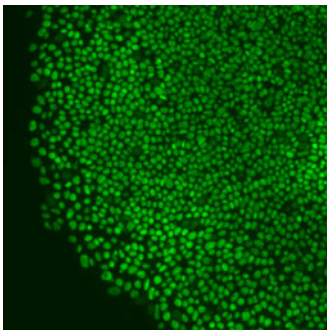
The Recovery of Cryopreserved H9 hESCs on the Corning® Synthemax™ Surface in Defined Medium



SnAPPShots

A brief technical report from the Corning Development Group

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Introduction

Scalable, reproducible, low cost and regulatory-friendly technologies must be developed to enable clinical use of hESC-based therapeutics. hESC culture methods typically include surfaces that use complex, animal-derived products, such as mouse feeder layers, Matrigel™, murine laminin or human-derived biological substances as surfaces to which the hESCs attach. Most of these materials are costly, of limited scalability, have batch to batch variability and are a potential source of adventitious agents.

To overcome these challenges, Corning Life Sciences in collaboration with Geron Corporation developed and commercialized the Corning Synthemax Surface, a fully synthetic, xeno-free, ready-to-use surface for the culture of hESCs. Previous studies demonstrated successful long-term self-renewal of multiple hESC lines (H7, H1, H9, BGO1v/hOG) in several defined commercially available media while maintaining stable proliferation rates, expression of phenotypic hESC markers (OCT4, Tra-1-60, SSEA4), normal karyotype and pluripotency. Corning Synthemax Surface also supported the directed differentiation of H7 cells to functional cardiomyocytes (CLS-AN-151 REV4).

The successful development of hESC-based therapeutics requires the production of master and working cell banks and development of cryopreservation and thaw methods. The objective of this study was to examine the recovery of cryopreserved hESCs on Corning Synthemax Surface in defined medium. Cryopreserved H9 cells were thawed and propagated for at least five serial passages on Corning Synthemax Surface and Matrigel-coated plates in mTeSR®1 medium.

Materials and Methods

- Cells: H9 hESC (WiCell® Line)
- Cell culture medium: mTeSR1 (Stem Cell Technologies, Cat. No. 05850)
  Note: mTeSR1 is a defined medium.
- Thaw medium: mTeSR1 medium containing 4% HSA (Human Serum Albumin, Grifols, Cat. No. TS1-165) and 1% HEPES (Invitrogen, Cat. No. 15630)
- Cell culture surfaces:
  - Corning Synthemax Surface 6 well plates (Corning, Cat No. 3877XX1 or 3979XX1)
  - Biological coating: GFR-Matrigel™ (BD Biosciences, Cat. No. 356231)
  - Tissue Culture Treated (TCT) 6 well plates (Corning, Cat. No. 3516)

hESC thaw method: H9 hESC were removed from liquid nitrogen and quickly thawed in a 37°C water bath by swirling until all ice crystals were melted. Cells were then transferred into a sterile 50 mL conical tube and room temperature thaw medium was added drop wise while gently swirling the cell solution to mix. The cell suspension was centrifuged for 5 minutes at 1,000 rpm and the supernatant removed. The cell pellet was resuspended in room temperature mTeSR1 medium, then brought up to the appropriate volume for seeding. Cells were seeded on Corning Synthemax Surface and GFR-Matrigel-coated TCT plastic 6 well plates (1:30 dilution in KnockOut™ DMEM, Invitrogen, Cat. No. 10829-018) at the density of 300,000 to 400,000 cells per cm^2. Culture vessels were then placed in a humidified incubator set at 5% CO_2 and 37°C.

