2014

Novel Conditioning Protocols Focusing on Oxygen Manipulation to Enhance Stem Cell Transplantation

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NOVEL CONDITIONING PROTOCOLS FOCUSING ON OXYGEN MANIPULATION TO ENHANCE STEM CELL TRANSPLANATION

by

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Bachelor of Science
University of South Carolina, 2013

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biomedical Engineering

College of Engineering and Computing

University of South Carolina

2014

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DEDICATION

I dedicate this dissertation work to my family, including my church family, and friends. My parents for their unwavering interest and support through their encouraging words and push. A special thanks to all of my friends for always being there to listen and encourage me through the frustrating moments.

ACKNOWLEDGEMENTS

I wish to acknowledge my committee members, Dr. Melissa Moss and Dr. Ehsan Jabbarzadeh, for giving their time and expertise. A special thanks to Dr. James O. Blanchette, my committee chairman and mentor for all of the hours he expended reflecting, reading and most important keeping his patience throughout the entire process. I would also like to acknowledge Dalton Galloway and April Everatt for proofreading and providing encouragement in times of doubt during this road. I would also like to thank my lab mates Suchit Sahai and Matt Skiles, who both provided me with the valuable skills that I have learned to reach these goals. I would also like to recognize Matt for the help he provided me with problem solving and critical thinking that was required to get through these projects.

ABSTRACT

Musculoskeletal tissue engineering involves the creation of multiple tissue types that interact together to perform a particular function related to motion and maintaining the body's frame. In order to create a fully functional musculoskeletal system, a concrete method for the creation of the different tissue types must first be completed. Of the different tissue components related to the function of a musculoskeletal system, these studies deal with preconditioning circumstances of stem cells that will differentiate into cartilage and bone. These studies also deal with methods for the creation of functional bone and cartilage to be combined for the creation of a musculoskeletal system. The idea of ischemic preconditioning, a solution for the improvement of ischemic cerebral and cardiovascular tissues, was adapted in this study to show that it eases the transition of cells implanted into an injury site to have increased engraftment and survival compared to current methods. It was also determined that HIFs are vital to this increased survival of stem cells in a toxic microenvironment of an injury site. That information translated to the second study dealing with HIF-2's involvement in the onset of hypertrophy. The contributions of this study show that the use of a HIF-2 inhibitor on ADSCs during chondrogenic and hypertrophic differentiation allows for increased survival and as a result increased collagen type II production. Overall, these two studies added information relating to how stem cells could be prepared for the creation of a musculoskeletal system for implantation into defect systems.

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CHAPTER 1

INTRODUCTION

Review of Musculoskeletal Tissue Engineering: Bone and Cartilage Engineering

1.1 MUSCULOSKELETAL TISSUE ENGINEERING

Tissue engineering is known as a science for the design and creation of tissue that can be used to restore impaired organs and systems that have been damaged or lost due to disease or trauma. [1,2] Tissue engineering is a complex process that is composed of many different components, each of different importance.[3] It can be stated that the main components of tissue engineering are cells, scaffolds, biological factors/signals, and preconditioning, through culture conditions or biomechanical loading.[2,4] All of these factors are related to the tissue type being regenerated only by the consideration that stem cells are the optimal cell choice for musculoskeletal tissue formation and the fact that they are all taken into account when performing tissue engineering for a specific tissue. Each component tends to have an optimal choice depending on the tissue being regenerated.[3,5] These components and their relationships are portrayed in figure 1.1. In the current state of tissue engineering, more specifically musculoskeletal tissue engineering, the focus of studies have shifted to tissue function because studies have shown the ability to produce adequate tissue formation, but without the expression of proper properties.

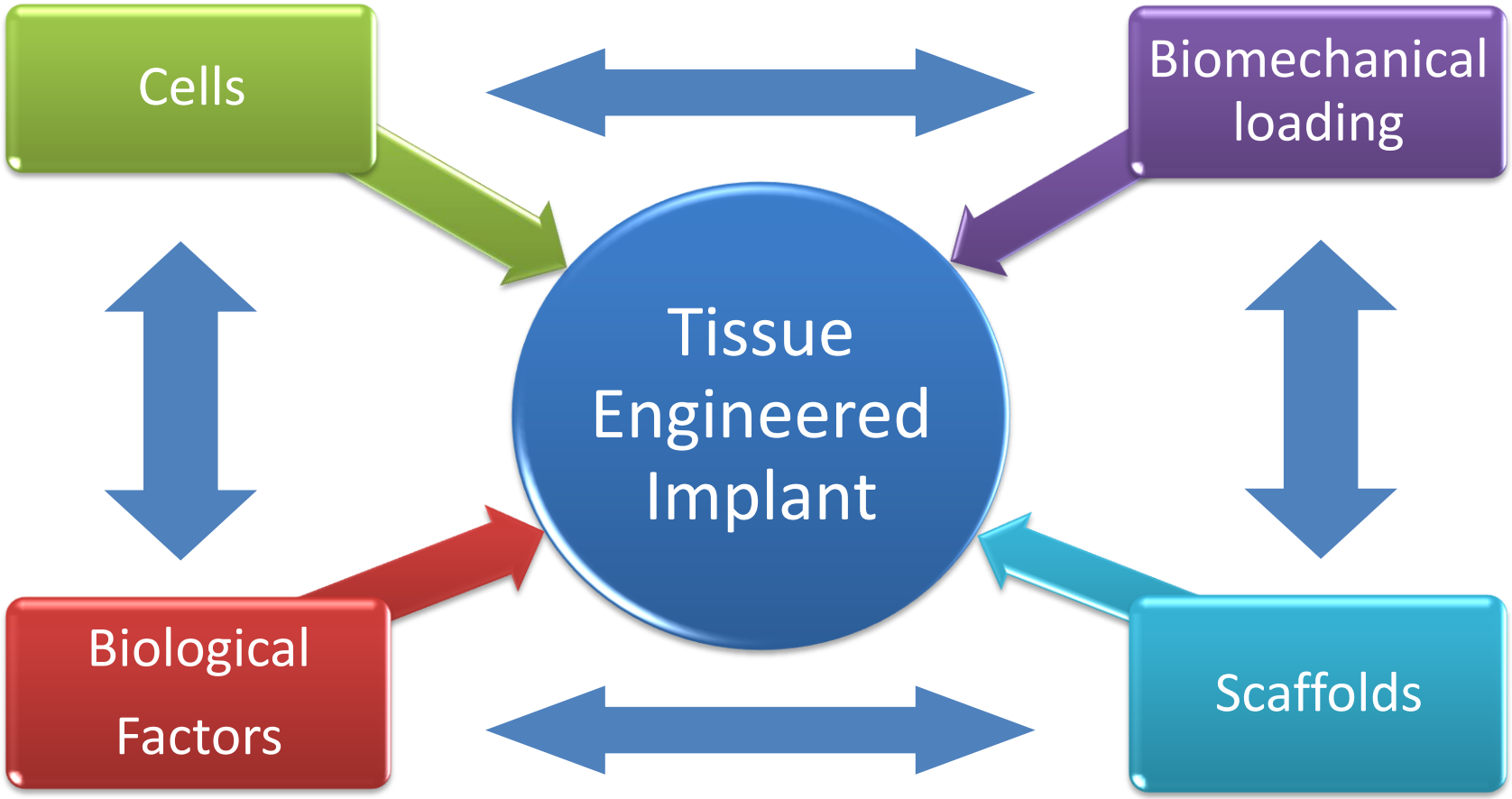

Figure 1.1: Diagram showing the general components encompassing a viable tissue engineered implant and the relationships between them.

Musculoskeletal tissues are interesting in how they integrate and interact with one another to provide a desired function in the body. For instance, cartilage and skeletal muscle both interact with bone to give different functions, cartilage for frictionless movement and support, while the muscle provides the actual power for the desired motion. Areas where the muscle or tendon insert are usually attained through the use of an interface composed of a specialized tissue called fibrocartilage. As seen in this example, engineering of musculoskeletal tissue entails a variety of different tissue types that must integrate with one another to create a fully functional musculoskeletal system. Bone, cartilage, ligaments, muscles and tendons are all different components and tissues that make up a musculoskeletal system. It is interesting to note that bone is a type of tissue which is considered to have one of the highest potentials for regeneration of any tissue in the body, while cartilage on the other hand is one of the most difficult tissues to regenerate in the body.[2] An explanation for this stems from the concept that highly vascularized tissues will have a greater ability for regeneration due to higher availability of nutrients, while tissues of less vascularization will regenerate slower or not at all because of lack of nutrients. This could help to explain the speed at which bone and cartilage regenerate. Bone is a tissue that is highly vascularized from its initial formation and receives nutrients constantly and proper concentrations for regeneration. Cartilage on the other hand, is known to be an avascular tissue, therefore it does not have nutrients as readily available to help its regeneration. Of the tissues that make up a musculoskeletal system, bone and cartilage will be focused on during the rest of this

chapter to create an understanding of these two tissues to support the studies that were completed.

1.2 TISSUE ENGINEERING BONE

Bone is a tissue in the body that has multiple functions in the body, such as providing support, protecting vital organs, and maintaining the body's frame. If there is an issue with the bone due to disease or trauma there is a need to repair the tissue to restore it to its full functionality. Current tissue engineering of bone tissue is aimed at repairing bone problems related to birth defects or critical-size bone defects (CSDs). CSDs are a consequence of a multitude of different causes, such as tumors, trauma, or infection. When bone encounters one of these issues the result is considered a CSD only when the disease or trauma has left the bone in a state where it cannot perform self repair or regeneration. In eyes of current tissue engineers, a practical way of repairing CSDs is through the implantation of scaffolds seeded with cells to accelerate the formation and repair of bone.[6–9] Vasculature is an important physiological factor in tissue, no matter the extent that the tissue is vascularized. At the site of a CSD there is a great loss of vasculature because of the disease or injury. It has been reported that at a CSD site the O_2 concentration can be reduced to levels between 0% and 3%.[10–12] Levels of O_2 in this range are considered to be hypoxic, $[O_2]<5\%$. It is known that hypoxia has a considerable influence on cell behavior because O_2 has multiple affects at different concentrations.[13–15]

The ultimate goal of tissue engineered bone is a vascularized bone in the subject that serves all the functions of native bone. When engineering new bone there are

multiple choices that must be made: *in vivo* or *in vitro*, cell type, vascularization before or after implantation, among many others. Ideally, a completely formed and vascularized bone would be created *in vitro* then implanted and integrated to be fully functional, but that is a difficult goal to reach. It is much more realistic to form the bone *in vivo* where the body would undergo vascularization in the bone as it would normally do for any tissue. When going about the design to engineer bone in the body the cell type is a very vital factor due to limitations on the capabilities of certain types of cells. Stem cells have shown to have promising results in the tissue engineering field, including their use for tissue engineering bone, because of their increased capacity of differentiation into different tissues and other cellular phenotypes. While many studies have employed bone-marrow derived mesenchymal stem cells, adipose-derived mesenchymal stem cells (ADSCs) have shown the possibility of being a better source for tissue engineering because they are easier to harvest.[16–18] It is important to note that through the use of stem cells for tissue engineering, there is a requirement for the cells to differentiate at some point into the cells of the specific desired tissue type. Most literature on osteogenesis states that hypoxia has a very detrimental role in osteogenesis, if not completely inhibiting it, while others have shown the opposite effect on osteogenesis. [19–26] There are various explanations for differing results. These explanations range from cell source, because older subject's stem cells portray reduced phenotypes and differentiation capabilities, to the exposure time or the specific O_2 concentration used.[27]

Current hurdles in tissue engineering of any kind, more prominent in bone, are the engraftment and survival of implanted tissue. The current method for bone tissue engineering studies is culture of the cells at atmospheric conditions with culture or

differentiation medium based on the study being performed. When the desired tissue has formed *in vitro* the cells are transplanted into the animal subject of interest and after some period of time the viability of the implanted tissue is examined. The result of this type of study is typically poor engraftment and low viability. Typical explanations for this relate to the drastic change from the exposure of atmospheric O_2 concentration (20% O_2) during culture to the hypoxic O_2 levels of the injured tissue which is typically around 1% O_2 or less. This drastic change results ultimately numerous effects such as inflammatory response and ischemia-reperfusion (IR), but the main cause of low survival and engraftment is apoptosis.[28–34] As a result of this effect, there is a need for the formulation of approaches to improve cell survival to help increase engraftment and capabilities for their differentiation.

One such strategy has been proposed that would result in numerous responses including activation of different survival signaling pathways for protection in the toxic environment of an injury. Ischemic preconditioning (IPC) is the proposed method and has been studied for transplanting cells into different tissues such as infracted myocardium and ischemic brain and limb tissue.[35–48] IPC has shown a cytoprotective effect of stem cells through the upregulation of VEGF, anti-apoptotic proteins Bcl-2, Bcl-xl, and erythropoietin (EPO), which are all considered to be survival proteins.[24,40,48–50] While those proteins are considered anti-apoptotic, there are also proteins that have an agonistic effect on the apoptotic pathway called pro-apoptotic proteins. Some of the proteins viewed more frequently in literature are active caspase-3and more importantly the pro-apoptotic protein involved with Bcl-2 called Bax. The analysis of pro-apoptotic proteins has been used as a way to determine how much apoptosis occurs. Therefore, Bax

levels are used to determine conditional effects on survival. As previously stated, it has been well studied that hypoxia has adverse effects on osteogenic capabilities.[19,21,25,51] An injury site is a hypoxic environment, therefore no matter what type of implant is created, it will have to be implanted into a hypoxic environment.[10,11,52] An important question posed through this statement is prior to transplantation whether stem cells should be cultured under atmospheric or ischemic conditions similar to those experienced at the CSD site.

