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Pretreatment of periosteum with TGF-β1 *in situ* enhances the quality of osteochondral tissue regenerated from transplanted periosteal grafts in adult rabbits

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SUMMARY

Objective: To compare the efficacy of *in situ* transforming growth factor-beta1 (TGF-β1)-pretreated periosteum to untreated periosteum for regeneration of osteochondral tissue in rabbits.
Methods: In the pretreatment group, 12 month-old New Zealand white rabbits received subperiosteal injections of 200 ng of TGF-β1 percutaneously in the medial side of the proximal tibia, 7 days prior to surgery. Control rabbits received no treatment prior surgery. Osteochondral transverse defects measuring 5 mm proximal to distal and spanning the entire width of the patellar groove were created and repaired with untreated or TGF-β1-pretreated periosteal grafts. Post-operatively the rabbits resumed normal cage activity for 6 weeks.
Results: Complete filling of the defects with regenerated tissue was observed in both the TGF-β1-pretreated and control groups with reformation of the original contours of the patellar groove. The total histological score (modified O'Driscoll) in the TGF-β1-pretreated group, 20 (95% Confidence Interval (CI), 19–21), was significantly higher ($P = 0.0001$) than the control group, 18 (16–19). The most notable improvements were in structural integrity and subchondral bone regeneration. No significant differences in glycosaminoglycan or type II collagen content, or equilibrium modulus were found between the surgical groups. The cambium of the periosteum regenerated at the graft harvest site was significantly thicker ($P = 0.0065$) in the TGF-β1-pretreated rabbits, 121 μm (94–149), compared to controls, 74 μm (52–96), after 6 weeks.
Conclusions: This study demonstrates that *in situ* pretreatment of periosteum with TGF-β1 improves osteochondral tissue regeneration at 6-weeks post-op compared to untreated periosteum in 12 month-old rabbits.

Introduction

While tissue engineering and regenerative medicine approaches are very promising, their full potential has yet to be realized, especially in the area of cartilage repair. In this pursuit, the use of autologous cell sources such as cartilage, bone marrow, perichondrium, synovium, adipose, and periosteum have been explored[1–8]. There are advantages and disadvantages associated with each of these autologous sources, but the most efficacious cell source for durable cartilage repair is yet to be determined. Ultimately, randomized prospective clinical studies may provide a definitive answer[9]. However, it is more likely that an individualized approach to cartilage regeneration will be needed, which will require the availability of multiple treatment options. Therefore, it is important to continue to use preclinical models to investigate the optimal conditions for cartilage regeneration with each available cell source.

The clinical potential of conventional periosteal transplantation, which is performed using an unaltered periosteal graft, has been reported with varying results[10–19]. However, the regenerative capacity of all periosteum is not equal. It is dependent on the number of mesenchymal stem cells (MSCs) in the tissue which varies depending on the graft donor site[20], and the age of the patient or laboratory animal[21–24]. This is likely to explain many of

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the inconsistencies in reported results[10–13]. Despite these facts, few attempts to optimize the regenerative capacity of periosteum prior to tissue harvest have been reported[25–28].

Previously, we reported the effect of age on cambium cellularity and *in vitro* periosteal chondrogenesis in rabbits[23]. In that study, the number of cambium cells and the amount of cartilage formed *in vitro* from the periosteum were significantly decreased in 6, 12, and 24 month-old rabbits compared to 1.5–2 month-old rabbits[23]. We subsequently demonstrated that it is possible to significantly increase the number of cambium cells and the amount of cartilage formed *in vitro* from periosteum in 6, 12, and 24 month-old rabbits using local subperiosteal injection of transforming growth factor-beta1 (TGF-β1) with or without Insulin-like growth factor-I (IGF-1)[28]. In that study, the effect of the growth factor injections was dependent on the type of growth factor, the concentration and the amount of time between injection and tissue harvest[28]. In 12 month-old rabbits, the greatest response was observed when periosteum was harvested 7 days after the injection of 200 ng TGF-β1, which resulted in more than a 4-fold increase in cambium cellularity and over a 2-fold increase in cartilage production *in vitro*[28]. The values for cambium cellularity and cartilage yield after 200 ng TGF-β1 injection in 12 month-old rabbits were similar to values previously obtained from rabbits between 2 and 4 months old[23,28]. Therefore, in terms of the number of cambium cells present and the ability of the periosteum to form cartilage, the periosteum from older rabbits can be rejuvenated by local injection of TGF-β1[28]. In addition, subperiosteal TGF-β injection results in increased extracellular matrix production and initiation of cartilage formation[28–30]. Thus, after injection with TGF-β1, the periosteal graft is primed for transplantation *in vivo* by increasing cell number and initiating chondrogenesis prior to harvest.

Therefore, we hypothesized that *in situ* pretreatment of periosteum with TGF-β1 would improve the outcome of periosteal transplantation for osteochondral tissue regeneration. In this study, we compared the efficacy of TGF-β1-pretreated periosteal grafts vs untreated control grafts to regenerate osteochondral tissue in the patellar groove of 12 month-old rabbits using a previously established model[21,22].

