Defining the Baseline Transcriptional Fingerprint of Rabbit Hamstring Autograft

Mario Hevesi MD[1], Christopher R. Paradise[1,2,3], Carlo A. Paggi MS[1,4], Catalina Galeano-Garces[1], Amel Dudakovic PhD[1,5], Sanjeev Kakar MD[1], Timothy E. Hewett PhD[1], Aaron J. Krych MD[1], Andre J. van Wijnen PhD[1,5], Daniel B. F. Saris MD PhD[1,6]

1. *Department of Orthopedic Surgery, Mayo Clinic, Rochester, MN, USA 55905*
2. *Mayo Clinic Graduate School of Biomedical Sciences, Rochester, MN USA, 55905*
3. *Mayo Clinic Center for Regenerative Medicine, Rochester, MN, USA, 55905*
4. *Department of Regenerative Medicine, University of Twente, Enschede, the Netherlands*
5. *Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA 55905*
6. *Department of Orthopedics, University Medical Center Utrecht, Utrecht, Netherlands*

Corresponding Author:

Daniel B.F. Saris, MD, PhD
Department of Orthopedic Surgery
200 1st St SW
Rochester, MN 55905, USA
saris.daniel@mayo.edu

ABSTRACT

Anterior cruciate ligament (ACL) injuries are common and of high relevance given their significant effects on patient function, quality of life, and posttraumatic arthritis. To date, investigators have reported on the expression of genes classically associated with tendon and ligament reconstruction, including decorin (DCN) and collagen type 1 (COL1A1 and COL1A2). However, the transcriptional fingerprint for hamstring tendons, one of the most common autografts used for ACLR, remains to be determined. The purpose of this study was to characterize the baseline transcriptional state of semitendinosus autografts in a rabbit model for ACLR and to employ such characterization to guide scientifically-driven target gene selection for future analyses.

Next generation RNA sequencing was performed on whole semitendinosus autografts from four New Zealand White rabbits (mean age: 193 ± 0 days, mean weight: 2.78 kg ± 0.15 kg) and subsequently analyzed using gene enrichment and protein-protein interaction network analysis. Decorin, Secreted Protein Acidic and Cysteine Rich (SPARC), Collagen type 1, and Proline and Arginine Rich End Leucine Rich Repeat Protein (PRELP) and were determined to be the highest expressed genes with tendon-associated ontology. These results strengthen the association between genes such as DCN, COL1A1, and COL1A2 and tendon tissues as well as provide the novel addition of further high-expression, tendon characteristic genes such as SPARC and PRELP to provide guidance as to which molecules serve as high-signal candidates for future ACL research. In addition, this paper provides open-access to the expression fingerprint of hamstring autograft for ACLR in New Zealand White rabbits, thus providing a readily-accessible collaborative reference, in alignment with ethical animal research principles.

Keywords: ACL, RNA Sequencing, transcriptional fingerprint, SPARC, PRELP, rabbit

INTRODUCTION

Anterior crucial ligament (ACL) injuries are of high clinical relevance given their frequency, effects on patient function, and potential for associated meniscus and cartilage injury.[1-3] Given their high incidence and prolonged recovery, ACL research expenditure is amongst the highest in orthopedics.[4, 5, 3] While methods of ACL injury prevention are increasingly recognized and employed, the rate of ACL injuries continues to rise.[2, 6]

A key aspect of ACL injury research has been the development of various biomaterial and biologic adjuncts to ACL reconstruction (ACLR) and associated animal models.[7-9] Subsequently, rabbits have emerged as providing the gold standard for animal research models.[9-12] Mouse models have been described, but there are limitations in the amount of material for subsequent molecular, histological, and biomechanical analysis, which has made rabbits the preferred species for research in this field.[13, 14] Furthermore, with the use of rabbit models, a semitendinosus autograft can be harvested at the time of surgery, which provides a hamstring-based reconstruction, much as is performed clinically in humans.[15, 16]

As sequencing technologies and downstream bioinformatic pipelines rapidly improve, the transcriptomic state of cells and tissues can be accurately and precisely assessed. Our group has successfully utilized RNA sequencing (RNA-seq) to characterize cell types, tissues, and disease states across a wide range of *in vitro* and *in vivo* orthopedic applications[17-21]. In doing so, we have come to appreciate the value of such datasets in describing cells and tissues, phenotyping animal models, as well as characterizing human disease states.

