

Effects of canine myoblasts expressing human cartilage-derived morphogenetic protein-2 on the repair of meniscal fibrocartilage injury

WEN-HUI ZHU¹, YU-BIN WANG¹, LIANG WANG¹, GU-FENG QIU² and LIANG-YU LU¹

¹Department of Sports Medicine, Shanghai East Hospital, Tongji University School of Medicine;

²Institute for Nutritional Sciences, Chinese Academy of Sciences, Shanghai 200120, P.R. China

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Abstract. The aim of the present study was to explore the effects of human cartilage-derived morphogenetic protein-2 (hCDMP-2)-expressing canine myoblasts on the repair of meniscal fibrocartilage injury. Purified canine myoblasts were infected with lentiviruses carrying an empty vector or the hCDMP-2 gene. The following four experimental groups were established to study the *in vivo* meniscal repair in a canine model of meniscal injury: Group A, suture only; group B, suture with the addition of the recombinant hCDMP-2 on a polylactic acid/polyglycolic acid (PLA/PGA) scaffold; group C, a PLA/PGA scaffold with canine myoblasts carrying the empty vector; and group D, a PLA/PGA scaffold with canine myoblasts expressing hCDMP-2. Samples of the regenerated tissue were extracted at weeks 3, 8 and 12 post-repair and analyzed by morphological observation, immunohistochemistry (IHC) and quantitative analysis. At week 12 post-repair, the scaffold material had completely dissolved in the control groups and no changes were observed at the injured area, while regenerated tissue was observed in group D only. Hematoxylin and eosin and Safranin-O staining techniques further revealed cartilage lacunae and fibers present at the red-red zone of the repaired tissue, while cartilage lacunae without fibers were observed at the white-white zone in group D. In addition, IHC studies demonstrated that collagen I and II, and the S-100 protein were expressed at the red-red and the white-white zones of the repaired tissue in group D. It was concluded that purified canine myoblasts expressing the hCDMP-2 gene were able to promote meniscal fibrocartilage healing by regenerating fibrocartilage-like tissue. The tissue in the red-red zone was regenerated more rapidly than that in the white-white zone. Further studies are required to

identify the best way to combine hCDMP-2 growth factor with myoblasts for use in the clinic due to the limitations regarding the clinical use of lentiviral infections.

Introduction

Meniscal fibrocartilage injury of the knee joint is one of the most common sports injuries, and is the focus for numerous specialists. If not treated in a timely manner, meniscal fibrocartilage injury may lead to serious consequences for patients (1). However, due to the low content of chondrocytes, the poor self-repair capabilities and the deficient local blood supply in the meniscal fibrocartilage, current treatments, including the widely recognized arthroscopic meniscal repair techniques, do not lead to satisfactory outcomes (2). For example, it remains unknown whether certain areas or types of meniscal injury repaired using sutures are capable of healing (3). Yoon *et al* (4) presented a novel technique designed for the reduction and repair of bucket-handle meniscal tears to make an avenue to success of the surgery. Wang *et al* (5) developed a novel arthroscopic technique of direct repair for the radial tear of the posterior root medial meniscus using a posterior trans-septal portal which could avoid disturbing the normal meniscal movement during flexion in a loaded condition. In spite of the different techniques used by the aforementioned surgeons, the ultimate results of meniscal healing are not certain. Our previous study showed that canine myoblasts can be induced to transform into chondrocytes by cartilage-derived morphogenetic protein-2 (CDMP-2) and transforming growth factor β 1 (TGF- β 1) *in vitro* (6). The present study explores the effects of canine myoblasts expressing human (h)CDMP-2 on the repair of meniscal fibrocartilage injury *in vivo*.

Materials and methods

Animals and reagents. A total of 10 one-year-old male canines were provided by the College of Agriculture at the Shanghai Transportation University (Shanghai, China; permit no. 2007-0004). Study approval was obtained from the Shanghai Animal Care and Use Committee (Shanghai, China) prior to performing the study. The following reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA):

Correspondence to: Dr Wen-Hui Zhu, Department of Sports Medicine, Shanghai East Hospital, Tongji University School of Medicine, 150 Jimo Road, Pudong New Area, Shanghai 200120, P.R. China
E-mail: dr_zhu2008@126.com