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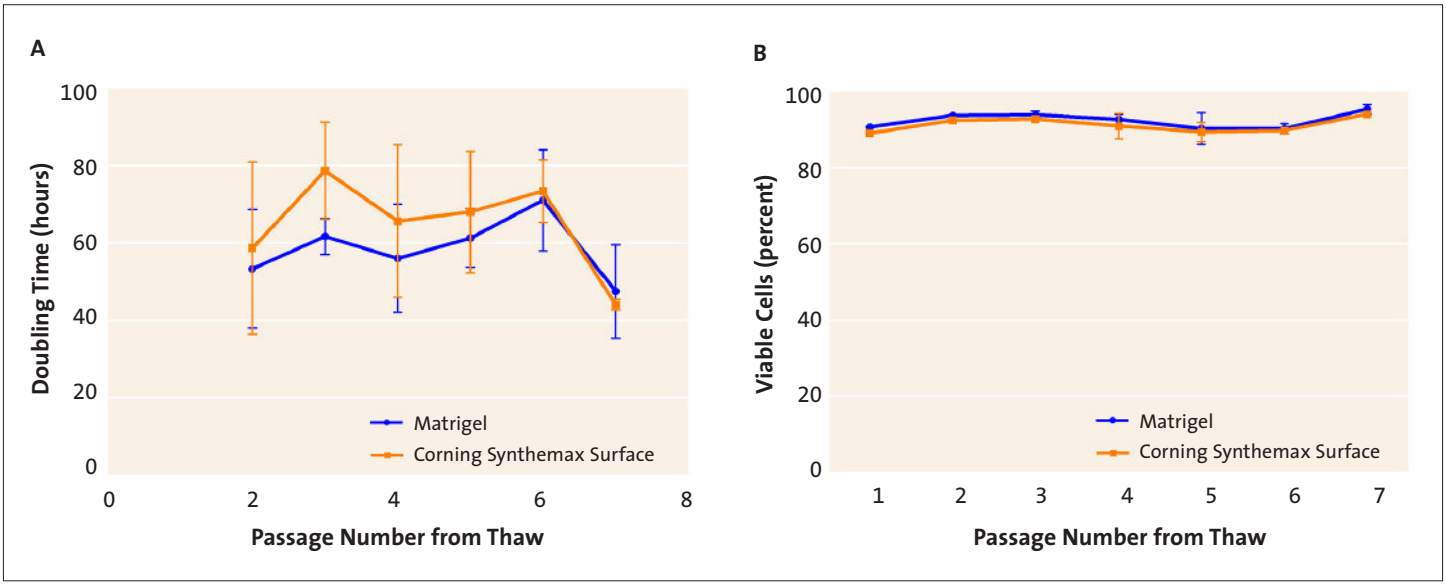

Figure 1. H9 hESC recovery from thaw for 7 sequential passages in defined medium. Doubling time and viability are shown for H9 hESCs cultured on Corning® Synthemax™ Surface and GFR-Matrigel™ for seven sequential passages after thaw on the corresponding surface in defined mTeSR1 medium. The data represents the average of three experiments (n = 3). Proliferation of H9 cells cultured on Corning Synthemax Surface is comparable to cells cultured on Matrigel-coated TCT plates.

Note: H9 hESCs were maintained in mTeSR1 medium on Matrigel™-coated TCT cultureware prior to cryopreservation. For recovery, cells were thawed directly on Matrigel or Corning® Synthemax™ Surface in mTeSR1 medium with no adaptation.

hESC multi-passage study: H9 hESC were propagated on Corning Synthemax Surface and Matrigel-coated TCT 6 well plates in chemically-defined mTeSR1 medium. Cultures were passaged every 4 to 5 days, as cells reached 80% confluence, by incubation with 200 U/mL collagenase IV, followed by a brief dPBS (Invitrogen, Cat. No. 14190-144) wash and gentle scraping (small plastic scraper, Corning Cat. No. 3010). Seeding density was about 100,000 to 120,000 cells/cm^2. Cells received fresh medium every day, except for the day after passaging.

Cultures were routinely examined microscopically for cell and colony morphology. Cell viability and number were assessed at the end of each passage by harvesting one well of each 6 well plate with trypsin/EDTA (0.25%, Invitrogen Cat. No. 25200-114) followed by cell counting with an automated cell number/viability analyzer, Vi-Cell™ (Beckman Coulter). Expression of hESC markers was assessed by flow cytometry (FACSCalibur, BD Biosciences) at the end of each passage. To monitor genomic integrity, cell samples for all experimental conditions were submitted for karyotyping analysis by G-banding (Cytogenetics Laboratories) at passage 6 - 8.

