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# Bioengineering approaches for tissue engineering of bone cartilage

Chunyu Cao
*Haidian Foreign Language Academy*

***Abstract:*** *Cartilage is a connective tissue and doesn't contain blood vessel or nerve, which let it become a forgotten organ. When people feel the painful sense from cartilage, it has already been a dangerous place. Before the exploration in bioengineering, transplantation is one of solutions to treat this disease. However, patients have to face the hurt sense of rejection reaction. Nowadays, HIF-1alpha was found, which is transcriptional factor of vascularization and is a possible way to repair the damaged cartilage. In order to regulate this molecule, scientists have already conducted many experiments on it to find its dependable factors. Besides the existence of oxygen, three ways of mechanical stress and pH-value also can affect the HIF-1alpha.*

***Keywords:*** *Cartilage, HIF-1alpha, stiffness, pH-value*

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## I. Introduction

Structure of the bone cartilage interface:

High performing athletes can often damage their cartilage as well as senior citizen that suffer from osteoporosis. This have sparked interest in creating artificial bone cartilage devices that can be used in tissue engineering. However, it is needed to have a good understanding of the bone interface. The bone-cartilage interface is composed of several layers like tidemark, calcified zone of cartilage, which locates in the area between deep-cartilage level and underlying subchondral bone1. Inside of the zone of calcified cartilage, there are abundant type X collagen, hypertrophic chondrocyte, and other enzymes, alkaline phosphatase. (as shown in Figure 1 )

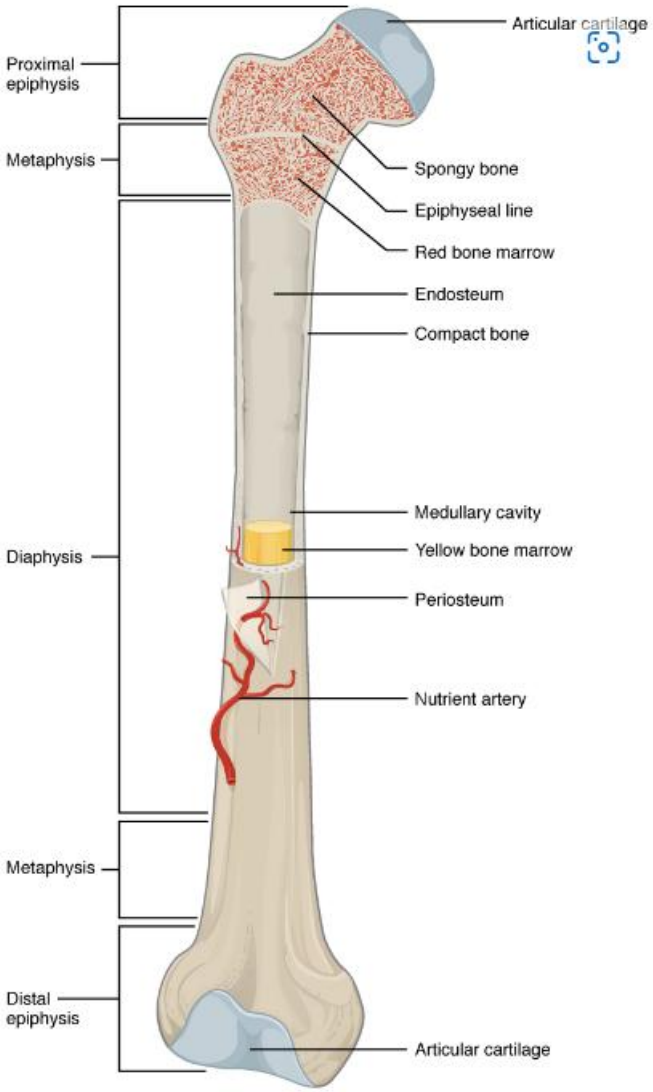


**Figure 1 The structure of bone.**
**Taken from: https://opentextbc.ca/anatomyandphysiologyopenstax/chapter/bone-structure**

Although both bone and cartilage belong to connective tissue, they have significantly different structures and components. The most striking difference is the cell type in the tissue. It is defined that either cells in the bone or in the cartilage, they all come from MSCs (Mesenchymal Stem Cell) which are stem cells that perform differentiation of cells and regeneration of injuries in the body. However, MSCs differentiate into osteoprogenitor cells in the bone marrow and eventually become osteocytes. This is a process highly controlled by the factors present in the extracellular matrix of tissues. In the youth people, MSCs form osteoblast which is a productive cell that synthesizes most of substances in bone's extracellular matrix and serves the function of generating osteocytes. Osteocytes are the proper cells in the bone that perform most critical functions to keep the homeostasis of this type of tissue. Osteocytes can be considered mature osteoblasts and they are surrounded by a thick and significant mass known as extracellular matrix. This mass is made mostly of collagen type I. There is another type of cell, osteoclast, which are derived from monocytes and perform bone resorption. These cells are also very important for bone functioning. However, older people used to have an unbalanced proportion of osteoblast versus osteoclast, which means that much more bone is destroyed than produced. Consequently, bones get thinner and more prone to fracture.

