

# Quantitative assessment of neural elements in rat model using nerve growth factor after remnant-preserving anterior cruciate ligament reconstruction: a hematoxylin and eosin stain and immunohistochemical study

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## Research article

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# Abstract

**Background:** Immunohistochemical analyses of anterior cruciate ligament (ACL) allografts following remnant-preserving ACL reconstructions using Achilles tendon allografts have provided evidence for the presence of neural elements. In this study, we aimed to examine the expression of neural elements and quantify the presence of neural cells in ACL remnants and Achilles allografts using nerve growth factor (NGF) therapy after remnant-preserving ACL reconstruction.

**Methods:** Experiments were conducted on 5 pairs of rats (approximately 8 weeks old and weighting 320 g at the time of surgery). Longitudinally-split Achilles tendons from the paired rats were freshly frozen and later defrosted with warm saline and allografted onto the right ACL of the other, which was partially detached at the femoral attachment site. A sham operation was conducted on the left knee to be used as Control. NGF was injected in both the knee joints 1 week after surgery. The presence of neural cells in the ACL of the sham-operated knee, allografted Achilles tendon, and ACL remnant was examined 6 weeks post surgery using H and E and immunohistochemical staining.

**Results:** H and E staining did not reveal neural cells in any of the three groups. However, immunohistochemical analysis showed the presence of nestin-positive neural elements in normal ACL as well as ACL remnants. Additionally, neural elements were examined in 7 of the 8 (87.5%) allograft tissues. Quantitative analysis showed no difference in the number and area of nuclei among the three groups. However, the number and area of neural cells in Achilles allograft were significantly lower than in the other two groups ( $p=0.000$  and  $p=0.001$ , respectively).

**Conclusion:** Our observations indicate that ACL remnants promote new ingrowth and persistence of neural cells. We suggest that the ingrowth of neural elements could support the persistence and new ingrowth of mechanoreceptors, thereby enhancing the functional stability of knee joints. Moreover, the expression of neural cells in Achilles allograft was lower than that of normal ACL or ACL remnants in the quantitative evaluation, thereby confirming the essential role of ACL remnants in knee joint functionalization.

**Key terms:** anterior cruciate ligament, remnant preservation, immunohistochemistry, nerve growth factor

## Background

The functional instability of a knee joint is majorly caused due to the lack of coordinated muscle stabilization, thereby inciting pertinent interest in unraveling methods to protect mechanoreceptors in the knee joints [1, 2]. It has been reported that remnant-preserving ACL reconstruction technique preserves mechanoreceptors and renders positive results [1, 3,4,5,6,7]. The current study aimed to determine whether the remnant preservation technique preserves the existing mechanoreceptors or leads to re-innervations.

Evidence has demonstrated the growth of mechanoreceptors in reconstructed ACLs. For instance, Barrack et al. reported increasing of mechanoreceptors in ACL grafts in dogs 6 months after surgery compared to their normal patellar tendon [8]. Denti et al. reported that mechanoreceptors were present in the bone-patellar tendon-autografts in the knees of sheep 3 months after surgery, and also in human knees with failed autograft at 9 and 10 years after surgery [9]. These findings suggested that ACL remnants are a possible source of re-innervation of the tendon graft. However, previous histological studies failed to validate the presence of mechanoreceptors in ACL allografts [10]. In addition, few studies have quantitatively evaluated neural elements in ACL remnants and ACL allograft.

The nerve growth factor (NGF) is widely used clinically for its significant roles in supporting neuronal survival, peripheral nerve growth, nutritional adaptation, nerve regeneration and fracture repair [11]. However, it has not been delineated whether these receptors effect on re-innervation or proprioception. However, the it is important that the persistence of mechanoreceptors after remnant-preserving ACL reconstruction. Thus, stimulating the expression of neural cells using NGF, we tried to validate the superiority of remnant preservation technique.

The aim of present study was to investigate the effects of administering NGF following remnant preservation technique by assessing the presence of nerve cells immunohistochemically in the allograft and remnant ACL. In addition, neural elements in ACL remnants and ACL allografts were quantitatively evaluated using ImageJ particle analysis. We hypothesized that remnant tissues following ACL reconstruction as well as Achilles allografts would contain neural cells, which was identified using immunohistochemistry.

