Expression of Signaling Molecules Involved in Embryonic Development of the Insertion Site Is Inadequate for Reformation of the Native Entesis

Evaluation in a Novel Murine ACL Reconstruction Model

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Background: Since healing of anterior cruciate ligament (ACL) grafts occurs by formation of a fibrovascular scar-tissue interface rather than by reformation of the native fibrocartilage transition zone, the purpose of our study was to examine expression of various signaling molecules and transcription factors that are known to be involved in embryologic insertion-site development following ACL reconstruction. We also aimed to characterize a murine model of ACL reconstruction to allow future study of the molecular mechanisms of healing.

Methods: Seventy-nine mice underwent reconstruction of the ACL with autograft. Healing was assessed using histology in 12 mice and quantitative real-time polymerase chain reaction (qRT-PCR) gene-expression analysis in 3 mice at 1 week postoperatively (Group-1 mice) and by biomechanical analysis in 7, histological analysis in 7, immunohistochemical analysis in 5, microcomputed tomography analysis in 5, and qRT-PCR analyses in 8 at 2 weeks (Group-2 mice) and 4 weeks (Group-3 mice) postoperatively. Fifteen additional mice did not undergo surgery and were used for biomechanical (7 mice), qRT-PCR (3 mice), and immunohistochemical (5 mice) analyses to obtain baseline data for the native ACL.

Results: Histological analysis demonstrated healing by formation of fibrovascular tissue at the tendon-bone interface. Immunohistochemical analysis showed a positive expression of proteins in the Indian hedgehog, Wnt, and parathyroid hormone-related protein (PTHrP) pathways. There was minimal Sox-9 expression. Gene-expression analysis showed an initial increase in markers of tissue repair and turnover, followed by a subsequent decline. Mean failure force and stiffness of the native ACL were 5.60 N and 3.44 N/mm, respectively. Mean failure force and stiffness were 1.29 N and 2.28 N/mm, respectively, in Group 2 and were 1.79 N and 2.59 N/mm, respectively, in Group 3, with 12 of 14 failures in these study groups occurring by tunnel pull-out.

Conclusions: The spatial and temporal pattern of expression of signaling molecules that direct embryologic insertion-site formation was not adequate to restore the structure and composition of the native insertion site.

Clinical Relevance: Development of a murine model to study ACL reconstruction will allow the use of transgenic animals to investigate the cellular, molecular, and biomechanical aspects of tendon-to-bone healing following ACL reconstruction, ultimately suggesting methods to improve healing in patients.

Function of an anterior cruciate ligament (ACL) graft is dependent on secure healing in a bone tunnel^{1,2}. Prior work has demonstrated that healing at the tendon-bone interface in ACL reconstruction occurs by formation of a fibrovascular scar-tissue interface that does not recapitulate the micro-

scopic architecture of the native entesis²⁻⁶. Bone resorption along the tunnel (tunnel widening) may also compromise attachment strength, contributing to recurrent knee laxity^{7,8}. Accordingly, research has focused on understanding the biologic healing mechanism in an attempt to optimize healing²⁻⁶.

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Fig. 1
Allocation of animals to the intact-ACL and ACL-reconstruction groups. IHC = immunohistochemistry.

Animal models of ACL reconstruction have provided insight into the basic biologic and biomechanical factors that affect graft healing⁹⁻¹⁵. However, a complete understanding of the cellular signaling pathways that regulate ACL graft incor-

poration is still lacking. Murine models provide the ability to study the role of specific molecular pathways in healing using genetically modified strains. Accordingly, we have worked to develop and validate a murine model of ACL reconstruction that would allow study of the role of important cell-signaling molecules in graft healing, given the availability of transgenic animals and biologic assays for molecular investigation.

Several of the signaling molecules critical to the initiation and regulation of insertion-site formation during embryogenesis include scleraxis, Mohawk, Indian hedgehog, EGR1 and EGR2 (early growth response 1 and 2), Sox-9, Wnt, and PTHrP (parathyroid hormone-related protein)^{16,17}. Scleraxis and Sox-9 are factors that play a fundamental role in insertion-site formation in the developing embryo, as tendon attachment-site progenitor cells express both scleraxis and Sox-9¹⁸⁻²⁰. It seems reasonable that recapitulation of the events that occur during embryogenesis would lead to optimal insertion-site healing. In order to begin to study the role of these signaling molecules in graft healing, the purposes of this work were to develop a murine model of ACL reconstruction and to define the healing process at the histological and molecular levels as well as to test the hypothesis that signaling molecules that direct enthesis formation during embryogenesis will not be expressed in the fibrovascular scar response at the healing tendon-bone interface.

