MEDS Clinical Medicine (2022)
Clausius Scientific Press, Canada

DOI: 10.23977/medsc.2022.030505
ISSN 2616-1907 Vol. 3 Num. 5

Extracellular Factors Regulating HIF-1A Activation and Contributing to Cartilage Regeneration and Fracture Healing

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Keywords: Regulating HIF-1A, Cartilage Regeneration, Fracture Healing

Abstract: Cartilage is an essential component of human body tissues. However, due to the insufficient oxygen and nutrition supply, cartilage is generated slowly by human body. In order to repair cartilage fractures, researchers are now looking for ways to stimulate cartilage regeneration artificially. It is known that hypoxia inducible factor 1A, known as HIF-1A, upregulates the chondrocyte formation. Under normoxia, HIF-1A degrades while under hypoxia, HIF-1A molecules accumulates, and chondrocytes are formed. Currently, researchers are investigating cartilage regeneration by increasing the amount of HIF-1A inside human cells. In this paper, the basic understanding of cartilage is provided, and I evaluate the influence of mechanical factors and acidified environments to the presence and stabilization of HIF-1A.

1. Introduction

Biology of the Bone and Cartilage

Bones and cartilages are connective tissues that have significant influences on our body. Cartilage covers up the joint surfaces and renders the bones to move freely, which increases the flexibility of the joints [1-2].

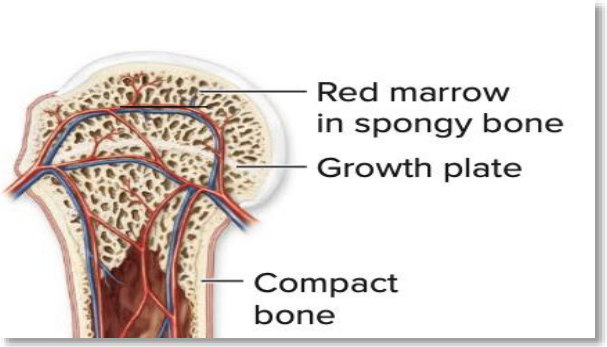

Figure1: [2] The basic structure of bones

The structure of the bone from the inside out consists of the medullary cavity, the spongy bone and the outer compact bone. In addition, the bone marrow consists of blood vessels and nerves which are responsible for nutrient supply.

The bone is composed of bone matrix, which is an important structure inside the bone making up compact bones and spongy bones. Both compact bones and spongy bones are essential component of bones. The main differences between compact and spongy bones are location and mineralization. Specifically, the spongy bones are in the middle and less mineralized with calcium crystal [3].

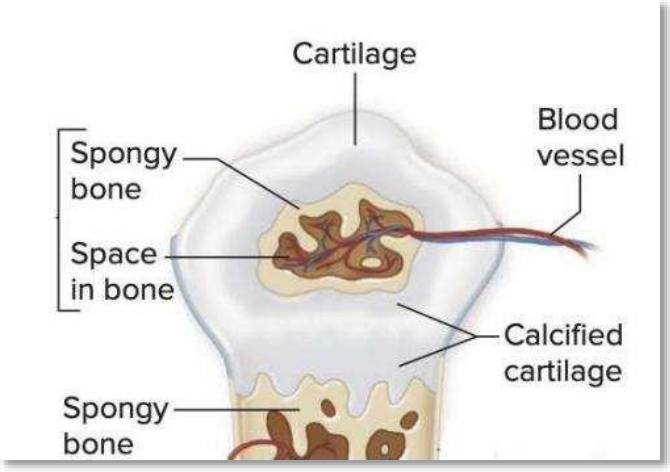

Figure 2: [2] The basic structure and location of cartilage

Cartilage-the main type of connective tissue presents in the human body, with tough but flexible texture. It provides structural support to lightweight movements.

Cartilage is mainly composed of a rigid ground substance that keeps it firm and difficult to compress. Moreover, there is an interface between the bone and the cartilage which contains both bone cells and cartilage cells. The interface is the transition between the bone and cartilage.

