

Specific transcription factors distinguish umbilical cord mesenchymal stem cells from fibroblasts

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SUMMARY

Now at the forefront of scientific research, stem cells play a crucial role in regenerative medicine and cell therapy. Although noted for their great promise in understanding organismal development and potential as a treatment for various diseases, stem cell research and applications have been limited by ethical and political concerns. However, recent research regarding induced pluripotent stem cells (iPSCs) has revolutionized the principle of stem cell-based treatment, especially since certain ethical controversies are no longer applicable. In 2009, scientists discovered that differentiated somatic cells could be induced to their stem-cell state by expressing transcription factors specific to self-renewal and potency. Since iPSCs are derived from one's own somatic cells, they bypass ethical and political concerns. Our objective is to further investigate whether specific protein markers, inherent to stem cells and their properties of self-renewal and potency, can be used to identify umbilical cord mesenchymal stem cells (UC-MSCs). We compared fibroblasts as a control cell type because of their similar physical structure to stem cells and their lack of self-renewal and potency-specific markers. We cultured both cell lines and measured protein levels of four selected factors (β -actin, Klf4, Nanog, and Sox2) using western blot techniques. Our results revealed that these selected proteins were expressed exclusively by UC-MSCs and not by fibroblasts, successfully demonstrating that specific protein markers can be used to distinguish UC-MSCs.

INTRODUCTION

Stem cells are cells with the unique potential to differentiate, or specialize, into cell types with various specific functions. For example, while a retina cell of the eye or a β -cell of the islets of Langerhans can only divide into retina cells or β cells respectively, a stem cell has the potential to differentiate into a muscle cell, neuronal cell, etc. Stem cells can be divided into three large subgroups – embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs) (1). Our investigation focused on umbilical cord mesenchymal stem cells (UC-MSCs). Mesenchymal stem cells (MSCs) are multipotent ASCs that are able to differentiate into cells of a mesodermal origin: osteoblasts, chondrocytes, and adipocytes (2).

Two properties essential to a stem cell include self-renewal and potency (3,4). Self-renewal describes the process of cell

replication specific to stem cells. Through self-renewal, stem cells undergo replication while maintaining pluripotency, thus increasing the number of stem cells. Moreover, stem cells have potency, the ability to specialize into different types of cells. The potency of stem cells ranges from totipotent, meaning the cells are able to differentiate into over 200 cell types, to unipotent, meaning the cells are able to differentiate into only one type of cell, depending on where the stem cells were extracted and their stage of development.

To manage and ensure the potency and self-renewal of the cell, stem cells express specific proteins that maintain its immature state (5). The expression of certain proteins in differentiated, mature cells causes these cells to revert into undifferentiated stem cells. Using this principle, Shinya Yamanaka pioneered iPSC technology and discovered that four transcription factors could be used to generate pluripotent stem cells from mature, differentiated somatic cells. Specifically, Yamanaka successfully derived iPSCs from mature fibroblasts by introducing pluripotency-associated “reprogramming factors” – Nanog, Sox2, Klf4, and cMyc – into this somatic cell type (6).

In essence, expressing these protein markers can cause a differentiated, somatic cell to revert into a stem cell, regaining its potency and self-renewal capabilities. Our study involves three of these reprogramming factors (Nanog, Sox2, and Klf4) due to issues of availability and access. Nanog is a transcription factor that maintains pluripotency in ESCs by repressing the expression of cell determination factors. Sox2 is also a protein essential in maintaining the potency of undifferentiated stem cells (7). Klf4, or kruppel like factor 4, is involved in the proliferation, apoptosis, and reprogramming of somatic cells (8). In ESCs and MSCs, Klf4 is an indicator of stem cell capacity. Because all abovementioned factors contribute to stem-cell like properties, we hypothesized that these four factors would be present in all stem cells, including UC-MSC. As these factors have, in effect, given somatic cells the characteristics of a stem cell, it is possible to deduce that these four factors are present in all stem cells, including UC-MSC.

