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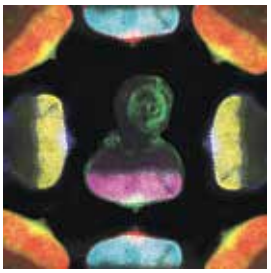
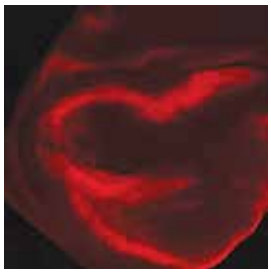
Proceedings of the 2013 Berry Summer Thesis Institute

University of Dayton. University Honors Program



Berry Summer Thesis Institute



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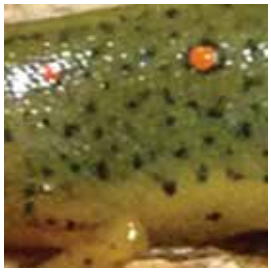

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LETTER FROM THE DIRECTOR

Dear Reader:

On behalf of the students and staff of the University Honors Program, welcome to this first edition of the Proceedings of the Berry Summer Thesis Institute. Our founder, Dr. Patrick F. Palermo, created the University Honors Program nearly 35 years ago, motivated by a conviction that a Catholic and Marianist institution of higher education was obligated to bring students of exceptional academic ability into active participation in the creation of new knowledge under the guidance of faculty advisors. The Honors thesis at the University of Dayton has remained the most distinguishing characteristic of the Program. Since December 1982, nearly 1,200 students have completed the 2-year process of working with a faculty advisor to identify a research question, gather data and disseminate results, thereby exemplifying the power of the Marianists' emphasis on learning in community while contributing significant scholarship in diverse fields of knowledge.

Two years ago we wondered what would happen if we attempted to deepen this experience by extending the project timeline and more intentionally linking scholarship to service and leadership in the community. Through a generous gift from the Berry Foundation and Berry Family, we were able to attempt this in the summer of 2012, when we selected the first cohort of the new Berry Summer Thesis Institute. The cohort spent the summer on campus between their sophomore and junior years, initiating their thesis projects ahead of the standard timeline, learning about the community and matching their talents and interests in service to community needs. We selected a second cohort for 2013 and these *Proceedings* are the fruit of their academic labors. They represent our latest attempt to teach students about the process of scholarly inquiry and the dissemination of results through the process of peer review.

I am confident that you will be impressed and inspired. Happy reading!

David W. Darrow, Ph.D.
Director
University Honors Program
University of Dayton

Thanks to a gift from the Berry Family Foundation and the Berry family,
the UHP offered eleven rising juniors the opportunity
to participate in the 2013 Berry Summer Thesis Institute.

First initiated in the summer of 2012, the Institute introduces students
with a proven record of academic success and interest in research
to intensive research, scholarship opportunities and professional development
while earning Honors credits towards their Honors Program diplomas.

Students selected for the Institute were nominated by faculty mentors
and competitively selected for participation
by the University Honors Program review committee.

Each student pursued a 12-week summer thesis research project
under the guidance of a UD faculty mentor.

In coordination with the Center for Social Concern, Campus Ministry
and the Fitz Center for Leadership in Community,
the students also learned about civic engagement and servant leadership
by volunteering with local community partners.

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Dorsal-Ventral Differences in Expression of Genes Related to Wnt Signaling Pathway

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Abstract

Background:

Humans cannot regenerate damaged tissues which causes loss of tissue function. However, amphibians have the ability to regenerate a variety of organs. Among them, the red spotted newt, *Notophthalmus viridescens*, regenerates the lens after complete removal. Lens regeneration occurs through the process of transdifferentiation of dorsal iris pigment epithelial cells into lens cells. Natural regeneration does not occur in the ventral iris, which serves as the control. Previous *in vitro* studies have concluded that the Wnt signaling pathway is responsible for the transdifferentiation process.

Objective

The aim of our study is to examine genes of the Wnt Signalling pathway in order to discover their roles in the dorsal and ventral iris during regeneration.

Methods

Lentectomy was used in combination with dorsal and ventral iris sample collection at 0, 4, and 8 days. RNA was extracted and cDNA was synthesized. Semi-quantitative PCR was used and expression of genes was visualized through gel electrophoresis.

Results

The expression of eight genes was studied. Four genes: KANK1, MACF1, LRRFIP2, and GRK5 did not show any expression in these time points. The remaining four genes: CTR9, ARL6, LRP5, and AXIN1 showed expression in all the time points, with similar intensity in dorsal and ventral samples.

Significance

Understanding the mechanism of lens regeneration in newts could potentially be used in regenerating tissues in humans. Studying the Wnt signaling pathway could give rise to therapeutic approaches in treating human injuries.

Introduction

The human body regenerates injuries only partially, mainly through a wound healing response that includes scar formation to reconstitute body function. On the other hand, other vertebrates, such as amphibians, can repair any physical injury through regeneration including the eye lens and the retina. Due to this unique ability, many studies have been conducted on amphibians, in particular the red-spotted newt *Notophthalmus viridescens*, in hope of understanding the process of regeneration.

Newts can regenerate many body parts including the brain, heart, and lens, while humans are limited in their regeneration abilities: the liver, skin and bone[1]. The difference in regeneration between species is due to the process that the cells undergo during regeneration. The brain and heart of amphibians are able to regenerate when cells dedifferentiate, and then redifferentiate into a brain or heart cell. In humans, regeneration occurs by cell proliferation of specialized cells rather than dedifferention. The type of regeneration in amphibians is different from humans which allows for a larger variation in regenerating body parts. Lens regeneration in newts occurs through a process

of dedifferentiation, transdifferentiation, and pattern formation[1]. This process is imperative to the study of lens regeneration because the lens can be completely removed from the newt and regeneration still occurs (*Figure 1*). The lens regenerates specifically from the dorsal iris and not from the ventral iris even though they are the same iris tissue and contain the same genes[2]. The reasoning for this occurrence is based on differential gene regulation in dorsal and ventral iris. For instance, dorsal and ventral genes are regulated through different pathways, and previous studies have shown that the Wnt/ß-catenin signalling pathway is active during lens regeneration[3,4,5,6].

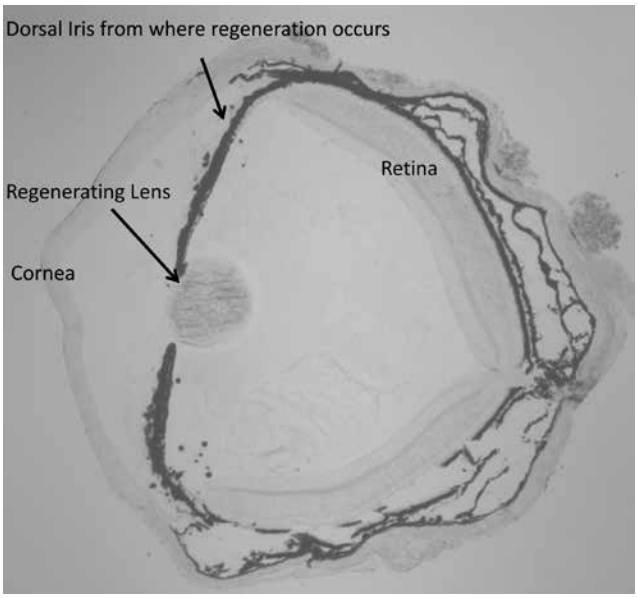

Figure 1. Cross-section of a newt eye during lens regeneration. Lens regeneration occurs from the dorsal iris. Arrows and text indicate several parts of the eye.

In general, the Wnt pathway follows these steps. First, the Wnt pathway must be activated by regulatory proteins. When the Wnt pathway is activated, the Wnt signalling is "on" (*Figure 2*). During Wnt pathway activation, secreted glycoproteins, named Wnts, bind low-density lipoprotein receptor-related proteins (LRP5 and LRP6) and frizzled seven transmembranespan receptors to stabilize the protein, ß-catenin (*Figure 2*). The binding is enabled through the G protein-coupled receptor kinase 5 (GRK5) because it is a kinase for the single transmembrane receptor LRP6 during Wnt signalling.[10] Next, disheveled protein inhibits the ß-catenin degradation by the displacement of glycogen synthase kinase 3ß (GSK3ß) from Axin1, so ß-catenin can enter the nucleus[7]. Moreover, additional regulation in the cytoplasm by microtubule-actin crosslinking factor 1 (MACF1) which is involved in the translocation of Axin1 and its associated complex[9], while LRP5 binds to Axin1 and activates the Wnt pathway[14]. Furthermore, ADP-ribosylation factor-like protein 6 (ARL6) is involved with cilia assembly and also regulates the Wnt pathway[12, 15].

While ß-catenin is in the nucleus transcription of Wnt target genes occurs. Leucine-rich repeat in Flightless interaction protein 2 (LRRFIP2) interacts with disheveled to increase the cellular levels of ß-catenin and activate ß-catenin transcriptional activity within the nucleus[11]. Another protein that induces ß-catenin transcription is Kn motif and ankyrin repeat domain-containing protein 1 (KANK1) by acting as a nucleo-cytoplasmic shuttling protein. This protein is responsible for the relocalization of ß-catenin to the nucleus to activate ß-catenin-dependent transcription by binding to ß-catenin and regulating the subcellular distribution of ß-catenin[8]. Ctr9 protein, a subunit of the Parafibromin complex, is required for the transcription of Wnt target genes[13].

However, when ß-catenin cannot reach the nucleus transcription of the genes cannot occur, so the Wnt pathway is considered inactive. There are no activating Wnts present, so the Wnt pathway is considered "off" (*Figure 2*). Secretion factors, like Cerberus, bind to Wnts and inhibit binding to frizzled7. Furthermore, Dickkopf inhibits binding of LRP. Without the binding of Wnts, the ß-catenin is phosphorylated by Casein

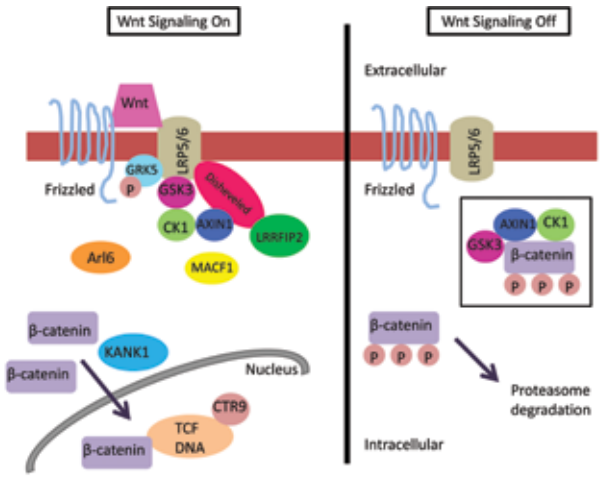

Figure 2. Comparison of active and inactive state of the Wnt Signalling pathway. The right side shows the binding of proteins that activated the Wnt signaling pathway. This activation is termed "on". During activation, ß-catenin enters the nucleus and transcription occurs. The left side shows that there are no proteins activating the pathway, so the pathway is termed "off". The degradation of ß-catenin occurs. Modified from 16.

kinase 1-alpha (CK1). Next, phosphorylation of ß-catenin occurs in the multiprotein complex with scaffold protein Axin1, ideally causing Axin to be upregulated in the ventral iris. At last, GSK3ß to phosphorylate serine/threonine, which in turn ubiquinates ß-catenin causing proteasome degradation[7].

The Wnt pathway is conserved in all vertebrates and has been shown to play role during regeneration in different model systems. KANK1 may have a part in axon regeneration which could be important in brain deteriorating diseases, such as Alzheimer's Disease[17]. MACF1 has been studied in regards to periodontal regeneration[18]. GRK5 is a critical regulator of pathological cardiac growth after ventricular pressure[19]. LRRFIP2 has been found to cause regeneration in hair follicles[20]. Previous studies have found LRP5 to be responsible for epithelial cell regeneration[21]. Axin1 has been studied in regards to liver regeneration[22]. These genes have potential for future studies in humans if their regulation in the Wnt pathway can be understood through the differences in regeneration between the dorsal and ventral iris.

A previous study using the transcriptional analysis had identified the eight genes chosen in our study to be regulated during lens regeneration in the newt[23]. Our hypothesis is that when the Wnt pathway is active (on), lens regeneration occurs in the dorsal iris and when the Wnt pathway is inactive (off) lens regeneration does not occur in the ventral iris. Thus, we expect genes activating Wnt pathway to be upregulated in the dorsal, while genes inhibiting the Wnt pathway to be upregulated in the ventral (*Table 1*).

Table 1. Genes studied within the Wnt pathway. The regulation explains whether the genes would be up-regulated in dorsal and ventral. The role explains whether the gene activates or inhibits the pathway.

Full Name	Gene Name	Regulation in Iris	Role in Pathway
kn motif and ankyrin repeat domain-containing protein 1	KANK1	Up in Dorsal	Activator
microtubule-actin crosslinking factor 1	MACF1	Up in Dorsal	Activator
g protein-coupled receptor kinase 5	GRK5	Up in Dorsal	Activator
leucine-rich repeat flightless-interacting protein 2	LRRFIP2	Up in Dorsal	Activator
ctr9 protein	CTR9	Up in Dorsal	Activator
adp-ribosylation factor-like protein 6	ARL6	Up in Dorsal	Activator
low-density lipoprotein receptor-related protein 5	LRP5	Up in Dorsal	Activator
axin-1	AXIN1	Up in Ventral	Inhibitor

Materials and Methods

Approval: Obtained by the University of Dayton Institutional Animal Care and Use Committee (IACUC; Protocol ID: 011-02).

Design

Thirty newts were used for this study. Newts were distributed in two groups: Group A with 10 newts and Group B with 20 newts. First, group A had their whole eyes removed and their dorsal and ventral irises separated for the 0 day control samples. Group B with 20 newts received lentectomies. 10 newts were placed in container 1 for 4 days, and 10 newts were placed in container 2 for 8 days. These newts will be used for the 4 day and 8 day time points.

Iris Collection

Newts were anesthetized in 0.1%(w/v) ethyl-3-aminobenzoate methanesulfonic acid (MS222; Sigma) in phosphate buffered saline. During anesthesia, group B newts a scalpel and scissors were used to cut an incision across the entire length of the cornea. Following the incision, tweezers are used to pull out the lens from within the eye. After 4 days, iris pieces are attained and separated into dorsal and ventral samples from the 10 newts. After 8 days the final 10 newts receive an eye extraction to obtain the dorsal and ventral iris pieces. Similarly, the group A iris samples were collected but without lentectomy. The ventral iris can be identified from the V-shaped morphology and the dorsal iris from black dots in the dorsal sclera. The samples were placed within microcentrifuge tubes in RNAlater® Solution (Applied Biosciences) and then were centrifuged.

RNA Extraction and cDNA Conversion

RNA was extracted via TRIzol® Reagent protocol (Applied Biosciences) for 500 µl of reagent or the aqueous phase was transferred to RNA Clean & Concentrator™ (Zymo Research) columns. The quality and quantity of isolated RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). A sample peak at 260nm and at A260/A280 ratio was evidence that the sample was of good quality. DNA contamination was determined by including –RT reactions into the reverse transcription protocol. –RT reactions contained the inactivated enzyme so that no cDNA synthesis occurred. DNA contamination in these samples can be determined by the following PCR reaction with RPL27 expression in positive controls, TBX5 expression in dorsal, and VAX2 expression in ventral.

Gathering Gene Expression Data

Table 1 shows the selected genes for study in this experiment. Primers were made to select for these genes using Primer-BLAST program (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/*). The designed binding of the primers on the known sequence determines the length of the expected band as given from the program. The primer sequences, annealing temperatures, and expected bands are as follows:

KANK1 Forward=ATG ACG GGA GTA CAG CAT TG
KANK1 Reverse= GCA CTT TCC TAC CAA GCC TC

The annealing temperature for KANK1 was 53.0°C and the expected band was 134 base pair units.

MACF1 Forward = CAA ATG TTG TCC ATC CGA CC
MACF1 Reverse = TGG ATT TTA GCC TTT TCC GC

The annealing temperature for MACF1 was 53.0°C and the expected band was 176 base pair units.

GRK5 Forward =TTT TCC ACA GTC AAG GGT GT
GRK5 Reverse =CAC TCC GTC TCT ACC ATC TC

The annealing temperature for GRK5 was 53.0°C and the expected band was 113 base pair units.

LRRFIP2 Forward =TTA GAA ATG AGC GGG ACG AA
LRRFIP2 Reverse =GAC ACC ACA GTA ATT GCA CC

The annealing temperature for LRRFIP2 was 53.0°C and the expected band was 154 base pair units.

CTR9 Forward =TGA TGT AGA GGC ATG GAT CG
CTR9 Reverse =GGA CAT CAG CTT GCA CTT TT.

The annealing temperature for Ctr9 was 53.0°C and the expected band was 141 base pair units.

ARL6 Forward =TCG CTA ACA AGA TGG ACC TG
ARL6 Reverse=AAC CCC TCA CCT TTA AGT CC

The annealing temperature for ARL6 was 54.0°C and the expected band was 127 base pair units.

LRP5 Forward =TCC AGA AGA TAC AGC GAT GG
LRP5 Reverse =GAC ATG CTT TGT TGC TCA CT

The annealing temperature for LRP5 was 53.5°C and the expected band was 119 base pair units.

AXIN1 Forward =GGC CTT ACA TTA TCC GTG GG
AXIN1 Reverse =GAG GGT ATG GGT CAG AGT CA

The annealing temperature for Axin-1 was 55.0°C and the expected band was 138 base pair units.

Expression of selected Wnt-related genes was found using a polymerase chain reaction (PCR) and visualized using gel electrophoresis. A PCR was completed with 12 PCR tubes containing 25 µL Premix Taq™ DNA polymerase (TaKaRa), 1 µL 1mM of forward primer, 1 µL 1mM of reverse primer, 21 µL of DNA nuclease free water, and 2 µL the template. The settings include 40 cycles including 95°C for 30 sec, annealing temperature for 30 sec and 72°C for 30 sec, with specific annealing temperatures for each primer. There was a last extension in the PCR program at 72°C for 10 minutes. PCR samples were separated by agarose gel electrophoresis using a 1% gel and run at 200 volts for 30 minutes. The gel was stained with ethidium bromide for 15 minutes and photographed through vision works software and camera (BioSpectrum).

Results

The results of the gel electrophoresis were inconclusive (*Figure 3*). Our results are of three types. Firstly, the correct band size was not found in the gels. That potentially indicates incorrect design of the primers that were used or the gene is not expressed in the samples. Secondly, expression of genes was in both positive and negative samples showing DNA contamination leading to inaccurate results. Finally, genes were expressed in all the time points tested with equal intensity in dorsal and ventral iris samples. This shows that the primers used are correct but it does not allow for further examination of dorsal versus ventral regulation.

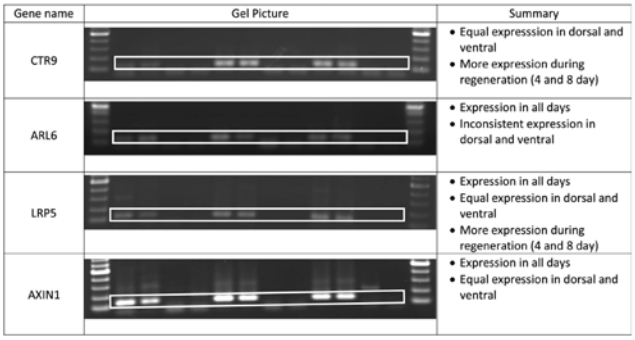

Figure 3. Semi-quantitative PCR results using Gel Electrophoresis to visualize the regulation of the genes. The white box on each gel indicates the correct band size for each gene. From left to right the title of the columns are as follows: 100 base pair ladder/ 0 Day Dorsal/ 0 Day Ventral/ -0 Day Dorsal/ -0 Day Ventral/ 4 Day Dorsal/ 4 Day Ventral/ -4 Day Dorsal/ - 4 Day Ventral/ 8 Day Dorsal/ 8 Ventral/ -8 Day Dorsal/ -8 Day Ventral/ 100 base pair ladder.

KANK1 and MACF1 did not display the correct size band. The bands displayed are around 100 base pairs, but the correct band was not amplified (134 bp and 176 bp respectively). The gel for GRK5 showed poor resolution. The majority of the bands displayed are not the correct size. However, a faint correct band (113 bp) is visible in ventral 8 days post-lentectomy. There were bands greater than 500 base pairs and smaller than 100 base pairs in the LRRFIP2 gel, but no bands of the correct size (154 bp).

CTR9 gel results showed more expression in the 4 day and 8 day samples versus 0 day samples. However, there is an equal amount of expression between the dorsal and ventral samples. ARL6 showed slightly more expression in the 0 day ventral than 0 day dorsal. There is slightly more expression in the 4 day dorsal than ventral.

There is a small yet equal amount of expression in the 8 day dorsal and 8 day ventral. LRP5 has more expression in the 4 day and 8 day samples than the 0 day, with equal amounts of dorsal and ventral expression throughout the time course. There was high expression of AXIN1 in all time points with equal amounts in dorsal and ventral samples.

Discussion

KANK1, MACF1, GRK5 and LRRFIP2 genes were not found to be expressed in the newt iris in all time points tested. While incorrect design of primers cannot be excluded, these results indicate that these genes are not responsible for regulating the Wnt pathway during lens regeneration. CTR9 and LRP5 genes were found to be more expressed during 4 and 8 day post lentectomy. Thus, they have a role during regeneration though without differences between dorsal and ventral iris and cannot be responsible for their discrepancy in the regeneration potential. ARL6 showed slight differences between dorsal and ventral samples though the results were not consistent between the time points. This result cannot be used in determining the potential role of this gene during lens regeneration. AXIN1 has a strong equal expression in all samples; a result that is supported by the fact that AXIN1 appears in both the "on" and "off" states of Wnt pathway.

The genes tested did not give us strong dorsal or ventral differential expression. Thus, they cannot be responsible for the dorsal and ventral differences during lens regeneration. However, previous *in vitro* studies show that the Wnt signaling pathway is in particular interest in newt lens regeneration as discussed previously. More Wnt-related genes need to be investigated in order to discover the gene that is responsible for lens regeneration. These studies can provide the necessary information needed for regeneration studies in higher vertebrates, including humans.

Future Studies

To move forward, new primers could be created for KANK1, LRRFIP2, and GRK5. Also, exploring different annealing temperatures for the PCR machine could produce the correct bands from either the current primers or the new primers. During the gel electrophoresis process, completely dissolving the agarose in the 1X TAE will decrease the amount of bubbles which will yield better resolution. If these techniques do not resolve the problems, different genes from the Wnt pathway could be chosen to be studied and the same process repeated in hope of understanding the dorsal-ventral differences in expression of genes related to Wnt Signaling pathway.

Acknowledgements

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Notophthalmus viridescens. Photo courtesy of Jessica L. Beebe, 2013.

Protein Homeostasis Via the Ubiquitin Proteasome System

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Abstract

Protein homeostasis is paramount in cell life and in maintaining the cell's integrity, making control of the synthesis and degradation of vital proteins. There are two pathways through which proteins can be degraded, the lysosomal and proteasomal pathways. The proteasomal pathway is one in which three enzymes work together to attach chains of ubiquitin molecules to targeted substrates, tagging them for degradation. The E3 ubiquitin ligase is responsible for selecting targets and attaching ubiquitin molecules to the substrate. There are many E3 ubquitin ligases, but the most common family of E3 ligases is the Cullin family. Cullins are active in the G1 to S phase transition in the cell cycle as well as in other cellular functions. These proteins act as scaffolds to align all the elements necessary to ubiquitinate specific proteins and tag them for the correct fate. This is just one of the proteins responsible for tagging proteins for degradation. The system of protein homeostasis, especially degradation, is a complex network that must work flawlessly to maintain the life of a cell and therefore all life.

Introduction

The instructions for life are found in DNA, which is housed in the nucleus of every cell. According to the central dogma of biology, proteins are expressed by the transcription of DNA to RNA followed by the translation of RNA into the corresponding protein. These proteins carry out extremely diverse functions and must be present at optimal levels, which vary greatly, for its function. Maintaining these homeostatic conditions requires coordination of the synthesis, via the cell cycle, and degradation, via lysosomic or proteasomal pathways, of proteins at certain times and in diverse conditions [Goldberg, 1995; Hochtrasser, 1995]. The required proteins change throughout the life of a cell. After they are used, proteins are no longer needed and are subsequently degraded to prevent accumulation of proteins [DeMartino *et al*, 1989]. The amino acids from the degraded proteins are recycled and form new proteins required by the cell. The remainder of this review will focus on protein degradation, mediated proteasomal degradation, and its importance in every cell's viability and ability to reproduce. A specific family of ligases, the Cullins, which are involved in proteasomal degradation, and their functionality, will be discussed as well.

Protein Homeostasis

Protein degradation is important for the life of the cell for several reasons. When proteins are formed incorrectly, they are immediately degraded so they do not inhibit the functionality of the cell. Surprisingly, as many as thirty percent of proteins formed are immediately degraded due to misfolding. Functional proteins are degraded due to changing conditions and variable optimal protein levels [Lodish, *et al*. 2008]. Proteins are required to be degraded for normal cellular function, preventing their accumulation. Some examples include cell cycle regulation, cell development and differentiation, apoptosis, angiogenesis, and cellular signaling [Hochtrasser, 1995; Ciechanover, 1994; Orlowski, Dees, 2003]. Protein degradation is also implicated in several human diseases [Eyal, Ciechanover, 2006].

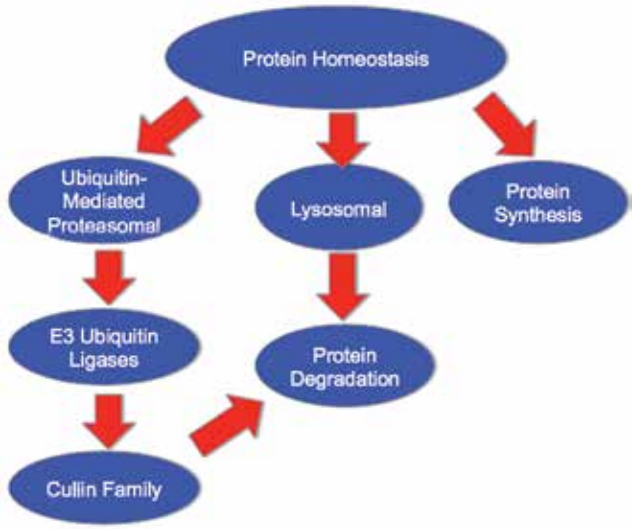

Figure 1. Simple explanation of relationships of parts of this review. The overarching theme of the review is protein homeostasis. To achieve protein homeostasis, proteins must be synthesized and degraded appropriately. Proteins can be degraded via the lysosomal or proteasomal pathways. Ubiquitin-mediated proteasomal degradation involved three different families of ligases, one of which is E3 ubiquitin ligases. The Cullin family of proteins is a highly conserved family of E3 ubiquitin ligases.

As stated earlier, maintaining protein homeostasis is important for cells to survive, but it is also necessary for cells to divide and form new cells. In the cell cycle, when forming daughter cells, a single cell moves through four stages: G1 (growth), S (chromosomal replication), G2 (growth), and mitosis (division into two cells), to form a cell identical to that of the original cell. These stages of growth, duplication, and division are well regulated in order to maintain genetic integrity. The cell cycle is controlled primarily by cyclin activity. Cyclin dependent kinases (CDK) are proteins which bind to a few select cyclins to phosphorylate and activate their substrates. The specific cyclin that binds to the CDK determines the substrate to be phosphorylated [Ciechanover, 1994].

The cell cycle assuredly does not move from one phase to the next before the previous phase is complete due to Cyclin-CDK activity [Lodish, *et al*. 2008]. For example, there are G1 Cyclin-CDK complexes that phosphorylate and regulate transcription factors involved in replication which is done in the S phase of the cell cycle. The enzymes that are needed to perform the next phase aren't synthesized until the end of the previous phase. It is very important that the ubiquitin proteasome pathway then degrades these cyclins before the next phase of the cycle in order to maintain the cell's integrity. Once degraded, the substrates that are phosphorylated by the cyclin-CDK activity are no longer active, an important component of transitioning to the next phase of the cycle. Another target of proteasomal degradation is CDK inhibitory proteins, which demonstrates another great importance for this pathway of protein in degradation [Adams, 2004]. The cell cycle must be performed perfectly to avoid mutation or cell death.

Pathways of Protein Degradation

Before Alfred Goldberg proposed the existence of a second protein degradation pathway, it was thought that lysosomes were responsible for all protein degradation [Golderg, 1995]. The lysosomal pathway degrades extracellular, short-lived proteins that are imported into the cell [Hochtrasser, 1995]. In 1977 when Etlinger and Goldberg suggested an ATP-dependent pathway for protein degradation, it was thought that only non-functional proteins were degraded by this pathway [Etlinger, Goldberg, 1977]. However, we now know this is not true and that normally formed, long-lived, cellular proteins are degraded by the ubiquitin-proteasome pathway as well as non-functional proteins [Hershko, *et al*. 2000]. This pathway is what we now call the ubiquitin-proteasome system. There are as many as 30,000 proteasomes per mammalian cell and the cell will use as much as thirty-percent of the energy needed to synthesize the cell to degrade it, showing its paramount importance in metabolism cell [Lodish, *et al*. 2008]. Another difference between lysosomal and proteasomal

degradation is that lysosomal degradation is fairly non-specific, whereas proteasomal degradation is selective [Hochtrasser, 1996].

