing 1 % penicillin/streptomycin (PAA Laboratories GmbH), 2 % nystatin (Sigma-Aldrich Chemicals) and 10 % FBS (Life Technologies).

Bioluminescence imaging (BLI) was conducted 1, 2 and 3 d after *ex vivo* transfection, using a Xenogen IVIS® Spectrum *in vivo* imaging system (Caliper Life Sciences, Waltham, MA, USA). D-Luciferin substrate (S039, SYNCHEM, Felsberg/Altenburg, Germany), diluted in Dulbecco's PBS (1 \times), was added to the cell culture flasks (100 μ g D-Luciferin/mL medium) 1 h prior measurement of luciferase activity. Living Image® 2.50 software (Caliper Life Sciences) was used for data analysis.

Histological processing of *ex vivo* cultivated tendon explants

β -galactosidase staining was performed 1 d after *ex vivo* transfection, according to Dai *et al.* (2003). Tendons were fixed on ice for 30 min in 0.5 % glutaraldehyde (Carl Roth, Karlsruhe, Germany) and 2 mM magnesium chloride hexahydrate (Carl Roth) in PBS and adjusted to pH 7.4. Subsequently, they were washed twice for 30 min at room temperature in rinsing buffer [100 mM HEPES, 5 mM DTT (Dithiothreitol, Sigma-Aldrich Chemicals), 1 mM MgSO₄ (Magnesium sulphate heptahydrate, Carl Roth), 2 % Triton X-100 (Carl Roth), pH 8.0] and then rinsed for 1 h at 50 °C in the same buffer. Next, tendons were incubated overnight in a non-CO₂ incubator in X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside) staining solution [1 mg/mL 5-Bromo-4-Chloro-3-indolyl β (Sigma-Aldrich Chemicals), 2 mM MgCl₂, 5 mM K₃Fe(CN)₆ (Potassium ferricyanide (III), Carl Roth), 5 mM K₄Fe(CN)₆ (Potassium hexacyanoferrate(II) trihydrate, Carl Roth) in PBS pH 7.4]. Finally, tendons were fixed in 4 % buffered formaldehyde solution (Roti Histofix, Carl Roth), dehydrated, embedded in paraffin and stained with haematoxylin and eosin.

For hBMP-7 immunohistochemistry (IHC), tendons were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek, Staufen, Germany), snap-frozen in liquid nitrogen and stored at -80 °C. Cryosections were prepared and immunohistochemistry was performed by an external laboratory (Sophistolab AG, Muttenz, Switzerland) using a rabbit polyclonal hBMP-7 antibody (ab56023, abcam, Cambridge, UK) as primary antibody.

In vivo studies in intact and injured rat common calcaneal tendons

All experiments were approved by the local ethics committee for animal experiments and adhered to the international guidelines for the care and treatment of laboratory animals. Female Sprague Dawley rats (4 months of age, bodyweight 300-340 g, Janvier Labs, Saint-Berthevin, France) were group-housed under a 12 h light/dark cycle, with food and water *ad libitum*.

To determine luciferase reporter protein expression in intact rat common calcaneal tendons, animals were anaesthetised by isoflurane inhalation using air and O₂

as carrier gases (3-5 vol % isoflurane for induction in an inhalation chamber, followed by 1.5-2.5 vol % for maintenance using an inhalation mask). Different doses of cmRNA^{LUC} (0, 10, 20 and 40 µg; dose volume 10 µL) were injected into the right common calcaneal tendon ($n = 4$ rats/dose group). At day 1, 2 and 7 post cmRNA^{LUC} injection, *in vivo* bioluminescence imaging was performed using PhotonIMAGERTM (Biospace Lab, Nesles-la-Vallée, France). BLI was conducted 15 min after that D-Luciferin substrate (L-8220, dissolved in saline; Biosynth, Staad, Switzerland) was administered intraperitoneally (150 mg/kg, 5 mL/kg). Images were acquired using 10 min as integration time. No filter was used. Quantification was performed using a region of interest defined manually (common calcaneal tendon) and the results were expressed as total counts. M3Vision software (Biospace Lab) was used for data analysis. At day 7, rats were sacrificed using a CO₂ chamber, tendons were explanted immediately and luciferase activity was measured again in tendon explants.

Therapeutic cmRNA^{hBMP-7} was applied directly into injured, *i.e.* dissected and surgically repaired, common calcaneal tendons to investigate BMP-7 influence on

early tendon healing. Prior to surgery, rats received a subcutaneous injection of 0.05 mg/kg of buprenorphine (Temgesic[®], Reckitt Benckiser, Wallisellen, Switzerland). Surgery was performed under inhalation anaesthesia, as described above. The left leg was prepared for aseptic surgery and then fixed into a custom-made holder to stabilise the ankle into a 90° position, with the foot facing downwards. A skin incision was made along the longitudinal axis, on the plantar side of the right lower hind limb. Common calcaneal tendon was exposed and fully dissected along the transversal axis, in the mid-portion between the calcaneus and the insertion of the triceps surae muscle. An end-to-end anastomosis of the tendon stump was performed applying a three-loop pulley pattern suture using a non-absorbable monofilament (Prolene[®] 5-0, 3/8 reverse cutting needle P3, Ethicon, Johnson & Johnson Medical, Norderstedt, Germany). Subsequently, either cmRNA^{hBMP-7} (100 µg) or vehicle were administered into the tendons, proximal and distal to the anastomosis, with a dose volume of 5 µL per injection site (30-G insulin syringes, BD Micro-Fine). Next, the subcutaneous layer and the skin were closed by a continuous suture technique

Table 1. Solutions used for cmRNA dilution. A selection of primary ingredients is listed. ¹ commercially available, ² standard chemical solutions made in house, HEPES: 2-(4-(2-hydroxyethyl)-1-piperazinethansulfonic acid.

Description	Label	Primary Ingredients	Final concentration of ingredient in tested vehicle solution
Nitrogen compound containing	Ammonium acetate solution ¹	Ammonium acetate	3.8 %
	Aminosteril plus ¹	Total aminoacids 100 g/L, K, Ca, Mg, Cl, phosphate, acetate	7.5 %
	Aminoven 15 % ¹	Total aminoacids 150 g/L	11.25 %
Colloidal solutions	HAES steril 10 % ¹	Hydroxyethyl starch 100 g/L, Na, Cl	7.5 %
	Gelafundin 4 % ¹	Gelatine polysuccinate 40 g/L, Na, C	3 %
Glucose containing	HEPES buffered glucose 2.5 % ²	12.5 mM HEPES, glucose 25 g/L	2.5 %
	HEPES buffered glucose 3.75 % ²	18.75 mM HEPES, glucose 37.5 g/L	3.75 %
	HEPES buffered glucose 5 % ²	25 mM HEPES, glucose 50 g/L	5 %
	HEPES buffered glucose 10 % ²	50 mM HEPES, Glucose 100 g/L	10 %
Electrolyte containing	Isotonic sodium chloride ²	Glucose, Na, Cl	0.9 %
	Ringer acetate ¹	Na, K, Ca, Mg, Cl, acetate	0.75 %
	Ringer lactate ¹	Na, K, Ca, Cl, lactate	0.75 %
Electrolyte and glucose containing	Isotonic sodium chloride containing 5 % glucose ²	Glucose 50 g/L, Na, Cl	0.9 % (saline) 5 % (glucose)

with an interrupted intra cutaneous suture using an absorbable synthetic thread (Safil® 6-0, B. Braun). Then, animals were allowed to wake up on a heating pad to aid the return to normal body temperature. Buprenorphine was administered every 8-12 h post-surgery for up to 2 d.