In reviewing the ACL literature, investigated molecular markers are often selected and reported on the basis of academic precedence, with quantification of genes such as decorin (DCN) and collagen type 1 (COL1A1 and COL1A2). [22-26] However, to date, the overall molecular fingerprint of rabbit hamstring tissue has yet to be characterized through modern methods such as RNA sequencing. Therefore, it would be of significant knowledge to both characterize the baseline transcriptional state of such ACL reconstructive tissues and also to use this characterization for the selection of genes for future investigation.

Furthermore, a central tenet of ethical animal research is the maximization of benefit while minimizing unnecessary duplication of previous research. Given that a large portion of musculoskeletal rabbit experiments are carried out using the New Zealand White species[27, 10, 28,

[23, 29, 30], there exists practical and ethical value in describing the basal transcriptional state of rabbit hamstring tendons. By publishing open-access mRNA sequencing data for the most commonly used rabbit breed from one of the world's largest suppliers of Specific Pathogen Free (SPF) rabbits (Covance, Princeton, NJ), data can subsequently be employed for post-reconstruction RNA sequencing comparisons as well as for the discovery and establishment of target genes for in-laboratory RT-qPCR.

Therefore, the authors' open-access investigation of New Zealand White rabbit semitendinosus grafts is of significant research relevance given the paucity of literature on the baseline transcriptional state of hamstring tissues, large volume of publications in this area, ethical goals of animal studies, and the status of rabbits as the gold standard for small animal ACL research.

MATERIALS AND METHODS

Hamstring Harvest Technique

Under sterile conditions, rabbit semitendinosus autografts were harvested employing a midline incision centered over the anterior aspect of the knee for four rabbits (mean age: 193 ± 0 days, mean weight: 2.78 kg ± 0.15 kg). A medial flap was developed along the fascial plane of the patellar tendon by exposure of the medial collateral ligament (MCL). Subsequently, a transverse incision was made in the muscular fascia just posterior and medial to the MCL and the medial edge of the quadriceps was lifted to expose the semitendinosus. The distal insertion of the tendon was released and retracted to allow for mobilization of the tendon to its proximal aspect. Thereafter, the proximal aspect of the tendon was divided, providing 3-4 cm of tendon autograft for subsequent reconstruction. For samples to be used for RNA sequencing, muscle was debrided from the tendon surface employing gentle perpendicular sweeps of a clean scalpel blade. Thereafter, tendon was rinsed in sterile PBS and frozen at -80° C until mRNA isolation and sequencing.

mRNA Isolation Procedure

Frozen tendon biopsies were removed from -80° C and kept in liquid nitrogen at all times during processing. Individually, tendons were ground into a fine powder using a mortar and pestle set

on dry ice while re-applying liquid nitrogen as needed (approximately every 30 seconds). Powder was then transferred to a sterile 1.5ml Eppendorf tube and 700µl of TRI Reagent (Zymogen Research) was added. Total mRNA was extracted using a Zymogen Research Direct-zol RNA Kit (Zymogen Research) and quantified using the NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware).