Ubiquitin-Mediated Proteasomal Protein Degradation

The most important job of the ubiquitin-proteasome degradation machinery is to degrade regulatory proteins in the cell [Hershko, *et al*. 2000]. Even though there are many types, the best understood proteasome machinery is called the 26S proteasome [Ravid, Hochtrasser, 2008]. The proteasome is made of four stacked rings. Each ring is composed of seven subunits; the outer rings of alpha subunits and the inner rings of beta subunits (*Figure 2*). The beta subunits contain active sites. These stacked discs are arranged symmetrically with the intersection of the two beta rings forming the line of symmetry. The substrate enters the proteasome after it is activated, causing a gated channel to be opened (*Figure 2*) [Groll, *et al*. 2000]. Proteasomal degradation is basically performed in two steps (*Figure 3*): ubiquitination of the targeted substrate and degradation of the substrate, by way of the 26S proteasome, into the substrate's corresponding polypeptides and amino acids [Ravid, Hochtrasser, 2008].

The ubiquitination of a protein is a process involving three enzymes E1, E2, and E3. The E1 enzyme is coined the activating enzyme because it initiates the process of ubiquitinating the substrate after activation by ATP. The E1 enzyme attaches the ubiquitin molecule to the E2 enzyme, the conjugating enzyme, which attaches the ubiquitin molecule to the E3 or ligating enzyme (*Figure 3*) [Hershko, Ciechanover, 1998]. Only one E1 enzyme exists while there are dozens of E2 enzymes and hundreds of E3 ligases [Seol, *et al*, 1999; Tan *et al*., 1999].

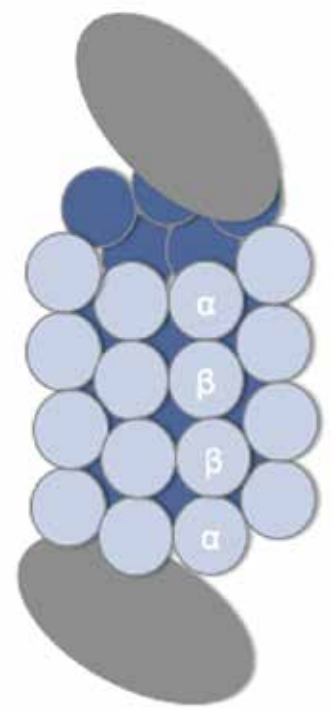

Figure 2. Schematics of the 26S proteasome. The 26S proteasome is where degradation of substrates takes place. Four rings are stacked on top of each other that compose the proteasome. Each sphere represents one of the seven sections that compose a ring. The outer rings are alpha rings and the inner rings are beta rings, which contain the active sites. At the top and bottom of these stacked rings are gate-like structures that open and close to allow specific substrates to enter [Groll, *et al*. 2000].

E3 Ubiquitin Ligases

The E3 ubiquitin ligase binds directly to the substrate and is solely responsible for substrate selection [Nagy, Dikic, 2010; Kamura, *et al*., 1999]. After selecting the substrate, the E3

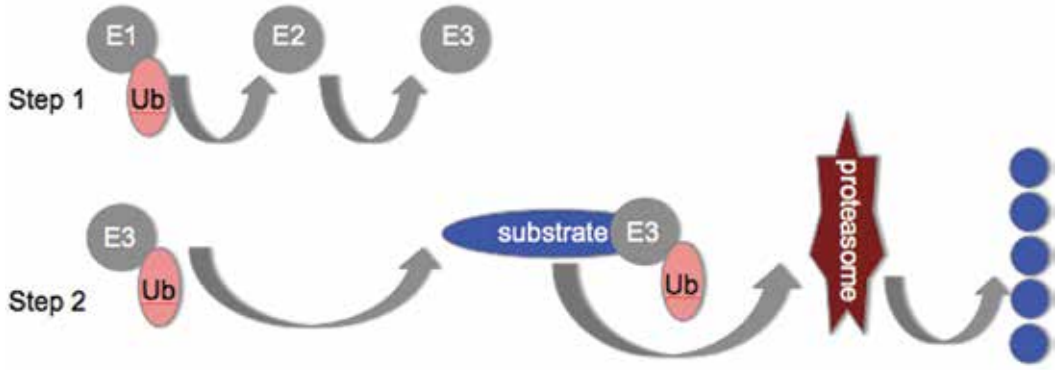

Figure 3. Cartoon depicting the two steps of ubiquitin-proteasomal degradation. In step 1, the E1 ubiquitin ligase begins the process and the ubiquitin molecule is attached. The ubiquitin is moved from the E1 to E2 ubiquitin ligase. The E3 ubiquitin ligase aligns all the necessary components to ubiquitinate the substrate of choice. In step 2, the E3 ubiquitin ligase binds to the substrate and attaches the ubiquitin molecule. The substrate is then recongnized by the proteasome and degraded [Jackson, Xiong, 2009].

ubiquitin ligase leaves a lysine residue or pattern of ubiquitin molecules that tags the substrate for a specific function (*Figure 4*). While most substrates are tagged for degradation by the proteasome, some are tagged for other cellular functions. Generally, the k48 polyubiquitin chain tags the substrate for degradation. Proteins with other lysine residues are destined for cellular function. For example, k63 ubiquitinated proteins have been shown to affect functions such as cellular signaling, ribosomal functions, and DNA replication and repair [Zhou, *et al*. 2013]. Yet another ubiquitin pattern, simply monoubiquitination, is involved in other biological functions, such as membrane transport and transcriptional regulation [Hicke, 2001]. The pattern of

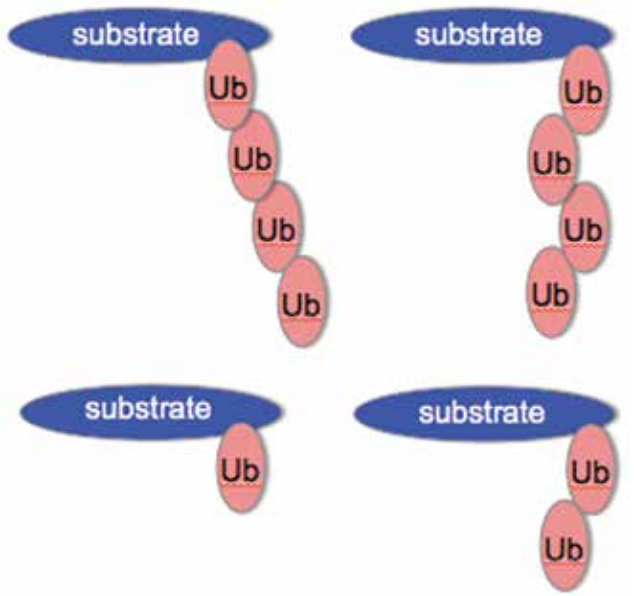

Figure 4. Differing lysine residues attached to substrates. Different E3 ubiquitin (Ub) ligases target specific substrates. These substrates are then tagged with a specific lysine residue for a specific function. Different ligases tag different substrates and each ligase tags their substrates for different functions. These varied ubiquitin tags destine the substrates for these diverse functions [Zhou *et al*. 2013].

the ubiquitin chain attached to the substrate depends on the E3 ubiquitin ligase which selects the substrate and ubiquitinates it accordingly [Zhou, *et al*. 2013].

The variety of E3 ubiquitin ligases allows the ubiquitin-proteasome pathway to degrade a variety of proteins. Four classes of E3 ubiquitin ligases exist: HECT (Homologous to E6-AP C-terminus), RING (Really Interesting New Gene), N-end Rule, and APC/C (Anaphase-Promoting Complex/Cyclosome) ligases [Zhao, Sun, 2012]. The Cullin family of E3 ubiquitin ligases is a family of RING ligases and the

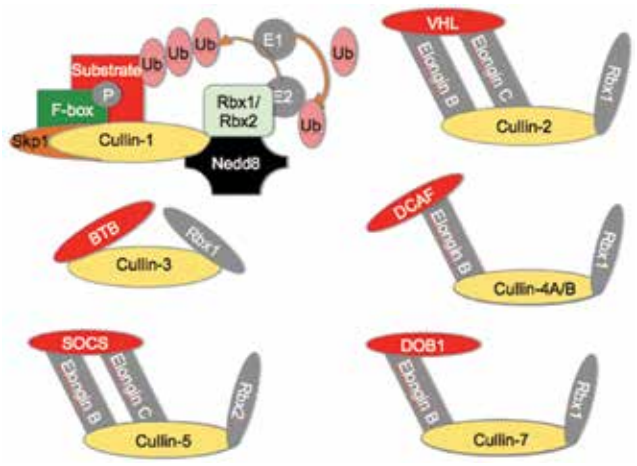

Figure 5. The Cullin family of E3 Ubiquitin ligases in mammals. The Cullin family is a family of scaffolding proteins that align the necessary parts in order to ubiquitinate substrates. They all serve different functions, target different substrates, and tag them for different functions [Zhou, *et al*. 2013].

largest family of E3 ubiquitin ligases [Deshaies, Joaziero, 2009; Sarikas, et. al, 2011; Willems, *et al*. 2004].

Cullins

The highly conserved Cullin family is active in the G1 to S phase transition in the cell cycle [Mathias, *et al*., 1996]. In mammals, there are eight members of the Cullin family: Cul-1, 2, 3, 4A, 4B, 5, 7, and 9 (*Figure 3*) [Sarikas, et. al, 2011]. These proteins form a scaffold that aligns all the necessary components to ubiquitinate a substrate for its intended purpose [Zhou, *et al*. 2013]. Notice each Cullin has a different set of components which participate in the ubiquitination of the substrate targeted by that specific protein. The specificity of these E3 ubiquitin ligases is evident when knock-out

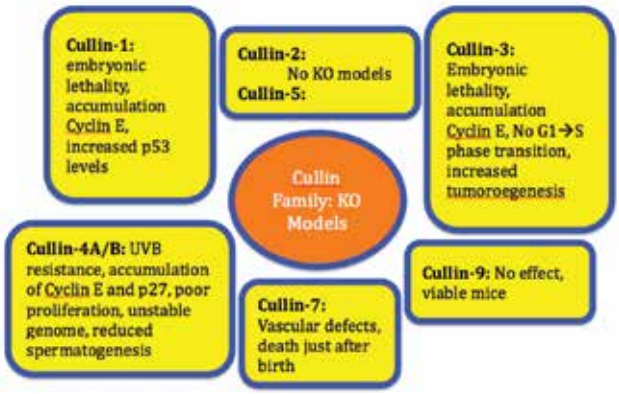

Figure 6. Mouse knockout (KO) models show the function of each Cullin through loss-of-function studies. By producing a loss-of-function clone, it is possible to see the role of each Cullin by studying the changes in the affected progeny [Zhou, *et al*. 2013].

(KO) models, which display a loss-of-function of the specific component, of some of these scaffolding proteins are examined, as their effects are different on the resulting progeny (*Figure 4*). For example, Cullin-1 and 3 KO mice show embryonic death with an increase in Cyclin E [Dealy, *et al*., 1999; Singer, *et al*., 1999]. This suggests that Cyclin-3 is a target of Cullin-1 and 3.

The Study of Protein Homeostasis in *Drosophila melanogaster*

For several reasons, *Drosophila melanogaster*, or the fruit fly, is a perfect model organism for genetic studies. The *Drosophila* and human genomes are remarkably conserved and the genetic machinery of both species is identical. This means that results from *Drosophila* studies can be directly applied to humans and higher organisms, making it an excellent model to study human diseases [Bier, 2005]. The *Drosophila* genome is completely sequenced and our ability to easily manipulate its genome using simple genetic tools is another reason data is easily attainable from *Drosophila*. The Upstream Activator Sequence (UAS) Gal 4 system, derived from yeast, is one of the simple genetic tools used in *Drosophila* to overexpress a gene of interest [Brand, Perrimon, 1993]. By over or under expressing a protein using the UAS Gal-4 system, information about how it is regulated and if this protein plays a role in the regulation of any other proteins can be attained by examination. Phenotypic as well as immunohistochemical examinations help us to determine the role of the specific protein.

Because of the relatively short life cycle of *Drosophila*, only 12-15 days, data can be attained quickly. Fly appendages develop from imaginal discs that are studied in larval stages to show the regulation of growth and differentiation [Cohen, 1993]. *Drosophila* have compound eyes that also develop from imaginal discs, called eye-antennal discs, which are composed of 800 unit eyes called ommatidia. Each ommatidium is made up of eight photoreceptors [Ready *et al*., 1976; Wolff, Ready, 1993; Kumar, 2011]. The eye forms as the result of a series of events: specification, growth and patterning (the generation of dorsal-ventral, anterior-posterior, and proximal-distal axes), proliferation, and differentiation [Pappu and Mardon, 2004; Kumar, 2010; Singh *et al*., 2012]. Using immunohistochemical approaches, we can monitor the levels of proteins at varying stages of development. All of these techniques make *Drosophila* an invaluable tool for protein homeostasis studies.

Implications of Protein Homeostasis Studies for Humans

After understanding how protein homeostasis, their importance at the cellular level, and how the studies are performed, it's important to understand the bigger picture and how these studies can directly affect humans. Some diseases that plague humans are caused by a lack of protein homeostasis, more specifically a problem in which proteins are being degraded at an inappropriate rate, which either leads to degradation or accumulation of proteins. An accumulation of proteins in a cell may cause a process, which requires a specific protein to be active at all times. If proteins are degraded too quickly, a lack of necessary proteins would mean so processes aren't active enough. Some of the most common diseases in which a problem with the UPS is implicated include neurodegenerative diseases, malignant cancers, and inflammatory diseases [Eyal, Ciechanover, 2006].

In neurodegenerative diseases, like Parkinson disease (OMIM- #168600) and Alzheimer Disease (OMIM- #104300), an accumulation of proteins that are normally degraded by the UPS have been recorded [Ciechanover, Brundin, 2003]. This is an example of a disease that is caused by an accumulation of proteins, causing cell death. Malignant cancers, such as HPV-induced cervical cancer, are also the result of improper protein degradation by the UPS. In HPV-induced cervical cancers, the tumor suppressor protein p53 is low, whereas in non-HPV-induced cervical cancers, this is not the case [Werness, *et al*., 1990; Scheffner, *et al*., 1991]. Since the lower levels of a tumor suppressor, p53, is the cause of the cancer, HPV-induced cervical cancer is a disease caused by rapid degradation of proteins.

Many other diseases; colon cancer, breast cancer, ovarian cancer, Angelman Syndrome, Liddle Syndrome, just to name a few, can be, but aren't necessarily, caused by inappropriate protein degradation by the UPS [Eyal, Ciechanover, 2006]. The mutation in UPS function can be genetic or viral [Eyal, Ciechanover, 2006].

Conclusion

Protein homeostasis is vital for the maintenance of life. Many human diseases are caused by inappropriate degradation of proteins. These proteins are then present at elevated or decreased levels in the cell, causing a disease state. A more in-depth understanding of protein degradation and its effects on human disease is important. Continued research is necessary to make efforts for human therapies targeting inappropriate protein homeostasis possible. The diseases in which inappropriate protein degradation is implicated are some of the most common and most deadly diseases for humans. Understanding these processes will bring us closer to a cure.

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Notation

As per *Drosophila* nomenclature, gene names and symbols are italicized and after first time the names of the genes are abbreviated while proteins names and symbols are written in uppercase letters (http://flybase.org/static_pages/docs/nomenclature/nomenclature3.html#1).

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Drosophila melanogaster. Photo courtesy of Timothy L. Cutler, 2013.

Review: The Roles of Hippo Signaling and JNK Signaling in Alzheimer's Disease in *Drosophila melanogaster*

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Abstract

Alzheimer's Disease (AD; OMIM#104300) is the most prevalent cause of dementia in the world. An estimated 5.2 million individuals have AD and in 2013 it is expected to cost the nation $203 billion dollars. AD is characterized by accumulations of Aβ42 peptides, which are toxic to neuronal cells and lead to cell death. *Drosophila melanogaster*, the fruit fly, is a highly studied and well-elucidated model for neurodegeneration that can be used to study conserved pathways, such as the Hippo signaling pathway and the c-Jun amino-terminal (NH_2) kinase (JNK) signaling pathway. Through the use of *D. melanogaster* the functions of these pathways in neurodegeneration, and their interactions with one another in the same context, has been better defined.

Introduction

Alzheimer's Disease (AD) has plagued humans for most of recorded history, even prior to its recognition. In fairly recent history, the amyloid hypothesis of AD neurodegeneration has emerged and focused the scope of AD research (Hardy, 2009). Perhaps, due in part to this, research in AD has accelerated and revealed numerous mechanistic insights, implicating different signaling pathways in the progression of AD using various models such as *Drosophila melanogaster* (Hardy, 2009). Since signaling pathways are conserved between *Drosophila* and higher organisms, findings from one experiment can be extrapolated in other model systems as well, with the end result being applicable to humans. One such conserved pathway is the c-Jun amino-terminal (NH_2) kinase (JNK)/ Mitogen activated protein kinase (MAPK) signaling pathway which is involved in cell signaling specificity. The Hippo signaling pathway has recently been shown to interact with JNK to induce cell death (Verghese *et al*., 2012), making their relationship in different contexts relevant. Here, I will give overviews of each of these overarching topics: AD, *Drosophila melanogaster*, JNK signaling, Hippo signaling and the relationship between the two pathways. I will specifically focus on the literature which pertains to AD and on the literature which demonstrates what is currently known about the relationship between JNK and Hippo signaling.

Alzheimer's Disease

AD is an age-related, progressive, neurodegenerative disorder that primarily affects the elderly and to date has no cure. Neurodegenerative diseases are defined by progressive loss of

neurons leading to deficiencies in cognition, behavior and overall health (Beal *et al*., 2005). It is estimated that 5.2 million Americans have AD (Herbert *et al*., 2013). The number of cases in individuals over 65 is expected to triple by 2050 (Herbert *et al*., 2013).

Alois Alzheimer first noted AD in 1907 (Alzheimer, 1995). He described a general confusion and lack of memory in Auguste Deter, a 55-year-old patient in an insane asylum. Her cognitive and physical condition declined over time until her death, upon which Alzheimer performed an autopsy. He noted neurofibrillary tangles (NFT), which have been shown to be hyper-phosphorylated forms of tau, a microtubule-associated protein (O'Brien & Wong, 2011), as well as military foci, or neuritic plaques. He described "a special substance" in the brain (Alzheimer, 1995), which was later shown to be a peptide 40 or 42 amino acids in length (Glenner & Wong, 1984). Glenner and Wong's prediction that the peptide resulted from cleavage of a larger precursor protein was proven true by Kang *et al*. when they cloned the Amyloid Precursor Protein (APP; 1987). The peptide later came to be called the Amyloid Beta (Aβ) peptide. Currently, the amyloid hypothesis dictates that accumulation of Aβ42 initiates a pathogenic cascade leading to the neurodegeneration present in AD (Hardy, 2009). There is evidence that an oligomeric assembly of Aβ accounts for its toxicity as well (Shankar *et al*., 2008). Recent research has established neurotoxicity of Aβ in vitro and in vivo (Yankner *et al*., 1989; Irizarry *et al*., 1997; Chen *et al*., 2000; Kamenetz *et al*., 2003; Spires *et al*., 2005; El Khoury *et al*., 2007). Initially, Aβ fibrils were shown to be toxic to neurons in culture (Yankner *et al*., 1989). It was then shown that mice which overexpress human APP develop Aβ deposits causing neuronal deficiencies (Hardy, 2009; O'Brien & Wong, 2011). Using APP transgenic mice, Aβ was shown to cause decreased synaptic density and other synaptic dysfunction (Kamenetz *et al*., 2003; Shankar *et al*., 2008), loss of terminals and spine density (Irizarry *et al*., 1997; Spires *et al*., 2005), cognitive deficiencies and loss of memory in learned behaviors (Chen *et al*., 2000; Shankar *et al*., 2008), and inflammation (El Khoury *et al*., 2007). Additionally, soluble Aβ plays a role in cleavage and phosphorylation of Tau, which is part of the generation of NFT (Hardy, 2009; O'Brien & Wong, 2011). Despite this success using mice models, another model has emerged as extremely valuable in AD research.

Drosophila melanogaster as a Model

Drosophila melanogaster, the fruit fly, is a protostomian, ecdysozoan arthropod (Bier, 2005; Hirth, 2010). It belongs to a sub-species of Drosophilidae, dipteran insects that inhabit a majority of the world. Though the arthropod lineage separated from humans millions of years ago, genetic analysis suggests humans and *D. melanogaster* are related (Bier, 2005; Hirth, 2010). Nearly three quarters of human genetic diseases have related sequences in *D. melanogaster*, and approximately 70% of human disease genes are estimated to be sufficiently conserved for appropriate analysis in *D. melanogaster* (Bier, 2005; Sang & Jackson, 2005), including genes encoding APP and Tau, two proteins involved in AD (Hirth, 2010). *D. melanogaster* is advantageous as a model for a myriad of reasons. Its entire genome is sequenced and contains approximately 13,600 genes located on 4 chromosomes, significantly less functionally redundant than vertebrates (Adams *et al*., 2000). *D. melanogaster*'s fundamental cell processes, including neural communication and cell death, are similar to humans (Hirth, 2010). In addition, *D. melanogaster* are smaller in size, allowing them to be easily kept in large quantities. They also mature and reproduce quickly, allowing several generations to be studied in a single month. Another valuable characteristic of *D. melanogaster* as a model is that domain specific mutants can be created so that lethal mutations may be studied while allowing the organism to survive (Hirth, 2010). One such tissue that is commonly used in the creation of domain specific mutations is the eye-antenna imaginal disc. In fact, the first developmental mutant discovered in *D. melanogaster* was an adult eye mutant, found in Dr. T.H. Morgan's lab in 1913 (Butterworth & King, 1965). Since then, interest in this model has increased exponentially.

The eye-antenna imaginal disc is one of several types of imaginal discs found in the developing larvae of *D. melanogaster* (Kumar, 2011; Singh *et al*., 2012). It is a monolayer epithelium which gives rise to an adult eye, antenna and head cuticle (Atkins & Mardon, 2009; Kumar, 2010). During embryogenesis, specific numbers of cells are set aside for each of the discs in *D. melanogaster* (Bryant & Schneiderman, 1969; Postlethwait & Schneiderman, 1971; Gehring *et al*., 1976; Lawrence & Morata, 1977; Madhaven & Schneiderman, 1977). In the eye-antenna disc, 20 founder cells are set aside and ultimately result

in approximately 44,000 cells in the third instar disc, which gives rise to the adult eye (Kumar, 2011; Singh *et al*., 2012). These cells develop at different rates from the rest of the embryo and between the different types of discs, allowing for disc specific tissues. When fully formed, the adult compound eye is comprised of approximately 800 ommatidia, each containing 8 photoreceptor neurons and associated structures (Kumar, 2011; Singh *et al*., 2012).

Domain-specific misexpression, in the eye-antenna imaginal disc for instance, can be accomplished by using the Gal4/UAS system (Brand & Perrimon, 1993). Targeted misexpression using the Gal4/UAS system is accomplished by inserting the gene that encodes the yeast transcriptional activator Gal4 into the genome so that the enhancer, in this case Glass Multiple Repeat (GMR; Moses & Rubin, 1991), can drive expression of Gal4. The intended target gene can then be subcloned downstream of a Gal4 binding sequence called an upstream activator sequence (UAS). Alone, the UAS and subcloned target gene remains inactive or "silent." A fly expressing Gal4 can be mated to a fly containing the target gene of interest which is under control of the UAS so that Gal4 can then bind the UAS site and activate transcription of the downstream target gene (Brand & Perrimon, 1993). Using this GMR-Gal4 driver in *D. melanogaster*, the JNK signaling pathway has been shown to be involved in Aβ42 mediated neurodegeneration (Tare *et al*., 2011).

JNK Signaling

The JNK signaling pathway belongs to a sub family of MAPKs that are evolutionarily conserved and are involved in numerous signal specific processes, including apoptosis, proliferation, differentiation, and morphogenesis (Davis, 2000; Chang & Karin, 2001; Weston & Davis, 2002; Adachi-Yamada & O'Connor, 2004; Stronach, 2005; Dhanasekaran & Reddy, 2008). This sub family was first described in the early 1990s and also plays a role in multiple diseases such as diabetes, neurodegenerative diseases and liver

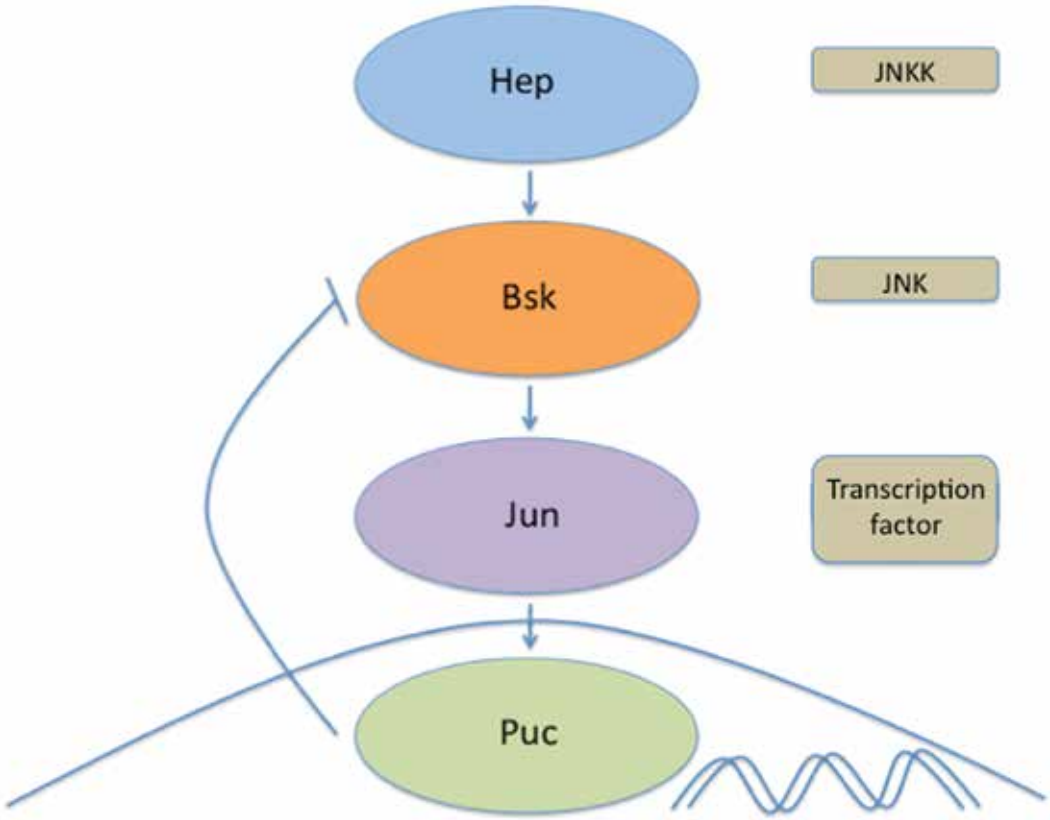

Figure 1. Schematic of the JNK signaling pathway. The components of the pathway and their hierarchy are shown as discussed in the text. Ovals mark the pathway components, while tan boxes identify the nature of the component. The curved line at the bottom of the figure marks the nucleus (adapted from Stronach, 2005).

disease (Bogoyevitch *et al*., 2010). MAPKs are a group of serine/threonine kinases that mediate responses to extracellular stimuli, for instance stress in JNK signaling (Morrison & Davis, 2003). In MAPK signaling modules, a MAPKKK phosphorylates and activates a MAPKK, which ultimately results in the phosphorylation and activation of a MAPK, creating the kinase cascade (Schaeffer & Weber, 1999; Chang & Karin, 2001). Activation of JNK signaling causes caspase activation which results in cell death, although capase independent cell death also occurs (Adachi-Yamada *et al*., 1999; Adachi-Yamada & O'Connor, 2002; Moreno, 2008). Numerous studies have demonstrated that, in culture, JNK signaling reponds to numerous stresses like arsenite, ceramide, and hyperosmotic conditions (Sluss *et al*., 1996; Botella *et al*., 2001; Boutros *et al*., 2002; Chen *et al*., 2002; Sathyanarayana *et al*., 2002; Silverman *et al*., 2003). In addition, JNK signaling has been shown to respond to reactive oxygen species (ROS), which are free radicals characteristically involved in AD, from which Wang *et al*. theorized that JNK acts in a protective manner in response to ROS (2003).

In *D. melanogaster*, the JNK signaling pathway kinase cascade ultimately phophorylates Jra, a Jun related antigen (referred to as Jun; *Figure 1*) on its N-terminal domain (Riesgo-Es-covar *et al*., 1996; Sluss *et al*., 1996; Kockel *et al*., 2001). Upstream, *basket (bsk)* encodes the only Jun kinase regulated by phosphorylation in *D. melanogaster*, which is the substrate for the JNKK encoded by *hemipterous (hep;* Glise *et al*., 1995; Sluss *et al*., 1996; Holland *et al*., 1997).

