

Expansion and Differentiation of H7 Stem Cells in Chemically Defined Medium Using Corning® Synthemax™ -T Surface

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Application Note

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Introduction

Cultures of hESC have two distinct properties which make them appealing for the development of cellular therapies: self-renewal and the potential to differentiate into all major lineages of somatic cells in the human body. To enable the commercialization of hESC-derived therapeutic cells, tools are required which will allow researchers to develop methods without concern of lot variability and the potential of contamination. The development of these culture tools is critical for the utility of the hESC lines to be truly understood and studied. Corning has developed one such tool in the form of a synthetic surface which will allow continued expansion and differentiation of hESC lines.

Historically, hESCs have been maintained in complex culture systems. These culture systems often consisted of mouse or human feeder cell layers, medium containing fetal bovine serum (or serum replacement) to provide an extracellular matrix (ECM)-rich environment for cell adhesion, and soluble growth factors for self-renewal (1-3). Several researchers have developed feeder-free culture systems for hESCs by substituting the feeder cell layers with Matrigel™, a basement membrane preparation from mouse sarcoma (4), human serum (5), or purified ECM proteins (4,6,7). However, most of these biological materials are expensive to manufacture, have limited scalability, and may have problematic batch-to-batch variability. In addition, animal-derived materials are subject to costly testing to ensure freedom from pathogens.

The rapidly expanding interest in alternatives for stem cell culturing options that are more amenable to scalability, and ultimately use in cell therapy applications, has led to the development of a synthetic surface for the growth and differentiation of hESCs. Here, we describe application of the Corning Synthemax-T Surface to support long-term self-renewal and differentiation of H7 hESCs in chemically defined medium.

Materials and Methods

Reagents

X-VIVO™ 10 (Lonza Cat. No. 04-743Q); hrbFGF (R&D Systems® Cat. No. 234-FSE-025/SF), hrTGF-β1 (R&D Systems Cat. No. 240-B002); Dulbecco's PBS (DPBS) (Invitrogen Cat. No. 14190250), KnockOut™ DMEM (KO-DMEM) (Invitrogen™ Cat. No. 10829-018), Collagenase IV (Invitrogen Cat. No. 17104); Matrigel (BD Biosciences Cat. No. 356231); Antibodies: Oct4 (Millipore® Chemicon® Cat. No. MAB4401), TRA-1-60 (Millipore Chemicon Cat. No. MAB4360), SSEA-4 (Millipore Chemicon Cat. No. MAB4304); α-actinin (Sigma® Cat. No. A7811), Nkx2.5 (Santa Cruz Biotechnology® Cat. No. SC-14033), all corresponding secondary antibodies (Invitrogen/Molecular Probes).

hESC Culture

H7 hESCs were provided by Geron Corporation under collaborative agreement. The cells were maintained in chemically defined xeno-free medium (X-VIVO™ 10 basal medium supplemented with 80 ng/mL human recombinant (hrbFGF) and 0.5 ng/mL human recombinant TGFβ (hrTGF-β) according to the protocol for hESC culture on Corning® Synthemax™-T Surface (CLS-AN-148). Cultures were briefly passaged every 4 to 6 days. Once the cells approached 80% confluency, they were detached by incubation with 200 U/mL collagenase IV for 2 to 3 minutes, followed by a brief wash with DBPS and gentle scraping. For each passage, H7 cells were subcultured into 6 well Corning Synthemax-T Surface plates and Matrigel™ (1:30 dilution in KO-DMEM) coated Corning tissue culture treated plates (Corning Cat. No. 3506) at the density of 100,000 cell/cm² in chemically defined medium described above. Culture medium was replaced every day except for the day after passage. Microscopic examination of cell and colony morphology was performed daily. Cell viability and number were assessed at each passage by harvesting duplicate wells with collagenase IV/EDTA treatment followed by cell counting with an automated cell number/viability analyzer, Vi-Cell™ (Beckman Coulter). Expression of hESC-specific markers was assessed by flow cytometry and immunofluorescent staining at the end of each passage for >10 serial passages. To monitor genomic integrity, cell samples were submitted for karyotyping analysis by G-banding (Cytogenetics Laboratories, Children's Hospital, Oakland, CA) at passages 0, 5, and 10 on Corning Synthemax-T Surface.

Teratoma Formation Assay

Cell pluripotency was assessed by teratoma formation assay. After 10 passages on Corning Synthemax-T Surface and Matrigel surface, H7 cells were harvested and resuspended in PBS at the concentration of 1x10⁵ cells/mL. Prior to each injection, 100 μL of cells was mixed with 100 μL of Matrigel. The mixture was immediately injected into a hind limb muscle of SCID/bg mice. Each group (Corning Synthemax-T Surface and Matrigel) contained 8 animals. The animals were terminated case by case, depending on the visible masses in the muscle. The masses were fixed *in situ* using 10% neutral formalin and paraffin embedded using standard histological methods. Sections were cut at 5 to 8 μm and stained using hematoxylin and eosin. Tissues were examined under brightfield optics using a ZEISS® Axioskop™ 2 Plus photomicroscope (Carl Zeiss, Inc., Göttingen, Germany) and digital microscopy images were captured using a Pixera® