The Effect of Sirtuin-1 on Chondrocyte Progenitor Cell Activity in Acute Cartilage Injury

Orthopaedic Trauma Association Resident Research Grant Application

I. Specific Aims

Post-traumatic osteoarthritis (PTOA) after intra-articular fracture is a debilitating disease leading to synovial joint destruction from abnormal mechanical loading, inflammation, and disordered chondrocyte metabolism. In osteoarthritis mitochondrial dysfunction of chondrocytes is well-documented, and seems to be mediated by a sirtuin-1 (SIRT1) dependent pathway.[1-3] This NADPH-dependent deacetylase regulates several targets in well-characterized mitochondrial biogenesis pathways and cellular metabolism.[3] Interestingly, SIRT1 also serves an important role in cell migration and adhesion.[4]

Despite advances in intra-articular fracture management, the risk of PTOA after articular surface injury remains high.[5-6] Recent investigation has revealed a partial response after cartilage injury by resident tissue stem cells, termed chondrogenic progenitor cells (CPCs).[7] Discovery of a role for SIRT1 modulation of chondrocyte function suggests that this gene may also play a role in CPC response.

Hypothesis: SIRT1 expression directly correlates with chondrogenic progenitor cell (CPC) defect infiltration and repair of post-traumatic articular cartilage defects.

Specific Aim 1: Determine whether manipulation of the SIRT1 pathway produces respective changes in CPC activation and mobility *in vitro* after both scratch and impact injuries. *Hypothesis:* Increases in SIRT1 increase CPC viable cell numbers and mobility whereas decreases in SIRT1 decrease these values.

Specific Aim 2: Determine whether CPCs activated by SIRT1 gene expression lead to defect filling, and increased total cell seeding *in vitro* in post-traumatic articular cartilage from surgical discards. *Hypothesis:* SIRT1 expression levels will directly correlate with successful defect filling.

II. Background and Significance

Post-traumatic osteoarthritis (PTOA) is a common sequela of intra-articular fracture that negatively impacts the quality of life of millions of people, leading to disability and high healthcare expenditures.[8] The pathogenesis of osteoarthritis is a multi-faceted disease process involving mechanical, genetic, metabolic, and inflammatory components that irreparably damage the chondral surface.[9-10] Cartilage repair poses a unique problem due to its lack of neural, lymphatic, and hemogenous tissues and subsequent low potential for self-regeneration.[11-12] Disabling PTOA is common despite surgical intervention to restore the articular surface suggesting the need to identify alternative modalities to prevent progressive joint destruction after injury.

It is well-established that chondrocytes exhibit myriad metabolic perturbations in PTOA and osteoarthritis.[13] Damage-associated molecular signals

serve not only as chemotactic agents, but also activate chondrocyte progenitor cell (CPC) migration over cartilage surfaces near sites of chondrocyte damage.[7] Sirtuin-1 (SIRT1) seems to mediate mitochondrial biogenesis and function in cartilage damage, and may represent a key gene in modulation of the healing process.[1-3]

Enhancing native CPC response to chondral damage has been under robust investigation with notable success.[14] CPCs exist in both healthy cartilage and late-stage osteoarthritis.[15] Stem cell-based treatments have been under investigation for adult human articular repair, and harnessing the regenerative potential of CPCs has attracted significant attention as a method to alleviate the disease burden associated with PTOA.[16-18]

If successful, this study will become the basis for further experiments studying the mechanism of PTOA progression as mediated by the SIRT1 pathway and aid in understanding of mitochondrial function as driven by SIRT1 expression as a critical mediator of CPC activity. In the future, viral transgene induction may become a promising therapeutic option for patients who are at risk for developing PTOA. Ultimately, we hope this research will contribute to the development of a perioperative intervention that affects SIRT1 gene regulation in CPCs and chondrocytes. In so doing, we aim to improve the healing of traumatically injured cartilage and reduce the disease burden of PTOA.