Bsk was thought to be phosphorylated by another JNKK in addition to Hep (Stronach, 2005) and recently another JNKK, Mkk4, was identified as playing a non-redundant role parallel to Hep/Mkk7 in activating Bsk (Geuking *et al*., 2009). The ligand responsible for initiating the pathway is Eiger (Egr), the homologue of the human Tumor Necrosis Factor (TNF) and its receptor, Wengen (Wgn) (Igaki *et al*., 2002; Kanda *et al*., 2002; Moreno *et al*., 2002; Kauppila *et al*., 2003). The link between JNK and Egr was established when induced Egr expression upregulated the dual specificity phosphatase encoded by *puckered (puc)*, the transcriptional target of the pathway that also negatively regulates the pathway. Further evidence to support this came when it was shown that coexpression of Puc or

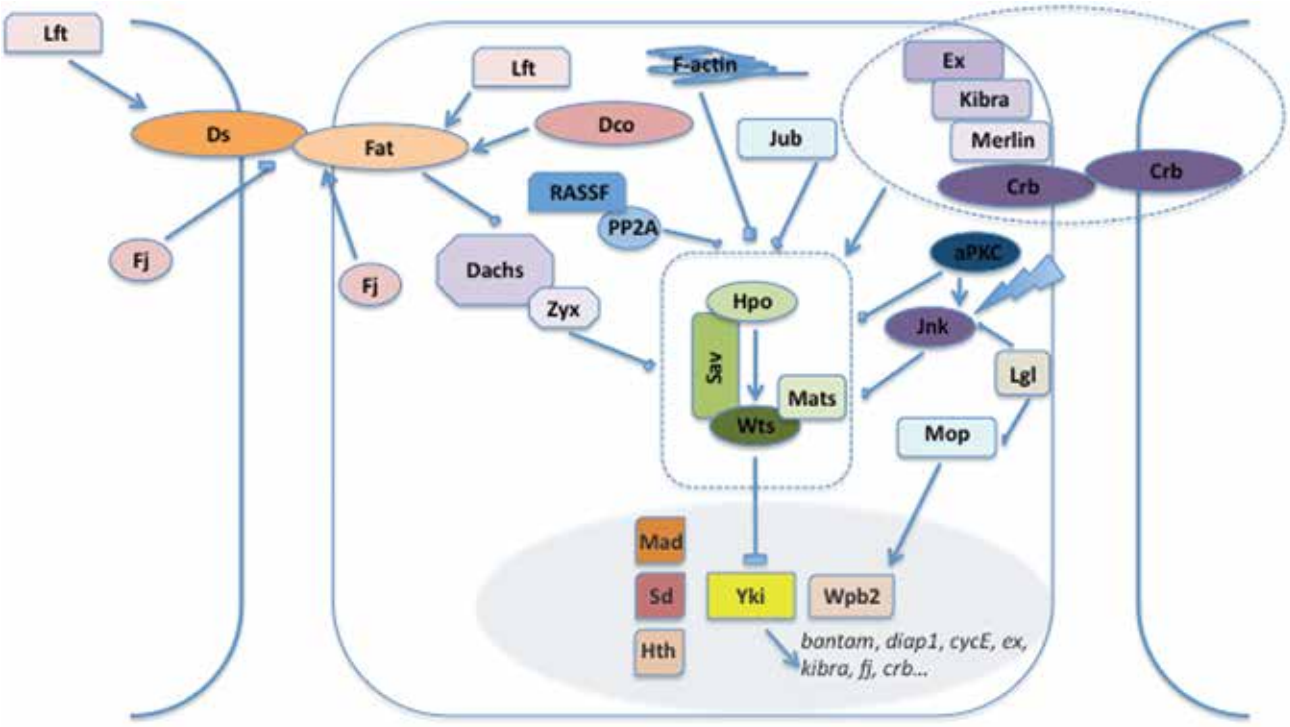

Figure 2. Schematic of the Hippo signaling pathway and network. The components of the pathway discussed in this text are shown, as well as other components generally identified as part of the Hippo network that were outside the scope of this text. Arrows show activation and block arrows show inhibition. The core kinase cassette is shown in green, the Mer-ex complex is shown in purple, the Fat branch is shown in orange and transcription factors are shown in the shaded region (adapted from Pan, 2010; Staley & Irvine, 2011). Abbreviations: Jub: dJuba, Mop: Myopic; PP2A: dSTRIPAK PP2A, Zyx : Zyx102.

loss of function of one of the pathway members, i.e. Hep or Bsk, resulted in the suppression of Egr induced apoptosis (Igaki *et al*., 2002; Moreno *et al*., 2002).

There are two suggested models by which JNK induces apoptosis. The first involves up-regulation of the pro-apoptotic genes *reaper (rpr)* and *head involution defective (hid)*, which repress *Drosophila* inhibitor of apoptosis (DIAP1) to allow the caspase-9 (DRONC) complex to become active. Experiments have also demonstrated that Rpr knockdown fails to block Egr induced apoptosis, leading to the conclusion that there are other caspase independent mechanisms to carry out cell death through JNK signaling (Stronach, 2005). Evidence suggests that this mechanism involves mitochondrial translocation of the cleaved portion (known as jBid) of Bid, a proapoptotic protein (Dhanasekaran & Reddy, 2008). In addition, UV irradiation has been shown to induce caspase independent cell death, but the specific biochemical mechanisms behind caspase independent cell death still remain unclear (Dhanasekaran & Reddy, 2008).

Interestingly, JNK has been shown to be associated with cell death in neurons, prompting research on inhibitors of the pathway as therapeutic drugs for neurodegenerative diseases like AD (Stronach, 2005; Bogoyevitch *et al*., 2010). Recently, Tare *et al*. demonstrated activated JNK signaling in Aβ42 mediated cell death (2011). Since it has been shown that misexpression of human Aβ42 in the *D. melanogaster* brain results in accumulation of plaques, loss of neurons, age dependent deficincies in learning and memory, and shorter life spans (Iijima *et al*., 2004; Cao *et al*., 2008), Tare *et al*. used the GMRGal4 driver to misexpress human Aβ42 in the developing eye of *D. melanogaster*. The model exhibited progressive loss and degeneration of neurons similar to AD neuropathology. When the loss of neurons was shown to be due to cell death, JNK signaling was investigated. By manipulating the JNK pathway members Hep, Bsk, Jun and Puc to either gain or lose function of the pathway, JNK signaling was shown to modulate the Aβ42 neurodegenerative phenotype. Inhibition of JNK activity partially rescued neurodegenerative phenotypes, while activation of the pathway enhanced them (2011). As these rescues were only partial, it remains to be seen what other pathways are involved in this type of neurodegeneration.

Hippo Signaling

The Hippo signaling pathway is central to growth control during organ development and regeneration (Kango-Singh & Singh, 2009; Pan, 2010; Halder & Johnson, 2011; Staley & Irvine, 2012). First discovered in *D. melanogaster* tumor suppressor screens, the core of the Hippo pathway is composed of four proteins, Hippo (Hpo), Salvador (Sav), Warts (Wts), and Mob-as-tumor-suppressor (Mats), referred to as the Hippo kinase cassette (*Figure 2*; Justice *et al*., 1995; Xu *et al*., 1995; ; Tapon *et al*., 2002; Harvey *et al*., 2003; Jia *et al*., 2003; Pantalacci *et al*., 2003; Udan *et al*., 2003; Wu *et al*., 2003; Lai *et al*., 2005). If any of these components lose their function, overgrowth results from increased proliferation and compromised apoptosis. Gain of function in Hippo signaling results in cell death (Verghese *et al*., 2012). The gene *hpo* encodes a serine-threonine protein kinase and *wts* encodes a nuclear Dbf-2-related (NDR) family kinase; both are activated by phosphorylation (Justice *et al*., 1995; Harvey *et al*., 2003; Jia *et al*., 2003; Pantalacci *et al*., 2003; Udan *et al*., 2003; Wu *et al*., 2003). There is evidence for intermolecular autophosphorylation of Hpo (Glantschnig *et al*., 2002; Lee and Yonehara, 2002), which, in complex with Sav, phosphorylates Wts in complex with Mats (Wu *et al*., 2003; Wei *et al*., 2007). Sav can bind to both Hpo and Wts, acting to link Hpo and Wts together (Wu *et al*., 2003). Hpo phosphorylates Mats, an essential co-factor of Wts, to increase its affinity for Wts, allowing Wts to fulfill its essential function: targeting Yorkie (Huang *et al*., 2005; Lai *et al*., 2005; Wei *et al*., 2007).

After identifying the core kinase-cassette, additional tumor suppressors, which are all thought to converge on Hpo, were identified (Kango-Singh & Singh, 2009; Pan, 2010; Staley & Irvine, 2012). Two FERM domain containing proteins, Merlin (Mer) and Expanded (Ex), along with a WW and C2 containing protein, Kibra (Kbr), function together to promote Wts phosphorylation. They physically interact with the Hpo-Sav complex. Ex is regulated by Crumbs (Crb), which has a short intracellular domain that contains a juxtamembrane FERM-binding motif (FBM). Fat, a large transmembrane protein, acts as a cell polarity regulator and as a receptor for Fat-Hippo signaling. Genes that work within the Fat branch of Hippo signaling separately from Ex/Mer/Kbr are Dachsous (Ds), Four Jointed (Fj), Discs overgrown (Dco),

Dachs, Approximated (App) and Lowfat (Lft) (Kango-Singh & Singh, 2009; Pan, 2010; Staley & Irvine, 2012). In addition to hyperplastic tumor suppressors such as *fat*, *ex*, *wts* and *dco*, the neoplastic tumor suppressor *lethal giant larvae* (*lgl*) has been linked to Hippo signaling (Sun & Irvine, 2011). Lethal giant larvae (Lgl) acts with Discs large (Dlg) and Scribble (Scrib), two other proteins at the basolateral membrane of epithelial cells, to regulate apical basal polarity. This complex targets the Crb complex and the atypical Protein kinase C (aPKC) to restrict them to apical membranes (Staley & Irvine, 2012). All of these upstream regulators of the pathway feed into the core kinase-cassette whose functional target is Yorkie (*Figure 2*).

After nearly a decade of search for the unidentified target of the Hippo pathway, a yeast two-hybrid screen for a Wts binding protein identified Yorkie (Yki), a transcriptional coactivator, as the direct target of Wts (Huang *et al*., 2005). Yki promotes cell proliferation and inhibits apoptosis, thus functioning as an oncogene. Yki is phosphorylated at the sites Ser168, Ser111 and Ser250, Ser168 being the most critical, to create the binding sites 14-3-3, 14-3-3ε and 14-3-3ζ (Dong *et al*., 2007; Zhao *et al*., 2007; Oh & Irvine, 2008; Zhang *et al*., 2008; Ren *et al*., 2010). Wts can then bind Yki and, in doing so, negatively regulate Yki by localizing it to the cytoplasm (Dong *et al*., 2007; Oh & Irvine, 2008; Zhang *et al*., 2008). Evidence also supports possible nuclear export as a mechanism of regulating Yki independently of 14-3-3 (Ren *et al*., 2010). With loss of function of Hippo signaling (Dong *et al*., 2007; Oh & Irvine, 2008) or loss of the 14-3-3 binding site (Zhao *et al*., 2007; Ren *et al*., 2010), Yki accumulates in the nucleus.

In the nucleus, Yki indirectly promotes growth by partnering with Scalloped (Sd; Wu *et al*., 2008; Zhang *et al*., 2008; Ren *et al*., 2010), a TEAD/TEF family transcription factor (Campbell *et al*., 1992) that also acts to localize Yki to the nucleus. Additional DNA binding partners for Yki include Mothers-Against-DPP (Mad) and Homothorax (Hth) in complex with Teashirt (Tsh; Staley & Irvine, 2012). The Yki/Sd complex directly targets *Myc* and *bantam*, both promoters of growth, and Diap1, an apoptosis inhibitor (Nolo *et al*., 2006; Thompson & Cohen, 2006; Wu *et al*., 2008; Zhang *et al*., 2008; Peng *et al*., 2009; Neto-Silva *et al*., 2010; Oh & Irvine, 2011). Other targets that play roles in cell proliferation include E2F1 (Goulev *et al*., 2008) and *cyclin E* (Tapon *et al*., 2002), both cell cycle regulators. Another class of targets includes upstream components of the Hippo pathway like Kbr, Ex, Crb and Fj (Pan, 2010; Staley & Irvine, 2012). This allows for negative feedback regulation of the signaling pathway. The third class of downstream targets are components of other pathways, for instance Serrate, a Notch ligand. Additional targets of this class are E-Cadherin, Wingless, Vein (an EGFR ligand), Dally and Dally-like. This class of targets potentially allows for cross talk and communication between Hippo and other pathways (Pan 2010; Staley & Irvine, 2012). Recently another pathway Hippo interacts with, the JNK signaling pathway, has been identified (Sun & Irvine, 2011).

Hippo and JNK Signaling in Regeneration

D. melanogaster imaginal discs can regenerate by increasing proliferation to replace cells that have been damaged through a process called compensatory cell proliferation (Fan & Bergmann, 2008). Recently, Grusce *et al*. and Sun and Irvine showed hyperactivity of Yki induced by damage in wing discs (2011; 2011). In fact, Yki was demonstrated to be required for regeneration when it was shown that fruit flies with only one copy of Yki develop normally, but have a highly impaired ability to regenerate after imaginal disc damage when compared to flies with both copies of Yki (Grusche *et al*., 2011; Sun & Irvine, 2011). Grusche *et al*. found that though the Fat-Hippo pathway plays some role in Yki hyperactivation, it does not completely account for Yki hyperactivation (2011). Interestingly, the JNK pathway, known for its role in apoptosis, was not only implicated in Yki-required regeneration, but was also found to be critical for Yki-required regeneration after tissue damage (Sun & Irvine, 2011). Also interestingly, the JNK Bsk was found to be necessary for Yki required regeneration even though it is not required for normal development, demonstrating the concept of context specific activity and inputs (Igaki, 2009; Sun & Irvine, 2011). While interactions between JNK and Hippo have been demonstrated in regeneration, future research will have to answer the question of their involvement in other contexts.

Future Directions

Research on AD has accelerated in recent years, partially due to increased urgency as the number of individuals affected by AD rise, and partially due to increased insight into animal models, like *D. melanogaster*, used to study neurodegenerative diseases. However, Hardy, a leader in AD research, argues that the enormous accumulation of research on AD based on the amyloid hypothesis is still somewhat inconclusive and it is necessary that the normal function of APP be better understood before dramatic conclusions can be made (2009). Despite this, the strides made in understanding the function of individual pathways in AD, like JNK, are invaluable in defining the genetic machinery responsible for AD neurodegeneration. Currently, a better understanding of the biochemical mechanisms within these pathways is needed. This understanding is lacking in both JNK, which has been implicated in AD, and Hippo signaling, which has been related to JNK signaling. To date, JNK inhibitors have been developed and somewhat successfully used in inhibiting neurodegeneration (Bogoyevitch *et al*., 2010), however, JNK is involved in various processes in normal cell signaling. This raises the question of the damage done by inhibiting normal JNK signaling in developing cells, rendering the understanding of the interactions between JNK and other pathways that much more valuable so that only neurodegenerative specific signaling can be targeted. Finally, understanding the contexts which bring about specific signaling and specific pathway interactions will be necessary to continue the current pace of research in this field.

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Notation

As per *Drosophila* nomenclature, gene names and symbols are italicized and after first time the names of the genes are abbreviated while proteins names and symbols are written in uppercase letters (http://flybase.org/static_pages/docs/nomenclature/nomenclature3.html#1).

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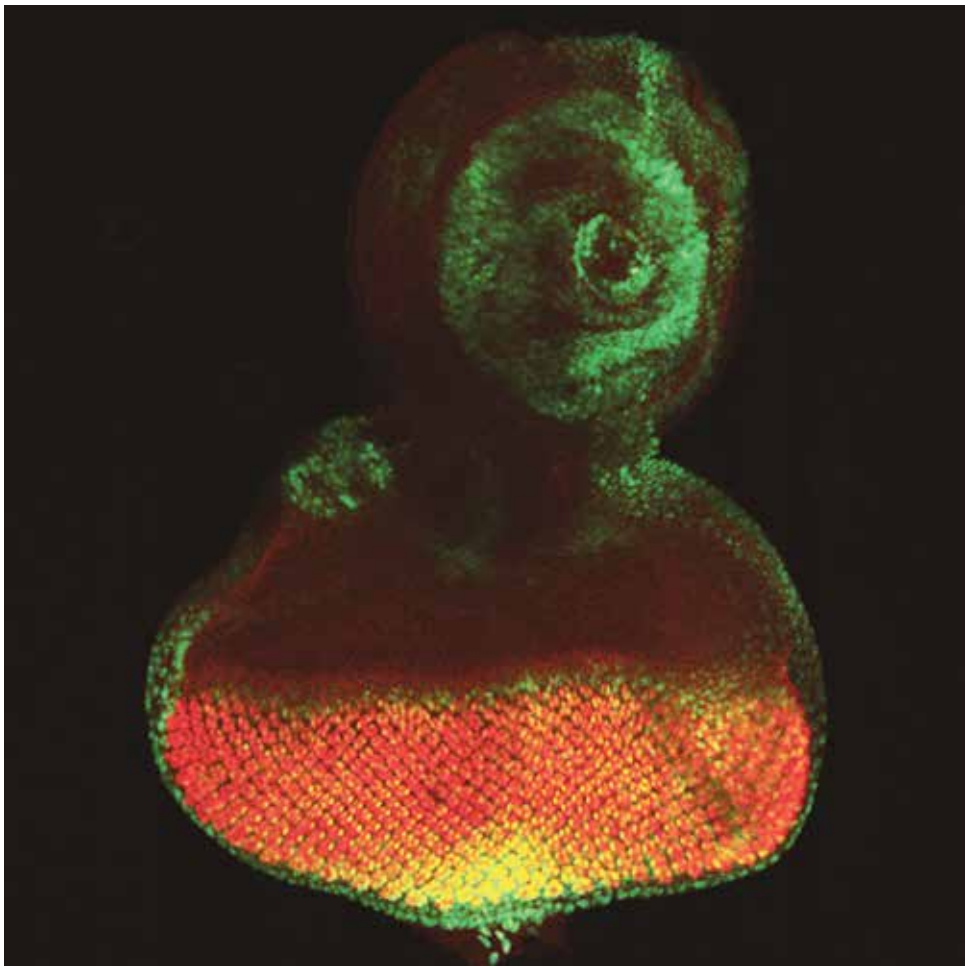

Drosophila melanogaster imaginal disc. Photo courtesy of Madison Irwin, 2013.

Src and Hippo Signaling: The Intersection of Two Tumor Suppressor Pathways

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Abstract

Growth of a single-celled zygote to a fully developed organism requires three processes: cell division, cell differentiation and quiescence following morphogenesis. Developmental genetic pathways control all of these processes. The molecular underpinnings of these pathways (*e.g.*, the MAPK, p53, Hippo, TSC/TOR pathways) are beginning to emerge. It is clear that not only the genes comprising these pathways play essential roles during development, but loss of these genes is responsible for several diseases including cancer. Two such pathways, *viz.*, Hippo and Src which comprise a network of tumor suppressor genes and oncogenes, are the focus of this review. These pathways control tissue and organ size during development by regulating cell proliferation, cell death, cell migration, and cell adhesion. Overall, the chief components and known interactions of the two pathways are discussed.

Introduction

The transformation of a single-celled zygote into a complex multi-cellular organism requires three processes: cell division, cell differentiation, and morphogenesis. A fertilized egg, called the zygote, undergoes multiplicative or embryonic growth via mitosis, dividing into a multi-cellular organism (Raff, 1992; Conlon and Raff, 1999). After multiplication, these cells specialize in different structure and function due to differential gene expression, and organize themselves into three-dimensional organs. Yet, growth of an organism does not stop after embryogenesis. The growth of certain body parts, such as the muscle, is due to auxesis, in which the size of cells increases while the number of cells remains the same. In a mature organism, the differentiated cells have lost the capacity of undergoing division, but undifferentiated cells present at some locations keep dividing mitotically to replace worn-out cells; this production of reserve cells from an increase of intercellular material is called the accretionary growth. Evidently, ongoing growth is restricted to the formation of cells that are needed to be replenished. As opposed to a tightly controlled growth during development, the phenomenon of unregulated growth is named cancer, one of the major leading causes of death worldwide.

Cancer arises from tumor cells that continually divide due to their inability to recognize growth regulatory signals, including signals controlling apoptosis (Kerr *et al.*, 1972; Jacobson *et al.*, 1997; Wyllie *et al.*, 1980) and contact inhibition. Cells that repetitively undergo cell cycle are likely to become cancerous, due to multiple errors that have accumulated from the numerous DNA replications. Indeed, most cancer cells contain at

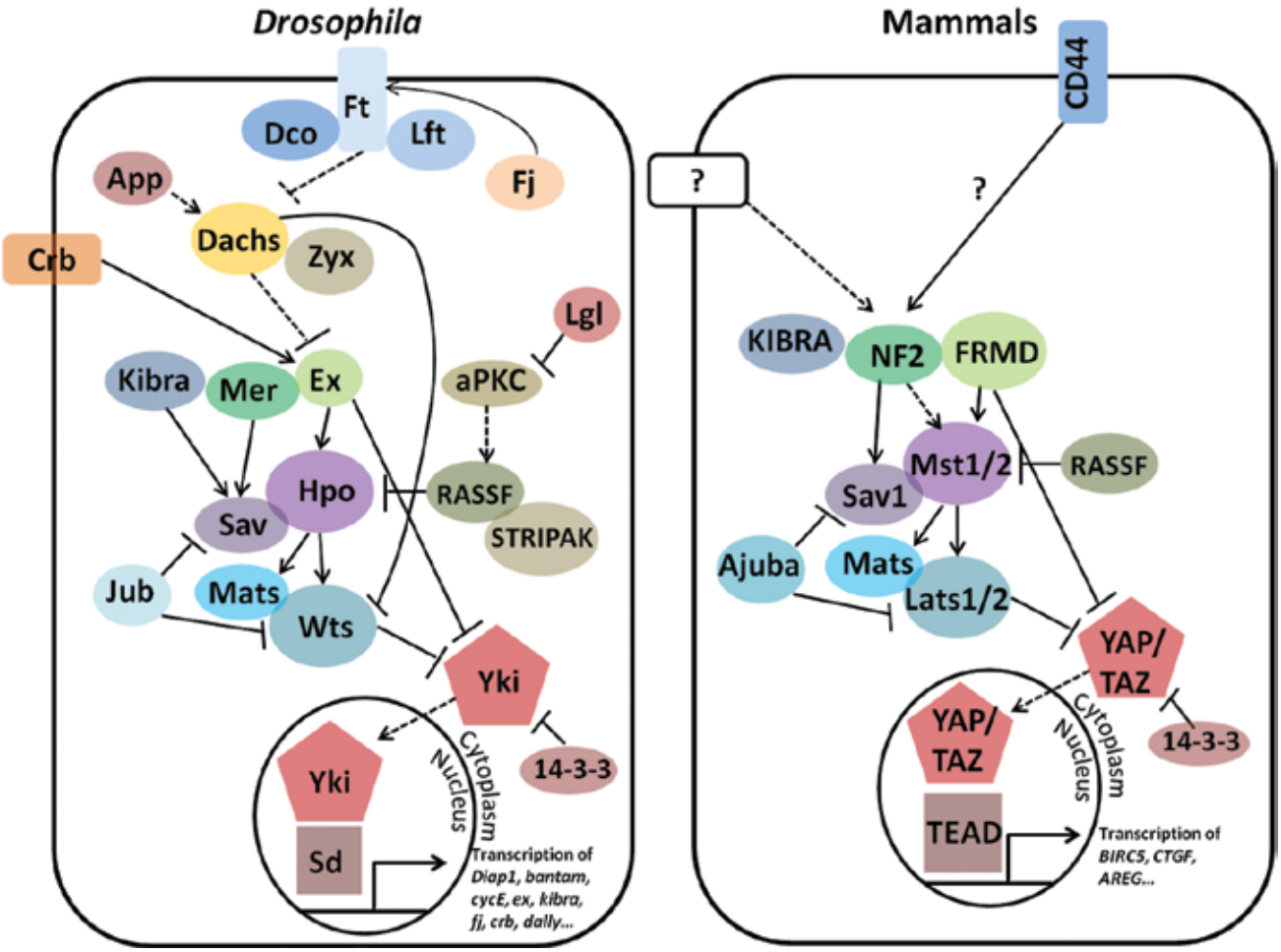

Figure 1. The Hippo Signaling Network in *Drosophila* and Mammals: Corresponding proteins in *Drosophila* and mammals are indicated by matching colors and shapes. Solid lines indicate direct biochemical interactions, whereas dashed lines show indirect interactions. Arrowed or blunted end indicates activation or inhibition. Selected target genes are shown.

least one mutated gene, or an oncogene, which can permanently activate the cell cycle and cause malignancy. In addition, inactivation or deletion of both copies of a tumor suppressor gene can also give rise to cancerous cells. These malignant tumors evade apoptosis, or programmed cell death, and continue to proliferate by increasing in their number. The more serious problem occurs when the malignant tumors spread throughout the body via metastasis, inducing the surrounding cells to also become tumorous. Studies to identify genes and genetic mechanisms that are involved in tumorigenesis and metastasis have led to the discovery of several tumor-suppressor networks (*e.g.*, the MAPK, p53, Hippo, TSC/TOR pathways) whose genes are often mutated in cancers. These studies reveal that only some cells in the tumor acquire the ability to metastasize, suggesting that additional mutations are responsible for metastatic defects. Amongst the pathways involved in tumorigenesis, the recently identified Hippo pathway has garnered the most attention. Originally identified by studies in *Drosophila*, the Hippo signaling pathway largely contributes to organ size regulation (Harvey and Tapon, 2007) in both invertebrates and vertebrates (*Figure 1*). Loss-of-function of the genes within this pathway leads to potent tumorigenesis in flies and humans.

The Hippo Signaling Pathway

The Hippo signaling pathway is a complex network of tumor suppressor genes and oncogenes, whose mutations lead to large, Hippopotamus-like phenotype (reviewed by Edgar, 2006; Pan, 2007; Saucedo and Edgar, 2007; Kango-Singh and Singh, 2009). It regulates organ size by inhibiting cell proliferation and promoting apoptosis (*Figure 2*). At the core of the pathway reside two kinases, Hippo (Hpo, the serine/threonine Ste20-like kinase) (Harvey *et al*., 2003;

Figure 2. Roles of the Hippo Pathway: The Hippo signaling pathway regulates various cellular activities, including cell growth, proliferation, cell death, cell cycle, morphogenesis, and cell-to-cell interactions.

Jia *et al*., 2003; Pantalacci *et al*., 2003; Udan *et al*., 2003; Wu *et al*., 2003) and Warts (Wts, the nuclear Dbf-2-related (NDR) family kinase; also known as Lats) (Justice *et al*., 1995; Xu *et al*., 1995), and two adaptor proteins, Salvador (Sav, the WW domain scaffolding protein) (Kango-Singh *et al*., 2002; Tapon *et al*., 2002) and Mob as Tumor Suppressor (Mats) (Lai *et al*., 2005). Initially, Hpo forms a complex with Sav to phosphorylate Wts. Wts, in turn, forms a complex with Mats to phosphorylate Yorkie (Yki) (Huang *et al*., 2005), a transcriptional coactivator, retaining Yki in the cytoplasm. In the absence of Hippo signaling, the dephosphorylated Yki translocates into the nucleus and binds to the transcription factors (*e.g*., Scalloped, Sd), inducing the expression of target genes that (1) promote cell proliferation (*e.g*., bantam microRNA, myc), (2) induce cell cycle progression (*e.g*., E2F1, cyclins A,B, E), or (3) inhibit apoptosis (*e.g*., diap1), which results in tissue overgrowth (Huang *et al*., 2005) (*Table 1*).

Multiple points of signal integration have been found in the Hippo Pathway, suggesting that the core kinase cassette responds to diverse stimuli. Examples of the upstream regulators include Expanded (Ex), Merlin (Mer) (Hamaratoglu *et al*., 2006), Kibra, Fat (Ft, the protocadherin) (Bennett and Harvey, 2006; Cho *et al*., 2006; Silva *et al*., 2006; Willecke *et al*., 2006), Tao1 (Boggiano *et al*., 2011; Poon *et al*., 2011), Crumbs (Crb), Ajuba (Jub), and Scribble (Scrib) (Verghese *et al*., 2012). Ex, Mer, and Kibra function together to activate the Hpo kinase cascade by directly binding to the Hpo-Sav complex (Yu *et al*., 2010). Additionally, Ex is known to directly repress Yki. The Ex-Mer-Kibra branch is modulated by the Fat/Dachsous branch, which does so by regulating Ex levels. The Fat/Dachsous branch consists of the atypical cadherins, Ft and Dachsous (Ds), and the downstream effectors Discs overgrown (Dco, a serine-threonine kinase; aka casein kinase 1ε), Dachs (D, an atypical myosin), Approximated (App, a palmitoyltransferase), Lowfat (Lft), and Zyxin (Zyx) (Grusche *et al*., 2010; Rauskolb *et al*., 2011). Tao1, a sterile 20-like kinase, is found to phosphorylate and activate Hpo. Furthermore, the Hippo pathway activity is thought to be also modulated by cell polarity, cell adhesion, and cell junction proteins, such as Crb, Jub, Scrib. The cell junction proteins exist in the epithelial cells of *Drosophila melanogaster* at the sub-apical region (SAR), adherens junction (AJ) or septate junction (SJ). Notably, the Hippo pathway activity is also affected by the modulation of the apical level of filamentous actin (F-actin). High accumulation of F-actin inhibits the Hippo signaling, thereby activating Yki.

Yki is the major downstream target of the Hippo signaling pathway. Upon phosphorylation by Wts, Yki creates a binding site for 14-3-3 proteins; this binding restricts Yki in the cytoplasm and leads to its degradation. Without proper phosphorylation, however, Yki can bind to several transcription factors, such as Scalloped (Sd), Homothorax (Hth) (Peng *et al*., 2009), Teashirt (Tsh), and Mothers against DPP (Mad) (Oh and Irvine, 2011), to promote tissue growth. In addition, the activation of Yki results in autoregulation of some upstream genes, such as *ex*, *mer*, *kibra*, *crb*, and *fj* via a positive feedback loop. Such broad spectrum of target genes confers tremendous versatility to Hippo signaling. Emerging evidence demonstrates that the Hippo pathway crosstalks with other signaling pathways to regulate different target genes.

