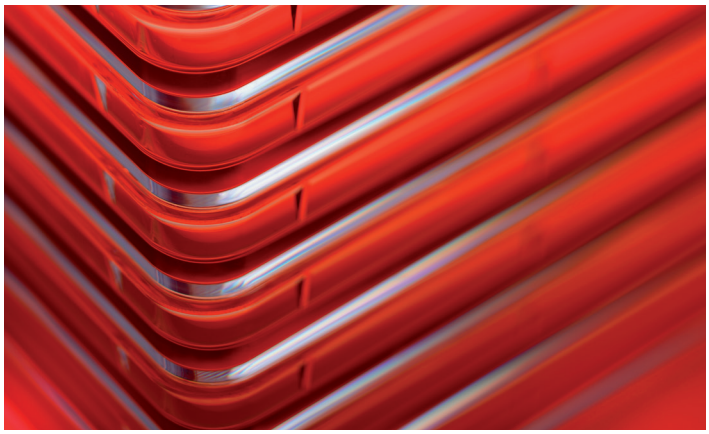
Culture of human mesenchymal stem cells in Nunc Cell Factory systems

Key words: Cell culture, cell culture scale-up, Nunc Cell Factory system, hMSC, expansion and differentiation, mesenchymal stem cell basal medium, Nunclon Delta

Abstract

Thermo Scientific™ Nunc™ Cell Factory™ systems are an effective format for the easy and rapid expansion of human mesenchymal stem cells (hMSCs). The Thermo Scientific™ Nunclon™ Delta surface is a fully synthetic chemical surface available with many options for size, shape, and surface area. hMSCs were removed from liquid nitrogen and plated directly onto the surfaces to be evaluated. T-25 cell culture flasks were used to determine the optimal seeding density prior to expansion of hMSCs on larger formats. For expansion, a Nunc Cell Factory 1-layer system, also with the Nunclon Delta surface, was seeded from a fresh cryovial. These cells were in turn used to seed a 4-layer Cell Factory systems. To ensure that the cells maintained multipotency after expansion, the expanded cells were seeded in Thermo Scientific™ Nunc™ Multidish 48-well plates and differentiated into osteoblasts or adipocytes. The seeding density chosen for expansion was 350 cells/cm² because it was the lowest seeding concentration displaying good exponential growth. The reported expansion protocol was able to increase the cell number 136-fold; however, since Cell Factory systems are available with up to 40 layers, the expansion potential is virtually limitless. Expansion of hMSCs on the Nunclon Delta surface using mesenchymal stem cell basal medium significantly enhances the osteogenic and adipogenic potential of hMSCs and number of differentiated cells, relative to α-MEM medium. Additionally, the use of a trypsin alternative for cell passage gave a slightly higher yield of adipocytes compared to cells passed using trypsin. The methodology presented here can be applied to the large-scale expansion of other cell types for use in various applications using Cell Factory systems.



Introduction

hMSCs are good candidates for clinical or research use because they are readily expanded in culture, have immunomodulatory potential, and can differentiate into the osteogenic, chondrogenic, and adipogenic lineages. The therapeutic potential of hMSCs is currently being studied as part of clinical trials to treat diseases such as graft-versus-host disease[1], osteoarthritis [2], and for the regeneration of cardiac muscle following myocardial infarction [3].

Whether the requirements are for clinical or research use, obtaining a substantial number of cells can constitute a bottleneck for the investigator. hMSCs display some plasticity in their culture conditions, but several investigators report a higher growth index and increased differentiation potential at lower seeding densities [4,5].

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We present a protocol enabling the clinician or researcher to rapidly expand a population of hMSCs on the Nunclon Delta surface utilizing the potential of the Nunc Cell Factory system and mesenchymal stem cell basal medium. The Nunclon Delta surface is a fully synthetic, chemically modified surface that makes the otherwise very hydrophobic polystyrene surface more hydrophilic, thus facilitating cell attachment and growth. The Nunclon Delta surface is available with many options for size, shape, and surface area. hMSCs can be expanded on a single surface, in vessels ranging from a T-25 cell culture flask up to a Cell Factory 40-layer system. The combination of Nunclon Delta surfaces, mesenchymal stem cell media, and our in-house protocol allows for easier expansion and differentiation of hMSCs.