1.3 TISSUE ENGINEERING CARTILAGE

Cartilage serves different purposes related to its location in the body and the type of cartilage it is composed of. Cartilage serves to create smooth surfaces for frictionless motion to reduce damage, provide support and rigidity, and also to define and maintain shape. Currently 70% of Americans over the age of 65, approximately 12.4 million, are affected by osteoarthritis (OA), a cartilage disease that changes the ability of cartilage to complete the roles that it serves stated above.[53,54] Looking to the future, the baby boomer population is approaching this age and that means a dramatic increase in the number of joint injury cases that will need to be repaired.[55]

The three types of cartilage are elastic, fibrocartilage, and hyaline cartilage. Articular cartilage, found in thin layers between bones, creates a relatively frictionless surface for the ends of the two long bones to move ensuring no damage occurs. Synovial fluid present in the joints also facilitates this process. Another important purpose of the synovial fluid is to provide chondrocytes with nutrients, even though it is a limited reservoir. Chondrocytes are the resident cell type of cartilage, but they are so sparsely

found in the tissue that cartilage is considered to be acellular. Along that same note, cartilage is also considered to be avascular because it lacks a true blood supply which feeds the chondrocytes. As a result cartilage tissue shows a low capacity for quick repair, if repair at all.[56] Another result of a limited nutrient supply that does not come directly from the blood, the physiological oxygen concentration found in cartilage is significantly lower than found in other tissues of the body. Physiological O_2 concentrations range from approximately 1-7%. Cartilage is found on the lower end of that range around 1% or 2%, an environment considered to be hypoxic.

Majority of cartilage tissue engineering studies focuses on cartilage defects of the articular layer between bones, i.e. dealing with hyaline cartilage.[55] Current attempts to engineering articular cartilage have met numerous drawbacks. One result of tissue engineered cartilage is a tissue expressing markers of fibrocartilage. Fibrocartilage also shows up as a repair mechanism for injured cartilage, but it has been shown that the mechanical properties, load-bearing capabilities and ability to handle stress, of this cartilage is not sufficient to be used to replace damaged hyaline cartilage. This becomes a problem in development of engineered cartilage because it results in the development of larger problems that could form chronic issues if not properly repaired.[4,53,56–58] As far as marker expression, the main difference between hyaline cartilage and fibrocartilage is that fibrocartilage has collagen type I with collagen type II, whereas hyaline cartilage only expresses collagen type II. This explains why there is a reduction in mechanical properties of fibrocartilage compared to hyaline. Chondrocytes in any type of cartilage are responsible for production and maintenance of the ECM. The composition and amount of each ECM components depends on the cartilage type and site in the body.

ECM of hyaline cartilage usually consists of collagen, proteoglycans, and other matrix proteins. Collagen type II is the prominent type of collagen in hyaline cartilage for its shock absorbing capabilities and it makes up a good portion of the ECM along with proteoglycans, which have glycosaminoglycans (GAGs) attached.[59]

Current treatment options for cartilage defects are limited, but also not complete repairs of the defect in the current state of cartilage repair. There are a number of possible options that can be explored to repair cartilage damage. Some of these treatment strategies are autologous chondrocyte implantation (ACI), fairly well known; mosaicplasty, and microfracture. The success of these treatments has been shown to vary, but on a long-term scale they are considered to be unsatisfactory because they do not fully solve the problem or they just delay the further onset of the disease.[60–65] The current issue with treatment for repairing damaged cartilage are the components of native cartilage and the ability to maintain sufficient mechanical capabilities that have been lost in regenerated tissue.[66] Therefore, the new cartilage is insufficient and will undergo degradation as a result of the high loads and stress encountered. ACI is more frequently used for cartilage repair, but this treatment does have some flaws that could be solved by a procedure for creating of a functional cartilage implant that can maintain its properties. ACI hurdles one of the largest debates in these types of surgeries, cell source. The reason cell source is very important here is the same reason seen in any other surgery which uses an implant. The implant must be biocompatible and not illicit an immune response. In ACI, cells are harvested from a non-load-bearing portion of the cartilage and then expanded *ex vivo* until they have reached a volume large enough for re-implantation. By using the patient's own cells they are able to disregard the issues of immuno-rejection one

would possibly face with implanting primary chondrocytes or stem cells from another source. Although they do not trigger an immune response, they include an extra surgery in order to harvest the cartilage from the patient and then a final surgery to implant them into the patient. This increases the expense of the procedure and if a method for the creation of viable cartilage implants were created it would have the possibility of reducing the cost of the procedure, let alone result in a reduction in the number of times the patient would have to go under the knife. There have been multiple comparative studies of the different defect treatments which have yielded good comparisons. ACI was found to be more beneficial than mosaicplasty when examined after one year via arthroscopy. Two year post-surgery assessment yielded little difference between the two treatments based on a scoring system used for assessment. Also at two years after the surgery, the tissue composition found in the ACI treated cartilage resulted in mainly fibrocartilage or a mix of fibrocartilage with hyaline cartilage. Mosaicplasty on the other hand, showed qualities similar to that of the defective tissue found prior to surgery.[65,67–69] As multiple studies have been performed to understand the true effectiveness of the surgeries it has been shown that ACI has the possibility of having both satisfactory and unsatisfactory results, but one thing is known, that the issue is not being completely fixed. The biggest factor seen in this range is the different complications or conditions of the trial subjects. If a patient has already had surgery they were not used. Table 1.1 shows a comparison of some important observations of the three different cartilage defect treatment.

Table 1.1: Comparison of the treatment solutions for cartilage defects.

	Surgery Type		
	ACI	Mosaicplasty	Microfracture
Regenerated Tissue Composition	Fibrocartilage and Hyaline mix	Similar to original defective tissue	Not determined
Further Complications/Surgeries	Yes	Yes	Yes
Post-Surgery Function Rating (Cincinnati Method)	88%	69%	Not determined
One Year Post-Surgery Arthroscopy	82% good repairs	34%	Not determined
Cost	~$25,000	~18,000	>$7,000

Although ACI is the most expensive surgery, it has been remarked that ACI is most cost-beneficial of the three surgeries. One drawback with ACI, and possibly the other treatments, is that the possibility for further surgeries down the road to shave off hypertrophic cartilage around the defect area is a greater possibility. It is also interesting to note that these treatments tend to not be performed on defect areas showing signs of OA.[68] This makes the need for a suitable tissue engineered cartilage implant even more important because it would have the ability to solve the defects currently treated with ACI, mosaicplasty, or microfracture, while also treating those that are unable to be repaired through those surgeries; i.e. osteoarthritic tissue.

Currently, the field lacks a set protocol for creating cartilage that has been accepted or has FDA approval. There are multiple factors which go into producing an implantable tissue construct, beyond the ideologies that the implant must be biocompatible, preserve function, have a stable composition, and maintain itself while integrating with surrounding tissue. Four general factors which make an implant perform and satisfy the requirements are portrayed in figure 1.1 and are as follows: biological

factors, cells, biomechanical forces and scaffolds. Biological factors are chemicals, any compound that has an effect on cellular behavior, whether their production is induced or they are supplemental. Typically for tissue engineering biological factors either inhibit tissue from forming a nonfunctional phenotype or are used to drive a cell toward the desired phenotype. Biological factors can also be used to simulate a physiological environment *in vitro* which is unattainable because it is not produced by the tissue. For example, hypoxia-inducible factor 1α (HIF-1α) is a direct regulator of vascular endothelial growth factor (VEGF), if HIF-1α were knocked-out or inhibited to stop specifically the production of another gene, there would be a need for a VEGF supplement to maintain the physiological levels. The next component of cartilage tissue engineering, scaffolds, is a constituent which has a lot of variability based on its composition. The designer has the option to pick a variety of different materials to create the scaffold which will be seeded with cells to be implanted into the injury site. Ideally this material will be biocompatible at least if not also biodegradable, which does reduce the overall variety of choices to some extent. Physiologically, cartilage in the human knee undergoes thousands of loading/unloading cycles per day. These are biomechanical forces which can be utilized during the creation of the cartilage implant to prepare it for the defect site. Therefore, biomechanical preconditioning will prepare the construct to withstand the same types of forces it will encounter at the injured joint. Biomechanical forces are also responsible for regulating gene expression which can be advantageous if they are desired. For example, studies have shown that specific mechanical stimuli will cause a down regulation of proteins associated with the chondrocyte phenotype, i.e. collagen II and Sox9.[70] Biomechanical force studies could show that forces supplied in

different forms leads to production of specific genes for survival, such as the anti-apoptotic factors, or the production of the proper extracellular matrix, ECM, components, like collagen type II, that would give the engineered tissues properties visible in native tissue. The final components for creating a cartilage implant are the cells used for its creation. There are a number of sub-components determining what this category is. The cell type must first be determined so that the source of these cells can then be decided. For example, if the cell type used is primary chondrocytes, which have a higher immune response if used between different subjects, then the ideal choice of source would be from the person in whom the engineered construct will be implanted. Selecting stem cells opens up a larger box because then the type of stem cell is also to be questioned, whether it could be mesenchymal stem cells, induced pluripotent stem cells (iPS), or embryonic stem cells. Taking into consideration how all of these components interact is important because they affect each other. More details on specifics of cell types will follow later.

In a way, there is a current tissue engineering method for cartilage repair used to treat defects. ACI can be viewed as such because cells are harvested from the patient and expanded to a larger population before being implanted at the defect site. Although, no other alteration is done to harvest cells they are expanded for re-implantation. This is also known as an *ex vivo* process. There have been other attempts to solve the unsatisfying results, but have run into a few issues which have restricted advances in the field. The biggest disadvantage is the onset of hypertrophy in cells of *in vitro*, *in vivo*, or *ex vivo* studies, which seems to be inevitable through the current culture systems.[4] Another issue is visible during the expansion phase of the *ex vivo* repair system (ACI), where the cells

tend to dedifferentiate. The cells will undergo re-differentiation during integration at the defect site, or as part of the progression towards physiological cartilage in experimental studies. This re-differentiation tends to produce cells which portray a phenotype that is deficient in the mechanical properties of cartilage compared to the target tissue deeming it unusable. The problem with the re-differentiated tissue is that it secretes a matrix shown closer in relation to either that of fibrocartilage or of hypertrophic cartilage. Hypertrophic cartilage is characterized by the swelling of the cells, commonly seen in the development and formation of long bones in a process called endochondral ossification, and the production of collagen type X, alkaline phosphatase (ALP), or other secreted bone factors.[71–81] This supports why ACI tissue is composed of fibrocartilage-like components or possibly some hypertrophic cartilage. Therefore, it is a simple statement to say that the optimal cartilage implant would be one that encompasses all of the properties of the physiological cartilage; its load-bearing capabilities, ability to maintain homeostasis, and exhibits the same ECM components, while integrating into the healthy tissue surrounding the defect area. This has proved to be a much harder task as there are many variables that make up this healthy tissue.

Chondrocyte differentiation and culture is a well-studied topic, but there is still more that must be studied to determine a concrete method, to maintain the chondrocytes for implantation and integration at the defect site, that will be more successful compared to the current cartilage repair strategies. When creating anything there is a step which must be done before starting anything else and that is a very thorough analysis of the topic of creation. This analysis, in the case of cartilage, would start with a characterization of chondrocytes and their differentiation process along with a

characterization of what is not desired, hypertrophy. The current status of research on chondrocytes has yielded results revealing a lot about the cell type, but as all knowledge goes, discovery of one thing just leads to a new question. It has been well documented that Sox9 is important in chondrogenesis and chondrocytes, so important that many refer to this gene as the master regulator gene for chondrogenesis. It has been shown that this gene is required for early chondrogenesis, but further studies are required to determine what else it contributes to in order to truly understand it.[82] It is also interesting to note that Dy and colleagues also showed direct contribution with Mef2 in the activation of collagen X, the main collagen produced in hypertrophic chondrocytes. They also saw the need for growth plate chondrocytes to have Sox9, which could mean Sox9 also have some contribution to hypertrophy, but more studies are needed to clear up this unanswered question. A study performed on maturation and terminal differentiation of chick chondrocytes showed that as time passed, HIF-2α levels were elevated above normal levels. This led to speculation that HIF-2 has a very crucial role in chondrocyte maturation and possibly in their terminal differentiation.[73,83] Other known proteins produced by chondrocytes are aggrecan, GAGs, collagen II, among others. As far as chondrocytes go, studies differ in results implicating HIFs as regulating chondrogenesis or hypertrophy; therefore, more in depth studies are required to truly determine their specific roles in each process to move the field forward.

Understanding different roles that hypertrophy plays in the body can also help draw conclusions to be used in formulating a method for engineering cartilage. Hypertrophy has a crucial role in limb development; as long bones grow, cartilage undergoes hypertrophy to make room for osteocytes to invade and create bone. Multiple

studies have examined gene levels during this process and HIF-2α levels are increased in the epiphyseal growth plate where endochondral ossification takes place.[84] That study implicated HIF-2 as a regulator of endochondral ossification, but stating more specifically its role in hypertrophic induction of chondrocytes in that process. Hypertrophic chondrocytes have significant phenotypic changes along with morphological changes that accompany them. As the onset of hypertrophy occurs, chondrocytes began to greatly increase in size. The purpose of this change is to create a space that the osteocytes can invade and create bone during endochondral ossification. Phenotypically, there is a shift from the normally factors produced by chondrocytes to factors that are not functional in this type of cartilage. The factors produced when chondrocytes shift to hypertrophy are VEGF, collagen X, degrading proteins, such as matrix metalloproteinases (MMPs), and different osteogenic related proteins, including alkaline phosphatase (ALP), osteonectin, and osteocalcin, which are used in the creation and maintenance of bone. VEGF is factor produced by cells responsible for the recruitment of blood vessels to newly formed bone from pre-existing vessels. Collagen X is a protein produced by hypertrophic chondrocytes and is a main component in the ECM that replaces the main ECM component of healthy cartilage, collagen II. MMPs are responsible for the degradation of ECM to allow creation of ECM with new components or to remove damaged components. ALP is a factor produced by hypertrophic chondrocytes and bone tissue and is involved with calcification. ALP and collagen X have been studied and used to identify hypertrophic phenotypes in current literature. Figure 1.2 shows the different proteins produced as chondrocytes move toward a terminally differentiating state.

As previously stated, the issue with cartilage tissue engineering currently is the frequent onset of hypertrophy in engineered cartilage. The important conclusion to make in understanding how to fix this process is that with the onset of hypertrophy comes new protein production. As a result the tissue cannot survive in the joint due to poor mechanical properties. More studies are required to determine how oxygen relates to the progression of hypertrophy. Once it is clearly understood there will be much clearer ways to solve the current problems seen with the creation of cartilage. Using conclusions drawn from other studies that say HIF-2 is involved in the onset of hypertrophy, a study to determine the effects on viability of ADSCs differentiated towards a chondrogenic phenotype was formulated. It was hypothesized that HIF-2 inhibition during and after chondrogenesis would result in a phenotype representative of native, functional cartilage opposed to the hypertrophic phenotype seen in *in vitro* studies.

1.4 HYPOXIA INDUCIBLE FACTOR

O_2 is a very important regulator of the group of vital transcription factors called hypoxia-inducible factors (HIFs). As stated in the name, these factors become active in states of reduced O_2. HIFs are regulated through the fluctuations of O_2 encountered between physiological tissue and injured tissue. When hypoxia is encountered, there is a reduction in the amount of available oxygen molecules present in and around the cell. As a result, the α-subunits do not undergo proteosomal degradation via prolyl hydroxylation and dimerize with the ever-present β-subunits. Upon creation of the heterodimer, binding

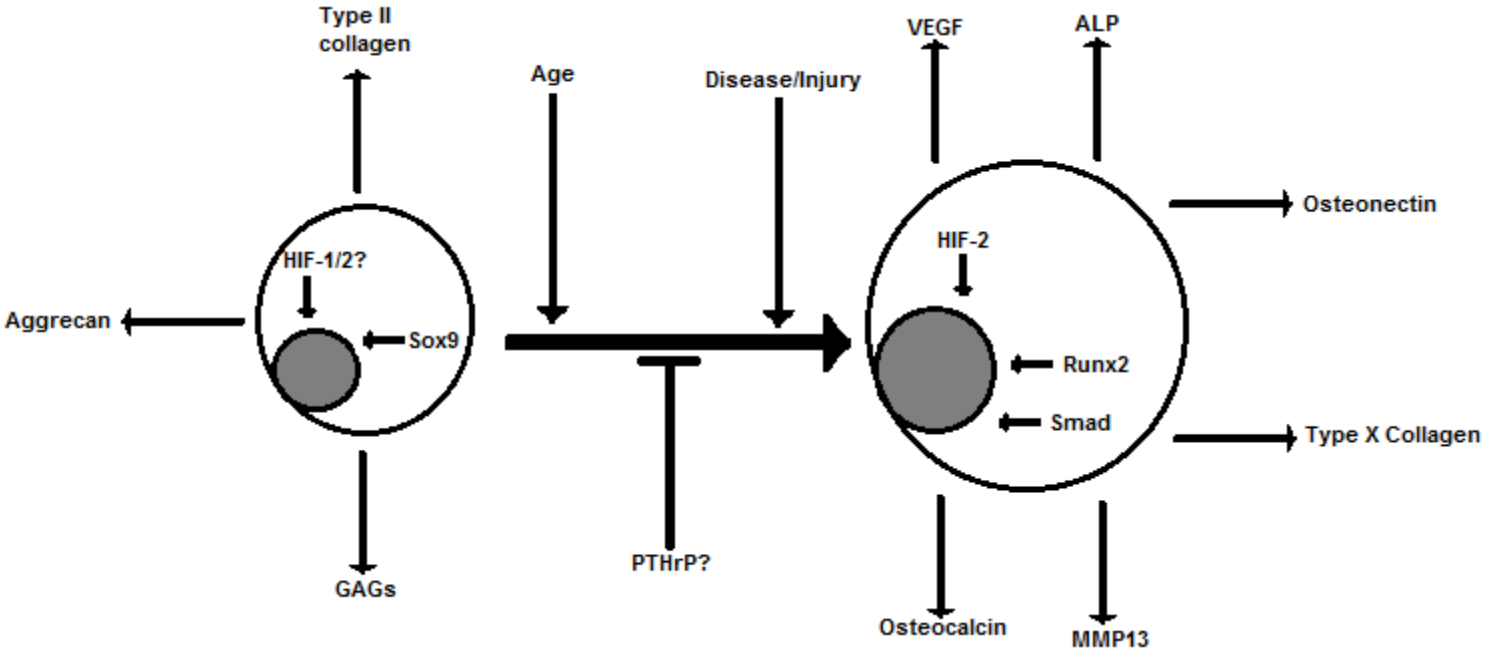

Figure 1.2: Schematic showing different transcriptional and cases of the process of chondrocytes moving to a hypertrophic phenotype. Also showing the main components produced by each phenotype.

to the hypoxia response elements (HRE) of target genes allows direct regulation of gene activating pathways to allow the cell to survive in the toxic and stressful environment created by the reduced oxygen concentration.[85–87] Figure 1.3 shows the process of transcriptional regulation under hypoxic conditions. The name hypoxia-inducible factor is misleading by producing the thought that the factors stabilization only occurs under hypoxia. Studies have also shown that HIFs stabilize under chemical regulation that blocks the prolyl hydroxylation of the α-subunits and also under hyperoxic conditions, oxygen concentrations higher than normal.[88]

HIFs have been implicated as potent regulators of a number of different pathways that are involved in different tissues. Bone and cartilage are both different tissues that are affected through the pathways set off by the HIF family both directly and indirectly. It has been well studied that HIFs have a major part in angiogenesis, formation of vasculature from preexisting vessels, through its regulation of vascular endothelial growth factor (VEGF).[89] HIF has also been shown to have an impact on bone formation and repair, most likely though its role in angiogenesis, but it is difficult to discern angiogenesis and osteogenesis because they are so closely related through development and repair.[90,91] Due to HIF-1s distinct role in angiogenesis it can be stated that bone repair and, in turn, angiogenesis will be greatly hindered with the inhibition of HIF-1 activity, something that is not a desirable outcome for bone tissue engineering.

To understand how these pathways act, a thorough understanding of HIFs is desired. There are three different α-subunits, the unit degraded in the presence of oxygen, known as HIF-1α, HIF-2α, and HIF-3α. Each α-subunit binds to a β-subunit, which does not undergo degradation in response to oxygen abundance as seen with the α-

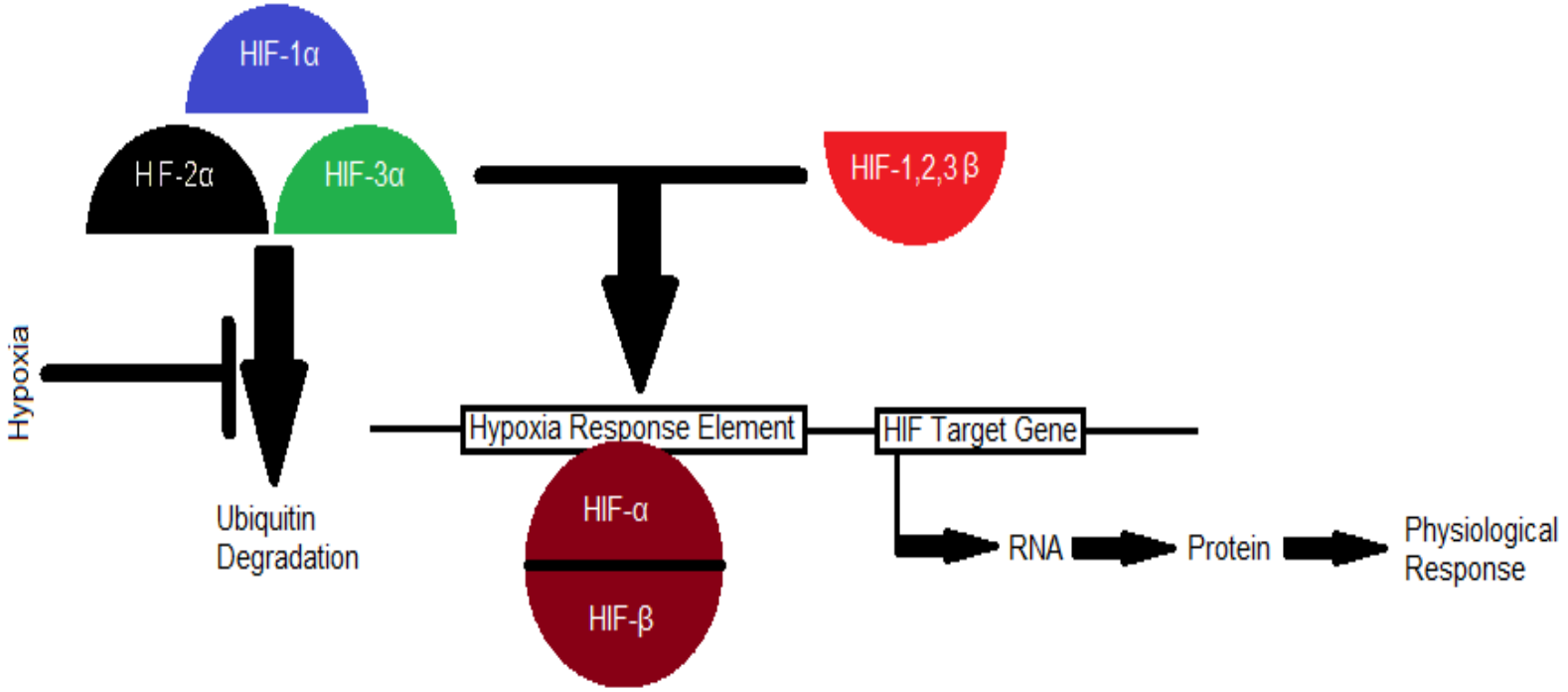

Figure 1.3: Transcriptional Regulation of the HIF Pathway.

subunits. The complex stabilized under these conditions regulates a wide variety of protein transcription. Studies have shown that they can act together or alone to affect specific gene regulation, but there are still a lot unknown information about the HIFs. Semenza was the first to discover HIF-1α almost two decades ago and because it was the first to be discovered it also has the most known about it, HIF-2α is the next most studied of the three, with HIF-3α having the most unknown.

As stated above, hypertrophy or the hypertrophic phenotype is more prevalent in cells that are differentiated *in vitro* or re-differentiated and implanted into the injury site. *In vitro* the cells tend to lack a "stop signal" to keep them from the onset of hypertrophy.[4] HIF function could play a role in this among other detrimental pathways that occur in musculoskeletal tissues. HIF-1α and HIF-2α have both been debated as having crucial roles in chondrogenesis and related to the onset of hypertrophy. One example of the relationship between different HIF molecules is the conclusion drawn from OA. It is known that elderly subjects are more predisposed to have issues related to OA. Studies have shown that with increasing age and OA onset HIF-2 levels decrease, whereas HIF-1 levels increase. Concluding that this reduction in HIF-2 activity is related to the increase in HIF-1 expression, remarking that it is antagonist to HIF-1s degenerative cartilage effects.[92] Although this study indicated that HIF-2α reduction was related to the onset of hypertrophy, many studies have found contradicting results. Based on the differences seen in studies there is a need to determine the true roles played by the HIFs regarding the onset of hypertrophy and OA. Saito et al. studied the role of HIF-2 during skeletal growth and OA development. HIF-2 activity was linked to collagen type X (col X), matrix metalloproteinase 13 (MMP-13), and VEGF due to their localization in the

hypertrophic zone of samples studied. HIF-2 showed direct regulation of hypertrophic indicators due to their reduction found in HIF-2 insufficient samples. Also in that study, over 100 transcription factors allegedly involved in hypertrophy of chondrocytes were analyzed from hypertrophic cartilage samples and HIF-2α was shown to have the highest expression, labeling it to be highly linked to the onset of hypertrophy.[72,78] Runx2, IHH, PTH1R, MMP-3, and MMP-9 were also shown to be regulated by HIF-2α, which could give mixed conclusions. Other studies have also shown that HIF-2α elevation is higher in terminally differentiating chondrocytes , i.e. undergoing hypertrophy, and OA cartilage.[72,73,78,84,93] While some studies will support the argument that HIF-2α causes OA, other studies have drawn conclusions that it actually acts to prevent it, as seen in the study that showed that HIF-2 decreases with age which is when OA increases, but without any other studies to back to support this conclusion seems baseless. Some studies have also shown evidence that HIF-2α does help to suppress cartilage degradation through down-regulation of proteins and enzymes which cause the breakdown to occur.[94] A good question posed from these results is whether this role is played by HIF-2α or hypoxia as a whole. These heterodimers regulate such a vast number of protein production, therefore it is a given that some will be positive and others negative. One negative result of HIF activation is the production of reactive oxygen species (ROS) which is related to OA degradation. Although, the exact mechanisms in which HIF causes ROS production is unknown, studies to determine a way to prevent it are required to take steps toward OA prevention and repair.[95]

In conclusion, the current repair methods for musculoskeletal systems involve the creation of multiple separate components that must not only integrate with the person it is

implanted in, but it also requires the integration of the individual components to ensure proper function. The current results of implantation of engineered constructs results in low survival and engraftment. This is hypothesized to be a result of the traumatic change from atmospheric culture to the deadly, hypoxic conditions encountered at the defect site.

CHAPTER 2

Ischemic Preconditioning of ADSCs to Accelerate Bone Defect Repair

2.1 BRIEF INTRO TO IPC

Stem cells present a very promising technique for repairing organ defects. The largest drawback with the use of stem cells for defect repair is the efficiency related to low survival and engraftment of the transplanted cells at the target site. The reason for the poor survival and engraftment is cellular apoptosis related to the dramatic shift from atmospheric culture of the stem cells to the hypoxic environment encountered at the defect site. IPC, for engineering any tissue, is a suggested strategy that could supply the desired stimulus resulting in numerous responses including an increase in protein expression related to survival pathways allowing protection from the hypoxic environment. Another problem that IPC could solve, related to tissue engineering bone, is that it could prime stem cells prior to differentiation into bone. Studies have shown that osteogenesis is hindered under hypoxia. It is desired to have stem cells be implanted into the defect site prior to differentiation to allow for vascularization of the newly formed bone through angiogenesis. The idea here is that IPC would create a transition period for the stem cells to begin to produce factors that will allow them to have a higher capacity to differentiate into bone. The aim of this study is to show that IPC enhances survival gene expression and to establish HIF-1s role in these survival pathways. Another aim of this study is to establish the positive relationship between IPC and osteogenic differentiation under hypoxic conditions to help enhance and accelerate bone formation.

2.2 MATERIALS AND METHODS

2.2.1 ADSC CULTURE

Passage 1 (P1) ADSCs were purchased from a supplier (Invitrogen, Carlsbad, CA). Cells were seeded and cultured in 75 cm^2 culture flasks in humidified environments of 5% CO_2, and 95% air at 37°C (referred to as 20% O_2 conditions). Cells were cultured in MesenPro basal medium supplemented with MesenPro growth supplement, 1% penicillin/streptomycin (Mediatech, Manassas, VA) and 2 mM L-glutamine (MP Biomedicals, Irvine, CA). Once cultured cells reach between 80 and 90% confluence, they were trypsinized (Corning Life Sciences, Manassas, VA) and a hemocytometer was used to count the number of cells per milliliter to obtain the proper number of cells for the study. Cells between P2 and P5 were used to ensure consistent cellular behavior, per the manufacture's recommendation.

2.2.2 HIF-1 & 2 INHIBITION

To inhibit the activity of HIF-1 and HIF-2, a HIF-1 inhibitor (Calbiochem, USA) and a HIF-2 inhibitor (Millipore, Billerica, MA) were used. The inhibitors selectively block hypoxia-induced accumulation of cellular HIF-1α and HIF-2α, respectively. For both 1% and 4% O_2 samples, a concentration of 60 μM of the HIF-1 inhibitor and 10 μM of the HIF-2 inhibitor were sufficient to reduce accumulation of HIF-1α and HIF-2α levels below detection through western blotting. Cell viability tests showed no loss of viability of ADSCs exposed to the inhibitors at their working concentrations.

2.2.3 ISCHEMIC PRECONDITIONING

Constant O_2 levels of 1%, 2%, and 4% were maintained using nitrogen-purged, programmable incubators. for the hypoxic studies (Napco Series 8000 WJ, Thermo Electron). Cells were seeded into 6-well plates (100,000 cells/well) and placed in the atmospheric oxygen incubator to allow for attachment over a 12 hour period. Following the 12 hour attachment period, plates were moved to 1, 2 or 4% oxygen incubators for ischemic exposure or left at atmospheric conditions. At each 24 hour time point following, a new set of plates is moved from the atmospheric incubators to the hypoxic incubators. This continues until 108 hours after initial seeding took place to create cells that were exposed to 1, 2 or 4% O_2 for 24, 48, 72 and 96 hours. To ensure exposure hypoxic conditions were maintained for as much time as possible, all media, buffered salt solutions, and fixatives used with the cells were allowed to pre-equilibrate in vented tubes in the hypoxic incubator for 24 hours prior to use. Our lab has, in previous studies, determined that is sufficient time to allow for equilibration of the dissolved oxygen concentration.[96] Also to limit exposure to higher O_2 levels, media changes and imaging procedures were kept under 10 minutes per day. It was determined in dissolved oxygen studies that a 10 minute exposure to atmospheric conditions was small enough to allow reestablishment of the oxygen level within 10 minutes of return to the incubator.

2.2.4 ANALYSIS OF ADSC STEMNESS AND APOPTOSIS BY FLOW CYTOMETRY

ADSCs were trypsinized and washed with a washing buffer. A hemocytometer was then used to add 200,000 cells were added to each polystyrene flow cytometry tubes

while on ice. Cells were stained with FITC conjugated CD44, PE conjugated CD105 and a combination of both (BD Biosciences, San Diego, CA, USA). DeadEnd™ Fluorometric TUNEL System was performed following the manufacturer's instructions to assess apoptosis of ADSCs (Promega, USA). The Beckman Coulter-Cytomics FC 500 and CXP Analysis software were used to analyze the labeled cells.

2.2.5 PROTEIN EXTRACTION AND WESTERN BLOTTING

RIPA lysis buffer (Thermo Scientific, Rockland, IL) containing a protease and phosphatase inhibitor (Thermo Scientific, Rockland, IL) was used to collect total cell lysates from the samples. When added to the samples, they were stirred on ice for 10 minutes and sonicated for 15 minutes. The lysates were then centrifuged at 14000 rpm at 4°C for 15 min. Supernatants were collected and protein concentration was then quantified with a Coomassie Plus Bradford Assay kit following the manufacturer's instructions (Thermo Scientific, Rockford, IL).

Western blotting was then performed on the lysates by SDS-PAGE. Approximately 50 μg of protein lysate was loaded per lane of 4-20% gradient gels. Transfer to PVDF membranes (Millipore, Billerica, MA) followed after electrophoresis. Following transfer to the membrane, a blocking phase followed. The membrane was first incubated in 5% dry, milk, 1% bovine serum albumin (BSA) diluted in HBSS for 1 hour at room temperature. The membrane was then washed for 15 minutes in a wash solution (0.1% Tween-20, 0.1% dry milk, 0.1% BSA in HBSS). Next, the membrane was incubated with rabbit anti-HIF-1α antibody (1:1000)(Santa Cruz Biotechnology, Santa Cruz, CA), rabbit-anti-pAKT (1:1000), rabbit anti-bcl-2 antibody (1:1000), rabbit anti-

Bax antibody (1:1000), rabbit anti-active Caspase-3 antibody (1:1000), rabbit anti-Osteonectin antibody (1:1000), rabbit anti-Osteopontin antibody (OPN)(1:1000), or rabbit anti-β-actin antibody (1:1000)(Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The next step involved 3 washes of 15 minutes each in the previously mentioned wash. Then membranes were incubated in a secondary antibody, peroxidase-conjugated, goat, anti-rabbit IgG (1:5000)(Rockland Immunochemicals, Gilbertsville, PA) for 2 hours on an orbital shaker for 2 hours. Following incubation in the secondary antibody, membranes were washed 3 times for 15 minutes in a new wash (0.1% Tween-20 in HBSS). Prior to imaging, the membranes were exposed to a chemilluminescence reagent, SuperSignal West Pico Substrate (Thermo Scientific, Rockford, IL) for 5 minutes. ChemiDoc™ XRS+ System with Image Lab™ image acquisition and analysis software was used to for band detection (Bio-Rad, Hercules, CA). Image J program was used for densitometry analysis. For densitometry analysis, the expression of all proteins was normalized to β-actin to account for % IOD (intensity optical density). All incubation steps took place on an orbital shaker set to continuously rotate at 200 rpm. The statistics shown represent results from three independent studies.

2.2.6 OSTEOGENIC DIFFERENTIATION

Induction of osteogenic differentiation was through culture in StemPro® Osteocyte/Chondrocyte Differentiation Basal Medium and StemPro® Osteogenesis supplement (Invitrogen, USA). Cells were monitored over a 4 week period at 2% O_2, exchanging the used medium for fresh preconditioned media every 3 days. After completing the initial IPC studies it was determined based on optimal survival, the Bcl-

2/Bax ratio, that the best results were in the 72 hour IPC condition. Therefore IPC performed prior to osteogenic differentiation was performed for 72 hours at all O_2 concentrations. All preconditioned cells and one set of cells cultured in 20% O_2 were differentiated in 2% O_2.

2.2.7 ALP ACTIVITY AND DNA QUANTIFICATION

Media samples were taken on days 7, 14, 21 and 28 to analyze for ALP activity. An ALP colorimetric assay kit (Abcam, USA) with p-nitrophenyl phosphate as the substrate was used to determine the activity. ALP activity was normalized to cellular DNA content using a PicoGreen dsDNA quantitation kit (Molecular Probes, USA) following the manufacturer's instructions.

2.2.8 QUANTIFICATION OF MINERALIZATION VIA ALIZARIN RED STAINING

Samples were collected on days 14 and 28 to quantify mineral deposition using an Alizarin Red staining procedure. Cells were first rinsed with calcium and phosphate-free HBSS, and fixed with 70% ethanol for 1 hour on ice. Following another brief wash with HBSS, cells were stained for 20 minutes with 40 mM Alizarin Red staining solution (pH 4.2) at room temperature. Cells were then rinsed with 2 exchanges for 15 minutes in HBSS to reduce nonspecific staining. Next, a 10% acetic acid solution was added to the samples for 30 minutes. Samples were collected by scraping and were then headed at 80°C for 10 minutes. Extracts were centrifuged at 8000 rpm for 20 minutes and quantified using a microplate reader (Biotek, USA) at 570 nm. Calcium concentrations

were calculated from the standard curved generated using a serial dilution of a calcium standard solutions and then values were normalized to DNA contents.

2.2.9 STATISTICAL ANALYSIS

Statistical analysis was performed with Microsoft Excel and Graphpad Prism 4.01 (GraphPad Software Inc., San Diego, CA). Experimental results are expressed as the mean ± standard deviation and were analyzed by two-way ANOVA for comparisons and p-values <0.05 were defined to be statistically significant.

2.3 RESULTS

2.3.1 FLOW CYTOMETRY FOR STEMNESS

Flow cytometry revealed that the percentage of ADSC population after preconditioning for the longest period of time (96 hours) in 1%, 2%, 4% and 20% O_2 were 99. 5±0.05 %, 99% ±1%, 98.8% and 99% ±1%, respectively, as shown in Appendix 1.

2.3.2 IPC PROTEIN EXPRESSION

In the 1% O_2 preconditioning set up, the pAKT expression was 7.61±0.43, 8.52±0.61, 12.16±0.56 and 14.13±0.71 at 24, 48, 72 and 96 hours respectively. The preconditioning set up under 2% O_2 expressed pAKT as 6.82±0.32, 10.24.52±0.73, 12.37±0.36 and 12.56±0.66 at 24, 48, 72 and 96 hours respectively.

Active caspase-3 expression is as follows. In the 1% O_2 preconditioning set up the expression was 0.91±0.52, 12.14±0.81, 13.43±0.76 and 18.24±0.92 at 24, 48, 72 and

96 hours respectively. In the 2% O_2 preconditioning set up was 0.83±0.31, 0.85±0.24, 13.43±0.69 and 13.53±0.82 at 24, 48, 72 and 96 hours respectively.

Protein expression of Bcl-2 and Bax is expressed as a ratio of Bcl-2 to Bax. This is a standard ratio in literature looking at the enhancing cell survival. Bcl-2 is an anti-apoptotic protein, while Bax is an apoptotic protein. Therefore this ratio is viewed as a representation of anti-apoptotic proteins to pro-apoptotic proteins to determine whether there has been an increase of survival. In 1% $O_2$the Bcl-2/Bax ratio expression was 1.34±0.31, 3.15±0.36, 3.41±0.46 and 3.53±0.23 at 24, 48, 72 and 96 hours respectively. In 2% O_2 condition, the Bcl-2/Bax ratio expression was 2.62±0.43, 3.23±0.27, 4.36±0.21 and 4.16±0.35 at 24, 48, 72 and 96 hours respectively.

In 1% O_2 preconditioning set up the HIF-1α expression was 8.92±2.51, 9.12±1.23, 10.52±1.34 and 14.12±1.46 at 24, 48, 72 and 96 hours respectively. In 2% oxygen condition, the HIF-1α expression was 7.31±1.31, 8.76±0.83, 9.85±1.25 and 14.9±1.37 at 24, 48, 72 and 96 hours respectively.

All protein expression is represented in %IOD fold difference against the samples in 20% oxygen. Figure 2.1 shows the collected results for all of the protein expression data and appendix 2 shows a representative image of the gel.

2.3.3 EFFECTS OF IPC ON OSTEOGENIC DIFFERENTIATION

ALP activity of samples taken on day 28 in 2% O_2 after no preconditioning, 1% O_2, 2% O_2 and 4% O_2 was 19.31± 1.23, 24.21± 4.11, 28.21±1.9 and 29.32± 3.14 μmol/ml/min/mg DNA, respectively. Quantification of mineralization under the same conditions as above was 36.87± 2.76, 42.81± 2.36, 39.72±3.91 and 39.22± 4.12 mg

mineral/mg DNA on day 28, respectively. Protein expression for the osteogenic markers osteonectin (ON) and OPN were taken on day 28.