Table 1. Function of the Hippo Pathway Target Genes

Function	Example Target Gene(s)
Cell cycle exit	*expanded*
Cell growth and survival	*homothorax (hth), diminutive (myc), bantam microRNA (miRNA)*
Proliferation	*cyclins A, B, E, E2F transcription factor (E2F1), hth, bantam miRNA*
Apoptosis	*merlin (mer), diap1*
Morphogenesis	*dally, dally-like*
Planar Cell Polarity	*crumbs, fat*
Contact Inhibition	*Mer*

The Src Pathway and Csk

The Src-family protein tyrosine kinases (SFKs) are implicated in various cellular processes, such as cell cycle exit, cell proliferation, survival, differentiation, adhesion, and cytoskeletal rearrangement. For instance, abnormal activation of the SFKs is involved in proliferative disorders such as cancer, particularly in liver, breast and colon (Masaki *et al*., 1999; Bougeret *et al*., 2001; Cam *et al*., 2001; Frame, 2002), and is associated with metastatic behavior (Yeatman, 2004). The SFKs were originally identified in the transforming gene of the Rous-Sarcoma virus, v-src. *Drosophila melanogaster* has two SFK members, Src42 and Src64 (Simon *et al*., 1985; Potter *et al*., 2000). The SFK activity is inhibited by phosphorylation of their Carboxyl-terminal (C-terminal) region by C-terminal Src kinase (Csk) (Cole, 2003). Flies have one Csk homolog, *dCsk* (Read, 2004; Stewart, 2003), which functions similarly to the mammalian Csk (Hoffmann *et al*., 1983; Simon *et al*., 1985; Takahashi *et al*., 1996). In fact, Csk was first identified by its ability to negatively regulate the SFK activity. Reduced levels of *dCsk* activate Src kinases (Thomas and Brugge, 1997; Schwartzberg, 1998; Bjorge *et al*., 2000), including Jun N-terminal kinase (JNK), Stat, and Btk29A (Pedraza, 2004, Read, 2004); this Src activation results in organ size increase, lethality of the organism, and overproliferation due to extra cell cycles (Read, 2004; Stewart, 2003). Thus, Csk acts as a tumor suppressor through the Src pathway regulation (Read, 2004), while also negatively regulating the JNK pathway.

Csk is known to have Src-independent functions as well (*Figure 3*). Independently of the SFK activity, Csk links G-protein signaling to the actin cytoskeleton (Lowry *et al*., 2002). In addition, Csk phosphorylates a number of other downstream molecules (Autero *et al*., 1994; Hildebrand *et al*., 1995; Cloutier and Veillette, 1996; Tremblay *et al*., 1996). Within the past decade, *dCsk* has been reported to regulate cell proliferation by genetically modifying wts tumor suppressor gene, a core component of the Hippo pathway, by direct phosphorylation (Stewart 2003). Presumably, mammalian Wts molecules may also be substrates of Csk, considering that the C-terminal *dCsk* phosphorylation site is conserved in other Wts homologs.

Broad loss of *csk* results in overproliferation (*Figure 4G*), inhibition of apoptosis, and decreased cell adhesion. However, local inactivation of *dCsk* in discrete patches surrounded by normal cells does not cause overgrowth (*Figure 4 A-E*), as *dCsk* mutant cells begin to delaminate and disperse from the growing imaginal disc tissue, leading to their elimination by macrophages (Vidal *et al*., 2006). Although loss-of-function phenotype of *dCsk* is strikingly similar to that of hpo, wts, sav, and mats (or gain of Yki function), clonal patches of *dCsk* cells fail to survive to adulthood, unlike cells containing the others four genes. Instead, these cells spread among the wild-type cells while simultaneously undergoing apoptosis, which may reflect the function of Src in promoting motility and invasion (Langton, 2007).

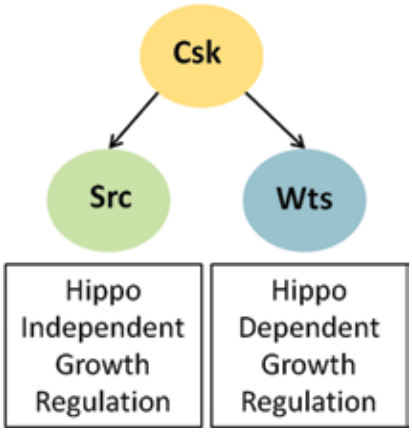

Figure 3. Csk at the Intersection between Src and Hippo Signaling Pathways: Certain genes in each Hippo and Src pathway (i.e., *wts* and *src*) have been shown to be regulated by a common kinase, Csk, suggesting that there might be a molecular link among the three.

More recently, Src has been reported to control tumor microenvironment by JNK-dependent regulation of the Hippo pathway (Enomoto and Igaki, 2013). Clone cells of Src overexpression activate the Rac-Diaphanous and Ras-mitogen-activated protein kinase (MAPK) pathways, which induces accumulation of F-actin, one of the upstream regulators of the Hippo pathway. Highly accumulated F-actin inhibits the Hippo signaling, thus activating Yki (Fernandez *et al*., 2011; Richardson, 2011; Sansores-Garcia *et al*., 2011) in both Src mutants and wild-type cells. Simultaneously, Src activates the JNK pathway, which signals the propagation of Yki activity to surrounding wild-type cells; the surrounding tissue is overgrown as a result. On the other hand, activated STAT acts independently of the Hippo pathway (Rodrigues, 2012). Src is known to interact with STAT. Thus, it is important to continue investigating the new, independent roles of Src and Csk.

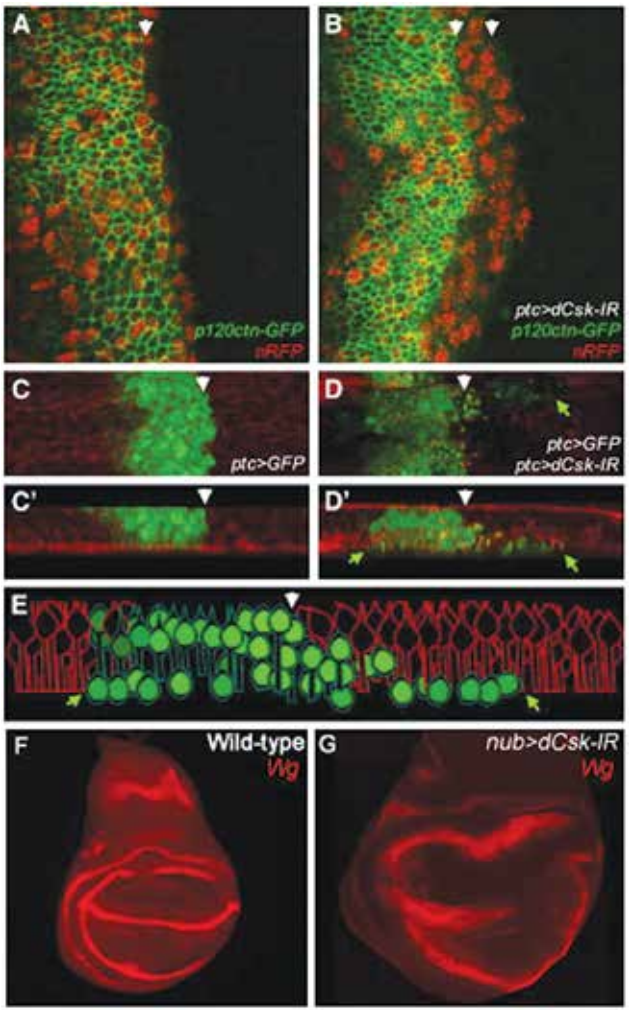

Figure 4. The Outcomes of the Local and Broad Loss of *dCsk*: (Panels A-E from Vidal *et al.*, 2006; F-G from Kwon *et al.*, *manuscript in preparation*.) Arrows mark the anterior/posterior boundary in panels A-E. (A-E) Loss of *dCsk* in discrete patches resulted in epithelial exclusion, invasive migration through the basal extracellular matrix (green arrows in D'), and eventual apoptotic death; these events occurred exclusively at the boundary between *dCsk* and wild-type cells. (F-G) Wing discs were stained with Wingless. (F) Wild-type wing imaginal disc is shown. (G) Broad loss of *dCsk* in the developing wing (nub>*dCsk*) resulted in overproliferation and disruption of anterior/posterior polarity.

Discussion

Emerging evidence supports that tumor development is regulated by cell-to-cell communication through multiple signals, namely Hippo and Src. Certain genes in each Hippo and Src pathway (*i.e.*, *wts* and *src*) have been shown to be regulated by a common kinase, Csk, suggesting that there might be a molecular link among the three (*Figure 3*). Although Csk activity is known to regulate metastatic behavior by decreasing cell adhesion, reduced *csk* expression alone is not sufficient to direct stable tumor growth. The fact that discrete patches of *dCsk* fail to maintain survival of migrating cells calls for further investigation of the Src-independent role of Csk in metastasis, specifically in relation to how important the Hippo signaling input is to Csk-mediated growth regulation.

Csk may have specific roles as a tyrosine kinase by modifying proteins within the Hippo Pathway, a hypothesis that remains untested. These modifications may reveal the molecular circuitry that enhances the migratory behavior of certain types of tumors, which can then be targeted for therapeutic intervention. For example, suppressing Csk activity could revert defects, or disrupting the binding domains of Csk with the Hippo Pathway components could prevent the cellular changes required for metastasis. Therefore, understanding the genetic relationship between Csk with the Hippo Pathway would enhance the therapeutic approaches to relevant diseases such as cancer.

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Notation

In keeping with *Drosophila* nomenclature, gene names are italicized (*e.g.*, *dCsk*), proteins coded by the corresponding genes are capitalized (*e.g.*, *dCsk*). Species names are also italicized (*e.g.*, *Drosophila*).

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Review: The Role of p53 in Regeneration and Wound Healing

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Abstract

P53 is one of the major tumor suppressor proteins in control of cell survival and cell-death mechanisms. Through its interactions with different regulatory proteins it can modulate different steps of regeneration and wound healing through regulation of cell cycle, adhesion, and in response to wound hypoxia. This review will cover the role of p53 in regeneration and wound healing.

Introduction

The tumor suppressor protein p53 is activated in stressed or injured cells. Stress conditions include reactive oxygen species- (ROS) and UV light-induced DNA damage, ribonucleotide imbalance, extracellular matrix alterations, hypoxia and the activation of oncogenes such as ras or myc [Vogelstein, Lane & Levine, 2000; Villiard *et al*., 2007]. These stressors can activate ataxia telangiectasia (ATR), ataxia telangiectasia mutated (ATM), checkpoint kinase 1, and checkpoint kinase 2 (CHK2) through phosphorylation which then activates p53. Activated p53 triggers inhibition of the cell cycle which stops cell division [Vogelstein, Lane & Levine, 2000]. The effects of p53 are facilitated by a vast majority of different genes divided into four categories: cell cycle inhibition, apoptosis, genetic stability, and inhibition of blood-vessel formation. The inactivation or a mutation in p53 is the most frequent alteration that is found in cancer and tumor formation. Quantitatively, p53 is found mutated in half of the tumors associated with cancer, however, some of the pathways associated with p53 have not yet been fully understood [Vogelstein, Lane & Levine, 2000; Villiard *et al*., 2007].

P53 Mechanism of Action

Besides having a minor role in site specific pathways in the cell, the major function of p53 is to inhibit the cell cycle or initiate apoptosis in response to cell stress. Under regular conditions, mdm2 acts as a p53 negative feedback regulator by targeting p53 for ubiquitin-mediated proteolysis [Vogelstein, Lane & Levine, 2000]. Under stress conditions, mdm2 becomes phosphorylated and is no longer a p53 inhibitor. The corresponding upregulation of p53 activity results in binding to the specific region of the mdm2 gene initiating upregulation of mdm2 transcription. The new mdm2 protein binds to the p53 protein and promotes feedback inhibition of p53 by ubiquitination to decrease p53 levels in the absence of cell stress [Meek, 1999; Prives & Hall, 1999; Giaccia & Kastan, 1998]. The mdm2-p53 feedback loop can also be modulated through kinases that specifically phosphorylate and stabilize the p53 protein. The stabilized p53 protein can then modulate p53-dependent pathways including activation of p21 for cell cycle arrest in the G1 phase, promotion of the 14.3.3σ protein to induce cell cycle arrest in the G2 phase, and activation of Bax/Redox genes for induction of apoptosis [Vogelstein, Lane & Levine, 2000].

1. Role of p53 in Cell Cycle Regulation during Wound Healing and Regeneration

Regeneration of injured tissue requires activation of the cell cycle for cell proliferation. The cell cycle has 4 phases consisting of the G1, S, G2, and M phase. In the G1 phase, the cell increases in size and makes proteins needed for the subsequent phases. In the S phase, DNA is synthesized for cell transition into the G2 phase where it becomes ready for mitosis. During mitosis (M phase) the chromosomes condense and the cell duplicates. The cell cycle is regulated through two major processes: protein phosphorylation and ubiquitin-mediated degradation. The corresponding regulatory proteins are divided into three categories including cyclins, cyclin-dependent kinases (CDK), and cyclin-dependent kinase inhibitors [Heber-Katz *et al*., 2012].

CDK/cyclin complexes are needed for cells to progress through the cell cycle. Different cyclins are present at different points in the cell cycle. For instance, cyclin E becomes activated before cyclin A in the transition from G1 to S. Specifically, the cyclin dependent kinase 2 (CDK2)/cyclin A complex is used to drive the transition into the S-phase [Heber-Katz *et al*., 2012]. During regeneration CDK2 has been demonstrated to be upregulated in parallel with cell proliferation and decreased apoptosis [Tsonis *et al*., 2004]. On the contrary, using a cyclin dependent kinase inhibitor, p27, CDK2 becomes downregulated during regeneration [Albrect *et al*., 1998]. The transcription factor E2F is used to activate genes involved in DNA synthesis. When it exists in unbound form, it is able to activate any of the over 800 genes that are involved in DNA synthesis including transcription of cyclin E. Cyclin E interacts with CDK2 to aid in the transition of G1 to S [Heber-Katz *et al*., 2012].

During regeneration p53 ensures that only healthy undamaged cells proliferate for participation in wound healing. The p53 protein can interfere with the cell cycle at two different steps including G1 arrest and G2 arrest. When p53 activates p21 it inhibits the cyclin A/CDK2 complex and the cell cycle is unable to transition from the G1 phase into the S phase. In detail, p21 binds to the cyclin A/CDK2 complex which is important for Rb (Retinoblastome protein) phosphorylation. The cell is unable to transition into the S-phase until Rb becomes phosphorylated, releasing the transcription factor E2F and allowing the cell to continue into the S phase. This prevents other genes to be activated and synthesize DNA [Heber-Katz *et al*., 2012]. In response to DNA damage, CHK1 and p53 are activated. CHK1 phosphorylates cdc25 which can then interact with 14.3.3σ. Alternatively, when p53 interacts with 14.3.3σ it transports the phosphorylated cdc25 into the cytosol causing cell cycle inhibition through G2 arrest [Fu, Subramanian & Masters, 2000; Hermeking *et al*., 1997].

2. Role of p53 in Wound Hypoxia

Oxygen is a molecule used in cell metabolism and the production of energy. There is higher energy consumption in cell proliferation during regeneration [Hunt, Zederfeldt & Goldstick, 1969]. Through an oxygen dependent process, adenosine-tri-phosphate (ATP) is formed in the mitochondria and is used in intracellular processes [Tandara & Mustoe, 2004]. Post-injury, a wound is essentially free of oxygen due to the vascular disruption causing an oxygen shortage [Hunt, Niinikoski & Zederfeldt, 1972; Niinikoski, Hunt & Dunphy, 1972]. Oxygen levels normalize as revascularization begins to take place and as the wound continues to heal. Therefore, low oxygen levels are associated with the initiation of tissue repair or angiogenesis [Tandara & Mustoe, 2004]. One major factor of oxygen-dependent induction of wound healing and angiogenesis is hypoxia-inducible factor 1 (HIF-1) [Fels & Koumenis, 2005]. HIF-1 is a heterodimeric transcription factor comprised of two subunits, HIF-1α and HIF-1β [Wang *et al*., 1995; Ravi *et al*., 2005]. During wound hypoxia, HIF-1 is regulated through the stabilization of the HIF-1α subunit [Ravi *et al*., 2000]. The central molecular mechanism involved during hypoxia consists of HIF-1 binding to the promoter region activating the transcription of different target genes [Tandara & Mustoe, 2004].

HIF-1α is able to become stabilized under mild or moderate hypoxia to induce regeneration while under more extreme conditions p53 accumulates to induce cell death [Fels & Koumenis, 2005]. During this hypoxic p53 mediated apoptosis, p53 was demonstrated to directly interact with HIF-1α for ubiquitin-mediated degradation of HIF-1α through the use of mdm2, an E3 ubiquitin-protein ligase [Sánchez-Puig, Veprintsev & Fersht, 2005]. Correspondingly, ubiquitin-dependent degradation of HIF-1α through p53 results in the inhibition of HIF-1 activity.

Under the special circumstances of tumor hypoxia, HIF-1 activation causes p53 to

accumulate in hypoxic tissue regions [Zhong *et al*., 1999; Graeber *et al*., 1996; Fels & Koumenis, 2005]. Thus, when p53 is mutated in tumor cells increased in HIF-1α levels are able to activate genes associated with angiogenesis, such as vascular endothelial growth factor (VEGF), promote tumor cell survival, and trigger neovascularization to begin [Sanchez-Puig, Veprintsev & Fersht, 2005; Forsythe *et al*., 1996].

3. Role of p53 in Regulation of Cell Adhesion

When tissues are injured, cell adherence is disrupted causing a change in cell signaling and communication. Cadherins are cell adhesion molecules responsible for the control and maintenance of many tissues and structures [Lui *et al*., 2002]. Cadherins can be separated into three subgroups including classical cadherins, desmosomal cadherins, and protocadherins. Members of the cadherin family are involved during regeneration. For example, in axonal regeneration there is increased expression of both N and R cadherin in the central nervous system [Faulkner-Jones, Godinho & Tan, 1999; Lui *et al*., 2002]. In addition to adhesion, cadherins are involved in cell sorting and tissue morphogenesis through interactions with other proteins. These proteins include protein kinases and phosphatases involved in signaling pathways affecting gene expression [as reviewed Halbleib & Nelson, 2006]. The survival of cells in absence of functional adherens junctions can trigger cell-detachment and tissue metastasis. The inactivation of E-cadherin in combination with p53 mutations is associated with tumor invasiveness and resistance to apoptotic signals. When both E-cadherin and p53 were inactivated a synergistic effect occurred through inducing malignant cells and metastasis [Derksen *et al*., 2011].

Conclusion

The tumor suppressor protein p53 intereacts with a variety of proteins to participate in a range of cellular functions to support cellular and tissue processes, including wound healing and regeneration. Mutations in the p53 gene are commonly found in human cancers, thus indicating a role for p53 in maintaining cellular homeostasis. Further experimentation is needed to more clearly elucidate the molecular and mechanistic pathways in which p53 is involved in ormal and disease states.

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Notophthalmus viridescens. Photo courtesy of Jessica L. Beebe, 2013.

Analysis of Premium Food and Beverage Trends in the North American "Big Four" Sports Venues

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Abstract

The purpose of this exploratory study is to understand the trends in premium food and beverage based on a statistical analysis of menus. By gathering data on pricing, region, and vendor, the insight gained on trends will be helpful to the industry as it looks for ways to grow. How do regional differences impact menu offerings? What food and beverage items are most included in premium seating areas, and which are seen more sporadically? We investigated a selection of 30 items, narrowed down by industry experts. The items were divided into categories: traditional fare, up and coming favorites, and beverages. Information about the items studied was recorded, and results were delivered on what is currently trending in the industry. Menus were collected from 69 of the 113 (61%) venues that host National Basketball Association (NBA), National Football League (NFL), National Hockey League (NHL), and Major League Baseball (MLB) events.

Background

Premium seating can account for up to 25% of local revenue for sport organizations (Connelly, 2011). A critical variable in the premium experience is food and beverage, an area in which more and more organizations are subcontracting due to convenience and financial considerations. Of the "Big Four" leagues, NHL organizations outsource food and beverage least often at 83%, while 97% of MLB organizations outsource their food and beverage, making the MLB the highest (Lee & Lee, 2011, p. 4).

The Food and Beverage Manager is directly connected to the Marketing Director and the Suite Administrator, but he has little to no contact with two important stakeholders, the Suite Owner and Director of Premium Seating (Titlebaum & Lawrence, 2009, p. 130). This situation is likely to create a void between the parties involved.

A more cohesive team can be created in the luxury suite service department through an understanding of the value of promoting packages around a theme. Some examples of this are a barbeque spread, a selection of regional specialties, or a menu based on a particular diet. This concept helps staff by consolidating orders through common items, simplifying waste, and improving overall satisfaction (Titlebaum, Titlebaum & Dick, 2011, p. 490). Therefore, menus should be designed in a way that reflects this concept.

Sports fans' tastes are growing more luxurious as premium food and beverage becomes a more sought-after industry. It is not unusual to find "wine pairing dinners at racetracks, dining on made-to-order sushi meals or [fans] participating in game-time cooking demonstrations in specially equipped suites" (Elan, 2006, p.4). Mainstream food and beverage trends are being adopted by food and beverage decision makers in the sports world. Local eating is one of the trends for Summer 2013 in Nation's Restaurant News; from using local ingredients to offering local craft beers, restaurants and venues alike are participating (Thorn, 2013a). Even the food fringe groups: the vegans, vegetarians and gluten-free or nut-free groups, are being represented on menus in the food world with increasing frequency. Gluten-free is a growing culinary trend which has gained representation in sport venues' premium seating menus (Thorn, 2013b).

Sport venues can be seen as "varied-menu restaurants" because they serve a broad range of clientele and need to have something on the menu for everyone. This restaurant category is developing a greater appreciation for global flavors, particularly Latin and Southeast Asian flavors in appetizers and starters, such as soup. In traditional restaurants, bundling is seen as a "value" for customers so restaurants provide "two-for and three-for appetizer/entrée bundles" to appeal to patrons. Sandwiches are another area where premium trends are breaking through. "Unique flavor profiles and high-quality foods" are being added to sandwiches to appeal to the premium customer. (Harvey, 2012, p. 12-16)

On the beverage side, there is a significant push, driven mostly by the younger demographic, to offer craft alcoholic beverages. As the demand grows, more venues are offering the option to purchase craft beers, particularly local brews. Similarly, the demand for a craft cocktail is also growing. Venues are looking to offer a few easy but unique cocktails to interest customers. Centerplate™ Senior Vice President, Greg Fender, expressed his company's focus on bringing high-quality wines to more customers this year. (Muret, 2013, p.16)

Table 1. Food and Beverage Menu Analysis Criteria
For each item, the following were recorded: Venue, Vendor, Price – Low, Price – High, Price – Mean, Serving Size, Region.

Traditional Fare	Up-and-Coming	Beverages
Hotdogs and Sausages	Gluten-free	Wine – Sparking
Macaroni and cheese	Vegan	Wine – White
Chicken Tenders	Salad – Vegetarian	Wine – White Zinfandel
Pretzels	Salad – with Protein	Wine – Red
Ice Cream	Flatbread	Beer – Domestic
Chicken Wings	All-Inclusive Package	Beer – Craft
Hamburgers/Cheeseburgers	Local Food	Beer – Import
Nachos	Organic	Spirits
Popcorn	Regional Specialty	Soft Drinks
Pizza	Sushi	Water

For decision-makers in sports, food and beverage pricing remains an issue. Wholesale beer costs continue to rise, but customers have a threshold where prices are considered to be unreasonable. The key is to find a creative solution that sweetens the value proposition for the customer, such as an all-inclusive ticket. The exclusive premium offerings have more value for fans and generally garner a higher price tag while still satisfying the fan. (Muret, 2013, p.16)

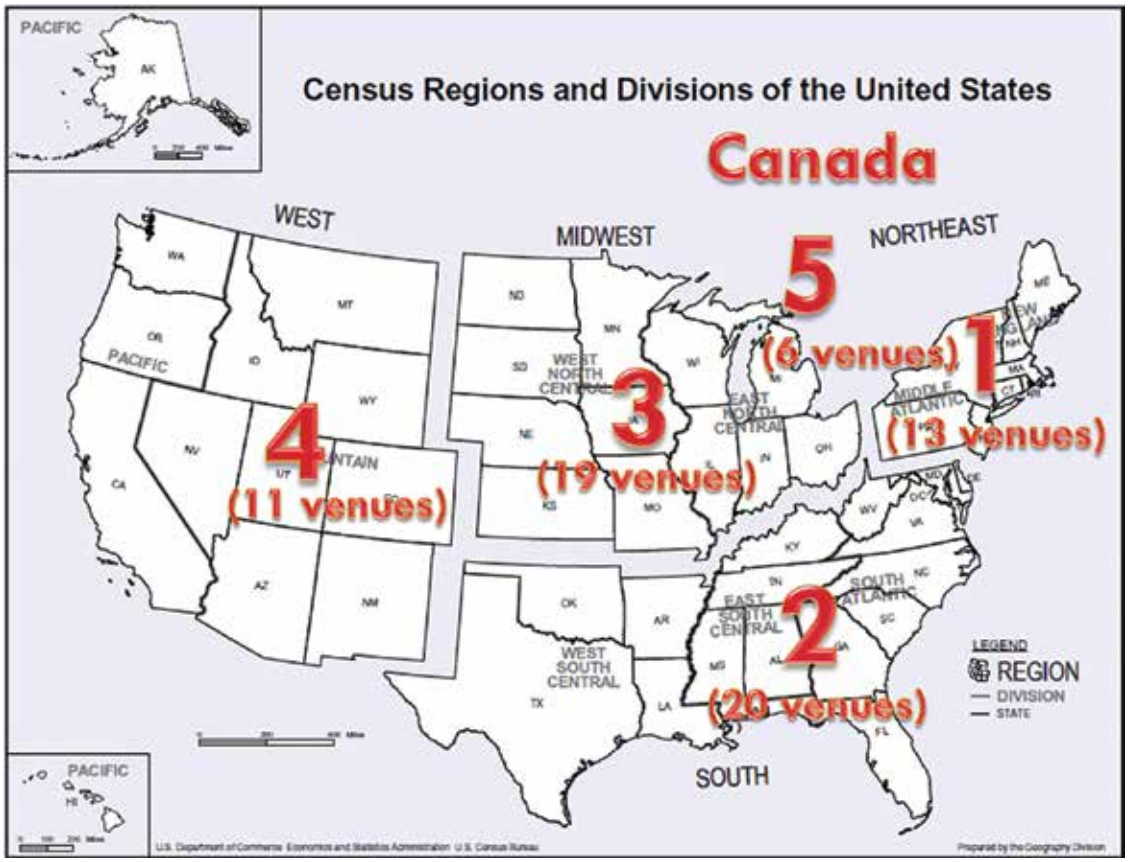

Figure 1. US Census Regional Divisions. United States Census Bureau. (2013). Census regions and divisions of the United States. Retrieved from http://www.census.gov/geo/maps-data/maps/pdfs/reference/us_regdiv.pdf.

Methodology

This study is a continuation of a pilot study of food and beverage in luxury suites, "Food and beverage industry takes a bite out of U.S. luxury suite market," which explored the industry gaining insights through ten phone interviews (Titlebaum, 2011, p.488). The format of the current exploratory study is supported by statistical analysis. The researchers developed a sample set of menu items and a list of criteria by which to analyze them (*Table 1*). Both the items and criteria were reviewed by a panel of experts that included the Executive Director of the Association of Luxury Suite Directors (ALSD) and the President of Bigelow Companies. Their feedback focused primarily on the clarification of sources of information with no significant modifications to the criteria. The menu items were divided into three categories: traditional fare, up-and-coming options, and beverages. In each category, ten items were selected for the study. The criteria focused on pricing, region, and company to determine trends in the industry.

Menus were obtained through internet searches and email requests. Of the 113 venues in MLB, NFL, NBA and NHL, menus were received from 69 venues (61%); 23 MLB venues, 15 NFL venues, and 32 NBA and/or NHL menus were received (*Table 2*). One venue hosts both MLB and NFL events so it was represented twice in the previous count. Menus were limited to premium seating areas, including suites, club areas, and restaurants. While menus were collected from May to June 2013, they included a combination of menus from 2012 and 2013, depending on availability of the organizations. Data was input and sorted by unit price, region, and company to see trends. Regional divisions were based on the four areas designated by the US Census (see *Figure 1*), with the fifth region being Canada.