Materials and methods

Cultivation of hMSCs

hMSCs (Lonza, USA) were cultured on the Nunclon Delta surface in either α-MEM containing 10% FBS, 1% penicillin/streptomycin, and 2 mM UltraGlutamine supplement, or mesenchymal stem cell basal medium with 10% stem cell growth supplement. Cells were cultivated at 37°C with a humidified atmosphere of 5% CO_2 in a Thermo Scientific™ Revco™ Ultima™ II Series CO_2 Incubator.

Growth curves

To determine the optimal seeding density for the larger formats, growth curves of hMSCs grown in mesenchymal stem cell and α-MEM growth media were established in T-25 flasks. hMSCs in passage 2 (P2) were plated onto the Nunclon Delta surface. Cultures were placed in an IncuCyte™ Plus system (Essen Instruments, USA) to measure cell proliferation. The IncuCyte Plus system is located inside an incubator and eliminates the need to remove the cells from the culture environment. Cell proliferation is measured kinetically and phase-contrast images are acquired relying on the iebedded contrast-based confluence algorithm to determine monolayer confluence for each image at each time point.

Expansion protocol

5 x 10^5 hMSCs in P2 were thawed onto two Nunclon Delta– treated Cell Factory 1-layer systems with a seeding density of 350 cells/cm^2 with either mesenchymal stem cell or α-MEM growth medium. The cells were cultivated for 8 days, and the medium was changed on days 3 and 7. The Cell Factory 1-layer systems were harvested with either trypsin or a trypsin-EDTA alternative cell dissociation agent and were expanded onto Nunclon Delta–treated Cell Factory 4-layer systems with the same culture conditions used for the Cell Factory 1-layer systems. The remaining cells were banked cryogenically.

Expansion protocol

After the hMSCs were expanded onto the Nunclon Delta–treated Cell Factory 4-layer systems, cells were harvested with either trypsin or a trypsin-EDTA alternative cell dissociation agent. Harvested cells were plated on Nunc Multidish 48-well plates with a seeding density of 5,000 cells/cm^2 in mesenchymal stem cell basal medium or α-MEM. To induce hMSC differentiation into adipocytes or osteoblasts, the medium was changed to either adipogenic or osteogenic differentiation medium supplemented with a stem cell growth supplement. The medium was changed every 4–5 days and the cultures were assayed on days 3, 7, and 18 with commercial kits. The OsteoImage™ assay, which utilizes the OsteoImage PA-1501 kit (Lonza, USA), measures specific staining of the fluorescent reagent on the hydroxyapatite portion of the bone-like nodules deposited by cells. For adipogenic differentiation, the AdipoRed™ PT-7009 kit (Lonza, USA) was used. The kit utilizes Nile Red to dye the intracellular lipids formed inside the differentiating adipocytes. The accumulation of intracellular triglycerides is often used as a marker of adipocyte differentiation. Cells were counted using the NucleoCounter™ automated fluorescence microscope (ChemoMetec). The manufacturer's instructions were followed.