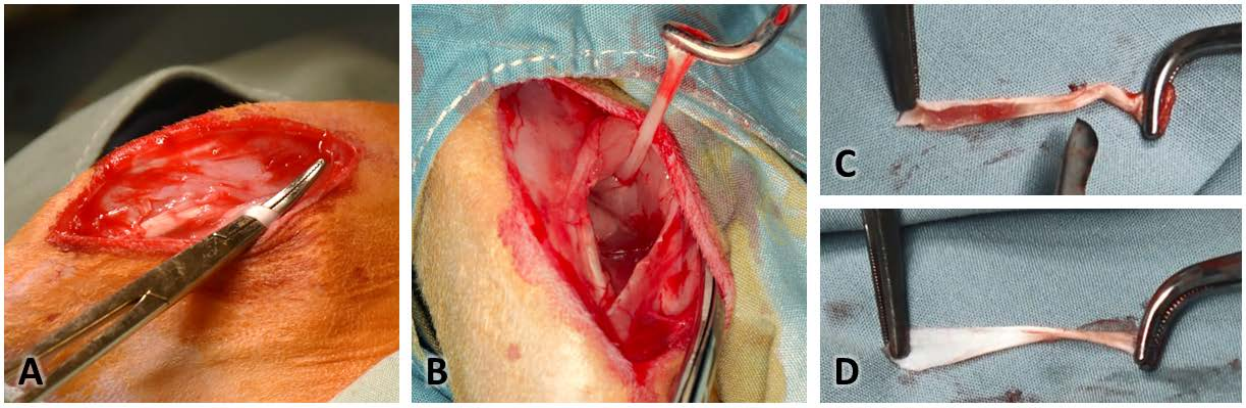

Figure 1: Semitendinosus graft harvest and preparation. The semitendinosus is identified on the medial side of the knee (A), divided distally and isolated along its proximal course (B), atraumatically cleared of muscle using a fresh scalpel (C), and prepared for final washing in PBS (D).

RNA-sequencing

RNA sequencing and subsequent bioinformatic analysis were performed in collaboration with the Mayo Clinic RNA sequencing and bioinformatics cores, as has been previously described in detail[31, 32]. RNA integrity was assessed using the Agilent Bioanalyzer DNA 1000 chip (Invitrogen, Carlsbad, CA). Only samples with an RNA Integrity Number (RIN) and DV200 score greater than our Sequencing Core's minimum cutoff (RIN >6 and DV200 > 50%) were used for sequencing. In brief, library preparation was performed using the TruSeq RNA library preparation kit (Illumina, San Diego, CA). Polyadenylated mRNAs were selected using oligo dT magnetic beads. TruSeq Kits were used for indexing to permit multiplex sample loading on the flow cells. Paired-end sequencing reads were generated on the Illumina HiSeq 4000 sequencer. Quality control for concentration and library size distribution was performed using an Agilent Bioanalyzer DNA 1000 chip and Qubit fluorometry (Invitrogen, Carlsbad, CA). Sequence

alignment of reads and determination of normalized gene counts were performed using the MAP-RSeq (v.1.2.1) workflow, utilizing TopHat 2.0.6[33], and HTSeq[34]. Normalized read counts were expressed as reads per kilobasepair per million mapped reads (RPKM). Data have been deposited in the GEO Database.

Tertiary Analysis

Gene Ontology term overlap was conducted using the Compute Overlap tool in the Molecular Signature Database (MSigDB) v6.2 suite on the Gene Set Enrichment Analysis (GSEA) website[35-37]. Protein-protein interaction networks were generated using STRING Database version 10.5[38, 39].

RESULTS

To assess the quality of the dataset and offer a general description for investigators, we first created a standard plot of average RPKM values for all annotated genes across the four samples (Figure 2A). Supporting the efficacy and validity of our sequencing data, we note the classic distribution of reads with few genes receiving a large number of reads while most of the genes received 10's-100's of mapped reads. Because an expected small proportion of genes received a large majority of the mapped reads, we investigated these genes specifically given that they represent genes of potential biologic significance as well as targets for measurement in future studies (Figure 2B). Genes classically involved in tendon formation (i.e., DCN, COL1A1, and MGP) received 10% of the total reads. Concurrently, we noted that several of the genes receiving the most reads were markers of mitochondria and muscle (14% and 3% of total reads, respectively), as is to be expected given the intimate relationship of tendon and muscle.

To better understand the molecular signature of the tendon samples, we conducted Gene Ontology keyword overlap using the Gene Set Enrichment Analysis (GSEA) Compute Overlap online tool. The top 25 expressed genes were used to compute overlaps with Gene Ontology terms to produce a bubble chart (Figure 2C). Gene Ontology terms related to extracellular matrix (ECM) production demonstrated the most significant enrichment and largest number of genes (i.e., COL1A1, COL1A2, PRELP, SPARC, DCN) overlapping with the input gene list. This same gene list was utilized to construct a protein-protein interaction network using STRING

online software (Figure 2D) and resulted in clustering of tendon- and muscle-specific genes into distinct nodes.