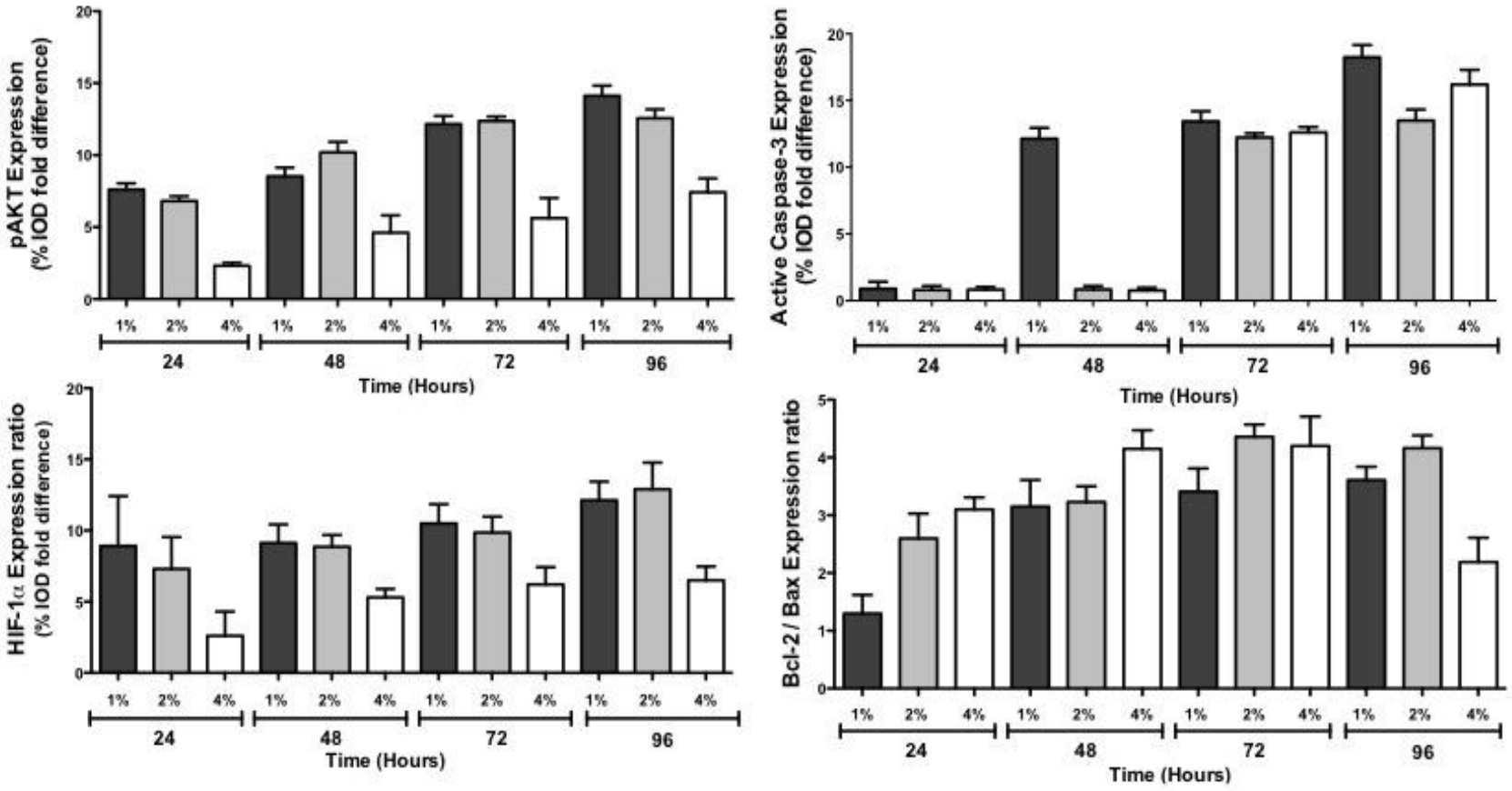

Figure 2.1: Quantification of the Expression of Apoptotic Proteins in 1%, 2% and 4% O_2 Environments through all four IPC time points.

ON expression of samples taken on day 28 from 2% O_2 after no preconditioning, 1% O_2, 2% O_2 and 4% O_2 was 2.61±0.21, 3.56±0.53, 3.81±0.34 and 3.96±0.57 %IOD fold difference was observed. OPN expression of samples taken on day 28 in 2% O_2 after no preconditioning, 1% O_2, 2% O_2 and 4% O_2 was2.11±0.34, 3.73±0.62, 3.42±0.82 and 3.53±0.31 %IOD fold difference was observed. All of these results can be seen portrayed in figure 2.2.

2.3.3 EFFECTS OF IPC ON OSTEOGENIC DIFFERENTIATION

ALP activity of samples taken on day 28 in 2% O_2 after no preconditioning, 1% O_2, 2% O_2 and 4% O_2 was 19.31± 1.23, 24.21± 4.11, 28.21±1.9 and 29.32± 3.14 μmol/ml/min/mg DNA, respectively. Quantification of mineralization under the same conditions as above was 36.87± 2.76, 42.81± 2.36, 39.72±3.91 and 39.22± 4.12 mg mineral/mg DNA on day 28, respectively. Protein expression for the osteogenic markers osteonectin (ON) and OPN were taken on day 28.

ON expression of samples taken on day 28 from 2% O_2 after no preconditioning, 1% O_2, 2% O_2 and 4% O_2 was 2.61±0.21, 3.56±0.53, 3.81±0.34 and 3.96±0.57 %IOD fold difference was observed. OPN expression of samples taken on day 28 in 2% O_2 after no preconditioning, 1% O_2, 2% O_2 and 4% O_2 was2.11±0.34, 3.73±0.62, 3.42±0.82 and 3.53±0.31 %IOD fold difference was observed. All of these results can be seen portrayed in figure 2.2.

2.4 DISCUSSION

Analysis of the results showed that the anti-apoptotic and pro-apoptotic markers were regulated by IPC in both a time and O_2 dependent manner. It was noticed that ADCSs that experienced IPC in the 1% and 2% O_2 concentrations expressed higher levels of apoptotic markers than those expressed from ADSCs that underwent IPC in 4% O_2. It was also shown that with the use of the HIF-1 inhibitor, the Bcl-2/Bax ratio was reduced with no effect on the levels of pAKT and active caspase-3. Figure 2.3 shows the results of HIF-1 inhibition on the Bcl-2/Bax ratio. With regards to osteogenic differentiation, cells that underwent any form of IPC exhibited enhanced osteogenic marker expression compared to cells that did not experience any form of IPC.

Survival of transplanted cells is a known problem in the field of tissue engineering. Many strategies have been examined in attempts to promote survival and engraftment at the injury site. Some of these strategies are, but not limited to, knocking down pro-apoptotic genes, genetic manipulation through anti-apoptotic protein and gene over expression, and infusion of neurotrophic factors continuously.[97–100] Although these studies have proposed great strategies for increasing survival, limitations have become more apparent, such as clinically impracticality, concerns with the efficacy of targeting a single gene or signaling cascade, and the safety issues connected with permanent gene modification. Compared to the previously listed proposed solutions for survival and engraftment at defect sites, IPC has produced some promising results. In order for stem cells to surmount stresses encountered at the injury site, optimization of IPC conditions is of extreme importance for *in vitro* activation of ADCSs.

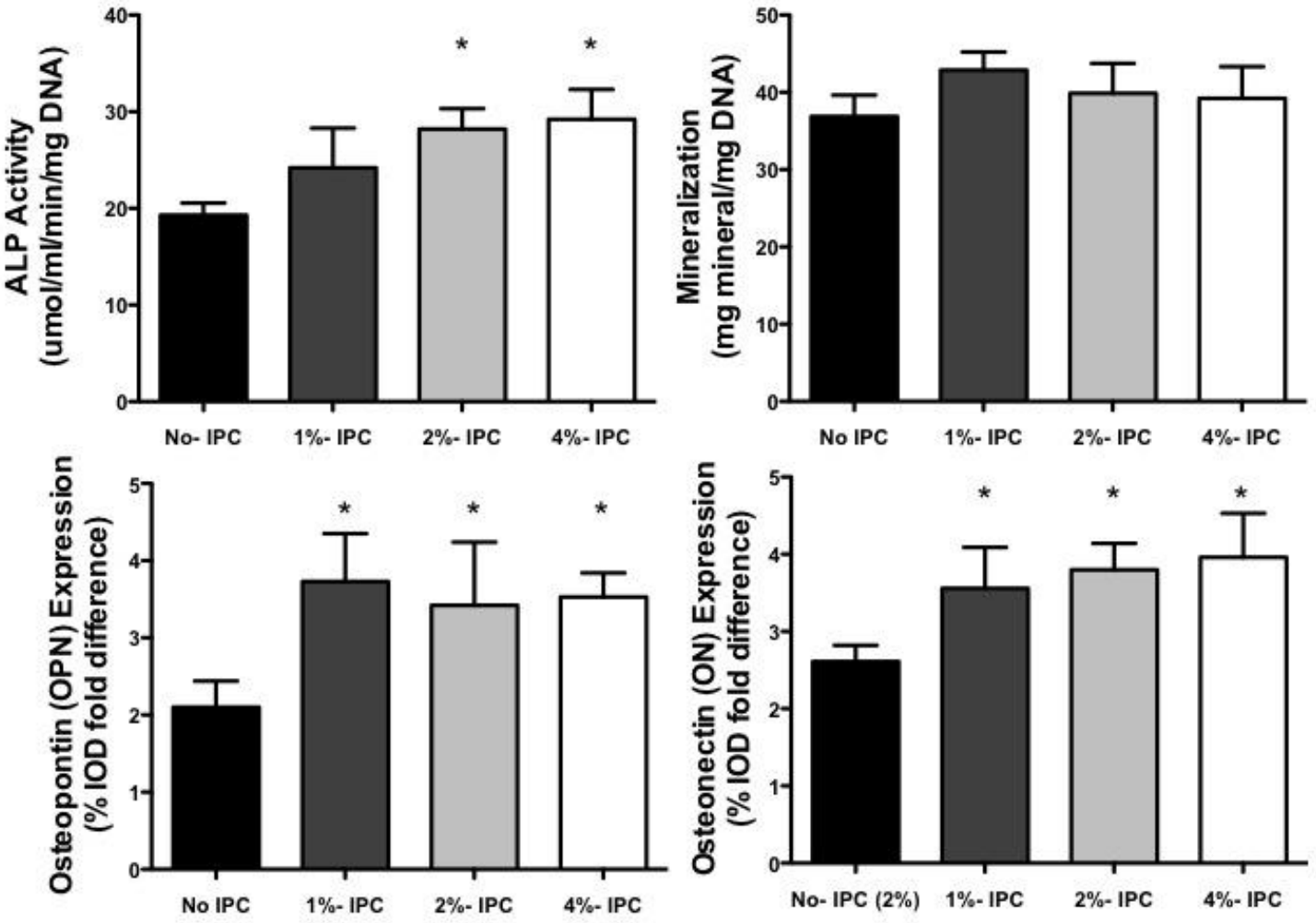

Figure 2.2: Effect of IPC on Osteogenic Differentiation of ADSCs.

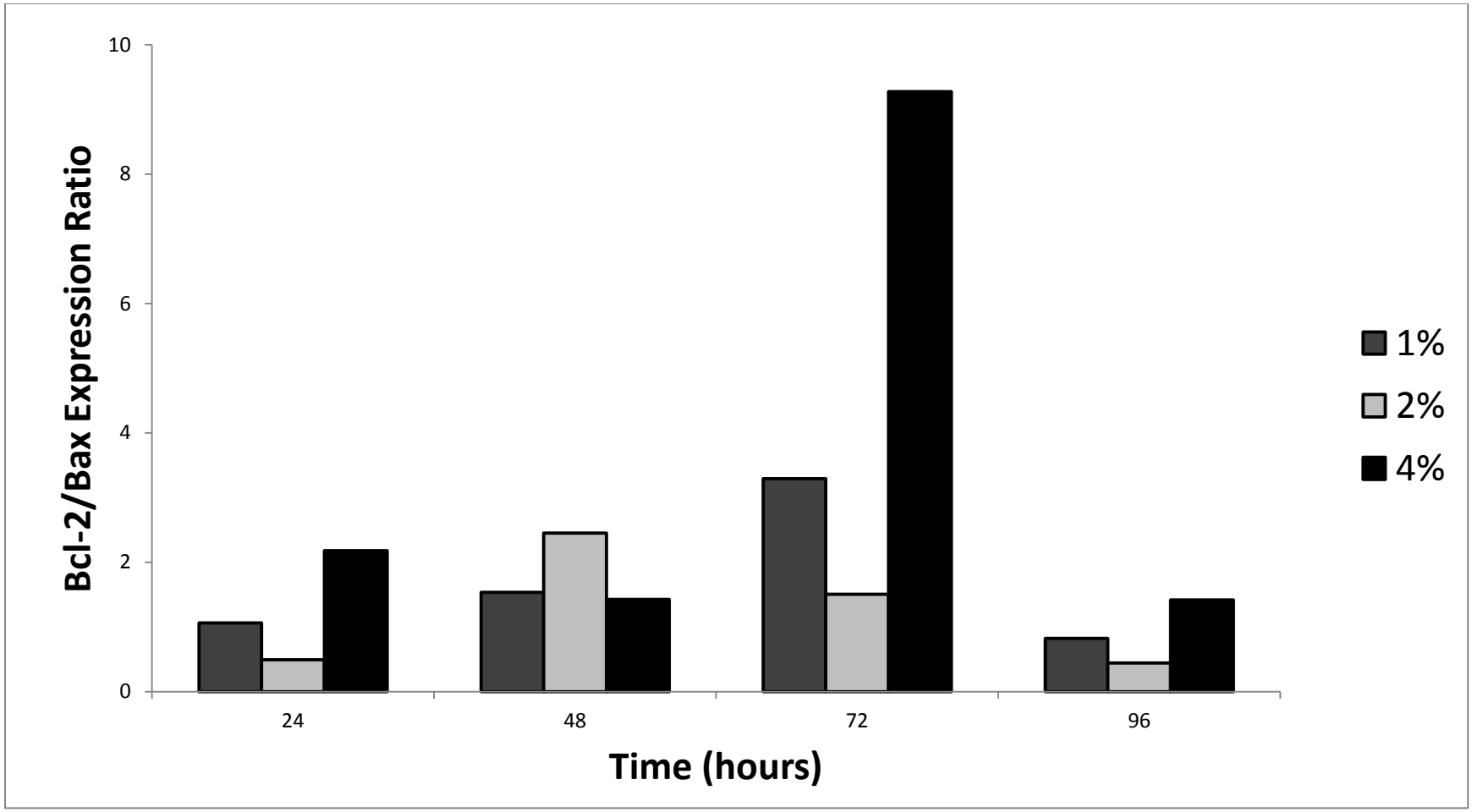

Figure 2.3: Effect of HIF-1 Inhibition on Survival Criteria Proteins to Gauge HIF-1's Role in Survival.