## Methods

### Animals

Experiments were conducted on 10 adult male rats (*Rattus norvegicus albinus*, Samtako®, Korea), approximately 8 weeks old, and weighing 320 g at the time of surgery. All the rats were numbered using ear tags. Rats were housed in accordance with the National Institutes of Health guidelines, kept in a vivarium, maintained at 20–25 °C and 60% humidity with 12 hours alternating light–dark cycles (7 am–7 pm), and provided food and water ad libitum.

### Surgical procedure

#### The remnant-preserving ACL allograft

Arthrotomy was performed after intraperitoneal (IP) administration of general anesthesia [a mixture of Zoletil (50 mg/kg, Virbac Laboratories, France) and Rompun (10 mg/kg, Bayer, Korea)]. In pairs, the Achilles tendon was obtained from a donor rat and used as an allograft for ACL reconstruction in the right knee of the recipient rat. Briefly, an approximately 1.5 cm longitudinal skin incision was made on the Achilles tendon insertion site in the left ankle. After longitudinally splitting the 1.5 cm sized Achilles

tendon of each rat, it was fresh frozen at -80 °C for 5 minutes in a deep freezer. Then, the Achilles tendon was defrosted with 47 °C warm saline for 5 minutes (Fig. 1) and used as allograft.

For the ACL reconstruction, an approximately 1.5 cm incision was made on the right knee. Then, the joint was exposed by transposing the patella laterally after a parapatellar incision was made with the knee in flexion. The ACL was detached at femoral insertion, but tibial insertion was maintained. Subsequently, the Achilles tendon was allografted onto the right ACL which was partially detached at the femoral attachment site. Suture fixation was used on Femoral, tibial ACL anatomical attached site (Fig. 2). Irrigation was performed on the joints, and the capsule and skin were closed with interrupted sutures.

#### The Sham operation

A sham operation was conducted on the left knee as a control. The knee joints were exposed with the same incision. Also irrigation was performed on the joints, and the capsule and skin were closed with interrupted sutures.

#### NGF injection and sample collection

10 µl of recombinant rat NGF (50 µg dissolved in 500 µl ddH<sub>2</sub>O, Invitrogen #50385MNAC50, CA, USA) was injected in both the knee joints 1 week after operation using 0.3 cc insulin syringe. The presence of neural cells in the control group (sham operation), allografted Achilles tendon, and ACL remnants was examined 6 weeks post-surgery using hematoxylin and eosin (H and E) staining and immunohistochemical staining with anti-nestin antibody. The presence of neural cells was then compared among the Achilles allografts, ACL remnants, and normal ACL (sham operation) tissues.

#### H and E staining

Tissue samples were collected from femoral insertion site of normal ACL, detached site of ACL remnants and femoral insertion site of Achilles allograft. After dehydration with alcohol and washing with a tissue processor (Leica TP 1020, Leica, Germany), tissue pieces were fixed with masked formalin solution (mask form 2A, DANA Korea) for 24 hours and embedded in paraffin. The tissues were sectioned sequentially into 4 µm thick slices and stained with H and E. Mechanoreceptors are classified into four types according to prior study (type I, a spherical or ovoid Ruffini corpuscle; type II, a columnar concentric circular Pacini corpuscle; type III, a spindle-shaped Golgi corpuscle; and type IV, a non-myelinated free nerve ending) [12]. H and E stained sections were assessed for the presence of mechanoreceptors following the stated classification.

#### Immunohistochemical staining

Sliced tissues on the coverslips were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilization with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. After washing three times with PBS, sections were blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature. Sections were incubated with anti-nestin antibody

(Sigma, St. Louis, MO) (diluted in blocking solution (1% BSA in PBS)) for 1 hour at room temperature in a shading box. Subsequently, the tissue sections were washed three times with PBS and incubated with secondary antibody, Alexa Fluor 555-conjugated rabbit anti-goat antibody (Invitrogen, Grand Island, NY) for 1 hour at room temperature. Alexa Fluor 488-conjugated phalloidin (Invitrogen, Grand Island, NY) was used for F-actin staining. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnologies, Santa Cruz, CA). Images were captured using confocal microscope (Olympus, FV-1 mm).