Results and Discussion

The first focus of the research looked at the regional prevalence of 30 items (*Table 3*). Analysis has shown the prevalence of regional specialties in the South and West. In the South barbeque is indigenous; therefore it is reflected on menus. The West remains cutting edge in its food choices, setting trends such as the sushi craze that later spread across the country. The Midwest and East continue to prefer classic favorites. Only two venues across the country listed organic items on their menu, therefore it is concluded that, for the vast majority, organic fare is not a value driver. In contrast, locally-sourced items are readily listed in all 5 regions, with 43 menus (62%) boasting this claim. The data suggests customers put more value

Table 2. Venues used in this study and their vendors for the 2012/2013 seasons.
*switched to ARAMARK for 2013-14 season

Venue	Vendor	Sport
Air Canada Centre	Pinnacle Caterers	NBA/NHL
Amway Center	Levy Restaurants	NBA
Arrowhead Stadium	Levy Restaurants	NFL
AT&T Park	Bon Appetit	MLB
Bank of America Stadium	Delaware North	NFL
Bankers Life Fieldhouse	Levy Restaurants	NBA
Barclays Center	Levy Restaurants	NBA
BB&T Center	Centerplate	NHL
Bell Centre	Levy Restaurants	NHL
BMO Harris Bradley Center	Levy Restaurants	NBA
Busch Stadium	Delaware North	MLB
Chase Field	Levy Restaurants	MLB
Chesapeake Energy Arena	Levy Restaurants	NBA
Citizens Bank Park	ARAMARK	MLB
Comerica Park	Delaware North	MLB
Consol Energy Center	ARAMARK	NHL
Coors Field	ARAMARK	MLB
Dodger Stadium	Levy Restaurants	MLB
Fenway Park	ARAMARK	MLB
First Niagara Center	Delaware North	NHL
FirstEnergy Stadium	ARAMARK	NFL
Georgia Dome	Levy Restaurants	NFL
Great American Ballpark	Delaware North	MLB
Heinz Field	ARAMARK	NFL
HP Pavilion at San Jose	ARAMARK	NHL
Jobing.com Arena	ARAMARK	NHL
Joe Louis Arena	Olympia Entertainment	NHL
Lambeau Field	Delaware North	NFL
LP Field	Centerplate	NFL
Lucas Oil Stadium	Centerplate	NFL
Marlins Park	Levy Restaurants	MLB
MetLife Stadium	Delaware North	NFL
Miller Park	Delaware North	MLB
Minute Maid Park	ARAMARK	MLB
Nassau Veterans Memorial Coliseum	SAVOR Catering	NHL
Nationals Park	Levy Restaurants	MLB
Nationwide Arena	Delaware North	NHL
New Orleans Arena	Centerplate	NBA
O.co Coliseum	Levy Restaurants	MLB/NFL
Oracle Arena	Levy Restaurants	NBA
Oriole Park at Camden Yards	Delaware North	MLB
Petco Park	Delaware North	MLB
Philips Arena	Levy Restaurants	NBA
PNC Arena	VAB Catering	NHL
PNC Park	Levy Restaurants	MLB
Progressive Field	Delaware North	MLB
Prudeintial Center	Centerplate	NHL
Ralph Wilson Stadium	Delaware North	NFL
Rangers Ballpark	Delaware North	MLB
Raymond James Stadium	Levy Restaurants*	NFL
Reliant Stadium	Connoisseur	NFL
Rexall Place	Delaware North	NHL
Rogers Arena	ARAMARK	NHL
Rogers Centre	ARAMARK	MLB
Scotiabank Saddledome	Calgary Flames Food Services	NHL
Scottrade Center	Levy Restaurants	NHL
Sports Authority Field at Mile High	Epicurean Entertainment	NFL
Staples Center	Levy Restaurants	NBA/NHL
Sun Life Stadium	Centerplate	NFL
Tampa Bay Times Forum	Delaware North	NHL
Target Field	Delaware North	MLB
TD Garden	Delaware North	NBA/NHL
The Palace at Auburn Hills	Levy Restaurants	NBA
Tropicana Field	Centerplate	MLB
United Center	Levy Restaurants	NBA
US Cellular Field	Levy Restaurants	MLB
Verizon Center	Levy Restaurants	NBA/NHL
Wells Fargo Center	ARAMARK	NBA/NHL
Xcel Energy Center	Levy Restaurants	NHL

Table 3. Regional Breakdown of Food and Beverage Items

	Region					
	Northeast	South	Mid-West	West	Canada	% of
# of Venues	n=13	n=20	n=19	n=11	n=6	Acceptance
Hotdogs and Sausages	12	20	17	10	4	94%
Macaroni and cheese	10	15	17	7	0	71%
Chicken Tenders	11	18	18	9	5	88%
Pretzels	10	12	19	9	3	77%
Ice Cream	5	8	3	4	4	35%
Chicken Wings	12	18	18	10	6	93%
Hamburgers/Cheeseburgers	10	11	11	8	4	64%
Nachos	9	14	10	6	2	59%
Popcorn	11	20	18	11	5	94%
Pizza	9	10	10	4	5	55%
	Region					
	Northeast	South	Mid-West	West	Canada	% of
# of Venues	n=13	n=20	n=19	n=11	n=6	Acceptance
Gluten-free	7	11	15	5	1	57%
Vegan	3	7	5	3	1	28%
Salad – Vegetarian	13	19	18	10	6	96%
Salad – with Protein	13	19	18	10	5	94%
Flatbread	2	9	10	4	0	36%
All-Inclusive Package	11	18	18	11	6	93%
Local Food	5	15	13	6	4	62%
Organic	0	0	2	0	0	3%
Regional Specialty	10	12	11	6	3	61%
Sushi	8	8	3	8	5	46%
	Region					
	Northeast	South	Mid-West	West	Canada	% of
# of Venues	n=13	n=20	n=19	n=11	n=6	Acceptance
Wine - Sparkling	11	20	19	9	5	93%
Wine – White	11	20	19	9	6	94%
Wine - White Zinfandel	9	18	19	7	2	80%
Wine – Red	11	20	19	9	6	94%
Beer – Domestic	9	20	19	9	5	90%
Beer – Craft	9	18	17	9	4	83%
Beer – Import	11	20	19	9	5	93%
Spirits	10	20	19	9	6	93%
Soft Drinks	12	20	19	8	5	93%
Water	11	20	19	8	5	91%

on using local ingredients. It is interesting to note the lack of ice cream made available across the country; only 24 (35%) of the menus collected included ice cream. This could be attributed to lack of demand or the lack of the proper storage facilities. Finally, in Beverages, fewer venues offered White Zinfandel than any other beverage. This data can be attributed to the specific nature of this category, because it is a narrower varietal of wine rather than the broader categories of whites and reds.

Hotdog and sausage pricing (*Table 4*) varies across companies. The multiple pricing levels on this item make it appealing to different customers, from those looking to save money to those looking to impress. Macaroni and cheese is a consistent option among the majority of the venues the food and beverage vendors serve. However, it is served most by Levy Restaurants™ and Delaware North Companies™, making it a staple of their menu structure. Chicken tenders are served by most of the companies in this study, 60 out of 69 venues (87%); however, most offer this item at a basic pricing level. This is evident because there is very little price variance between low-end chicken and high-end chicken. Two companies offer chicken tenders at one price level. Similar to chicken tenders, chicken wings have limited price variance across all of the companies in this study. The classic and reliable chicken-based foods are a common item that food and beverage vendors have left uncomplicated. Pretzels have proven to be an affordable snack for customers, with the highest average price at $4.32 per person. Nachos, another staple food at sporting events, are fairly static in pricing at the premium level. Some companies are offering a higher priced nacho, while others have gone for a slightly less expensive nacho; regardless of price, companies seem to pick one or the other, deciding for the customers at what level they will be eating.

In the "Up and Coming" menu section, the prevalence of gluten-free items is noted. The majority of venues have gluten-free items listed on their menu. Interestingly, Centerplate™ has chosen to leave gluten-free items unidentified on their menu. While most people who abide by this difficult diet constraint are aware of what foods they can and cannot eat, it makes it difficult for those who are hosting gluten-free guests to know what their guests can consume. Another fringe diet, vegan, is represented in a handful of menus; however, there is a significant gap in pricing. Levy Restaurants™' prices are significantly higher than any other company offering advertised food for vegans. Levy Restaurants™ also has the most venues offering advertised

Table 4.1 Breakdown of Pricing Per Person by Vendor.
* Delaware North Companies™ ** Levy Restaurants™

	Vendor (prices in dollars)				
	ARAMARK	Centerplate	DNC*	Levy**	Others
Hotdogs and Sausages	n=10	n=6	n=18	n=21	n=8
Low price	7.53	7.38	7.06	6.84	6.43
High price	9.64	10.69	9.13	12.88	9.01
Mean price	8.59	8.99	8.19	9.33	7.70
Macaroni and cheese	n=4	n=2	n=17	n=21	n=5
Low price	7.44	7.49	7.20	6.64	4.86
High price	13.17	7.49	8.01	10.12	5.43
Mean price	9.45	7.49	7.72	8.54	5.15
Chicken Tenders	n=10	n=5	n=18	n=21	n=6
Low price	8.42	11.48	8.58	9.91	10.31
High price	8.42	12.56	8.58	11.40	10.52
Mean price	8.42	11.91	8.58	10.64	10.42
Pretzels	n=11	n=4	n=14	n=20	n=4
Low price	3.52	2.54	3.71	1.06	3.53
High price	4.32	3.17	3.88	3.59	3.53
Mean price	3.92	2.85	3.80	2.33	3.53
Ice Cream	n=7	n=6	n=6	n=2	n=3
Low price	6.21	5.12	6.44	3.88	5.83
High price	6.73	9.58	7.10	7.25	6.50
Mean price	6.45	7.33	6.77	5.56	6.17
Chicken Wings	n=10	n=6	n=18	n=23	n=7
Low price	9.21	10.92	8.55	11.04	10.06
High price	10.92	10.92	8.83	12.08	10.78
Mean price	9.55	10.92	8.76	11.73	10.42
Hamburgers/Cheeseburgers	n=12	n=5	n=15	n=6	n=6
Low price	9.81	12.52	9.45	13.31	9.83
High price	10.99	15.77	9.93	13.31	11.19
Mean price	10.47	14.12	9.69	13.31	10.56
Nachos	n=5	n=4	n=8	n=19	n=5
Low price	8.77	11.82	6.08	9.89	9.27
High price	8.77	11.82	6.75	10.51	9.31
Mean price	8.77	11.82	6.45	10.19	9.29
Popcorn	n=11	n=6	n=18	n=22	n=8
Low price	2.62	2.62	2.94	1.85	2.48
High price	2.94	2.62	3.32	2.40	2.52
Mean price	2.80	2.62	3.13	2.10	2.50
Pizza	n=11	n=5	n=14	n=2	n=6
Low price	6.60	6.35	4.62	6.83	5.52
High price	7.39	6.90	5.10	6.83	6.74
Mean price	6.92	6.64	4.91	6.83	6.09

vegan foods. Salads are the ever-popular healthy offering. The options in the premium seating areas studied remain highly variable for both vegetarian and protein choices. With a broad price range, customers have many options for their greens.

Flatbread is very popular with Levy Restaurants™, offered at 23 venues, while it is rarely seen anywhere else. It is interesting to see how one company has adopted an ambiguous food with a trendy name and provided customers a spectrum of ways to enjoy it. Pricing for flatbread broadly varies averaging from $8.98 per person on the low end to $14.19 per person at the high. Many of Levy Restaurants™' customers can fit this item into their budget.

Local foods with the four main companies in this study are reasonably priced, hovering around $9 to $10 per person on average. Conversely, in the "other" category, the price for local foods shoots up to $17.88. These results could be attributed to the strength of relationships with suppliers in each area and the amount of product the company is able to purchase. Sushi, a popular item with ARAMARK in particular, has some variance in price on each company's menu, but there are still limited options for this item. Interestingly, a high proportion of "other" companies offer sushi compared to the larger companies.

In the beverages category, pricing remained similar for each item. One category that stood out is the Spirits section. Not only does each company have a fairly diverse offering of pricing levels, but the price range across all of the companies is fairly large as well. Sparkling wines also had a diverse selection of different qualities of sparkling wine and Champagne. Champagne, the "big ticket item" in this section, was very interesting because prices went all the way up to $1,200 for a single bottle. The other categories of wine were much closer in price, although there were a significant number of higher priced reds (over $100). Beer fluctuated somewhat in price, but the per can cost did not vary more than $2 in the majority of instances. Soda and water did not have much of a difference in price on each menu; in most cases, the price was the same for all types of soda and there was only one option for regular water.

Conclusions and Limitations

This research can be utilized by professionals in the industry to gain a better understanding of what is really going on in premium food and

Table 4.2 Breakdown of Pricing Per Person by Vendor.
* Delaware North Companies ** Levy Restaurants

	Vendor (prices in dollars)				
	ARAMARK	Centerplate	DNC*	Levy**	Others
Gluten-free	n=5	n=0	n=13	n=20	n=1
Low price	10.28	-	3.36	10.28	12.00
High price	24.34	-	16.95	11.02	15.00
Mean price	14.44	-	7.87	10.69	13.50
Vegan	n=2	n=2	n=1	n=14	n=0
Low price	1.63	4.09	6.25	24.70	-
High price	7.15	7.27	6.25	25.63	-
Mean price	4.65	6.33	6.25	25.16	-
Salad - Vegetarian	n=12	n=5	n=18	n=23	n=8
Low price	6.53	4.28	5.74	7.27	5.01
High price	8.11	7.24	6.95	8.64	6.32
Mean price	7.29	5.65	6.36	7.98	5.66
Salad – with Protein	n=12	n=6	n=17	n=23	n=7
Low price	7.57	9.13	7.45	7.89	7.58
High price	10.36	10.06	8.51	11.65	10.35
Mean price	9.12	9.58	7.99	10.14	8.98
Flatbreads	n=1	n=1	n=0	n=23	n=0
Low price	8.75	7.75	-	8.98	-
High price	11.25	7.75	-	14.19	-
Mean price	10.00	7.75	-	11.05	-
All Inclusive Package	n=11	n=7	n=15	n=24	n=7
Low price	49.00	31.53	39.01	26.79	35.21
High price	80.70	56.41	64.10	60.33	62.16
Mean price	63.84	43.65	50.04	43.74	49.35
Local Foods	n=5	n=2	n=17	n=14	n=5
Low price	7.97	8.98	8.73	9.69	14.43
High price	10.39	8.98	10.45	11.38	26.10
Mean price	9.18	8.98	9.56	10.39	17.88
Organic	n=0	n=0	n=0	n=2	n=0
Low price	-	-	-	7.50	-
High price	-	-	-	7.50	-
Mean price	-	-	-	7.50	-
Regional Specialty	n=9	n=5	n=10	n=13	n=5
Low price	7.31	14.30	7.04	19.96	5.62
High price	18.94	17.27	14.04	35.60	8.32
Mean price	10.30	15.48	10.18	26.42	7.07
Sushi	n=11	n=3	n=4	n=7	n=7
Low price	19.78	24.44	11.11	26.54	14.28
High price	25.42	24.44	11.11	35.29	14.97
Mean price	22.40	24.44	11.11	30.89	14.57

Table 4.3 Breakdown of Pricing Per Person by Vendor.
* Delaware North Companies™ ** Levy Restaurants™

	Vendor (prices in dollars)				
	ARAMARK	Centerplate	DNC*	Levy**	Other
Wine - Sparkling	n=10	n=7	n=17	n=22	n=8
Low price	42.10	48.86	47.35	34.64	49.88
High price	175.00	248.57	159.18	274.55	198.88
Mean price	92.93	120.08	93.89	110.72	108.77
Wine - White	n=11	n=7	n=17	n=22	n=8
Low price	35.45	29.71	29.71	29.73	30.16
High price	82.55	67.57	78.94	89.55	87.25
Mean price	50.81	43.40	43.46	45.37	45.72
Wine – White Zinfandel	n=8	n=7	n=13	n=21	n=6
Low price	31.25	28.14	30.15	30.14	31.50
High price	31.25	28.71	30.85	30.14	36.67
Mean price	31.25	28.43	30.50	30.14	33.87
Wine - Red	n=11	n=7	n=17	n=22	n=8
Low price	34.73	29.71	30.41	32.55	30.78
High price	124.18	114.14	146.94	130.95	153.50
Mean price	58.24	48.49	53.82	53.82	59.89
Beer - Domestic	n=11	n=7	n=17	n=22	n=7
Low price	5.13	4.69	4.90	4.62	5.06
High price	5.19	4.90	5.62	5.00	5.06
Mean price	5.14	4.78	5.12	4.75	5.06
Beer - Craft	n=11	n=5	n=16	n=19	n=6
Low price	5.70	4.68	5.42	5.55	6.01
High price	6.03	4.82	5.74	5.69	6.28
Mean price	5.86	4.76	5.60	5.59	6.13
Beer - Import	n=11	n=7	n=17	n=22	n=6
Low price	5.47	5.06	5.67	5.29	5.25
High price	6.44	5.68	5.69	5.97	7.04
Mean price	5.80	5.54	5.38	5.51	6.39
Spirits	n=11	n=7	n=17	n=21	n=8
Low price	43.69	50.36	50.76	45.62	42.75
High price	144.09	178.21	150.06	174.86	224.88
Mean price	74.77	80.18	78.36	74.87	81.87
Soft Drinks	n=11	n=7	n=14	n=22	n=8
Low price	2.79	2.83	2.65	2.58	2.60
High price	2.79	2.83	2.57	2.58	2.60
Mean price	2.79	2.83	2.65	2.58	2.60
Water	n=11	n=5	n=14	n=22	n=6
Low price	3.18	2.99	3.02	3.07	3.04
High price	3.18	2.99	3.02	3.07	3.13
Mean price	3.18	2.99	3.02	3.07	3.08

beverage. Many statements can be made about trends, but very little research is done on the subject. By analyzing the quantitative research presented, professionals can understand and implement practices based on these trends. Currently, there is a disconnect between service staff and food and beverage providers. By giving those involved in the food and beverage industry a better idea of what is going on, fan experience can be improved. Clearly many companies see some benefit to outsourcing their food and beverage services, but sport organizations must recognize that this arrangement takes away touch points with the fans.

The fact that an item is listed in the food and beverage menu only reveals what the food and beverage industry believes the customers wants. Due to this limitation, we only know what is being offered, not what is purchased. We also do not know the reasons that certain items are listed and others are not. Conclusions can be drawn from current trends, but the reasoning behind the menu offerings are not listed in the menu. If we explored more items from the menus, it might expand our understanding of regional differences.

It is worthwhile to mention the question of menu length. There are up to 40 pages in a menu for a premium seating area, which is a daunting stack of information for those tasked with preordering food for events. This menu provides ample information about policies, procedures, and menu items. However, many suite administrators have many other responsibilities and end up ordering the same package for the entire year (personal communication, July 23, 2013). This makes the decision easy for the suite administrators but it means that the menu is largely obsolete because people are not reading it or changing their options.

Future Research

The next step is to explore best practices in food and beverage by interviewing food and beverage professionals in the sports and entertainment venues. Questions of interest will be pricing, ordering procedure, and new trends. Additional research of this nature will provide the opportunity to understand the "why" behind the data collected in this study. The more insight into practices around the industry, the more informed decisions industry members can make. This will allow food and beverage to continue to prosper and remain a stable part of annual revenues for sport organizations.

Acknowledgements

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Notation

Data collected though personal communication with vendors and sport organizations from May to June 2013.

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Nontraditional arena foods. Photo courtesy of Danielle D. Kloke, 2013.

The Use of a Compact Elliptical Trainer in an Individual with Chronic Stroke: A Case Study

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Key words: stroke, exercise, elliptical, electromyography, range of motion

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Abstract

Background
Stroke is a leading cause of long-term disability. Following rehabilitation, there are limited opportunites for individuals with stroke to maintain or improve their fitness in the community setting. Primary barriers to community-based exercise include time, cost, transportation and saftey. Recently, a compact-ellipitcal trainer has become comercially available that can be used safely in the home setting. However, the use of this device in individuals with stroke has never been explored.

Purpose
The purpose of this case study was to evaluate the use of the compact elliptical trainer on leg motion, muscle activity and perceived effort.

Methods
One individual with chronic stroke was tested. The subject used the elliptical trainer under six different conditions. During each condition the following was measured: ankle muscle activity using surface electromyography, ankle joint range of motion using 3D analysis, and percieved mental and physical effort using the Task Load Index.

Results
Maximum range of motion values for using the device were -6.8° of dorsiflexion and 37.9° of plantarflexion. Maximum tibialis anterior muscle activation occurred when pedaling backward with a foot strap and verbal encouragement. Maximum muscle activation of the gastrocnemius occurred pedaling backward with no foot strap or verbal encouragement. Percieved effort was highest with pedaling with a foot strap and verbal encouragement.

Conclusion
A compact ellipitcal trainer may be a feasible device for use with individuals with chronic stroke. Clinicians should consider ankle ROM requirements and muscle fatigue when prescribing exercise parameters and patient positioning for use of the trainer.

Background and Purpose
Stroke is one of the leading causes of long-term disability. People recovering from stroke typically participate in a variety of therapies including speech therapy, occupational therapy, and physical therapy. A lifetime of the medical care and rehabilitation can add up to about $140,048.[7] One of the most significant disabilities commonly experienced following a stroke is a loss of the ability to ambulate independently. In a study done by Barer, *et al*, fewer than 50% of the individuals studied had the capabilities of walking.[2] The ability to walk is important for maintaining independence and overall health and physical activity of the individual. As a result, many people with stroke become sedentary and continue to lose fitness.[5] This can become a vicious cycle in which a lack of mobility and fitness reduces the ability of the individual to participate in the necessary activities to improve their fitness, leading to even further losses of fitness.

The most common reasons for which adults with chronic stroke do not get adequate exercise after leaving a formal rehabilitation setting include the cost of the program, a lack of awareness of a fitness center in the area, no means of transportation to a fitness center, no knowledge of how to exercise, and no knowledge of where to exercise.[6] Because the cost of joining a fitness facility and transportation are major barriers for persons with chronic stroke, a safe and cost-effective form of exercise that can be performed in a home setting is an important aspect of any proposed long-term post-stroke care plan. As a result, there is significant need for home-based exercise opportunities that are safe, effective and low cost.[6]

Exercise equipment currently available at home includes treadmills, stationary bikes, and standard elliptical machines. Treadmills assist in the recovery of muscular strength and activity in the lower extremities and help persons with stroke regain the muscle memory of walking.[3] In addition, treadmill use also has the potential to increase a person's walking speed due to the fixed, steady cadence that the equipment promotes.[1] The negative side to such exercise equipment is that they pose a safety risk as some people have trouble walking upright with very little to hold onto. Without a therapist present or a harness system, the risk of falling increases significantly.[3] Stationary bikes are beneficial because they promote muscle firing of the hip/knee muscles that aid in walking.[4] In some cases, however, people with stroke do not have the necessary core strength to hold themselves in the proper position for cycling. As a result, accommodations need to be made to the machine to make it more usable, or a therapist needs to be present to assist. Standard elliptical machines promote continuous motion similar to that of typical walking. The pedals promote greater stability of the lower extremities, and the controlled track of the pedals provide consistent motion on both sides of the body.[3] Like

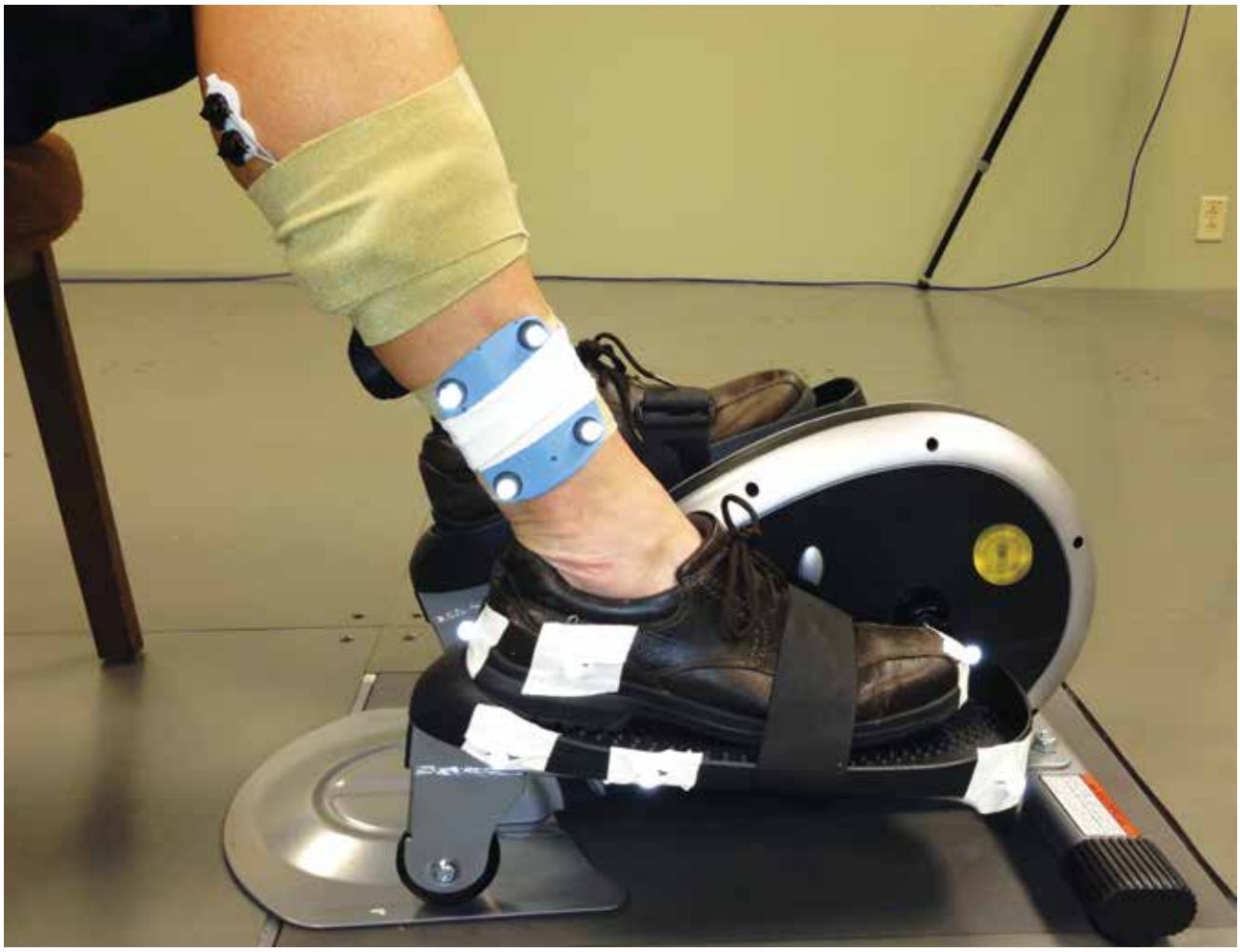

Figure 1. Experimental set-up. Photo courtesy of Mary A. Willarad, 2013.

treadmills and stationary bikes, standard elliptical machines cost anywhere from four hundred to four thousand dollars to purchase. In addition, all of these pieces of exercise equipment take up a significant amount of space in the home.

Recently an affordable compact elliptical trainer (*Figure 1*) has become commercially available through major retailers. Potential benefits of the compact elliptical trainer include the absence of transfers, eliminating a safety risk. A patient can just roll his/her wheelchair up to the device and begin using it from there. The compact elliptical trainer is a cost-effective device that can be utilized in the home setting as a way to initiate some form of physical activity for persons with chronic stroke. The purpose of this case study was to determine the extent to which a compact elliptical trainer influences ankle motion and muscle firing of the lower leg muscles in individuals with chronic stroke. Another purpose of this study is to use the information gathered to determine whether the compact elliptical trainer is a feasible option for home use.

Methods

Subject

A 75 year old male who is 6'2" 214 lb and has suffered chronic stroke since 2005 was tested in this study. Inclusion criteria were: ability to sit in a chair independently and the absence of any other neurological or orthopaedic conditions that would interfere with the ability to use the elliptical. Additionally, participants were required to have a resting systolic blood pressure of less than 180 and a resting diastolic blood pressure of less than 100. Before testing began, the participant was briefed on the activity to be performed and signed an informed consent approved by the University of Dayton Institutional Review Board.

Instrumentation

The Stamina InMotion compact elliptical trainer is a commercially available device that provides a smooth elliptical pedaling motion. In this study, it was only used in seated position. Vicon Nexus cameras and software were used to acquire the data for this study. Joint kinematics was derived using Visual 3D (C-motion, Bethesda, MD, USA) software. A TeleMyo DTS (Noraxon, Scottsdale, AZ, USA) wireless EMG system was used to collect EMG and muscle activity data.

Testing Procedures

The subject recruited for the study came to the University of Dayton for a single testing session. Measurements were taken that include height, weight, blood pressure, and heart rate. Range of motion of the ankle was measured using a standard goniometer during the screening process. Surface EMG electrodes were placed on the skin just off of the motor points of the tibialis anterior and lateral head of the gastrocnemius. To prepare for the motion analysis, reflective markers were placed on the distal foot, proximal heel, lateral heel, top lateral shank, top medial shank, bottom lateral shank, and bottom medial shank (*Figure 1*). The subject was then familiarized with the compact elliptical trainer to ensure that they could perform the basic pedaling motion. Maximal voluntary isometric contractions (MVIC's) of the tibialis anterior and lateral gastrocnemius were recorded for the involved lower extremity with the subject seated with his knee flexed to 90 degrees and his foot stabilized with a strap on the pedal of the compact elliptical trainer that was locked in place. The subject then pedaled the compact elliptical trainer in a seated position with no resistance for 30 seconds under six different conditions: 1) pedal forward 2) pedal backward 3) pedal forward with foot strap, 4) pedal backward with foot strap, 5) pedal forward with foot strap and verbal cue to "push with toes", 6) pedal backward with foot strap and verbal cue to "pull up with toes". After every two conditions, the subjects completed the NASA Task Load Index, and rating scale that evaluated the perceived mental and physical demands of the activity.