Results and discussion

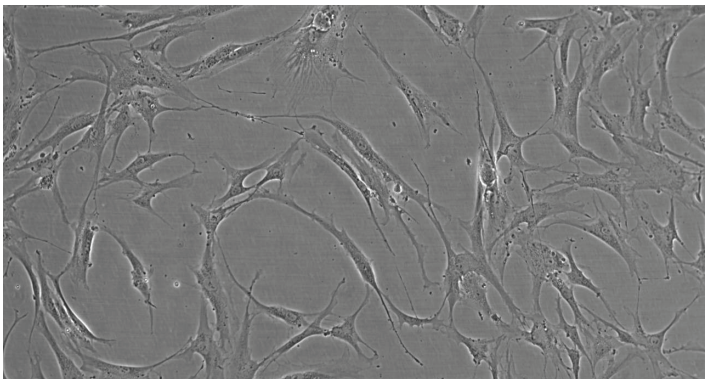
hMSC growth curves

The growth of hMSCs seeded on the Nunclon Delta surface in either α-MEM or mesenchymal stem cell basal medium with three seeding densities was evaluated to determine the optimal seeding density for the expansion protocol. hMSCs were cultured for 12 days at 37°C in a humidified atmosphere of 5% CO_2. hMSC morphology and growth were monitored in the incubator using an IncuCyte Plus imager. hMSCs grown in mesenchymal stem cell basal medium displayed normal morphology on the Nunclon Delta surface after 7 days of incubation (Figure 1A).

For the first 50 hours, after seeding at 1,000 and 4,000 cells/cm^2, hMSCs in α-MEM and mesenchymal stem cell basal medium displayed similar growth curves (Figure 1B), but then the cultures diverged. The growth rate of hMSCs in α-MEM declined and reached a plateau at approximately 80% confluence after approximately 150 hours, for cultures seeded at 1,000 and 4,000 cells/cm^2. The cultures grown in mesenchymal stem cell basal medium continued to grow past 80% confluence and were able to reach 96–99% confluence.

At the lowest seeding density of 350 cells/cm^2, the cultures seeded in α-MEM reached a plateau at around 75% confluence after 180 hours, and cells seeded in mesenchymal stem cell basal medium reached the same level after approximately 250 hours. The purpose of our protocol is to expand a relatively low number of cells; we thus chose a seeding density of 350 cells/cm^2, which displayed good exponential growth (Figure 1B).