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pcDNATM6.2-GW/EmGFP, plenti6/v5, one shot Stbl3 chemically competent *Escherichia coli*, BLOCK-iT polymerase II (Pol II) expression vector kit with emerald green fluorescent protein (EmGFP) and BLOCK-iTTM lentiviral Pol II expression system. The following reagents were also used: AxyPrep plasmid purification kit (Axygen, Union City, CA, USA), polylactic acid (PLA) and polyglycolic acid (PGA) (Sigma, St. Louis, MO, USA), recombinant human CDMP-2 (PeproTech, London, UK), 0.25% F-10 basic culture media and Safranin-O dye (Shanghai Reagent Company, Shanghai, China), toluidine blue dye (Shanghai Reagent Company), anti-collagen-I antibodies (mouse and canine; Abcam, Cambridge, UK), anti-collagen-II antibodies (mouse anti-canine; Neomarkers, Fremont, CA, USA), anti-S-100 antibodies (rabbit anti-canine; Neomarkers) and Alcian Blue staining buffer (Pierce Biotechnology, Inc., Rockford, IL, USA).

Lentiviral infection of canine myoblasts. Canine myoblasts were purified and cultured, as previously described (7). The cells were infected with lentiviruses containing the empty vector or the hCDMP-2 gene for two days. GFP⁺ clones were selected for further studies. The expression of GFP and hCDMP-2 genes in different clones were quantified by quantitative polymerase chain reaction (qPCR) using total RNA. The sequences of the primers were: GAPDH forward, 5'-GACAACTTTGGTATCGTGGAAGG-3' and reverse, 5'-CCAGTAGAGGCAGGGATGATGT-3'; GAPDH probe, fam-5'-CTCATGACCACAGTCCATGCCATCACT-3'-tamra; GFP forward, 5'-AAGCAGCACGACTTCTCAAGTC-3' and reverse, 5'-TCGCCCTCGAACTTCACCTC-3'; GFP probe, 5'-CATGCCCGAAGGCTACGTCCAGGAG-3'; hCDMP-2 forward, 5'-CAGAAAGTATTTGTTTGATGTGTCCA-3' and reverse: 5'-AAAGGCAAGGGAAGAGCTGCA-3'; and hCDMP-2 probe, 5'-CAGACAAAGAAGAGCTGGTGGCGC-3'.

Western blot analysis. Western blot analyses were performed with total protein extracts prepared using T-PER[®] tissue protein extraction reagent (Pierce). Filters were probed with anti-CDMP-2 (mouse anti-human; Sigma).

In vivo repair of meniscal fibrocartilage injury in canines. The PLA/PGA (Sigma) scaffold was made using a specific mold. The canine model of meniscal injury was established by cutting across the entire meniscus (0.5 cm from the front edge, with a width of 2 mm, and a thickness of 2 mm at the meniscocapsular junction and 1.5 mm at the other side) (Fig. 1A). The injury encompassed all three zones (the red-red zone, red-white zone and white-white zone). The animals were divided into four groups: Group A, suture only; group B, suture with the addition of the recombinant hCDMP-2 on the PLA/PGA scaffold; group C, PLA/PGA scaffold with canine myoblasts carrying the empty vector; D, PLA/PGA scaffold with canine myoblasts expressing hCDMP-2 (six menisci for each group) (Fig. 1B and C). Samples of repaired tissue extracted at weeks 3, 8 and 12 post-repair were subjected to gross morphological studies and hematoxylin and eosin (H&E) and Safranin-O staining. Immunohistochemistry (IHC) studies were performed to assess the expression of collagen I and II and S-100 protein. Quantitative assays were also performed