Even though bones and cartilages belong to the same tissue, they are different from each other in many aspects. Firstly, they are comprised of distinctive types of cells. The cells that form the bone are osteoblasts, osteocytes, and osteoclasts. Osteoblasts are the immature bone cells that contribute to the formation and reconstruction of bones. Osteocytes regulate bone homeostasis and uptake minerals from blood. Osteoclasts continuously break down osteocytes and dissolve minerals in the bone matrix. The cells that form cartilage are called chondrocytes [3].

Secondly, the matrix that makes up bone and cartilage is different. One of the differences of their matrix is the composition. 90% of the matrix is composed of collagen, which is a kind of tough protein fibers, and the types of collagens in bone and cell matrix are different. Collagen I make up the extracellular matrix in the bone whereas the matrix that consists of collagen II, named lacuna, makes up extracellular matrix in the cartilage.

Thirdly, cartilage is an avascular tissue since it lacks blood vessels and nerves. The underlying bones support the metabolic needs of cartilage by providing necessary nutrients and oxygen. However, bones are vascularized and contains nerves the bone marrow.

Mesenchymal Stem Cells (MSCs) are multipotent cells [2] (difference between multipotent and pluripotent) produced in the bone marrow, differentiate into specialized cells of tissues. MSCs can be formed into adipocytes (fat cells), osteocytes (bone cells), and chondrocytes (cartilage cells). The differentiation of MSCs is determined by many environmental conditions. For example, MSCs will transform into osteocytes under normoxia and transform into chondrocytes under hypoxia. This is regulated by the HIF-1A factors inside our body cells [4].

2. The Importance of HIF-1A and Its Regulation in Cells and Tissues

HIF-1A stands for hypoxia inducible factor-1, which is an oxygen-sensitive transcriptional activator. It mediates many transcriptional inductions of the adaptions to low oxygen stress in cells

and tissues. It is an essential factor for cartilage differentiation and regeneration and maintaining the formation of chondrocytes as well as their matrices. Because of the avascular nature of cartilage, limited amount of oxygen is supplied, and HIF-1A is upregulated in the cartilage because HIF-1A accumulates in hypoxic condition. The abundance of HIF-1A stimulates MSCs to differentiate into chondrocytes. The lower the oxygen level is, the more HIF-1A is regulated, thus more chondrocytes will form.

In normoxia, the proteins of HIF-1A are degraded substantially through the regulation of oxygen. Firstly, HIF-1A experiences a process named hydroxylation, where the hydroxide ions are attached to the HIF-1A molecules. After that, pVHL (von Hippel Lindau) protein targets HIF-1A by ubiquitination, labeling HIF-1A for degradation. Finally, proteosome recognizes labeled HIF-1A and degrades it.

In hypoxia, HIF-1A molecules are stabilized and transported from cytoplasmic matrix into the nuclei, where both HIF-1A and HIF-1B are combined with HRE (hypoxia responds elements) target genes [5].

