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Defining the baseline transcriptional fingerprint of rabbit hamstring autograft

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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.

1. 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 (Nessler et al., 2017; Hewett et al., 2016; McArdle, 2010). Given their high incidence and prolonged recovery, ACL research expenditure is amongst the highest in orthopedics (McArdle, 2010; Samitier et al., 2015; Zaffagnini et al., 2015). While methods of ACL injury prevention are increasingly recognized and employed, the rate of ACL injuries continues to rise (Hewett et al., 2016; Webster and Hewett, 2018).

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 (Crispim et al., 2018; Crispim et al.,

Abbreviations: ACL, Anterior Cruciate Ligament; ACLR, Anterior Cruciate Ligament Reconstruction; ACTA1, Actin, Alpha, Skeletal Muscle 1; COL1A1, Collagen, Type I, Alpha1; COL1A2, Collagen, Type I, Alpha2; COL4A1, Collagen Type IV Alpha 1 Chain; DCN, Decorin; ECM, Extracellular Matrix; GSEA, Gene Set Enrichment Analysis; MCL, Medial Collateral Ligament; MGP, Matrix Gla Protein; mRNA, Messenger RNA; PCR, Polymerase Chain Reaction; PRELP, Proline/Arginine-rich End Leucine-rich Repeat Protein; RNA, Ribonucleic Acid; RNA-Seq, RNA Sequencing; RPKM, *Reads Per Kilobase Million*; RT-qPCR, Quantitative Real Time Polymerase Chain Reaction; SPARC, Secreted Protein Acidic and Rich in Cysteine; SPF, Specific-pathogen-free; TPM2, Tropomyosin 2; VIM, Vimentin

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2017; Parry et al., 2018). Subsequently, rabbits have emerged as providing the gold standard for animal research models (Parry et al., 2018; Wang et al., 2018; Chen et al., 2018; Liu et al., 2018). 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 (Deng et al., 2018; Camp et al., 2017). 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 (MARS Group, 2014; Kaeding et al., 2017).

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 (Dudakovic et al., 2018; Paradise et al., 2018; Samsonraj et al., 2018; Galeano-Garces et al., 2017; Dudakovic et al., 2017). 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) (Juneja and Veillette, 2013; Hoyer et al., 2016; Haslauer et al., 2014; Kato et al., 2015; Kaynak et al., 2017). 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 (Wang et al., 2018; Hoyer et al., 2016; Wang et al., 2017; Papachristou et al., 1998; Sekiguchi et al., 1998; Bachy et al., 2016), 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.

2. Materials and methods

2.1. 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) (Fig. 1). 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.

2.2. 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 s). Powder was then transferred to a sterile 1.5 ml Eppendorf tube and 700 µl 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).

2.3. 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 (Dudakovic et al., 2014; Kalari et al., 2014). 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 (Kim et al., 2013), and HTSeq (Anders et al., 2015). Normalized read counts were expressed as reads per kilobasepair per million mapped reads (RPKM). Data have been deposited in the GEO Database (accession#: GSE125360).

2.4. 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 (Subramanian et al., 2005; Liberzon et al., 2011; Liberzon et al., 2015). Protein-protein interaction networks were generated using STRING Database version 10.5 (Szklarczyk et al., 2015; Szklarczyk et al., 2017).

3. 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 (Fig. 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 (Fig. 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

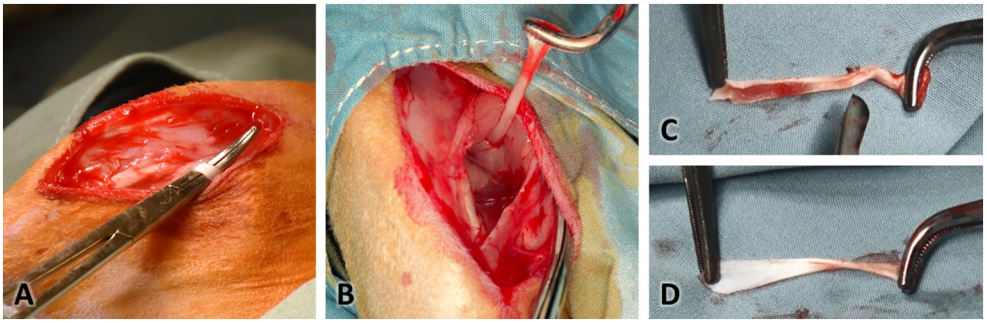

Fig. 1. Semitendinosus graft harvest and preparation. The semitendinosus is identified on the medial side of the knee (A), divided distally and isolated along its proximal course (B), atraumatically cleared of muscle using a fresh scalpel (C), and prepared for final washing in PBS (D).