Chapter 3

Inhibition of HIF-2α during Chondrogenesis for Cell Survival Leading to Prevention Hypertrophy

3.1 BRIEF INTRO TO HIF-2A'S ROLE IN HYPERTROPHY

Hypertrophy is a step in the formation of bone, known as endochondral ossification, where chondrocytes swell to create a space for osteoblasts to invade and begin bone formation. Studies have shown that levels of HIF-2α are decreased in the hypertrophic zone of ossifying bone during development.[78] Saito et al. also showed that through HIF-2α inhibition there was a reduction in cartilage degradation and osteophyte formation, but these studies were oriented more towards development. To aid in the formation of implantable tissue constructs, *in vitro* studies of HIF-2α's effects on apoptosis during chondrogenesis are required. The development of a method to prevent hypertrophy, or apoptosis, during *in vitro* chondrogenesis would provide more insight to regeneration of cartilage for cartilage defect or OA cartilage repair. This study aims to determine the effects of a HIF-2 inhibitor at different stages of *in vitro* chondrogenesis (middle and end) on apoptosis and hypertrophic marker expression ADSCs in 3D culture.

3.2 MATERIALS AND METHODS

3.2.1 ADSC CULTURE

Passage 1 (P1) ADSCs were purchased from a supplier (Invitrogen, Carlsbad, CA). Cells were seeded and cultured in 75 cm^2 culture flasks in humidified environments

of 5% CO_2, and 95% air at 37°C (referred to as 20% O_2 conditions). Cells were cultured in MesenPro basal medium supplemented with MesenPro growth supplement, 1% penicillin/streptomycin (Mediatech, Manassas, VA) and 2 mM L-glutamine (MP Biomedicals, Irvine, CA). Once cultured cells reach between 80 and 90% confluence, they were trypsinized (Corning Life Sciences, Manassas, VA) and a hemocytometer was used to count the number of cells per milliliter to obtain the proper number of cells for the study. Cells between P2 and P5 were used to ensure consistent cellular behavior, per the manufacture's recommendation.

3.2.2 ADSC SPHEROID FORMATION

A similar approach to ADSC spheroid formation was performed previously and described in a preceding paper.[101] Once cells reached 80-90% confluence trypsin (Corning Life Sciences, Manassas, VA) was used to detach them from the flask. A hemocytometer was used after resuspension in MesenPro medium (Invitrogen, Carlsbad, CA) to reach a cell density of 1,000,000 cells/mL. Media containing either 20,000 (20k), 60,000 (60k), or 200,000 (200k) cells was added to 0.8 mL, siliconized screw-cap microcentrifuge tubes (VWR, Radnor, PA) and spun at 500 rcf for 2 minutes. Screw caps were then loosened to allow gas transfer and the tubes were placed in an incubator set at 20% O_2 overnight. After 24 hours for initial formation of 3D spheroids through spontaneous self-assembly, wide-mouth tips were used with a micropipette, to prevent shearing, to carefully collect the pellets and use them for the study.

3.2.3 PELLET SIZE EXPERIMENTS

To determine the pellet size to use for the HIF-2 inhibition study over an extended period of time, apoptosis studies were set up. Following cohesion of pellets of sizes: 20k, 60k, and 200k, the pellets were all placed in a 2% O_2 incubator for 7 days. Media was exchanged each day, adding 200 μL of preconditioned ADSC media. On day 7 pellets were placed in zinc formalin (VWR, Radnor, PA) for 24 hours to fix them for paraffin embedding.

3.2.4 EFFECTS OF HIF-2A ON ADSC VEGF PRODUCTION

Spheroids were formed and allowed 24 hours for cohesion. Pellets were then divided evenly into six different categories based on pellet size and +/- 10 μM HIF-2α inhibitor as follows: 20k - inhibitor, 20k + inhibitor, 60k - inhibitor, 60k + inhibitor, 200k - inhibitor, and 200k + inhibitor. Media was collected around the same time each day for 8 days and frozen. Following media collection from the pellets, a VEGF ELISA kit was performed, per the manufacturer's instructions (Invitrogen, Carlsbad, CA).

3.2.5 HIF-2 INHIBITION STUDY

Spheroids were formed and allowed 24 hours for cohesion. Pellets were distributed evenly 9mm petri dishes (VWR, Radnor, PA) labeling each with one of six different conditions as follows: 20k and 200k pellets with no addition of HIF-2 inhibitor (called no), 20k and 200k pellets with the addition of HIF-2 inhibitor at the midpoint of induced chondrogenesis (day 14, called early), and 20k and 200k pellets with the addition of HIF-2 inhibitor at the initiation of hypertrophy (day 28, called late). Upon

distribution to the petri dishes, 3 mL of preconditioned media is added to each petri dish and they are placed into the 2% O_2 incubator. Every 3 days the media approximately 80% of the media is removed using wide mouthed pipette tips to reduce chances of tearing the pellets. Chondrogenic media (Invitrogen, Carlsbad, CA) is added to the pellets through day 28. After day 28, hypertrophic media, based on a formula from another study[74], is added to the pellets through day 49. After day 14, only the groups labeled early received 10 μM of HIF-2 inhibitor to the chondrogenic media from this day forward. After day 28, the groups labeled late received inhibitor for the rest of the study. Therefore, after day 28 the only groups without any addition of HIF-2 inhibitor in the media was the group labeled "no". Media samples were collected on days 7, 14, 21, 28, 35, 42, and 49 for alkaline phosphatase analysis. On day 49 the pellets were placed in zinc formalin (VWR, Radnor, PA) for 24 hours to fix them for paraffin embedding.

3.2.6 ALP ANALYSIS

ALP samples collected on days 7, 14, 21, 28, 35, 42, and 49, were analyzed using an ALP colorimetric assay (Abcam, Cambridge, MA), per the manufacturer's instructions.

3.2.7 PARAFFIN EMBEDDING

Following fixation the pellet goes through an embedding preparation process, including dehydration, clearing, and infiltration. This process includes the following steps: was in running water, submerge in 80% alcohol, then in 95% alcohol for 3 changes for 15 min each, 3 changes in absolute alcohol for 15 min each, submerge in 50/50 absolute alcohol and xylene for 15 min, 2 changes of xylene for 15 min each, 3 changes

of paraffin for 15 min each, and finally in paraffin under vacuum for 15 min. After the final step embedding may take place. After embedding in paraffin the block must be cut with beveled edges for ease of cutting on the tome.

3.2.8 PELLET SECTIONING

Pellets were cut using a microtome (Reichert-Jung 2030) at a thickness of 5 μm. The newly cut section is then laid in a warm-water bath (Triangle Biomedia Sciences) to release wrinkles prior to placement of glass microscope slides (Superfrost Plus).

3.2.9 HEMATOXYLIN AND EOSIN STAINING

Following deparaffinization and hydration of slides in distilled water, the slides go through the following progression of submersion: xylene for 3 min, xylene for 3 min, 100% alcohol, 100% alcohol, 95% alcohol, 80% alcohol, water wash, hematoxylin for 7 min, water wash, acid alcohol, water wash, ammonia water, water wash, eosin for 2 min, 95% alcohol, 95% alcohol, 95% alcohol, 100% alcohol, 100% alcohol, then three dips in xylene. After the progression is completed the slides should be cover slipped.

3.2.10 SAFRANIN O STAINING

Following deparaffinization and rehydration to water, the slides are stained with Weigert's iron hematoxylin working solution for 7 minutes. Next running tap water is used to wash the slide for 10 minutes and a fast green stain solution (0.1g fast green in 1 L of water) is applied for 3 minutes. A quick rinse of approximately 10 seconds is performed, following the fast green stain, with a 1 % acetic acid solution. Safranin O

solution (1g of safranin O and 100 mL of water) is then added to stain the slide for 5 minutes, followed by dehydration and clearing with 95% ethyl alcohol, 100% ethyl alcohol, and xylene through 2 changes of 2 minutes each. Following rinsing the slides were mounted with a coverslip for microscopy.

3.2.11 STATISTICAL ANALYSIS

Statistical analysis was performed with Microsoft Excel and Graphpad Prism 4.01 (GraphPad Software Inc., San Diego, CA). Experimental results are expressed as the mean ± standard deviation and were analyzed by two-way ANOVA for comparisons and p-values <0.05 were defined to be statistically significant.

3.3 Results

3.3.1 EFFECTS OF HIF-2 INHIBITION ON VEGF PRODUCTION IN ADSCS

The analysis of VEGF production of ADSCs was measured using a VEGF ELISA kit. Results indicated that there was no significant difference between VEGF production of cells with and without the addition of HIF-2 inhibition. It is worthy to note also that after the initial release of VEGF, the amount present after every day later reduces. Figure 3.1 shows the results of the VEGF ELISA to determine if HIF-2 has any effect on the production of VEGF in ADSCs.

3.3.2 EFFECT OF HIF-2 INHIBITION ON ALP PRODUCTION

Results of the colorimetric ALP assay used to asses ALP levels during the HIF-2 inhibition study showed that ALP levels were similar for each group. Typically, ALP

levels were high in pellets that received late induction of HIF-2 inhibitor, but the difference was deemed insignificant. Results of the ALP analysis are shown in Figure 3.2.

3.3.3 HEMATOXYLIN AND EOSIN STAINING

H&E staining was performed on pellets of 20k and 200k after 7 days of hypoxic culture in ADSC medium and at day 49 following culture in hypertrophic and chondrogenic medium.

3.3.3.1 7-DAY HYPOXIC CULTURE

Viewing sections of 200k pellets after 7 days of hypoxic culture, as the cuts became closer to the center of the pellet significantly more necrosis occurred. Viewing 3 sections of the same pellet, at a magnification of 10x, each approximately 120 μm apart, the final section being the most central representation, necrosis increased from approximately 25% to 40% to 65%, respectively. Three 20k pellets were viewed approximately 20 μm apart each, the final being the central most cut. When viewing the 20k pellets at 10x magnification, there was very little, if any, visible necrosis with the possibility of >5% in the most central cut. Figure 3.3 shows the visual representation of each of these pellet sections.