Results

Ankle Motion

The kinematic data for each pedaling condition is displayed in *Table 1*. Average maximum plantar flexion and dorsiflexion range of motion (ROM) values for each condition were determined from the last 5 pedaling revolutions. Maximum

Table 1. Ankle range of motion values (degrees) for each condition.

* During this condition the subject used a foot strap and was verbally encouraged to push forward with forefoot forcefully.
** During this condition the subject used a foot strap and was verbally encouraged to pull back with the toes forcefully.

ROM	Forward	Backward	Forward strap	Backward Strap	Forward Verbal*	Backward Verbal**
Plantarflexion	28.5	29.6	36.4	38.1	36.9	37.9
Dorsiflexion	-8.9	-10.1	-6.9	-9.4	-7.2	-7.7
Total ROM	19.4	19.5	29.5	28.6	29.7	30.2

plantar flexion ROM values ranged 28.5° to 38.1° and maximum dorsiflexion ROM values from -6.0° to -10.1°.

Muscle Activation

EMG signals from each trial were processed within MATLAB which included calculating root-mean-square amplitudes of the full-wave-rectified EMG signal and using a low-pass filter (10Hz). *Table 2* displays the normalized muscle activation represented as a percentage of maximum voluntary isometric contraction (MVIC) for each condition. Values for the tibialis anterior ranged from 50% - 133.3% of MVIC while gastrocnemius values ranged from 14.8% - 120.2% of MVIC.

Table 2. Normalized muscle activation as % of maximum voluntary isometric contraction for each condition.

Muscle	Forward	Backward	Forward strap	Backward Strap	Forward Verbal	Backward Verbal
Tibialis Ant.	72.9	102.1	50.0	95.8	50.0	133.3
Gastrocnemius	108.1	120.2	14.8	114.8	78.3	94.5

Perceived Effort

The NASA Task Load Index was used to evaluate perceived level of effort required to use the compact elliptical. This data was collected for the conditions when no foot strap was used, for the conditions when the foot strap was in place and when both a foot strap and verbal encouragement were used. These results can be found in *Table 3*.

Table 3. NASA Task Load Index values.

Task Load Index	Without Strap	With Strap	With Strap and Verbal Cue
Mental	.75	2.5	3
Physical	1.5	3.5	4.5
Temporal	1	1	1.5
Performance	2	1	4
Effort	.5	3	3
Frustration	.5	1.5	1
Composite	6.25	12.5	17

Discussion

Ankle Motion

Following a stroke it is common to develop limitations in range of motion (ROM) in the involved lower extremity, especially ankle dorsiflexion. Limitations in dorsiflexion can cause impairments in gait and functional mobility. One goal of regular exercise for individuals with stroke is to improve or maintain ROM. Our findings showed that when using the elliptical trainer the maximum ankle ROM elicited was -6.9° of dorsiflexion and 38.1° of plantar flexion. This finding raises several clinical considerations and concerns. The elliptical trainer does not provide sufficient dorsiflexion ROM to maintain normal dorsiflexion ROM of 20°. While this may be considered a negative for some patients, those with long term plantarflexion contractures of the ankle may be able to use this device when other devices may require too much dorsiflexion initially. The trainer does provide adequate plantarflexion ROM to maintain near normal values of 40-50°, however our subject had some difficulty initially tolerating this amount of plantar flexion ROM especially during the backward pedaling with strap condition. However, by simply placing the front legs of the elliptical trainer on elevating blocks or an inclined surface the amount of ROM required could easily altered to accommodate most individual patient's needs. Doing this would effectively require more doriflexion and less plantarflexion ROM.

Muscle Activation

Following a stroke it is common for individuals to demonstrate weakness and poor voluntary contraction of distal lower extremity muscles such as the tibialis anterior and gastrocnemius. Inability to lift the foot during the swing phase of gait, often referred to as "drop foot" is common with weakness of the tibialis anterior. Additionally, persons with stroke often demonstrate temporal overlap when contracting agonist and antagonistic muscle such as the tibilias anterior and gastocnemius making it difficult to isolate movement and to move quickly. Ideally, an exercise device such as the compact elliptical trainer would require good isolated activation of lower extremity muscles. The subject demonstrated the greatest activation (133% of MVIC) of the tibialis anterior when pedaling backwards with a foot strap and verbal cues. This result was expected because the foot strap allows the foot to be pulled upward and backward more effectively since it is secured in place and verbal encouragement has been shown to increase force output. The subject demonstrated the greatest activation (120.2% of MVIC) of the gastrocnemius when pedaling backwards without a foot strap or verbal cues. This result was somewhat unexpected as we predicted that pedaling forward with verbal encouragement would show the greatest activation.

Perceived Effort

Perceived level of difficulty is an aspect of any exercise or activity that can impact the likelihood

that a patient demonstrate long-term adherence to the activity Activities that are too easy may not be perceived as having value while exercises that are too difficult will lower the chances of long-term compliance. In this study we used the NASA Task Load Index to measure the perceived effort of using the elliptical trainer. We found that pedaling the trainer without a foot strap demonstrated the lowest composite Task Load Index values (6.25/60) while pedaling with a foot strap and verbal encouragement demonstrated the highest (17/60). The greatest difference between these conditions was in the sub-category of "Physical Performance". This indicates that when using a foot strap and receiving verbal encouragement the subject perceived a greater physical effort. The subject reported a 4.5/10 for this condition which would indicate an appropriate level of effort for most individuals.

Conclusion and Clinical Implications

Based on our findings we feel that a compact elliptical trainer may be a feasible exercise option for individuals with stroke to use in the home setting. However, the following clinical implications should be considered when prescribing use of the device.

- To use the device in a seated position as intended (flat on ground) requires a minimum of -7° of dorsiflexion and 38° of plantar flexion. Therefore, ROM should be measured prior to recommending use or purchase to ensure adequate ankle motion is available. However, ROM requirements could be altered by placing the device on an incline which would increase the amount of dorsiflexion ROM needed and decrease the amount of plantarflexion ROM required.
- Pedaling backward with a foot strap and verbal encouragement generated the greatest amount of muscle activation of the tibialis anterior. Since the elliptical trainer does not include a foot strap this would needed to be added by the individual using the trainer. Pedaling backward without a strap generated the greatest amount of gastroc activity.
- Based on the previous point, if the primary goal for using the elliptical trainer is to increase muscle activity the therapist would likely recommend pedaling backward with or without a strap. However, if severe muscle weakness or fatigue was limiting a patient's ability to initially use the device, they might benefit from starting by pedaling forward with a foot strap which created the least amount of muscular demand. Alternating between forward and backward pedaling could be another strategy to allow a patient to use the device for longer periods of time while avoiding excessive local muscle fatigue.
- This study did not assess the ability of the elliptical trainer to generate a sufficient workload to create a cardiovascular demand that could lead to improved aerobic fitness. This is important area for future study. It is possible that a device such as this may provide a sufficient stimulus for lower fitness individuals but not for those with higher levels of fitness.

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Quasi-Plagiarism vs. Human Universality in the Dystopian Genre

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Abstract

Dystopian literature characteristically addresses the plight of the "everyman" as he copes with the oppression imposed by a totalitarian regime. Touchstone writers of the genre known for novels including *Nineteen Eighty-Four*, *Brave New World* and *Anthem* have, however, been scrutinized for creating uncannily similar plots. While scholars have linked the writers' ideas back to a Russian predecessor, the novel *We*, this research explores how a charge of quasi-plagiarism is a shallow explanation. The great question being considered in any dystopian novel is whether government can save mankind from itself by eradicating individual will. The commonalities among that individual will dictates the appearance of a world without it. It is because of human universals such as love, family, and a desire for knowledge that these dystopian novels focus on the prevention of love through the regulation of sex, communal rearing of children, and thought-level censorship of ideas.

> *Society attacks early, when the individual is helpless. It enslaves him almost before he has tasted freedom. The 'ologies' will tell you how it's done. Theology calls it building a conscience or developing a spirit of selflessness. Psychology calls it the growth of the superego. Considering how long society has been at it, you'd expect a better job. But the campaigns have been badly planned, and the victory has never been secured.*
>
> --B. F. Skinner, *Walden Two*

Wikipedia states on a webpage dedicated to the Russian writer and Bolshevik dissident Yevgeny Zamyatin that dystopian novels including *Nineteen Eighty-Four*, *Brave New World* and *Anthem* were "directly inspired" by Zamyatin's 1921 novel, *We*. Wikipedia, used heartily and guiltily by time-starved knowledge-seekers, accurately represents the consensus among scholars of dystopian literature[1] that extreme similarities throughout the genre are caused by repeated incidences of idea-level plagiarism. The web entry even uses euphemistic language consistent with that wielded by the scholars to dismiss the seriousness and mask the improbability of the charges of plagiarism they are making against the renowned writers George Orwell, Aldous Huxley and Ayn Rand. The fatal flaw with this explanation is that is assumes that a correlation between plot elements of these four novels is grounds for citing plagiarism as causation. An alternative is explored in the present study, suggesting that the options for realistic plotlines are made narrow by the common purpose to persuade that dystopian writers share. A dystopian writer argues that the social contract made when people enter society is a necessary evil and that experimentation in search of an unachievable utopic state will only lead to the suppression of individuals' free will. Dystopian writers employ a form of operant conditioning on their readers, dissuading them from revolution by crafting worst-case-scenario societies. The human universals most strongly associated with evolutionary survival

[1] Proponents of this idea include Jerome Meckier in "Poetry in the Future, the Future of Poetry: Huxley and Orwell on Zamyatin," Zina Gimpelevich in "'We' and 'I' in Zamyatin's We and Rand's Anthem," Michael Beehler in "Yevgeny Zamyatin: The Essential, the Superfluous and Textual Noise," and Peter Saint-Andre in "Zamyatin and Rand"

instincts such as knowledge, sex and family must be endangered in every dystopian novel in order for the readers to be conditioned. The endangerment of these universals must logically develop in the same way in *We*, *Brave New World*, *Anthem* and *Nineteen Eighty-Four* due to the consistency of human nature; it is for this reason, as opposed to widespread plagiarism, that a common dystopian template has emerged.

The concept of a dystopian plagiarism epidemic being taught to the common *Wikipedia*-reader is a tragedy that cannot be fully explained without first detailing how the similarities between the texts are profound enough for controversy. It is best to begin with a synopsis of the precursor, *We*, in order to compare it to the three other novels in question. A skeletal outline is as follows: in the wake of a world-wide revolution, society has been overtaken by a totalitarian regime run by the "Benefactor." The regime, called the "One-State," restricts individual freedoms for the sake of collective harmony. D-503 is the male protagonist and narrator. The book is his secret journal, and in his first entries, he speaks as a perfectly conditioned drone. He eats and sleeps according to the schedules mandated in the "Tablet" and even has sex only after obtaining the proper pink slip. He has no family because children are reared by the state. However, D-503 begins to feel, dream and love after falling into a relationship with a woman who defies the laws and introduces him to an underground revolution. She takes him beyond the "Green Wall" which separates the civilized One-State from the savage outside. However, they are apprehended by the Guardians and lobotomized to treat the soul illness with which they are diagnosed. The novel ends with D-503 indifferently watching his lover being tortured for information about the revolution.

The Englishmen who wrote *Nineteen Eighty-Four* (1949) and *Brave New World* (1932) accused each other of stealing thoughts from *We* (1921), so it is logical to begin with a juxtaposition of these works, leaving Rand's *Anthem* for later. Orwell and Huxley created futuristic worlds that arise from revolution, each creating terminology that parallels Zamyatin's. In *Nineteen Eighty-Four*, Zamyatin's Benefactor is Orwell's "Big Brother," the One-State is "Airstrip One," and the land beyond the Green Wall is the "Golden Country." Additionally, the novel unfolds almost identically with the protagonist, Winston Smith, meeting a girl, Julia, who introduces him to the revolutionaries. Orwell's novel ends with the government discovering the lovers' insurrection and torturing Winston until he no longer cares about Julia but says he truly loves Big Brother. In *Brave New World*, Zamyatin's Benefactor is "the Ford," children are reared by the state to prevent nuclear families from forming as an autonomous entity, and sex is regulated by encouraging orgies that prevent lovers from feeling intimacy. Bernard is the equivalent to Zamyatin's D-503 and Orwell's Winston Smith; each has a physical insecurity and a dangerous awareness of his own humanity that separates him from his fellow citizens.

A review of *We* that Orwell wrote for the *London Tribune* three years before his own book was published is offered by scholars as evidence that he plagiarized elements of his Russian forerunner's novel. In the review, he states, "This is a book to look out for when an English version appears." However, Orwell had been forced to read Zamyatin's book in French because the English version was only available in the United States, so it is possible that he was referring to the appearance of a translated copy in England. Regardless, the review divulges that Orwell was aware of the open market for such a book. Ironically, it was in that same review that Orwell states, "Aldous Huxley's *Brave New World* must be partly derived from [*We*.] Both books deal with the rebellion of the primitive human spirit against a rationalized, mechanized, painless world, and both stories are supposed to take place about six hundred years hence." According to a conversation documented in Zamyatin's biography, Huxley denied having read *We* when speaking to Zamyatin in 1923 (Shane 140). Zamyatin accepted Huxley's alibi, replying "Certain ideas are in the stormy air we breathe" (Lefevre 8). This attitude has been shared by scholars who suggest that the template copied by dystopian writers was actually created by the revolutionary atmosphere of the first half of the twentieth century. It is a suggestion made frequently when discussing Zamyatin and his relationship to Ayn Rand.

Rand was attending Petrograd University in Russia at a time when Zamyatin was at his most influential, and it is her novel, *Anthem* (1938), that shares the most direct similarities with *We* (Saint-Andre 1). As Russian literature scholar Zina Gimpelevich states, "There are too many coincidences in the philosophical approaches to the literature of Zamyatin and Rand to consider them as merely

accidental. Zamyatin's influence on Rand is evident in every chapter of *Anthem*" (13). Rand chose to call her characters by numbers, created a male protagonist who narrated the story via his journal, decided the protagonist would find his individuality through love with a woman, ended with the discovery of a free land beyond the walls of the city, alluded to Prometheus just as Zamyatin, and emphasized the significance of the words 'we' and 'I'. The last line of the novel has the protagonist and his lover discovering a library full of books, and in one of those books, the sacred word – 'EGO' – suggesting that life fulfillment lies in the acknowledgement of the self rather than the whole or the "we" (Rand 105).

There is no evidence proving that Rand read *We*, but it is likely based on a letter she sent her agent in which she writes, "I have watched very carefully all the literature on new Russia that has appeared in English" (Berliner 4).[2] Rand expert Peter Saint-Andre responds to the evidence incriminating Rand in the same way that Zamyatin responded to the charges against Huxley, saying, "These similarities may provide evidence that Rand was aware of and influenced by Zamyatin, or merely that both thinkers breathed the same intellectual air in post-Revolutionary Russia" (3). This represents a second theory that the impetus for these dystopian writers to create similar worst-case-scenario societies was their shared European socialization at a time when the continent was faced with a socialist revolution. This is, however, an oversimplification no better than the plagiarism theory. Even if there were absolute proof that Orwell, Rand and Huxley had read *We* before writing their novels and each declared that they had endeavored to write novels like it, quasi-plagiarism would still be an inadequate explanation for the continuities among them. Here is why: there are multiple other novels in the genre that follow the dystopian template in question. What is more, several of the novels in this vein were written in a time and place where socialism and revolution were no longer eminent, throwing the correctness of the second theory into question. It is imperative to move on to the true origin of the dystopian template as it is defined in this study. However, adequate time must be spent on a brief exposition of the novels that support the given counterargument to the theory that a common revolutionary socialization is the cause of the dystopian template.

In order to determine the grounds by which a novel can be considered similar, it is necessary to broadly summarize those similarities which have been specified via the direct comparisons of the texts. The similarity of the regimes in the novels is one issue. Each regime restricts its citizens through conditioning achieved by the thought-level censorship of ideas, the restriction of love through the regulation of sex, and the communal rearing of children to prevent the formation of autonomous nuclear families. A second issue is the similarity between the protagonists, always the common man (usually male) who becomes a dissident after a reawakening of the soul. Dystopian plots that implement at least two or more of the above mentioned traits include but are not limited to *The Machine Stops* (1909), *Player Piano* (1952), *Fahrenheit 451* (1953), *A Clockwork Orange* (1962), *The Handmaid's Tale* (1985), *The Children of Men* (1992), *The Giver* (1993), *The House of the Scorpion* (2005), *Uglies* (2005) and *The Hunger Games* (2008). The likelihood that this many successful and capable writers were of the mentality to directly copy their predecessors is minute, and because the novels span multiple decades, the suggestion that dystopian writers are alike due to a common renunciation of socialism is also weakened. The true cause of the dystopian template is that the writers need to condition readers for a common purpose. They are all responding directly to a specific idea: the idea that humanity is capable of returning to a utopic state.

The essential difference between a utopia and a dystopia is that utopias glorify the engineering of societies in which individualism is made secondary to communalism, whereas dystopias warn against it. Utopian writers see all of the problems in society as we know it, whereas dystopian writers see problems with the utopian alternative (Richter 4). Robert Baker includes a chart in his book that details the characteristics of "utopian" society in one column and that of "non-utopian" society (as we know it) in another. In the utopian column are traits such as "socialism, world state, limited sexuality, science and urbanization." In the non-utopian column are terms like "emotion, individualism, class hierarchy, capitalism and religion" (Baker 29). The latter represents a world

[2] It is more probable that Rand would have been able to obtain an English copy than Orwell since she moved to the United States in 1926, and *We* was translated into English In the United States in 1924.

that fosters humans' natural predispositions for self-promotion. The former equalizes by taking away human choice through conditioning. It was, in fact, the father of operant conditioning and radical behaviorism, B.F. Skinner, who wrote a utopia entitled *Walden Two*, in which he crafted his ideal society around his theory that behavior could be controlled. In *Walden Two*, "Freedom was not free will, but rather having the requisite repertoires and opportunities for attaining valued outcomes" (Altus and Morris). This is a pattern seen throughout utopic literature, tracing back to Plato's *Republic* in which the communal rearing of children and restriction to sex at only specific times of the year is promoted as idyllic (Plato 149-189).

The aforementioned methods of conditioning and restriction must then be used across dystopian literature because dystopias address the flaws in utopian ideology. Aldous Huxley specifically identified his goal as such. According to Robert Baker, "In a 1962 letter to Christopher Collins, Huxley wrote that Wells' [utopian] *Men Like Gods*, "annoyed me to the point of planning a parody, but when I started writing I found the idea of a negative utopia so interesting that I forgot about Wells and launched into *Brave New World*'" (25). This discredits the idea that *We* was the primary influence for Huxley since he is explicitly stating he was inspired by H.G. Wells. Further support that utopian thought dictates dystopian writing is offered in the article titled "By Underground to Crystal Palace: The Dystopian Eden" in which the author suggests that Yevgeny Zamyatin borrowed his ideas for *We* from an even older antecedent, Dostoevsky's reworking of the Garden of Eden story" (Sicher 1). The Garden of Eden is the ultimate utopia, but Dostoevsky clearly understood the impossibility of returning to such a state in his short story, "The Dream of a Ridiculous Man", as Sicher explains:

> "*In Dostoevsky's scheme of things, the church has been superseded by the city-state which continues to employ the same questionable utopian ends to justify similarly ruthless means. Yet, men and women, born free yet everywhere in chains, would not wish to give up the happiness provided by social order.*" (9)

The phrase "born free yet everywhere in chains" is borrowed from Rousseau's *The Social Contract* which states that man cannot return to the ideal "state of nature" because he has grown accustomed to a life of reliance upon his fellow man. Dostoevsky connects thoughts from *The Social Contract* to the Garden of Eden narrative in order to convey the message that a contractual society in which man can rely on man through government and society is the closest one can come to a utopic state. This is the same message conveyed by dystopian writers. The acceptance of the status quo for the sake of security is explored by Huxley, as stated by Myron, "Huxley's *Brave New World* attempts to examine why there has been so little controversy regarding the Protestant work ethic, which has become the driving force of capitalism, and which has forced human beings to consider idleness the playground or the workshop of the devil" (12). Consistency is safety, the authors seem to be saying. There is, however, even more direct evidence connecting dystopian works with the Garden of Eden narrative. In *We*, an allusion to Eden was deliberately made by Zamyatin when he chose to name the revolution Melphis, short for Mephistopholes, the devil from German folklore associated with knowledge (Zamyatin 133). Realizing that the dystopian plot structure is actually a parody of the Eden narrative provides an explanation for the emphasis on sexuality and predomination of male protagonists with moral dilemmas in dystopias. The question then must become, why are so many authors concerned with addressing the matters of knowledge, sex and family central to the Garden of Eden narrative?

A relatively new field of thought called Biopoetics provides the response that human universals brought about through Darwin's natural selection have molded templates for literary form. Leading researcher in the field Brett Cooke explains, "Since there is no distinction between a gene and its copy, a gene could be said to benefit itself in the body of its host's progeny. As a result, we are prompted to engage in sex and love our children, whether or not we understand why. And we like to read about affairs of the heart" (1). Freedom of thought is a main theme alongside sex and family in the dystopian genre because it is humans' greatest arsenal in the animal world. The acquisition and application of knowledge is the best means by which one is able to continue life. However, these explanations about universality may seem to suggest that the inclinations towards writing about knowledge, sex and family are so strong that every literary genre should have as many similar novels as the dystopian genre. There are two reasons that the dystopian writers are particularly

inclined to accentuate these universal themes: first, the extremities of the human condition are surfaced by worst-case-scenarios, and second, dystopian writers have an acute awareness of the most effective methods of conditioning and persuasion.

The truth of the first reason can be made evident with a brief return to the idea that the works of Orwell, Huxley and Rand were inspired by the revolutions of the first half of the twentieth century. Indeed, history books focusing on this time tend to read more like dystopian literature due to Stalin's dispersion of socialism and Hitler's capitalization on the impressionability of the German people after their country took the blame for WWI. Stalin became the face of socialism, forming a freedom-restricting government in the wake of revolutions that transpired in Russia after the abolition of serfdom and WWI caused an economic crisis (Beehler 32). Under his control, the political opposition was squelched in the Great Purges and atheism was promoted over religion with the clergy often being sent to labor camps or tortured. Censorship prevented the spread of competing ideas, and even travel was not permitted without government approval. All of these tactics speak to the effort to control the socialization of citizens by preventing anything from priming their consciousness that was not in accordance with government ideals. This is pertinent to explore because events in Russia laid out in the same sequence as in the dystopian template being studied here: war leads to revolution that causes a dictatorship which results in the conditioning of the masses. This may seem to support the previous argument that the novels were inspired by political events of their time, but the consistency of this pattern in historical events besides the Bolshevik Revolution suggests that history is just more evidence that specific human universals are stirred by the worst-case-scenario of war and revolution.

As an example to demonstrate this point, a detailed comparison of World War II and *Nineteen Eighty-Four* will show how Orwell's novel aligns even more closely with the specifics of Hitler's regime than Stalin's. Joseph Goebbels was given the title of Minister of Propaganda and National Enlightenment by the Fuhrer. This upbeat-sounding position entailed preventing the people of Germany from receiving any negative messages about the Nazi Party from media, film, education or books. Also, Goebbels was instructed to inundate the German people with messages that hoisted Hitler up as their savior. Looking at *Nineteen Eighty-Four*, the similarities are unmistakable. Isolating the population so that citizens were socialized only by the government's messaging was the method used both in the fiction and out. Technology was utilized in *Nineteen Eighty-Four* so that the government could speak to citizens through their televisions, just as Goebbels set up massive speakers in every public arena and mandated that radios should be made highly inexpensive so that every household would have the means to hear Hitler's speeches. In order to produce any form of art or publication in Nazi Germany, one had to belong to the Nazi Party, which would censor every work that threatened their mission. In *Nineteen Eighty-Four*, art and writing were simply forbidden.

One may explain the similarities between Nazi Germany and Nineteen-Eighty-Four by saying Orwell was inspired to publish his novel in 1949 by the events of WWII which had so recently posed a threat to his homeland of Britain. However, Orwell later said in his essay, "Why I Write," that he based the novel on his fear that socialism would overtake democracy in Britain and that his greatest worry about WWII was that Britain would be forced to become a socialist state in order to unite strongly enough against Hitler (3). He even refers to the form of government in Airstrip One as IngSoc, English Socialism (*Nineteen Eighty-Four* 7). What this demonstrates is that connections may be drawn repeatedly between dystopian works and history or dystopian works and their predecessors, but the connections that can be made extend in too many directions to say that correlation means causation, or in this case, inspiration. The concept of a human universal that makes all humans value the freedom to think freely and spread these thoughts on through sex and family is the only conclusion broad enough to encompass all of the facets of life that adhere to the template seen throughout dystopian literature. However, the second reason that dystopian literature is especially funneled into this pattern has still yet to be explored. Therefore, we now ask, how does dystopian authors' acute awareness of conditioning and persuasion make them inclined to write about human universals?

It was actually Hitler who coined the term for the psychological control necessary to create a totalitarian government: "weltanschauungkrieg." It translates, "worldview warfare" (Evans). The idea is to condition the people of a society to a standard way of thinking and submitting. It was in order to depict this method of control that dystopian writers had to learn how to employ it themselves. Brett Cooke applies the theory of Biopoetics to the writers, stating, "Only…traits (i.e. persons and texts) which win reproductive opportunity and/or our attention will persist and be able to shape the future. All of the works that influence what we now read were able to do what every viable work of art must accomplish: attract readers" (5). In order to attract readers, dystopian writers must focus on those themes which evolution has lead people to value the most: the freedom to gain and wield knowledge to promote the survival of genes within their own bodies and within the bodies of their heirs. Dystopian authors use operant conditioning to teach readers to associate experiments regarding the structure of society with the eminent loss of security. They teach the association between the utopic worlds and the eradication of free will. It may even be said that dystopian authors such as Zamyatin, Orwell, Huxley and Rand demonstrate how the Garden of Eden – where Eve was not free even to eat from a tree representative of knowledge – was the true dystopia. Dystopian authors are able to make convincing arguments that have extreme implications by targeting the human universals identified in this study. It is dangerous, therefore, if common *Wikipedia*-readers do not understand that the dystopian template is used because it is the most effective way of conditioning them, and not because of a genre-wide plagiarism epidemic.

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Deep Woods Illustration by John Cloud, 2013

Airships, Automatons and Amazing Things: Reconstructing the Hero's Journey

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To Summer and a Long Autumn

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Abstract

My thesis examines the concept of the hero's journey, as proposed by Joseph Campbell in his work The Hero with a Thousand Faces, by re-constructing the hero's journey through fiction. In doing so, I hope to produce my own modern day hero's journey. This excerpt introduces the young hero, Darren, and the setting of the novel. It explores the beginning of the cycle, before the hero receives the call to adventure. The excerpt also includes a short story about Nikola, Darren's eventual guide, and the villain Darren must face later in his journey. As a piece of writing, my thesis experiments with style, point-of-view, voice, scene, and dialogue. The entire thesis is divided into scenes, each employing or relying heavily on one of said elements.

Table of Contents

I. The Hero and His World

II. Hero's Guide and the Shadow of the Villain

I.
The Hero and His World

Darren loved the thrill of a good fight, even when he was the one being thrashed to the cold, stone ground.

He leapt to his feet and raised his fists, ignoring the sting in his side when he inhaled. The ring of boys and men cheered him on, slapping him on his bare back with their caps.

His opponent stood in front of him, a stout man with a wide jaw. The man's hairy body glistened in the dying light of the sun that crept its way into the wide alleyway. "Come at me young man," spat his bulky opponent. He raised his large, red hands, grubby from the oil of factory machines.

Darren wasn't beaten just yet. His opponent was tired. The man's labored breath was a songbird's tune to his ears. Darren sprang forward, staying low. The man swung a branch-like arm at Darren's head. Darren ducked, then swayed backward to avoid a left-handed hook. Another punch aimed right to his ribs. Darren dodged, keeping his eyes trained on the man's face, waiting for an opening, any opening. The punch-and-dodge dance continued, leading the two around the entire circle.

Sweat dripped down Darren's face, stinging his eyes and rolling off his chin in large droplets. His muscles screamed, and his lungs felt tight as they clawed for air. He glided away from another swipe, slower than the others. This was his chance. Darren unleashed a fusillade of jabs, striking at the man's stomach and head. One punch landed wrong, right below his opponent's eye. As the man staggered to the side, Darren bounced backward, shaking his pulsing left hand. Damn, that hurt.

Sore, he stumbled to the edge of the ring. A cacophony of cheers, jeers, and toots met him. "Towel," he muttered, extending a shaking arm towards the crowd. Someone threw him a grungy rag, which he wiped across his face and threw back into the mass of grungy faces with no recognition of its provider.

Darren turned towards the tall and burly man. "I say let's finish this, you?" He retook his stance and gave his opponent a little smile and wink.

"Yeah, I'll wipe that bloody smile clean off your face, pretty boy," said the man.

"Is it just me, Roger, or does this kid take the Huntley?" one of the man's friends called. Roger scratched his thick brow and spat, showing a couple missing teeth in his wide mouth.

Darren crept towards the center of the circle, meeting Roger face-to-face. For a second, the noise of the crowd was drowned out and all he could hear was the heavy breathing of him and his opponent. He exhaled, his fists tightened, his shoulders turned, and he swung. Roger absorbed the blow with the broad side of his arm and answered with a couple of jabs. Darren ducked under the first clumsy punch; the second caught him just under his right arm.

He winced under the pain of the punch, but it left Roger's hairy belly open to a response attack. Darren's opponent was vulnerable. Again, Darren let go a series of punches, first striking Roger in the stomach. Then, with all his might, he sent a jab straight for Roger's big nose. Yes! His fist connected and he could feel the satisfying crunch of breaking cartilage. A quick bounce back, Darren raised his left arm to deliver another shot.