### Quantification with particle analysis

After immunohistochemical staining, quantitative analysis was performed on each image using ImageJ (National Institutes of Health, Bethesda, MD). The number and area of nuclei that were stained with DAPI and neural cells that were stained with anti-nestin antibody were calculated. Several attached cells in each image were optically separated using a watershed separation tool provided by the ImageJ software. Following this, the area and number of cells were quantified using ImageJ particle analysis.

### Statistical Analysis

Statistical analysis was conducted using SPSS for Windows, version 12.0. Kruskal Wallis test was used to analyze the immunohistochemical data (with the 95% confidence level). P-values < 0.05 were considered significant. Where indicated, Mann-Whitney post-hoc analysis was performed after the Kruskal–Wallis test, with a significance level set at  $p < 0.16$ . The power of group comparison was analyzed using G\*Power 3.1.9.2, where this study achieved a power of 0.68 for detecting differences with  $\alpha = 0.05$ .

## Results

Two of 10 rats expired during the experiment (at 2 weeks and 4 weeks post-surgery). The experiment was completed with the remaining 8 rats.

H and E examination confirmed ligamentous ACL tissues and synovium in all the sections (Fig. 3). However, neural cells or mechanoreceptors could not be detected in normal ACL, ACL remnants, and Achilles allograft tissues through H and E staining (Fig. 3).

In the immunohistochemical study, nestin was expressed in all normal ACL tissues that were injected with NGF. In addition, nestin was expressed in all ACL remnants and in 7 of 8 (87.5%) allograft tissues that were processed with NGF (Table 1). Nestin-positive cells suggested the possible presence of neural cells in the ACL remnant tissue and Achilles allograft (Fig. 4). We did not observe any significant difference in neural cell expression among the normal ACL, ACL remnants, and Achilles allografts with the immunohistochemical study ( $p = 0.368$ ).

Table 1  
An immunohistochemical study on the presence of nestin-positive neural cells

Number	Presence of neural cells (nestin staining)		
	Normal ACL (sham operation)	Remnant ACL	Achilles allograft
#1	+	+	+
#2	+	+	+
#3	+	+	+
#4	+	+	+
#5	+	+	-
#6	+	+	+
#7	+	+	+
#8	+	+	+
+: expression of nestin, -: no expression of nestin			

Quantitative analysis showed no difference in the number and area of nuclei among normal ACL, ACL remnants, and Achilles allografts. However, the number and area of neural cells were significantly different among the groups. Post-hoc analysis revealed that the number and area of neural cells in Achilles allografts was smaller than that of normal and remnant ACLs ( $p = 0.000$  and  $p = 0.001$ , respectively). (Table 2) (Fig. 5).

Table 2  
Number and area of nuclei and neural cells in normal, remnant and allografted ACL

		Normal ACL (n = 8)	Remnant ACL (n = 8)	Achilles allograft (n = 8)	p
Nuclei	Number	178.6 ± 84.3	114.4 ± 49.8	179.3 ± 78.7	0.227
	Area (pixels)	77565.9 ± 56207.1	41082.9 ± 24237.0	76713.6 ± 37395.3	0.138
Neural cell	Number	1977.3 ± 1521.8	1668.5 ± 1015.6	89.9 ± 68.8	0.000
	Area (pixels)	36218.9 ± 33157.0	29613.4 ± 21547.3	1846.1 ± 1179.4	0.001
Data are presented as mean ± SD.					

## Discussion

This study used immunohistochemical analysis to shed light on a critical aspect of the localization of neural elements. We provide evidence that the neural elements are found in not only remnant ACL but Achilles allografts. In the quantitative test, neural cells of ACL remnants were not found to be different from those of normal ACL. These results indirectly demonstrate that ACL reconstruction with remnant preservation could better preserve proprioception and be a resource for re-innervation in reconstructed allografts.

Proprioception is a key factor in maintaining the stability of knee joint against functional deficiencies after ACL reconstruction. Many mechanoreceptors known to be distributed in ACL and remnant tissues contribute to the proprioceptive function of the knee [13, 14]. Some previous studies reported the presence of mechanoreceptors in ACL remnants after ACL reconstruction with remnant preservation technique [5, 15, 16, 17]. Previous study reported the presence of mechanoreceptors in ACL remnants 3 years after ACL rupture [17]. In another recent study, normal proprioceptive fibers and mechanoreceptors were reported in 50% of remnant ACL using neurofilament protein staining [16]. They also showed the difference between mechanoreceptors in healthy and injured ACLs and highlighted their clinical significance [15]. Lee et al. reported the presence of mechanoreceptors in about one-third of the ACL remnants, which was less than expected [5]. In this study, neural cells were identified in ACL remnants, which was not different from normal ACL upon quantitative evaluation. This outcome suggests that remnant-preserving ACL reconstruction could preserve proprioception and have a positive effect on knee joint functions.