Methods and materials

Study design

All work in this study was conducted with the approval of the Mayo Clinic Institutional Animal Care and Use Committee. A total of 19 New Zealand white rabbits (12 months old) were used in this study. The nine pretreated rabbits received subperiosteal injections of TGF-β1 in the medial side of the left proximal tibia 7 days prior to periosteal transplantation surgery. The 10 control rabbits received no pretreatment prior to surgery. Osteochondral defects were created and repaired based on a previously established model[21,22]. In order to mimic the clinical approach, as previously described[31], the donor periosteal grafts were harvested from the same limb as the recipient joint. After 6 weeks, the operated (left) and contralateral control knees were harvested and cut in half along the patellar groove. One half was processed for histology and analyzed using a modified ÓDriscoll histological score[22,32] and the International Cartilage Repair Society Score (ICRS) system[33]. The remaining half underwent mechanical testing followed by biochemical analyses for DNA content, collagen type II formation and glycosaminoglycan (GAG) content.

In situ periosteal pretreatment

Seven days prior to surgery, rabbits in the pretreatment group received subperiosteal injections of TGF-β1 as previously described[28]. Briefly, under general anesthesia, intravenous injection of acepromazine (0.75 mg/Kg), xylazine (5 mg/Kg), and ketamine (50 mg/Kg), the left legs of the rabbits were shaved and prepared with surgical scrub. Four injections (10 μl) of 200 ng TGF-β1 (R&D Systems, Inc., Minneapolis, MN) were then made percutaneously using a Hamilton syringe with a 30-gauge needle under the periosteum of the medial proximal tibia. The injections were standardized using the distal edge of the tibial tuberosity as a landmark. The first injection was always made 5 mm proximal to the distal edge of the tibial tuberosity. The four injections were distributed evenly within the 5×10 mm area of the medial proximal tibia to be used as the periosteal graft harvest site in the subsequent periosteal transplantation procedure.

Osteochondral defect

All operative procedures were performed under general anesthesia, which was induced by intravenous injection of acepromazine (0.75 mg/Kg), xylazine (5 mg/Kg), and ketamine (50 mg/Kg). With the rabbit in the supine position, one hind limb (left one) was shaved from the hips to the ankles, prepared with Techni-care scrub (Care-Tech® Laboratories, Inc., St. Louis, MO), and draped. The knee joint and the proximal tibiae were exposed by a 6 cm anterior incision and the joint was opened by a 3 cm transvastus approach, which allowed the patella to be dislocated laterally, gaining full access to the patellar groove. An osteochondral transverse defect, measuring 5 mm proximal to distal (Fig. 1), and spanning the entire width of the patellar groove, as previously described[22,34], was created using a scalpel and a conventional jewelry saw (Fig. 1). Measurements of all areas in the defects were done with a Vernier caliper. In contrast to the original contour of the patellar groove, the base of the defect was flattened with

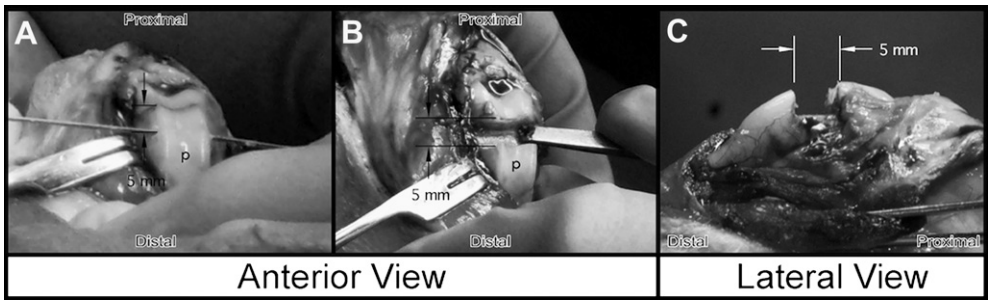


Fig. 1. An osteochondral transverse defect, measuring 5 mm proximal to distal and spanning the entire width of the patellar groove, was created using a scalpel and a conventional jewelry saw. Measurements of all areas in the defects were done with a Vernier caliper. In contrast to the original contour of the patellar groove, the base of the defect was flattened with a 5 mm wide file and was approximately 2 mm below the surface of the middle of the patellar groove.

a 5 mm wide file (Fig. 1) and was approximately 2 mm below the surface of the middle of the patellar groove. The depth at the medial and lateral edges was approximately 3–4 mm. A portable drill and a 0.64 mm drill bit were used to make a hole in each corner of the base of the defect and in the lateral and medial cortices adjacent to the defect for fixation of the periosteal graft as described below.