"Darren!" A shrill voice, a woman's, exploded through the obstreperous calls of the crowd.

Darren stopped, startled. He turned.

A fist, with knuckles as hard as brick, slammed into the side of Darren's face. He fell to the ground, his head cracking on the stone, his vision cutting black.

He opened his eyes to the same setting sun, low on the horizon beyond the buildings. He blinked once, twice, attempting to orientate himself. He was on the ground where the ring of people once stood. Groaning, he sat up. A heavy throb tore itself through the side of Darren's skull. Feeling the hard smash of the punch again, his hand shot to his cheek.

"Really did you good." It was his friend Wallace standing over him, hands deep in his gray trousers. He looked down at Darren, his green eyes peering behind his horn-rimmed spectacles.

"Fucking smarts," Darren said, keeping one hand on his face.

"If that man didn't already knock you into the ground, I'd do it myself right now," said a girl standing behind him. He knew that voice.

"And if you hadn't distracted me, I wouldn't be on the ground right now," he said, "so thanks a lot, Trixie."

Beatrice walked by him to join Wallace at his side. In her hands she held a small bag. "I got the ice," she said.

"What are you doing still going to these fights, Darren?" asked Wallace, taking the bag of ice from Trixie and tossing it to Darren.

Darren caught the ice with his free hand, "You guys cost me four silver pieces."

"Darren, Master Geoffrey said he didn't want you doing this anymore. It's just stupid," said Wallace.

"Excuse me, the Back Alley Pugilist Club is a fine organization," said Darren, carefully applying the bag of ice to the bad side of his face. The cold ice seared for a moment before his right cheek went numb.

"Back Alley Pugilist Club," Beatrice repeated the phrase as if she was chewing on something bitter and desperately needed to spit it out.

"Some of the finest men in St. Charlesburg," Darren said as he pushed himself slowly onto his feet. His back ached and his legs cried for him to sit back down.

"They're just street rats and dirty factory workers," Beatrice exclaimed.

"I could care less about the factory rats that fight here," said Wallace, cutting Beatrice off before she went on one of her rampages. "However, it's dangerous and Master Geoffrey won't have it."

"Well, if that's the case, I'll tell you what Geoffrey can have! He can take my-" Beatrice flicked the mighty bruise forming on his cheek. Darren exclaimed in pain.

"Your prizefighting days are over. Now grab your shirt and let us move on from here," she said.

Darren held the ice firmly to his face and stared at her for a few seconds. She was tall at sixteen and stood eye-level with him, her blue eyes colder than the ice on his cheek. Darren smiled, "Four silvers you lost me, Trixie."

He ditched the bag of half-melted ice after a couple of minutes and found his shirt and waistcoat lying on the ground nearby. Quickly, he threw on the shirt and tucked it into his trousers, the same gray as Wallace's. He didn't bother buttoning the waistcoat.

"How do I look?" he asked with a lopsided smile, the right side of his face still a bit swollen.

"That man's fist actually helped your complexion," said Beatrice.

"Forgot your tie," said Wallace, he picked up a navy necktie from the ground and tossed it to Darren.

"Thanks," Darren said, tossing the tie around his neck and wrapping it in a quick loop.

Beatrice yanked at the collar of his shirt, drawing his body close to hers. She began to fuss with his tie, "Don't even know how to tie a proper tie. What kind of man are you?"

"One who would rather not wear a tie," he breathed, catching a whiff of Beatrice's scent. It was something like lemongrass and ginger. He smiled, "You smell nice."

"Oh shut up," she said, pulling his knot up tightly, strangling him with his tie. She hit him on the chest as she stepped away from her knot-work, "Let's go."

"Yeah, before Master Geoffrey kills us," added Wallace.

The three left Horseshoe Alley, thus named because it actually looped around and connected with another wide alley way by means on a perpendicular back alley. It housed many pubs and brothels, so its residents were factory workers mostly, along with some of the men wary from long trips at sea or sky.

It was the perfect home to the Back Alley Pugilist Club, a good way for some of the boys and men to let go of some steam and make a few coins on the side. Darren loved Horseshoe. Once you made it past the mumpers burnt out on spice and the rough façade, it wasn't too bad. Wallace and Beatrice obviously didn't think so. They couldn't get away quick enough. Darren picked up his pace, trying to keep up with his friends who booked it west, up to the townhouses Beatrice and her crowd called home.

The smoke stacks peaked over the rest of the buildings, always watching him, Darren thought.
Illustration by Ramona R. Speranza, 2013

St. Charlesburg was built off of the coast. Past the docks and open markets was the smog-filled east side of the city, ruled by the tall and domineering factories. The smoke stacks peaked over the rest of the buildings, always watching him, Darren thought. As they walked west, the buildings, dilapidated and gray, shifted to well-kept lines of new and colorful houses.

Beatrice lived on top of the hill on the far edge of the city, a small neighborhood called Finchley. Her house was wide and white, with a green lawn, something rare even in the company of the other Finchley homes. Its shutters were blue, and the windows on the front of the house were long. The three stopped in front of the short, black-iron gate at the edge of her lawn.

"Thank you for walking me," Beatrice said, tucking a loose strand of her strawberry blond hair behind her right ear.

"Of course," smiled Wallace.

"Take care," Darren said. He gave her a small nod and a wide smile, even though it hurt his bad cheek.

"You're a danger to yourself," Beatrice said with a light smile. She turned to Wallace, "Keep an eye on him, please." With that she opened the gate, walked down the yellow brick path to her front door and into her house. She didn't walk like the other girls from Finchley, head up and every line of the body stiff and proper. No, Beatrice walked with a flare. Not quite like a boy, but something in her step, even in a dress, seemed powerful to Darren. She was a total tom, he thought.

"You know, I think she likes you," Wallace said, shaking his head and smirking.

"That's stupid," Darren replied. He and Beatrice had known each other since they were kids, running around causing havoc throughout Finchley. Darren's voice was composed, but something about Wallace's words twisted his guts and made a fuzzy feeling in the pit of his stomach.

"Well the way you two act sometimes, wouldn't be surprised," Wallace said. He started walking back down the hill towards the city. "We better hurry back, Master Geoffrey will be locking the library doors soon."

Something about the way Wallace spoke bothered Darren. "What if she did fancy me?" he asked.

"Well," Wallace began, "I think it'd be strange, honestly. With her family and your background." Wallace scratched his chin. "But I'm not saying it would be wrong or anything," he added quickly.

That's exactly what he was saying, thought Darren. He knew Wallace didn't approve of how close the two were. The twisted feeling in his gut unraveled and he picked up his step, "Already dark, let's hurry."

Darren, alongside Wallace, was an apprentice at the Astor Library, which sat just outside the industrial ring of St. Charlesburg. The two arrived at the front steps of the library just as city workers began to light the lampposts along the street.

The library was a prodigious building made of white marble. Large, pale pillars held up the pavilion of the building, which cast a large shadow over the three tall wooden doors that acted as the entrances. Darren flew up the long series of steps, passing one of the two lions that flanked the stairway. They were fierce creatures, lying with their paws crossed and their eyes looking forward in calm solitude. They were named Patience and Fortitude, according to Master Geoffrey. Supposedly, those were the two traits apprentices needed if they were going to succeed in their studies, the first of which Darren was always told he lacked. He didn't argue.

Wallace ran up the stairs and tried the door. To his surprise, it silently swung open. Darren issued a sigh of relief. The two slipped in through the door, Darren carefully closing it behind him. The dark room before them was thick with silence. Moonlight streamed through the large glass windows, casting a spectral light over the double staircase. The staircase rose to a balcony with a row of doors, each leading to different sections of the library.

"Think we're clear," whispered Darren, walking by Wallace, patting him on the shoulder.

"You're lucky I went to fetch you," Wallace hissed, "we could have been in trouble."

Darren stepped over to the stairway and tiptoed up the stairs onto the balcony. He leaned on the smooth railing, looking over the entrance and the increasingly flustered Wallace. "See," he said, "Geoffrey didn't even notice we were gone."

"Did he now?" came a low, throaty voice from the darkness beside him.

The hairs on the back of Darren's neck shot up as straight as soldiers. Master Geoffrey crept into the moonlight. He was a short and thin man, his long bony fingers held onto a wooden cane to help with his limp. The light in the room reflected off his clean, bald head. His frown was accentuated by his neatly curled mustache.

The click of his wooden cane was slow as he approached the frozen Darren. He hoped the moonlight didn't catch his bruised and swollen cheek. "I'm sorry, Master Geoffrey," Darren stuttered, ducking his head in a small bow. The man who approached him was like a menacing creature of the night, not taking his dark eyes off of Darren.

The library was a prodigious building made of white marble
Illustration by Ramona R. Speranza, 2013.

Master Geoffrey didn't say a word until he was inches from Darren. "Wallace," the old man's voice boomed, eyes still trained on Darren.

"Yes, sir?" Wallace answered humbly, hurrying to the base of the staircase.

"Go to bed," Master Geoffrey said, "I must have a talk with young Darren here."

"Yes, sir," Wallace said with a nod. He walked up the stairs, shooting glances at Darren. When he passed Darren he gave a little smirk. It screamed, 'I told you so." With another passing nod, Wallace hurried up a side staircase to the dormitory. Master Geoffrey's glance broke from Darren, his thick, gray eyebrows furrowed, making sure Wallace was gone.

"I see you've been fighting again," he said, turning his attention back to Darren.

"Yeah," Darren admitted.

"As a pupil here at this library, that is unacceptable behavior."

"I know, but can I explain, sir?"

"Here in this building, you are given more than many in the city."

"I know, I know."

"Clothes, an education, food, hope for more." Master Geoffrey turned and began walking away from him. Darren had space to breathe without the Head Librarian only inches from him.

"But sir-"

"Darren, ever since your mother brought you here, I've given you everything this establishment has to offer."

"Exactly," Darren practically shouted.

Master Geoffrey stopped, silent. He turned back towards Darren. Finally, Darren thought, he had the man's attention.

"I was given," Darren began again. "I didn't earn it like the others. You knew my mum and that's why I'm here."

The old man's eyes blinked in the darkness of one of the columns of the balcony, "And what difference does that make, boy?"

"Why does it matter if I fight? I'm trash, I'm a fuck-up," Darren said, "and that's all the others will see me as: a fuck-up."

"You're just as good as all the others, even if you're not the same," Master Geoffrey growled.

"I'm just not meant to spend my life among the bookshelves."

"You're barely fifteen, you've got plenty of your life to live, so quit whining."

He was a short and thin man, his long bony fingers held onto a wooden cane to help with his limp.
Illustration by Ramona R. Speranza, 2013

The last word flew through the air, a jab straight at Darren's ego. He wasn't whining. He wasn't a little kid to be talked down to by someone. He wasn't a book that could be picked up and understood. Darren stood in silence, fists clenched in anger.

Master Geoffrey hobbled over to Darren, reached up and clapped a hand on his shoulder, and said, "You're young, trust me, you'll have all the opportunities to travel, fight, and all that excitement later.

But for now, you are under my guidance and I expect you to listen to me from now on. Okay?"

Darren nodded.

"Your mother trusted me with the responsibility of ensuring that you have a fighting chance in that world out there, and I'm not going to fail her," he continued, patting Darren hard on the shoulder.

"Thank you, sir." Darren didn't know what else to say. Every time Master Geoffrey brought up his mother, he felt bad, guilty even.

"Promise me two things."

"Okay."

"No more fighting in those gutters, for one," Master Geoffrey said, raising a skeletal finger, "and that you'll come see me in my office tomorrow morning, after breakfast."

"For what, sir?" Darren asked. The next day was Sunday, and the library was closed. It was a day off for apprentices and librarians alike.

"I have something I want you to do for me." Master Geoffrey smiled crookedly, eyes gleaming.

"What is that?" Darren inquired.

"You'll find out tomorrow. The important thing is I don't want you coming back bloody and bruised."

"I'll try," Darren said, though he was unsure if he meant it or not. The whole conversation left him empty. He had been talked at the entire time and felt as if his voice dropped dead on Geoffrey's wrinkled ears.

"Now, hurry to bed. I expect you in my office early." With that, the Head Librarian turned on his heel and slowly walked off into the shadows and up a stairwell, up to his study.

Darren remained there for a minute, simply feeling the aching twitch of his right cheek. He got off without even a slap on the wrist, but he felt dumb and foolish. What am I doing here? he thought. No answer came to him. None at all. For the first time that night, he was able to feel the aches and twangs of his body from the fistfight. He was tired and wanted nothing more but the pillow on his bed.

It was like this every time he got in trouble. He would argue how he wanted more. More than the dusty bookshelves of the library, more than his studies with the librarians and tutors, more than St. Charlesburg and Finchley. Darren looked up out the window, at the dim glow of the stars in the muggy night sky. Once again, he felt empty. With a final sigh, he turned around and dragged his feet to the stairs, up to the dormitory, down the hall, and reluctantly to his bed.

Lying in bed, he thought about his fight. He thought about Beatrice and Wallace. He thought about the factory workers. He thought about anything but Master Geoffrey. Anything, but Astor Library.

II.
Hero's Guide and the Shadow of the Villain

Nikola Finbar was a bitter and cold man. Everyone in Lagado knew that, and Nikola didn't give a damn. For all he cared, all of the socialites could burn. He was content sitting near the back of the ballroom, but not so far away from the dance floor that he could not watch the guests, or hear the

band. Nikola loved to observe the dancers, and judge the players. He found delight in each misplaced finger, flat note, and missed beat.

Everyone who was anyone had gathered at the White Palace for the Equinox Ball. If it wasn't for the celebration, Nikola would have never guessed autumn was upon them. His fitted suit stuck to his sweaty body and the exorbitant ballroom became nothing more than an over-sized hot box. Nikola couldn't believe anyone wanted to be there. But it was the Equinox Ball, after all.

It was a ball alright. A damn huge party, filled with fakes and whores, Nikola brooded. He tugged at the collar of his shirt. Too damn hot.

"Pleasure you with some champagne, Dr. Finbar?" a waiter with a silver saucer of glasses that sparkled white offered.

"Yes," Nikola said, snatching two and then turning away from the man without further acknowledgement.

In a single gulp, he downed the first glass. He then placed it down on the table in front of him only so that he could take another drag of his cigarette. The other glass followed likewise. He liked to drink when he smoked.

The assemblage of the city's rich and elite made Nikola uneasy. His eyes darted around, looking for another glass of champagne, or someone with a cigarette, his was almost dead. He took a final drag, letting the smoke settle in his lungs for a moment before exhaling and killing the cigarette on the beautiful white table cloth. Someone would be very angry the next morning. Good.

If Nikola had his way, he'd be far away from the Palace, in his workshop, tinkering. He tapped his foot along with the music, trying to calm himself down. One and two and three and four, one and two and three and four, one and - There went that pudgy horn again, dammit!

He had been asked, or rather told, to attend the ball that night. They were orders. And if he learned anything in Lagado, it was that you followed orders. Else you would find yourself missing and wind up being found in the gutters days later.

Nikola observed the room with cold precision. He took in the different pockets of people, all speaking to each other with the felicity of a Sunday brunch. But he noticed small things. The woman, for example, who was subtly touching the Earl of Tannhauser's elbow as she laughed at whatever dry joke he gave: she would be in bed with him before the night was over, and if she was lucky, find herself with some kind of position in Tannhauser by season's end.

The nerves got worse, and the muscles in his stomach tightened. His eyes shot to the source of his nerves, up on one of the pale balconies that overlooked the ballroom. Curtains covered most of the balcony, and the rest was obscured in shadow, but Nikola knew who lurked there, watching the whole scene.

The magician, he thought.

"Does he scare you, Master?" asked Swanwick, who sat at his side. Nikola was pulled from his focus on the balcony, and the boisterous noises of the room flooded into his ears.

"Yes," he admitted, gazing up at the balcony as he got his hands on another glass of champagne from a passing-by servant.

Nikola looked at his creation. Swanwick was beautiful. His dark skin glistened in the light of the chandeliers suspended far above them. Nikola admired the strong lines of Swanwick's body, the large arms, the straight back, the perfectly chiseled jawline. He wanted to do nothing more but raise a hand and caress the cold, lifeless skin, or to lay his head near the prodigious figure's chest, and hear the steady ticking of his inner workings.

Swanwick sat perfectly still, staring forward in mocking attention, his lavender eyes seeming as though they were enjoying the scene before them. He was dressed similarly to most of the men in attendance: black suit, short waistcoat and white bowtie. Nikola had made sure Swanwick's hair, tight wiry locks, was tied back in a short tail. He almost regretted imbuing such ineffable glamor upon such a creature.

"Master, it is almost time," Swanwick said, tilting his head with the perfect curvature of his thick neck.

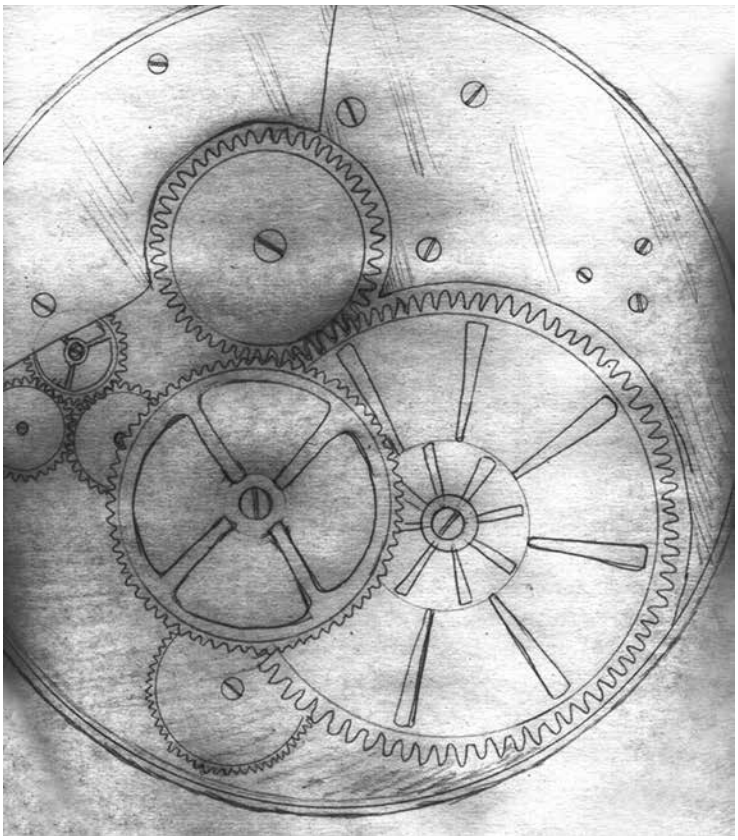
Nikola checked his watch, Swanwick was right. "Well, I suppose there is no use in prolonging the inevitable," he looked back at the shadows on the balcony, "our friend is waiting for us."

"Shall I lead the way, Master?"

"Please."

Swanwick rose from the table. He was at least a head taller than everyone nearby. Nikola let his gaze at the balcony linger for a second longer, before he stood and followed his creation through the crowd.

The magician waited.



He wanted to do nothing more but raise a hand and caress the cold, lifeless skin, or to lay his head near the prodigious figure's chest, and hear the steady ticking of his inner workings.
Illustration by Ramona R. Speranza, 2013

Phosphorus Adsorption Using Local Low-Cost Media

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Abstract

Phosphorus entering water systems is known to cause problems, such as eutrophication. A significant portion of this nutrient comes from agricultural runoff through fertilizer and other materials. Adsorption is a particularly efficient and potentially inexpensive wastewater treatment technique that could be utilized on a large scale to control nonpoint source pollution stemming from agricultural fields. In this study, oak sawdust, gypsum, granular activated carbon, cork, and corn residue were tested for phosphorus adsorption capacities using batch type adsorption tests. In their natural states, oak sawdust, cork, and corn residue showed minimal phosphorus adsorption capacities, either contributing additional phosphates or having a negligible effect on the solution. Gypsum and granular activated carbon both adsorbed phosphorus effectively, but cost, availability, and low kinetic sorption rates make both materials less than ideal. Research indicates several techniques that could increase sorption capacities of the organic materials, and additional research will be conducted, focusing on cheap and efficient ways to create effective adsorbents from the materials.

Introduction

The health of waterways are vital to many aspects of life, from maintaining aquatic biodiversity to providing fresh water for human consumption. Many efforts to achieve and maintain water quality lie in regulating point-source discharges. Nonpoint source pollution remains a major source of pollution in surface water and is extremely difficult to regulate. Large contributors of nonpoint source pollution include large areas of agriculture, surface mining, urban storm runoff, construction activities, forestry; these sources contribute massive amounts of sediment, heavy metals, nutrients including phosphorus (P) and nitrogen (N), pesticides, and salinity (Baker, 1992 and Farrel-Poe, 1995).

Agricultural fertilizer is a major source of nonpoint source pollution, and contributes large amounts of nutrients into water systems (Ryther and Dunstan, 1971 and Sharpley *et al.*, 2003). When entering water in excess, these nutrients promote algal blooms which lead to accelerated eutrophication, the aging of lakes and streams, which can lead to oxygen shortages in the water and adverse effects on water quality (Sharpley *et al.*, 2003). In many areas, either P or N, major components of agricultural fertilizer, are the limiting factors of algal blooms. (Ryther and Dunstan, 1971 and Sharpley *et al.*, 2003). By limiting the amount of phosphates (PO_4^{3-}) contained in agricultural runoff, the amount of algal blooms could potentially be reduced and water quality preserved.

Various wastewater treatment techniques have been tested and analyzed for potential to remove P from agricultural runoff — precipitation from metal salts, utilizing microorganisms, constructing wetlands, creating advanced biologic processes (de-Bashan and Bashan, 2004), crystallization, (Eggers *et al.*, 1991), and adsorption (Mortula *et al.*, 2007). Adsorption is a particularly appealing process, as it can have a low initial cost if materials are gathered locally and inexpensively, can remove pollutants with even very low concentrations, is simple to operate and maintain, and environmentally friendly (Foo and Hameed, 2010). Currently, many adsorption processes use activated carbon, an extremely effective adsorbent, to remove pollutants from both aqueous and gaseous solutions. However, activated carbons can be

prohibitively expensive to produce and utilize in large quantities for nonpoint source pollution, making them impractical for agricultural use (Foo and Hameed, 2009).

More recently, various low-cost materials have been investigated for adsorption capacities of P. These include ferric-impregnated granular ceramics (FGCs) (Chen *et al.*, 2013), modified Aleppo pine adsorbent (Benyoucef and Amrani, 2011), alum sludge, blast furnace slag, other industrial by-products, (Mortula *et al.*, 2007), and solid waste compost ash (Shams *et al.*, 2013). These materials were obtained in their respective local areas at minimal cost, and the adsorptive capacities measured. Waste compost ash and chemically modified Aleppo pine adsorbent displayed rapid kinetic adsorption rates, with most of the adsorption of phosphates occurring within the first 30 minutes of the experiment and maximum adsorbency capacities reached at approximately 40 minutes (Benyoucef and Amrani, 2011 and Shams *et al.*, 2013). FGCs had a high adsorption efficiency (99%) with lower concentrations of PO_4, but efficiency dropped with higher concentrations (>40%). The kinetic period and equilibrium times occurred over a much longer period of time, with the kinetic rates occurring over a period of 15 hours and maximum adsorbency reached after 40 hours (Chen *et al.*, 2013). Blast furnace slag and alum-dried sludge treated through several different processes had between 80 and 98% adsorption efficiencies at lower concentrations of PO_4^{3-}, but data was not recorded in regards to kinetic or equilibrium rates (Mortula *et al.*, 2007).

This study provides a screening of readily available environmentally friendly materials including corn residue, cork, oak sawdust, gypsum, and granular activated carbon to find ideal PO_4^{3-} adsorbents that could be used to reduce P runoff from agricultural pollution. Results of basic P adsorbency tests are discussed and reviewed in this study as well as directions for further research.

Materials and Methods

2.1 Adsorbents Studied

Corn Residue

Corn residue was obtained from a local farm in Spencerville, Ohio. The residue consisted of components of corn that were not used for food or other purposes including corn stalks, leaves, cobs, and other organic matter treated as waste in a corn field. The material was broken down into smaller pieces and sieved to a size of ¼ to ½ inches to prevent smaller particulate matter from affecting data measurements.

Cork

Used tea cork from wine bottles were obtained after a wine festival hosted by Valley Vineyards in Morrow, Ohio. Individual wine corks had been attached to plastic tops and contained a significant amount of red wine residue. Corks were separated from plastic tops and broken into approximately ¼ to ½ inch pieces.

Sawdust

Sawdust was provided from Ogonek Custom Hardwoods in Akron, Ohio. The material primarily consists of chainsaw shavings obtained from a single Red Oak tree that was taken down and used by the company. The material is thin and fibrous, ranging in size from approximately 1/2 inch up to 3 inches long and less than 1/8 inch wide.

Gypsum

A sheet of ½ - inch thick SHEETROCK Brand gypsum board was purchased at a local building supplies store. The board consisted of gypsum material between paper backings to hold it together. The gypsum was broken into smaller pieces ranging in size from ½ to ¾ inches in diameter. While most of the paper was removed while breaking up the material, some paper remained attached to the gypsum pieces used in the tests.

Activated Carbon

Activated carbon is a known adsorbent and used commonly for wastewater treatment. This experiment used Norit Hyrdrocarbo 4000 granular activated carbon (GAC). Because of the known efficiency of GAC as an adsorbent and the expense associated with the material, smaller amounts were used to compare results of a known adsorbent to the local materials tested.

2.2 Analytical Measurements

Analytical techniques were used from Hach DR/890 Colorimeter Procedures Manual, adapted from Standard Methods for the Examination of Water and Wastewater. A Hach DR/890 Colorimeter was used for measurements of total and orthophosphate. Total phosphate was measured using Hach Method 8190, PhosVer3 with Acid Persulfate Digestion at a wavelength

of 610 nm. Reactive phosphate (orthophosphate) was measured using Ascorbic Acid, Hach Method 8048, for lower concentrations, and Hach Method 8114, Molybdovanadate Method, for higher concentrations at a wavelength of 420 nm. Solutions were diluted appropriately, from a 1:1 ratio of solution to reverse osmosis (RO) water up to a 1:40 dilution if concentrations were outside of acceptable ranges.

2.3 Experimental Procedure

Control Testing

Control tests were necessary to establish consistent testing procedures and ensure instruments including vials, filters syringes, and sorbents would not interfere with experimental results. Negative controls were conducted with RO water, and positive controls with dilutions of a standard 50mg/L PO_4^{3-} solution (Fisher Scientific). All instruments and containers contributed or removed negligible P in solution, with less than 0.1 mg/L variance with positive and negative controls. Negative control tests were performed on materials with 2.5g of materials placed with 40mL of RO water in a 50mL Falcon Centrifuge Tube (Fisher Scientific) and reacted for 24 hours in a hybridization oven at 60 Spm and 26°C.

Adsorption Experiments

A stock solution of P was prepared by dissolving 1.4531g of $NaH_2PO_4 \bullet 2H_2O$ in 1.0L of RO water. Appropriate concentration solutions were diluted from the stock solution, then adjusted to a pH of 7.5 with dilute NaOH and HCl. Batch type adsorption experiments were conducted in 50 mL Falcon tubes, with 2.5g of sorbent material placed with 40mL of 45 mg/L PO_4 solution. Because of the known effectiveness of GAC, a 1250mg/L solution was used in the adsorption experiment, using 0.500 g GAC for 40 mL PO_4^{3-} solution. Sacrificial tubes were used, with multiple tubes per material run in parallel due to the relatively small volumes of solution used. PO_4^{3-} concentrations were measured at 0, 60 and 90 minutes for approximate kinetic rates and 3 and 24 hours for maximum adsorbency capacities. PO_4^{3-} was measured at each time interval after being centrifuged for 10 minutes and pipetted through a 0.2- µm filter.

Results and Discussion

3.1 Control Tests

Figure 1 shows the PO_4^{3-} concentration of RO water after 24 hours of contact with each material. GAC and gypsum released no chemicals or materials that registered as PO_4^{3-}, sawdust had small amounts of interference measured (2.4 mg/L) during the negative control test, and cork and corn residue both contributed PO_4^{3-} to RO water with 15.6 mg/L and 352 mg/L released into 40 mL of RO water respectively.