In the cartilage, MSCs differentiate to chondrocyte. The precursor of chondrocyte is chondroblast and creates extracellular matrix containing fibers and ground substances to surround itself and provide nutrition of growth. Cartilage has no blood vessels or nerves. Thus, this type of tissue needs to store much required nutrients in the extracellular matrix. Similarly as in bones, there are three types of cells in cartilages: Chondroblasts,

chondrocytes, and chondroclasts. All with equivalent functions in the cartilage.

Large number of people mistakenly think that bone is a non-living organ. In fact, comparing to the cartilage, there are vast numbers of blood vessel in or surrounding the bone. The central artery enters the bone through foramen, branches into abundant small arteries and arterioles to supply oxygen and other nutrition, and finally converged into vein to remove wastes produced by osteocytes2. And there are several specific arteries attach to the bone to work as the same function. These arteries and veins form an extensive network on bone and these vascular systems on bone consume 10-15 percent resting cardiac output3, 4. Circulated blood in the artery and vein provides necessities of bone formation or bone remodeling like oxygen and other nutrients. During fracture healing, blood vessel functions in the first step, inflammation, forming blood clot to eliminate damaged cell and to provide repair cells. After that, endothelial cells release vascular endothelial growth factors (VEGF) to proceed angiogenesis in fracture5, 6. Cartilage is an avascular tissue, because most of cells absorb their survival necessities by the mechanism of diffusion. After the synthesis of ground substance by chondroblasts, these ground substances enclose the chondrocyte inside and supply nutrition the cell needed to grow. Because of lack of blood vessels in cartilage, cartilage tissue continuously stays in a hypoxia condition and has a higher level of HIF-1 alpha than in the bone tissue.

Furthermore, the compositions of the extracellular matrix (ECM) in bone and cartilage are disparate. TheECM in cartilage consists mostly of collagen II, and sulphated Glycosaminoglycans (GAGs). The ECM in bone consists of collagen I, RUNX2, MMP13, and Sox9. This different characteristic is used in markers for differentiation and helps researches to distinguish bone and cartilage.

Hypoxia inducible factor 1 alpha subunit (HIF-1A) combines with HIF-1B to form a regulator of homeostasis during hypoxia. There is a common case that people who climb the mountain tend to become anoxic when they arrive at a high altitude. In this case, HIF-1A helps their host to stimulate the formation of red blood cells.

HIF-1A does not only work in hematopoiesis, but it also play an important role in articular cartilage. Chondrocytes originate from bone marrow cell. In the early stage, skeletogenesis, HIF-1alpha serves as an stimuli of SOX9 expression which converts the hypoxic prechondrogenic cells to chondrocyte7. Beside this, HIF-1alpha also maintains the survival of chondrocytes and the composition of extracellular matrix. In articular cartilage, chondrocytes normally survive in hypoxic conditions. They need to used anaerobic respiration to generate energy for live and production. However, the amount of free ATP in the chondrocyte that are cultured at high oxygen level is much lower than the chondrocyte cultured at hypoxic level with high concentration of HIF-1A8. The transcriptional factor HIF-1A also gets involve in the production of collagen, a kind of protein that fill full in ECM9, 10.

Under hypoxic condition, there is no oxygen that can be used to begin proteasomal degradation. The HIF-1alpha combines with the HIF-1beta to form a transcriptional factor of vascular endothelial growth factor (VEGF). After the vascularization in damaged bone, mesenchymal stem cells can be transported by the blood vessel to the facture, differentiate to osteoblast, and begin the osteogenesis, one of two process of bone formation.

It is unambiguous that HIF only can exist in hypoxic condition because of the oxygen-dependent HIF-1 alpha degradation. Although HIF-1 consists of HIF-1alpha and 1 beta, only HIF-1 beta can remain in normoxic condition. In the environment, PHD, an enzyme, that detects the HIF-1alpha, it hydroxylated the HIF-1 alpha on residues 402 and 564 two genic position. And then, pVHL adds a poly-ubiquitin tail, a label that can be detected by proteosome to perform proteasomal degradation. (as shown in Figure 2)

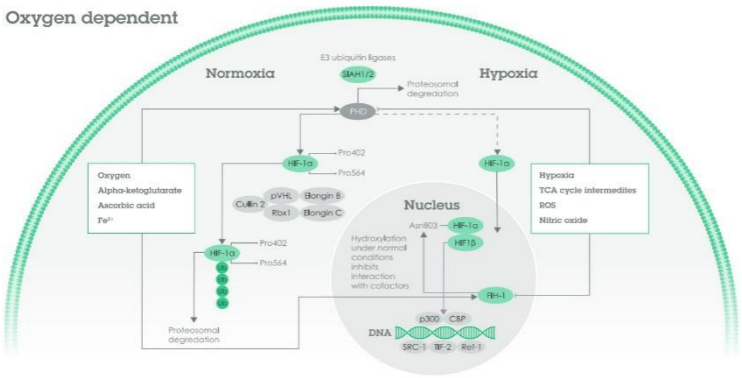


**Figure 2. oxygen independent HIF-1alpha degradation - Bing images**

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Although the existence of oxygen perishes the HIF-1apha by proteasomal degradation, oxygen is essential for the body health such as aerobic respiration. Therefore, in order to make sure HIF-1alpha and oxygen existed together, hypoxia mimicking compounds were found. Dimethylallyl Glycine is a kind of PHD inhibitor and is used as hypoxia mimicking compounds to maintain the amount of HIF-1alpha.

It is evident that DOMG can be acted as hypoxia mimicking compound in the experiment and make sure the existence of HIF-1alpha from proteasomal degradation. However, the drawback of using hypoxia mimicking compounds is distinct. It is hard to achieve its function in the treatment of human body, because this kind of compound like DMOG doesn't only act as PHD inhibitor, but it also shows other functions in body. This kind of uncertainty cannot be applied to medical care.

Apparently, low oxygen level is the direct factor on triggering the expression of HIF-1A. But under the hypoxic condition, it is unable for cells like MSCs to survive and differentiate to chondrocytes. Although using the hypoxia mimicking compounds to stimulate the HIF-1A expression seems to be feasible in vivo, this kind of compound also trigger other unexpected reactions in the human body. In order to regulate the expression of HIF-1A, scientists have already explored several natural physiological agents such as stiffness and pH-value, to direct chondrocyte differentiation

## II. Results

It was believed in the scientific community that the regulation of HIF-1A is related to the oxygen level. However, the environment surrounding cells in tissues are affected by many other factors such as temperature, acidity, and mechanical properties. These factors may also modulate HIF-1A in an oxygen independent manner. But it is impossible for cartilage regeneration to proceed in an ischemic condition. Furthermore, in the 21st century, a large number of scientists speculated there are other factors that can regulate the amount of HIF-1A.

### 1. Mechanical regulation of HIf-1A

In 2002, Kim and collaborators concluded that under mechanical stress in the mouse's left ventricle, the gene expression of VEGF is induced and increasing amount of HIF-1 alfa, which accumulates in the nucleus of myocardiocytes. In this experiment, they inserted soft balloon and inflated it to 35 mm Hg for 1 or 2 hours in the ventricle of an isolated heart. Then, (as shown in Figure 3) which represents the quantification of the VEGF mRNA, the bands in the graph became denser or more, as the stretch time extended from 0 hour to 2 hours. Also, when the stretching time is prolonged, the density of HIF-1 alfa protein become denser in the nuclei. (as shown in Figure 4Figure 5**Error! Reference source not found.**) This observation suggests that HIF-1A may be activated and in the nucleus.

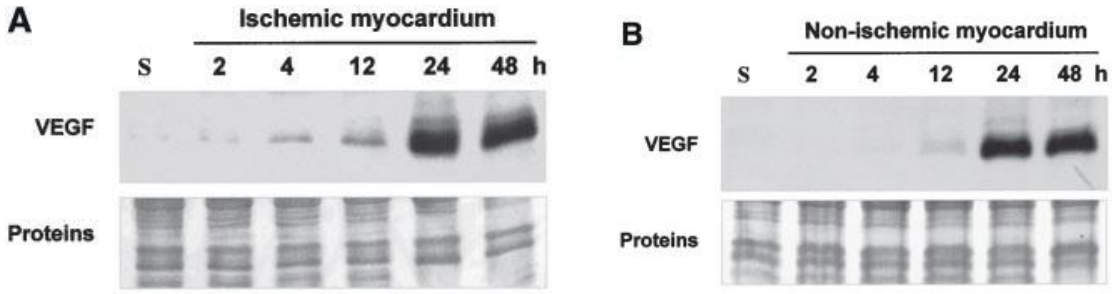


**Figure 3. Regulation of VEGF by mechanical stretching.**

In this figure we can see how vascular endothelial growth factor (VEGF) is upregulated after 12 h of mechanical stimulation. The bands represent the expression of protein taking by western blot (immunoassay). (A) Positive control for the regulation of VEGF in the absence of oxygen. (B) Mechanical regulation of VEGF occurs. After 24 h in myocardiac cells submitted to stretching. Adapted from Kim et al11.

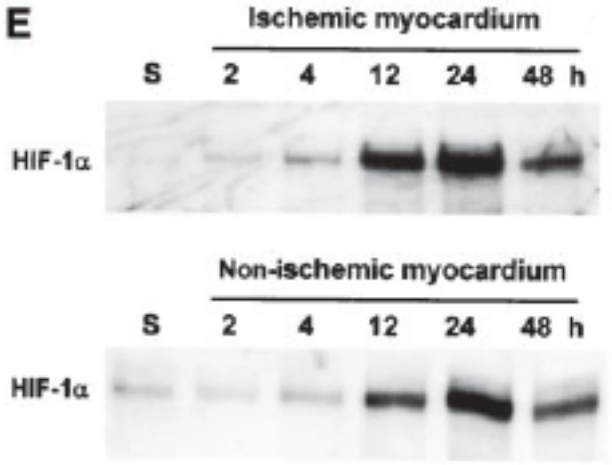


**Figure 4. Regulation of HIF-1a by mechanical stretching.**

HIF-1A is regulated at the protein level when cells are stretched after 12 h and this effect is maximum at 24 h. After this time, the effect stabilizes in a plateau. Adapted from Kim et al11.

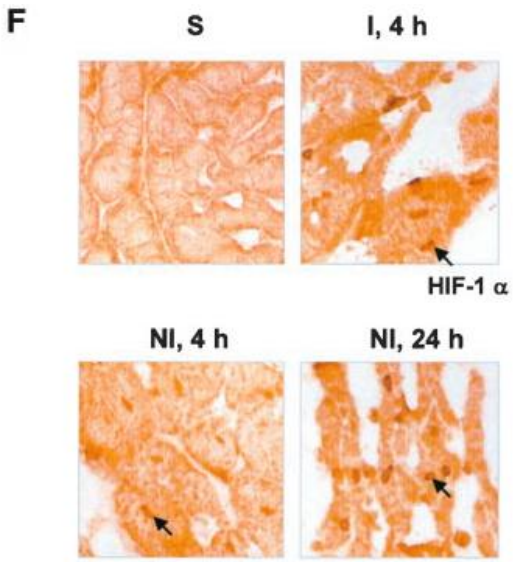


**Figure 5. HIF-1A is accumulated in the nucleus of tissues that are mechanically stimulated.**

(S) means sample of native tissue (control). I represents ischemia or lack of oxygen. This is a positive control to demonstrate that in the absence of oxygen HIF-1A localized in the nucleus. NI is non ischemic, and mechanically regulated. At 4h and 24h multiple nucleus in the tissues are filled with HIF-1A. Adapted from Kim et al11.

Besides Kim team's experiment, other experiments also provide the evidence that HIF-1 Alfa is regulated by mechanical stress in completely different ways.

**i. The experiment 2:**

Feng and collaborators showed that the activation of HIF-1 alfa at the atheroprone regions of artery is induced by mechanical shear stress. Comparing to the first Kim's experiment, they changed the location of HIF-1 alfa from mice's myocardium to endothelial cells,. In these experiments, authors used endothelial cells coming from the wall of blood vessels. This is different from the first experiment that used myocardyocytes in the heart of mice.

In these experiments, authors submitted the endothelial cells to low shear stress using unidirectional and multidirectional flow with a parallel plate and an orbital shaker, respectively.

First, authors quantified the amount of mRNA for HIF-1A and observed an upregulation of 50% for this molecule under low shear stress stimulation. (as shown in Figure 6)

Following this, authors used Immunofluorescence techniques to quantify the levels of HIF-1A in endothelial cells of blood vessels. They observed a five-fold increase in the expression of HIF-1A when cells are stimulated with low shear stress compared to high shear stress. (as shown in Figure 7)
In Biology, western blot is the standard technique used to quantify the expression of proteins. Authors used western blot to demonstrate that applying low shear stress using the orbital shaker (multidirectional stimulation) or the parallel plate (unidirectional stimulation), an increase in the expression of HIF-1A is observed under low shear stress. When authors used DMOG to mimic hypoxic conditions, the observed effect is much more pronounced. (as shown in Figure 8)