Results/Discussion

The results demonstrate efficient attachment and growth of cryopreserved H9 cells on Corning Synthemax Surface. Doubling time (Figure 1A), viability (Figure 1B), cell morphology (Figure 2), and expression of pluripotency markers (Figures 3 and 4) were comparable to cells recovered on Matrigel (SSEA4 and Tra-1-60 data not shown). Normal karyotype at the end of five passages was confirmed by G-banding analysis (data not shown).

Figure 1 shows the doubling time and viability of H9 cells during seven sequential passages after thaw on both the Corning Synthemax Surface and Matrigel-coated TCT 6 well plates in defined mTeSR1 medium. Doubling time and viability of cells recovered on the Corning Synthemax Surface follow the same trend and show no statistically significant difference as compared to the control Matrigel surface. Fold expansion of cells recovered on Corning Synthemax Surface and Matrigel were also comparable at an average of about 4-fold per passage (data not shown).

Figure 2 shows representative morphology pictures for H9 cells recovered on both the Corning Synthemax Surface and Matrigel-coated TCT 6 well plates. As expected, after thaw (p1) cells on both surfaces showed a higher population of differentiated cells around colony edges and slightly cystic colony centers. Within the next couple of passages, cells on both surfaces resumed typical hESC morphology with tight colonies, high nuclear to cytoplasm ratio, well-defined colony borders, and a lower differentiated cell population. This cell morphology was maintained throughout the rest of the study on both surfaces.

In Figure 3, immunofluorescent staining for pluripotency marker, OCT4, is shown for H9 hESCs thawed and propagated on Corning Synthemax Surface and Matrigel-coated 6 well plates for 7 sequential passages in mTeSR1 medium. Phase contrast images (left), Hoechst nuclear staining (middle), and OCT4 staining (right) are shown for repre-

sentative fields of cells maintained on corresponding surfaces. The majority of cells cultured on Matrigel™ and Corning® Synthemax™ Surfaces express OCT4 (green color) when compared to the total cell population by nuclear staining (blue color).

Figure 4 shows the quantitative analysis of the OCT4 expression in H9 cells thawed and propagated on Corning Synthemax Surface and Matrigel-coated 6 well plates for 7 sequential passages in mTeSR1 medium. Panel A shows typical flow cytometry histograms for H9 cells cultured on Matrigel or Corning Synthemax Surface at p1 and 5 post thaw. The lower population of OCT4 positive cells at p1 on both surfaces is consistent with the morphology images in Figure 2, demonstrating the presence of some differentiated cells during the first passage after recovery from cryopreservation. Within a couple of passages, the percent of OCT4 positive cells increased and remained high on both Matrigel and Corning Synthemax Surfaces (Panel B).

