MINISTRY OF EDUCATION AND TRAINING

VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

GRADUATE UNIVERSITY OF SCIENCE AND TECHNOLOGY

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# DIFFERENTIATION INTO HEPATOCYTE-LIKE CELLS FROM HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS

SUMMARY OF DISSERTATION ON BIOTECHNOLOGY
Code: 9 42 02 01

*Hanoi - 2024*

The dissertation is completed at: Graduate University of Science and Technology, Vietnam Academy Science and Technology

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# INTRODUCTION

**The necessity of the dissertation:**

The differentiation of stem cells into hepatocyte-like cell is an area of interest among scientists, they have significant for potential applications in cell therapy and in the screening of compounds or drugs in the form of individual functional cells or in combination to create organoids.

To differentiate stem cells into hepatocyte, various agents such as cytokines, growth factors, or gene transduction can be utilized. Certain cytokines and growth factors are known to have specific effects on the differentiation and development of hepatocyte under *in vitro* conditions. However, the use of these agents still presents differences and there is still a lack of a method to achieve the expected efficiency.

In order to contribute to futher scientific knownledge and explore new differntiation methods, as well as open up possibilities for research on the production of liver cells for the aforementioned purposes, we carried out the project titled **"Differentiation into hepatocyte-like cells from human umbilical cord mesenchymal stem cells".**

**The objectives:** (1) Successfully isolating and culturing umbilical cord Mesenchymal stem cells (MSCs) as a source material for cell differentiation, and obtaining information on conditions related to stability, differentiation potential, and maintenance of MSCs sources under *in vitro* culture conditions. (2) Inducing differentiation of umbilical cord MSCs into cells expressing some characteristic markers expressed by hepatocytes.

**Research contents:** (1) Isolation, culture, and *in vitro* expansion of umbilical cord tissue-derived MSCs from umbilical cord samples. (2) Investigation and assessment of the stability, differentiation potential, and maintenance capacity of MSCs sources under *in vitro* culture conditions. (3) Study on the differentiation of umbilical cord tissue-derived MSCs into cells

expressing some characteristic markers expressed by hepatocytes using various agents under *in vitro* conditions.

**Scientific and practical basis of the dissertation:**

The dissertation continues to carry out and further develop deeper research based on the studies conducted at the Institute of Biotechnology, as well as focusing on research directions that are of interest to many laboratories worldwide.

The completed research project will provide valuable information in the field of research on hepatic stem cells, from the methods of collection, isolation, and evaluation of the cell's potential to their differentiation capabilities. This will demonstrate the potential application of the results in the direction of creating liver cells for testing, screening biologically active substances, as well as constructing models for testing new drugs.

**The new contributions of the dissertation:**

The dissertation has provided comprehensive information on the differentiation potential of functional liver cells derived from MSCs sources isolated from the umbilical cord of newborns. The dissertation is the first study to utilize the Tet-ON system to overexpress the HNF4α gene for differentiating MSCs from the umbilical cord into cells with some liver cell functions.

# CHAPTER 1-LITERATURE REVIEW

## 1.1. Umbilical cord mesenchymal stem cell source, differentiation potential, and applications

### *1.1.1. The source of mesenchymal stem cells from the umbilical cord*

In various tissues throughout the human body, mesenchymal stem cells (MSCs) are present. The primary known source of MSCs is bone marrow; however, subsequent discoveries have revealed many alternative sources that yield MSCs in larger quantities than bone marrow, such as

adipose tissue, peripheral blood, deciduous teeth, umbilical cord blood, Wharton's jelly, amniotic membrane, endometrial lining, and placenta. Among these sources of MSCs, the umbilical cord is considered to be quite an ideal source.

#### *1.1.2. The differentiation potential of mesenchymal stem cells*

The strong proliferative capacity is a notable advantage of MSCs. Additionally, MSCs have the potential to differentiate into various cell types such as osteoblasts, chondrocytes, myocytes, adipocytes, neurons, hepatocytes, etc.

#### *1.1.3. Applications of mesenchymal stem cells*

The clinical application of MSCs through stem cell therapy is considered the most important potential application of MSCs. MSCs can generate new cell types and tissues to supplement or replace damaged or dysfunctional cells and tissues within organs.

### 1.2. The structure of the umbilical cord, the advantages of mesenchymal stem cells derived from the umbilical cord

#### *1.2.1. The structure of the umbilical cord*

The umbilical cord is enveloped by the amniotic membrane and consists of two arteries and one vein. Surrounding the umbilical cord is a gelatinous tissue layer called Wharton's jelly (WJ).

#### *1.2.2. The advantages of MSCs derived from the umbilical cord*

(1) Easy harvesting and processing of stem cells, without posing any health risks to both the mother and the baby. (2) Active control over infectious diseases through pre-birth testing of the mother for research samples. (3) Exhibiting excellent proliferation potential and the quantity of cells obtained directly or after *in vitro* expansion is substantial, no longer possess the ability to form malignant tumors like embryonic stem cells. (4) Umbilical cord MSCs can be stored long-term for use. (5) Express low levels

of HLA and are less likely to be rejected in transplantation. (6) Umbilical cord MSCs belongs to the category of multipotent stem cells, capable of differentiating into various cell types such as blood cells, osteocytes, cardiac muscle cells, chondrocytes, adipocytes, hepatocyte, etc.

## 1.3. The research situation

### *1.3.1. The current status of human stem cell research*

Stem cell research in Vietnam has been conducted since 1995, focusing on bone marrow-derived stem cells. Recently, there has been significant growth in stem cell research, with various types of cells being studied, such as umbilical cord blood stem cells. For sources of bone marrow-derived stem cells (primarily enriched from bone marrow), they have been used to treat various bone conditions. This includes clinical applications such as treating fractures, spinal fractures, and avascular necrosis of the femoral head.

### *1.3.2. The status of research on hepatocyte differentiation*

#### *1.3.2.1. The current status of research on hepatocyte differentiation*

Research by Mattiucci and colleagues (2018) has demonstrated that umbilical cord MSCs can differentiate and treat liver diseases.

Currently, there are several commercially available lines of hepatocyte-like cells differentiated from human pluripotent stem cells. Major providers are located in the United States and Japan (Yokohama; iCell; Otsu). These companies offer cell lines with specific characteristics and functions tailored for particular experiments.

#### *1.3.2.2. Differentiation pathways for generating hepatocyte-like cells*

*a. In vivo and in vitro research*

In 2013, a Japanese scientific team at Yokohama National University successfully transplanted multipotent stem cells isolated from human skin and blood into mouse livers. In October 2015, scientists at the

Hebrew University of Jerusalem (Israel) achieved a breakthrough by successfully culturing functional human liver cells. Also in 2015, the research group led by Dr. Roel Nusse at Stanford University in the United States identified a population of proliferative nuclei and self-renewing cells adjacent to the central veins in the liver.

*b. Using chemical inducers for differentiation*

Some hormones, cytokines, vitamins, ions such as Ca2+, and certain chemicals are commonly used in cell differentiation induction, including Dexamethasone, Indomethacin, Hydrocortisone, TGF-β, among others, with specific concentrations and ratios depending on the cell type.

*c. Differentiation through substrate materials.*

Differentiation through substrate materials relies on the interaction between cells and the substrate in cell culture *in vitro*. Cells function within the extracellular matrix (ECM) of the substrate. ECM contains high molecular weight compounds such as collagen, laminin, fibronectin. Apart from its structural role as a scaffold for cells, ECM also plays a physiological role as a microenvironment for cells.

*d. Differentiation through a combination of cytokines and chemicals.*

In most differentiation methods, MSCs can be directed towards hepatocyte-like cells through differentiation stages (initiated by factors like FGF, HGF, and others) and mature hepatocyte stages (induced by OSM and dexamethasone) to form a characteristic immature hepatocyte phenotype.

*e. Co-culture with differentiated cells.*

The research group led by Stecklum (2014) used umbilical cord blood stem cells co-cultured with mouse alpha liver cells (AML12) for their study. The authors pointed out that the stepwise investigation of cell differentiation induction can be achieved using co-culture methods.

*f. Differentiation through gene transfer methods.*

This method is often used to regulate the differentiation of embryonic stem cells. It involves introducing the desired gene into the cells to supplement certain active genes into the embryonic stem cell gene pool, initiating the differentiation of embryonic stem cells along the desired specialized cell lineage.

*1.3.2.3. The status of research on the application of stem cells in differentiation and treatment of liver diseases in Vietnam*

In Vietnam, in 2013, Doan Chinh Chung and colleagues differentiated MSCs derived from human umbilical cord lining membrane into hepatocyte like cells using a differentiation method based on changes in the culture environment.

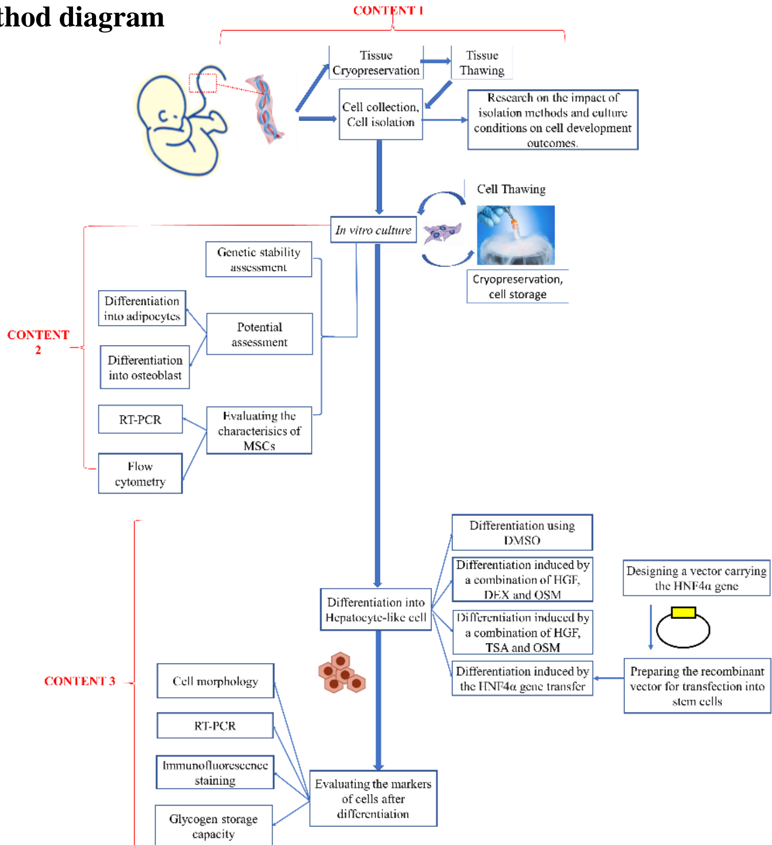
In 2014, Nguyen Thi Kim Nguyen and colleagues differentiated hepatocyte like cells *in vitro* MSCs collected from adipose tissue.

In 2015, Truong Thi Hai Nhung conducted experimental treatment for liver fibrosis using stem cell therapy on a mouse model of liver fibrosis. Following the results of this study, Nguyen Minh Thu and colleagues (2021) also conducted experiments injecting MSCs with a dose of $5x10^5$ cells/mouse into mice with liver injury due to bile duct ligation.

In a clinical study in 2021, Dao Truong Giang and colleagues evaluated the outcomes of autologous bone marrow stem cell transplantation for treating decompensated liver cirrhosis due to hepatitis B virus.

# CHAPTER 2- MATERIALS AND METHODOLOGY

## 2.1. Method diagram




## 2.2. Research materials and chemicals

### *2.2.1. Research samples*

The umbilical cord samples were obtained from healthy donors (full-term pregnancies, negative results for infectious disease screening tests, including HIV, hepatitis B virus, hepatitis C virus, cytomegalovirus, and syphilis) who consented to donation through voluntary consent forms

### *2.2.2. Research location*

The research for the thesis was conducted at the Animal Biotechnology Department - Institute of Biotechnology - Vietnam Academy of Science and Technology, located at 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi.

#### *2.2.3. Chemicals and kits used*

#### *2.2.4. Research equipment*

#### *2.2.5. Operating, culture, and storage environment*

### 2.3. Research methodology

#### *2.3.1. Cell collection, isolation, and culture*

#### *2.3.2. Cell transplantation method*

#### *2.3.3. Cryopreservation and cell thawing method*

#### *2.3.4. Chromosome staining method*

#### *2.3.5. Assessment method of the differentiation potential of isolated cells*

#### *2.3.6. Assessment method of the stem cell characteristics of isolated cells*

#### *2.3.7. Method for assessing cell growth rate*

#### *2.3.8. Method for hepatocyte differentiation*

#### *2.3.9. Method for evaluating cell markers post-differentiation*

#### *2.3.10. Statistical analysis of data*

## CHAPTER 3. RESULTS AND DISCUSSION

### 3.1. Harvesting, isolating, and *in vitro* culturing of MSCs from umbilical cord tissue samples

#### *3.1.1. Harvesting, isolating stem cells from umbilical cord tissue*

The umbilical cord tissue samples and vascular tissue samples with dimensions of approximately 1mm$^3$ were cultured in 4-well culture dishes (NUNC), in DMEM/F12 medium supplemented with other components. Monitoring the samples, we observed cell adhesion on the dish surface through cell outgrowth at the edges of tissue fragments from the 14th day of culture.

### *3.1.2. In vitro cell culture and cryopreservation of umbilical cord stem cells*

During *in vitro* development, the homogeneity of cell populations may not be high initially. However, from day 18 of culture, the stem cells proliferate rapidly, forming multiple layers that twist and stack upon each other (Figure 3.3).

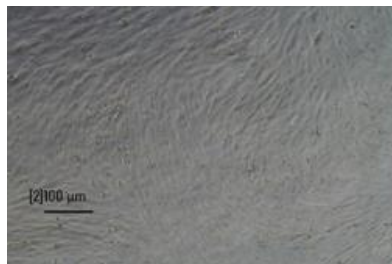



*Figure 3.3*. *In vitro* development process of isolated cells: Cells densely growing on the culture dish forming parallel, overlapping layers.

After 90 days of being stored in the cold bank, we randomly thawed one vial to assess the survival rate of the cells. The cell culture dish before freezing was also examined as a control for comparison.

*Table 3.1*. Cell counting results and cell viability ratio

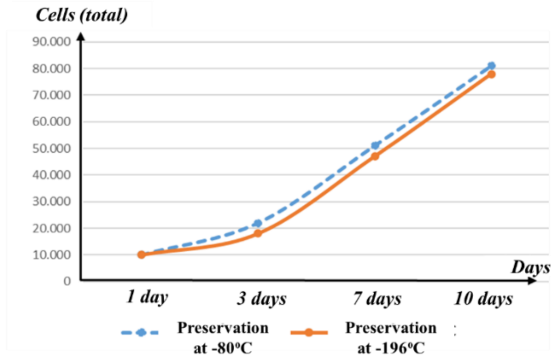
| **Sample** | **Cell density (Cell/ml)** | **Survival rate (%)** |
|---|---|---|
| Pre-cryopreservation MSCs | 295.000 ± 1.732[a] | 98,33 ± 0,58[a] |
| Post-thaw MSCs | 72.500 ± 1.322[b] | 85,29 ± 1,56[b] |

The table indicates that before freezing, the cell viability rate was relatively high (98.33 ± 0.58%). After thawing, the cell viability rate decreased to 85.29 ± 1.56%, although the surviving cells still exhibited the characteristic fibrous morphology of MSCs.

We also investigated the influence of two common storage conditions (−80°C freezer and liquid nitrogen at −196°C). The results showed relatively high cell proliferation rates in the thawed cells (from $10^4$

cells/cm$^2$ to 8×10$^4$ cells/cm$^2$ after 10 days of culture) under both temperature conditions examined (Figure 3.4).

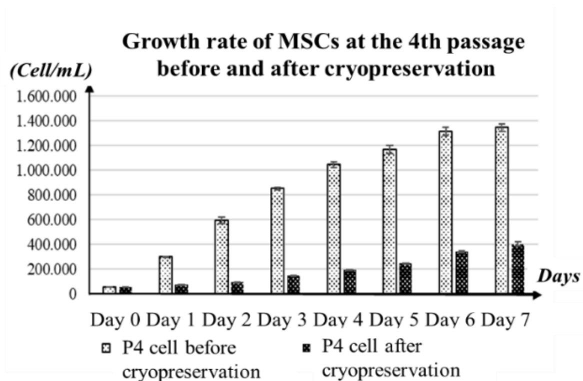
*Figure 3.4.* Effect of storage temperature on the growth capacity of thawed MSCs




To compare the isolation efficiency of MSCs from tissue samples before and after cryopreservation, we conducted two experimental batches. MSCS from the fresh tissue isolation group (Group 1) began to proliferate after 7-10 days of cultivation. The surface coverage of cell growth reached 50-60% after 14-18 days of cultivation. In contrast, MSCs isolated from frozen umbilical cord tissue (Group 2) started to proliferate after 15 days of cultivation. It took 20-25 days of cultivation for the surface coverage of cell growth in this group to reach 50-60%.

### *3.1.3. Assessment of cell growth rate*

*Figure 3.7.* Growth rate chart of P4 cells before freezing and P4 cells after freezing



According to the chart in Figure 3.7, we can observe that the growth rate of P4 cells before freezing increases rapidly in the first few days, from the first day to the fifth day. After the sixth day, this rate stabilizes, and the cell proliferation decreases. On the other hand, P4 umbilical cord-derived MSCs after thawing exhibit slow growth in the initial days, and then gradually accelerate from the fifth day onwards. However, the growth rate of cells before freezing is much faster compared to the growth rate of cells after thawing.

*Table 3.2*. Increase in MSCs from fresh and frozen umbilical cord tissue samples from passage 2 to passage 5.

| **Experimental batch** | **Cell doubling time (hours)** | | | |
|---|---|---|---|---|
| | P2 | P3 | P4 | P5 |
| **Fresh umbilical cord tissue** | 28,42 ± 0,60[a] | 26,34 ± 0,55[a] | 27,69 ± 0,20[a] | 25,71 ± 0,53[a] |
| **Cryopreserved umbilical cord tissue** | 36,00 ± 0,93[b] | 33,75 ± 0,55[b] | 40,00 ± 0,39[b] | 40,00 ± 0,63[b] |

The results in Table 3.2 indicate that in the group isolated from fresh umbilical cord tissue, the doubling time slightly decreased from passages 2 to 5. Meanwhile, in the group isolated from frozen umbilical cord tissue, the doubling time tended to increase later. At passage 2, the doubling time was 36,00 hours, but it increased to 40,00 hours at passages 4 and 5.

The evaluation of cell numbers between the two experimental groups showed that the cell concentration increased almost exponentially over 7 days of culture (Figure 3.8). In the first 3 days, the number of MSCs isolated from fresh umbilical cord tissue and frozen umbilical cord tissue was similar, then they exhibited a slight delay in expansion and proliferation compared to the number of cells originating from fresh umbilical cord tissue. By the 7th day, the proliferation rate gradually stabilized, and the cell proliferation rate was no longer high.

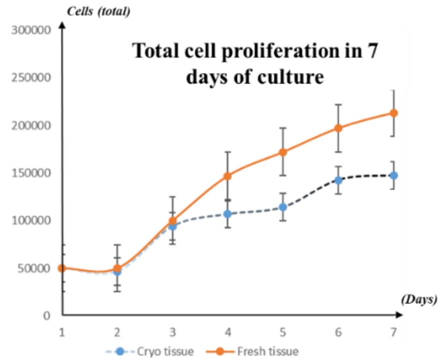



*Figure 3.8.* Total cell proliferation recorded daily over 7 days of culture in 2 experimental groups: fresh umbilical cord tissue and frozen umbilical cord tissue

## 3.2. Evaluation of stability, differentiation potential, and maintenance capacity of UC-MSCs under *in vitro* culture conditions

### *3.2.1. Evaluation of the genetic stability of isolated stem cells*

The obtained karyotypes after analysis all showed normal, typical characteristics. The chromosomes exhibited stable numbers, and their structures remained unchanged across passages 2, 10, and 15.

### *3.2.2. Evaluation of the differentiation potential of stem cells*

*3.2.2.1. Differentiation of stem cells into adipocytes*

The adipocyte-like cells with round morphology began to appear on the 7th day after induction of differentiation, whereas they were absent in the control cells. Upon staining with Oil Red dye (Merck), they consistently stained red, indicating lipid droplets in the majority of the cell mass. In contrast, the control cells showed no morphological changes, absence of lipid droplets, and did not stain with the dye

When comparing the adipogenic differentiation potential of MSCs between the group isolated from fresh umbilical cord tissue and the group isolated from frozen umbilical cord tissue, similar results were obtained.

*3.2.2.2. Differentiation of stem cells into osteoblasts*

The characteristic morphology of osteoblasts in differentiated cells begins to appear after 7 days of culture in the differentiation medium: cells transition from elongated and slender fibrous-like shapes to round and granular forms (Figure 3.13A). The presence of calcium ions (a characteristic property of osteoblasts) within the extracellular matrix of the cells, combined with the staining agent, forms an orange-red complex.

When comparing the differentiation ability of MSCs into osteoblasts between the group isolated from fresh umbilical cord tissue and the group isolated from frozen umbilical cord tissue, similar results were obtained.

***3.2.3. Evaluating the characteristics of mesenchymal stem cells***

*3.2.3.1. Assessing the characteristics of mesenchymal stem cells through flow cytometry cell counting*

The results show a high positive expression rate in cells with characteristic markers of MSCs, specifically: CD73 (99.90%), CD90 (98.20%), and CD105 (98.50%) (Figure 3.15). Most samples exhibited negative expression with a combination of characteristic markers not expressed in MSCS (CD14, CD20, CD34, CD45). For the MSCs isolation sources from the umbilical cord that we conducted, the overall rate for this combination in the samples isolated from the umbilical cord membrane tissue was 5.10%.

In the group of MSCs isolated from fresh umbilical cord tissue, the expression of CD90 (99.75%), CD105 (99.51%), CD73 (99.46%) was observed (Figure 3.17). In the group of MSCS isolated from cryopreserved umbilical cord tissue, the expression of these markers decreased, specifically CD90 (96.60%), CD105 (96.15%), CD73 (95.20%).

The intermediate MSCs lines that are more stable are still being maintained and stored by our research group. Table 3.3 shows that the *in*

*vitro* culture process does not affect the expression of surface markers, as evidenced by the consistently high positive rates. Key markers such as CD73, CD90, and CD105 all exhibit expression rates of over 95.03%, and they remain negative for the combination of CD14/CD20/CD34/CD45 markers.

*3.2.3.2. Characterization of MSCs using RT-PCR method*

*Table 3.6.* Expression of surface markers during *in vitro* culture

| Cell source | Marker | Size (bp) | Expression at P5 | Expression at P10 | Expression at P15 | Expression at P20 |
|---|---|---|---|---|---|---|
| Mesenchymal stem cells (MSCs) | *CD105* | 179 | + | + | + | + |
| | *CD90* | 265 | + | + | + | + |
| | *CD73* | 308 | + | + | + | + |
| | *CD34* | 367 | - | - | - | - |
| | *CD86* | 290 | + | + | + | + |
| Embryonic stem cells | *Eras* | 315 | + | + | + | + |
| | *Oct – 1* | 297 | + | + | + | + |
| Mesenchymal cells | *GATA4* | 290 | + | + | + | + |
| Liver cells | *AFP* | 216 | - | - | - | - |
| | *ALB* | 136 | - | - | - | - |
| | *HNF4α* | 350 | - | - | - | - |

The results in table 3.6 indicate that with the RT-PCR method, at passage 20, the markers still maintained positivity for MSCs-specific markers and negativity for functional markers of liver cells, similar to passage 5.

**3.3. Differentiation of mesenchymal stem cells into hepatocyte like cells**

***3.3.1. Differentiation using DMSO***

The umbilical cord-derived stem cells after 30 days of culture, despite showing certain morphological differences compared to cells before treatment, still do not exhibit similarity to HepG2 liver cancer cells when compared.

*Table 3.7.* Expression of molecular markers in cells after differentiation using DMSO

| Marker | Size (bp) | Expression of molecular marker before DMSO treatment | Expression of molecular marker after DMSO treatment (30 days) | | | The expression of markers in HepG2 cells |
|---|---|---|---|---|---|---|
| | | | 0,01% | 0,1% | 1% | |
| CD73 | 308 | + | + | + | + | + |
| CD34 | 367 | + | + | + | + | + |
| CD86 | 290 | + | + | + | + | + |
| CD90 | 265 | + | + | + | + | + |
| CD105 | 179 | + | + | + | + | + |
| *Eras* | 315 | + | + | + | + | + |
| *Oct-1* | 297 | + | + | + | + | + |
| *GATA4* | 290 | + | + | + | + | + |
| *HNF-4α* | 350 | - | - | - | + | + |
| *ALB* | 136 | - | - | - | - | + |
| *G6P* | 379 | - | - | - | - | + |

Table 3.7 shows that only the transcription factor *HNF4α*, related to liver cell function, was expressed in the stem cells after differentiation in the experimental batch treated with 1% DMSO for 30 days.

The assessment of glycogen storage ability in differentiated cells using the Periodic Acid Schiff (PAS) staining method revealed that the DMSO-induced differentiated cell batches exhibited glycogen storage capability after 3 weeks

***3.3.2. Differentiation induced by a combination of HGF, DEX and OSM***

The results indicate that the cells exhibit positive expression for *HNF4α* after 4 weeks of differentiation. However, previous RT-PCR analyses at 1 week, 2 weeks, and 3 weeks did not show any expression. Additionally, two characteristic markers of liver cells, ALB and G6P, were also not expressed similarly to DMSO-induced differentiation.

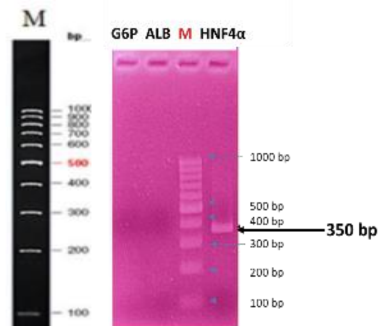



*Figure 3.25.* RT-PCR electrophoresis of cell cultures after 4 weeks of differentiation with a combination of HGF, DEX, and OSM (M: DNA Marker 100 bp).

Staining with Periodic Acid Schiff (PAS) revealed that in the cell batch differentiated by the combination of HGF + DEX + OSM, there was evidence of glycogen storage capability within the cells after 3 weeks.

***3.3.3. Differentiation induced by a combination of HGF, TSA và OSM***

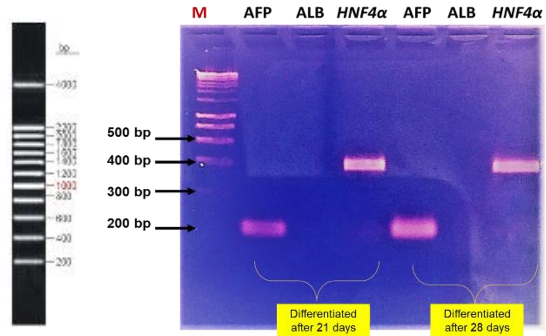



*Figure 3.28.* Electrophoresis results of RT-PCR samples cultured for 4 weeks after differentiation with a combination of HGF, TSA, and OSM (M: DNA Marker 200 bp)

The results showed that the differentiated cells began expressing AFP and *HNF4α*, markers related to liver cells, from the second week after exposure to the supplemented environment containing HGF, TSA, and OSM (Figure 3.28).

After performing Periodic acid Schiff (PAS) staining, we observed that the glycogen storage ability in the cells differentiated using the combination of HGF + TSA + OSM appeared after 3 weeks, similar to the cells differentiated using DMSO and the combination of HGF + DEX + OSM.

***3.3.4. Differentiation induced by the HNF4α gene transfer.***

*3.3.4.1. Designing a vector carrying the HNF4α gene for transfection into stem cells*

The coding sequence of the *HNF4α* gene and primer pairs for amplifying the *HNF4α* gene sequence were both synthesized according to specific sequences by Phu Sa Biochemical Company Limited. This sequence was amplified using PCR reaction with the primer pairs NheI-*HNF4α*-F

(Forward primer) and PacI-*HNF4α*-R (Reverse primer). After PCR, the products were electrophoresed on a 1.0% agarose gel.

After the digestion reaction, electrophoresis was performed to assess the purity of the digestion products on an Agarose gel for the two samples, *HNF4α* and pTRE-Tight-BI, with sizes approximately 1,359 bp and 2,856 bp, respectively.