The aim of our experiment was to determine whether we could distinguish UC-MSCs from fibroblasts on the basis of protein marker expression. We chose fibroblasts as a control cell type because of their similarity in morphology to that of UC-MSCs, in order to prevent conclusions resulting from differential physical appearance. Furthermore, we used β -actin, a housekeeping gene present in both UC-MSCs and fibroblasts, as a loading control to ensure that success

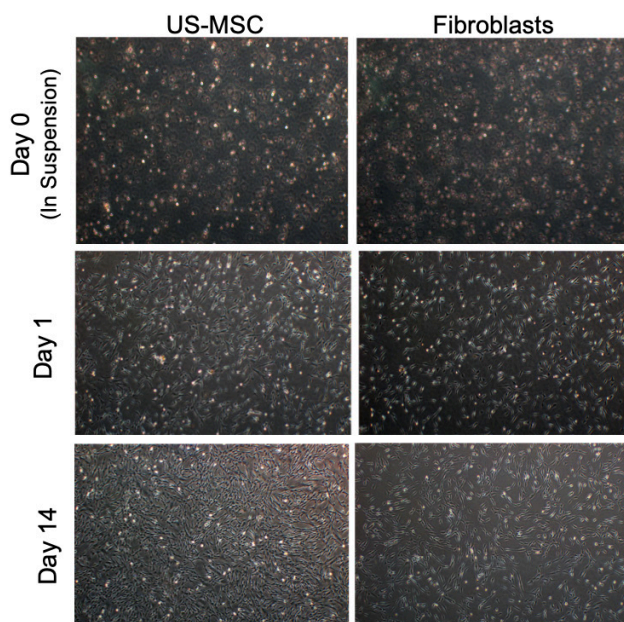


Figure 1. Growth of UC-MSCs and Fibroblast Cell Lines. Representative images of UC-MSCs (left) and fibroblasts (right) were imaged using a microscope at 20x magnification at Days 0, 1 and 14 of culture. After 24 hours (Day 1), it is apparent that the cell lines have settled.
of the methodology.

UC-MSCs are pluripotent cells, meaning that they express proteins necessary for self-renewal and potency, the two key aspects that define a stem cell. On the other hand, a fibroblast should not need proteins for such functions, because they are specialized cells. Therefore, we hypothesized that self-renewal and potency-specific protein markers would be expressed in UC-MSCs, but not in fibroblasts, as they lack stem cell specific properties such as self-renewal and potency.

In conclusion, our results from these experiments revealed that only UC-MSCs expressed Klf4, Nanog, and Sox2 and both cell types expressed β -actin. This has important implications regarding methods of differentiating between different cell lines without varying physical characteristics.

RESULTS

We performed our experiments on cultured UC-MSCs and fibroblast cells. To ensure the health of UC-MSCs and fibroblast cells and make sure they were growing appropriately, we maintained consistent observation and collection of qualitative data. We demonstrated successful proliferation of both cell lines throughout the process of cell culture and cell banking using microscopes to observe the proliferation rates of cells (**Figure 1**). We cultured both cell lines for two weeks before we lysed cells for protein analysis (**Figure 1**).

We initially observed and compared the results of western blot with literature reading and band values, and subsequently compared protein markers' expression in

UC-MSCs and fibroblasts

Western blot of Klf4 was conducted to examine its presence in both UC-MSCs and fibroblasts. (**Figure 2A**). Typically, Klf-4 has a band weight of approximately 56 kDa, as per the manual outlined on Cell Signal (CST), where the antibodies were purchased. UC-MSCs expressed Klf-4, while fibroblasts did not. Average band weights for Sox2 antibodies are approximately 35 kDa. The western blot results of UC-MSCs confirms the literature value of band weight, while fibroblasts did not express Sox2 (**Figure 2B**). Although not present in fibroblasts, Nanog is present in UC-MSCs and reinforces the literature weight of Nanog, approximately 40 kDa (**Figure 2C**).

Western blot of β -actin, a housekeeping gene present in both UC-MSCs and fibroblasts, served as a positive control to ensure that the methodology of the investigation was conducted without error. (9) We found expression of β -actin, appearing as a band weighing 40 kDa, in accord with the value suggested by the CST manual (**Figure 2D**). Because β -actin was present in both cell types and of similar band intensities, the experiment's methodology was accurately completed.

DISCUSSION

Based on the expression levels of β -actin and three transcription factors, Nanog, Sox2, and Klf4, specific to self-renewal and pluripotency of stem cells, we concluded that the UC-MSCs express the protein markers that were used to develop iPSCs. As expected, fibroblasts, being specialized cells, expressed only β -actin, a housekeeping gene, and showed lower levels of expression of the self-renewal and pluripotency related markers.

There were some instances where human error could have impacted the results. For instance, the blotting paper of western blot may have had imperfections, such as small bubbles, that might have prevented the effective migration of proteins across the membrane. Another source of error may have been the amount of time the western blot was conducted. Due to constraints with the facility, we performed the gel electrophoresis overnight, in which a lower voltage was used for a longer period of time. The voltage or time may not have been sufficient for proteins to completely travel down the gel. Moreover, fibroblasts expressed minute amounts of Nanog. It could be hypothesized that fibroblasts would express little to no Nanog as it is a differentiated cell. Fibroblasts may contain trace amounts of Nanog as it has been shown that fibroblasts contain some UC-MSCs like behavior (10). A potential source of error may have been during the experimentation, where pipettes may not have been completely sterile when transferring fibroblast and UC-MSCs cell lysate. However, given that predicted results were successfully obtained, we concluded that these aspects did not have a substantiable impact on results.