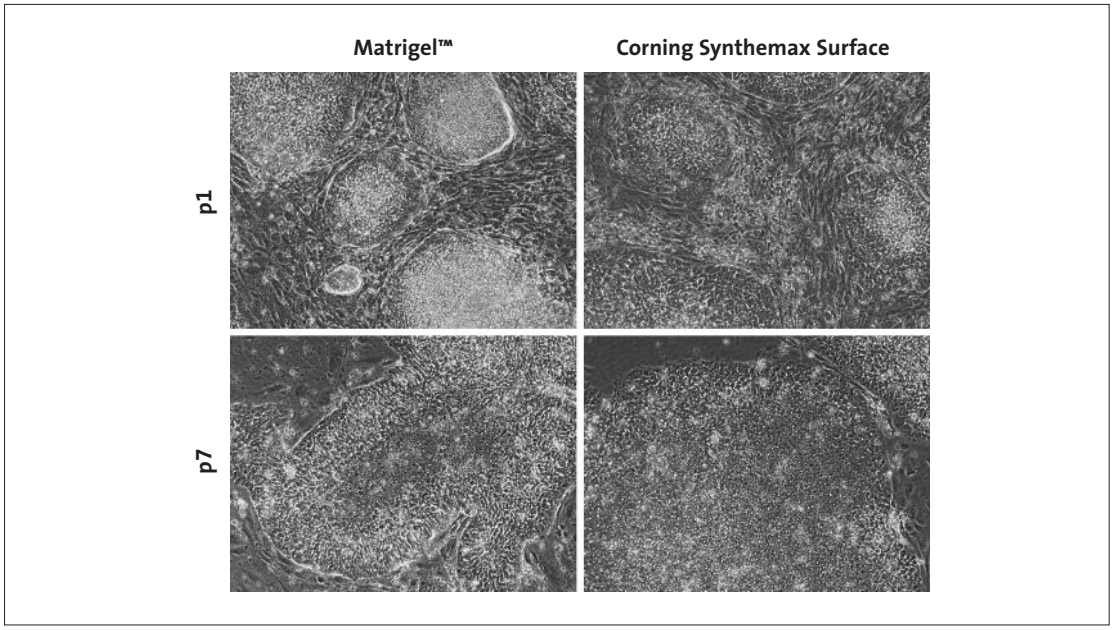

Figure 2. H9 hESC morphology at p1 and p7 post thaw on Corning Synthemax Surface and Matrigel in mTeSR1 defined medium. At passage 1 post-thaw (top), cells on both Matrigel and Corning Synthemax Surfaces exhibited a high proportion of differentiated/stromal cells. After 2 to 3 passages, colonies on both surfaces resume a typical hESC morphology with high nuclear to cytoplasm ratio, well-defined colony borders, and a lower population of differentiated/stromal cells.

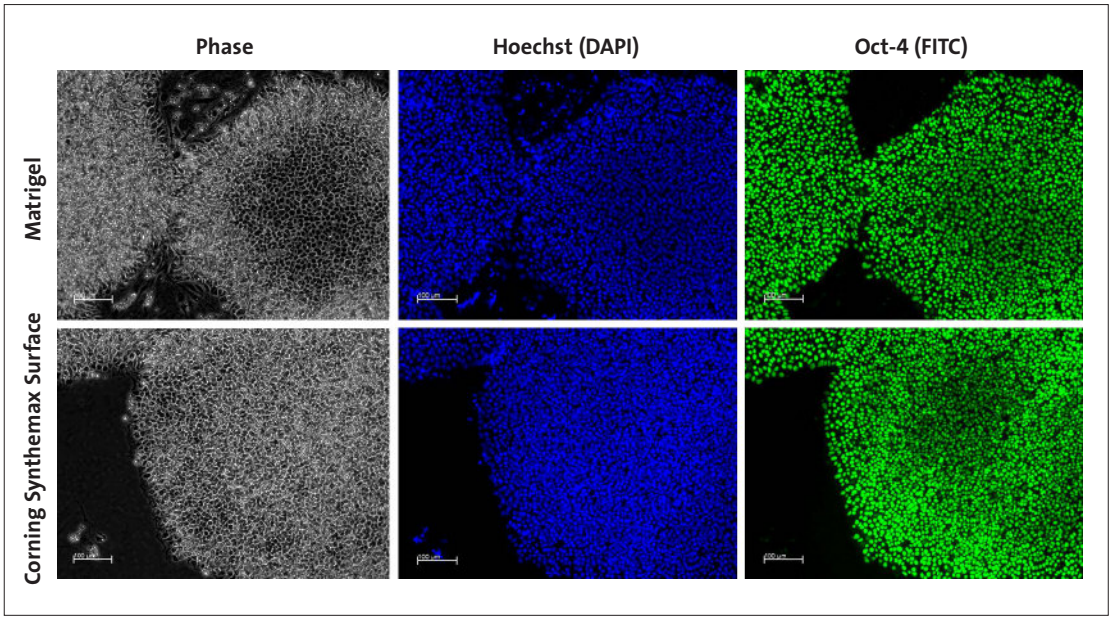

Figure 3. OCT4 immunofluorescent staining of H9 hESC thawed and propagated on Matrigel and Corning Synthemax Surfaces for 7 sequential passages in mTeSR1 medium. Phase contrast (left), Hoechst nuclear staining (middle) and OCT4 staining (right) is shown for representative fields on Matrigel and Corning Synthemax Surfaces at 10x magnification.