Given the presence of muscle markers in our RNA sequencing data following sample preparation including muscle debridement, we assessed the expression levels of specific muscle and tendon markers in our novel tendon samples compared to previously described muscle samples from the GEO Database (accession#: GSE60591) (Figure 2E). When comparing our tendon samples to those of well-described muscle specimens, we noted significantly lower expression of muscle markers ACTA1 ($p < 0.001$) and TNNC1 ($p = 0.027$) in rabbit hamstring tissues as compared to the isolated muscle samples, supporting that our obtained samples are representative of the tendon transcriptional fingerprint. In addition, we observed enhanced expression of tendon-related markers DCN ($p < 0.001$), SPARC ($p < 0.001$), COL1A2 ($p = 0.005$), and PRELP ($p < 0.001$) when comparing the tendon and muscle tissues side-by-side. Thus, although tendon and muscle are intricately related and there may be residual muscle contamination, RNA sequencing data presented is dominantly representative of isolated, debrided rabbit hamstring, as would be expected in the setting of ACL reconstruction.

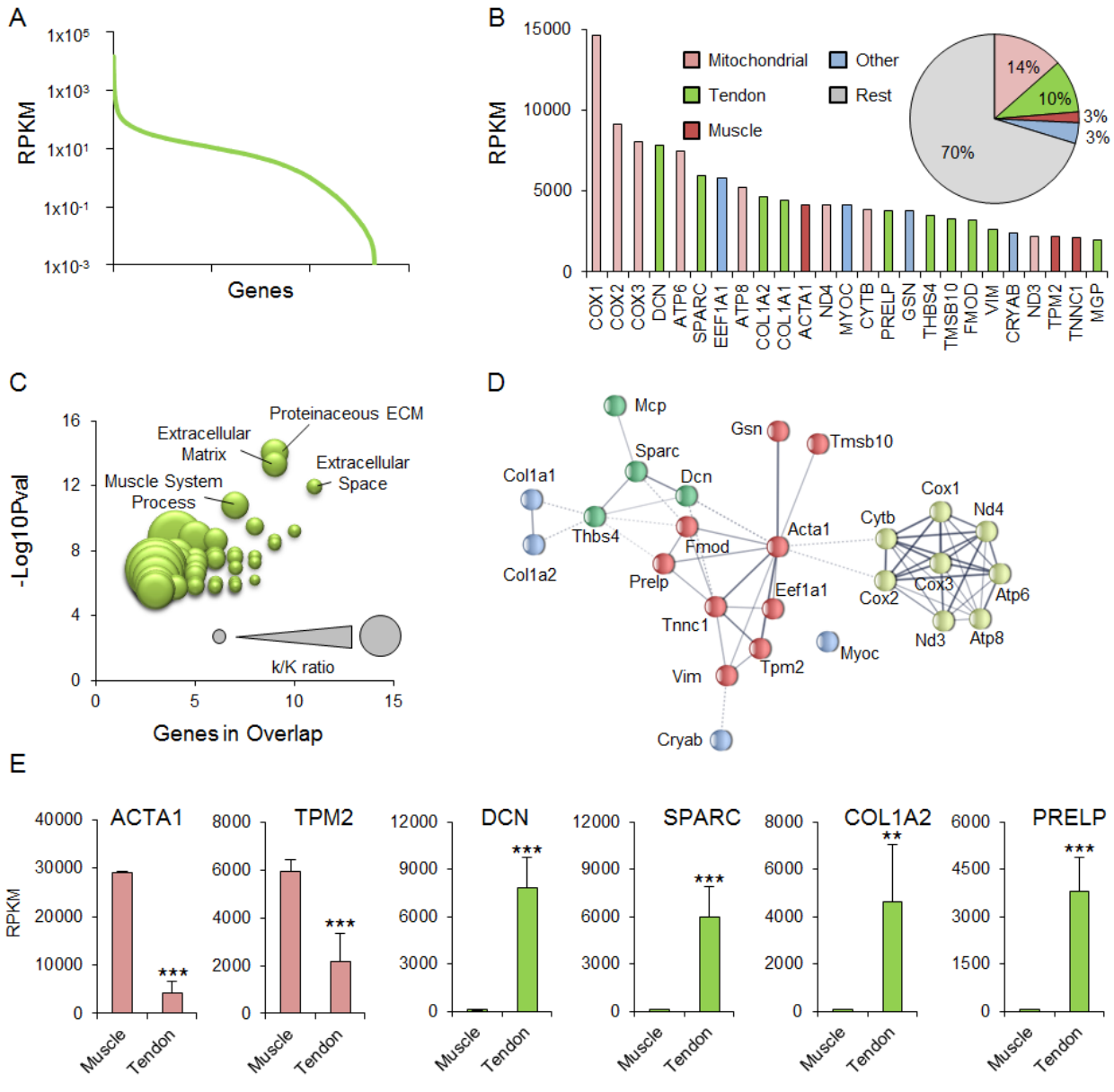

Figure 2: Tertiary analysis of RNA-seq derived from hamstring grafts prior to ACLR. Read counts were converted to reads per kilobase per million mapped reads (RPKM) and average expression across the four samples was evaluated for each gene **(A)**. The top 25 expressed genes were determined **(B)** and used for subsequent Gene Ontology keyword overlap **(C)** and STRING protein-protein interaction network analysis **(D)**. Expression levels of muscle markers (red) and tendon markers (green) were evaluated in pure muscle samples (Muscle) compared to our isolated hamstring grafts (Tendon) **(E)**.

DISCUSSION

Anterior cruciate ligament injury remains a point of focus in orthopedic research and clinical practice given its high prevalence and potential for subsequent meniscus and joint degeneration. [1-3] A key aspect of ACL research has been the creation of animal models for the evaluation of novel biomaterials and adjuncts for ACL reconstruction, with rabbit models providing the gold standard for ACLR given their clinically relevant hamstring-based technique and appropriate size for molecular, histologic, and biomechanical studies. This paper provides novel characterization and open-access availability of the transcriptional fingerprint of rabbit hamstring autograft, serving as a reference for future comparisons and a guide for establishing molecular research targets.

There is a current need in the literature for tendon transcriptional characterization, with few animal studies and no human studies characterizing hamstring graft gene expression. Furthermore, current studies with PCR-based analyses often analyze a subset of candidate genes which have been classically associated with tendons (i.e. COL1A1, DCN), however, the prioritization and selection of these molecular targets is often a matter of expert opinion and not rigorous scientific evaluation and prioritization.

DCN was determined to be the highest expressed tendon-specific gene in terms of RPKM counts and this gene has previously been well described in the setting of tendons in general as well as rabbit ACL models in particular. [22-24] Additionally, we observed a high basal level of COL1A1 and COL1A2, as has been previously well characterized.[23, 25, 26] However, SPARC was noted to be 2nd highest expressed tendon marker and the 6th highest overall gene, yet a paucity of data exists for this marker in the tendon and ligament setting.[40-42] This highlights the need for large RNA sequencing efforts prior to focused, PCR-based evaluation of tissues. Given its large role in basal hamstring expression, SPARC, which serves as a cysteine-rich acidic matrix-associated protein involved in cell growth and extracellular matrix synthesis, should be highly considered for evaluation in rabbit models of tendon healing.

In addition, PRELP, a leucine-rich protein involved in connective tissue extracellular matrix structure and molecular anchoring, provides a significant target for tendon studies. To date, the role of PRELP in tendon tissues has only been discussed in one paper focusing on bovine deep

flexor tendons.[43] The protein has been previously characterized to be the major proteinaceous component of flexor tendons along with type I collagen (85% dry weight) and decorin (DCN, 1% dry weight).[43, 44] In this study, PRELP's status as the 15th most expressed gene amongst 20,000+ genes and third highest tendon specific signal after DCN, SPARC, and COL1A1/COL1A2, place it as candidate for prioritized quantification when evaluating ACLR, especially given that previous papers have focused on and evaluated lower-signal genes such as VIM, MGP, and COL4A1.[45-48]

This paper has certain important limitations. First, as these grafts are intricately involved with muscle both on physical and molecular levels, we anticipate a small degree of muscle contamination, even following careful surgical debridement. Despite this, we have demonstrated that our samples are predominantly tendinous, with high tendon-specific signals such as DCN and SPARC and significantly decreased muscle markers such as ACTA1 and TPM2. Therefore, we are confident in presenting these samples as tendon biopsies with slight muscle contamination as to be expected after collection from the hamstring. Second, there may be differences in tendon gene expression with various suppliers of New Zealand White rabbits and other common species used in research. To this end, we have evaluated a well-established rabbit breed, as provided by one of the largest research providers of rabbits globally in order to improve generalizability and applicability for other laboratory groups. Finally, given that gene expression may vary with developmental status and age, we have provided the ages and weights of the evaluated rabbits for groups wishing to optimize and reproduce our experimental conditions.