A



B

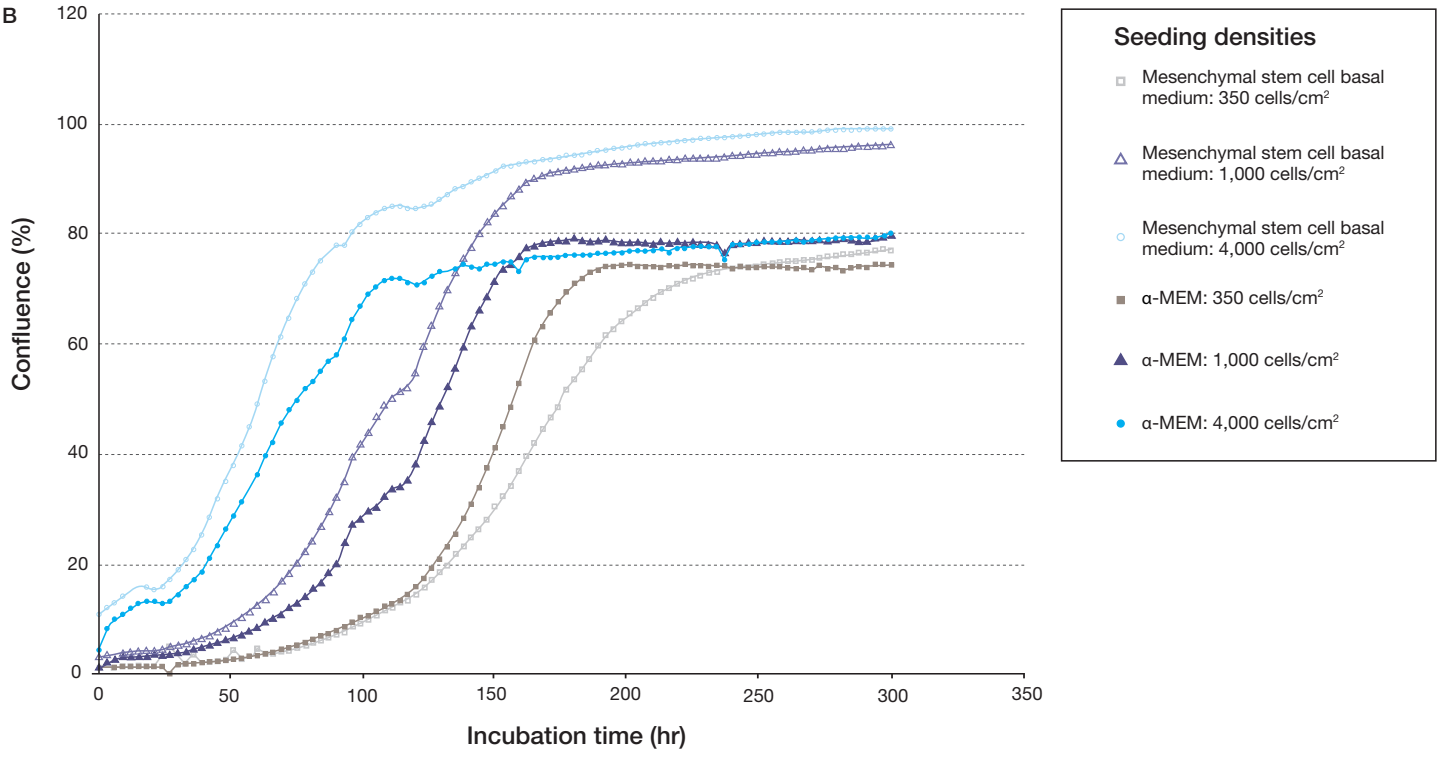


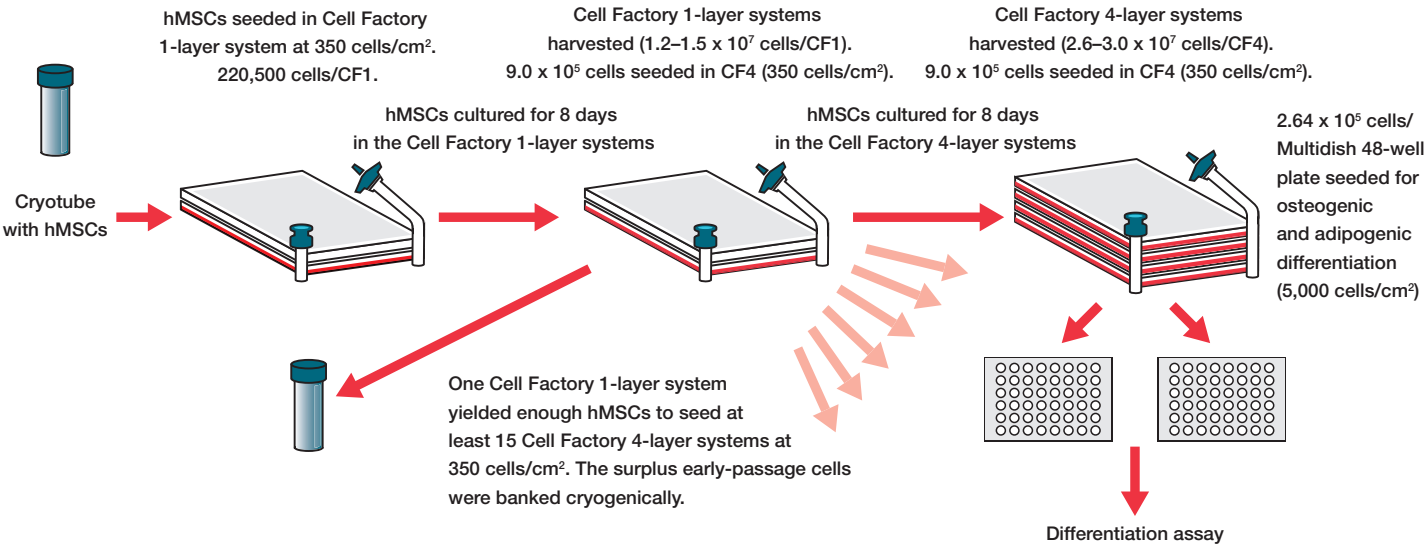
Figure 1. Growth of hMSCs on the Nunclon Delta surface in α-MEM or mesenchymal stem cell basal medium. Cells were seeded at three different densities: 350, 1,000, or 4,000 cells/cm^2. **(A)** Normal hMSC morphology in mesenchymal stem cell basal medium after 7 days of incubation. Seeding density: 350 cells/cm^2. **(B)** Cells were cultured for 12 days under standard conditions, and the media were renewed every 4 days. Each data point represents the mean of 50 measurements in one flask.

