BMEG 4998 – Thesis I, 2024-2025

Role of Hypoxic Conditions in Cartilage Tissue Engineering

Author: Chan Cheuk Ka

Student ID: 1155174356

Supervisor: Professor LI Zhong Alan

Associate Examiner: Professor CHAN Pui Barbara

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Abstract

Cartilage damage is a common ailment that can be caused by many factors. Due to the avascular nature of cartilage, its natural regeneration capacity is limited. Moreover, the phenotypic destabilisation into hypertrophy induced by trauma can lead to cartilage degradation, which harms joint articulation. Cartilage repair techniques, particularly *in vitro* cartilage culturing, are under heavy research; however, phenotypic instability is a significant challenge for such techniques. In previous studies, hypoxic conditions are suggested for incubation to mimic the *in vivo* environment of cartilage more closely. This study aims to further investigate the culturing technique by comparing the chondrogenic performance of mesenchymal stem cells (MSCs) between normoxia (5% O_2) and hypoxia (21% O_2). Samples are collected every seven days to examine the progress via microscopic observations, quantitative polymerase chain reaction (qPCR), and immunohistochemistry (IHC). While quantitative results have yet to be obtained, preliminary microscopic observations support the claim that hypoxia can enhance chondrogenesis. An improvement in cartilage culturing techniques can reduce the necessity of using factor and hormone cocktails for phenotypic control, simplifying the culturing process drastically.

Acknowledgements

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1 Introduction

1.1 Background Information

Cartilage damage is a common symptom of trauma, arthritis, and joint injuries [1]. However, the regeneration capacity of cartilage is very low due to its lack of vasculature, neural connection, lymphatic system, and progenitor cells [2].

Cartilage tissue can be classified into elastic cartilage, hyaline cartilage, and fibrocartilage. Hyaline cartilage is rich in type II collagen and can be found in joints to aid articulation due to its smooth nature and ability to resist compressive loads [3]. After cartilage injuries, especially those of high severity, fibrocartilage scar tissue can form during the healing process as a compensatory mechanism; however, fibrocartilage is a tough material, has inferior biomechanical properties compared to hyaline cartilage, and can remodel the extra-cellular matrix (ECM) into one unsuitable for joint articulation [3, 4, 5, 6]. Hence, cartilage damage can easily lead to cartilage breakdown and other diseases like osteoarthritis (OA) [4]. Current cartilage injury treatments fail to produce satisfactory therapeutic outcomes, and cartilage repair techniques often possess undesirable traits or exhibit side effects, including immunogenicity, allergenicity, and invasiveness [7].

1.2 Literature Review

As can be observed, there is a medical need for cartilage regeneration. The *in vitro* culturing of cartilage tissue has been a research focus. Mesenchymal stem cells (MSCs) have the potential to differentiate into chondrocytes and can produce ECM molecules that are important for cartilage function [7], making them a popular choice for cartilage tissue culturing.

However, there are still significant challenges that make cartilage repair difficult. Conventional culturing techniques can sparsely control the phenotype of the chondrocytes and often face the loss of the hyaline cartilage phenotype [8, 9]. Notably, there are currently 3D culturing

techniques, both with and without scaffolds, under research [1, 10, 11, 12], with some suggesting chondrocytes cultured using a hydrogel scaffold can be injected intra-articularly as a treatment [1]. However, these culturing techniques are often complicated, utilising many added factors to maintain phenotypic stability.

1.3 Study Overview

This study aims to investigate the effectiveness and feasibility of using hypoxic incubation to induce the differentiation of MSCs into the desired phenotype, namely hyaline cartilage. The study compares the degree of hyaline cartilage differentiation between normoxic (21% O_2) and hypoxic (5% O_2) incubation via microscope observation, quantitative polymerase chain reaction (qPCR), and immunohistochemistry (IHC).

2 Theory

2.1 Hypoxic Incubation

Due to the avascular nature of cartilage, it has a native oxygen concentration of 2% to 7% [7]. This environment facilitates the upregulation of hypoxia-inducible factor-1α (HIF-1α), which regulates genes associated with cartilage anabolism and chondrocyte differentiation like *Sox9* [13]. The existence of HIF-1α also stabilises the desired hyaline cartilage phenotype [14]. Under normoxia, HIF-1α is rapidly degraded via prolyl hydroxylation and proteasomal degradation [15] with a half-life of 5 to 10 minutes [16]. A hypoxic condition is, therefore, essential for chondrogenesis. Hypoxic incubations at both 2% O_2 [17] and 5% O_2 [18] have been found to facilitate chondrogenic differentiation.