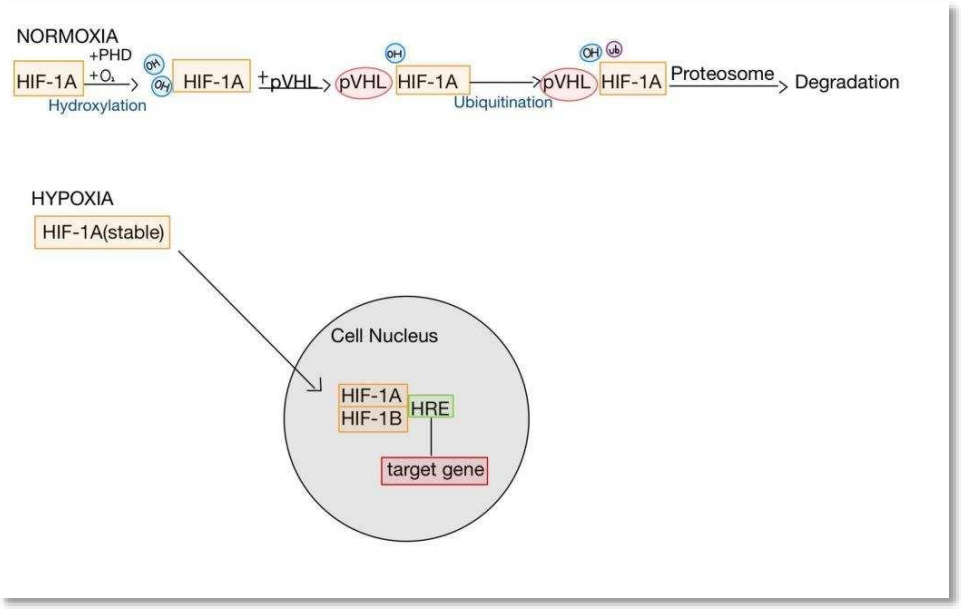

Figure 3: The activation and degradation of HIF-1A

In normoxia, HIF-1A will be degraded through hydroxylation and Ubiquitination. In hypoxia, HIF-1A will be accumulated, transported to the nucleus, and bond with HIF-1B to form a target gene where the HIF-1A is activated.

HIF-1A increases vascularization which is required for bone regeneration after fracturing. When HIF-1A is activated in hypoxia, it allows MSCs to differentiate which supports the attraction of the cell and remove the cell to the fracture gap. Also, HIF-1A improves the growth of the bone and vascularization at the time. The stable condition of HIF-1A not only enhances the calcium accumulation but also prompts angiogenesis which promotes the repair of epithelial tissues. As for chondrocytes regeneration, HIF-1A enables chondrocytes to adapt the oxygen-deprived environment.

When it comes to tissue engineering, it is critical to find multiple methodology to stabilize HIF-1A in order to proliferate chondrocyte from MSCs for cartilage regeneration. When human body generates chondrocyte spontaneously, HIF-1A is only activated in hypoxic condition. However, scientists and researchers have used tissue engineering techniques to regenerate HIF-1A in normoxia because cells cannot function well under hypoxia, which makes chondrocytes hardly exist, so the

strategy is to find ways to upregulate HIF-1A under normal oxygen level. Tissue microenvironment is complex, cells are surrounded by the extracellular matrix with different mechanical and physical properties such as pH, stiffness, temperature, ionic strength, etc. [6]

There are multiple strategies to regulate HIF-1A. For example, the inactivation of PHDs (prolyl hydroxylase domain) by compounds like 2-OG analogs may reduce the degradation of HIf-1A. Three types of PHDs (PHD1, PHD2, PHD3) has the different underlying abilities to hydroxylate HIF-1A. Among them, PHD2, known as limiting enzyme, is most capable of hydroxylating HIF-1A. The elimination of PHD2 by interfering its RNA in normoxia may increase the level of HIF-1A. Additionally, using iron chelators and metal irons (Co2+, Ni2+ and Mn2+) to decrease Fe2+ level may stabilize HIF-1A because of the inactivation of PHDs.

With the technique of increasing HIF-1A, tissue engineers have established several ways contributing to regenerating cartilage and fracture healing. The osteochondral interface is composed of the interaction of calcified cartilage and the underlying subchondral bone. Back in the days, scientists only invented the biphasic cartilage repair implant strategy with the potential to generate cartilage. However, the biphasic construct does not establish the interface between cartilage and the underlying bone, and the artificial cartilage was not as lubricant as the indigenous cartilage, so the effect is not ideal. Recently, doctors are using the triphasic gel method to implant cartilage where there is an interface between the cartilage and the bone. However, the natural intermediate layer shows unique traits because of the infiltration of the blood vessels, and the triphasic construct material cannot mimic such unique feature. Currently, tissue engineers are working on an advanced methodology, including a gradient matrix of bone and cartilage which imitates the human cartilage best. Thus, with the development of the technology, more and more people that suffer from osteoarthritis or exercise friction will be cured efficiently [7-8].

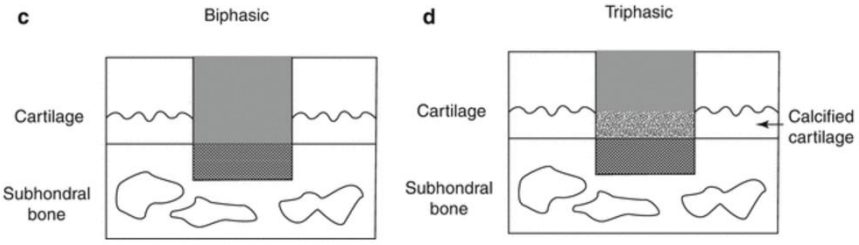

Figure 4: The biphasic cartilage repair implant strategy and the triphasic cartilage repair implant toward cartilage regeneration

(C) is the biphasic method which does not have the intermediate layer between the cartilage and bone. (D) The triphasic strategy has an interface that contributes to a fluent transition from cartilage to bone. More recently, investigators proposed a new strategy for treating people with osteocartilage damage. The continuous gradient scaffolds are a gradient of hydrogel. It creates a steady transition from the subchondral bone to the articular bone.

3. Hypothesis

Properties of the cellular microenvironment in tissues may be used to regulate HIF-1A in osteo-cartilage regeneration or fracture healing Under acidic environment, the stiff matrix, or in the presence of mechanical stimulation (shear stress and inflation), HIF-1A expression will be upregulated to have an impact on the cartilage regeneration and fracture repairment.

Synthetic materials such as gels that recapitulate the osteo-cartilage regeneration can be tailored based on pH and mechanical properties to improve osteo-cartilage regeneration and fracture healing.

4. Analysis of Results

4.1 Evidence 1

My first evidence of the influence of mechanical stresses on HIF-1A regulation is from an essay called Early Expression of Myocardial HIF-1A in Response to Mechanical Stresses [9]. It is mainly about the upregulation of HIF-1A when stretching rat's hearts. It is found that the expression of vascular endothelial growth factor (VEGF) is shown when the heart is stretched, and it is known that VEGF-A is upregulated by hypoxia-inducible factor-1 (HIF-1A) in ischemic tissues and growing tumors. Thereby, the researchers in this essay investigated the possible role of HIF-1A in stress-mediated induction of VEGF in the rat heart. They used three ways of raising the tension of left ventricular wall, namely, by inserting a balloon inside the ventricle of the mice, by inducing ischemia, and by producing bloodstream overflow using an aortocaval shunt (ACS). The essay suggests that the level of HIF-1A is related with mechanical stress, which points to the notion that my hypothesis is correct. Applying mechanical stresses to human tissues will increase the amount of HIF-1A inside cells.