The experimental results suggest that analyzing protein

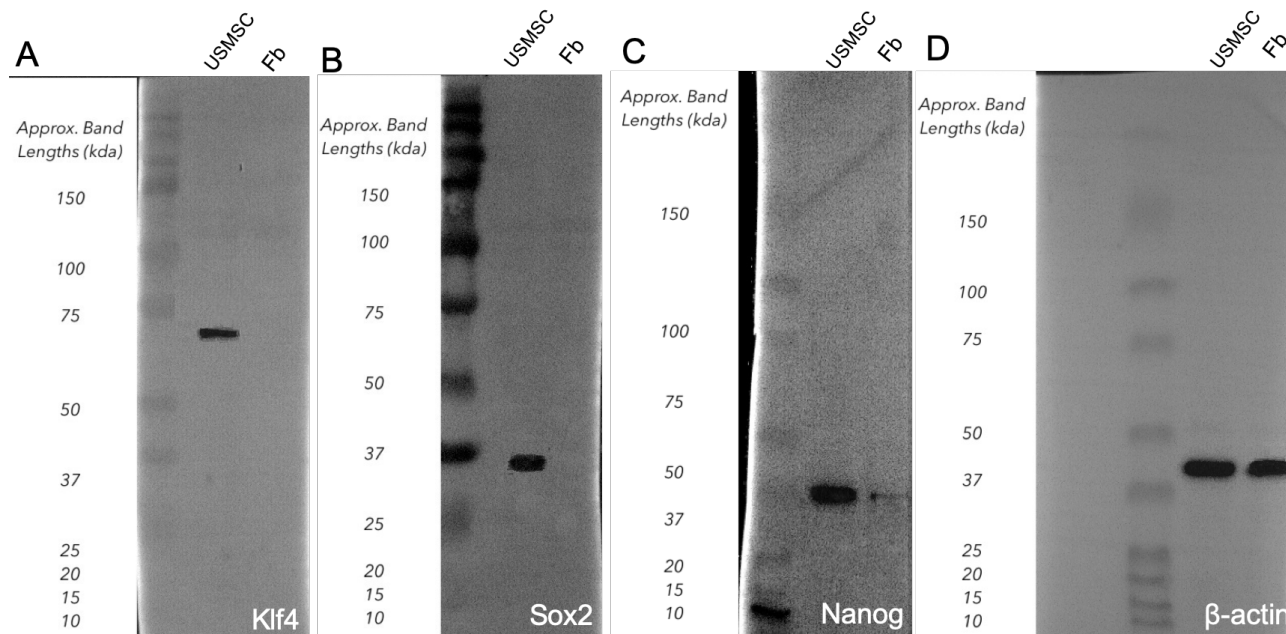


Figure 2. Comparison of protein levels of selected factors between US-MSC and fibroblasts. Protein levels in US-MSC and fibroblasts (Fb) were assessed by Western blot. (A) Klf4 is present in UC-MSC but not fibroblasts, confirming the hypothesis. (B) Sox2 is present in UC-MSC, but not fibroblasts affirming our hypothesis. (C) Nanog was expressed in UC-MSC, but not fibroblasts. (D) β -actin served as a control and similar band intensities of the same weight were observed.

expression using western blot is an effective technique of differentiating UC-MSC from other cell types. Therefore, western blotting proteins specific to a certain cell line seems to be a reasonable method of distinguishing certain cell lines.

Future studies can be conducted related to this experiment and field of study. The effect of stem cell age or time in growth medium on expression of self-renewal and potency factors could be examined. For example, do older stem cells express less transcription factors? This may have implications on future research, where younger stem cells are used to fully express the self-renewal and potency related characteristics of stem cells. The expression levels of potency transcription factors in other cell types, such as embryonic stem cells, could be examined as they are pluripotent and have a wider range of potency. Although likely legally restricted in many nations, this question presents an interesting study of pluripotency in different types of stem cells.

METHODS

Cell Culture

Umbilical cord mesenchymal stem cells and fibroblasts were cultured for two weeks at 37°C and 5% CO₂, allowing for proliferation to obtain the desired cell number for protein analysis. UC-MSCs were obtained from the CHA BioTECH, where there was a source of different cell lines.

Cells were grown in α -MEM containing fetal bovine serum (FBS), penicillin-streptomycin (P/S), and β -FGF2 (concentration 10 μ g/mL).

Vials of cells were thawed in a 37°C water bath,

centrifuged for five minutes at 1500 rpm, and the supernatant was removed. This process was repeated as necessary to ensure that only the pellet remained and all the supernatant was removed. Subsequently, the pellet was resuspended and added to a T-175 flask with media. To ensure successful cell seeding, a microscope with 40x magnification was used.

Cells from the flask were removed by first aspirating the pre-existing media and washing the cells using PBS. Then, 1X Trypsin-EDTA was added to the flask, which was placed in an incubator for one to two minutes. Fresh media was added to the flask and its contents were transferred to a new 15 mL tube. The tube was centrifuged at 1500 rpm for five minutes, and the supernatant was removed. A 9:1 ratio of FBS and DMSO was used to resuspend and transfer the cell pellet from the 15 mL tube to a vial. Vials were stored in a freezing box at -80°C for 24 hours and then placed in a liquid nitrogen tank.

Western Blot

The western blot analysis was performed to determine the presence of specific protein markers in UC-MSCs and fibroblasts cell lines. The process was split into three main steps – SDS-PAGE, antibody staining, and detection. To lyse the cells and extract their proteins, RIPA lysis buffer was added to the cell vials from the previous step and centrifuged for 15 minutes. The cell lysates were then placed on ice to minimize the effect of proteolysis and denaturation. Subsequently, SDS PAGE was conducted. In this particular experiment, an overnight electro transfer was used. Proteins were transferred from the gel to a polyvinylidene difluoride solution for greater accessibility to the antibodies.

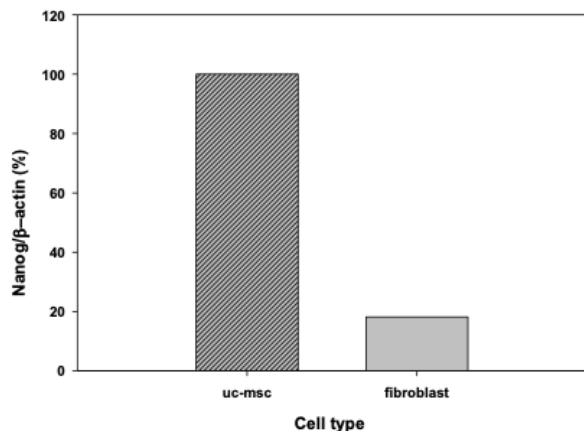


Figure 3. Ratio of Nanog to β-actin in UC-MSC and Fibroblasts
A considerably higher presence of Nanog was found in UC-MSC than in fibroblasts.