At day 1, 2 and 7 post-surgery, cmRNA^{hBMP-7}-treated and vehicle-treated rats were sacrificed with CO₂ and the injured and contralateral intact common calcaneal tendons were explanted. Tendons were fixed overnight in 4 % paraformaldehyde (Sigma-Aldrich Chemicals, Buchs, Switzerland), dehydrated with an ascending ethanol series and embedded in paraffin (Paraplast Xtra, Leica, Wetzlar, Germany). For immunohistochemistry, anti-BMP-7 antibody (ab56023, abcam), polyclonal anti-collagen I antibody (ab 34710, abcam) and polyclonal anti-collagen III antibody (ab7778, abcam) were used as primary antibodies. Biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories, Peterborough, UK) was used as secondary antibody. Staining was induced by peroxidase (biotin-labelled) activity. Sections were counterstained with haematoxylin (VWR International, Dietikon, Switzerland). In microscopy images, 3,3'-diaminobenzidine (DAB) staining was used as threshold and mean grey values of positive pixels were evaluated. Analysis was performed with Fiji/Image J Version 1.49k (National Institutes of Health, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>).

***In vivo* studies in an ovine model of acute tendon injury**

All experiments were approved by the local ethics committee for animal experiments and adhered to the international guidelines for the care and treatment of laboratory animals. Female mature 4-7 year old Merino sheep were housed in groups under a 12 h light/dark cycle. Hay and water were provided *ad libitum*. No animals exhibited clinical or ultrasonographical evidence of tendon injury.

For tendon defect induction, a previously described collagenase-gel model was chosen (Smith, 2008; Watts *et al.*, 2012). The metatarsal area of both hind limbs was clipped, shaved and aseptically prepared before either 500, 200 or 100 collagen digestion units (CDU) of collagenase type 1A (from *Clostridium histolyticum*; C0130, Sigma-Aldrich Chemicals) were injected into the DDFT of the left hind limb. Collagenase was diluted in Dulbecco's PBS (1×), filter-sterilised and mixed with 50 µL of thrombin solution (component 2, TISSUCOL Duo S Immuno, Baxter, Unterschleißheim, Germany). The injection was performed with a lateral approach in the mid-tarsal area under ultrasound guidance (Mindray DP 50 vet; Sonoring Schmitt-Haverkamp, Erlangen, Germany), using a 23-G needle and a DUPLOJECT System (Baxter). The collagenase-thrombin solution was combined with 50 µL of fibrinogen solution (component 1, TISSUCOL Duo S Immuno, Baxter) during injection.

Antibiotic (Veracin-compositum 3 mL/50 kg; Albrecht, Aulendorf, Germany) was administered intramuscularly every second day for 5 consecutive days. 0.3 mg/kg body weight of buprenorphin-hydrochloride (Buprenovet® Multidose 0.3 mg/mL; Bayer HealthCare, Leverkusen, Germany) were applied subcutaneously every 12 h for 2 d;

3 mg/kg body weight of ketoprofen (Romefen® PR 10 %; Merial, Hallbergmoos, Germany) were administered every 24 h for 5 d, as analgesics. Ultrasound and daily clinical examination were performed using a portable Mindray DP-50 vet ultrasound machine (Sonoring Schmitt-Haverkamp) with a linear probe (5-12 MHz). Diameter of the DDFT was measured in the mid-tarsal area, 2 cm below the applied suture. In group treated with 100 CDU, external thickness was measured from medial to lateral in the same mid-tarsal area by using a digital caliper.

6 d after collagenase-gel injection, cmRNA^{LUC}, diluted in 5 % HBG, was injected into healthy and damaged DDFT of both hind limbs. Procedure was performed under general anaesthesia. Test item (cmRNA^{LUC}) was injected in two separate injections of 150 µg = 300 µg in total (500 CDU group, *n* = 2), 200 µg = 400 µg in total (200 CDU group, *n* = 2) and 250 µg = 500 µg in total (100 CDU group, *n* = 2), under ultrasound guidance (proximal and distal area of the defect). At the end of the injection, some air was drawn into the syringe and injected to ensure that syringe and needle were completely emptied. During anaesthesia, ketoprofen 3 mg/kg (Romefen® PR 10 %; Merial) was administered intravenously.

On the next day, sheep were euthanised by intravenous overdose of pentobarbital (Euthadorm®; CP-Pharma, Burgdorf, Germany). Subsequently, tendons were harvested and immediately incubated for 1 h in D-Luciferin solution (100 µg D-Luciferin/mL PBS) at 37 °C and 5 % CO₂. BLI was performed using Xenogen IVIS® Spectrum *in vivo* imaging system (Caliper Life Science). Measurement was operated in the field of view A with high sensitivity (binning 16) and an exposure time of 1 min. Living Image® 2.50 software (Caliper Life Sciences) was used for the analysis. Tendons were fixed for 24 h in 4 % paraformaldehyde, dehydrated with an ascending ethanol series and embedded in paraffin. In intact tendons, five cmRNA^{LUC}-injected areas were chosen, as well as areas 3 cm proximal and distal to the injection site. In injured tendons, central and peripheral areas of the defect were selected and 3 µm thick sections were stained with haematoxylin and eosin for histopathological examination.

Blood was taken at day 0, day 6 (before mRNA application) and day 7 (before euthanasia) and full blood count as well as liver and kidney test values were analysed by an external veterinary laboratory.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 5. Results were presented as mean ± standard deviation (SD), with *n* equal to the number of samples per group. Differences between groups were analysed for statistical significance with significance attained at *p* < 0.05 using one-way ANOVA.

Results

Injection of naked cmRNA resulted in high protein expression in tendon explants

Naked cmRNA encoding for firefly luciferase (FFL) was dissolved in saline and injected into freshly explanted

tendons. Subsequently, injected explants were incubated and luciferase activity was measured 24 h later by BLI. Expression of luciferase was detected in specimen injected with cmRNA^{LUC}, but not in untreated control tendons or in tendons injected with saline solution only. Based on these promising findings, further experiments were conducted on explants of different animal species to investigate whether the concept of *ex vivo* transfection could be successfully applied to more mammals. Interestingly, besides expression in porcine tendon explants, injection of naked cmRNA^{LUC} resulted in distinct luciferase expression in explanted tendons of sheep, cattle, horses and rats (Fig. 1A). Notably, expression patterns revealed a particular distribution, with

higher luciferase activity at the injection site and a slim, tube-like distribution within the entire explant.