To verify whether the pTRE-Tight-BI-HNF4α vector was successfully ligated and transformed into E. coli, we utilized the bacterial colony PCR method with the primer pairs NheI – *HNF4α*-F (Forward primer) and PacI – *HNF4α*-R (Reverse primer). Electrophoresis of the PCR products showed a band corresponding to *HNF4α.*

The pTet-DualON vector will be amplified in large quantities using the heat shock transformation method with E. coli DH5α bacteria and then isolated using the GenJET Plasmid Miniprep Kit (Thermo) to obtain DNA. The vector will be emptied to receive the pTRE-Tight-BI vector after insertion of the *HNF4α* gene segment using the NsiI and PacI enzymes.

The next step involves cutting the pTRE-Tight-BI-HNF4α plasmid DNA, which contains the *HNF4α* gene, using the NsiI and PacI enzyme pair to separate the *HNF4α* gene segment.

We utilized the QIAGEN Gel Extraction Kit to purify the digestion products of pTRE-Tight-BI-*HNF4α* and pTet-DualON. The purified products were then subjected to agarose gel electrophoresis, resulting in distinct bands corresponding to the sizes of pTRE-Tight-BI-*HNF4α* and pTet-DualON, approximately 1,700 bp and 4,906 bp, respectively. The two digested products were ligated using T4 ligase to transfer the *HNF4α* DNA segment into the pTet-DualON vector, resulting in the final vector pTRE-*HNF4α*-ON.

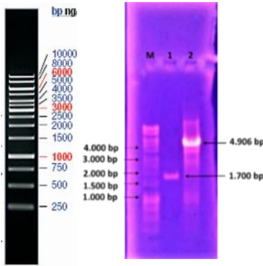



*Figure 3.35.* Result of purification of the digestion products of pTRE-Tight-BI-*HNF4α* and pTet-DualON with NsiI and PacI enzymes

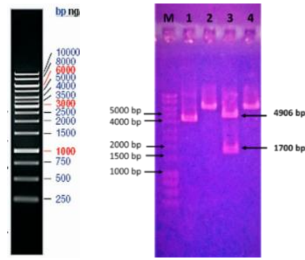



*Figure 3.37.* Results of checking the digestion products of the pTRE-*HNF4α*-ON plasmid with enzymes

Sequence analysis and translation into amino acids according to the obtained sequence confirmed the successful creation of the pTRE-*HNF4α*-ON vector, utilizing the improved Tet-ON gene expression system, containing the correct reading frame, start codon, and stop codon consistent with the design, serving the purpose of gene expression transfer in MSCs.

*3.3.4.2. Transfecting the vector expressing the HNF4α gene into stem cells.*

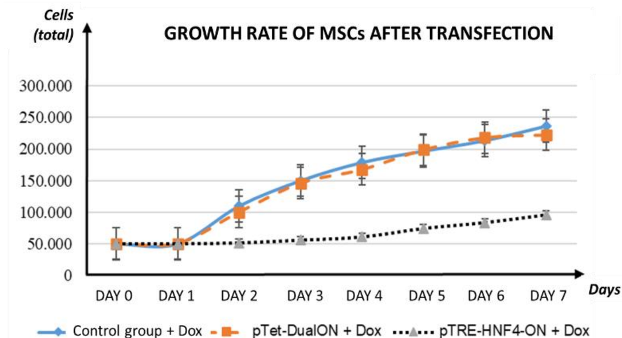
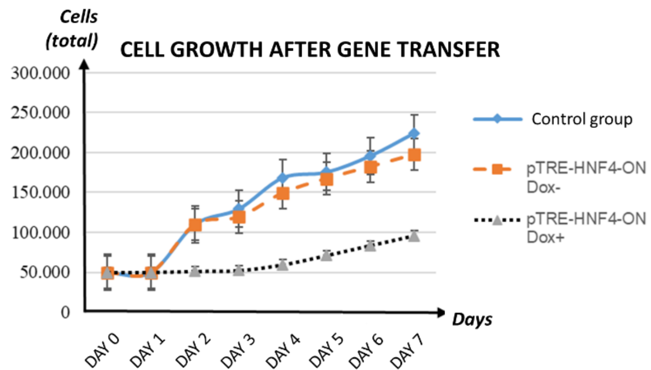



*Figure 3.38.* Growth rate of MSCs after transfection

Figure 3.38 shows that transfection with the pTRE-*HNF4*-ON recombinant vector does not promote MSCs proliferation but enhances differentiation, leading to a longer lag phase, indicating that MSCs cease rapid proliferation and begin differentiation.

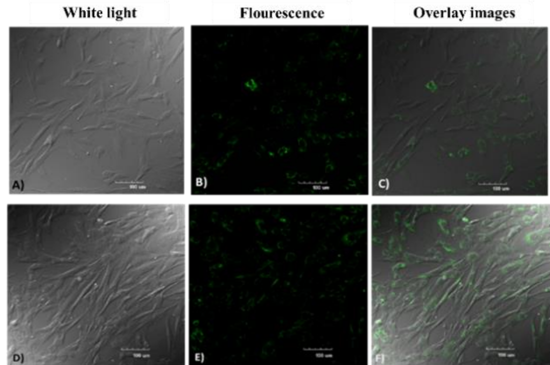
In another experiment to assess the influence of Dox on gene transfection, after 24 hours of transfecting the vector carrying the *HNF4α* gene, Doxycycline was added to the cell culture medium to activate the protein synthesis process. The results show that, in the group activated by Doxycycline, the cell proliferation rate decreased significantly, with cells maintaining a prolonged lag phase, indicating a significantly lower growth rate until day 8 (Figure 3.39).