CONCLUSION

By determining the RNA sequencing of whole rabbit semitendinosus autograft, this paper provides novel guidance as to which molecules serve as high-signal candidate genes for further analysis and pre- and post-intervention comparisons. In doing so, we have strengthened the association between genes such as COL1A1, COL1A2, and DCN and tendon tissues as well as provided the novel addition of further high-expression, tendon characteristic genes such as SPARC and PRELP. In addition, this paper provides open-access to the expression fingerprint

of hamstring autograft for ACLR in New Zealand White rabbits, thus providing a readily-accessible collaborative reference, in alignment with ethical animal research principles.

ACKNOWLEDGEMENTS

The authors acknowledge the generous philanthropic support of William and Karen Eby and thank the members of our laboratory including Janet M. Denbeigh PhD, Eric R. Wagner MD, and Joshua A. Parry MD, for stimulating discussions. The authors also acknowledge the support and assistance of Steve Krage and Joanne M. Pedersen.

DECLARATIONS OF INTEREST

MH: Moximed: Paid consultant

CRP, CAP, CG-G, and AD: None

SK: Arthrex, Inc: Paid consultant, Journal of Bone and Joint Surgery - American: Editorial or governing board, Journal of Bone and Joint Surgery - British: Editorial or governing board, Sonex Healthcare: Stock or stock Options

TEH: None

AJK: Aesculap/B.Braun: Research support, American Journal of Sports Medicine: Editorial or governing board, Arthrex, Inc: IP royalties; Paid consultant; Research support, Arthritis Foundation: Research support, Ceterix: Research support, Histogenics: Research support, International Cartilage Repair Society: Board or committee member, International Society of Arthroscopy, Knee Surgery, and Orthopaedic Sports Medicine: Board or committee member, JRF Ortho: Paid consultant, Minnesota Orthopedic Society: Board or committee member, Musculoskeletal Transplantation Foundation: Board or committee member, Vericel: Paid consultant

AJvW: GENE and GENE Reports: Editorial or governing board.

DBFS: Cartiheal: Paid consultant, Cartilage: Editorial or governing board, Ivy Sports: Research support, Smith & Nephew: Paid consultant; Research support

REFERENCES

1. Nessler T, Denney L, Sampley J: ACL Injury Prevention: What Does Research Tell Us? *Current reviews in musculoskeletal medicine* 2017;10:281-288.
2. Hewett TE, Myer GD, Ford KR, Paterno MV, Quatman CE: Mechanisms, prediction, and prevention of ACL injuries: Cut risk with three sharpened and validated tools. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2016;34:1843-1855.
3. McArdle S: Psychological rehabilitation from anterior cruciate ligament-medial collateral ligament reconstructive surgery: a case study. *Sports health* 2010;2:73-77.
4. Samitier G, Marcano AI, Alentorn-Geli E, Cugat R, Farmer KW, Moser MW: Failure of Anterior Cruciate Ligament Reconstruction. *The archives of bone and joint surgery* 2015;3:220-240.
5. Zaffagnini S, Grassi A, Serra M, Marcacci M: Return to sport after ACL reconstruction: how, when and why? A narrative review of current evidence. *Joints* 2015;3:25-30.
6. Webster KE, Hewett TE: Meta-analysis of meta-analyses of anterior cruciate ligament injury reduction training programs. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2018;36:2696-2708.
7. Crispim JF, Fu SC, Lee YW, et al.: Bioactive Tape With BMP-2 Binding Peptides Captures Endogenous Growth Factors and Accelerates Healing After Anterior Cruciate Ligament Reconstruction. *The American journal of sports medicine* 2018;46:2905-2914.
8. Crispim J, Fernandes HAM, Fu SC, Lee YW, Jonkheijm P, Saris DBF: TGF-beta1 activation in human hamstring cells through growth factor binding peptides on polycaprolactone surfaces. *Acta biomaterialia* 2017;53:165-178.
9. Parry JA, Wagner ER, Kok PL, et al.: A Combination of a Polycaprolactone Fumarate Scaffold with Polyethylene Terephthalate Sutures for Intra-Articular Ligament Regeneration. *Tissue engineering Part A* 2018;24:245-253.
10. Wang XM, Ji G, Wang XM, Kang HJ, Wang F: Biological and Biomechanical Evaluation of Autologous Tendon Combined with Ligament Advanced Reinforcement System Artificial Ligament in a Rabbit Model of Anterior Cruciate Ligament Reconstruction. *Orthopaedic surgery* 2018;10:144-151.
11. Chen B, Zhang J, Nie D, Zhao G, Fu FH, Wang JH: Characterization of the structure of rabbit anterior cruciate ligament and its stem/progenitor cells. *Journal of cellular biochemistry* 2018.
12. Liu S, Sun Y, Wan F, Ding Z, Chen S, Chen J: Advantages of an Attached Semitendinosus Tendon Graft in Anterior Cruciate Ligament Reconstruction in a Rabbit Model. *The American journal of sports medicine* 2018;46:3227-3236.
13. Deng XH, Lebaschi A, Camp CL, et al.: Expression of Signaling Molecules Involved in Embryonic Development of the Insertion Site Is Inadequate for Reformation of the Native Enthesis: Evaluation in a Novel Murine ACL Reconstruction Model. *The Journal of bone and joint surgery American volume* 2018;100:e102.
14. Camp CL, Lebaschi A, Cong GT, et al.: Timing of Postoperative Mechanical Loading Affects Healing Following Anterior Cruciate Ligament Reconstruction: Analysis in a Murine Model. *The Journal of bone and joint surgery American volume* 2017;99:1382-1391.