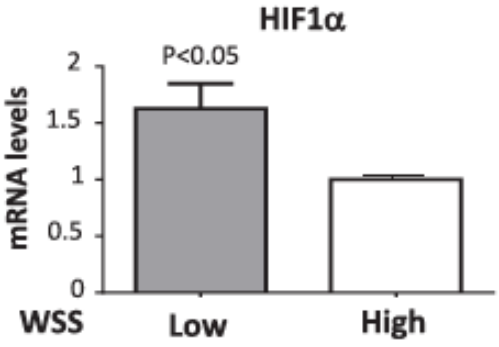


**Figure 6. HIF-1A is upregulated transcriptionally in blood vessels by shear stress.**

(A) mRNA levels of HIF-1A are increased 50% when low shear stress is used to mechanically stimulate rat aorta endothelial cells. Taken from Feng et al12.

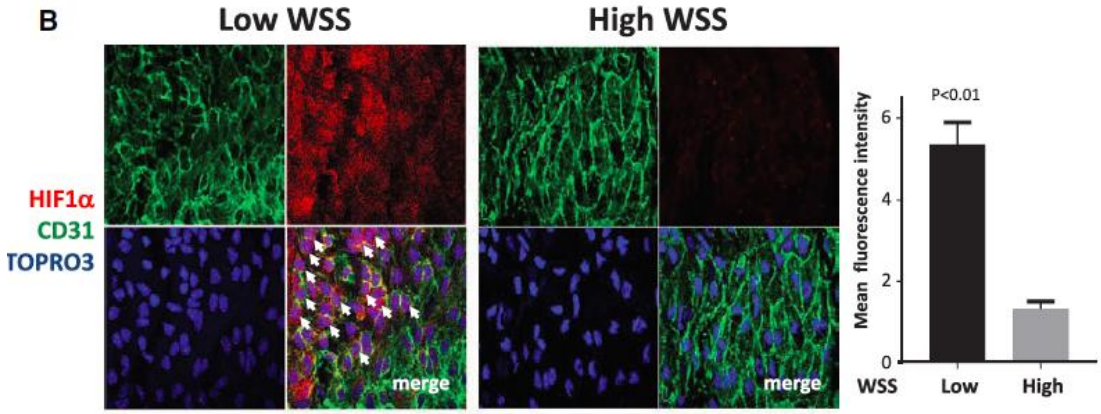


**Figure 7. HiF-1A is upregulated translationally in blood vessels by shear stress.**

When mechanically stimulated (low WSS) HIF-1A protein is significantly upregulate. CD31 is a marker of endothelial cells, TOPRO3 stain nuclei. Taken from Feng et al12.

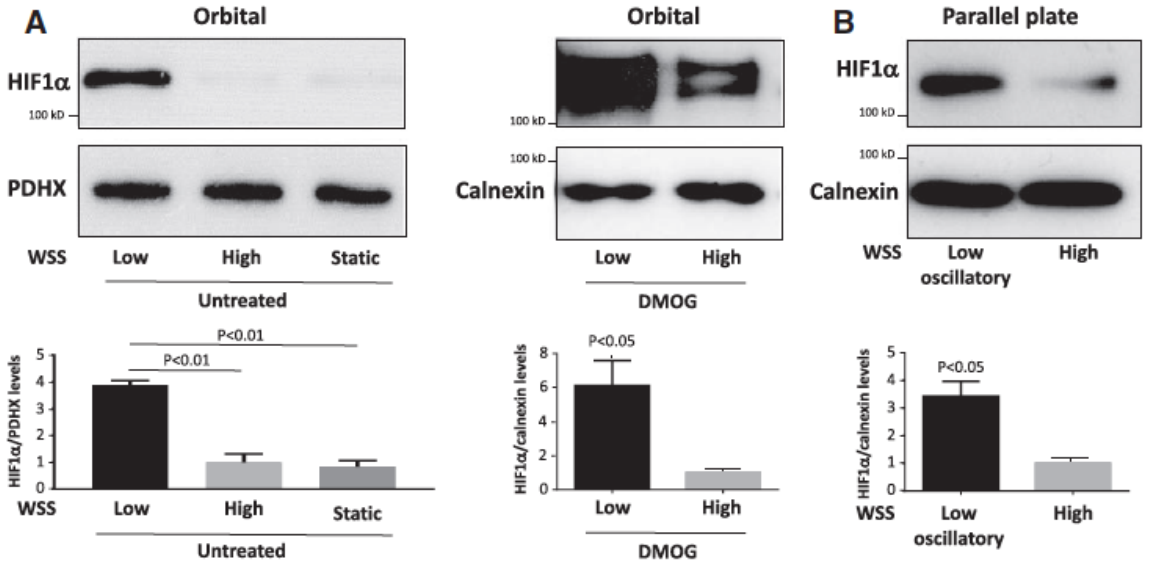


**Figure 8. Western blot is used to demonstrate that HIF-1A is mechanically stimulated by shear stress.**

In the left panel and middle panels, authors used an orbital shaker to demonstrate that HIF-1A is elevated when low shear stress forces are applied (mechanical stimulation).DMOG is a PHD inhibitor, which mimics hypoxia conditions. PDHX and calnexin are housekeeping proteins. Taken from Feng et al12.