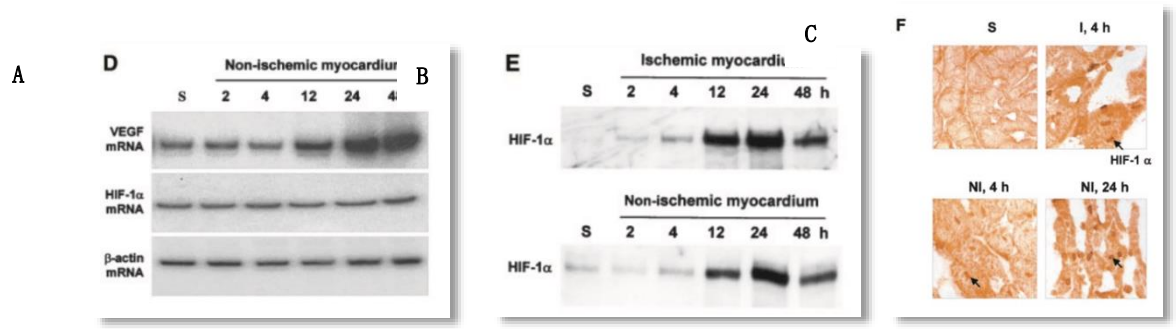

Figure 4: Mechanical stimulation increases the levels of VEGF and HIF-1A

(A) illustrates the mRNA of VEGF, HIF-1A and B-actin in non-ischemical myocardium after 2, 4, 12, 24, 48 hours using RT-PCR technique. (B) demonstrates HIF-1A expression in the 2, 4, 12, 24, 48 hours period by applying immunoprecipitation and immunoblotting. The pictures of regional ischemia cells are showed in (C), which takes the advantages of immunohistochemistry and shows the location of the cell, nuclei and HIF-1A.

From figure 4 panel A, shows that the mRNA of VEGF doesn't increase after 2, 4 hours. However, it soars from 4 to 12 hours and continues to grow after 24 and 48 hours. B-actin is introduced as a control, and the gene expression of HIF-1A does not change throughout the time. Therefore, the transcription of HIF-1A is stable whereas the protein of HIF-1A increases as the gene expression of VEGF rises.

Figure. 4(B) supports the conclusion in figure. 4(A) that the amount of protein in HIF-1A increases substantially after 12, 24 hours in both ischemic and non-ischemic myocardium. But it has a downward trend in 48 hours as the protein is degraded in that period.

Figure 4(C) further agrees the results in the previous paragraphs. HIF-1A antiserum is injected into the cell and stained with avidin-biotin-peroxidase. Therefore, the colored area shows immune response. Sham group is the control group that does not affect by the HIF-1A antiserum. On the contrary, HIF-1A increases over time as it is showed in the other three plots.

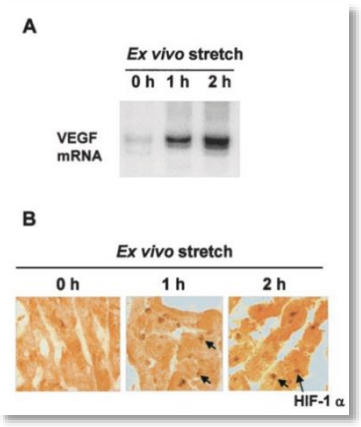

Figure 5: Mechanical stresses activate HIF-1A

(D) shows the genetic expression of VEGF of the myocardium in rats when researchers stretched it in vitro. (E) uses the immunohistochemistry technology and demonstrates the presence of HIF-1A in the nucleus (where HIF-1A is activated). The arrows point to the location of the nucleus. This figure was adapted from.

In figure 5(D), researchers took the rat's heart out of its bod, inserted a relax balloon, and inflated the stretch of 35mm Hg. After 1 or 2 hours, the gene expression of VEGF was measured. Total RNA was analyzed by semi-quantitative RT-PCR after extracting from the stretched ventricular myocardium. Overall, the mRNA of VEGF increased substantially after 1 and 2 hours.

Figure 5(E) illustrates the HIF-1A protein immunohistochemical analysis. The researchers stretched parts of the HIF-1A antiserum incubated heart tissue and used the avidin biotin to stain the cell. It shows that the area that had stained darker was the nucleus, and HIF-1A tended to accumulate in the nucleus, so HIF-1A was activated.

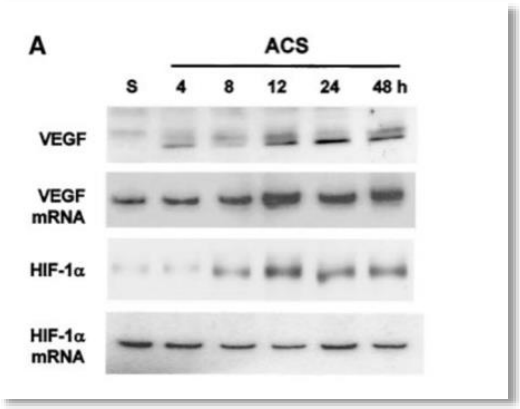

Figure 6: Further evidence for the effect of mechanical stumuli on HIF-1A regulation

(F) shows the VEGF and HIF-1A expressions in rat's stretched myocardium in vivo.

From figure 6, researchers used ACS to examine the quantity. As a result, both protein and gene expression of VEGF increased over time. However, though the protein of HIF-1A increased, the HIF-1A mRNA did not show apparent changes, which indicates that the transcription of HIF-1A was not the factor of the increased amount of HIF-1A.

4.2 Evidence 2

The study of the shear stresses regulating HIF-1A was from the essay called Mechanical

Activation of Hypoxia-Inducible Factor 1α Drives Endothelial Dysfunction at Atheroprone Sites [10]. It primarily says that when ligated arteries receive a shear pressure, the HIF-1A inside the cells will increase, which causes the hyperplasia of blood vessel because HIF-1A induces vascularization. This process can easily lead to thickening of the blood vessel walls and, eventually, arteriosclerosis. Shear stress upregulates HIF-1A through two ways. The first one is increasing transcription factor nuclear factor, which increases the gene expression of HIF-1A. The second one is reducing an enzyme called Cezanne, which terminates the process of HIF-1A degradation. Thus, this material has a great impact on my topic as it testified my hypothesis that under the influence of mechanical factors, low shear stress, HIF-1A will be increased.

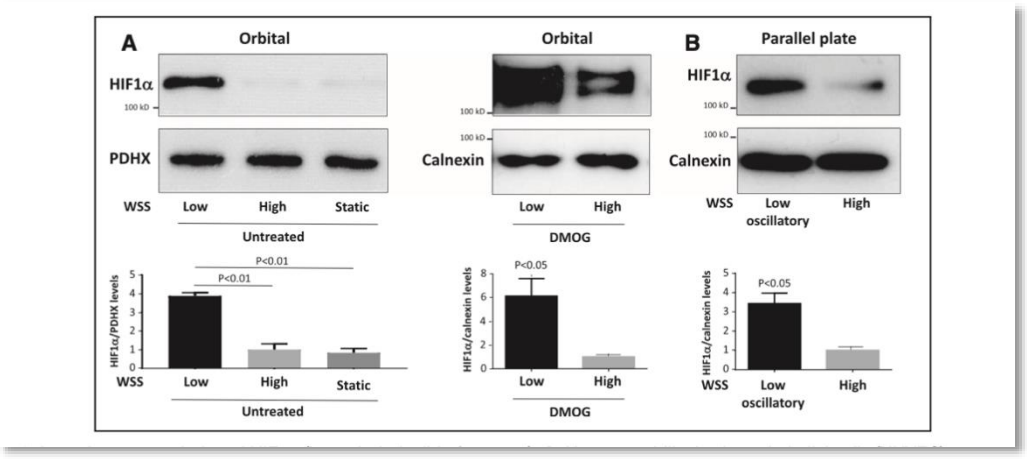

Figure 7: Orbital Shear stress activates HIF-1A

(A) shows that the human umbilical vein endothelial cells (HUVEC) were treated or untreated with dimethyloxalylglycine (DMOG) after 72h. Before that, they exposed the human umbilical vein endothelial cells (HUVEC) in low (5 dyn/cm2) or high (11 dyn/cm2) wall shear stress (WSS) with orbital force or kept in static. In (B), HUVEC was kept in parallel plate system for 72 hours. Noticeably, the levels of HIF-1A were presented by the western blotting which took the advantage of antibodies to perform. PDHX in (A) and Calnexin in (B 0are the control group in the experiment. The mean quantity of HIF-1A expression ±SEM is presented.

From figure (A), we can see that HIF-1A is only expressed in low WSS (wall shear stress) and untreated with DMOG. There was almost no expression for HIF-1A under high WSS and static condition. The quantity of the low WSS was nearly 4 HIF-1A /PDHX levels and had a P-value less than 0.01, which indicates significant differences.

From figure (B), it showed the similar results that the expression of HIF-1A was abundant at low WSS and expressed a little when the WSS was high. It also indicates significant difference between high and low WSS, as the P-value is less than 0.05[10].