While the sawdust in contact with RO water showed a small amount of PO_4^{3-} in solution, the

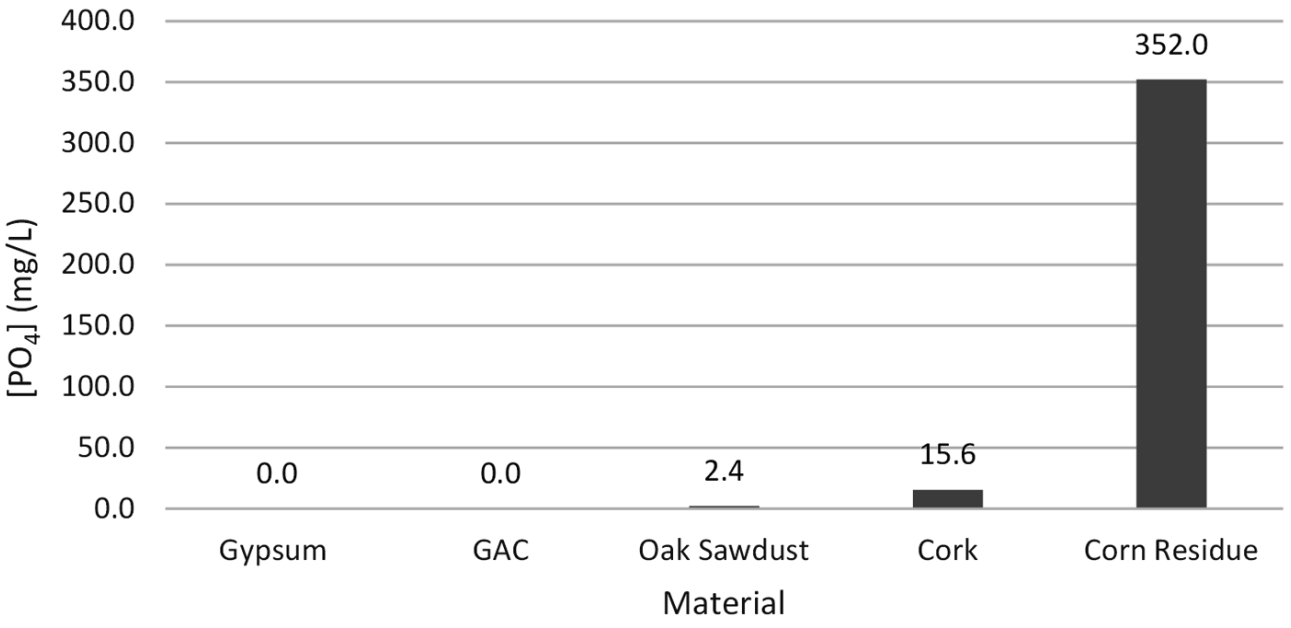

Figure 1. Phosphate concentration in RO water after 24 hours (negative control tests).

concentration is most likely measured from interference in the material. Oak wood contains chemicals, such as oak tannins, that can break down in the presence of water. This was shown very clearly in the Molybdovanadate method to test orthophosphate, where the reagent turned the RO- sawdust solution red and eventually green, while orthophosphate was measured by a yellow color in solution. The ascorbic acid method contained much less interference, but was unable to completely remove the interference from colorimeter tests. As a result RO-sawdust solution was used as a blank in the screening tests involving sawdust.

The PO_4^{3-} desorbed into RO water from cork probably came from the wine residue left on the majority of cork material used. Wine is known to naturally contain phosphates, which most likely adsorbed to the cork and was released when coming into contact with an aqueous solution.

Corn residue displayed the highest amount of PO_4^{3-} in solution when in contact with RO water. The phosphates were most likely absorbed onto or incorporated into the corn while in agricultural fields, and released when in contact with water. Other nutrients, such as nitrates and other fertilizer components, could be present in the corn residue and provide small levels of interference in the PO_4^{3-} tests.

3.2 Sorption Tests

While the negative control tests measured the release of PO_4^{3-} into RO water when in contact with each sorbent, the sorption tests measured the effects of each sorbent in removing PO_4^{3-} from a solution. The concentration of PO_4^{3-} remaining in solution was measured in each material over a period of 24 hours, as shown in *Figure 2*. Cork added some PO_4^{3-} to the PO_4^{3-} solution, raising the overall concentration from 47 mg/L to 60 mg/L over the experiment duration. Corn increased the concentration of PO_4^{3-} significantly, more than doubling the initial concentration over 30 minutes and increasing the concentration more than 5 times (220 mg/L) over the total experiment period. The cork required the use of the ascorbic acid method at dilutions of up to 1:40, and corn residue required dilutions of 1:10 for the molybdovanadate method. These dilutions were a significant source of error in measurements, as precise quantities were needed for such high dilutions and were difficult to measure accurately. Nevertheless, both showed minimal sorption in their natural states.

Oak sawdust was found to neither adsorb nor desorb PO_4^{3-} from the solution. The molybdovanadate method to test concentrations proved unreliable for testing the oak sawdust; as a result, the ascorbic acid method was used to measure PO_4^{3-} which required dilutions of up to 1:20 PO_4^{3-} solution to RO water. The dilutions required for this method provided variations in concentration

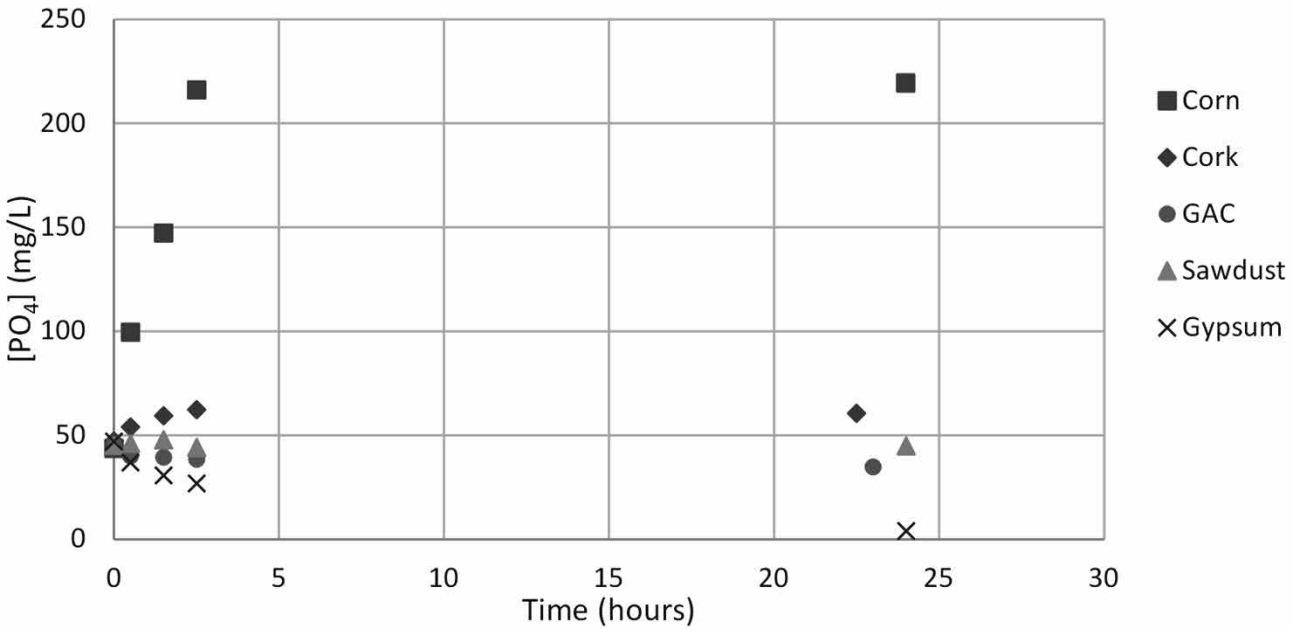

Figure 2. Phosphate concentration in solution over time

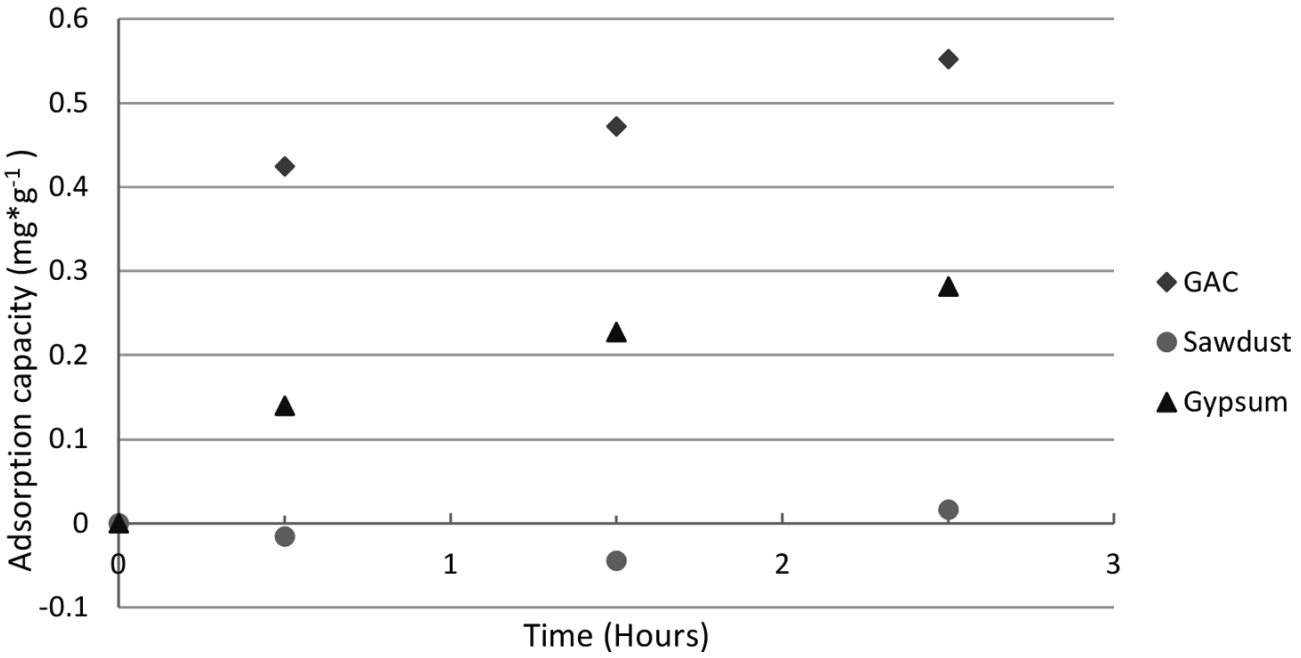

Figure 3. Kinetic P adsorption capacities of materials over time, mg sorbate/ g sorbent

found in the oak screening, varying from 44 to 48 mg/L. The adsorption capacity of the oak sawdust remained very small (*Figure 3*), and varied between -0.05 and 0.03 mg*g-1, small values in comparison to the other materials.

Both GAC and gypsum adsorbed orthophosphate and reduced the total concentration in solution. Gypsum had a rapid kinetic adsorption, adsorbing 35% of total PO_4^{3-} in the first 2.5 hours of the adsorption experiment. After a period of 24 hours, gypsum adsorbed over 90% of total PO_4^{3-} in solution and left only 4 mg/L in solution (*Figure 2*). The amount of GAC used adsorbed less PO_4^{3-} in comparison to gypsum, achieving only 23% efficiency over the experiment duration. The activated carbon had a more rapid kinetic adsorption rate, with the majority of adsorption occurring within the first 30 minutes of the experiment (*Figure 2*).

The capacity of adsorbents is generally measured in mass of sorbate adsorbed over mass of adsorbent used. Comparing the adsorption capacities of GAC and gypsum revealed that the carbon had a much higher per mass capacity to hold PO_4^{3-} (*Figure 3*), with a kinetic adsorption capacity approximately twice what gypsum could hold. Both materials were able to use the molybdovanate method to measure PO_4^{3-}, so the concentrations present required no dilutions and the results were the most accurate of the screening tests.

Conclusion

From the materials tested, only GAC and gypsum showed any significant adsorption capacities without any alteration of the material. GAC was the most efficient adsorbent per mass, but the amount of material needed to effectively utilize GAC in large areas paired with an expensive manufacturing process generally makes it unsuitable for use in agricultural fields for P uptake. Gypsum was an effective sorbent of PO_4^{3-} as well and could potentially be obtained inexpensively from housing projects or construction wastes. However, the environmental effects of gypsum when placed into the ground are not well documented and the kinetic sorption rate was low in comparison to GAC, making gypsum less than ideal for use in agricultural fields.

In general, the untreated organic materials tested either leached or were not able to adsorb any significant amounts of PO_4^{3-}. Unmodified corn residue would be unsuitable for use in agricultural fields, as it would most likely add PO_4^{3-} to agricultural runoff. Cork obtained without coming in contact with wine could adsorb PO_4^{3-} from fertilizer as it was shown to do when used in wine bottles, but the material does not naturally grow in the area and would be difficult to obtain amounts sufficient to utilize for agriculture.

Recent research has shown that simple processes, such as heating compost in an oven to convert

the material to ash or kiln-drying and using simple acids on pine sawdust can greatly increase the adsorption capacities of organic materials (Benyoucef and Amrani, 2011 and Shams *et al*., 2013). Biochar from wood shavings using inexpensive methods has been proven to effectively absorb crude oils and could potentially be used in the adsorption of P (Nguyen and Pignatello, 2013). Further research of selected materials will be directed towards finding simple and inexpensive methods to modify organic materials and enable them to effectively adsorb PO_4^{3-} from agricultural runoff. Faster kinetic adsorption rates will be stressed over equilibrium capacities as runoff will be flowing through material quite rapidly and will require materials to be able to quickly remove PO_4^{3-} from solution to effectively utilize the adsorbent for nonpoint source pollution.

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Effect of State Policy on Prison Population

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Abstract

States often follow trends when enacting sentencing policy. After a trend of get tough on crime policy which placed more and more offenders in prison, many states are turning to justice reinvestment policies, a reversal of these tougher policies. If a state passes a justice reinvestment policy, there is expected to be a decrease in the prison population for the state. Data was collected for every state's sentencing policy between 1979 and 2011, prison population between 1979 and 2011, as well as the crime rate for these years. Using a pooled time series regression test, it can be concluded that there is a negative correlation between justice reinvestment policies and prison population, indicating that the presence of a policy may cause a decrease in prison population. This is significant since the statistics test took into consideration various sentencing policies as well as crime rates, and despite this justice reinvestment still had a relevant impact upon the incarceration rate. Those who influence sentencing policy at the state level have to consider the precarious balance between community safety and the financial burden of prison terms. This research demonstrates the effectiveness of particular sentencing policies, which can help with this decision-making process.

Introduction

There is a relationship between the sentencing policy a state adopts, the crime rate, and the prison population. A particular sentencing policy may directly affect the prison population by increasing or decreasing the number of offenders sentenced or by altering the length of sentence, while the incarceration rate may directly affect the crime rate due to the imprisonment of dangerous offenders. At the same time, the crime rate may be a force which calls for a change in sentencing policy, such as a rising crime rate which may be thought to be lowered by passing more stringent sentencing policy. Although these three variables have many interactions, the concentration of this study is on the relationship between sentencing policy and prison population, specifically how a state's sentencing policy affects the prison population. Controlling for the crime rate helps to ensure that the change seen in the prison population is due to the sentencing policy, rather than an increase or decrease in crime.

The prison population quadrupled between 1977 and 1997 (Chen 1). This trend has drawn attention to the limited prison capacity which places a boundary on the growth of prison populations (Chen 39). Some believe that a very large increase in prison populations is required to achieve a significant decrease in crime (Greenwood *et al*. 25), but prison capacity issues call for state sentencing policies which are able to effectively curb this upward trend in prison population growth while still successfully combatting crime. Better strategies must be put into practice, by enacting sentencing policies which target the most dangerous offenders, rather than those who are generally of minimal risk to the safety of our communities.

Trends of sentencing policy are important when looking at their impact upon prison population. In the early 1970s, indeterminate sentencing was widely used, which "granted judges and parole boards broad sentencing and releasing discretion" (Wicharaya 1). As the rehabilitative goals of this system began to be questioned, there was a virtually nationwide shift to a determinate sentencing system, with the intent to ensure that punishments being given fit the offense and were given without discrimination between offenders, in hopes of achieving deterrence as a result

(Wicharaya 6). It was hoped that this deterrence would equate to crime control, which was "the underlying goal of many who voted for more determinate sentences in state legislatures" (Wicharaya 19).

During this shift to more determinate sentencing structures, a concentration on getting tough on crime began to take form. Tough on crime policies focus on imprisoning more offenders, so an increase in prison population may result. There are several different sentencing policies which fall under the category of "get tough on crime." Mandatory minimums, which were widely adopted in the 1980s (Mauer 50), involve a minimum year requirement which an offender must be sentenced to serve in prison when convicted of a particular offense, often resulting in enhanced sentences for felonies (Lowenthal 62). Three strikes laws, also known as habitual offender laws, call for a compulsory prison term for a third time felony offender (Chen 3). Truth in sentencing generally requires an offender to serve 85 percent of their prison term before being released on good time or through another release mechanism (Chen 16). Along with these policy changes, there was also a scaling back of parole release (Mauer 50), which can be considered a tough on crime policy as it is eliminating incarceration alternatives.

Other than sentencing policies which could be considered tough on crime, there are also policies which are less harsh and should show the effect of a decrease in prison population over time. Presumptive guidelines are guidelines which are set up for particular offenses, but judges may take aggravating or mitigating circumstances into consideration when sentencing an offender (U.S. Legal). Voluntary guidelines give an outline of the length of time which should be served for particular offenses, but judges do not necessarily need to follow them. Through this, judicial discretion is restored to some extent (Wooldredge 258). Alternative sentencing involves the increased use of alternatives to incarceration when sentencing, such as house arrest or intensive supervised probation (Wooldredge 282).

According to Wooldredge, there has been an increase of fifty percent in the incarceration rate despite a drop in the national crime rate as a result of the tough on crime policies of the previous two decades (256-286). These policies may be politically popular due to their appearance of cracking down on crime, yet they are damaging financially as states themselves are often forced to fund the resultant influx of prisoners. Due to the lack of evidence of positive effects from the application of get tough on crime laws, it appears the movement may have peaked and a reversal is necessary (Mauer 50). During the mid-2000s there has been a trend of turning to justice reinvestment policy.

Justice reinvestment was first introduced in 2003 in Connecticut, and is "aimed at reserving prison for serious offenders and making community corrections more effective" (Eisen and James 5). In addition to improving incarceration alternatives, justice reinvestment concentrates on lowering unnecessary spending in prisons (Lyons). It uses "data analysis to reduce prison populations and redirects dollars saved to strategies proven to decrease crime" (Eisen and James 5). States spend about 52 billion dollars a year on corrections alone (Enda 2011), making this an issue which must be addressed. Also, justice reinvestment often includes decriminalization, or the lessening of a previous punishment, of many offenses, particularly drug laws (Wooldredge 259). The intent of justice reinvestment, also known as "smart on crime" (Eisen and James 2) policy, is to reduce the rate of prison growth rather than the number of prisoners; length of stay must also be addressed in order to effectively curb the growing prison population (Austin *et al*. 8).

Research Design

In setting up an analysis of the effect of state sentencing policy on prison population, the independent variable is sentencing policy. Crime rate is a control variable, while state prison population is the dependent variable being studied. If a state passes a justice reinvestment policy, there is expected to be a decrease in the prison population for the state. Using a pooled time series regression test, it will be determined whether the individual sentencing policies have either a positive or negative impact upon the prison population.

The variables were coded first by state then by year, beginning in 1979 and ending with 2011. For sentencing policy, the data was largely obtained from previous compilations of sentencing policy trends, as well as state sites providing their policy enactments. (For a list of the state sentencing policies by year, see *Table*

1.) This data was then coded by year for each state as a dichotomous variable, with a 0 for a year in which a state did not have a particular certain sentencing policy, and a 1 in the year the policy was enacted as well as the following years the policy was present.

Some categories of sentencing policy are very similar in nature, so for the purpose of this study determinate sentencing and mandatory guidelines can be combined under the heading determinate sentencing, as the mandatory sentencing guidelines mirror the goals of determinate sentencing. Both call for more equality and less disparity in sentencing for the same offenses, so as a result they would have the same general effect upon prison population. In addition, the variable truth in sentencing reform was used if a state made a major change to its

Table 1.1. Sentencing Policy Enactment by State Abbreviation
1. If a state enacted a policy earlier than 1979 and it was still in effect at this time, the state will be listed under the 1979 category.

Year	Determinate Sentencing	Presumptive Guidelines	Three Strikes / Habitual Offender	Truth in Sentencing	Truth in Sentencing Reform
1979	AZ, CA, CO, IL, IN, ME, NM	CA, CO, IN, NM, NC	AL		
1980	AK, MN	MN			
1981	CT, NC				
1982	PA				
1983	FL, OH				
1984	WA	WA		WA	
1985				UT	
1986					
1987	MI				
1988					
1989	OR, TN	OR			
1990					WA
1991					
1992				MN	
1993	KS	KS	WA	AZ, KS, TX	
1994			CA, CO, CT, GA, IN, KS, LA, MD, NM, NC, TN, VA, WI	CA, GA, MI, MO, NC, VA, WI	
1995	VA		AR, FL, MT, NV, NJ, ND, PA, SC, UT, VT	AR, CT, FL, IL, LA, ME, MS, MT, NY, ND, OR, SC, TN	KS
1996				AK, DE, IA, OH, PA, SD	FL
1997				NJ	
1998				OK	
1999				NM	
2000					
2001					
2002					
2003					
2004			OH		
2005					AZ
2006			AZ		
2007					
2008					
2009					
2010					
2011					

truth in sentencing policy which was substantial enough to necessitate a second variable. This helps to see the direct effect of the policy since a major change in a different year may indicate a new trend in the dependent variable. Repeal of some mandatory minimums was also an additional variable, used when states repealed important mandatory minimum laws that were previously in place.

For crime rate, the data was obtained from the Federal Bureau of Investigation's Uniform Crime Reports. This data was entered for each state for the range of years, including both

Table 1.2. Sentencing Policy Enactment by State Abbreviation
1. If a state enacted a policy earlier than 1979 and it was still in effect at this time, the state will be listed under the 1979 category.

Year	Mandatory Minimums	Repeal of Some Harsh Mandatory Minimums	Parole Abolishment	Voluntary Guidelines	Alternative Sentencing	Justice Reinvestment
1979	AZ, CT, FL, HI, ID, IA, KS, KY, LA, MD, MA, MI, MS, MO, MT, NE, NV, NJ, NY, ND, OH, OK, OR, SC, SD, TX, VT, WY		CA, CO (reinstated in '85), IL, IN, ME, NM	UT		
1980	AL, AK, GA		MN			
1981	AR, NH, WV		CT (reinstated in '90)	PA		
1982	DE, TN			RI		
1983			FL (reinstated in '05)	MD		
1984			WA	MI (repealed in '00)		
1985				WI (repealed in '96)		
1986						
1987				DE		
1988						
1989			OR			
1990			DE			
1991				VA	AL	
1992				LA		
1993			KS	AZ, AR (repealed in '96)		
1994			AZ, NC			
1995			MS, VA			
1996			OH			
1997				MO		
1998		MI				
1999			WI			
2000					CA	
2001		CT, LA			MT	
2002					HI	
2003			AL			
2004					AZ, IN, MD, MI, MS, OK, PA, VA	CT
2005					AR, CT, IL, MN, MO, ND, OR, TX, UT, WA	
2006				AL	LA	
2007						KS, TX
2008					NJ	AZ, RI, VT, WA
2009						
2010						MI, SC, VA
2011						AR, CA, GA, KY, NE, NC, OH, OK

property crime rate as well as violent crime rate. For prison population, the data was obtained from the Bureau of Justice Statistics' Prisoners Series. This data was entered for each state for the years 1979 to 2011. Both of these variables were coded as interval level variables, using the values found for the particular year. Using the prison population data obtained, the incarceration rate for each state during these years was calculated using the states' population figures obtained from the Uniform Crime Reports. The incarceration rate was used as the dependent variable for the statistics test rather than prison population so that the results would not be skewed toward larger states.

A pooled time series regression test was then used on the complete set of data to determine whether state sentencing policy has an effect upon incarceration rate and whether it is statistically significant. This statistics test is useful due to its ability to compare a large amount of variables over time, since pooling data across both units and time allows for a cause to occur at different times (Stimson 16). A regression was also done for each individual state, looking at the effect of that states' policies upon its incarceration rate during this time period.

Table 2. Pooled Time Series Regression: Incarceration Rate Policy Predictor
* If P value < 0.10
** If P value < 0.05
*** If P value < 0.01

Independent Variable	Coefficient
Determinate Sentencing	9.61e-06
Presumptive Guidelines	-0.0004796
Three Strikes/Habitual Offender Law	0.0004679**
Truth in Sentencing	0.0010862***
Truth in Sentencing Reform	-0.0002435
Mandatory Minimums	0.0007404***
Repeal of Some Harsh Mandatory Minimums	0.0007859
Parole Abolishment	0.0003058
Voluntary Guidelines	0.0005846***
Alternative Sentencing	0.0005447***
Justice Reinvestment	-0.0004583*
Violent Crime Rate	3.12e-06***
Property Crime Rate	-4.92e-07***
Constant	0.0024083***

Results and Discussion

Based upon the results obtained from the statistics test done with all fifty states, it can be concluded that there is a negative correlation between justice reinvestment policies and incarceration rate, indicating that the presence of a policy may cause a decrease in prison population. The statistics test takes into consideration various sentencing policies as well as the possible effect of crime rates upon incarceration rate, so it is notable that the results for justice reinvestment were still statistically significant.

In looking at the statistical results, it can be predicted what correlation a particular policy should have in relation to the prison population. If a policy falls under the get tough on crime trend, or is designed to imprison more offenders, it should have a positive correlation, meaning the presence of a policy may cause an increase in the prison population. The policies which should theoretically have a positive correlation are three strikes/habitual offender law, truth in sentencing, truth in sentencing reform, mandatory minimums, parole abolishment, and determinate sentencing. If a policy falls under the reversal of get tough on crime, specifically the trend of moving toward sentencing alternatives, it should have a negative correlation, meaning the presence of a policy may cause a decrease in the prison population. The policies which are predicted to have a negative correlation are repeal of mandatory minimums, voluntary guidelines, alternative sentencing, presumptive guidelines, and justice reinvestment.

The results of the pooled time series regression demonstrate the impact of the various policies. (For the pooled time series regression performed with all fifty states, see *Table 2*.) Out of 11 sentencing policy variables being studied, seven yielded the predicted trend in relation to prison population when studied within all fifty states. Despite this, only four yielded both the expected effect as well as results which were statistically significant. Three strikes/habitual offender laws, truth in sentencing, and mandatory minimums showed a positive effect upon the prison population, indicating the presence of such a policy may cause an increase in the population. Justice reinvestment was the only sentencing policy which demonstrated an ability to decrease the population while still being statistically significant. This is notable as past policies such as alternative sentencing and voluntary guidelines may have been enacted

in hopes of lowering the prison population, but when looking at all fifty states over time, they do not seem to have done so. Justice reinvestment may be the key to finally reversing the trend of a growing prison population across the United States.

When looking at regression results for individual states, the effect of justice reinvestment upon individual state prison populations can be observed. Of the 18 states which had enacted justice reinvestment policies by 2011, seven demonstrated the projected trend of decreasing prison population with three being statistically significant (California, Kansas, and Michigan), but of those demonstrating the reverse trend only one was statistically significant. This indicates that even on a state by state basis, a justice reinvestment policy can potentially decrease the number of prisoners in the state system.

A few states' results are notable for their consistency with anticipated trends. Michigan had six sentencing policies (determinate sentencing, truth in sentencing, repeal of some harsh mandatory minimums, voluntary guidelines, alternative sentencing, and justice reinvestment) which were able to be tested against the dependent variable. All six policies yielded the expected direction of correlation, and of these four were statistically significant (*Table 3*). Texas had three sentencing policies (truth in sentencing, alternative sentencing, and justice reinvestment) which were looked at in regards to their effect upon prison population. All of these followed the predicted trend, with one being statistically significant (*Table 4*). Washington had five sentencing policies (determinate sentencing, three strikes/habitual offender, truth in sentencing reform, alternative sentencing, and justice reinvestment) which were measured against the dependent variable, and all but alternative sentencing showed the expected correlation. Three of the four which were as predicted were statistically significant (*Table 5*).

Conclusion

This is a pertinent topic of research as states must balance the financial burden of imprisoning criminals with the safety of the community. Policymakers must take into consideration the impact of different sentencing policies upon the prison population since the cost and space in the state prison system is of concern. The results show the detrimental effects of the get tough on crime policies, shown by the majority of these policies which may cause an increase in prison population, as well as the potential for justice reinvestment in solving the problem of prison overcrowding. Justice reinvestment could be a good solution as it still works toward keeping our communities safe by employing alternatives to imprisonment, but at the same time can decrease the number of incarcerated individuals.

There are some limitations to the current study. Different sources were used for different states' sentencing policy information in many cases, and policies are not always classified under broader categories in a consistent manner. This may also cause holes in the data, as some states may not have all of their policies accounted for due to the sources being used. Policies are often amended, so their classification may change as well as the

Table 3. Regression: Incarceration Rate Policy Predictor for Michigan
* If P value < 0.10
** If P value < 0.05
*** If P value < 0.01

Independent Variable	Coefficient
Determinate Sentencing	0.0011291***
Truth in Sentencing	0.0004903*
Repeal of Some Harsh Mandatory Minimums	-0.0000965
Voluntary Guidelines	-0.0000373
Alternative Sentencing	-0.0003427*
Justice Reinvestment	-0.000661***

Table 4. Regression: Incarceration Rate Policy Predictor for Texas
* If P value < 0.10
** If P value < 0.05
*** If P value < 0.01

Independent Variable	Coefficient
Truth in Sentencing	0.0041753***
Alternative Sentencing	-0.0000709
Justice Reinvestment	-0.0003394

Table 5. Regression: Incarceration Rate Policy Predictor for Washington
* If P value < 0.10
** If P value < 0.05
*** If P value < 0.01

Independent Variable	Coefficient
Determinate Sentencing	0.0002025***
Three Strikes/Habitual Offender Law	0.0002449***
Truth in Sentencing Reform	0.0005785***
Alternative Sentencing	0.0001128
Justice Reinvestment	-0.0001565

impact they have over time. Also, some policies have been adopted recently, particularly justice reinvestment, which is important to note as it may take a while to see any sort of effect upon the prison system. Sentencing policies generally target a specific offense, so the policy can only have an effect on the offenders who fall under that category, rather than a broader impact upon the whole sentencing system. This may explain the lack of impact from certain sentencing policies upon the incarceration rate.

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