Fig. 2
Figs. 2-A and 2-B For the ACL reconstruction, the mouse is placed in the supine position (**Fig. 2-A**) and the FDL is harvested through 2 small incisions in the hindlimb (**Fig. 2-B**). **Figs. 2-C and 2-D** A medial parapatellar arthrotomy is performed to access the knee (**Fig. 2-C**), and the femoral tunnel is drilled in the center of the footprint with use of a 23-gauge needle (**Fig. 2-D**). **Fig. 2-E** Once both the femoral and tibial tunnels are created, the graft is passed from the femoral tunnel into the knee, exiting the tibial tunnel. **Fig. 2-F** The clip provides suspensory fixation on the femoral side, and transosseous suture fixation is used on the tibial side.

Fig. 3

For biomechanical analysis, specimens are dissected free of all soft tissue other than the ACL, and the tibia and femur are potted in Bondo filler to allow fixation into the testing apparatus.

Materials and Methods

A total of 94 C57BL/6 male mice (age, 12 weeks; weight, 22 to 30 g) were utilized (Fig. 1). Biomechanical (7 mice), immunohistochemical (5 mice), and quantitative real-time polymerase chain reaction (qRT-PCR) (3 mice) analysis was performed on 15 mice without surgery to provide a baseline for comparison. Seventy-nine mice underwent unilateral ACL reconstruction. Postoperatively, animals were allowed free activity within the cage and received standard postoperative care. Three mice were killed on postoperative day 7 (Group 1) for qRT-PCR analysis, and 12 were killed for histological analysis. Half (32) of the remaining mice were killed on postoperative day 14 (Group 2), while the remaining 32 were killed on day 28 (Group 3). Within each of these last 2 study groups, 7 mice underwent biomechanical testing, 5 underwent micro-computed tomography (microCT), 12 were analyzed with histology (7, with routine histology and 5, with immunohistochemistry), and the remaining 8 were used for qRT-PCR analysis.

For the surgical technique, a novel murine ACL reconstruction model using loupe magnification and microsurgical techniques was developed²¹. We practiced extensively on cadaveric specimens to identify isometric graft-attachment points. Graft isometry was established by attaching the graft to a load cell in order to measure tension through knee range of motion. We verified that the surgeon could consistently demonstrate reproducibility of the technique and graft isometry on a minimum of 6 consecutive specimens before performing the procedure on live animals. The surgical procedures were performed by a fellowship-trained orthopaedic surgeon. (Video 1).

Animals were placed on a custom operative table (Fig. 2-A). The flexor digitorum longus (FDL) was harvested through 2 percutaneous incisions on the leg and plantar aspect of the foot. The FDL was freed proximally and retrieved through the distal incision. A surgical clip was applied to the distal end, and a 7-0 PROLENE suture (Ethicon) was tied to the proximal end (Fig. 2-B).

TABLE I Antibodies Used to Study the Tendon-Bone Healing Process

Antibody	Signaling Pathway	Manufacturer	Catalog No.	Dilution
Indian hedgehog	Indian hedgehog	Abcam	Ab-39634	1:500
Gli1	Indian hedgehog	Abcam	Ab-151796	1:800
Patched	Indian hedgehog	Abcam	Ab-53715	1:1,000
β-catenin*	Wnt	Abcam	Ab-79089	1:800
Wnt3A	Wnt	EMD Millipore	09-162	1:500
Sox-9	Sox-9	EMD Millipore	AB5535	1:400
PTHrP	PTH	LifeSpan Biosciences	LS-B12325	1:1,000

*The β-catenin antibody binds to both active and nonactive forms.

Fig. 4

Figs. 4-A and 4-B Photomicrographs taken under 10× magnification were imported into ImageJ analysis software. **Fig. 4-A** Because of variation in the tunnel width and absence of a sharp transition between the interface and the surrounding osseous structure, for each image a 760- μ m circle (arrow) was drawn and centered at the middle of the tunnel (drill-bit diameter, 640 μ m), to include both the interface and the adjacent bone. The graft, along with the interposing empty space, was then manually delineated and removed (arrowhead). **Fig. 4-B** The area between these 2 delineations, representing the interface and adjacent bone, was cropped and “thresholded” to cover DAB (3,3'-diaminobenzidine)-positive areas. The total percentage of DAB-positive area was then calculated using ImageJ measurement functionality.