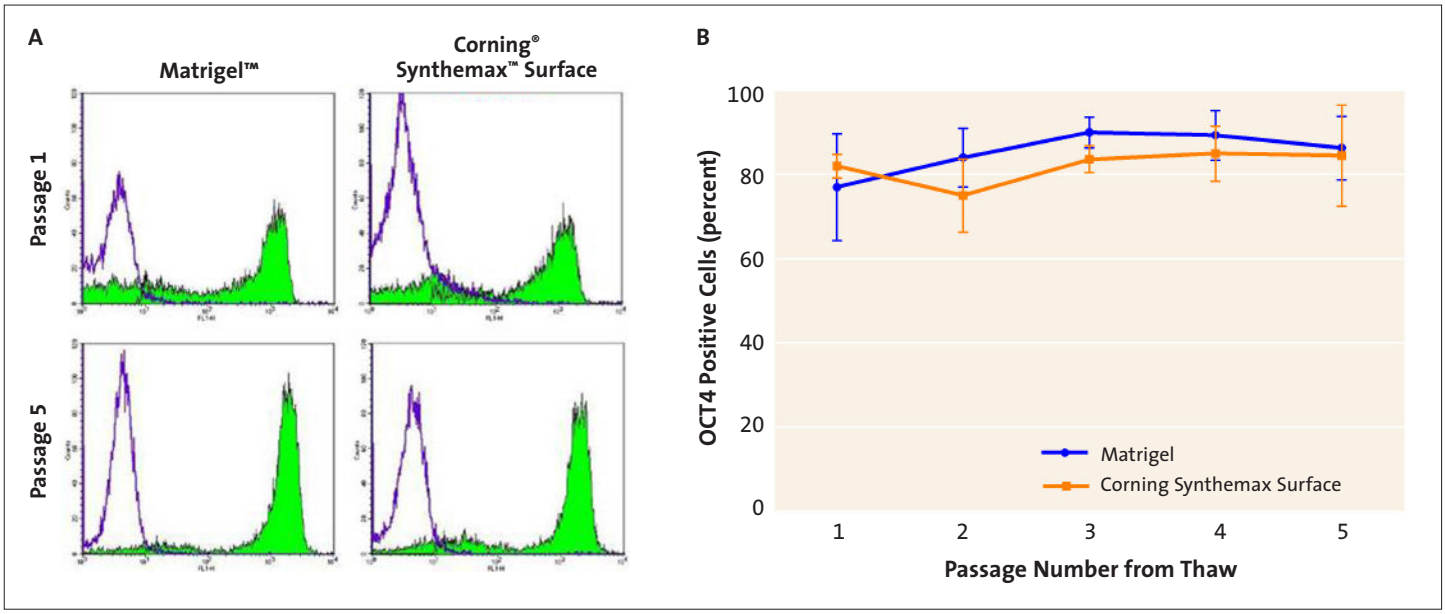

Figure 4. OCT4 pluripotency marker expression in H9 cells during recovery from cryopreservation. (A) Flow cytometry histograms for H9 cells at p1 and 5 post-thaw. Cells at p1 post-thaw show lower levels of OCT4 (top) on both surfaces due to a higher population of differentiated cells (consistent with cell images on the top of Fig. 2). After a few passages OCT4 levels are recovered (>85%, bottom) for both surfaces. (B) Graph shows OCT4 expression (average of three independent experiments, n=3) for H9 hESCs cultured for 5 sequential passages in mTeSR1 medium on both Corning® Synthemax® Surface and Matrigel. Note: The percentage of OCT4+ cells on Corning Synthemax Surface at p5 was considered an outlier for one experiment and removed from the data set. There were an insufficient number of cells for meaningful flow cytometry data.

As far as known, Corning® Synthemax™ Surface is the only commercially available, synthetic, non-biological surface that provides a complete solution for recovery, expansion, and differentiation of hESC in chemically defined media. Therefore, the Corning Synthemax Surface will be useful for both research applications and production of cells for cellular therapies.

Conclusions

- Corning Synthemax Surface supports the recovery from cryopreservation and multi-passage propagation of H9 hESCs in defined mTeSR1 medium.
- H9 hESCs recovered from cryopreservation on Corning Synthemax Surface retain stable doubling time, high viability, phenotypic marker expression, and normal karyotype.

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