Following SDS PAGE, primary and secondary antibodies were incubated for one hour at room temperature and washed using 3% BSA and TBST. The antibodies and proteins were placed on a microplate shaker for one hour, to ensure that the western blot was completed successfully. Finally, we added a detection solution (CST) to the membrane and used chemiluminescence to detect protein bands with a CCD camera. All antibodies used include anti-Nanog (CST), anti-Sox2 (CST), anti-Klf4 (CST), anti-β-actin (CST).

Data Analysis

We used ImageJ to quantify the intensity of each protein band. As observed in **Figure 3**, which represents the graphical data numerically, the expression of Nanog (as a ratio of Nanog: β-actin) in UC-MSCs and fibroblasts was 100:18. Therefore, expression of Nanog was considerably higher in UC-MSCs than in fibroblasts.

REFERENCES

- Jung, Sang Youn. "Adult Stem Cell Treatment for Rheumatoid Arthritis." *Journal of Rheumatic Diseases*, vol. 25, no. 3, 2018, p.158., doi:10.4078/jrd.2018.25.3.158.
- Murray, Iain R., and Bruno Péault. "Q&A: Mesenchymal Stem Cells — Where Do They Come from and Is It Important?" *BMC Biology*, vol. 13, no. 1, 2015, doi:10.1186/s12915-015-0212-7.
- Shenghui, He, et al. "Mechanisms of Stem Cell Self-Renewal." *Annual Review of Cell and Developmental Biology*, vol. 25, no. 1, 2009, pp. 377–406., doi:10.1146/annurev.cellbio.042308.113248.
- Singh, Vimal K., et al. "Describing the Stem Cell Potency: The Various Methods of Functional Assessment and In Silico Diagnostics." *Frontiers in Cell and Developmental Biology*, vol. 4, 2016, doi:10.3389/fcell.2016.00134.
- Matson, Jacob Peter, et al. "Rapid DNA Replication Origin Licensing Protects Stem Cell Pluripotency." *ELife*, vol. 6, 2017, doi:10.7554/elife.30473.
- Yamanaka, S. "Induction of Pluripotent Stem Cells from Mouse Fibroblasts by Four Transcription Factors." *Cell Proliferation*, vol. 41, 2007, pp. 51–56., doi:10.1111/j.1365-2184.2008.00493.x.
- Singh, Vimal K., et al. "Induced Pluripotent Stem Cells: Applications in Regenerative Medicine, Disease Modeling, and Drug Discovery." *Frontiers in Cell and Developmental Biology*, vol. 3, 2015, doi:10.3389/fcell.2015.00002.
- Mallanna, Sunil K., et al. "Proteomic Analysis of Sox2-Associated Proteins During Early Stages of Mouse Embryonic Stem Cell Differentiation Identifies Sox21 as a Novel Regulator of Stem Cell Fate." *Stem Cells*, vol. 28, no. 10, 2010, pp. 1715–1727., doi:10.1002/stem.494.
- Lin, Juntang, and Christoph Redies. "Histological Evidence: Housekeeping Genes Beta-Actin and GAPDH Are of Limited Value for Normalization of Gene Expression." *Development Genes and Evolution*, vol. 222, no. 6, 2012, pp. 369–376., doi:10.1007/s00427-012-0420-x.
- Chang, Yuqiao, et al. "Mesenchymal Stem Cell-Like Properties in Fibroblasts." *Cellular Physiology and Biochemistry*, vol. 34, no. 3, 2014, pp. 703–714., doi:10.1159/000363035.

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