**ii. Experiment 3:**

Cortes and collaborators examined a mechanical way to regulate the transcription and translation of HIF-1A, which is different with two experiments above. They showed that the expression of HIF-1A in the pancreatic ductal adenocarcinoma patient's extracellular matrix can be controlled by the degree of stiffness. These scientists studied this phenomenon by simulating the cancer microenvironment on mice.

Authors cultured pancreatic stellate cells (PSCs) on poly-acrylamide (PAA) and divided into three groups with different stiffness.

At first, authors observe the quantity of HIF-1A mRNA in 25 kPa PAA is 1.5 times of the HIF-1A mRNA quantity.Then, they designed an experiment in order to testify whether there is clear relationship between contractility and expression of LOX-L2 and HIF-1A. In the PAA, they transfer PSCs which contain active MLC-2, a protein works for increasing rigidity. Comparing to the control group, the fold change of groups with Active MLC-s has significant increment, especially LOX-L2. (as shown in Figure 9)

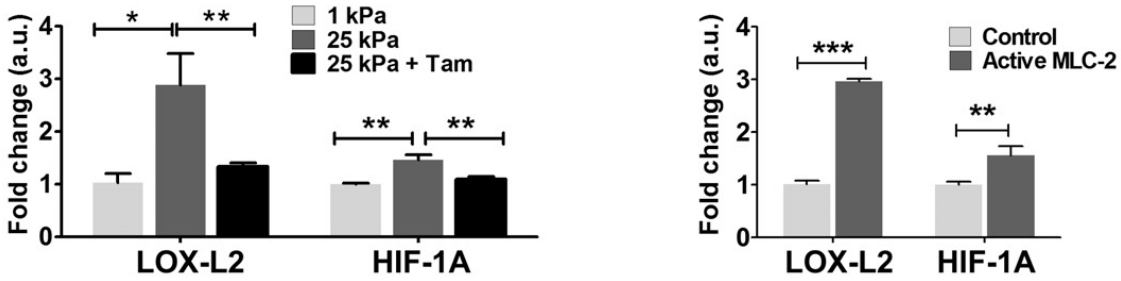


**Figure 9. HIF-1A is mechanically regulated by matrix stiffness and increased cell contractility.**

In other words, by external or internal mechanical stimulation. (A) Cells seeded in stiffer substrates (25 kPa) expressed much higher amounts of HIF-1A compared to cells seeded in soft subtrates (1 kPa). Tam is a drug that reduces tissue stiffness. It induces a reduction of the levels of HIF-1A and brings it back to the levels observed at 1 kPa (soft matrices). LOX-L2 means Lysyl oxidase Homologue 2 family, this is a gene that is regulated by HIF-1A. LOX-L2 control the crosslinking of ECM fibers. LOX-L2 levels follows the same trend observed for HIF-1a, which confirm that activation of HIF-1A gene in high stiffness. (B) Active myosin was introduced inside cells to induce cell contractility. This triggered much higher levels of HIF-1A expression. For all panels in this figure, mRNA levels are tested. Thus, the regulation is at transcriptional level. Adapted from Cortes et al13.

From Lachowski and collaborators' experiment, the result of relationship between stiffness and the expression of HIF-1A also be examined. Although they also simulated the tumor microenvironment, they used another method and gave a conclusion that whether high stiffness or low pH-value can upregulate the production of HIF-1A.

Authors cultured pancreatic ductal adenocarcinoma (PDAC) on PeptiGel matrix and used immunofluorescence to quantify the change of HIF-1A in protein level. The HIF-1A protein is stained to green color. Red and blue color represent actin, an protein in cytoplasm, and nucleus respectively. By comparing two different conditions, there is a significant upregulation of HIF-1A from soft matrix to stiff matrix. (as shown in Figure 10)

In addition, by using qPCR, researchers didn't find that the factor, stiffness, is related to the transcriptional level of HIF-1A.