Conversely, few studies suggest the possibility that ACL remnants could serve as a source of re-innervation for reconstructed allograft. Ochi et al. stressed that restoring knee function is important in terms of anatomical ACL reconstruction and mechanical restraint and graft sensory re-innervation could potentially improve the overall outcome [18]. Denti et al. found re-innervation of autologous bone-patellar tendon bone grafts in animals 3–6 months after surgery [9]. However, few studies suggest the possibility that ACL remnants could serve as a source of re-innervation for reconstructed allograft. Kim et al. proposed that no newly ingrown mechanoreceptors are present in Achilles allografts [10]. In addition, Chun et al. suggested that Achilles allograft ligaments do not show similar findings compared with biopsy samples from normal ACL [19]. Recently, Chun et al. identified the presence of neural elements in ACL remnant tissues after remnant-preserving ACL reconstruction using immunohistochemical evaluation with NGF application. However, in their study, neural elements were not observed in Achilles allograft tissues [20]. Therefore, we investigated the expression of mechanoreceptors in remnant tissues and allografts following remnant-preserving ACL reconstruction using NGF therapy. In the present animal study, the presence of neural elements was confirmed in remnant tissues and allografts using immunohistochemical methods with NGF, a well-known growth factor for nerve cell proliferation.

The presence of neural cells in Achilles allografts supports the potential regeneration of neural elements during remnant-preserving ACL reconstruction. This was, however, not supported in previous studies [10, 19].

Many studies have evaluated proprioception, as well as the function of mechanoreceptors in reconstructed ACLs [5, 10, 13, 14, 15, 16, 17]. However, it is difficult to compare outcomes between these studies because of variations in the experimental methods and other external factors. Recently, immunofluorescence and immunohistochemistry using specific antigen-antibody reactions have been used to detect nerve fibers, producing more reliable and relevant results [5]. In this study, we visualized mechanoreceptor-positive cells using immunohistochemical assessment with monoclonal antibodies against nestin. To identify the presence of nestin-positive cells in the remnant tissues, the cells were treated in vivo with NGF which was an important factor in the growth and maintenance of sensory and sympathetic neurons. Previous studies reported that NGF application could be used to promote the healing of ACLs [21]. They proposed that NGF promoted re-innervation and angiogenesis in healing rat ACLs. Therefore, we evaluated the expression of mechanoreceptors in remnant tissues and allografts after remnant-preserving ACL reconstruction using in vivo administration of NGF in rats. The results of the current study indicate that NGF promotes re-innervation of Achilles allografts.

Few studies have attempted to quantify the proprioceptive potential of the injured ACL stump [9, 16, 17]. However, none of these studies have quantified their immunohistochemical findings. We analyzed the number, area of nuclei and neural cells that were each stained with DAPI and anti-nestin antibody. The expression of neural cells in Achilles allograft was less than that of normal ACL or ACL remnants in the quantitative evaluation, although nerve growth was promoted by NGF. Therefore, to investigate the clinical significance of the expression of small amounts of neural elements in allografts, further research on the differences in proprioceptive function after remnant-preserving ACL reconstruction using allografts will be required.

## Conclusion

The results from this study demonstrate that ACL remnants promote the new ingrowth and persistence of neural cells. We suggest that the ingrowth of neural elements could support persistence and new ingrowth of mechanoreceptors and functional stability of the knee joints. However, the expression of neural cells in Achilles allograft was lower than that of normal ACL or remnant ACL in the quantitative evaluation.

## Abbreviations

ACL  
anterior cruciate ligament  
NGF  
nerve growth factor  
PBS  
phosphate-buffered saline  
BSA  
bovine serum albumin

DAPI

4,6-diamidino-2-phenylindole

## **Declarations**

### **Ethics approval and consent to participate**

This experimental study was approved by the Institutional Animal Care and Use Committee of Wonkwang University (WKU17-108). The procedures for animal experiments were in accordance with requirements in Declaration of Helsinki.