In a next set of experiments, we used explanted tendons from cattle (Fig. 1B), pigs and sheep (data not shown) to examine the dose-dependency of the reporter protein activity. As expected, transfection efficacy directly correlated with the amount of the injected test item (cmRNA^{LUC}) (Fig. 1B). To identify the transfected cells, cmRNA coding for the bacterial enzyme β -galactosidase was injected into porcine (Fig. 1C,D) and sheep (data not shown) tendon explants. Already after 1 d of *ex vivo* incubation, expression of β -galactosidase was detectable macroscopically by visual inspection. Microscopic analysis

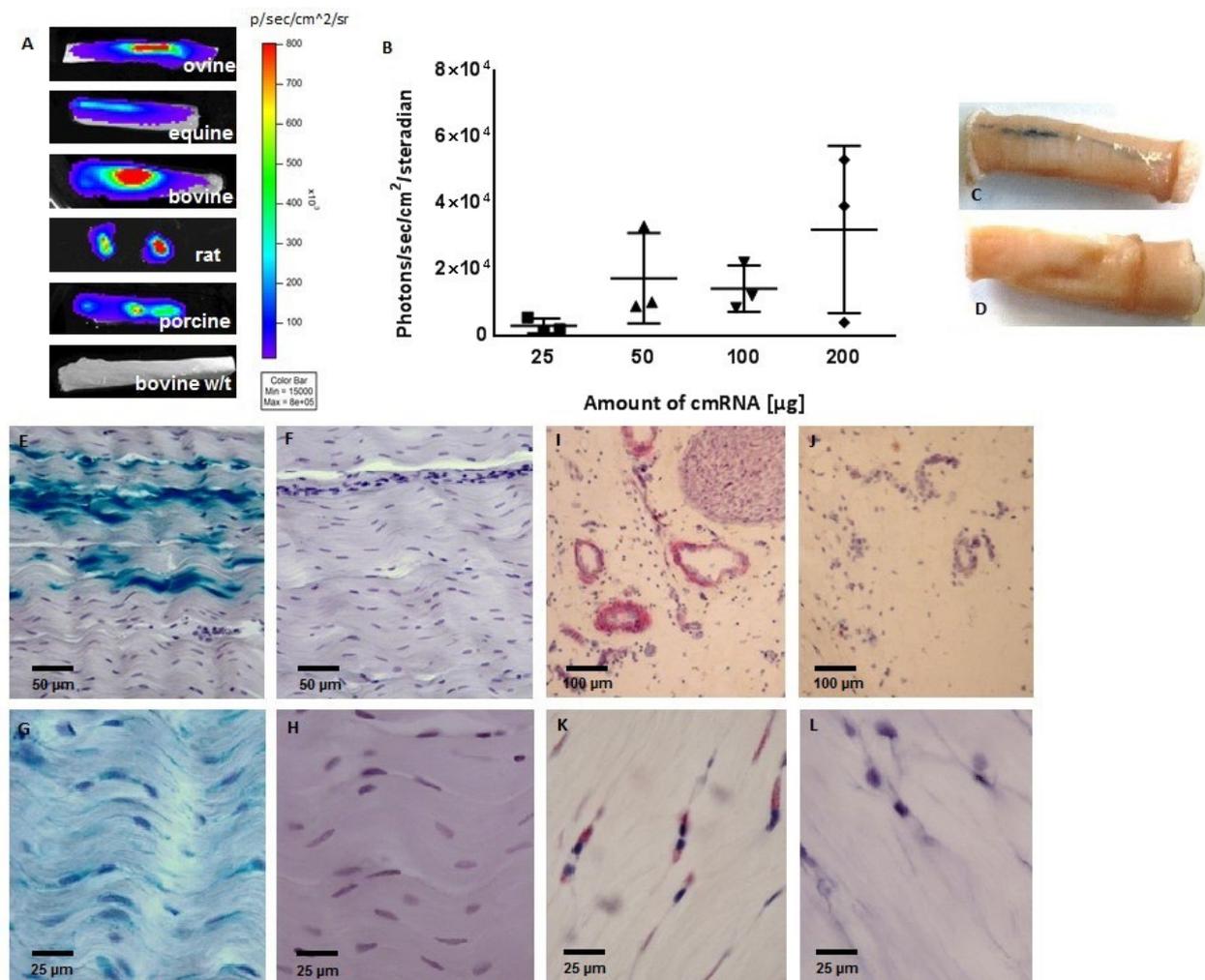


Fig. 1. Protein expression in intact tendon explants after naked cmRNA injection. (A) Bioluminescence imaging (BLI) 24 h after cmRNA^{LUC} injection in tendon explants of different species. Either 26.6 μ g [0.65 mg/mL] (rat), 50 μ g [0.5 mg/mL] (bovine, equine) or 100 μ g [1 mg/mL] (porcine, ovine) of naked cmRNA^{LUC} were injected. Expression of luciferase was detected in all species tested. (B) Luciferase expression was dose-dependent. Bovine tendon explants were injected with either 25 μ g, 50 μ g, 100 μ g or 200 μ g of cmRNA^{LUC} in 250 μ L of isotonic saline solution (NaCl). Mean expression \pm SD of 24 h time point is shown ($n = 3$). (C) 24 h after cmRNA^{LacZ} transfection, tendon explants showed macroscopically visible blue staining (indicating activity of β -galactosidase). (D) In contrast, no activity of β -galactosidase was found in control explants transfected with cmRNA^{LUC}. (E,G) Sections of LacZ stained tendon explants transfected with cmRNA^{LacZ} and (F,H) cmRNA^{LUC}. (I,K) Immunostaining for hBMP-7, in sections of cmRNA^{hBMP-7} transfected rat common calcaneal tendons, showed red staining (indicating evidence of hBMP-7) in (I) vascular walls and connective tissue and (K) tenocytes. (J,L) Controls transfected with cmRNA^{LUC} did not show remarkable evidence of hBMP-7.

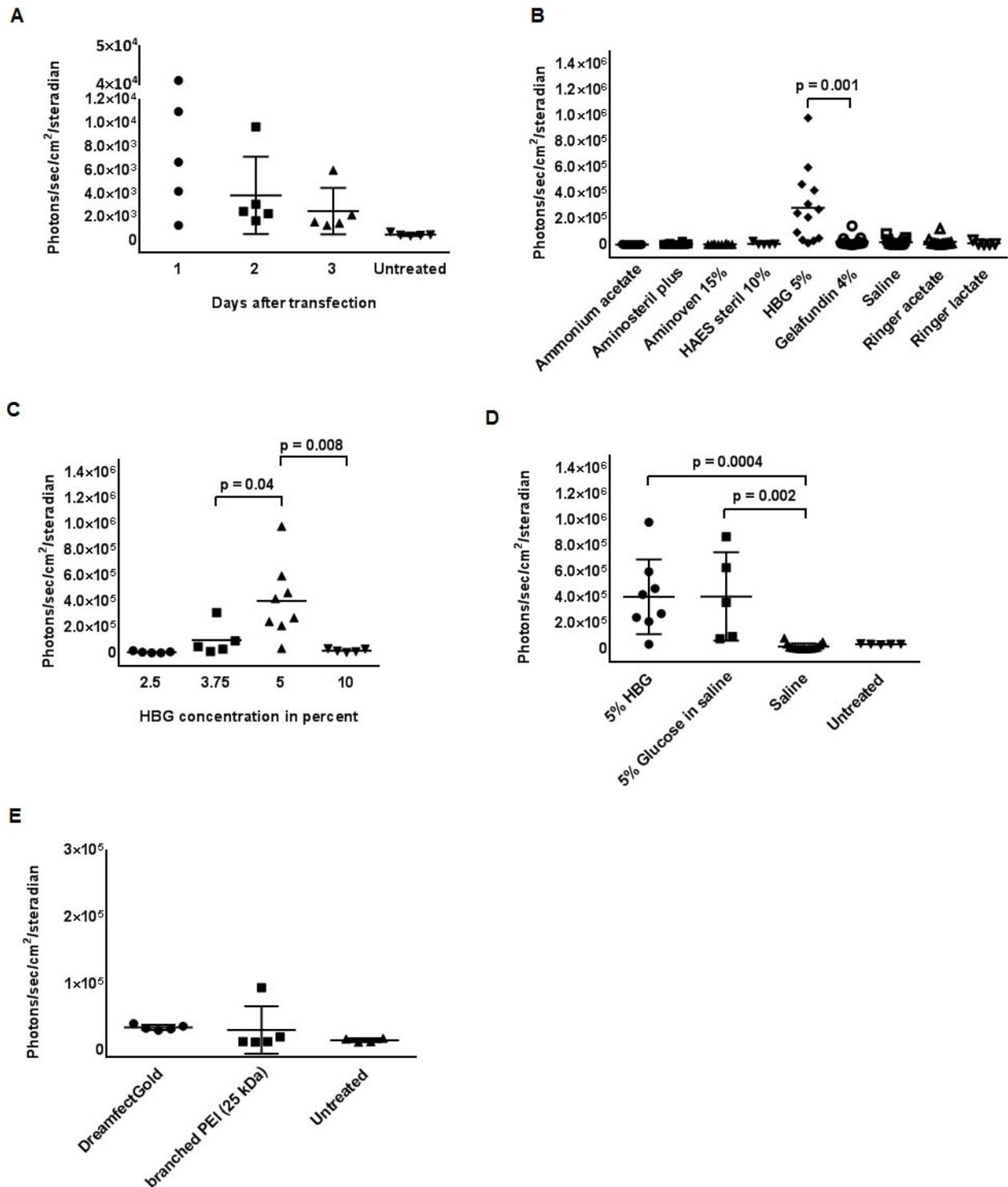


Fig. 2. Expression kinetics and comparison of different solvents used for naked cmRNA injection. (A) Expression kinetic of luciferase was evaluated in bovine tendon explants after transfection with 50 μg cmRNA^{LUC} [0.5 mg/mL] dissolved in saline. (B) 50 μg cmRNA^{LUC} [0.25 mg/mL] were diluted in different solvents and injected into bovine tendon explants ($n \geq 5$). Activity of luciferase was measured 1 d after *ex vivo* transfection revealing maximum expression upon use of HEPES-buffered glucose (HBG 5 %) as solvent. (C) Comparison of different HBG concentrations showed that maximum luciferase expression levels were reached using 5 % HBG as solvent. However, higher or lower concentrations of HBG resulted in statistically significant lower expression levels. (D) Comparison of glucose-containing saline and HEPES-buffered solution (HBG 5 %). (E) cmRNA^{LUC} complexed using DreamFect^{FM} Gold and branched Polyethylenimine carrier system.

showed strong β -galactosidase positivity (indicated by a blueish nuclear staining) in tenocytes and fibroblasts close to the site of injection and within the collagen fibre bundles (Fig. 1E,G). No β -galactosidase positive cells were found in control tendons injected with cmRNA coding for FFL (Fig. 1F,H).

Encouraging results with reporter genes (luciferase and β -galactosidase) prompted us to investigate the applicability of this technology to produce therapeutic proteins. BMP-7 was selected as a therapeutic target, due to its ability to increase healing processes by stimulating effects on tenocytes. Proof of concept experiments in rats aimed to evaluate the pharmacologic efficacy of human BMP-7 cmRNA. Moreover, using human BMP-7 cmRNA allowed us to quantify hBMP-7 protein, resulting from BMP-7 cmRNA, without significant cross-reactivity with the endogenous BMP-7. For these reasons, human BMP-7 was chosen for further *ex vivo* and *in vivo* studies. As further *in vivo* experiments were intended to be conducted in rats, cmRNA^{hBMP-7} was injected into rat common calcaneal tendons. IHC for hBMP-7 was performed after 1 d of cultivation. Using this methodology, hBMP-7 positive signals were found in vascular walls and connective tissue (fibroblasts and tenocytes) of cmRNA^{hBMP-7}-injected tendon specimens (Fig. 1I,K). Also control tendons were slightly positive, but only in vascular walls (Fig. 1J,L).

Vehicle composition strongly impacted transfection efficacy in tendon explants

Further experiments were carried out in bovine tendons, due to their easy accessibility from the local slaughterhouse. After establishing the expression dose-dependency (Fig. 1B), the subsequent study was initiated to explore the expression kinetics of cmRNA^{LUC} *ex vivo*. Results in bovine tendons showed a peak of expression 1 d after injection and a subsequent 2-fold decline over the next 2 d. 3 d after transfection, luciferase expression was reduced to background levels (Fig. 2A).

Hypothesising that electrolytes, sugars or amino acids might influence transfection efficacy, we investigated different commercially available and standard laboratory solutions as vehicles for cmRNA. We hypothesised that (1) osmotic gradients may increase intracellular electrolyte influx and thereby intracellular uptake of cmRNA, (2) providing cells with additional amino acids may enhance the translation of cmRNA, (3) precipitating the mRNA with ammonium acetate may result in a depot effect, thereby enabling extended expression. Furthermore, colloidal solutions were used to test the influence of large molecules on transfection of tendon tissue. As highest expression levels were observed 24 h after cmRNA^{LUC} injection, this time point was selected for the comparison of different solvents.

Our results revealed a strong influence of the used solvent/electrolyte on the resulting luciferase expression. This effect was most prominent when comparing electrolyte and/or glucose-containing solutions with solutions containing amino acids (Fig. 2B). Noticeably, glucose-containing solution, such as HEPES buffer containing 5 % glucose (HBG 5 %), resulted in higher luciferase expression compared to saline ($p < 0.001$) or

amino acids-containing solution (57-fold compared to Aminosteril plus; Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany). No luciferase expression was detected for cmRNA^{LUC} in ammonium acetate solution. Reasoning, whether concentration of glucose influenced transfection efficacy, we observed the highest luciferase expression at a glucose concentration of 5 %, which decreased several folds when glucose concentration was either increased (20-fold less) or decreased (50-fold) (Fig. 2C). To verify that these findings were transferable to other species, similar results were obtained in tendons derived from pigs and horses (data not shown).

Cationic lipids and polymers are commonly used carrier systems for facilitating cell membrane penetration and thereby enhancing transfection efficacy of nucleic acids *in vitro* and *in vivo*. Hence, the two standard transfection reagents, DreamFect^{FM} Gold (lipid based) and branched polyethylenimine (polymer based), were tested, assuming that these carriers would result in increased transfection levels. Surprisingly, both carriers did not result in any detectable luciferase activity, thereby confirming lack of transfection (Fig. 2E).

Naked chemically-modified mRNA transfected intact rat common calcaneal tendons *in vivo*

cmRNA^{LUC}, dissolved under optimised vehicle conditions (HBG containing 5 % glucose, adjusted to pH 7.4), was injected into the common calcaneal tendon of rats at 3 different doses (40, 20 and 10 μ g, *i.e.* 0.2, 0.1 and 0.05 mg/kg body weight) to explore whether our findings from *ex vivo* studies were translatable *in vivo*. Vehicle (HBG containing 5 % glucose, adjusted to pH 7.4) was injected as negative control. Expression of luciferase was measured using BLI 1, 2 and 7 d after injection of the test item (Fig. 3A). A dose-dependent luciferase expression and expression kinetics similar to our results in *ex vivo* studies using tendon explants were observed (Fig. 3A,B). Peak expression was observed 1 d after transfection, which declined to background levels within 7 d (Fig. 3B).

Naked cmRNA^{hBMP-7} positively affected early healing in injured rat common calcaneal tendons *in vivo*

The capability to overexpress a potential therapeutic protein using cmRNA was investigated in a rat model of acute tendon rupture. The common calcaneal tendon was surgically dissected and cmRNA coding for hBMP-7 (cmRNA^{hBMP-7}) was injected into both ends of the stumps. Subsequently, dissected stumps were re-adapted using surgical sutures and the animals recovered from anaesthesia. Content of BMP-7, collagen type I and III was measured 1, 2 and 7 d after surgery by IHC.

Higher levels of hBMP-7 in tendons of cmRNA^{hBMP-7}-treated animals could be measured at all time points, compared to vehicle treated animals (Fig. 3C). A statistically significant difference ($p = 0.03$) was observed 2 d after the application of the test item.

No difference was found at any of the examined time points between vehicle and cmRNA^{hBMP-7}-treated animals with respect to the content of collagen type I (Fig. 3E). However, significantly ($p = 0.03$) lower levels of collagen type III were found 7 d after tenotomy and injection

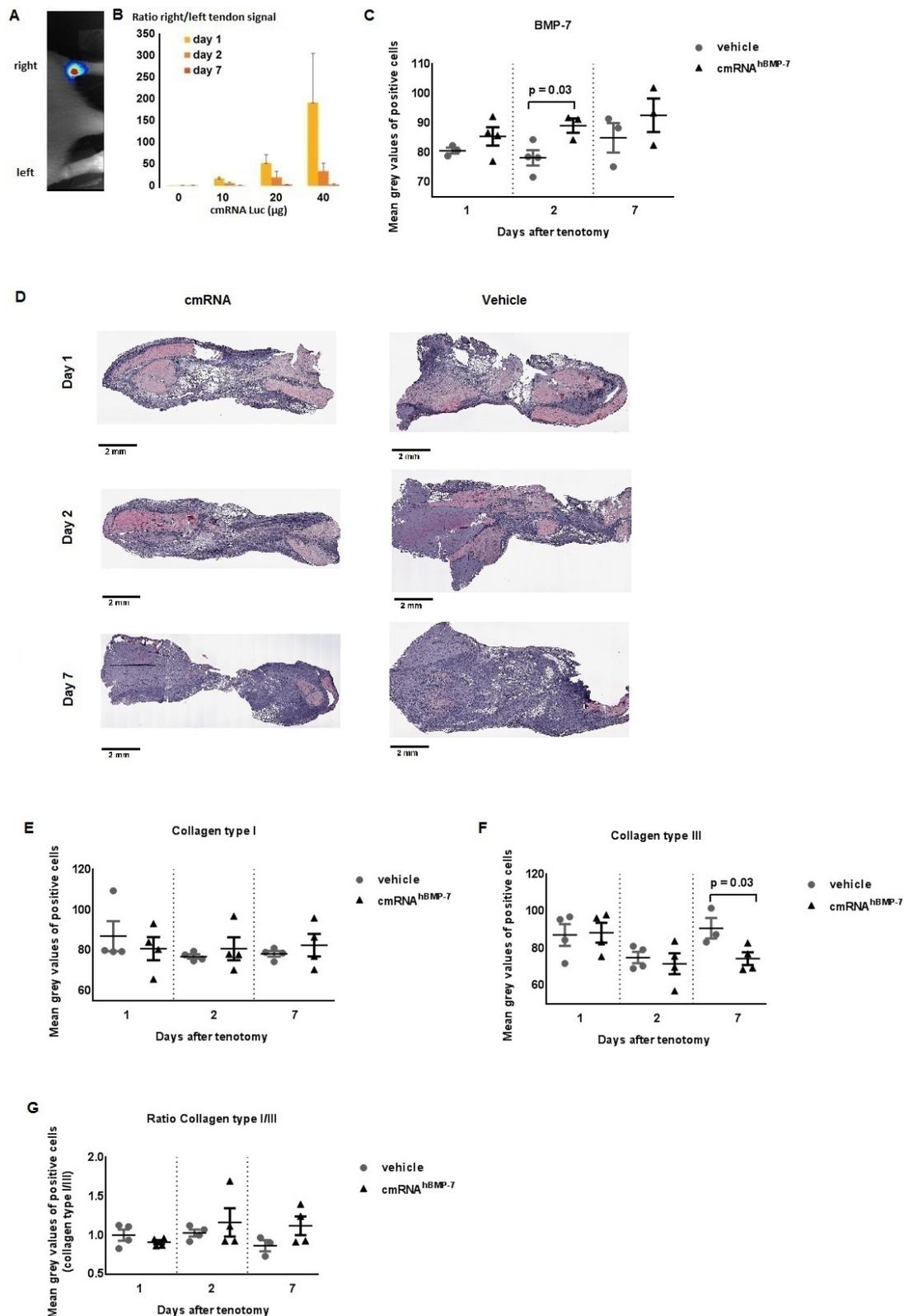


Fig. 3. Protein expression in intact and injured rat common calcaneal tendons upon injection of cmRNA *in vivo*. (A) *In vivo* luciferase activity was measured 1, 2 and 7 d after injection of 3 different doses of cmRNA^{LUC} into the common calcaneal tendon of rats ($n = 4$ per doses group). (B) Results revealed dose-dependent expression of luciferase with a maximum 1 d after test item application and declining expression up to 7 d post injection. (C) Immunostaining for hBMP-7 upon injection of cmRNA^{hBMP-7} into injured common calcaneal tendons of rats. (D) Histopathological examination of common calcaneal tendons treated with cmRNA or vehicle. (E) At any time point examined, immunostaining revealed no difference between the levels of collagen type I, upon treatment with cmRNA^{hBMP-7}, compared to vehicle. (F) Levels of collagen type III at day 1, 2 and 7 post treatments. (G) Ratios of collagen type I to collagen type III immunostaining showed no significant difference between cmRNA^{hBMP-7} and vehicle treated animals, at any time point examined.

of cmRNA^{hBMP-7} (Fig. 3F). The ratio between collagen type I and III showed no significant difference between cmRNA^{hBMP-7} and vehicle-treated animals, at any time point examined (Fig. 3G).

At day 1 and 2 post-surgery, histopathological examination of the injured common calcaneal tendon revealed an inflammatory reaction with migration of granulocytes and necrotic areas with haematoma in both groups. 7 d post-surgery, further proliferative progress was apparent in both groups (Fig. 3D).

Naked cmRNA efficiently transfected injured and intact tendons in large animals

Matrix disorganisation, fibre disorientation, hypercellularity and vascular ingrowth are characteristic histopathological findings during acute tendinopathy. As surgically injured rat tendons did not resemble these conditions, we additionally investigated cmRNA transfection efficacy in a large animal model of acute tendinopathy. Collagenase has already been

used to generate tendon defects in sheep (Martinello *et al.*, 2013). However, those defects do not closely resemble the morphology of acute tendon defects as seen in the clinics, as resulting inflammation and tissue destruction are distributed over the entire tendon. In contrast to this, clinical acute tendonitis is characterised by focal areas with loss of tissue structure, necrosis, inflammation and hematoma. Recently, Watts *et al.* (2012) described that injection of collagenase diluted in a fibrin and thrombin solution into superficial digital flexor tendons (SDFT) more closely resembled the clinical picture of acute tendinopathy in horses. We adapted this concept for establishing a sheep model of similar acute focal tendon defects. Therefore, under ultrasound guidance, collagenase diluted in fibrin and thrombin was injected with three different doses (100, 200 and 500 CDU) into the DDFT of anaesthetised sheep. Echography performed before, 3 and 5 d after collagenase-gel injection, showed clear inhomogeneity in all groups as early as 3 d after injection (Fig. 4A-F).

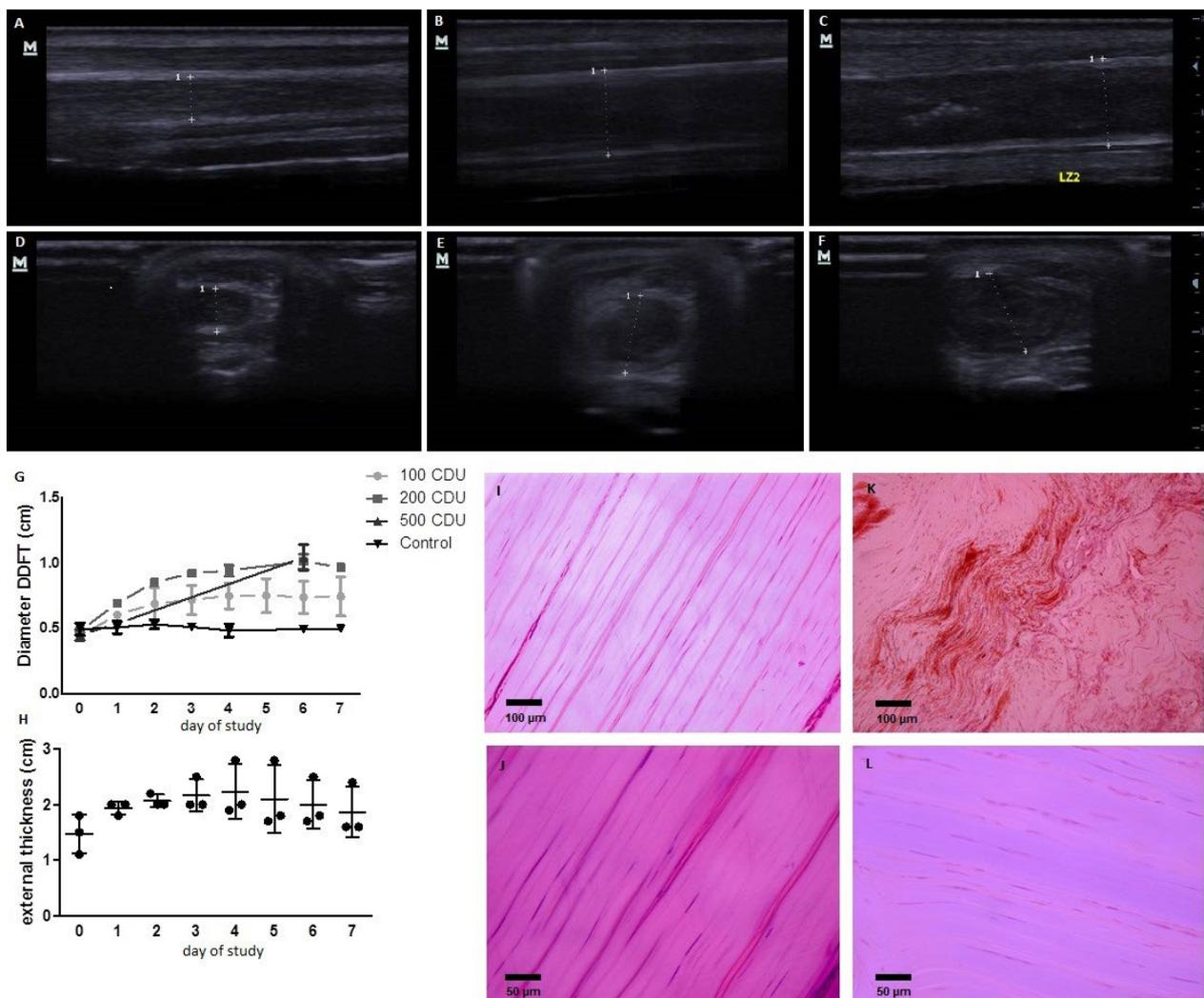


Fig. 4. Pathology of collagenase-gel-induced tendon defects in sheep. (A-C) Longitudinal (D-F) and transversal ultrasound images of a sheep's injured hind limb. Longitudinal and transversal ultrasound image at (A,D) day 0 before collagenase injection, (B,E) 3 and (C,F) 7 d after collagenase-gel injection (100 CDU). (G) Diameter analysis during experimental period of tendons injected with either 100, 200 or 500 CDU and untreated contralateral controls. Data are presented as mean \pm SD ($n = 2$). (H) External thickness analysis of sheep treated with 100 CDU during experimental period. Data are shown as mean \pm SD ($n = 2$). Histopathological examination of (I,J) intact and (K,L) injured sheep tendons.

Moreover, a dose-dependent increase of DDFT diameter (Fig. 4G) and limbs external thickness (1.5-fold within 4 d) was observed. DDFT diameter remained increased, whereas external thickness decreased back to untreated levels within 7 d (Fig. 4H).

Pathological examination of tendons showed macroscopically visible haematomas in up to one third of the entire tendon, upon the injection of 500 CDU of collagenase-gel and haematomas in up to one quarter of the entire tendon, upon the injection of 200 CDU of collagenase-gel. No haematoma was visible upon a

dosage of 100 CDU of collagenase-gel. For all tested doses, histological examination revealed conglomerates of red blood cells, granulocytes, necrosis and loss of fibre structure in the central area of all defects (Fig. 4K). In the periphery of the defects, adjacent fibres were swollen, connections between fibres were loosened and cell density was increased (Fig. 4L) compared to intact tendons (Fig. 4J).

cmRNA^{LUC} in 5 % HBG-containing buffer was injected with 3 different doses (300, 400 and 500 µg) into healthy and injured tendons (defects generated using 500, 200 and

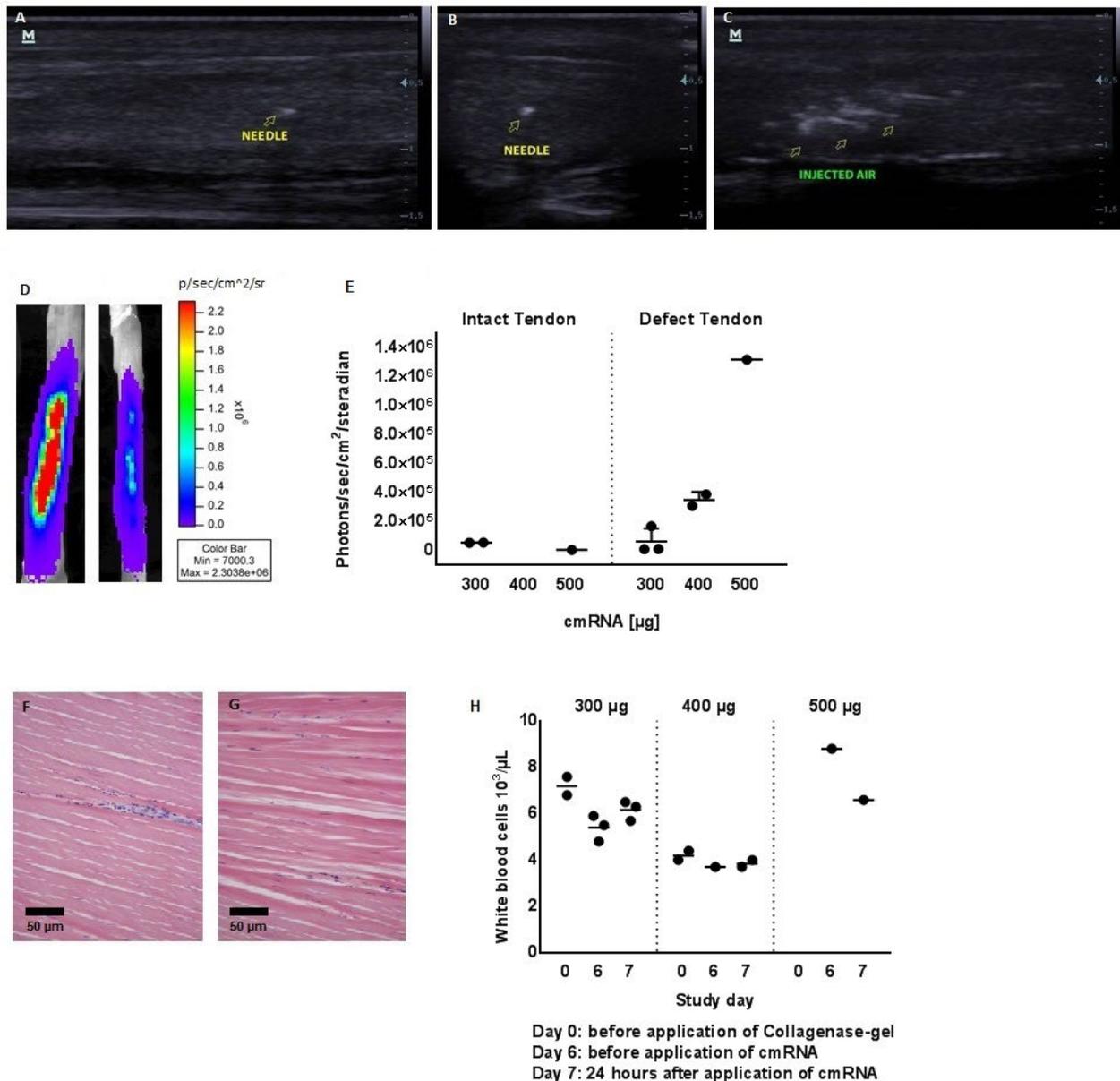


Fig. 5. Transfection of injured and intact tendons in sheep. (A) Longitudinal and (B) transversal ultrasound image of a damaged DDFT at day 6, with needle inserted in the central part of the tendon. (C) Longitudinal ultrasound image immediately after cmRNA^{LUC} injection. Injected air was visible in the central area of the tendon. (D) Exemplary *ex vivo* BLI of damaged (left) and intact (right) explanted contralateral ovine DDFT 24 h after *in vivo* injection of 400 µg of cmRNA^{LUC} in each DDFT. (E) *Ex vivo* luciferase activity 24 h after injection of increasing amounts of cmRNA^{LUC}. Each dot represented measured luciferase values in one individual animal. (F,G) Haematoxylin-eosin staining of intact cmRNA^{LUC}-treated tendon at (F) the injection site and (G) 3 cm proximal to the injection site. Original magnification 20×. (H) Analysis of white blood cell (WBC) from blood taken at day 0, day 6 and day 7.

100 CDU of collagenase-gel), under ultrasound guidance (Fig. 5A,B). Air was withdrawn in the syringe, together with the test item, to visualise the application (Fig. 5C). Luciferase expression was measured *ex vivo* 24 h after application, revealing luciferase activity in both, intact and injured tendons. Results showed no dose-dependent increase of luciferase expression in healthy tendons. On the contrary, increasing amounts of cmRNA^{LUC} led to increased luciferase expression (Fig. 5D,E).

Histopathological examination of cmRNA^{LUC}-treated intact tendons revealed predominantly intact tendon tissue without pathological findings, except mild cellular infiltrates of macrophages and granulocytes (Fig. 5F). Tissue distal and proximal to the injection site was without any abnormalities (Fig. 5G).

Blood was taken when the collagenase-gel injection was performed, before that cmRNA^{LUC} was injected and 24 h later, before the animals were euthanised. Haematology (white blood cell count, haematocrit, platelet count and red blood cell count) revealed no clinically significant abnormalities during the entire study in any of the experimental groups (Fig. 5H). Also, clinical chemistry (creatinine, blood urea nitrogen, aspartate aminotransferase, alanine aminotransferase, acute phosphatase, glutamate dehydrogenase) did not exhibit any clinically significant deviations at any of the time point examined. Moreover, also serum cytokines (interleukin-6, tumour necrosis factor α , interferon α and γ) did not show any relevant increase during the entire study (data not shown).

Discussion

For the treatment of various pathological conditions, chemically-modified mRNAs (produced by incorporation of the chemically-modified nucleotides 2-thiouridine and 5-methyl-cytidine in the *in vitro* transcription reaction) represent a novel technology for transfecting tissues and consequently expressing therapeutic proteins within the body (Kormann *et al.*, 2011; Zangi *et al.*, 2013). In the current proof of concept study, we reported the feasibility of using cmRNA technology as a potential therapeutic approach for expression of physiologically active proteins in intact and injured tendons. A self-established *ex vivo* transfection method enabled us to screen transfection efficacy of cmRNA in a variety of species (sheep, cattle, horses, hogs and rats), to optimise transfection conditions and to evaluate expression of several proteins (luciferase, β -galactosidase and BMP-7) in a cost and time efficient manner. Reporter protein expression was species-independent and expression levels were significantly higher when naked cmRNA was transfected, compared to cmRNA complexed with common lipid and polymer carriers. Furthermore, transgene expression could be further optimised by using naked mRNA dissolved in glucose-containing vehicle.

Results of our *ex vivo* studies were confirmed in 3 different *in vivo* models: intact rat common calcaneal tendons, injured rat common calcaneal tendons (dissected and surgically repaired) and collagenase-gel-induced tendon defect in sheep. Injection of cmRNA^{LUC} into healthy

rat tendons resulted in strong luciferase expression, peaking after 1 d and fading out within 7 d in a dose-dependent manner. These observations resembled the expression kinetics observed in our *ex vivo* studies.