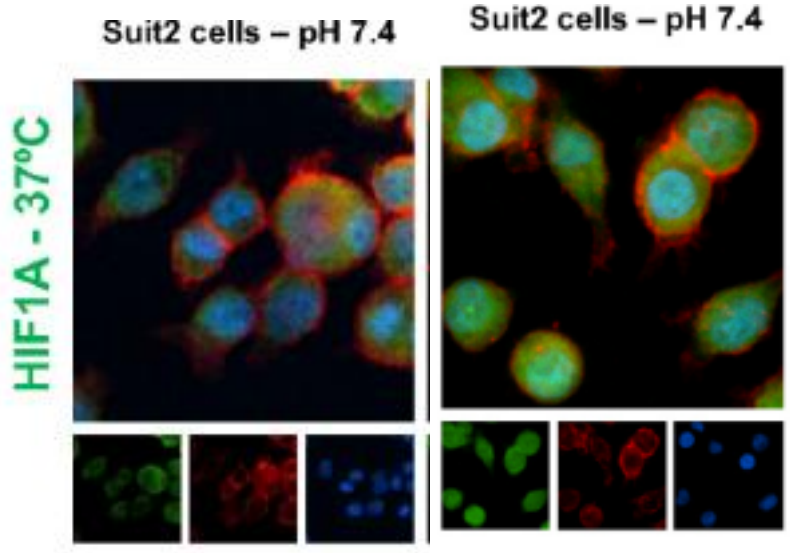


**Figure 10. HIF-1 is mechanically activated in cancer cells when they are seeded in stiff matrices compared to soft ones.**

Blue, red and green represent nuclei, cytoplasmic area, and HIF-1A protein, respectively. In can be observed that in stiff matrices (high tissue stiffness) HIF-1A is highly expressed and mostly accumulated in the nuclei. Adapted from Lachowski et al14.

Regulation of HIF-1A by acidosis or lowering pH.

Besides factors above, stiffness and oxygen level, the pH-value was also found to influence the levels HIF-1A.

In Meklhaid and collaborators' experiment, they demonstrated that pH-value triggers nucleolar sequestration of VHL, an enzyme that controls the degradation of HIF-1A.

In order to verify this hypothesis, they used the immunofluorescence technology to quantify the amount of HIF-1A, pVHL in more and more acidic conditions. The amount of pVHL doesn't change form 7.2 to lower pH-value. But, HIF-1A suddenly appears in cells below 7.2 pH-value and accumulates in lower pH. The measurement of Actin serves as a housekeeping protein in order to make sure the location of HIF-1A and pVHL. (as shown in Figure 12, Figure 13)

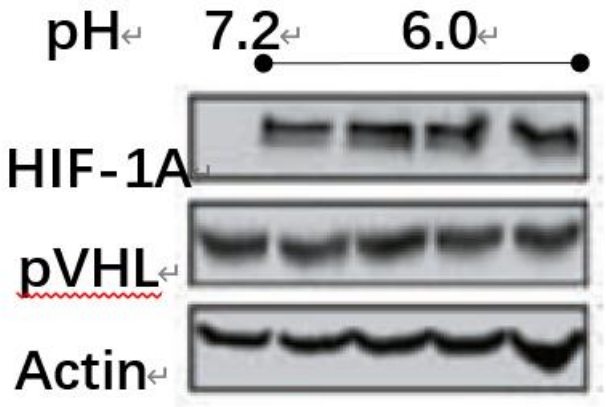


**Figure 11. Low pH values induce high levels of HIF-1A.**

At pH 6, we can see an upregulation of HIF-1A in comparison to pH 7.2. Intriguinly, the levels of pVHL expression (enzyme that degrades HIF-1A) does not change with pH. Actin is used as housekeeping protein to demonstrate that all bands have been loaded with the same amount of cells. Adapted from Mekhail et al15.