### **Consent for publication**

This paper is approved by all authors for publication.

### **Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests

### **Funding**

Not applicable

### **Author's contributions**

CCH designed and was a major contributor in this research. LSH, CHG, CKC performed all the experiments and prepared the initial draft of the manuscript. SJS performed the data analysis and interpreted the data. All authors reviewed, edited, and approved the final content of the manuscript.

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## **References**

1. Bali K, Dhillon MS, Vasistha RK, Kakkar N, Chana R, Prabhakar S. Efficacy of immunohistological methods in detecting functionally viable mechanoreceptors in the remnant stumps of injured anterior cruciate ligaments and its clinical importance. *Knee Surg Sports Traumatol Arthrosc* 2012;20:75-80.
2. Hogervorst T, Brand RA. Mechanoreceptors in joint function. *J Bone Joint Surg Am*. 1998;80:1365-1378.
3. Kim YK, Ahn JH, Yoo JD. A comparative study of clinical outcomes and second-look arthroscopic findings between remnant-preserving tibialis tendon allograft and hamstring tendon autograft in anterior cruciate ligament reconstruction: matched-pair design. *Clin Orthop Surg* 2017 Dec.;9:424-431. doi: [10.4055/cios.2017.9.4.424](https://doi.org/10.4055/cios.2017.9.4.424).
4. Ahn GY, Nam IH, Lee YH, Lee YS, Choi YD, Lee HH, et al. Factors Affecting the Extent of Graft Tendon Synovialization after Double-Bundle Anterior Cruciate Ligament Reconstruction: Based on Second-Look Arthroscopic Findings. *Clin Orthop Surg*. 2018 Dec;10(4):413-419. doi: [10.4055/cios.2018.10.4.413](https://doi.org/10.4055/cios.2018.10.4.413).
5. Lee BI, Min KD, Choi HS, Kwon SW, Chun DI, Yun ES, et al. Immunohistochemical study of mechanoreceptors in the tibial remnant of the ruptured anterior cruciate ligament in human knees. *Knee Surg Sports Traumatol Arthrosc* 2009;17:1095-1101.
6. Ochi M, Iwasa J, Uchio Y, Adachi N, Sumen Y. The regeneration of sensory neurones in the reconstruction of the anterior cruciate ligament. *J Bone Joint Surg Br* 1999;81:902-906.
7. Taketomi S, Inui H, Sanada T, Nakamura K, Yamagami R, Masuda H, et al. Remnant-preserving anterior cruciate ligament reconstruction using a three-dimensional fluoroscopic navigation system. *Knee Surg Relat Res* 2014 Sep;26:168-176. doi: [5792/ksrr.2014.26.3.168](https://doi.org/5792/ksrr.2014.26.3.168).
8. Barrack RL, Lund PJ, Munn BG, Wink C, Happel L. Evidence of reinnervation of free patellar tendon autograft used for anterior cruciate ligament reconstruction. *Am J Sports Med* 1997;25:196-202.
9. Denti M, Monteleone M, Berardi A, Panni AS. Anterior cruciate ligament mechanoreceptors. Histologic studies on lesions and reconstruction. *Clin Orthop Relat Res* 1994;308:29-32.
10. Kim SH, Chun CH, Chun KC, Jo HJ, Kim KM. Histological assessment of mechanoreceptors in Achilles allografts after anterior cruciate ligament reconstruction. *Am J Sports Med* 2012;40:2061-2065. doi: [1177/0363546512453303](https://doi.org/1177/0363546512453303).
11. Doerks T, Copley RR, Schultz J, Ponting CP, Bork P. Systematic identification of novel protein domain families associated with nuclear functions. *Genome Res* 2002;12:47-56.
12. Freeman MA, Wyke B. Articular contributions to limb muscle reflexes. The effects of partial neurectomy of the knee-joint on postural reflexes. *Br J Surg* 1966;53:61-68.
13. Nagai K, Araki D, Matsushita T, Nishizawa Y, Hoshino Y, Matsumoto T, et al. Biomechanical function of anterior cruciate ligament remnants: quantitative measurement with a 3-dimensional electromagnetic measurement system. *Arthroscopy* 2016;32:1359-1366.
14. Nakase J, Toratani T, Kosaka M, Ohashi Y, Tsuchiya H. Roles of ACL remnants in knee stability. *Knee Surg Sports Traumatol Arthrosc* 2013;21:2101-2106.