For this study, a hypoxic condition of 5% O_2 was used.

2.2 Culture Technique

Common *in vitro* culture techniques include 2D (monolayer) and 3D culturing. 2D culturing techniques are convenient and easily reproducible. However, they cannot replicate *in vivo* environments since there are more cell-plastic and cell-medium interactions than cell-cell interactions, which does not represent physiological conditions well [19, 20]. In contrast, 3D culturing promotes cell-cell and cell-ECM interactions and facilitates a more natural cell morphology [20]. In chondrogenic applications, 3D cultures are shown to induce more chondrogenic markers and proteins and less hypertrophic ones [21]. In cellular spheroid culturing, a common form of 3D culturing, cells can grow in three dimensions to aggregate and establish mutual contact, allowing the spheroid to create specific micro-environments to better simulate *in vivo* conditions [20].

In this study, cell spheroid culturing was used.

2.3 Well Plate Selection

In cell spheroid culturing, low-adherence plates must be used to encourage aggregation.

For this study, ultra-low round-bottom well plates (F) were used, as illustrated in *Figure 1* below. Ultra-low attachment plates prevent cells from adhering to the bottom of the well and forming a single layer, as seen in (A); instead, they aggregate and form a cell spheroid. The rounded bottom facilitates the formation of one single spheroid by funnelling all initial cell balls together at the bottom of the well.

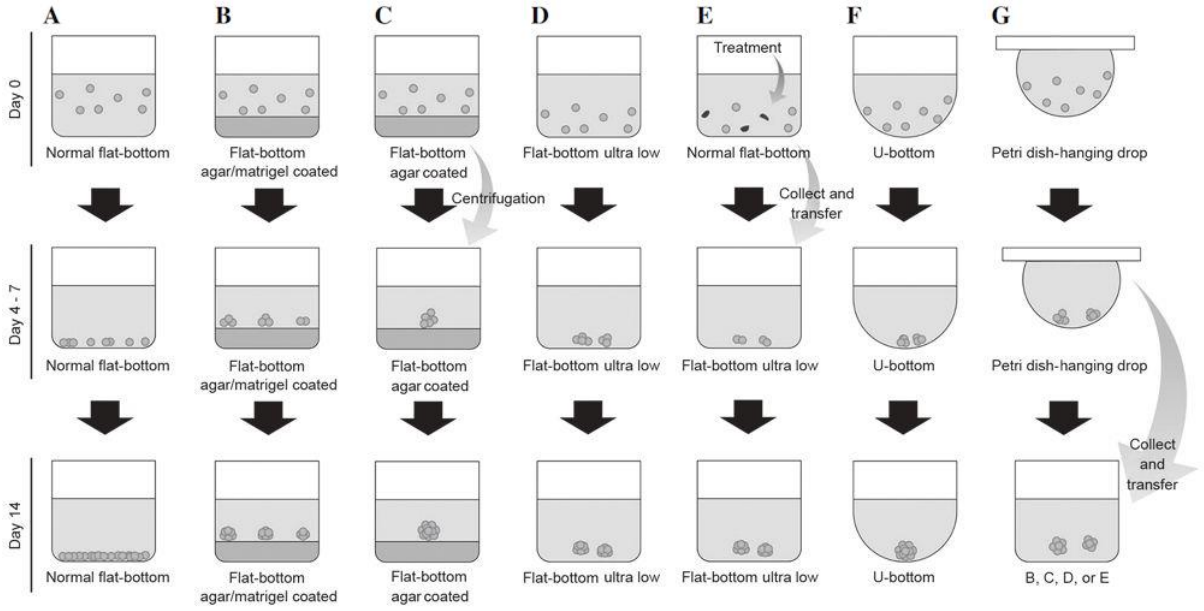


Figure 1: Comparison of cell morphology between cultures in different well shapes and types [22].

2.4 Chondrogenesis Evaluation

As mentioned briefly in *1.3 Study Overview* above, qPCR and IHC will be used to evaluate the degree of chondrogenesis in the cultures. Cells are harvested every 7 days for tests and observations to track the overall progress. *Col1a1*, *Col2a1*, *Col10a1*, *Acan*, *Hif1α*, *Sox9*, and *Mmp13* will be tested with qPCR, while COL2, COL10, and HIF-1α will be tested with IHC. These chondrogenic gene and protein markers can be used to compare the chondrogenesis

between the normoxic and hypoxic cultures. Their involvement in chondrogenesis and their expected observation results are detailed in *Table 1* below.

Table 1: The chondrogenic involvement and expected observation results of the chondrogenic gene and protein markers.

Gene	Protein	Chondrogenic Involvement	Expectation
Col1a1		Type I collagen	Lower in hypoxia
Col2a1	COL2	Type II collagen: major cartilage component	Higher in hypoxia
Col10a1	COL10	Type X collagen	Lower in hypoxia
Acan		Aggrecan: major cartilage component	Higher in hypoxia
Hif1α	HIF-1α	Regulates chondrogenic differentiation	Higher in hypoxia
Sox9		Maintains cartilage homeostasis	Higher in hypoxia
Mmp13		Degrades type II collagen	Lower in hypoxia

3 Experimental Results

3.1 Experimental Setup

For the study, MSCs at 6th passage (P6) were used. To obtain P6 MSCs, P5 MSCs were retrieved from liquid nitrogen and passaged in a growth medium in a T75 flask until there were enough cells for at least 2×10^5 cells per well, totalling 4.2×10^6 cells; this usually takes a week of culturing, changing the medium every three days (See *7.1 Protocol for Passaging MSCs* below). Afterwards, the P6 MSCs were cultured into spheroids in a chondrogenic medium in two well plates, with each plate having 21 occupied wells, to allow differentiation (See *7.2 Protocol for Making Cell Spheroids in Well Plates* below), and the cultures were incubated in normoxia (21% O_2) and hypoxia (5% O_2), respectively. The chondrogenic media were changed every two days. The culture was done for a total of 21 days, excluding passaging. During the 21 days, samples were collected every 7 days to examine the chondrogenesis progress, as mentioned in *2.4 Chondrogenesis Evaluation* above.

3.2 Results

As of the writing of this report, no successful round of RNA extraction was performed, so no results related to qPCR or IHC can be shown. However, some microscopic images were obtained during the passaging and culturing process.

3.2.1 MSC Passaging

The MSC passaging culture of P5 into P6 at days one and four of incubation can be observed in *Figure 2* below. Notice the increase in the number of cells in the latter image. ~80% of cell convergence can be observed in the latter image.

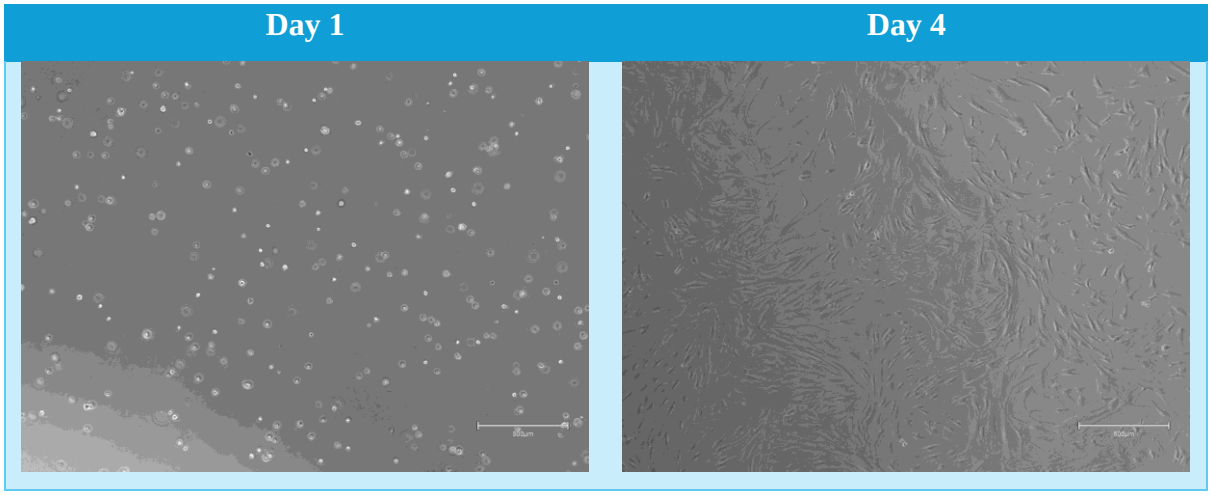


Figure 2: MSCs in T75 flask at days 1 and 4 of incubation, 4× magnification.

3.2.2 Chondrogenesis

3.2.2.1 Batch 1

Batch 1 chondrogenesis of MSCs at days 4 and 21 can be observed in *Figure 3* below. Notice that the cell pellet growths were more pronounced in hypoxia (~830µm → ~1380µm in diameter) compared to normoxia (~870µm → ~890µm in diameter), which aligned with our expectations. Note that only 5×10^4 cells were added per well in this batch.

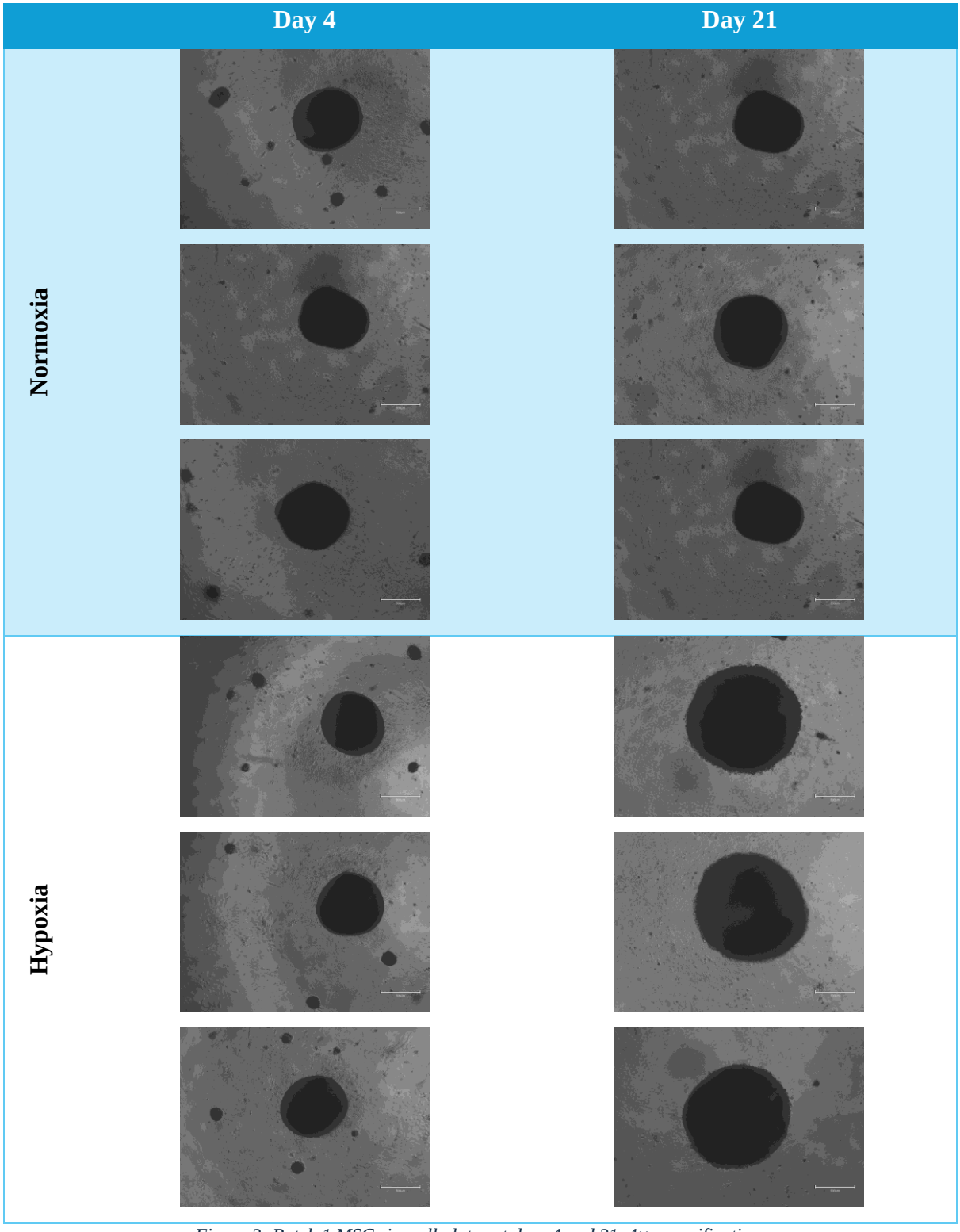


Figure 3: Batch 1 MSCs in well plates at days 4 and 21, 4× magnification.

3.2.2.2 Batch 2

Batch 2 chondrogenesis of MSCs at days 2 and 7 can be observed in *Figure 5* below. The growth differences are not yet very pronounced.

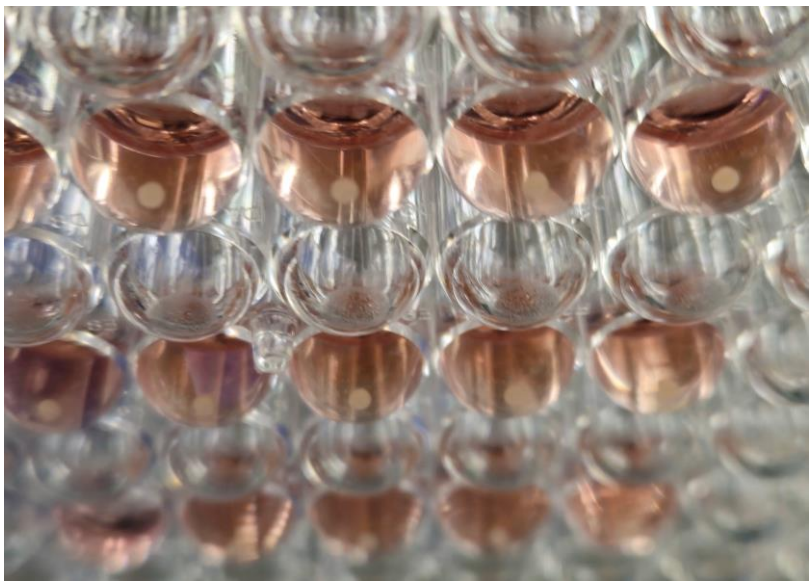


Figure 4: Bottom view of the well plate on day 2 of hypoxic chondrogenic incubation.

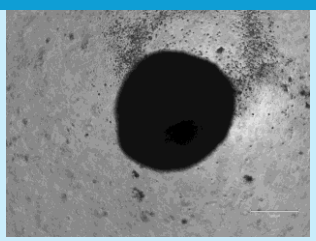
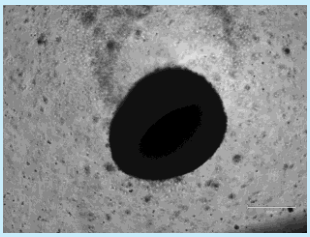
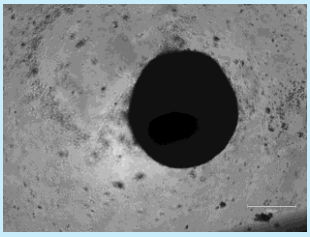
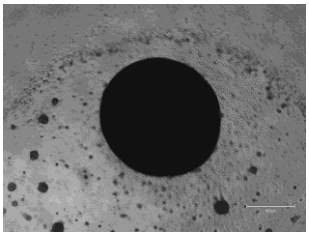
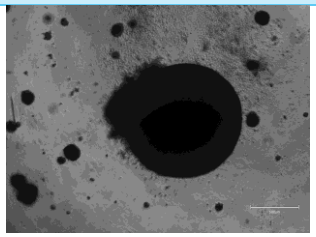
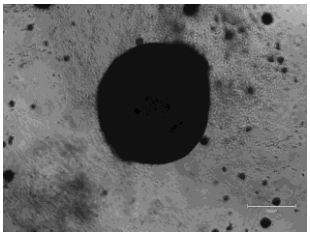
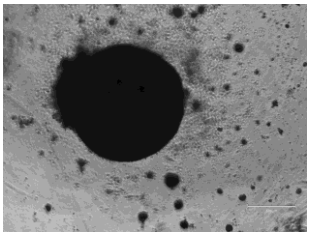
	Day 2	Day 7
Normoxia	/	  
Hypoxia		  

Figure 5: Batch 2 MSCs in well plates at days 2 and 7, 4× magnification.

4 Cost Summary

As of the writing of this report, no costs were incurred since hands-on experiments have yet to be allowed.

5 Discussion and Conclusion

Preliminary microscopic comparison between cultures in normoxia and hypoxia showed that cell pellet growth was more pronounced under hypoxia, indicating that hypoxic conditions at 5% O_2 can enhance chondrogenesis of MSCs, which confirmed the results of previous studies [18]. As of the writing of this report, qPCR and IHC have yet to be done, but they are expected to provide quantitative evidence to further support this claim.