4.3 Evidence 3

The study of Tamoxifen mechanically reprograms the tumor microenvironment via HIF-1A and reduces cancer cell survival [11]shows that Tamoxifen reduces the activation of HIF-1A through the inhibition of myosin dependent contractility and mechanical sensing of matrix stiffness. On top of that, Tamoxifen can also inhibit the gene expression of HIF-1A. The way of increasing the level of HIF-1A using Tamoxifen is by soften the contractility of the matrix.

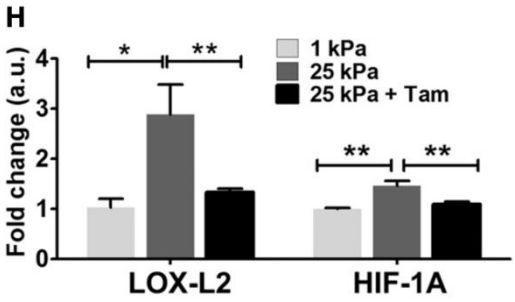

Figure 8: (H) LOX-L2 stands for lysyl oxidase homolog-2

Fig.8 has a similar resulting trend as HIF-1A after the experiment and it has an effect on treating cancers. Investigators put cells in different rigidities. 1kPa is the soft matrix while 25kPa is the stiff matrix. The third group was the stiff matrix with the presence of Tamoxifen. The result shows that HIF-1A will be deactivated in smooth matrix whereas upregulated in stiff tissues, and Tamoxifen is able to soften the tissue in order to decrease the level of HIF-1A. To sum up, the figures shows that when cells are plated on stiffer matrices or have increased endogenous contractility, the levels of HIF-1A gene expression increased.

4.4 Evidence 4

The evidence of how acidified environment influence the amount of HIF-1A was suggested in the essay named *HIF activation by pH-dependent nucleolar sequestration of pVHL* [12]It illustrates the relationship between HIF-1A and pH value. It is known that von Hippel-Lindau (VHL) molecules are used to degrade HIF-1A molecules. In acidic environment, von Hippel-Lindau (VHL) are sequestered into the cell nucleus where VHL and HIF-1A do not react with each other. VHL will only degrade HIF-1A in the cytoplasm. As a result, acidosis will inhibit the degradation of HIF-1A.

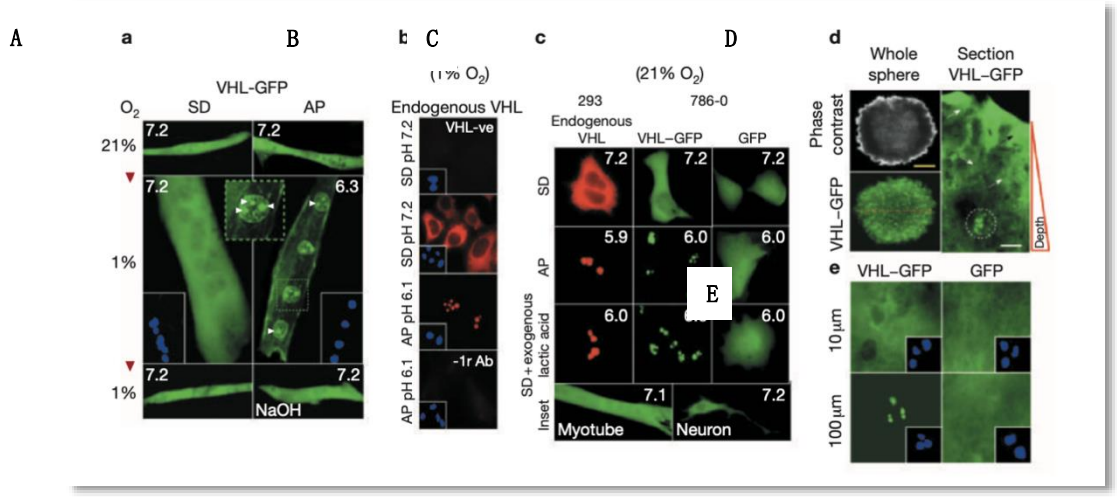

Figure 8: [12] Low extracellular pH sequesters pVHL in the nucleus and reduces HIF-1A degradation.