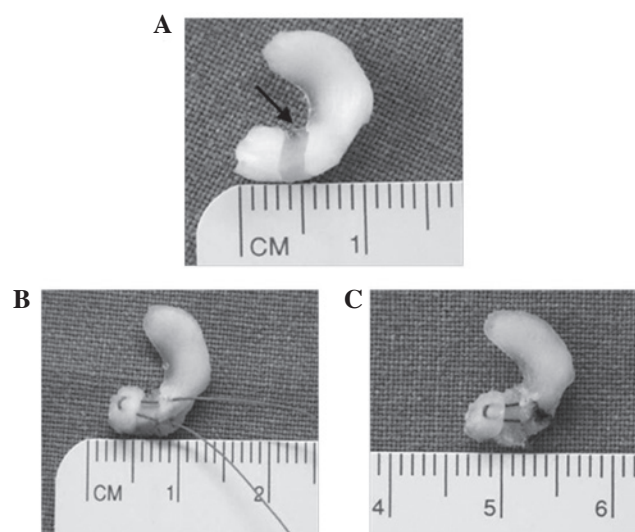


Figure 1. (A) Model of canine meniscal injury, indicated by arrow. (B) Treatment delineation of group A. (C) Treatment delineation of groups B-D. Group A, suture only; group B, suture with added recombinant hCDMP-2 on polylactic acid/polyglycolic acid (PLA/PGA) scaffold; group C, PLA/PGA scaffold with canine myoblasts carrying the empty vector; and group D, PLA/PGA scaffold with canine myoblasts expressing hCDMP-2. hcdmp-2, human cartilage-derived morphogenetic protein-2.

to assess the levels of collagen I and II (by ELISA), and the glycosaminoglycans (GAGs) (by Alcian Blue staining) in the red-red and white-white zones. The normal value was obtained by assessing the meniscal tissue at the uninjured part using the same methods as those used for the repaired tissue.

Statistical analysis. The data are expressed as the mean $\pm$ standard deviation. Statistical significance was determined using one-way analysis of variance and the level of statistical significance was set at a probability value of $P < 0.05$.

Results

Expression of hCDMP-2 in canine myoblasts following lentiviral infection. The infection efficiency was determined to be $71 \pm 0.088\%$ by qPCR, and the expression level of hCDMP-2 was 0.0015 ± 0.0005 . Western blot analysis confirmed the expression of hCDMP-2 protein upon lentiviral infection of the canine myoblasts.

H&E and Safranin-O staining for the evaluation of in vivo meniscal fibrocartilage injury repair. H&E and Safranin-O staining showed partial staining of the repaired tissue in group D at week 3 post-repair. A few cartilage lacunae mixed with the scaffold tissue were observed in the red-red zone, while no staining was observed in the white-white zone (Fig. 2A and B). At week 8, H&E staining showed increased cartilage lacunae in the red-red zone of group D, and fibrous structures started to appear (Fig. 2C), while a small amount of cartilage lacunae without fibrous structures were observed in the white-white zone (Fig. 2D). The red-red zone showed an increased intensity and volume of cartilage lacunae (Fig. 2E), while the white-white zone showed partial staining with Safranin-O (Fig. 2F). At week 12 post-repair, H&E staining showed obvious cartilage lacunae in the red-red zone, with increased surrounding fibrous