Periosteal transplantation

Using the same skin approach, the deep fascia overlying the medial aspect of the proximal tibia was incised and retracted. In the regions that were injected with TGF-β1, there was significant swelling and edema. In order to reach the plane of the periosteum, the edematous tissue was first removed. The distinct morphology of this inflammatory tissue and the use of surgical loupes allowed this to be done in a controlled manner without damaging the underlying periosteum. Once exposed, a rectangular graft of periosteum corresponding to the required size of the osteochondral defect was elevated as previously described[35]. Control rabbits received a periosteal graft that was not pretreated. The final thickness of the periosteal grafts were approximately 2 mm for the TGF-β1-pretreated grafts and 1 mm for the control grafts as judged by their position in the base of the osteochondral defects which were 3 mm deep at their shallowest point. In both surgical groups, the periosteal graft was placed on the base of the defect with the cambium layer facing up into the joint and secured to the base of the defect by passing a 4–0 Vicryl (polyglactin 910) suture through the holes made in the corners of the defect. After having sutured the ends of the graft on each side of the femur, the patella was relocated. Hemostasis was obtained with a cautery coagulator, and the joint was irrigated with normal saline prior to closing the articular capsule. The arthrotomy and fascia-muscle wounds were closed with single stitches of 4–0 Vicryl (polyglactin 910) suture and single knots of 3–0 Vicryl suture respectively. Once the arthrotomy was closed, passive flexion and extension movements were performed and patellar tracking was checked for lateral dislocations or excessive tension that could alter the articulation between the graft and the patellar joint surface. The remaining soft tissue from the periosteal graft donor site was repaired using a continuous locking 4–0 Vicryl suture. Inverted knots of 3–0 Vicryl suture were placed in the subcutaneous tissue to get a more secure closing. The skin was closed with subcuticular 3–0 Vicryl suture and the end knots were inverted and hidden under the skin. The wound was cleaned with saline and spray bandage was applied (Bard® Protective Barrier Film, C.R. Bard, Inc., Convington, GA). To alleviate post-operative pain, the rabbits received a single intramuscular injection of buprenorphine (0.1 mg/Kg). In order to avoid wound infection and as a prophylaxis of pasteurella pneumonia every animal received a single intramuscular injection of enrofloxacin (Baytril) at 5 mg/Kg. After surgery rabbits were allowed normal cage activity for 6 weeks. At the end of the experiment, the rabbits were sacrificed by an overdose of intravenous sodium pentobarbital.

Gross and histological analyses

The defect repair and corresponding contralateral sites were documented by digital photography. The defect repair and contralateral control sites were then excised. The osteochondral specimens were cut in half sagittally along the center of the patellar groove. Half of the specimens (divided equally between the medial and lateral specimens) were prepared for histology while the other half were analyzed mechanically and biochemically. The periosteal graft harvest sites and underlying bone were also harvested for histology. The specimens for histology were fixed for 2 days in 10% formalin followed by 2 days in Bouin's solution, decalcified in 10% ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, and sectioned at 5 μm. The osteochondral samples from the patellar groove were stained with safranin O/fast green. The periosteal graft harvest site samples were stained with haematoxylin and eosin (H & E). Three blinded observers scored the osteochondral samples using the O'Driscoll score modified to include scores for the regenerated bone tissue[22,32] (Table I). A blinded observer histologically measured the thickness of periosteum in the periosteal graft harvest site samples using an automated system as reported previously[28]. Briefly, three sections were obtained from each tissue sample and scanned at the periosteal harvest site using the Zeiss AuxioCam MRc at the same magnification with the periosteal layer line horizontal to the bottom edge of the scan's frame of reference. The cambium layer was outlined by hand. Maximum thickness was determined by comparing thicknesses in the outlined area from top to bottom.

Mechanical testing

Mechanical indentation testing was performed on the osteochondral samples using a method similar to Duda *et al.*[36]. The samples were placed, unconstrained, on a flat dish with the cartilage surface perpendicular to the 1.6 mm cylindrical indenter. Loading was applied at a rate of 5 N/min to a maximum force of 5 N (maximum applied stress = 2.5 MPa). A Dynamic Mechanical Analyzer (TA Instruments, New Castle, DE) was used to apply the load. The 5 N load limit compressed the cartilage beyond the toe region of the stress–strain curve. During testing the specimens were immersed in saline. Applied stress was calculated by dividing the applied force by the area of the indenter. Strain was defined as the indentation displacement divided by the original cartilage thickness in the tested area. After the biomechanical testing, cartilage was removed using a number 11 scalpel blade. Longitudinal incisions were made perpendicular to its surface down to the calcified cartilage zone. The cartilage tissue between these two cuts was excised by cutting along the calcified cartilage zone. The tissue was then weighed using a Mettler AT261 DeltaRange® balance accurate to 0.01 mg (Mettler-Toledo, Inc. Columbus, Ohio) and half of the sample was analyzed for percent collagen type II, while the other half was analyzed for DNA content, and GAG content.