15. MARS Group: Effect of graft choice on the outcome of revision anterior cruciate ligament reconstruction in the Multicenter ACL Revision Study (MARS) Cohort. *The American journal of sports medicine* 2014;42:2301-2310.
16. Kaeding CC, Pedroza AD, Reinke EK, et al.: Change in Anterior Cruciate Ligament Graft Choice and Outcomes Over Time. *Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association* 2017;33:2007-2014.
17. Dudakovic A, Camilleri ET, Paradise CR, et al.: Enhancer of zeste homolog 2 (Ezh2) controls bone formation and cell cycle progression during osteogenesis in mice. *The Journal of biological chemistry* 2018;293:12894-12907.
18. Paradise CR, Galeano-Garces C, Galeano-Garces D, et al.: Molecular characterization of physis tissue by RNA sequencing. *Gene* 2018;668:87-96.
19. Samsonraj RM, Paradise CR, Dudakovic A, et al.: Validation of Osteogenic Properties of Cytochalasin D by High-Resolution RNA-Sequencing in Mesenchymal Stem Cells Derived from Bone Marrow and Adipose Tissues. *Stem cells and development* 2018;27:1136-1145.
20. Galeano-Garces C, Camilleri ET, Riester SM, et al.: Molecular Validation of Chondrogenic Differentiation and Hypoxia Responsiveness of Platelet-Lysate Expanded Adipose Tissue-Derived Human Mesenchymal Stromal Cells. *Cartilage* 2017;8:283-299.
21. Dudakovic A, Gluscevic M, Paradise CR, et al.: Profiling of human epigenetic regulators using a semi-automated real-time qPCR platform validated by next generation sequencing. *Gene* 2017;609:28-37.
22. Juneja SC, Veillette C: Defects in tendon, ligament, and enthesis in response to genetic alterations in key proteoglycans and glycoproteins: a review. *Arthritis* 2013;2013:154812-154812.
23. Hoyer M, Meier C, Kohl B, Lohan A, Kokozidou M, Schulze-Tanzil G: Histological and biochemical characteristics of the rabbit anterior cruciate ligament in comparison to potential autografts. *Histology and histopathology* 2016;31:867-877.
24. Haslauer CM, Proffen BL, Johnson VM, Murray MM: Expression of modulators of extracellular matrix structure after anterior cruciate ligament injury. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 2014;22:103-110.
25. Kato S, Saito M, Funasaki H, Marumo K: Distinctive collagen maturation process in fibroblasts derived from rabbit anterior cruciate ligament, medial collateral ligament, and patellar tendon in vitro. *Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA* 2015;23:1384-1392.
26. Kaynak M, Nijman F, van Meurs J, Reijman M, Meuffels DE: Genetic Variants and Anterior Cruciate Ligament Rupture: A Systematic Review. *Sports medicine (Auckland, NZ)* 2017;47:1637-1650.
27. Wang R, Xu B, Xu HG: Up-Regulation of TGF-beta Promotes Tendon-to-Bone Healing after Anterior Cruciate Ligament Reconstruction using Bone Marrow-Derived Mesenchymal Stem Cells through the TGF-beta/MAPK Signaling Pathway in a New Zealand White Rabbit Model. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2017;41:213-226.
28. Papachristou G, Tilentzoglou A, Efstathopoulos N, Khaldi L: Reconstruction of anterior cruciate ligament using the doubled tendon graft technique: an experimental study in rabbits. *Knee Surgery, Sports Traumatology, Arthroscopy* 1998;6:246-252.