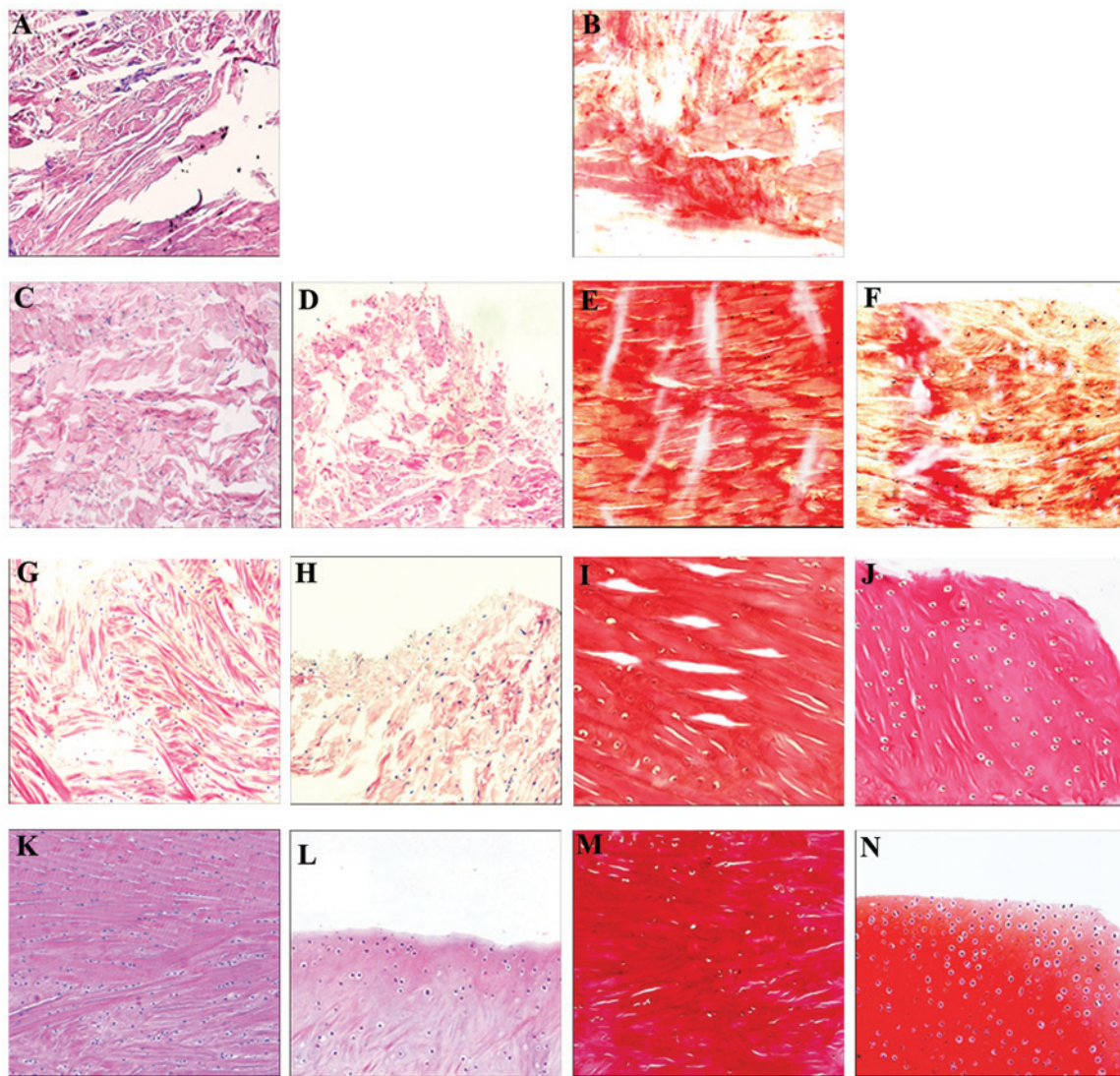


Figure 2. Morphology of the repaired tissue at the red-red zone in treatment group D at (A and B) week 3, (C and E) week 8 and (G and I) week 12 post-repair (A, C and G, H&E staining; B, D and I, Safranin-O staining; magnification, x100). Morphology of the repaired tissue at the white-white zone in group D at (D and F) week 8 and (H and J) week 12 post-repair (D and H, H&E staining; F and J, Safranin-O staining; magnification, x100). (K and L) H&E staining of the normal meniscus at the red-red and the white-white zones, respectively (magnification, x100). (M and N) Safranin-O staining of the normal meniscus at the red-red and the white-white zones, respectively. H&E, hematoxylin and eosin.

structures (Fig. 2G), while the presence of cartilage lacunae was also increased in the white-white zone, with no fibrous structures (Fig. 2H). Safranin-O staining showed the increased intensity and volume of the regenerated tissues in the red-red and the white-white zones at week 12 (Fig. 2I and J).

Examination of the protein expression of collagen I, collagen II and S-100. IHC studies revealed low expression levels of the cartilage-specific proteins, collagen II, collagen I and S-100, in the red-red zone in group D at week 3 post-repair, while no expression was observed in the white-white zone (Fig. 3A-C). At week 8 post-repair, positive expression of these proteins was detected in the repaired tissue in the red-red and white-white zones (Fig. 3D-I). At week 12, the expression of these proteins remained positive in the two zones (Fig. 3J-O).

Quantitative assessment of collagen I, collagen II and GAG proteins. The expression of collagen I and II was further

quantified by ELISA, while the GAGs were assessed by Alcian blue staining. At week 3 post-repair, the expression of collagen I, collagen II and the GAGs was detected in the repaired tissue of the red-red zone, while no expression of these proteins was observed in the white-white zone (Tables I-III). At week 8, the expression of these proteins was detected in the red-red and white-white zones (Fig. 4). Similar results were obtained at week 12 (Fig. 4). Statistical analyses indicated that the expression of these three proteins increased with increasing time, and the differences between each pair of time-points were significant ($P < 0.01$). In addition, significant differences were revealed between the red-red and white-white zones at the same time-point ($P < 0.01$).