Collagen typing

Quantitative collagen typing was performed using a published technique for measuring the relative amount of type II collagen with respect to type I collagen in tissue samples[37]. This technique has been modified to permit the analysis of very small samples (1–10 mg) without initial purification of the collagen[38]. Samples were weighed, and the collagen peptides were cleaved with 0.5 ml 5% cyanogen bromide (CNBr) in deaerated 88% formic acid. In preparation for electrophoresis, the samples were dissolved in a sample buffer containing 0.063 M Tris–HCL, pH 8, 3.3% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, at a concentration of 8 μg (wet weight) of sample per microliter of sample buffer. A 1 μl volume of sample was loaded onto 20% gels, and SDS-polyacrylamide gel electrophoresis was carried out using a Phast System (Pharmacia LKB, Uppsala, Sweden). As a control, cartilage was harvested from the patellar groove of the contralateral limb and analyzed in the same manner as the repair tissue. These control samples were run alongside the repair tissue samples and served as a standard to ensure that the banding pattern for 100% type II collagen was accurately represented and could be located for each experimental sample. The gels were stained with Coomassie blue and scanned on a laser densitometer (Pharmacia LKB). The percentage of type II collagen with

Table I
Detailed breakdown of the histological scoring (modified O'Driscoll) results and comparison between the defect repairs in the untreated controls and TGF-β1-pretreatment groups. The scores are the mean values from three blinded observers for the number of specimens receiving the designated score. Mean percentages of the total number of samples receiving a specific score are shown in parentheses. Scoring categories that were significantly different ($P < 0.05$) are marked with * and shown in bold

		Possible score	TGF-β1 pretreated ($n = 9$)	Untreated control ($n = 9$)
Original O'Driscoll histological score				
Cellular morphology	Hyaline articular cartilage	4	2.3 (26%)	2 (22%)
	Incompletely differentiated mesenchyme	2	6.6 (73%)	6.3 (70%)
	Fibrous tissue or bone	0	0 (0%)	0.6 (7%)
Safranin-O staining	Normal or nearly normal	3	1.3 (14%)	1.6 (18%)
	Moderate	2	4.6 (51%)	5.3 (59%)
	Slight	1	3 (33%)	2 (22%)
	None	0	0 (0%)	0 (0%)
Surface regularity	Smooth and intact	3	5 (56%)	4 (44%)
	Superficial horizontal lamination	2	2 (22%)	1 (11%)
	Fissures-25–100% of the thickness	1	2 (22%)	2.6 (29%)
	Severe disruption, including fibrillation	0	0 (0%)	1.3 (14%)
Structural integrity* ($P = 0.0003$)	Normal	2	**6 (66%)**	**3 (33%)**
	Slight disruption, including cysts	1	**3 (33%)**	**2.6 (29%)**
	Severe disintegration	0	**0 (0%)**	**3.6 (40%)**
Thickness	100% of normal adjacent cartilage	2	9 (100%)	9 (100%)
	50–100% of normal cartilage	1	0 (0%)	0 (0%)
	0–50% of normal cartilage	0	0 (0%)	0 (0%)
Bonding to adjacent cartilage* ($P = 0.0047$)	Bonded at both ends of graft	2	**1.6 (18%)**	**1 (11%)**
	Bonded at one end, or partially at both ends	1	**6.3 (70%)**	**4.6 (51%)**
	Not bonded	0	**1 (11%)**	**3.3 (37%)**
Hypocellularity* ($P = 0.0015$)	Normal cellularity	3	**4 (44%)**	**2.3 (26%)**
	Slight hypocellularity	2	**3.3 (37%)**	**2 (22%)**
	Moderate hypocellularity	1	**1.6 (18%)**	**3.6 (40%)**
	Severe hypocellularity	0	**0 (0%)**	**1 (11%)**
Chondrocyte clustering	No clusters	2	1.6 (18%)	2.6 (29%)
	<25% of the cells	1	5.3 (59%)	5 (56%)
	25–100% of the cells	0	2 (22%)	1.3 (14%)
Freedom from degeneration of adjacent cartilage	Normal cellularity, no clusters, normal staining	3	0.6 (7%)	2 (22%)
	Normal cellularity, mild clusters, moderate staining	2	4.3 (48%)	4.6 (51%)
	Mild or moderate hypocellularity, slight staining	1	3.6 (40%)	1.6 (18%)
	Severe hypocellularity, poor or no staining	0	0.3 (3%)	0.6 (7%)
Scoring criteria for bone layer reconstruction				
Subchondral bone alignment	Level	2	0.3 (3%)	0 (0%)
	Depressed	1	8.6 (97%)	9 (100%)
	Elevated or none	0	0 (0%)	0 (0%)
Bone integration* ($P = 0.0001$)	Integrated	2	**3.6 (40%)**	**2 (22%)**
	Partially integrated	1	**5 (56%)**	**4 (44%)**
	Not integrated	0	**0.3 (3%)**	**3 (33%)**
Bone infiltration into defect area* ($P = 0.002$)	Nearly complete or complete	2	**0.3 (3%)**	**0 (0%)**
	Partial	1	**8 (89%)**	**4 (44%)**
	None or minimal	0	**0.6 (7%)**	**5 (56%)**
Tidemark continuity* ($P = 0.0059$)	Present, no gaps	2	**2.6 (29%)**	**0.3 (3%)**
	Present, with gaps	1	**3.3 (37%)**	**2.6 (29%)**
	Absent	0	**3 (33%)**	**6 (66%)**

respect to type I collagen was determined by measuring the ratio of the α1(II)CB10 to the α1(I)CB7, 8 and α1(II)CB11 peaks in each lane[37,38].