*Figure 3.39.* Cell growth after gene transfection activated by Doxycycline




We observed the expression of target genes using laser scanning confocal microscopy 24 hours after transfecting cells with the *HNF4α*-carrying vector to detect whether MSCs was successfully transfected or not. The results showed that most cells exhibited expression of the ZsGreen1 gene. ZsGreen1 is a commercial fluorescent protein that serves as an indicator of the Tet-ON system. Fluorescence appeared in the cytoplasm of cells 2 days after gene transfection and activation by Doxycycline in both the pTet-DualON transfection group (Figure 3.40A, Figure 3.40B, Figure 3.40C) and the pTRE-*HNF4α*-ON transfection group (Figure 3.40D, Figure 3.40E, Figure 3.40F)

*Figure 3.40.* Fluorescence appearance in the cytoplasm of cells 2 days after gene transfection and activation by Doxycycline: A), B), C) pTet-DualON transfection group; D), E), F) pTRE-*HNF4α*-ON transfection group




*3.3.4.3.. Assessing the differentiation potential of cells following gene transfer.*

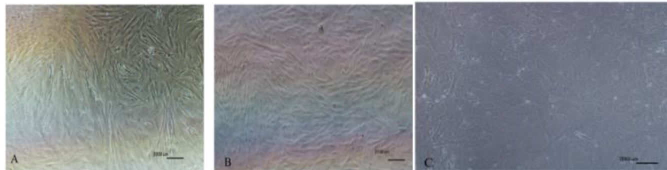



*Figure 3.41.* Cellular morphological changes during gene transfer process

Following the first week post-gene transfer, we observed that cells initially exhibited a characteristic morphology resembling primitive fibroblast-like cells (Figure 3.41A), which subsequently underwent shortening along their longitudinal axis (Figure 3.41B). Transitioning into the second week, polygonal-shaped cells reminiscent of liver parenchymal cells emerged, with an increased ratio of cellular cytoplasm to nucleus volume (Figure 3.41C). Additionally, we noted the presence of cells with 1-3 nuclei, a characteristic feature indicative of hepatic cells.

After approximately 21-28 days of culture, total RNA was extracted and subjected to reverse transcription PCR. The results indicated the expression of the *HNF4α* gene in the genetically modified liver cell line (MSCS). The expression of the *HNF4α* gene is known to promote the

expression of other genes associated with liver cells. Additionally, electrophoresis results showed another characteristic marker of liver cells, alpha-fetoprotein (AFP) expression (Figure 3.42).

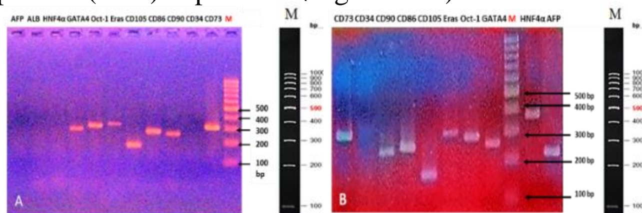



*Figure 3.42*. Expression of markers before and after gene transfer. A. Before gene transfer. B. After gene transfer

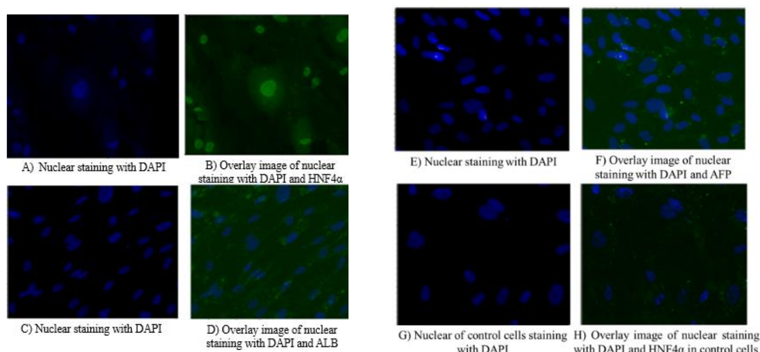



*Figure 3.43.* Immunofluorescence staining of differentiated cells after 2 weeks: A, B: Cells stained with *HNF4α*; C, D: Cells stained with ALB; E, F: Cells stained with AFP; G, H: Undifferentiated control MSCs stained with *HNF4α* (Magnification 100X)

Figure 3.43 illustrates that within 2 weeks post-induction, cells began to exhibit positivity for characteristic functional markers of liver cells (*HNF4α*, ALB, and AFP). This expression was evident in differentiated cells (Figures 3.43A, 3.43B, 3.43C, 3.43D, 3.43E, 3.43F) compared to control cells (Figures 3.43G, 3.43H). Indocyanine green (ICG) fluorescence was only observed in differentiated cells after 1 hour of incubation. Cell nuclei stained with DAPI exhibited a blue color. In the batch of cells stained with

*HNF4α* (Figures 3.43A, 3.43B), ICG fluorescence was observed in the cell nuclei, indicating successful translocation of *HNF4α* into the cell nuclei.

The MSCs transfected with pTRE-*HNF4α*-ON and activated with Dox were analyzed using qPCR to assess the mRNA expression levels of *HNF-4α*, AFP, ALB, and CYP3A4 at different time points. Among these genes, the expression of *HNF-4α* mRNA showed an increase in the early stages (day 7 - after 1 week), followed by a significant increase on day 14 (after 2 weeks), and then a significant decrease on day 21 (after 3 weeks) (Figure 3.44A). The expression level of the CYP3A4 gene did not show a statistically significant increase compared to the control. The highest increase was recorded on day 14 (2,08 times), followed by a slight decrease on day 21 (2,01 times) (Figure 3.44B). The expression level of the ALB gene showed a progressive increase compared to the control with fold changes of 2,31 after 7 days, 2,33 after 14 days, and the highest at 9,29 after 21 days (statistically significant) (Figure 3.44C). The expression level of the AFP gene increased by 1,74 times compared to the control after 7 days. However, there were no significant differences in the expression level of the AFP gene after 14 and 21 days, with fold changes of 1,76 and 1,84 compared to the control, respectively (Figure 3.44D).