29. Sekiguchi H, Post WR, Han JS, Ryu J, Kish V: The effects of cyclic loading on tensile properties of a rabbit femur–anterior cruciate ligament–tibia complex (FATC). *The Knee* 1998;5:215-220.
30. Bachy M, Sherifi I, Zadegan F, Petite H, Vialle R, Hannouche D: Allograft integration in a rabbit transgenic model for anterior cruciate ligament reconstruction. *Orthopaedics & Traumatology: Surgery & Research* 2016;102:189-195.
31. Dudakovic A, Camilleri E, Riester SM, et al.: High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. *Journal of cellular biochemistry* 2014;115:1816-1828.
32. Kalari KR, Nair AA, Bhavsar JD, et al.: MAP-RSeq: Mayo Analysis Pipeline for RNA sequencing. *BMC bioinformatics* 2014;15:224.
33. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL: TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology* 2013;14:R36.
34. Anders S, Pyl PT, Huber W: HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)* 2015;31:166-169.
35. Subramanian A, Tamayo P, Mootha VK, et al.: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:15545-15550.
36. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdottir H, Tamayo P, Mesirov JP: Molecular signatures database (MSigDB) 3.0. *Bioinformatics (Oxford, England)* 2011;27:1739-1740.
37. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P: The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell systems* 2015;1:417-425.
38. Szklarczyk D, Franceschini A, Wyder S, et al.: STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic acids research* 2015;43:D447-452.
39. Szklarczyk D, Morris JH, Cook H, et al.: The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic acids research* 2017;45:D362-d368.
40. Maillard C, Malaval L, Delmas PD: Immunological screening of SPARC/Osteonectin in nonmineralized tissues. *Bone* 1992;13:257-264.
41. Gagliano N, Pelillo F, Chiriva-Internati M, et al.: Expression profiling of genes involved in collagen turnover in tendons from cerebral palsy patients. *Connective tissue research* 2009;50:203-208.
42. Gehwolf R, Wagner A, Lehner C, et al.: Pleiotropic roles of the matricellular protein Sparc in tendon maturation and ageing. *Scientific reports* 2016;6:32635.
43. Vogel KG, Meyers AB: Proteins in the tensile region of adult bovine deep flexor tendon. *Clinical orthopaedics and related research* 1999:S344-355.
44. Koob TJ, Vogel KG: Site-related variations in glycosaminoglycan content and swelling properties of bovine flexor tendon. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 1987;5:414-424.
45. Park SA, Kim IA, Lee YJ, et al.: Biological responses of ligament fibroblasts and gene expression profiling on micropatterned silicone substrates subjected to mechanical stimuli. *Journal of bioscience and bioengineering* 2006;102:402-412.

46. Kuo CK, Marturano JE, Tuan RS: Novel strategies in tendon and ligament tissue engineering: Advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT* 2010;2:20-20.
47. Smith KD, Vaughan-Thomas A, Spiller DG, Clegg PD, Innes JF, Comerford EJ: Variations in cell morphology in the canine cruciate ligament complex. *Veterinary journal (London, England : 1997)* 2012;193:561-566.
48. Jiang YY, Park JK, Yoon HH, Choi H, Kim CW, Seo YK: Enhancing proliferation and ECM expression of human ACL fibroblasts by sonic vibration. *Preparative biochemistry & biotechnology* 2015;45:476-490.