DNA and GAG content

After being weighed, the cartilage tissue samples to be analyzed for DNA and GAG content were digested in 1 ml of 50 μg/ml proteinase K in 100 mM K_2HPO_4 (pH 8) at 60°C for 16 h. The reaction was stopped by placing the samples in a 90°C water bath for 10 min. The resulting digest was used for both the DNA and GAG assays. Double stranded DNA (dsDNA) content was determined using Quant-iT PicoGreen dsDNA Kit (invitrogen Eugene, OR) with a Fluorostar Plate Reader (BMG Labtechnologies, Offenburg, Germany). GAG content was determined using the dimethylmethylene blue assay (DMMB, Blyscan™ Sulfated Glycosaminoglycan Assay Kit; Biocolor Ltd., NI, UK). GAG content was normalized to dsDNA content. All samples were analyzed in duplicate and their values averaged.

Statistical analysis

In this study, the analysis outcomes included quantitative GAG content, collagen typing, cartilage scoring, and mechanical properties of the newly formed repair tissue. The experimental factor was treatment (TGF-β1 pretreated vs control periosteal grafts). The

data were analyzed using 2-factor analysis of variance. All statistical tests were two-sided and *P*-values less than 0.05 were considered statistically significant. Data are expressed as means with 95% Confidence Intervals (CIs).

Results

Gross observations

In order to minimize manipulation of the periosteal grafts during surgery and duration of the procedure, precise measurements of the size of the graft, and assessment of the mechanical properties of the grafts were not taken. However, the following general observations were made during the surgical procedure, which are worth noting. Consistent with our previous study[28], the TGF-β1-pretreated periosteum was obviously thicker and easier to elevate from the bone compared to control periosteum. Also, the TGF-β1-pretreated periosteum did not appear to shrink after graft harvest, while the untreated periosteum did shrink noticeably as expected. In addition, the TGF-β1-pretreated periosteum was firmer while retaining flexibility and in general was easier to handle than the control periosteum during the surgical procedure.

At 6-weeks post-op, contractures or intra-articular adhesions were not observed in the joints. Complete filling of the defects with regenerated tissue was observed in both surgical groups with integration into the surrounding tissue and reformation of the original contours of the patellar groove (Fig. 2). Also, the periosteal graft harvest site was replaced with regenerated periosteum in both the TGF-β1-pretreated and control groups.

Histological results

As illustrated in Fig. 2, neocartilage was formed in both the TGF-β1-pretreatment and control groups. However, the structural integrity, integration and bone regeneration in the repair tissue appeared to be better in the TGF-β1-pretreated group [Fig. 2(B)] compared to the control group [Fig. 2(A)]. These observations are reflected in the histological scores (Fig. 3 and Table I). The score for structural characteristics (Fig. 3), which includes scores for surface regularity, structural integrity, thickness, and bonding to adjacent cartilage, was significantly higher ($P = 0.0002$) in the TGF-β1-pretreated group, 7.2 (6.4–8.0), compared to the untreated control, 5.6 (4.8–6.3). As shown in Table I, 40% of the control samples were scored as severely disintegrated compared to none in the TGF-β1-pretreated group. The mean scores for the TGF-β1-pretreatment group were also significantly higher for structural integrity ($P = 0.0003$), bonding to adjacent cartilage ($P = 0.0047$), and hypocellularity ($P = 0.0015$).

The total bone histological score (Fig. 3) was also significantly higher ($P = 0.003$) in the TGF-β1-pretreatment group, 3.9 (3.3–4.5), compared to untreated controls, 3.0 (2.4–3.7). Within the total bone score, the TGF-β1-pretreatment group had significantly higher mean scores for integration ($P < 0.0001$), infiltration ($P = 0.002$) and tidemark continuity ($P = 0.0059$).

The total histological scores (Fig. 3) were also significantly higher ($P = 0.0001$) in the TGF-β1-pretreatment group, 20.2 (19.0–21.4), compared to the control, 17.5 (16.3–18.7). Importantly, all histological scores for both defect repair groups were significantly lower than the contralateral controls (Fig. 3). Overall, similar results were observed when the histological samples were scored using the ICRS scoring system (data not shown).

Biochemical and biomechanical results

As shown in Table II, no significant difference in DNA content was observed between the defect repair groups. However, the DNA content in both the defect repair groups was significantly higher than the respective contralateral controls ($P = 0.0002$). Likewise, no significant difference in percent collagen type II was observed between the defect repair groups (Table II). However, if pooled (untreated plus TGF-β1-pretreatment groups), the percent collagen type II in the defect repair groups was significantly lower than the contralateral controls ($P = 0.0409$). No significant differences in normalized GAG content were observed between any of the groups (Table II).

While the mean equilibrium modulus of the cartilage for the TGF-β1-pretreatment groups were higher than the controls, the differences were not statistically significant (Table II).