Discussion

To facilitate the repair of injury to meniscal fibrocartilage, which lacks a sufficient blood supply, numerous methods have been assessed in the clinic, including the establishment of a

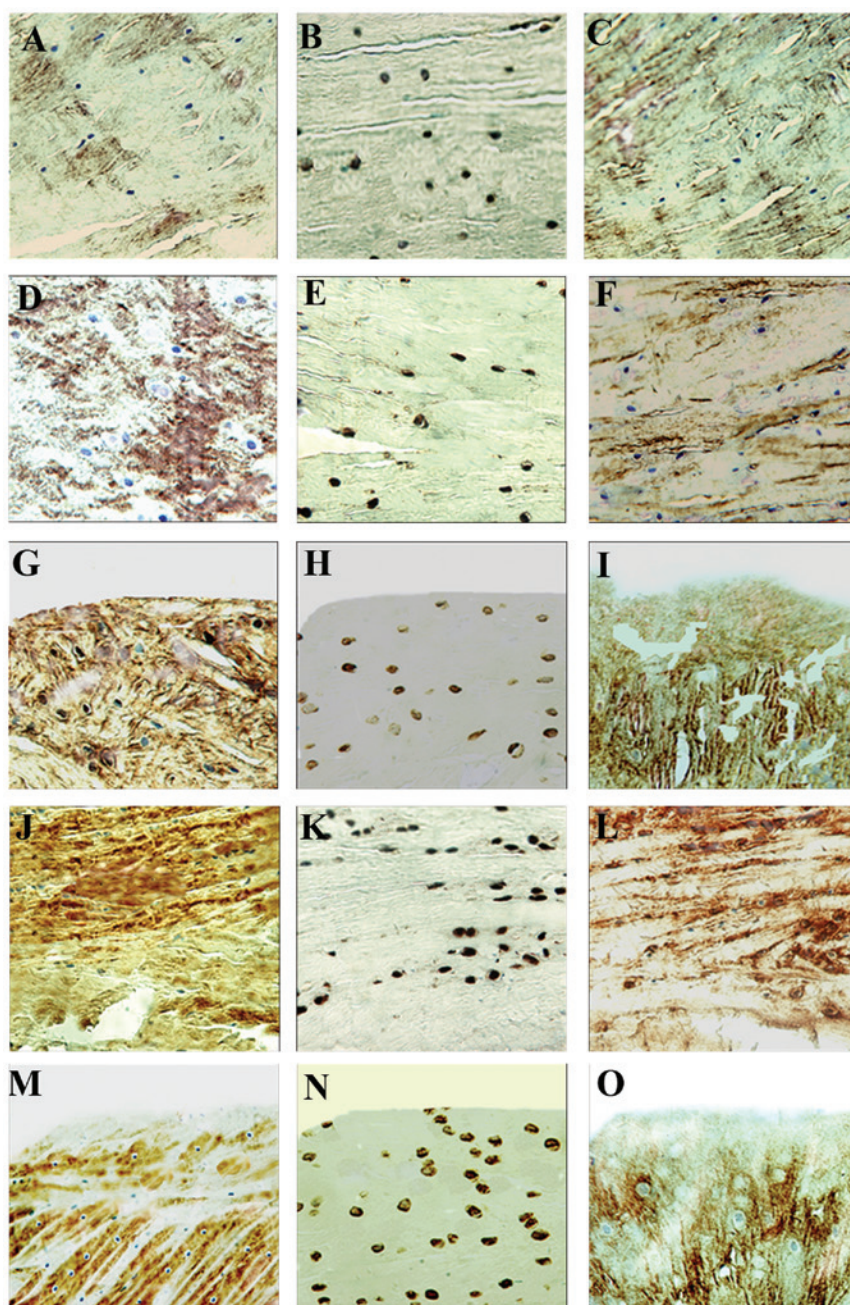


Figure 3. Expression of collagen-II, S-100 and collagen-I in the repaired tissue at the red-red zone in treatment group D at (A-C) week , (D-F) week and (J-L) week 12 post-repair (immunohistochemical staining; magnification, x100). Expression of these proteins at the white-white zone in treatment group D at (G-I) week 8 and (M-O) week 12 post-repair (immunohistochemical staining; magnification, x100).