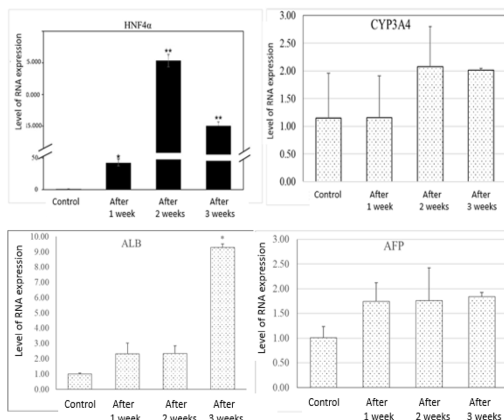



*Figure 3.44.* qPCR results determining the mRNA expression levels of *HNF4α* (A), CYP3A4 (B), ALB (C), and AFP (D) at different time points. The experiment was repeated three times, and the results are statistically significant at *P < 0.01; **P < 0.001

Cells did not stain with PAS (Periodic Acid-Schiff) in the batch of cells not activated with Doxycycline (Figure 3.45A). Conversely, in the batch of cells activated with Doxycycline to express *HNF4α*, the cells exhibited a pink-red staining indicative of PAS staining (Figure 3.45B)

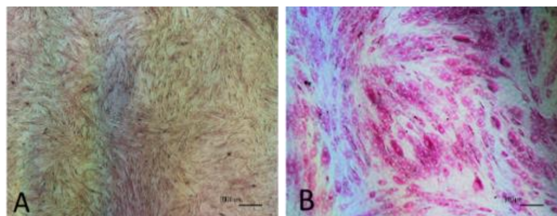



Figure 3.45. PAS staining results of *HNF4α* gene-transfected cell group after 2 weeks.

## CONCLUSIONS AND SUGGESTIONS

### CONCLUSIONS

**1.** MSCs from the umbilical cord has been successfully isolated, cultured, and preserved, exhibiting characteristic markers of MSCs such as positive expression of CD73, CD90, and CD105. Surface marker profiling also revealed that the isolated cell population had a positive expression rate for MSCs characteristic markers ranging from 95.03% to 99.90%.

**2.** The cells exhibit stable proliferation under *in vitro* conditions and have the potential to differentiate into adipocytes and osteoblasts. The freeze-thaw process does not compromise the morphological stability of umbilical cord MSCs; however, it reduces the cell viability to 85.29±1.56% compared to the viability of 98.33±0.58% for pre-freeze MSCs. Post-thaw growth potential and positivity for characteristic MSCs markers such as CD73, CD90, CD105 decrease compared to pre-freeze MSCs.

**3.** Successful differentiation of umbilical cord into hepatocyte-like cells. Following differentiation, cells underwent morphological changes, with the earliest detection observed on day 7, and by day 21, the majority of cells

transformed from fibroblast-like morphology to polygonal-shaped cells, characteristic of liver cells. Differentiation via *HNF4α* gene transduction through the Tet-ON expression system proved to be the most efficient and rapid method compared to differentiation methods using DMSO, a combination of HGF + DEX + OSM, and a combination of HGF + TSA + OSM. Specifically, RT-PCR analysis revealed expression of two liver cell-related markers, AFP and *HNF4α*, from day 21 (*HNF4α* gene transduction batch) and day 28 (other differentiation method batches). Immunofluorescence staining of differentiated cells in the gene transduction differentiation group showed expression of *HNF4α*, ALB, and AFP from day 14 post-differentiation. Evaluation of glycogen storage capacity in differentiated cells demonstrated higher glycogen storage levels on day 14 (gene transduction differentiation batch) and day 21 (other differentiation method batches) compared to the negative control group of undifferentiated cells.

**SUGGESTIONS**

**(1)** Further long-term monitoring is required to investigate the impact of freezing temperature and storage conditions on the growth of cells post-isolation;

**(2)** Continued research is needed to quantitatively compare protein levels in cells using Western blot imaging or by secretion into the culture medium (ELISA);

**(3)** Further research and additional necessary evaluations should be conducted, along with extending the differentiation period to mature liver cells, to serve the purpose of screening biologically active compounds and drug testing.

**(4)** Continued research and additional evaluations are needed to monitor both stem cells and liver cells *in vivo* through experimental mouse models.

## LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. **Do Trung Kien**, Nguyen Van Hanh, Nguyen Thi Thanh Nga, Nguyen Quynh Anh, Tran Thi Oanh, Nguyen Huu Đuc, Hoang Nghia Sơn, Chu Hoang Ha, *Expression of specific mesenchymal stem cell markers of stem cell derived from human umbilical cord and placenta*, Vietnam Journal of Biotechnology, 2017, 15(3A), 1-7.
2. Nguyen Van Hanh, **Do Trung Kien**, Nguyen Thi Thanh Nga, Nguyen Quynh Anh, Hoang Nghia Son, Chu Hoang Ha, *Evaluation of the stability of umbilical cord mesenchymal stem cells during in vitro culture,* Vietnam Journal of Biotechnology, 2017, 15(3A), 9-13.
3. **Trung Kien Do**, Van Hanh Nguyen, Thanh Nga Nguyen, Dinh Minh Pham, Trung Nam Nguyen,Thi Thanh Huyen Tran, Thi Thu Huong Ngo, Hoang Ha Chu, *Efficient Isolation and Long-term Red Fluorescent Nanodia-mond Labeling of Umbilical Cord Mesenchymal Stem Cells for the Effective Differentiation into Hepatocyte-like Cells*, Brazilian Archives of Biology and Technology, 2020, 63(e20200082), http://dx.doi.org/10.1590/1678- 4324-2020200082.
4. Nguyen Thi Nhung, **Do Trung Kien**, Nguyen Thi Hiep, Tran Thi Huong Giang, Nguyen Van Hanh, *Evaluation the ability to isolate and culture stem cells from frozen human umbilical cord tissue*, VNU Journal of Science: Natural Sciences and Technology, 2022, 38(2), 17-22.