Cambium thickness in the regenerated periosteum

As shown in Fig. 4, 6 weeks after harvesting periosteal grafts for transplantation, the cambium layer of the regenerated periosteum in the TGF-β1-pretreatment group, 121 μm (94.1–148.5 μm), was

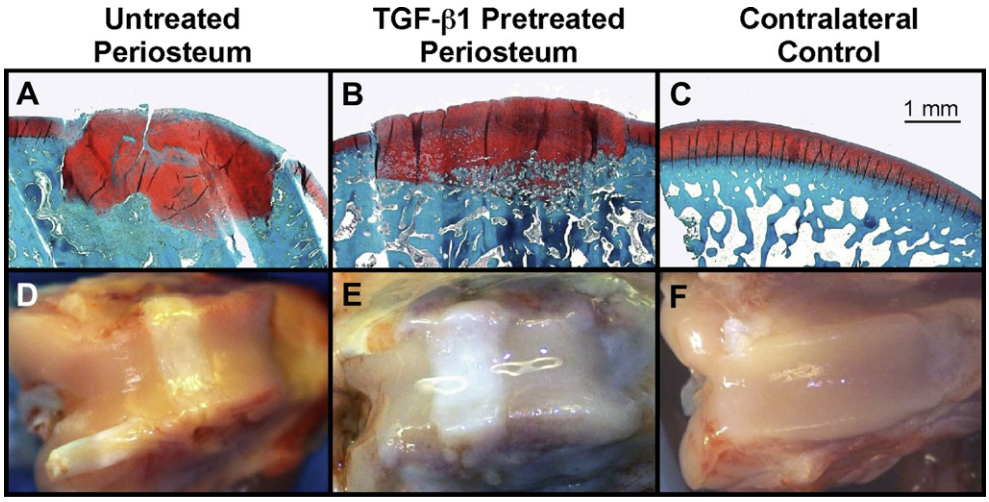
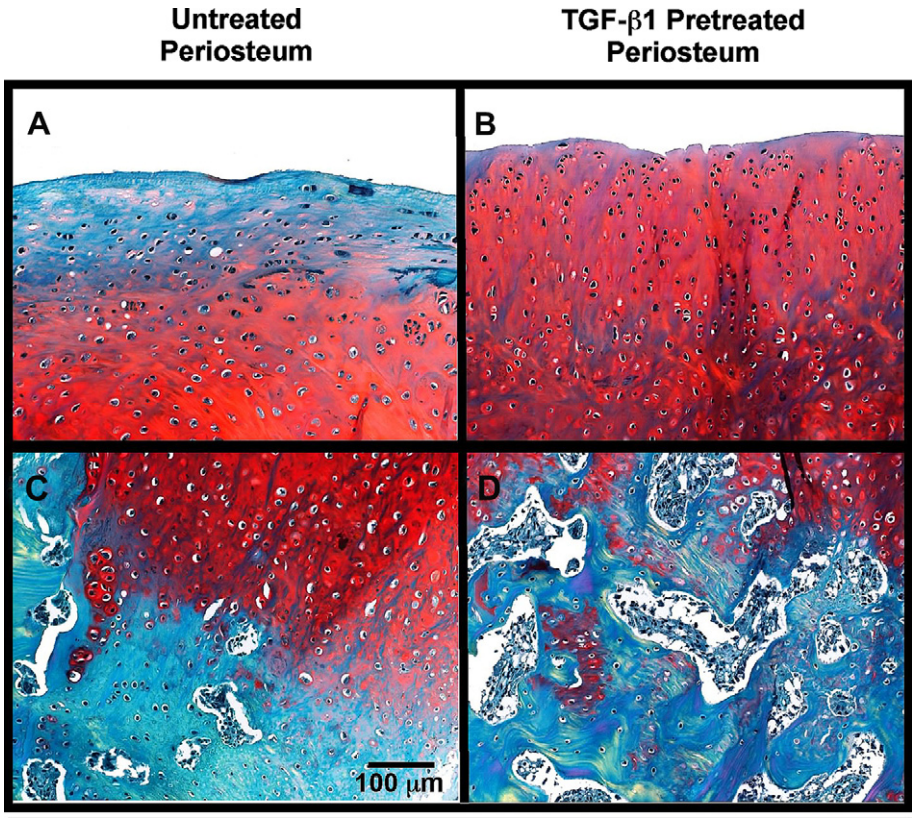


Fig. 2. Safranin O/fast green stained histological sections (A–C) and corresponding gross images (D–F) of defects repaired with untreated periosteum (A & D), TGF-β1-pretreated periosteum (B & E), and contralateral controls (C & F). The specimens are typical for each group at 6-weeks post-op. Improvements in the structural characteristics and bone regeneration in the TGF-β1-pretreatment group (B) compared to the untreated periosteum group (A) are clearly visible.