blood supply channel, blood clots and implantation of pedunculated synovial flaps, isolated synovia, porous polymers and fibrin clots (8,9). In recent years, certain studies have employed purified growth factors, including epidermal growth factor, TGF, platelet-derived growth factor, basic fibroblast growth factor, hepatocyte growth factor, bone morphogenetic protein 2 and interleukin 1 to facilitate the *in vitro* expansion of chondrocytes from meniscal fibrocartilage (10,11). Certain studies have used gene transfer technology to improve the *in vitro* growth of chondrocytes from meniscal fibrocartilage (12), while others have combined scaffold materials with growth factors to facilitate the healing of meniscal fibrocartilage (13). Further studies have explored stem cell technologies in combination with scaffold materials (13,15). These studies have indicated that it

is crucial to select appropriate cell types and an effective route of treatment in order to achieve the most favorable outcome for meniscal fibrocartilage injury repair.

CDMP-2 is able to facilitate cartilage repair without local tissue ossification and also to promote the ectopic generation of new tendons and ligaments (containing mainly type I collagen similar to fibrocartilage), and improve the quality of fibrocartilage tissue repair (16).

A previous study reported that myoblasts acted as adult stem cells for skeletal muscle, and that they maintained similar functions to multipotent mesenchymal cells that exhibit myogenic, osteogenic and adipogenic differentiation (17). Myoblast gene therapy has been successful in the treatment of muscle diseases and bone and joint problems.

Table I. Expression of collagen I (ng/ml) at the red-red and white-white zones in group D.

Zone	Time post-repair			Normal
	3 weeks	8 weeks	12 weeks	
Red-red	91.238±4.623 ^{a,b}	205.719±3.180 ^{a,b}	592.213±7.979 ^{a,b}	996.228±2.691 ^{a,b}
White-white	0.000±0.000	75.125±3.098 ^b	105.300±3.105 ^b	501.077±4.768 ^b

^aP<0.01 comparing red-red and white-white zones at the same time-point; ^bP<0.01 comparing the same zone at different time-points.

Table II. Expression of collagen II (ng/ml) at the red-red and white-white zones in group D.

Zone	Time post-repair			Normal
	3 weeks	8 weeks	12 weeks	
Red-red	51.030±2.905 ^{a,b}	79.820±4.720 ^{a,b}	100.215±3.790 ^{a,b}	187.119±7.288 ^{a,b}
White-white	0.000±0.000	62.915±3.330 ^b	85.752±3.122 ^b	279.182±6.173 ^{a,b}

^aP<0.01 comparing red-red and white-white zones at the same time-point; ^bP<0.01 comparing the same zone at different time-points.

Table III. Expression of GAGs (μg/ml) at the red-red and white-white zones in group D.

Zone	Time post-repair			Normal
	3 weeks	8 weeks	12 weeks	
Red-red	1.758±0.125 ^{a,b}	2.626±0.067 ^{a,b}	3.365±0.121 ^{a,b}	5.019±0.178 ^{a,b}
White-white	0.000±0.000	1.696±0.106 ^b	2.807±0.070 ^b	9.688±0.320 ^b

GAGs, glycosaminoglycans. ^aP<0.01 comparing red-red and white-white zones at the same time-point; ^bP<0.01 comparing the same zone at different time-points.