Expansion of hMSCs on Nunclon Delta–treated Cell Factory systems

Culturing of hMSCs in α-MEM and mesenchymal stem cell basal medium on Nunclon Delta–treated Cell Factory systems was effective in generating large populations of hMSCs for either differentiation or cryogenic storage. Cells were removed from liquid nitrogen and were plated on two Cell Factory 1-layer systems at a seeding density of 350 cells/cm^2. One Cell Factory 1-layer system yielded enough hMSCs to seed at least fifteen 4-layer systems. Here, the cells were harvested from the 1-layer system with either trypsin or a trypsin-EDTA alternative cell dissociation agent and were used to seed one 4-layer system. The remaining cells were banked cryogenically. Figure 2 displays the actual number of cells seeded and the actual yield obtained, together with the potential yield of 4.50 x 10^8 cells if all cells had been used for scale-up.

hMSCs maintain their multipotency after large-scale expansion in Nunclon Delta–treated Cell Factory systems

To verify that the cells had maintained their ability to differentiate after expansion in both types of growth media in Nunclon Delta–treated Cell Factory systems with both dissociation agents, the cells were differentiated into osteoblasts or adipocytes on a Nunc Multidish 48-well plate with a seeding density of 5,000 cells/cm^2. Differentiation was induced using either osteogenic or adipogenic differentiation media and was monitored on days 3, 7, and 18 using commercial kits.



	Cell Factory 1-layer system seeding population	Cell Factory 1-layer system harvest population	Cell Factory 4-layer system seeding population	Cell Factory 4-layer system harvest population
In our experiment	220,500	1.50 x 10^7	9.60 x 10^5	3.00 x 10^7
Potential			1.44 x 10^7	4.50 x 10^8

Figure 2. Schematic of our in-house hMSC expansion protocol using Nunclon Delta–treated Cell Factory systems.

Marked differences between hMSCs expanded in mesenchymal stem cell basal medium and in α-MEM were observed during differentiation. hMSCs expanded in mesenchymal stem cell basal medium displayed a 59% higher signal using the OsteoImage assay ,compared to cells expanded in α-MEM (Figure 3). Regarding adipogenic differentiation, hMSCs expanded in mesenchymal stem cell basal medium produced significantly more adipocytes than cells expanded in α-MEM. Additionally, cells passaged with the trypsin-EDTA alternative dissociation agent displayed a slightly higher signal using the AdipoRed assay, compared to cells passaged with trypsin. This can be interpreted as an increased yield of hMSCs that successfully differentiated into adipocytes, resulting from the use of the trypsin-EDTA alternative dissociation agent (Figure 4).