A medial parapatellar arthrotomy was performed (Fig. 2-C), and the patella was subluxated laterally. With the knee in flexion, the femoral tunnel was drilled using a 23-gauge needle (diameter, 0.64 mm) (Fig. 2-D), beginning in the center of the native ACL footprint and exiting the femur laterally, just proximal to the trochlea. The native ACL was cut with a number-11 blade. Soft tissue from the anteromedial surface of the tibia was removed and the tibial tunnel was drilled using a 23-gauge needle, beginning at the anteromedial aspect of the tibia, entering the joint in the footprint of the native ACL.

The graft was passed through the femoral and tibial tunnels using the attached suture (Fig. 2-E). The clip provided extracortical (suspensory) fixation on the femoral side. With the knee extended, the graft was pretensioned using a 50-g weight and fixed to the tibia using a transosseous 4-0 ETHI-BOND suture (Ethicon) passed through the anterior crest of

the tibia (Fig. 2-F). The arthrotomy site and skin incisions were closed in layers.

For biomechanical assessment, the femur and tibia were independently placed in 2.0-mL tubes of Bondo Lightweight Filler 265 (3M), and all soft-tissue structures other than the ACL were dissected. The surgical clip and suture used for the initial fixation were removed. The specimen was placed into the biomechanical testing device in a position that allowed distraction in line with the axis of the graft (Fig. 3). The specimen was loaded to failure at a rate of 10 mm/min (167 μ m/s). Load-to-failure data (N) were recorded, and stiffness (N/mm) was calculated from the load-deformation curves. The site of graft failure (femoral tunnel, midsubstance, or tibial tunnel) was recorded.

For microCT analysis, soft tissue from the leg was removed and a Scanco μ CT 35 system (Scanco Medical) was used. Imaging was performed with a 15- μ m voxel size, at 55

TABLE II Oligonucleotides Used in qRT-PCR

Gene	Forward	Reverse	Accession No.
Aggrecan	CCGCTTGCCAGGGGAGTTG	CCTGCAGCCAGCCAGCATCA	NM_007424.2
Collagen 1 α 1	AATGGCACGGCTGTGTGCGA	AACGGGTCCCCTTGGGCCTT	NM_007742.3
Collagen 3 α 1	TGACTGTCCACGTAAGCAC	GAGGGCCATAGCTGAAGTGA	NM_009930.2
GAPDH	GGGCTCATGACCACAGTCCATGC	CCTTGCCACAGCCTTGGA	NM_001289726.1
MMP3	TGTGTGCTCATCCTACCATTCG	CCCTGTCTCTCCAACCCGAGGA	NM_010809.2
MMP13	ATGGTCCAGGCGATGAAGACCCC	GTGCAGGCGCCAGAAGAATCTGT	NM_008607.2
MMP14	AACCTCAGCCCCGAAGCCTGG	ACAGCGAGGGCGCCTCATGG	NM_008608.3
Mohawk	ACGCTAGTGCAGGTGTCAAA	AGCGTTGCCCTGAACATACT	NM_177595.4
Scleraxis	CCTCAGCAACCAGAGAAAGTTGAGCA	GCCATCACCCGCTGTCCATC	NM_198885.3
Sox-9	AAGCTCTGGAGGCTGCTGAACGAG	CGGCCTCCGCTTGCCGTTCT	NM_011448.4
Tenomodulin	ATGAGCAATGGGTGGTCCCGC	ACAGACACGGCGGCAGTAACG	NM_022322.2

Fig. 5

Bar graphs showing mean ultimate force to failure (**Fig. 5-A**) and stiffness (**Fig. 5-B**) for all groups. An asterisk indicates a significant difference ($p < 0.05$), and the error bars indicate the standard deviation.

kVp, a 0.36° rotation step (180° angular range), and a 400-ms exposure per view. Scanco μ CT software (DECwindows Motif 1.6; Hewlett-Packard) was used for viewing, image analysis, and 3-dimensional reconstruction, by identifying a cylindrical volume of interest with a diameter of 0.64 mm (the same diameter as the needle used to create the tunnels) and a height of 1 mm. The amount of newly formed bone within the tunnel was measured at the aperture, midtunnel, and tunnel exit and was reported as bone volume (BV), total volume (TV), and directly measured bone volume fraction (BV/TV [voxels/mm³]).

