intro

Good morning everyone, I am Chan Cheuk Ka, and today I am here to present about my final year project on the role of hypoxic conditions in cartilage tissue engineering.

Here is my table of contents.

First, I will introduce the background of the topic.
Then, I will discuss the experimental rationale.
Next, I will talk about the experimental protocols.
Finally, I will present the results and conclusions of my study.

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background

Now, for the background of the topic,

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let's look into the problem with cartilage damage.

Cartilage damage is a very common ailment plaguing many individuals worldwide, especially the elderly. It is a common effect of trauma, arthritis, and joint injuries and can lead to severe pain and even disability.

One of the major reasons for this is that, cartilage, by its nature, lacks vasculature, neural connection, lymphatic system, and progenitor cells. This means that it has a very limited capacity for self-repair.

The cartilage that can be found normally in joints are called hyaline cartilage. This type of cartilage is rich in type 2 collagen and is very smooth, which allows for low friction between bones to help joint articulation.

However, when this cartilage is damaged, especially when the damage is severe, it is usually replaced by fibrocartilage scar tissue, which is rich in type 1 collagen. But this type of cartilage is not as smooth and has inferior biomechanical properties and cannot help joint articulation. Moreover, it can release enzymes to remodel the extra-cellular matrix, which can lead to further damage. We can see that cartilage damage can, therefore, very easily lead to cartilage breakdown and eventually other diseases like osteoarthritis.

While there are currently some treatments available, such as microfracture surgery and joint replacement, they all have their limitations and they often fail to produce satisfactory therapeutic outcomes, in addition to introducing side effects like immunogenicity, allergenicity, and invasiveness.

And therefore, we can see that there is currently a medical need for cartilage regeneration.

Currently, some of the most recent and promising treatment options involve the *in vitro* cultivation of cartilage tissue, before implanting it into the patient. For example, stem cells can be obtained from the patient, and then differentiated into chondrocytes within an injectable hydrogel. The hydrogel can then be injected intra-articularly into the patient, where it will form a new cartilage tissue.

Cartilage cultivation usually involves the use of mesenchymal stem cells, MSCs, since they are easy to obtain. MSCs can differentiate into chondrocytes in our application.

However, the major obstacle in these techniques is the inherent phenotypic instability of chondrocytes. When chondrocytes are cultured *in vitro*, they tend to dedifferentiate into fibroblasts, which can lead to the formation of fibrocartilage instead of hyaline cartilage. This exhibition of a hypertrophic phenotype is similar to that found in OA.

To cultivate chondrocytes *in vitro*, current techniques often have to involve a cocktail of growth factors and hormones to maintain the phenotypic stability. However, these techniques are often complicated and expensive with limited efficacy.

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theory

For the design rationale of my project,

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considering the avascular nature of cartilage, its native oxygen concentration *in vivo* is 2-7%.
And incubating chondrocytes in a hypoxic condition can better mimic the *in vivo* environment.

In fact, a hypoxic condition is actually essential for chondrogenesis.
Under a normoxic condition, which is 21% oxygen, a transcription factor called HIF-1α only has a half-life of 5-10 minutes since it is rapidly degraded via prolyl hydroxylation and proteasomal degradation by oxygen-dependent hydroxylases.
Under hypoxia, however, the hydroxylases are inhibited, leading to HIF-1α stabilisation.

But it is important for chondrogenesis since it regulates differentiation and stabilises the chondrocyte phenotype.

During the initial stages of chondrogenesis, HIF-1α is required for MSC condensation, chondrocyte proliferation, and ECM synthesis due to its ability to regulate genes associated with cartilage anabolism and chondrocyte differentiation, primarily Sox9, the master transcriptional regulator of chondrogenesis.
The downstream targets of Sox9 include Collagen 2a and Aggrecan, which are the major components in cartilage ECM. The existence of HIF-1α stabilises the desired articular hyaline cartilage phenotype.

Importantly, HIF-1α is not the sole affecter of chondrogenesis and ECM composition. If oxygen tension increases, the relative levels of Hif2α expression increases, triggering hypertrophic differentiation from its upregulation of osteoblastic Collagen 10a and catabolic genes like Mmp13 which destroys the ECM. These effects are also observable in OA cases.

Therefore, we can see that HIF-1α is a key regulator of chondrogenesis, and that hypoxia is essential for chondrogenesis.

However, for my project, I was not allowed to use a hypoxic incubator. Instead, hypoxia was simulated using a hypoxia mimetic agent, cobalt chloride, which is a popular choice due to the low cost and relative stability

compared to actual hypoxic conditions.