III. Research Design and Methods

IIIa. CPC Procurement and Activation Following Injury

12 mature bovine stifle joints will be obtained immediately after slaughter from a local butcher. Osteochondral explants will be prepared by manually sawing a 20 mm by 20 mm square of the articular surface and immediately incubating in culture medium (DMEM in 10% FBS with 50 ug/mL L-ascorbate, 100 U/mL penicillin, 100 ug/mL Fungizone) at 37^{o}C in an atmosphere of 5% CO_2 in and 5% O_2.[17] Twenty-four hours after harvest, osteochondral explants will be scored with a scalpel in a uniform fashion, keeping specimens submerged in culture medium at all times. This scoring has previously been shown to stimulate CPC activation.[7] CPCs will be harvested after 5-7 days by submerging explants in 0.25% trypsin-EDTA in HBSS and incubated for 10 minutes to detach migrating progenitor cells. Confirmation of CPC identity will be assessed by morphology and multipotent differentiation.[19,7]

Two populations of differing SIRT1 gene expression will be established from each osteochondral explant in order to compare normal SIRT1 gene expression (SIRT1-wildtype) and overexpression of SIRT1 (SIRT1-overexp). SIRT1-overexp CPCs will be achieved by lentiviral transfection. Lentivirus containing SIRT1 gene packaging (Cell Biolabs, Inc.) will be used to transfect SIRT1 and luciferase following the established protocol; SIRT1-wildtype will be transfected with luciferase-only containing lentivirus. Stable viral transfection will be validated by western blot.

IIIb. *Hypothesis 1*: SIRT1 expression is correlated with CPC activity

CPC activation will be determined by proliferation and differentiation potential. Three stifle joints will be processed as in IIIa. SIRT1-overexp and SIRT1-wildtype will be established from each explant as described in IIIa. SIRT1-wildtype bovine CPCs and SIRT1-overexp bovine CPCs will be incubated in culture medium at 37 ^{o} C in an atmosphere of 5% CO2 in air. After 12, 24, and 72 hours, cell proliferation will be assessed using calcein AM and ethidium homodimer as alive/dead markers and imaged using a confocal microscope.

Differentiation potential of CPCs will then be assessed by culturing cells under chondrogenic, osteogenic, and adipogenic conditions. Three stifle joints will be processed as in IIIa, and SIRT1-overexp and SIRT1-wildtype will be established from each explant as described. Each SIRT1-wildtype bovine CPCs and SIRT1-overexp bovine CPCs will be incubated in culture medium to induce chondrocyte, osteoblast, or adipocyte cellular differentiation. Specifically, chondrocyte differentiation will be induced by incubating 1 million SIRT1-wildtype bovine CPCs or SIRT1-overexp bovine CPCs in chondrogenic medium (DMEM, 10 ng/ml TGFB1, 0.1 uM dexamethasone, 25 ug/mL pyruvate, 50 mg/ml ITS + Premix, antibiotics and antifungal agents). Cells will be analyzed for matrix formation using Safranin O/fast-green staining.

Cell migration of SIRT1-wildtype bovine CPCs and SIRT1-overexp bovine CPCs will then be performed using a CytoSelect 96-Well Cell Invasion Assay kit (Cell Biolabs), as described by the manufacturer. Three stifle joints will be processed as in IIIa, and SIRT1-overexp and SIRT1-wildtype will be established from each explant as described. SIRT1-overexp bovine CPCs and SIRT1-wildtype bovine CPCs will be seeded on plates using conditioned medium from mechanically injured explants. The data will be presented as percentage of migrating cells. Conditioned medium will be made by incubating scored explants overnight in 10mL of serum-free medium and then concentrated by ultracentrifuge in Amicon Ultra centrifugal 10K filters.

IIIc. *Hypothesis 2*: SIRT1 expression levels will directly correlate with successful defect filling in surgical discards from patients with intra-articular fractures.

20 surgical discards from patients with non-reconstructable intra-articular fractures will be obtained from procedures within our department. A 15 mm x 15 mm square (or largest possible) of articular surface will be prepared from each sample and maintained in culture overnight (see IIIa). A 4 mm full-thickness circular defect will be made in

the articular surface with a scalpel while submerged in culture media. Should a defect already exist, it will be enlarged to preserve uniform preparation of the samples. Each sample will then be incubated in culture media overnight. A composite interpenetrating polymer network (IPN) of fibrin and hyaluronic acid impregnated with SDF-1 will be fabricated in 4 mm diameter x 2 mm thickness disks as previously described[16] and fit into the defect and maintained in culture media for an additional 24 hours.

Virus encoding SIRT1 and luciferase will be applied to the 10 surgical discards; sham vector containing only luciferase will be applied to 10 control samples. After 3 weeks, histological analysis will probe for SIRT1 expression and analyzed for ECM formation using Safranin O-fast green staining. Hematoxylin-eosin staining will also be utilized to quantify CPCs and chondrocytes within the defect. Luciferase expression will be assessed to ensure virus uptake and vector expression.

IIId. Solutions to potential pitfalls

The scoring method of inducing CPC activity may not injure the articular surface enough in the explants to produce a robust CPC response. In this case, we would change the induction method by using a 7 J drop tower impact after scratch assays, which is well known to cause significant injury and already used reliably in our lab.

In our surgical discards, it is possible the lentivirus vector will not adequately transfect and produce significant levels of SIRT1. In this case, we would independently culture CPCs with confirmed SIRT1 overexpression and add cells to the osteochondral explant culture medium. Additionally, we could utilize bovine explants and use a lentivirus co-culture to induce SIRT1 overexpression and monitor CPC migration and overall Safranin O/green-fast staining in the cartilage defect.

IIIe. Statistical Analysis

Statistical significance between studies of quantification will be evaluated by student t-test or ANOVA. Additional statistical analysis will be performed with the guidance of on-staff statisticians at our institution.

IIIf. Clinical Impact

In sum, these studies will be valuable in determining the significance of SIRT1 as a mediator of CPC migration, proliferation, and post traumatic defect healing potential. If the results are positive, as we expect, these

studies will provide information that will lead to additional experiments and eventually help identify a new therapeutic gene target that will improve cartilage healing and decrease the occurrence of PTOA.

IV. Role of resident

I have always enjoyed the challenge of asking questions and seeking answers. My interest in basic science research began as an undergraduate, where I earned a combined bachelor's and master's degree in Molecular Biophysics and Biochemistry studying protein dynamics of intrinsically disordered proteins. In medical school, I continued to pursue basic and translational research, examining bone metastatic disease in neuroblastoma, and later identifying a skeletal stem cell that is activated in fracture healing and adult skeletal bone repair. To me, the most interesting questions surround mechanistic pathways for pharmaceutical development to improve patient outcomes. The question in this research project was developed with the assistance of the authors listed. With their guidance, I formed the hypothesis and specific aims. I performed the literature review, designed experiments, and wrote this grant in its entirety. The outcomes will be collected with the help of our lab technicians. Data analysis will be performed with the help of staff statisticians. I plan to compose the manuscript with the authors listed. This application is in an area of significant interest to our lab and there will be abundant support to accomplish the project. However, the experiments proposed are novel and represent an original research effort.

V. References

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OTA Resident Research Grant Budget Sheet
The Effect of Sirtuin-1 on Chondrocyte Progenitor Cell Activity in Acute Cartilage Injury

Budget cannot exceed $20,000

Submitting a budget over this amount disqualifies your application for consideration

- Salaries and Wages: Enter name, percentage of time on project and salary requested as well as fringe benefits
  charged to the grant. Please also state what each person will be doing.
- Permanent Equipment: Justification to be appended.
- Consumable Supplies: Excludes animals and animal care.
- Animals and Animal Care: Justify all requests where need is not apparent.
- All Other Expenses**:** Charges for overhead are not covered by OTA Grants. No indirect costs will be funded.

SALARIES AND WAGES (List all personnel for whom money is requested)	% Of Time on this project	Requested from OTA Funds (Omit Cents)
Lab technician – will be required for aiding Dr. Compton with obtaining explants and performing cell culture work	1%	$703
Histologist – will be required for the immunohistochemical and histological investigations	5%	$4439
Staff statistician – will be required for statistics expertise throughout the project	1%	$980
	%	
Fringe Benefits 35.85% of Salaries and Wages Salaries and Wages plus Fringe Benefits	TOTAL	$6122

PERMANENT EQUIPMENT (Justification to be appended)	
Subtotal	$0

CONSUMABLE SUPPLIES (Exclude animals and animal care)	
Bovine explants	$848
Specimen cups	$259
6-well plates	$109
Culture media and multipotency items	$3280
SIRT1 gene packaging and transduction	$1000
Viability stains	$627

Cell migration assays		$927
Conditioned medium Amicon filters		$1000
Histology (IHC SIRT1, luciferase, Saf-O, H&E)		$2346
Human sample IPN+SDF gel treatments		$2139
WB antibodies and supplies		$1000
	Subtotal	$13,535

ANIMALS AND ANIMAL CARE		
NA		
	Subtotal	$0

ALL OTHER EXPENSES		
Publication costs		$343
	Subtotal	$343

TOTAL DIRECT COSTS $20,000