For histological analysis, specimens were fixed in 10% formalin for 24 hours, decalcified in Immunocal (Decal Chemical), dehydrated, and embedded in paraffin. Tissues were sectioned in a sagittal plane into 5- μ m-thick slices and were stained with hematoxylin and eosin as well as Safranin-O/Fast Green. Images were obtained using a Nikon Eclipse microscope with transmitted light at 4 $\times$ and 20 $\times$ magnification.

Sections were stained for tartrate-resistant acid phosphatase (TRAP) to localize osteoclasts. The slides were deparaffinized in xylene, rehydrated, and placed in sodium-acetate buffer (pH, 5.0) for 30 minutes. They were then treated with a prewarmed TRAP staining solution mixture (sodium nitrite, basic fuchsin, sodium-acetate buffer, tartaric acid, naphthol phosphate, and dimethylformamide) and incubated at 37°C for 15 minutes. The sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted.

For immunohistochemical analysis, the sections were incubated with primary antibodies specific to Indian hedgehog, Gli1, Patched, β -catenin, Wnt3A, Sox-9, and PTHrP for 1 hour at 37°C after endogenous peroxidase activity was quenched with 3% hydrogen peroxide. The specimens were then incubated with a biotinylated secondary antibody for 1 hour. Bovine serum albumin was used as a negative control by replacing the primary

Fig. 6

Fig. 6-A and 6-B Two-dimensional and reconstructed 3-dimensional microCT images following ACL reconstruction. The yellow circle has a diameter of 0.64 mm (the same diameter as the needle that is used to drill the tunnel) and indicates the region of interest (ROI). The amount of new bone formed within this ROI was quantified at the aperture, midportion, and exit of each tunnel. **Fig. 6-C** Mean bone volume fraction formed within the tunnels at the time of animal sacrifice for each of the study groups. TV = total volume, BV = bone volume, and BV/TV = bone volume fraction. The horizontal line is the median, the box represents the first quartile and third quartile, and the bars represent the minimum and maximum.

antibody. The tissues were developed using a goat avidin-biotin peroxidase system with 3,3'-diaminobenzidine chromogen (DAB; Dako). The sections were examined using light microscopy (Eclipse E800; Nikon). Digital images were made using a SPOT RT camera (Diagnostic Instruments). Table I provides information on the antibodies that were used.

Photomicrographs taken under 10× magnification were imported into ImageJ analysis software (National Institutes of Health) (Fig. 4). A 760- μm circle was drawn on each image and was centered at the middle of the tunnel (drill-bit diameter, 640 μm) to include both the interface and the adjacent bone. The graft, along with any interposing empty space, was then manually delineated and removed. The area between these 2 delineations, representing the interface and adjacent bone, was cropped to cover DAB-positive areas. The total percentage of DAB-positive area was then calculated.

For gene-expression analysis, tissue from the tendon-bone interface inside the tunnels was collected. The tissue was immersed in RNAlater (Thermo Fisher Scientific). Total RNA was isolated using a mirVana miRNA Isolation kit (Ambion). Complementary DNA (cDNA) was generated from 50 ng of total RNA using a QuantiTect Reverse Transcription kit (Qiagen). qRT-PCR was performed on a CFX96 Touch real-time PCR detection system (Bio-Rad) using Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific) and 10 mouse-specific primers relevant to tendon healing (Table II). The data were normalized to a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]), and the relative expression was analyzed using the delta-delta cycle threshold ($\Delta\Delta\text{Ct}$) method, where the fold differences of gene expression were analyzed by $2^{-\Delta\Delta\text{Ct}}$. Temporal changes in gene expression were calculated using the intact FDL tendon as a control.

For statistical analysis, 1-way analysis of variance testing was used to compare mean differences across groups (native-ACL group and Groups 2 and 3). Mean differences are reported with 95% confidence intervals (CIs). Post-hoc pairwise comparisons were performed using a Tukey test, and significance was set at $p < 0.05$.

Results

All animals underwent ACL reconstruction without complication and resumed normal cage activity. They all survived until their scheduled sacrifice date without complication.