Results of *in vivo* studies using reporter proteins prompted us to investigate the potential of cmRNA for expressing potentially therapeutic proteins. BMP-7 was selected as a potential therapeutic target, as it has been shown to play a crucial role in the healing process at the interface of tendons and bones (Forslund and Aspenberg, 1998). A rat model of surgically dissected common calcaneal tendons was selected to investigate the influence of hBMP-7-coding cmRNA in a pathological environment. Injection of cmRNA^{hBMP-7} into injured tendons, resulted in increasing hBMP-7 levels compared to vehicle as early as 2 d after application and up to day 7. It needs to be mentioned that BMP-7 was also detected in vehicle treated tendons at all time points. Measured levels were constant during the entire study. Most likely, these background levels could be attributed to cross reactivity of anti-hBMP-7 antibody with rat BMP-7.

Catabolism of collagen type I and excessive replacement by collagen type III are considered to be important events in the early inflammatory phase (day 1 to day 7 post injury) of tendon injury (Loiselle *et al.*, 2012; Sharma and Maffulli, 2005a). By time, collagen type III is either converted to full functional collagen type I (resulting in tissue regeneration) or to scar-like tissue, leading to loss of tensile strength and elasticity, compared to intact tendon tissue (Sharma and Maffulli, 2005b). In the present study, no differences were found with respect to the content of collagen type I in tendons, between animals treated with cmRNA^{hBMP-7} or vehicle, at any of the examined time points. However, 7 d after injury and injection of the test item, significantly ($p = 0.03$) less collagen type III was found. Summarising, our findings indicated that cmRNA^{hBMP-7} contributed to increased levels of hBMP-7 and reduced formation of collagen type III. Because only early time points were investigated, it might be too early to draw final conclusion whether cmRNA^{hBMP-7} positively impacts physiological regeneration of tendons. Studies from other authors reported significant changes of collagen type I content and morphological changes towards regeneration as early as weeks or months post injury (Martinello *et al.*, 2013). To finally answer the question, whether overexpression of hBMP-7 leads to increased content of collagen type I, further long term studies have to be conducted with examinations of the tendons at later time points.

In clinics, patients frequently report acute pain and/or limitation of the affected tendon in performing physiological movements. For example, partial rupture of the supraspinatus tendon leads to the inability to perform an abduction of more than 90° of the affected extremity (Woodward and Best, 2000). In veterinary medicine, acute tendinitis of the SDFT represents a frequent cause of severe lameness in horses (Thorpe *et al.*, 2010). However, before these clinical symptoms become noticeable, a long process of microtraumatic fibre disruption and subsequent inflammation is already present (Kannus, 1997). Patients frequently report unfavourable movements prior to the acute onset of the characteristic

symptoms of an acute tendinopathy. Histopathologically healthy tendons differ significantly from tendons affected by acute tendinopathy. The latter is characterised by disintegration of the extracellular matrix, necrosis and presence of inflammatory cells, such as neutrophils and macrophages. Results of our *ex vivo* studies in healthy cadaver tendons showed reporter protein expression in a slim tube-like pattern along the entire tendon (Fig. 1C-H). To investigate cmRNA's capability of transfecting not only healthy, but also tendons resembling the characteristics of acute early tendinopathy, cmRNA was injected into healthy and collagenase-gel-injured DDFT of sheep. At day 1 after injection, *ex vivo* BLI confirmed luciferase expression in healthy and inflamed DDFT. Interestingly, injured tendons exhibited higher expression levels upon higher doses of cmRNA, whereas expression levels remained in a similar range, independent of the applied dose. These observations could possibly be explained by the altered composition of the extracellular matrix and presence of inflammatory cells in injured tendons. Disintegrated matrix might allow a wider spread of cmRNA throughout the tissue, as the limiting connective tissue sheaths surrounding the tendon fibres are destroyed. This leads us to the assumption that therapeutic protein encoding cmRNA could possibly represent an interventional strategy for the transfection of acute tendon lesions.

These results prompt us to believe that cmRNA might not only be a tool for expressing proteins in healthy tendons, but also in acute inflamed tendons, with the aim to attain tendon regeneration and reduce scar tissue formation. Our results with BMP-7, indicate that therapeutic proteins can indeed be expressed in injured tendons. To address their therapeutic potential, future studies, investigating tendon healing process (later time points), need to be performed.

Conclusion

Naked cmRNAs in glucose-containing solutions were efficient in transfecting both healthy and injured tendons in a species-independent manner. The resulting protein was biologically active and localised to the site of injection. It is conceivable that cmRNAs can be utilised to encode any therapeutic protein of interest for the treatment of tendon disorders.

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Discussion with Reviewers

Kasia Whysall: How do you envisage a clinical application of the method described in terms of tendinopathies?

Authors: Therapeutic mRNAs could be used during surgery to positively influence the process of re-adaption (similar to the common calcaneal disruption model in rats). This may be useful in cases of partial or complete disruption of one of the rotator cuff tendons. Moreover, we foresee potential applications in the field of equine veterinary medicine. Many horses suffer from superficial digital flexor tendon impairments. In such cases, therapeutic mRNAs could be directly injected into the tendon defect using ultrasound guidance, as described in the collagenase-gel-induced tendinitis in sheep.

Gundula Schulze-Tanzil: What about the stability of cmRNA against endogenous RNases and antigenicity of this xenogenic RNA?

Authors: Though *in vivo* naked cmRNA are degraded upon injection into tendons, we believe that stability might be strong enough for them to keep their biological functionality. However, studies must be done to examine exactly this issue. With respect to antigenicity of the xenogenic RNAs, we cannot provide any information as we have not examined this issue.

Gundula Schulze-Tanzil: What is the advantage of cmRNA transfection compared to simple injection of BMP-7 [Ozeki *et al.*, (2013)]?

Authors: Recombinant growth factors are characterised by limited half-lives. In our present study, we have shown that proteins can be expressed for a period up to 3 d. Moreover, mRNAs can be used to express every protein desired. Hence, we see applications utilising the expression of transcription factors or long non-coding RNAs in order to influence cellular function.

Gundula Schulze-Tanzil: When testing the effect of different agents on cmRNA transfection efficacy, how about mediators increasing the blood flow in tendons? I think this could not be tested in explants but rather in an appropriate *in vivo* model. Also, *in vivo*, the blood flow might be increased by biomechanical stimulation.

Authors: We fully agree. This might be an interesting topic for future investigations. Morphological alterations of the tissue, but also changes in blood flow might influence transfection efficacy of mRNAs. In our present work, we observed that expression seems to be more localised to fibre bundles in intact tendons and tending to spread more intensively in inflamed tendons.

Gundula Schulze-Tanzil: The effects expected by BMP-7 in tendons are not thoroughly discussed. After injection of BMP-7 in a rat model, Ozeki *et al.*, (2013) reported fibrocartilaginous transdifferentiation of Achilles tendon fibroblast into fibrochondrocytes. Perhaps markers of cartilage expression, such as type II collagen (or aggrecan and Sox9), should be included in the analysis of the healing tendons, to exclude unwanted fibrocartilage formation.

Authors: We did not perform this analysis in the present work because we monitored the expression of hBMP-7 only for a short period (one week). This is long enough to show that there is protein expression, but not long enough to observe effects like ectopic bone formation. Bone formation upon application of BMP-7 could be useful in the re-adaption process of lacerated tendons. However, it might as well be possible that tissue environment influences which particular effect (bone formation or not) could be induced through BMP-7. All these are questions for very interesting future studies.

Additional Reference

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Editor's note: The Scientific Editor responsible for this paper was Juerg Gasser.