Given this claim, it is suggested that future *in vitro* cartilage culturing can be done under hypoxia, as doing so can effectively control the phenotype of the cartilage tissue without the necessity for complex factor and hormone cocktails. This can prove to be an improvement to current cartilage engineering techniques and reduce a significant difficulty in cartilage repair, namely phenotypic instability.

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7 Appendix

7.1 Protocol for Passaging MSCs

Below is the protocol for passaging MSCs from P5 to P6. Refer to *Table 2* below for the components of the medium used and their functions. The experiment should be done in a biosafety cabinet (BSC).

1. P5 MSC recovery.
    a. Retrieve P5 MSCs from liquid nitrogen storage.
    b. Warm the MSCs to 37°C with a water bath for recovery.
    c. Dilute the cells and the freezing medium 5 times using the growth medium.
    d. Centrifuge the sample at 300×*g* for 5 minutes and discard the supernatant.
    - This is necessary since the cells will die if they stay in the freezing medium.
2. Resuspend the cell pellet with 1mL of PBS.
3. Cell count measurement.
    - An Invitrogen™ Countess™ 3 Automated Cell Counter [23] is used for the measurement.
    a. Isolate 10μL of the resuspended sample into a centrifuge tube.
    b. Add 10μL of Trypan Blue into the isolated sample.
    c. Load 10μL of the dyed sample onto each of the A and B sides of the slide.
    d. Measure and calculate the initial cell count of the whole sample.
4. Centrifuge the sample at 300×*g* for 5 minutes and discard the supernatant.
5. Resuspend the cell pellet with 1mL of growth medium.
6. Transfer the sample into a T75 flask.
7. Add 19mL of growth medium into the flask to top the total volume to 20mL.
8. Add 0.8μL of FGF2 into the flask.
9. Rock the flask back and forth slightly to spread the medium evenly.

The cells are to be incubated at 37°C until there are enough cells for at least 2×10^5 cells per well, totalling 4.2×10^6 cells. This usually takes a week of culturing, changing the medium every three days. Four separate flask cultures are usually necessary to achieve the required cell numbers. Passaging can be started at P4 if there aren't enough P5 MSCs.

Table 2: Components of the medium and their functions.

Component	Ratios	Function
Growth medium:		
α-MEM or low DMEM	100mL	Mammalian cell culture base
FBS	10mL	Provides hormone factors for cell proliferation and growth
P/S/F	1mL	Antimicrobials to prevent contamination
Additional component:		
FGF2	0.04 µL/mL	Modulates proliferation and differentiation

7.2 Protocol for Making Cell Spheroids in Well Plates

Below is the protocol for making cell spheroids in well plates. Refer to *Table 3* below for the components of the medium used and their functions. The experiment should be done in a biosafety cabinet (BSC).

- Let all reagents come to room temperature before using.

1. Retrieve 4 P6 cell cultures in T75 flasks from the incubator. Observing under an optical microscope, check that they reach ~80% cell convergence.
2. Wipe the exterior of the flasks with ethanol.
3. Discard the culture media from the flasks using a pipette. Be careful not to disturb the bottom of the flasks since the cells are adhered to the bottom.
4. Wash each flask with 4mL of PBS to remove the lingering media. Gently rock the flasks back and forth to mix. Discard the PBS afterwards.
5. Squirt 1mL of trypsin to the bottom of each flask to resuspend the cells. Knock on the bottom of the flasks to aid the resuspension.
6. Incubate the flasks at 37°C for 3-5 minutes until ~80% are resuspended as observed under the microscope. Add trypsin as necessary.

- Trypsin is an enzyme that can help resuspend cells by digesting cell proteins. However, it can kill the cells if exposed for too long.

7. Add culture medium equivalent to 2.5 times the volume of trypsin used into each flask to stop the digestion.
8. Repeatedly squirt the medium onto the bottom of each flask to detach as many cells as possible.
9. Mix the contents of every flask and transfer them into one falcon tube.
10. Centrifuge the sample at 300×*g* for 5 minutes and discard the supernatant.
11. Resuspend the cell pellet with 2mL of PBS.