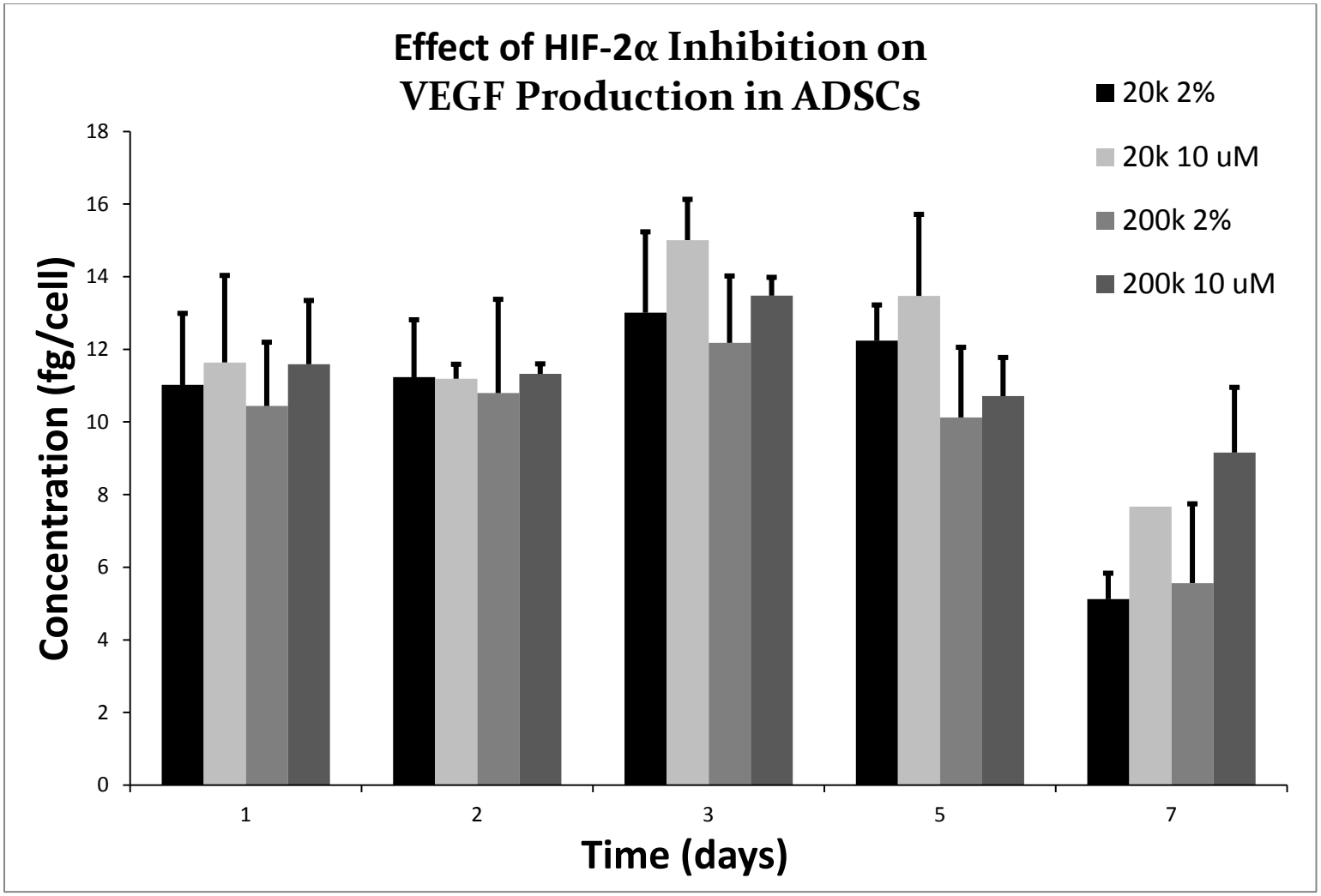

Figure 3.1: VEGF production in ADSCs with and without HIF-2 inhibitor.

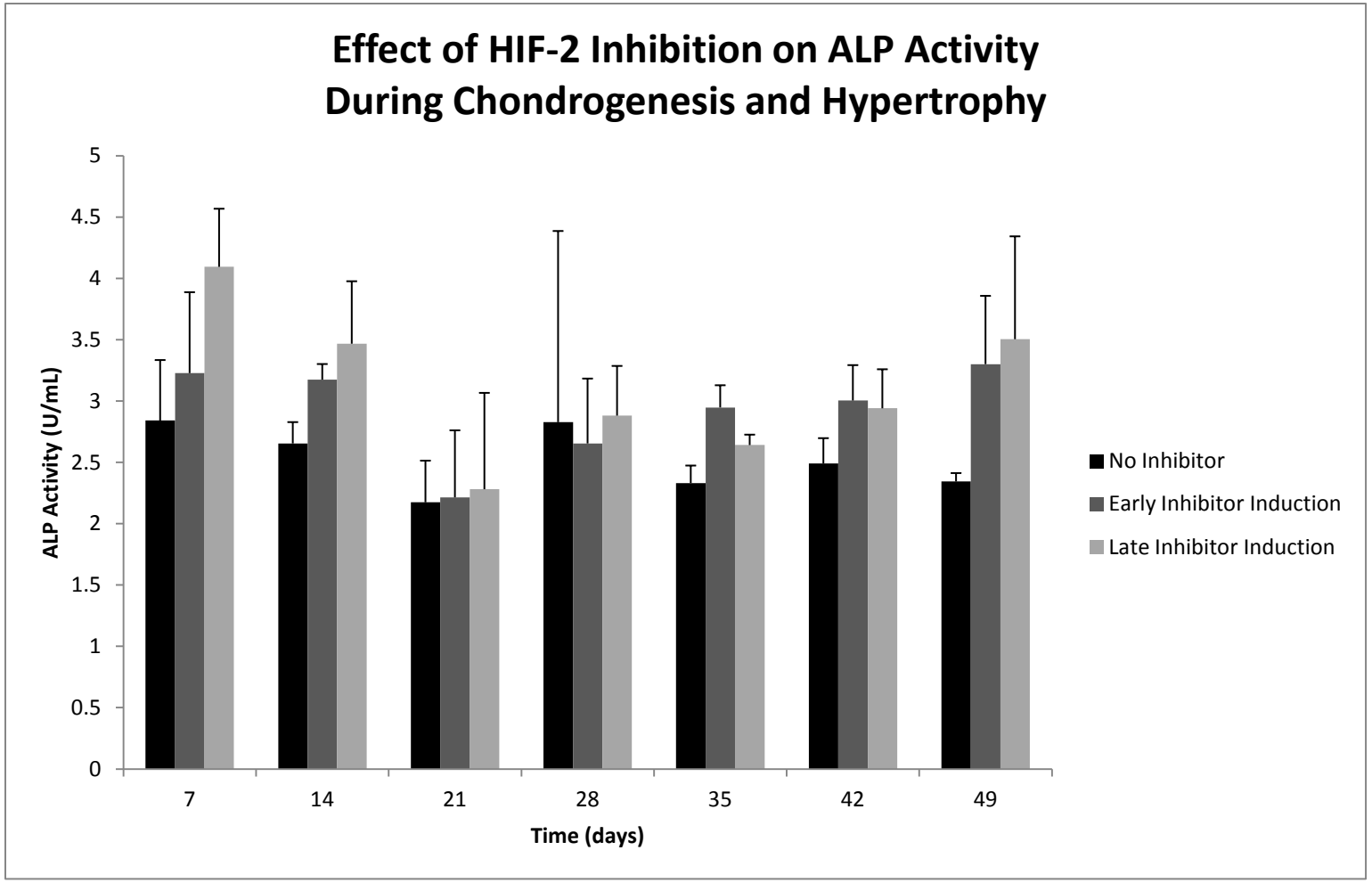

Figure 3.2: ALP activity with different induction points of the HIF-2 inhibitor.

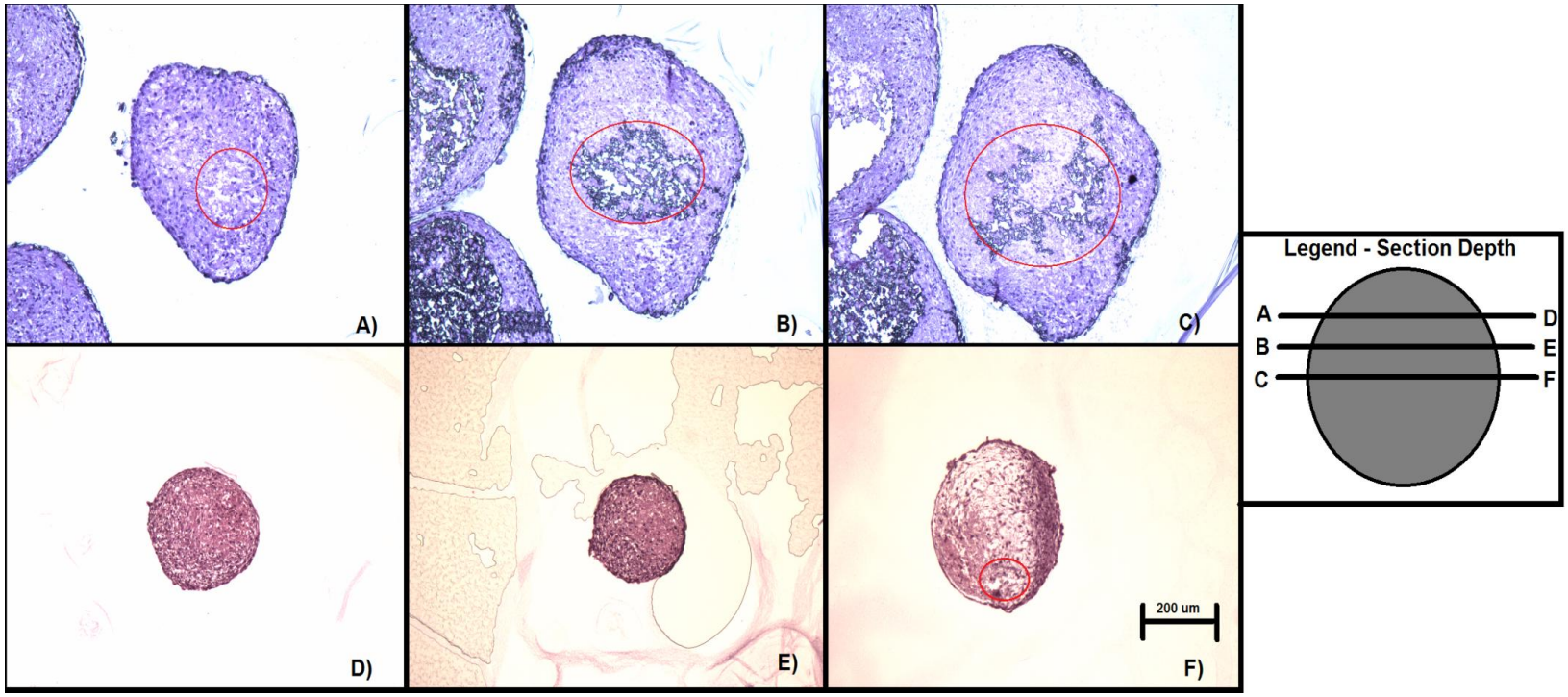

Figure 3.3: Pellet sections of a 200k pellet (A, B and C) and a 20k pellet (D, E and F) after 7 days of hypoxic culture. Red circles indicate areas of necrosis visible by the darker purple stain and areas of cells pulling away from each other. Legend shows the depth at which each section was approximately cut at.

3.3.3.2 49-DAY STUDY OF ADSCS UNDERGOING CHONDROGENIC AND HYPERTROPHIC DIFFERENTIATION

H&E staining was performed on 200k pellets that were exposed to the HIF-2 inhibitor and pellets that were not exposed to HIF-2. Figure 3.4 shows the effect of the HIF-2 inhibitor on apoptosis. The 200k pellet that was not exposed to the HIF-2 inhibitor had a much larger degree of necrosis including loss of the original size of the pellet compared to 200k pellet exposed to the HIF-2 inhibitor.

3.3.4 SAFRANIN O STAINING

Safranin O staining, with a counter stain of hematoxylin, of 20k and 200k pellets exposed to no HIF-2 inhibitor, exposure to HIF-2 inhibitor after 14 days of chondrogenic induction (early), and exposure to HIF-2 inhibitor after 28 days of chondrogenic induction (late). There is significantly more necrosis in 200k pellets compared to 20k pellets based on the hematoxylin counter stain. It is visually evident that pellets introduced to the HIF-2 inhibitor earlier, had more collagen shown by the amount of safranin O stain picked up, as shown in figure 3.5.

3.4 DISCUSSION

It is well known that HIF-1 has a direct role in the regulation of VEGF secretion of stem cells.[51,78,101,102] HIF-2's role in VEGF production, on the other hand, is much less documented. Here it was shown that HIF-2 had no visible regulation on the production of VEGF over a known period where VEGF secretion is its highest. Therefore, if HIF-2

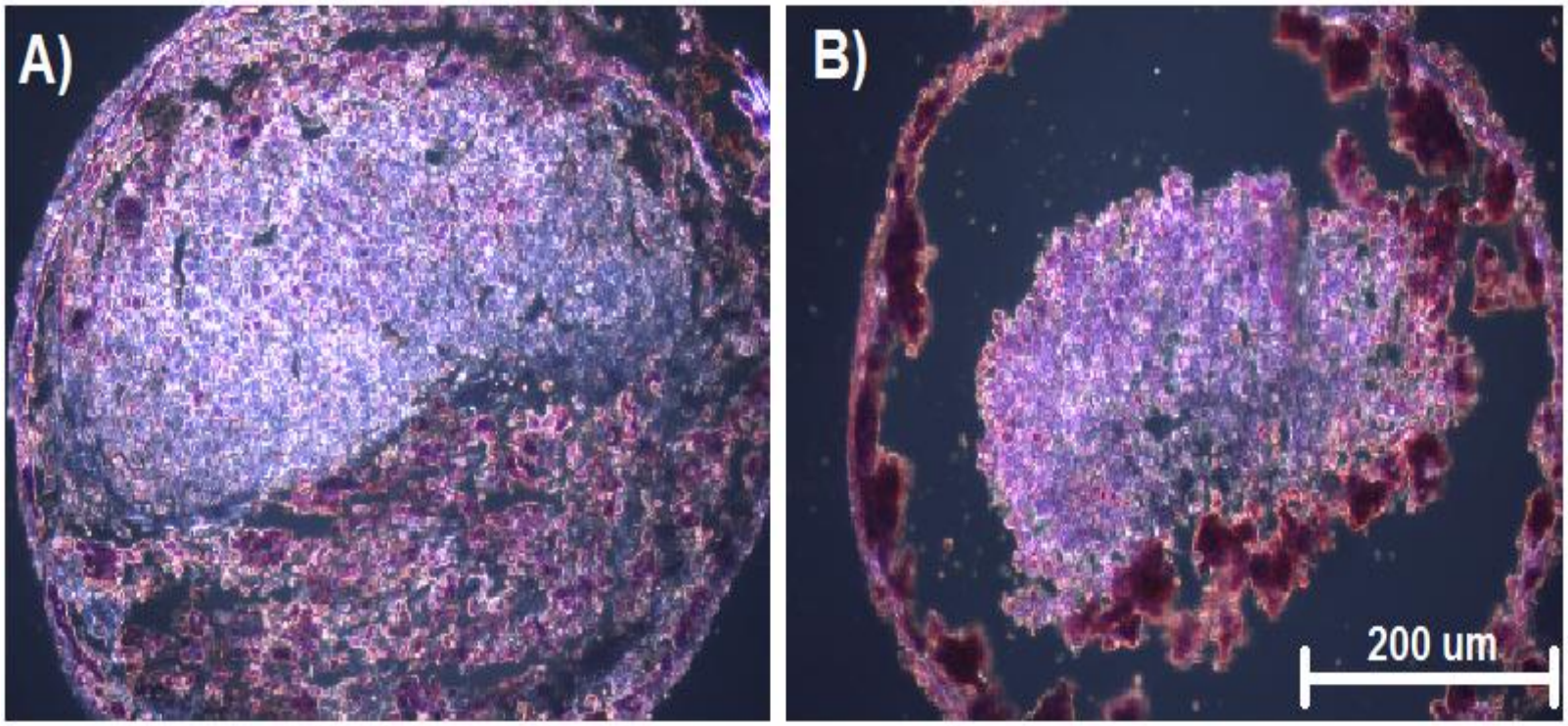

Figure 3.4: H&E sections of 200k pellets after 49 days of chondrogenic and hypertrophic differentiation. A) Shows a 200k pellet differentiated under HIF-2 inhibition compared to B) Showing a 200k pellet section differentiated without the HIF-2 inhibitor.