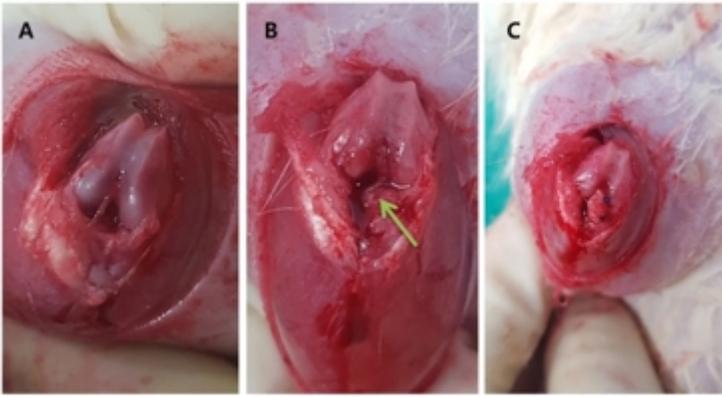
15. Dhillon MS, Bali K, Prabhakar S. Differences among mechanoreceptors in healthy and injured anterior cruciate ligaments and their clinical importance. *Muscles Ligaments Tendons J* 2012;2:38-43.
16. Dhillon MS, Bali K, Vasistha RK. Immunohistological evaluation of proprioceptive potential of the residual stump of injured anterior cruciate ligaments (ACL). *Int Orthop* 2010;34:737-741.
17. Georgoulis AD, Pappa L, Moebius U, Malamou-Mitsi V, Pappa S, Papageorgiou CO, et al. The presence of proprioceptive mechanoreceptors in the remnants of the ruptured ACL as a possible source of re-innervation of the ACL autograft. *Knee Surg Sports Traumatol Arthrosc* 2001;9:364-368.
18. Ochi M, Adachi N, Deie M, Kanaya A. Anterior cruciate ligament augmentation procedure with a 1-incision technique: anteromedial bundle or posterolateral bundle reconstruction. *Arthroscopy* 2006;22:463.e1-5.
19. Chun CH, Han HJ, Lee BC, Kim DC, Yang JH. Histologic findings of anterior cruciate ligament reconstruction with Achilles allograft. *Clin Orthop Relat Res* 2004;421:273-276.
20. Chun KC, Lee SH, Kim JW, Jin EJ, Kim KM, Chun CH. Immunohistochemical and immunocytochemical study of mechanoreceptors in anterior cruciate ligament reconstruction with the remnant-preserving technique using Achilles tendon allografts. *J Orthop Surg Res* 2017 Jun 14;12:93. doi: [1186/s13018-017-0593-0](https://doi.org/10.1186/s13018-017-0593-0).
21. Mammoto T, Seerattan RA, Paulson KD, Leonard CA, Bray RC, Salo PT. Nerve growth factor improves ligament healing. *J Orthop Res* 2008;26:957-964.