Figure (A) demonstrates how the modification of oxygen level and acidity influence the situation of location of VHL-GFP. (B) and (C) tested the location for endogenous VHL, VHL-GFP, and GFP using the same method as figure (A). (D) and (E) specify the precise area when VHL was inside the nucleus, which is the spheroid.

In figure (A), researchers compared two groups which were standard (SD) and (acidification

permissible) AP. The C2C12 differentiated myotubules expressing adenovirus introduced VHL-GFP were placed in the two media. At first, they were in the same oxygen level, 21%, with the pH of 7.2. It was apparent that the cell is completely colored with green, so VHL molecules were in the cytoplasm. Then, the researchers changed the environment by degrading the level of oxygen from 21% to 1%. The location of VHL-GFP was the same as in normoxic condition. However, as they lower the pH value in AP medium, the regions of the nucleus were stained with more color, which indicated that VHL-GEP had transported into the nucleus. The last step was the inverse process of the beginning. The pH value was modified back to 7.2 by adding NaOH. The consequence was that the location of VHL-GFP went back to the cytoplasmic matrix, which indicated that the relocation of VHL is reversible.

Figure (B) and figure (C) showed the similar results in terms of the relationship between acidity and the location of VHL. This time, the researchers used endogenous VHL, VHL-GFP, and GFP to compare. The result shows that both endogenous VHL and VHL-GFP relocated when pH value was lowered, which indicates that not only VHL produced in human body but also other types of VHL could relocate under acidified condition. The result is supported both in myotube and in neuron cells.

5. Discussion of Results

In this essay, I introduced the basic structure and function of cartilage as a result of illustrating the factors that regulates chondrocytes. After reading essays about regeneration of cartilage and fracture healing, I was aware that the regulation of cartilage is all linked to the level of HIF-1A—hypoxia inducible factor 1A. So, the next step I did was that I searched literature about factors that influence HIF-1A. Then, I devised the hypothesis which matches every factor that I collected, illustrated the principle of each factor, summarized the experiment that other researchers conducted, and finally, analyzed the results of each experiment to see if the conclusion agrees with my hypothesis or negate with it.

For the first evidence, I focused on the effect of mechanical stresses on HIF-1A regulation. Researchers used three ways to strengthen the tension of rat's hearts and examined the level of HIF-1A in order to tell if these two are linked. It shows that the amount of HIF-1A will be increased by applying mechanicalstresses to human tissues. In the second evidence, I found another stress that regulates the activation of HIF-1A, which is shear stresses. As a result, HIF-1A promotes when ligated arteries receive a shear pressure. This will lead to the possibility of thickening the wall of blood vessels. The third evidence was about the stiffness that influences the generation of HIF-1A. This essay was aimed at finding the possible ways of reducing the survival of cancer cells. However, both cartilage regeneration and cancer cells are related with the regulation of HIF-1A, thus I chose this essay as another factor to analyze. The results demonstrate that when cells are plated on rigid materials, the levels of HIF-1A gene expression increased. Finally, I worked on the relationship between pH and HIf-1A. The conclusion was clear that acidic environment will degenerate the level of HIf-1A.

In conclusion, the first three of my evidence agrees with the previous hypothesis whereas the last evidence negates with it. This led me to my next thinking—the actual treatment for people who have suffered painfully from cartilage related diseases such as cartilage damage or even choriocarcinoma. Cartilage implant is a treatment for damage condition. I have listed the traditional way of treating it (biphasic and triphasic) in the introduction part. However, I would like to emphasize the upcoming method of fracture healing which is hydrogel gradient. It can mimic the gradients of HIF-1A activation and boost pH-driven chondrogenic differentiation in MSCs. If the strategy comes into use, it will improve the treatments.

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