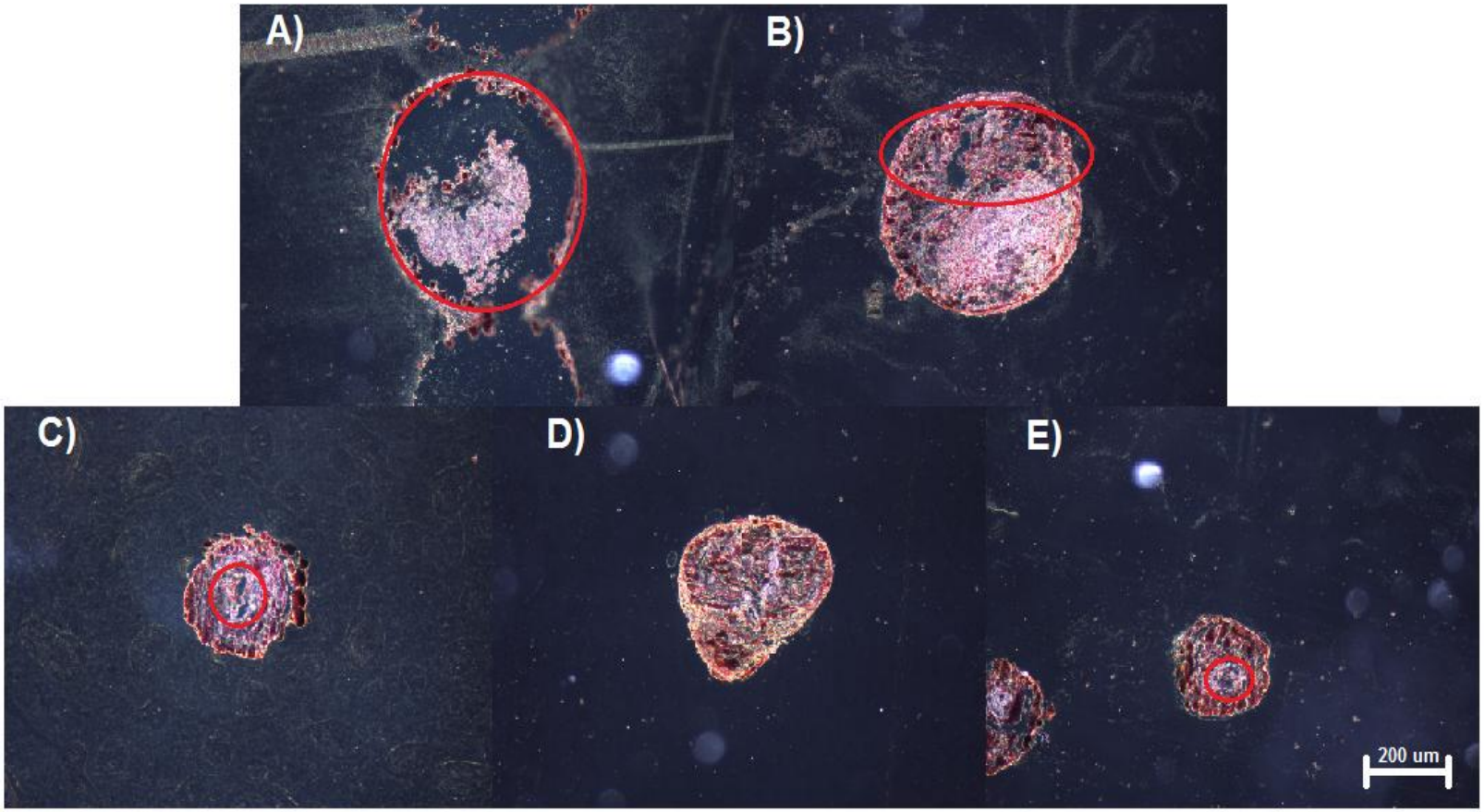

Figure 3.5: Safranin O stained sections of 200k (A and B) and 20k (C, D and E) pellets. Pellets A and C were cultured without any HIF-2 induction at all, while B, D and E was differentiated with HIF-2 inhibition, D had inhibition after day 14 and B, E had inhibition after day 28. There is significantly more visible necrosis in 200k pellets compared to 20k pellets, but comparing pellets that had HIF-2 inhibition it is visible that the earlier the inhibitor was added, the less necrosis occurred.

inhibition is found to have a crucial and definite role in the hypertrophy and cell death, then its inhibition would not warrant the addition of supplemental VEGF.

As ALP activity was measured over the 7 week span, there was no significant difference in the values received between the different groups. HIF-2 has been linked in literature to a role in ALP regulation, a known hypertrophic marker, so it would be odd that through inhibition of HIF-2 there was not a visible difference.[51,56,71,74,78] One possible explanation this could be the cell death that was seen in the safranin O and H&E staining.

As shown by the qualitative results obtained through safranin O and H&E staining, there is a clear advantage in the use of HIF-2 inhibitor even when producing larger pellets. The initial pellet size study, for determining the size of pellet to use for the longer study, revealed similar results to similar studies produced in our lab.[101] Although it was determined that larger pellets are more difficult to use due to issues of necrosis, a set of 200k pellets was performed during the long study to determine if HIF-2 inhibition had positive effects on sustaining cell viability in pellet culture. Not only was this idea confirmed in 200k pellets, but it was also evident that in 20k pellets that were exposed to the HIF-2 inhibitor earlier in the study. This was also similarly seen regarding collagen and GAG content through safranin O staining. One explanation for why there was more collagen in the early inhibitor pellet sets is that there is not as much apoptosis and necrosis present therefore more collagen can be produced. From this study it is learned that there is a possible link between HIF-2 inhibition and survival in 3D ADSC pellets during chondrogenic differentiation no matter the size of the pellet.

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APPENDIX A – ISCHEMIC PRECONDITIONING

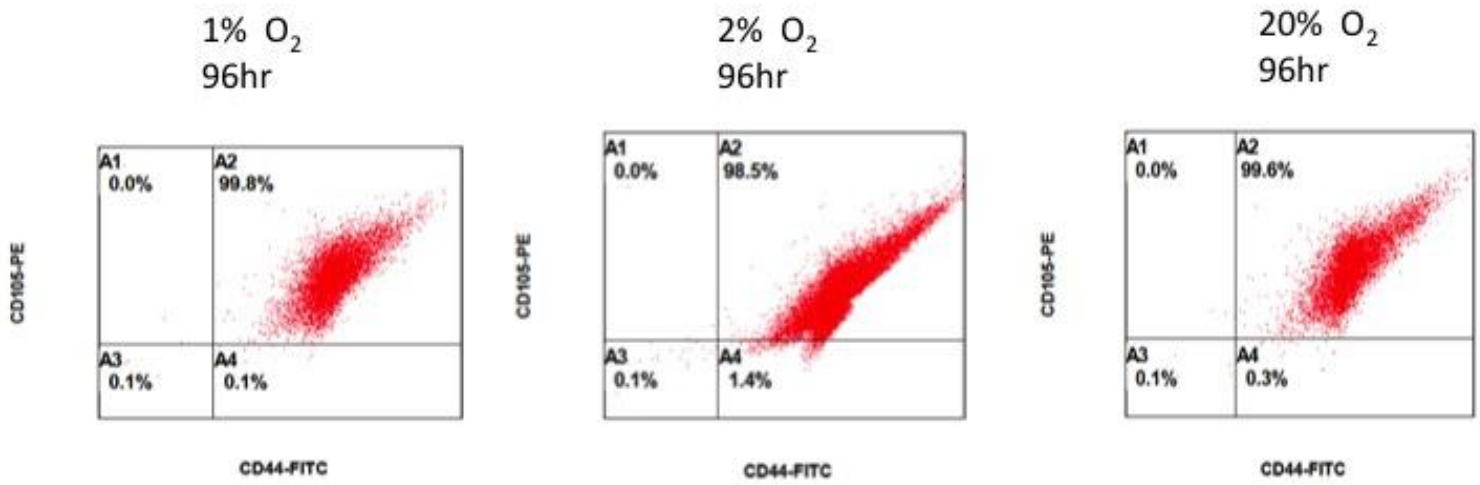

Figure A.1: Flow cytometry of ADSCs after 96 hours of IPC at the three O_2 concentrations of interest.

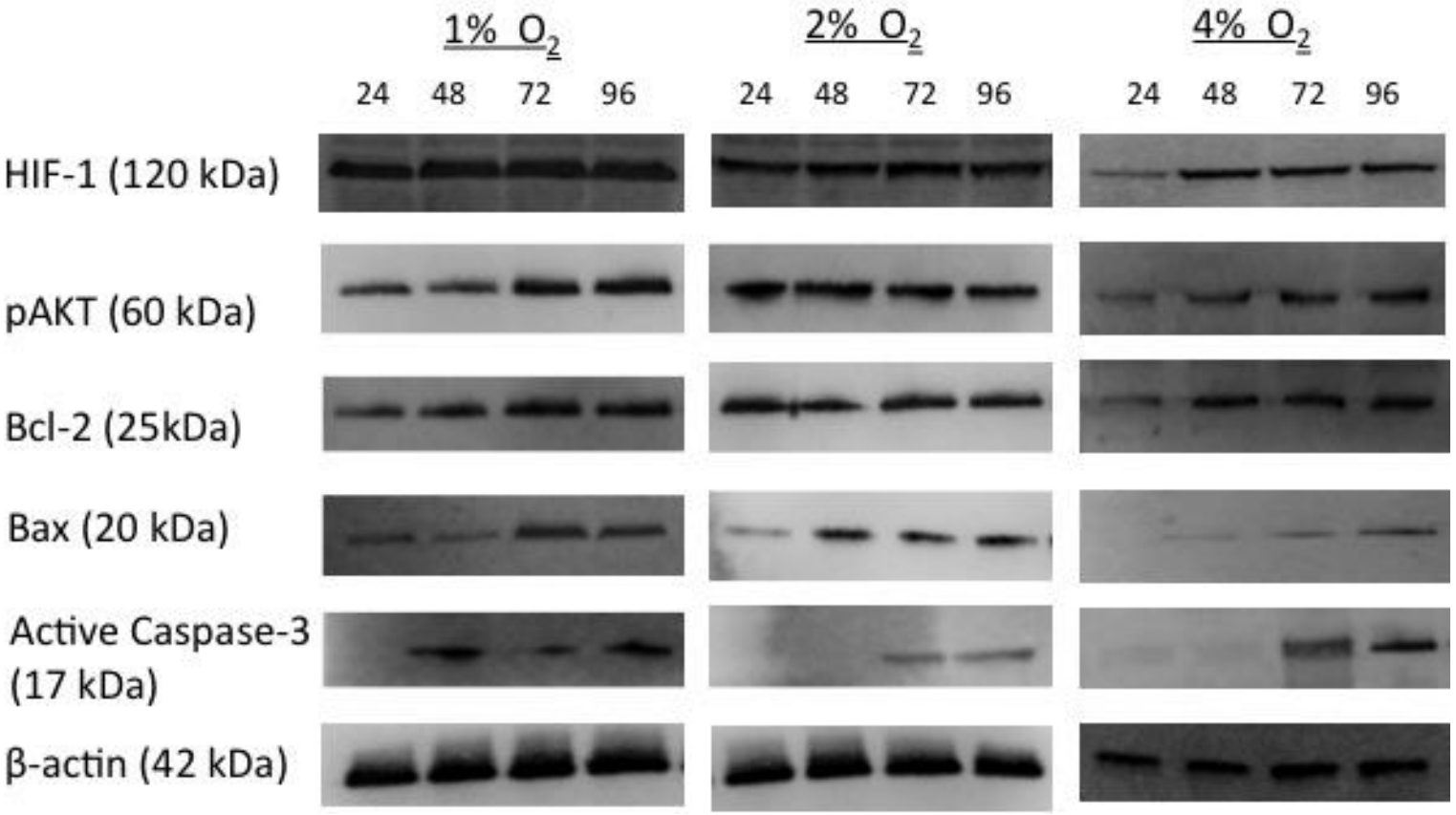

Figure A.2: Western blots showing apoptotic protein expression in the three O_2 concentrations of question at the different IPC time points.