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 (Fig. 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 (Fig. 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 (accession#: GSE125360) compared to previously described muscle samples from the GEO Database (accession#: GSE60591) (Fig. 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

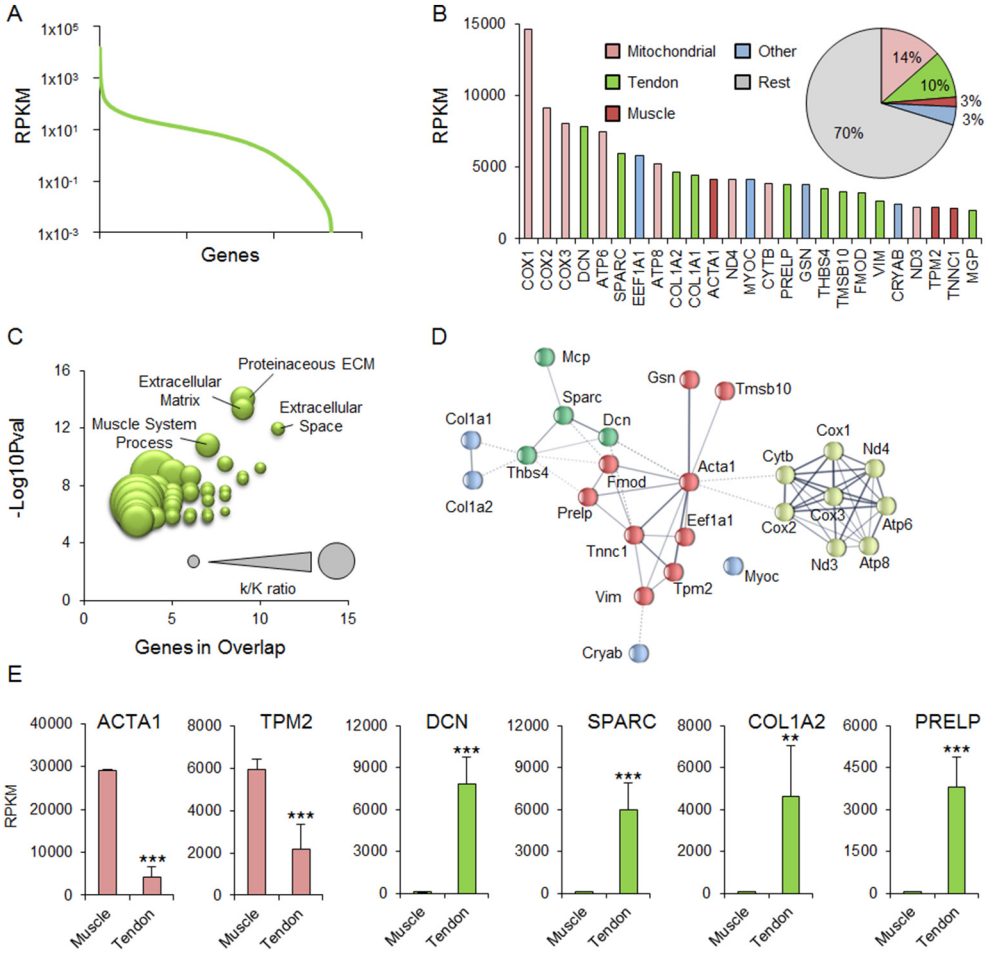

Fig. 2. Tertiary analysis of RNA-seq derived from hamstring grafts prior to ACLR. Read counts were converted to reads per kilobase per million mapped reads (RPKM) and average expression across the four samples was evaluated for each gene (A). The top 25 expressed genes were determined (B) and used for 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

representative of isolated, debrided rabbit hamstring, as would be expected in the setting of ACL reconstruction.

4. 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 (Nessler et al., 2017; Hewett et al., 2016; McArdle, 2010). 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 (Juneja and Veillette, 2013; Hoyer et al., 2016; Haslauer et al., 2014). Additionally, we observed a high basal level of COL1A1 and COL1A2, as has been previously well characterized (Hoyer et al., 2016; Kato et al., 2015; Kaynak et al., 2017). 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 (Maillard et al., 1992; Gagliano et al., 2009; Gehwolf et al., 2016). 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 (Vogel and Meyers, 1999). 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) (Vogel and Meyers, 1999; Koob and Vogel, 1987). 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 (Park et al., 2006; Kuo et al., 2010; Smith et al., 2012; Jiang et al., 2015).

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.

5. 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.

Declarations of interest

MH: Moximed: Paid consultant.

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

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