$CoCl_2$ can inhibit the prolyl hydroxylases by occupying their binding sites, therefore protecting HIF-1α from degradation. This leads to the accumulation of HIF-1α, which emulates the effects of hypoxia.

Following the work of another study, the $CoCl_2$ concentration of 100μM was chosen since they found it gave the highest cell viability after 48 hours of incubation, as well as inducing a 4-fold increase in HIF-1α expression.

Next, 3D culturing techniques can promote cell-cell and cell-ECM interactions, and encourage a more natural morphology by allowing cell aggregation and the subsequent formation of micro-environments. Studies have shown that 3D techniques can induce more chondrogenic markers and proteins and reduce hypertrophic markers.
While it is my original plan to use 3D spheroid culturing techniques, it was eventually changed since I don't have access to certain equipment.
Despite not being able to fully mimic *in vivo* environments, 2D culturing gave the advantage of being more convenient and replicable.

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materials & methods

Next, allow to briefly discuss the experimental protocols and methods.

Here is the general flow of the experiment.

First, P4 MSCs were thawed and cultured until confluency in T75 flasks under normal conditions.
Then, the cultures are transferred into two well plates, and again cultured until confluency.
Next, the medium is switched to chondrogenic medium with the experimental group having 100μM $CoCl_2$ and the control group being normal chondrogenic medium.
They are then incubated for 14 days before being harvested for analysis using qPCR and staining.

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qPCR will be used to measure Hif1α, Col1a1, Col2a1, Col10a1, Acan, Sox9, and Mmp13 gene expression levels. It is expected that collagen 2, aggrecan, HIF-1α and Sox9 levels, which are the ones shaded in green, will be higher in the hypoxic culture, while collagen 1 and 10 and Mmp13 levels, which are the ones shaded in red, will be lower.

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results & discussion

Let's move on to the results.

For this trial, the MSCs took much longer than expected to reach confluency after thawing, 9 days instead of 5, which suggested poor initial cell health and viability. This delayed the experiment a bit.

But after transferring them to the well plates, they reached confluency in 5 days, which is consistent with the previous trial.

The rest of the chondrogenesis process was unnoteworthy, and the cells were harvested after 14 days.

As mentioned earlier, staining was performed to assess the chondrogenic differentiation of the MSCs. For this project, Alcian blue was used to stain for glycosaminoglycans, which are the major components of cartilage ECM. The dye was supposed to mainly bind to the sulphate groups found in the mucin and proteoglycans of the ECM. However, the staining was not very successful, and the results were not very clear. As you can observe, there is not really an appreciable difference between the two groups. This is likely due to the insufficient chondrogenesis of the MSCs, which I will explore more in the qPCR section.

Before performing qPCR, I first extracted the RNA from the cells. The extraction wasn't really that good, and the yield was quite low.
One possible reason is that I had to transport the RNA for a far distance between the extraction and the qPCR, which may have led to degradation. This was also the case in the previous trial, where similarly low yields were observed. We can also see that the purity ratios deviated significantly, which suggested there was contamination.

Out of the 6 samples, the best ones from each group were chosen for qPCR, as highlighted here.

The RNA was reverse transcribed into cDNA, and qPCR was performed to measure the expression levels of the genes of interest. As mentioned earlier, a total of 7 genes of interest were measured.

As we can see there is a large variance in some of the samples, likely due to the low RNA yield. However, we can still see that the Hif1α expression levels were ~3.7 times higher in the hypoxic group, which is lower than the 4.4 times increase obtained in the study I referenced earlier. This difference between the groups has a p-value of 0.054, which is very close to the significance threshold of 0.05. However, similar conclusions cannot be drawn for the other genes. In fact, the expression level of Col10a1 was actually higher in the hypoxic group with a p-value of 0.024, which is opposite to initial expectations. The difference in the rest were not statistically significant.

When combined with the staining results, we can conclude that the MSCs did not undergo chondrogenesis as expected, or that they were less responsive to the $CoCl_2$ concentration as anticipated. In the future, I think the experiment should be repeated with a higher $CoCl_2$ concentration.

In conclusion, hypoxia is essential for chondrogenesis, and HIF-1α is a key regulator of chondrogenesis. Although the results of this study were not as expected, I believe it warrants further investigation to acquire more conclusive results. However, based on literature, hypoxic culturing of cartilage tissue *in vitro* is viable, since it is an effective and efficient method of phenotypic control and is more controllable than the use of many factor and hormone cocktails.

end

Here are a list of the references that I used throughout this presentation.

This is the end of my presentation.
Thank you for your listening.