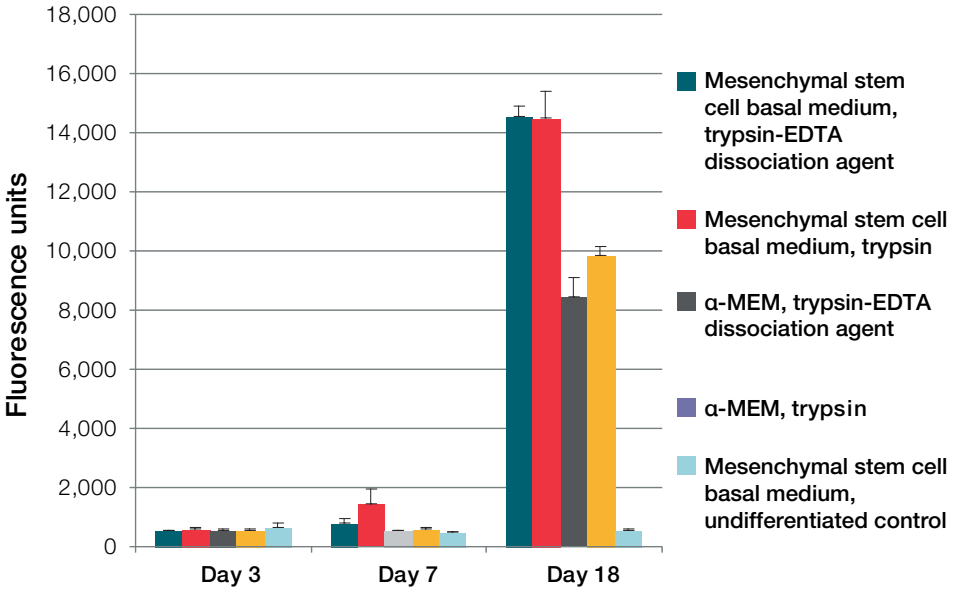


Figure 3. Osteogenic differentiation of hMSCs expanded in a Nunc Cell Factory 4-layer system. hMSCs differentiated to osteoblasts on Nunc Multidish 48-well plates with osteogenic differentiation medium containing stem cell growth supplement were assayed for differentiation with the OsteoImage PA-1501 kit on days 3, 7, and 18.

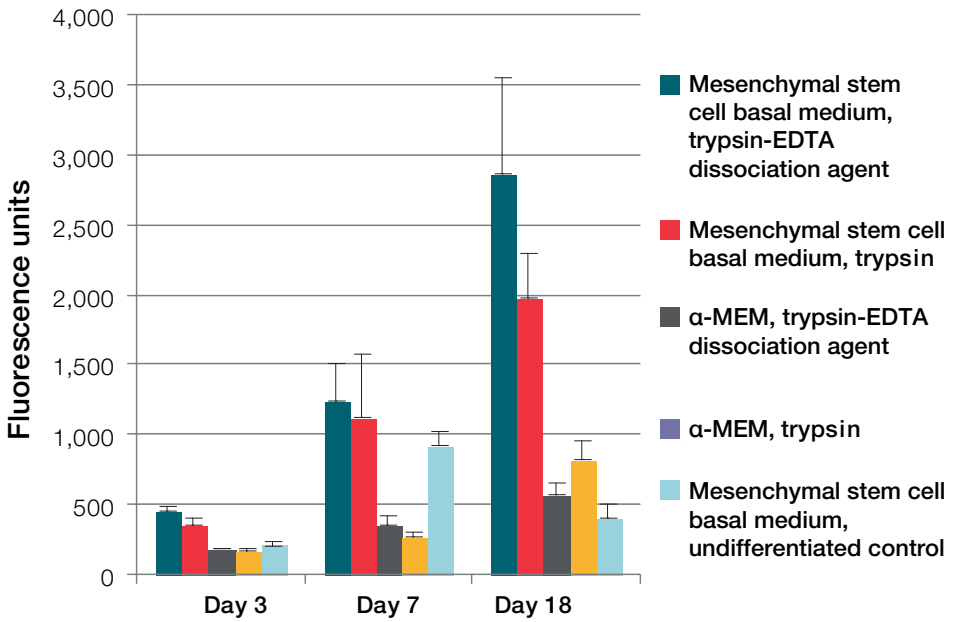


Figure 4. Adipogenic differentiation of hMSCs expanded in a Nunc Cell Factory 4-layer system. hMSCs differentiated to adipocytes on Nunc Multidish 48-well plates with adipogenic differentiation medium containing stem cell growth supplement were assayed for differentiation with the AdipoRed PT-7009 kit on days 3, 7, and 18.

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Conclusions

- Nunclon Delta–treated Nunc Cell Factory systems are an effective format for easy and rapid expansion of hMSCs
- Expansion of hMSCs on the Nunclon Delta surface using mesenchymal stem cell basal medium significantly enhances their osteogenic and adipogenic potential and the number of differentiated cells , compared to α-MEM
- Harvesting cells with a trypsin-EDTA alternative dissociation agent increases the yield of adipocytes compared to harvesting with trypsin
- The methodology presented here can be applied to the large-scale expansion of other cell types for use in various applications using Nunc Cell Factory systems

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