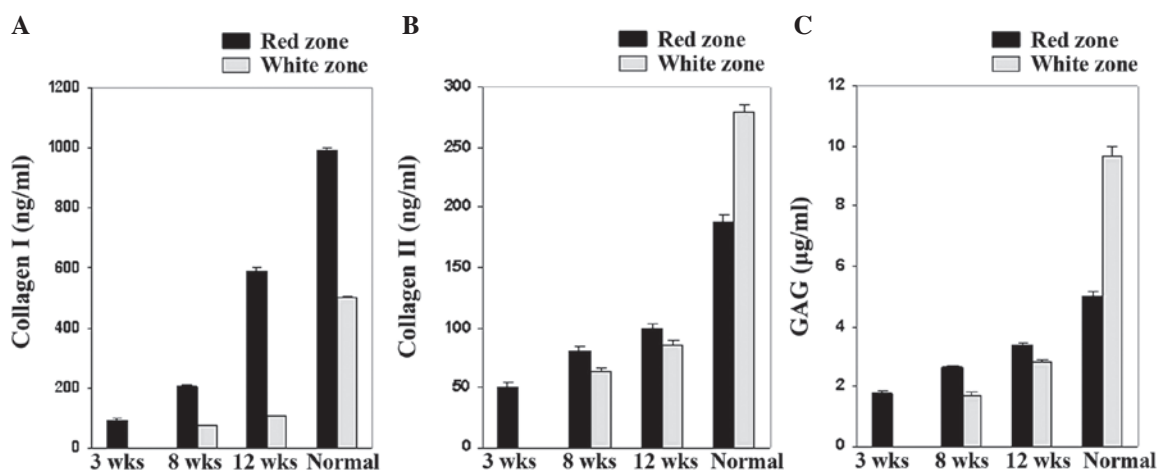


Figure 4. Expression of (A) collagen I, (B) collagen II and (C) GAGs at the red-red and white-white zones in treatment group D at the varying time-points post-repair. GAGs, glycosaminoglycans.

The present study showed that canine myoblasts are able to produce hCDMP-2 RNA and protein *in vitro* following lentiviral infection with the hCDMP-2 gene. The infected canine myoblasts were transplanted into the meniscal injury site via a

PLA/PGA scaffold in order to observe their effect on meniscal injury repair. H&E histology studies and Safranin-O staining, which assess cartilage GAGs in cells or tissue, showed that the treatments in groups A, B and C did not lead to the regeneration

of new tissue in the canine meniscal injury region. By contrast, group D showed newly repaired tissue in different regions (red-red and white-white zones) with the characteristics of fibrocartilage tissue, demonstrating that the treatment of group D was able to promote the meniscal repair by fibrocartilage-like tissue regeneration. Furthermore, quantitative assessment of the fibrocartilage tissue-specific components, including collagen I, collagen II and GAGs, indicated that the red-red zone was regenerated more rapidly than the white-white zone during the fibrocartilage-like tissue repair of meniscal injury. This is consistent with the previous hypothesis, and may be due to the differences in the blood supply of these two regions.

In all four groups, the PLA/PGA scaffold completely dissolved at 8 weeks post-transplantation, indicating that the PLA/PGA scaffold used in this study did not affect meniscal injury repair. The scaffold material can be degraded *in vivo*, and the time for the complete degradation is approximately eight weeks.

Meniscal fibrocartilage has the characteristics of cartilage tissue as well as fibrous tissue. The white-white zone is more similar to the cartilage tissue (18), while the red-red zone resembles the fibrous tissue (19). Collagen fibers in the red-red zone are generally distributed longitudinally in a horizontal plain and aligned in a 'C' shape, while collagen fibers in the white-white zone exhibit a radial distribution (20). The IHC results of the present study revealed that the regenerated collagen fibers in the red-red zone exhibited a 'C'-shaped distribution. Collagen I was aligned along the fibers, while collagen II was scattered irregularly among the mesenchyma of the tissue irrelevant to the fiber alignment. These observations are consistent with the previous description of the histology and structure of the meniscal fibrocartilage, indicating that the treatment adopted in the present study resulted in the meniscal regeneration by tissue that was similar to normal meniscal fibrocartilage tissue. Furthermore, a previous study indicated that the structure and components of the red-red and white-white regions are different from each other, as the former contains collagen I, which forms large beam fibers, while the latter contains high levels of collagen II and proteoglycans (21). These components have distinct functions to confer meniscal biomechanical properties. The content proportion of collagen I and II and the GAGs in the normal meniscal tissue obtained in the present study was similar to that of previous studies. However, the amounts and distribution of each component of the repaired tissue from group D showed differences from the normal tissue. For example, the red-red zone showed higher levels of the assessed proteins as compared to the white-white zone at all the examined time-points. Further evaluation is, however, required to assess whether these differences are capable of affecting the function of meniscal fibrocartilage.

The present study indicated that myoblasts infected with the hCDMP-2 gene are able to facilitate the regeneration of meniscal fibrocartilage-like tissue in the canine model of local meniscal injury, and that the repair of the red-red zone is more rapid than that of the white-white zone. Further studies are required to address the mechanism of the hCDMP-2-mediated effect on meniscal fibrocartilage injury repair. In addition, due to the limi-

tation in the clinical use of lentiviral infections, further studies are required to explore the best way to combine the hCDMP-2 growth factor with myoblasts to be used in the clinic.

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