Biomechanical analysis showed that the mean failure force and standard deviation of the native, intact ACL was 5.60 ± 0.75 N and the stiffness was 3.44 ± 1.47 N/mm (Fig. 5). All 7 native ligaments failed in the midsubstance. For Group 2 (2 weeks), the mean failure force and stiffness were 1.29 ± 0.47 N and 1.72 ± 0.75 N/mm, respectively. Five of the 7 failed at the tibial tunnel, 1 failed at the femoral tunnel, and 1 failed in the graft midsubstance. In Group 3 (4 weeks), the mean failure force and stiffness were 1.79 ± 0.40 N and 2.59 ± 0.87 N/mm, respectively. Three of the 7 failures occurred at the femoral tunnel, 3 occurred at the tibial tunnel, and 1 occurred in the midsubstance. While failure force differed significantly across the 3 groups ($p < 0.001$), stiffness did not ($p = 0.241$). Post-hoc Tukey testing revealed significant differences in failure force between the intact-ACL group and Group 2 (mean difference, 4.31 N; 95% CI, 3.55 to 5.08 N; $p < 0.001$) and between the intact-ACL group and Group 3 (mean difference, 3.81 N; 95% CI, 3.06 to 4.58 N; $p < 0.001$) but not between Groups 2 and 3 (mean difference, 0.50 N; 95% CI, -0.265 to 1.265 N; $p = 0.244$).

MicroCT analysis showed new bone formation at the tendon-bone interface along the bone tunnel. The parameters

Fig. 7

Fig. 7-A Safranin-O staining showing the hypercellularity of the interface tissue at 2 weeks (A and A' [inset]) and progressive graft healing by 4 weeks. Scale bar = 100 μm . **Fig. 7-B** Decreased cellularity and the appearance of chondrocyte-like cells proliferating within the tendon at 4 weeks (B and B' [inset]). Scale bar = 100 μm . (Link to [hematoxylin and eosin](#) as well as [safranin O](#)-stained whole slide images.) **Fig. 7-C** A histological section of the native murine ACL insertion site (10×), shown for comparison, demonstrates a well-organized fibrocartilage interface with columnar organization of the chondrocytes. **Fig. 7-D** Hematoxylin and eosin and safranin-O staining (D and D' [inset]) showing an ACL reconstruction with a graft in both the femoral and tibial tunnels at 4 weeks post-surgery.

Fig. 8

Representative sagittal histological sections of the native ACL insertion site showing immunohistochemical staining for Wnt, β -catenin, Gli1, PTHrP, Patched, and Indian hedgehog (Ihh). F = femur, and T = tibia.

of the newly formed bone in the tunnels are summarized in Figure 6. Although the amount of bone was greater for most measures in Group 3, these differences did not reach significance ($p > 0.05$ for all).

Histological analysis showed that healing occurred by formation of fibrovascular tissue between the tendon and bone.

There were occasional chondrocytes at the healing interface, but a regular fibrocartilaginous enthesis was not seen (Fig. 7 [Link to [hematoxylin and eosin](#) as well as [safranin O](#)-stained whole slide images]). There was progressive maturation of this fibrovascular interface tissue. TRAP histochemical staining demonstrated osteoclasts along the tendon-bone interface. The number of

Fig. 9

Representative axial histological sections of graft, interface, and surrounding bone showing immunohistochemical staining for Wnt, β -catenin, Indian hedgehog (Ihh), Gli1, Sox-9, PTHrP, Patched, and TRAP, at the 1, 2, and 4-week time points. Graphs next to each row plot the percentage of DAB (3,3'-diaminobenzidine)-positive area at each time point. For TRAP, the number of positively stained osteoclasts was highest at 1 week and declined over time (10 $\times$ magnification, specific antibody staining with use of DAB as a chromogen and hematoxylin as a counterstain). I-bars indicate the standard deviation.

Fig. 10

qRT-PCR analysis of scleraxis (SCX), Mohawk (MKX), tenomodulin (TNM), COL3, COL1, Sox-9, aggrecan (ACAN), MMP3, MMP13, and MMP14 gene expression at the bone-tunnel interface following ACL reconstruction, using the native FDL as the control. An asterisk indicates a significant difference ($p < 0.05$). I-bars indicate the standard deviation.

positively stained osteoclasts was highest at 1 week and declined over time.

Immunohistochemical analysis showed that there was positive expression of mediators in the Indian hedgehog, Wnt, and PTHrP pathways in both the native insertion site and the healing tissues. Positive staining was seen in fibrochondrocytes at the native insertion site and was generally weaker than the staining in the repaired tissues (Fig. 8). We found positive localization of signaling proteins that are part of the Wnt, Indian hedgehog, and PTHrP pathways in cells in the healing graft-bone interface. The number of positively staining cells was generally similar at each time point among most markers (Fig. 9).

qRT-PCR analysis revealed no change in expression of the transcription factors scleraxis and Sox-9 compared with the time-0 control tendon. We found that matrix metalloproteinases (MMPs) 3 and 13 were transiently upregulated on postoperative days 7 and 14, respectively, and declined thereafter (Fig. 10). MMP14 expression increased by postoperative day 14 ($p = 0.137$) and remained elevated on day 28. There was a trend toward increased expression of collagen type 1 α 1 (COL1) over time ($p > 0.05$) and significantly increased aggrecan (ACAN) ($p = 0.020$) (Fig. 10).