## Figures



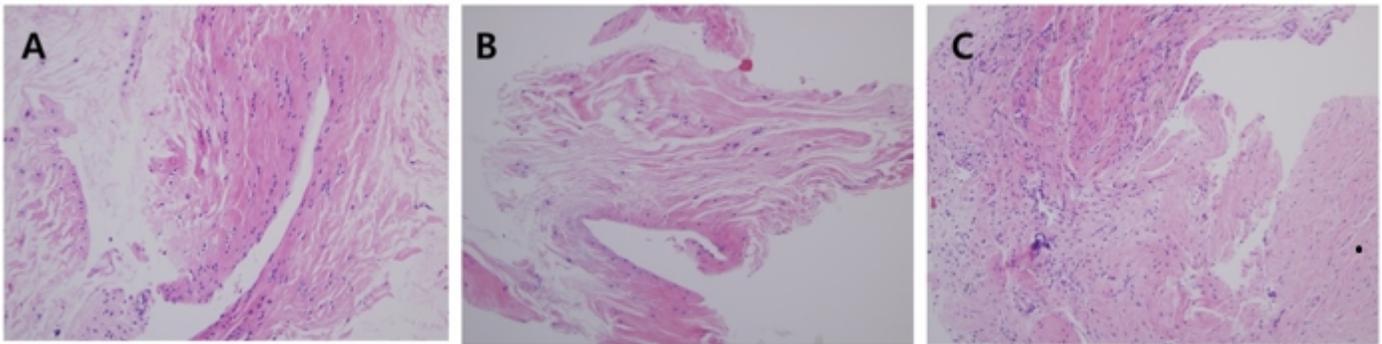
**Figure 1**

Achilles tendon was grafted for ACL reconstruction. A: Achilles tendon exposure, B, C: split Achilles tendon, D: frozen Achilles tendon thawing in warm saline (47 °C for 5 minutes)



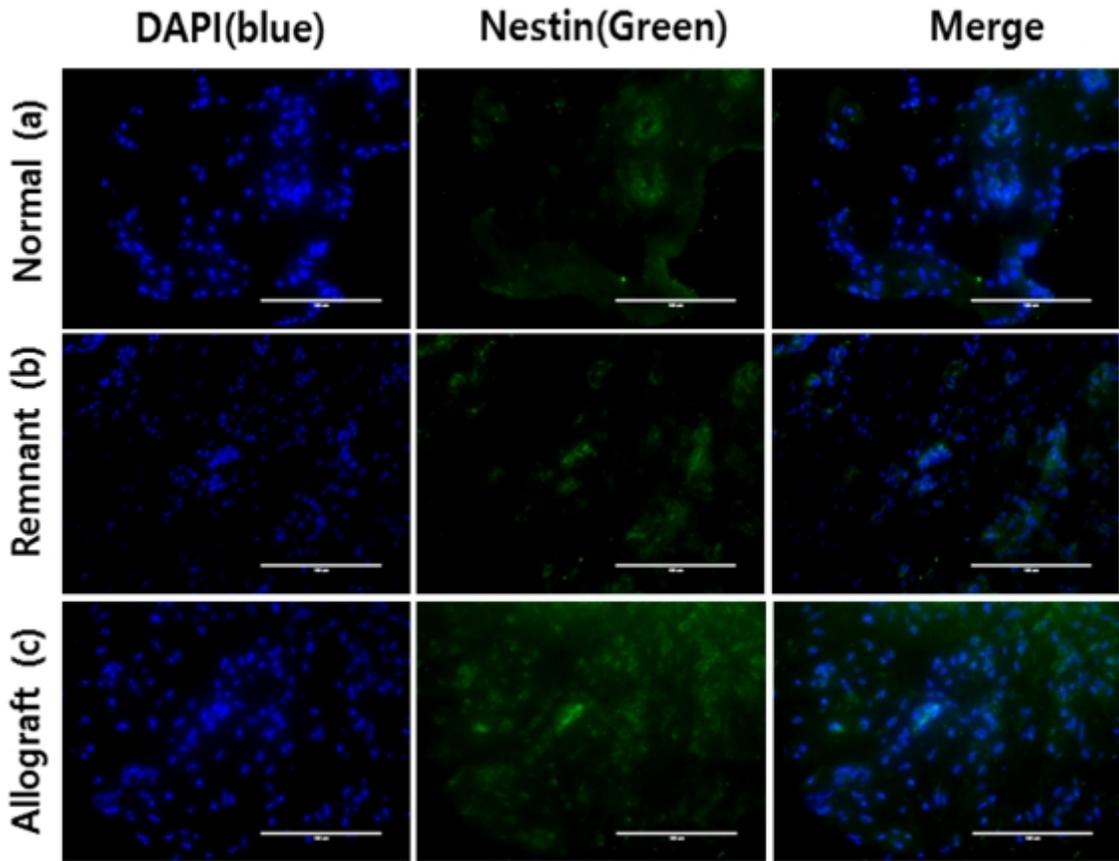
**Figure 2**

Achilles tendon was allografted to each other's left ACL. A: ACL exposed, B: ACL is partially detached (green arrow), C: Achilles tendon grafted to ACL