Histological Scores				
	Contralateral Control		Defect Repair	
	Untreated	TGF-β1 Pretreated	Untreated	TGF-β1 Pretreated
Cartilage Structural Characteristics	8.7 (8.3-9.2)	8.9 (8.8-9.0)	5.6 (4.8-6.3)	7.2* (6.4-8.0)
Total Bone Score	8.0 (8.0-8.0)	8.0 (8.0-8.0)	3.0 (2.4-3.7)	3.9* (3.3-4.5)
Total Histological Score	31 (30.5-31.4)	31.3 (30.8-31.8)	17.5 (16.3-18.7)	20.2* (19.0-21.4)

Fig. 3. Higher magnification images of Safranin O/fast green stained histological sections of the articular surfaces (A and B) and the base of the defects (C and D) from osteochondral defects repaired with untreated (A and C) and TGF-β1-pretreated periosteum (B and D) and histological scores at 6-weeks post-op. *Histological scores for cartilage structural characteristics ($P = 0.0002$) and total bone ($P = 0.003$), and the total histological scores ($P = 0.0001$) for the TGF-β1-pretreatment group were significantly higher than the untreated control group based on *post-hoc* testing using least squares means differences Student's *t* test. However, the scores for both of the defect repair groups were significantly lower than the contralateral controls. The scores are the mean values from three blinded observers. The data presented are means with 95% CI ($n = 9$).

significantly ($P = 0.0065$) thicker than the regenerated periosteum in the untreated control group, 74 μm (52.0–96.3 μm).

Discussion

This study demonstrates that *in situ* pretreatment of periosteum with TGF-β1 improves the quality of osteochondral tissue regenerated from transplanted periosteum *in vivo* at 6-weeks post-op compared to untreated periosteum in 12 month-old rabbits. These findings support our previous *in vitro* results demonstrating that subperiosteal injection of TGF-β1 increases the number of cambium cells in the tissue and increases *in vitro* cartilage production from periosteum explanted from adult rabbits[28]. This approach could provide a simple and effective method to partially overcome the age-related decline in the regenerative capacity of periosteum[21–24].

The main aspects of the regenerated tissue that showed improvement with TGF-β1-pretreatment were the structural characteristics of the cartilage and the bone regeneration. No significant differences were observed in the DNA content, percent type II collagen, or GAG content between the defect repair groups. Therefore, these biochemical parameters fail to explain the histological observations. We speculate that other components of the cartilage matrix that were not measured in this experiment may be differentially expressed leading to increased matrix organization and the observed improvement in structural integrity found with the TGF-β1-pretreated periosteum. Additional studies are needed to address this issue. Also, no significant increase in equilibrium modulus of the neocartilage was observed with TGF-β1-pretreated periosteum, although the means were higher. As expected, the tissue regeneration process is incomplete at 6-weeks post-op, and

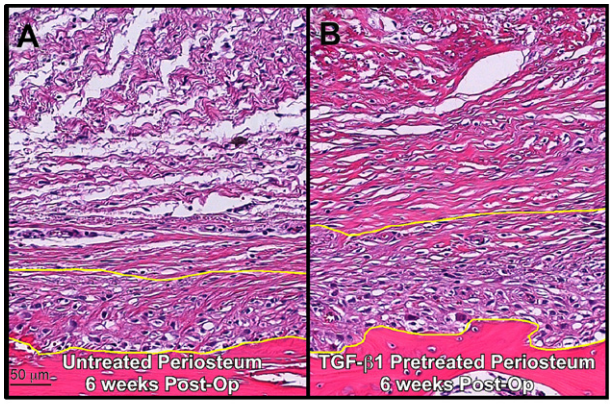
Table II
Quantitative analyses for DNA content, percent collagen type II, normalized GAG and equilibrium modulus in regenerated tissue from untreated or TGF-β1-pretreated periosteum and contralateral controls at 6-weeks post-op. No significant differences were detected between the defect repair groups. However, the DNA content in the defect repair groups was significantly higher than the contralateral controls ($P = 0.0002$), whereas, if pooled, the percent collagen type II was significantly lower in the defect repair groups ($P = 0.0409$) compared to the contralateral controls, based on *post-hoc* testing using least squares means differences Student's *t* test. The data presented are means with 95% CI ($n = 9$ or 10).

	Contralateral control		Defect repair	
	Untreated	TGF-β1 pretreated	Untreated	TGF-β1 pretreated
Biochemical and biomechanical analyses				
DNA content (μg)	0.057 (0.033–0.081)	0.055 (0.030–0.079)	0.13 (0.084–0.17)	0.13 (0.085–0.17)
Collagen type II (%)	68.0 (46.7–89.3)	77.2 (50.5–103.8)	47.0 (26.6–67.4)	53.6 (32.1–75.1)
Normalized GAG content (μg GAG/μg DNA)	95.2 (42.7–147.7)	99.3 (46.9–151.8)	109.0 (62.4–155.5)	68.8 (19.8–118.0)
Equilibrium modulus (MPa)	0.30 (0.17–0.43)	0.53 (0–1.1)	0.44 (0–0.98)	0.65 (0.13–1.2)

both defect repair groups were inferior to the normal contralateral limbs. Additional studies are currently being conducted to examine the outcome of this technique at 6 and 12 months post-op. These forthcoming results may provide insight into the maturation and durability of the regenerated tissue. It is also important to note that although TGF-β1 was not injected into the joint, we cannot rule out the possibility of a direct effect of TGF-β1 on the operated joint through systemic circulation or diffusion into the joint.