10. Cell count measurement.
    - An Invitrogen™ Countess™ 3 Automated Cell Counter [23] is used for the measurement.
    a. Isolate 10μL of the resuspended sample into a centrifuge tube.
    b. Add 10μL of Trypan Blue into the isolated sample.
    c. Load 10μL of the dyed sample onto each of the A and B sides of the slide.
    d. Measure and calculate the initial cell count of the whole sample.
    e. Make sure that at least 21 wells can be filled, each with 2×10^5 cells, for each of the two well plates (See *Figure 6* below).
12. Centrifuge the sample at 300×*g* for 5 minutes and discard the supernatant.
13. Resuspend the cell pellet with 1mL of chondrogenic medium.
14. Add more chondrogenic medium until the total amount is sufficient for 150μL per well.
15. Add 1μL of ascorbate and 1μL of TGF-β1 for every 1mL of medium.
16. Pipette 150μL of the mixture into each well. Use a separate well plate for normoxia and hypoxia.
17. Centrifuge the well plates at 300×*g* for 1 minute.

The plates are to be incubated at 37°C with 20% O_2 and 5% O_2, respectively, for 21 days, changing the medium every two days. Note that 200μL of the medium should be added for each well during medium changing. One row of samples will be collected every 7 days for examination (See *2.4 Chondrogenesis Evaluation* above).

Table 3: Components of the medium and their functions.

Component	Ratios	Function
Chondrogenic medium:		
DMEM (w/ Na-Pyruvate)	100mL	Mammalian cell culture base
P/S/F	1mL	Antimicrobials to prevent contamination
ITS+ (1 mg/mL)	1mL	Basal medium supplement
Proline (40 mg/mL)	100µL	Enhances mitochondrial clearance and cell metabolism
Dexamethasone (10^{-3}M)	10µL	Decreases cell proliferation but increases ALP activity and collagen synthesis
Additional component:		
Ascorbate (50 mg/mL)	1 µL/mL	Protects esterified and non-esterified unsaturated fatty acids from peroxidation
TGF-β1 (10 µg/mL)	1 µL/mL	Regulates cell growth and differentiation

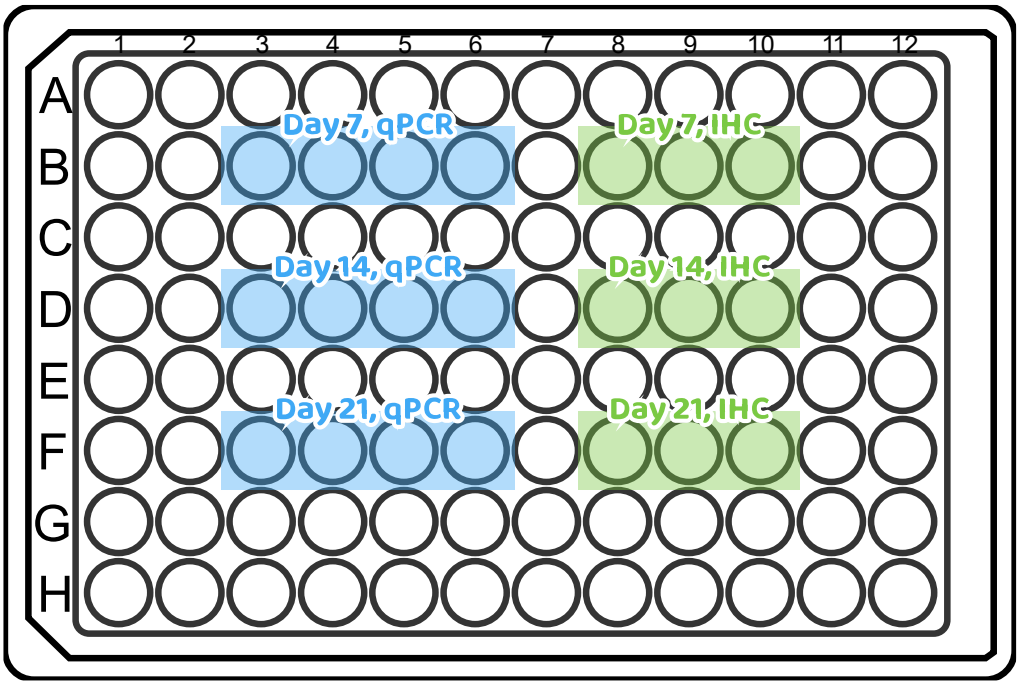

Figure 6: Diagram of minimum 96-well plate occupation and their purposes.

7.3 Presentation Video Download Link

The presentation video can be reviewed via this link: 1155174356_CHAN_CHEUK_KA_video.mp4.