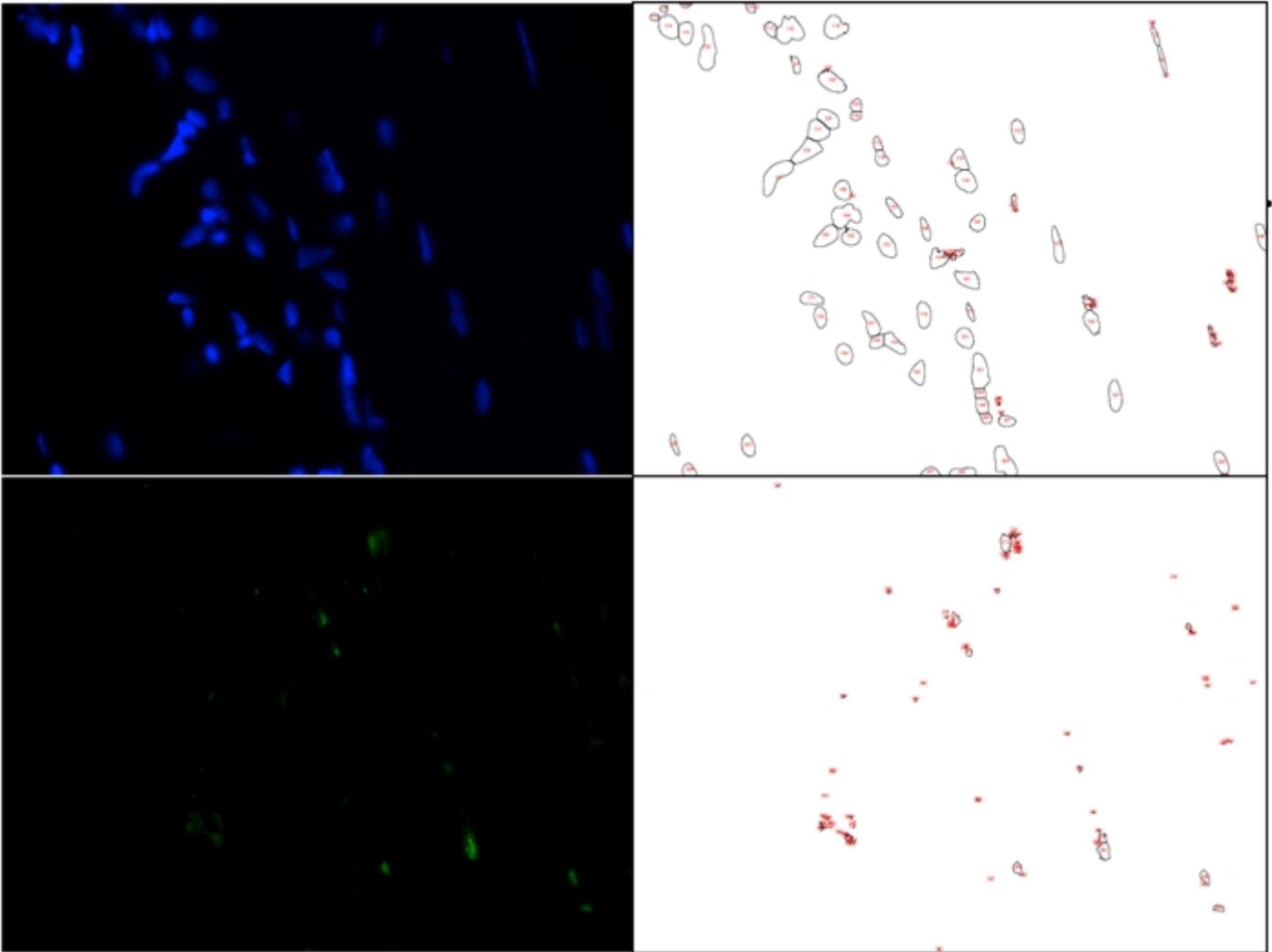
**Figure 3**

. In all cases, no mechanoreceptors were present on H&E staining. A: normal ACL, (control specimen) B: remnant ACL specimens, C: Achilles allograft specimens (H&E stain,  $\times 20$ ).



**Figure 4**

Immunohistochemical findings of nerve cells. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnologies, Santa Cruz, CA). Nerve cells were stained with anti-nestin antibody in (a) normal ACL tissue, (b) remnant ACL specimens, and (c) Achilles allograft specimens.



**Figure 5**

Several attached cells in each image were separated using the watershed separation tool based on the ImageJ software. Then, the area or number of cells was measured with ImageJ particle analysis.