Interestingly, the regenerated periosteum from the graft harvest site in the TGF-β1-pretreatment group was significantly thicker than the control group at 6-weeks post-op. This observation suggests that the effect produced by TGF-β1 injection 7 days prior to surgery was prolonged at the graft donor site. This may be due to stimulation of endogenous TGF-β expression in the surrounding periosteum by the exogenous TGF-β1 as previously observed *in vitro*[39,40].

It is important to recognize that the post-operative care in this study was normal cage activity, which is demonstrated to be inferior to the use of continuous passive motion (CPM) for the regeneration of osteochondral tissue by periosteal transplantation in rabbits[21,22,41]. Therefore, it is conceivable that combining TGF-β1-pretreatment with CPM post-operatively might produce an added improvement in periosteal transplantation. At a minimum, producing a periosteum with a thicker cambium layer should make harvesting a periosteal graft with viable cambium cells technically easier as observed in this study. Previous studies have documented the importance of procedure-specific training for successfully harvesting chondrogenic periosteum[35]. Providing a thicker periosteum that is easier to elevate and handle during transplantation may improve the learning curve.



Cambium Thickness (μm)	
Untreated	TGF-β1 Pretreated
74.1 (52.0-96.3)	121.3* (94.1-148.5)

Fig. 4. Representative H & E stained histological sections of regenerated periosteum from the untreated (A) and TGF-β1-pretreated (B) periosteal graft harvest sites 6 weeks after graft harvest. The periosteal tissue was regenerated in both groups, however, the cambium layer in the regenerated periosteum from the TGF-β1-pretreatment group was significantly thicker than the untreated group ($P = 0.0065$), based on *post-hoc* testing using least squares means differences Student's *t* test. The cambium layers are between the yellow lines, with the bone on the bottom and the fibrous layer on the top in panels A & B. The data presented are means with 95% CI ($n = 9$ or 10).

As summarized in Table I, the subchondral bone alignment was below the level of the normal (surrounding) tissue in all samples suggested a lack of mineralization into the normal cartilage layer at this early time point. However, it has previously been documented that endochondral ossification of transplanted periosteal grafts can extend beyond the normal subchondral bone, especially in the absence of appropriate mechanical stimulation[21,22,42]. Because CPM was not used on the rabbits in this experiment, it is possible that mineralization beyond the normal subchondral bone will occur over time.

Rejuvenated periosteal grafts could be suitable in the clinical application for cartilage and bone repair not only because they meet the three primary requirements for tissue engineering[43] (i.e., provides a matrix, source of stem cells and growth factors) but also because they can be harvested as a whole autologous tissue graft with minimal morbidity at the donor site in the same surgical procedure. In addition, no *ex vivo* culture step is required for the pretreatment approach described in this study. The versatility of periosteum is also a great asset. Periosteum can be used alone as a whole tissue graft[10–13], or the graft can be combined with another biological or synthetic graft[31,32,41,44,45].

Recent results also suggest that extracorporeal shockwaves (ESW) may be used as a non-invasive method to increase the number of cambium cells in periosteum[46]. However, whether pretreatment with ESW results in improved regenerative capacity of periosteum has not yet been reported. Such continued efforts to better exploit the regenerative capacity of periosteum are worthwhile considering the advantages of autologous periosteal grafts described above. Although the number of cambium cells is reduced with age, they are still present and maintain multipotency, and the ability to proliferate and respond to growth factors throughout adult life[47,48].

The *in situ* pretreatment approach described herein could be considered a 'second generation' technique of periosteal transplantation that offers improvement over conventional periosteal transplantation. However, we recognize that this approach is only one step towards achieving optimal, durable cartilage regeneration and inclusion of other factors such as mechanical stimulation will likely be needed to achieve this larger goal.

Contributions

A. Olivos-Meza was the lead surgeon for the study and contributed to the acquisition, analysis and interpretation of the data, drafting and critically reviewing the manuscript and final approval of the submitted version.
J.S. Fitzsimmons contributed to conception and design, obtaining funding, acquisition, analysis and interpretation of data, drafting and critically reviewing the manuscript and final approval of the submitted version.
M.E. Casper contributed to acquisition, analysis and interpretation of data, drafting and critically reviewing the manuscript and final approval of the submitted version.
Q. Chen contributed to acquisition, analysis and interpretation of data, drafting the manuscript and final approval of the submitted version.
K-N. An contributed to study design, revising the manuscript and final approval of the submitted version.
T.J. Ruesink contributed to acquisition of data, revising the manuscript and final approval of the submitted version.
S.W. O'Driscoll contributed to conception and design, obtaining funding, critically reviewing the manuscript and final approval of the submitted version.
G.G. Reinholz contributed to conception and design, obtaining funding, acquisition, analysis and interpretation of the data, drafting and critically reviewing the manuscript and final approval of the submitted version, and accepts responsibility for the integrity of the study as a whole from inception to finished article (reinholz.gregory@mayo.edu).

Conflict of interest

Mayo Clinic has filed a patent application related to this study on behalf of authors GGR, JSF and SWO. The remaining authors have no conflict of interests to declare.

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