Discussion

Our hypothesis that signaling molecules that direct enthesis formation during embryogenesis would not be expressed at the healing tendon-bone interface was not supported, as we found expression of mediators in the Indian hedgehog, Wnt, and PTHrP pathways in the healing tissues. We found that healing proceeds by formation of a fibrovascular scar-tissue interface between the tendon graft and bone, similar to published findings in larger animal models where the normal enthesis microstructure is not reformed. Our microCT findings suggest that graft incorporation occurs by infiltration of new bone into the outer tendon, consistent with findings in prior animal studies. Osteoclast accumulation with subsequent bone resorption is consistent with the phenomenon of tunnel-

widening that is seen in humans. These findings demonstrate that expression of signaling molecules that direct enthesis formation during prenatal and early postnatal development is not sufficient to direct reformation of the complex microstructure and composition of the native attachment site.

The graft failure force reached approximately one-third of the native ACL failure load at 4 weeks, similar to findings in prior animal models^{11,12}. The majority of graft failures occurred at 1 of the tunnels, in contrast to the native ACL, where failure occurs in the midsubstance. These findings verify that the healing tendon-bone interface represents the weakest link in the healing process^{11,15,21-25}.

The signaling molecules that we examined are known to play a fundamental role during insertion-site development in embryogenesis. Our findings are consistent with prior work in our laboratory, where we reported Indian hedgehog immunostaining at the healing tendon-bone interface in a rat ACL-reconstruction model and a rat rotator cuff-repair model^{26,27}. Our data indicate that factors other than these signaling molecules are required for formation of the complex microstructure and composition of the enthesis, and the data support the prevailing concept that recapitulation of developmental events in the animal in the postnatal period is difficult.

The failure to reform a native insertion site is likely due to one of several factors. First, it is not known which temporal pattern, spatial pattern, and magnitude of expression of these signaling molecules are necessary to direct insertion-site formation. We found no change in gene expression of the transcription factors scleraxis (tendon-specific) and Sox-9 (cartilage-specific), although our study was likely underpowered to find a difference. These factors are known to play a fundamental role in insertion-site formation in the developing embryo¹⁸.

Second, we hypothesize that there is an absence or insufficiency of appropriate responding cells present at the healing site. A progenitor cell population is necessary for tissue regeneration.

There are likely important differences in the phenotype and function of cells present in the animal in the postnatal period compared with the cells present during embryogenesis.

A third factor that may play a role in the failure of insertion-site formation is the local mechanical environment during healing. Mechanical loads have a profound effect on the biologic events of tissue formation and remodeling. The important role of mechanical loading is illustrated by studies showing abnormal insertion-site formation in animals where the developing limb was paralyzed with botulinum toxin in the immediate postnatal period²⁸. It is likely that the mechanical forces acting on cells in the bone tunnel differ from those present during embryonic development.

A final factor that may account for the failure of insertion-site formation is the role of inflammation postoperatively in the animal in the postnatal period. The presence of cell populations and inflammatory mediators associated with the inflammatory process may well have a profound effect on tissue regeneration. Wounds heal by tissue regeneration in the developing embryo prior to the development of a functional immune system and the associated inflammatory response rather than by the typical scar formation that is seen in the animal in the postnatal period. The presence of inflammation in our animal model likely has a considerable effect on the function of these signaling molecules.

The major limitation of this work is the small size of the murine knee and the unknown translation of quadruped models to humans. The procedure requires development of microsurgical skills and has a substantial learning curve. We recommend extensive cadaveric practice sessions and quantitative biomechanical evaluation of the ability to achieve graft isometry and secure graft fixation before studying live animals. The small number of specimens analyzed with PCR and the large variability across samples limits our ability to make firm conclusions about gene expression.

In conclusion, we have developed a reproducible and realistic murine model of ACL reconstruction that simulates techniques used in human ACL reconstruction. Despite expression of several signaling molecules that direct embryologic insertion-site formation, the spatial and temporal pattern of expression was not adequate to restore the structure and composition of the native insertion site. This clinically realistic murine model of ACL reconstruction will allow for eventual use of genetically modified animals to study the role of specific molecular pathways in healing in order to identify interventions that improve healing in our patients^{16,18,29}. ■

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