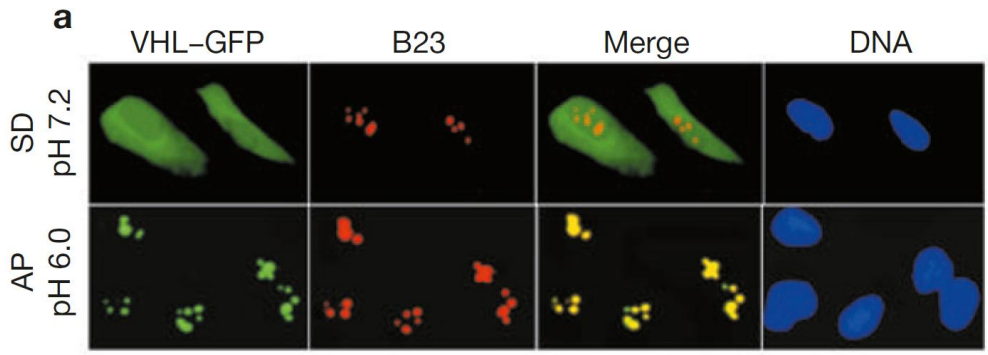


**Figure 12. Nuclear localization of VHL (pVHL) in low pH.**

At low pH (pH 6), VHL is localized in the nucleus as it can be seen by the co-localization with B23. B23 is a nuclear marker. It can be seen that the green VHL colocalizes with the red B23 to produce yellow clusters inside the nuclei. Adapted from Mekhail et al15.

In Lachowski and collaborators' experiment, they don't only prove the relationship of stiffness and the amount of HIF-1A, but they also the testify that the pH-value also involve in. By watching the epifluorecent images of HIF-1A for cells on stiff BIOGEL, the green part in pH 6.0 becomes much denser than in pH7.4, which demonstrates that as the pH-value decreases, the amount of HIF-1A is upregulated. (as shown in Figure 13)

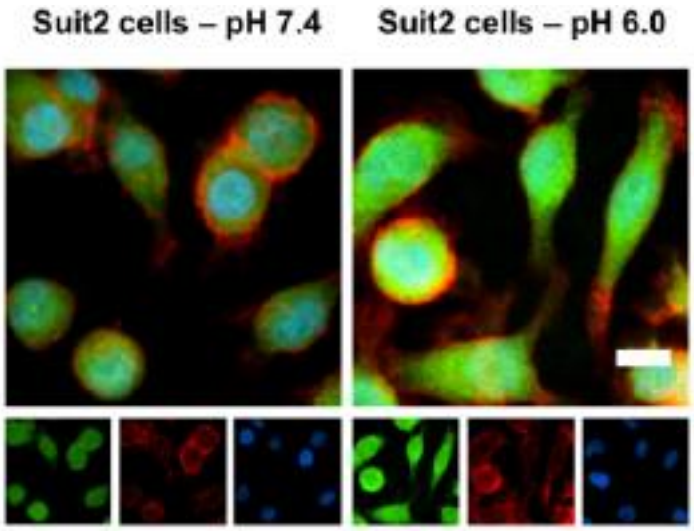


Figure 13. HIF-1A expression in pancreatic cancer cells is elevated at pH 6 compared by neutral pH 7.4. These images represent 3 colors: red is actin which marks the cell's area. Blue marks nuclei. Green shows the levels of HIF-1A. This image is adapted from Lachowski et al14.

## II. Discussion

HIF-1A plays a critical role in many physiological and pathological processes. It was largely known that HIF-1A is regulated by oxygen. However, the ECM surrounding cells are controlled by many factors such as mecanical cues, pH, and temperature. The papers discussed in this report have shown strong and compelling evidence to support the idea that HIF-1A can be controlled by mechanical and chemical factors.

Mechanical regulation of HIF-1A: We have seen three main lines of evidence to support the mechanical regulation of HIF-1A. All investigations have used different types of cells such as cardiomocytes, endothelial, or cancer cells to demonstrate the effect of stretching, low shear stress, or matrix rigidity on the expression of HIF-1A. In the first line of evidence, authors demonstrated increased levels of HIF-1A under mechanical stretching, which induced the expression of vascular endothelial growth factor (VEGF), which controls angiogenesis. This is critical for the formation of new bone after a fracture. In the second line of evidence, author used low shear stress to stimulate HIF-1A expression in endothelial cells. Finally, this mechanical effect on HIF-1A was confirmed culturing cancer cells in matrices of different rigidities.

Biochemical regulation of HIF-1A by acidosis: Many healthy tissues (i.e. cartilage) and pathological tissues such as fibrotic or inflammatory tissues have a very low pH values, which can condition the expression of HIF-1A. In this context, we have presented two lines of evidence that support the idea that low extracellular pH can regulate the levels of HIF-1A inside cells. This regulation occurs by affecting the interaction of HIF-1A with VHL in the cytoplasm of cells. VHL needs to interact with HIF-1A in the cytoplasm. However, under acidic conditions (low pH values), VHL is sequestered inside the nucleus and is unable to reach HIF-1A in the cytoplasm.

The fact that HIF-1A can be regulated by mechanical factors and pH offer the opportunity to used these ECM properties to control the level of HIF-1A in many tissues. One of these examples can be the regulation of MSC differentiation mediated by HIF-1A into chondrocytes inside cartilage. This will indeed open new possibilities for tissue engineering of the bone cartilage interface.

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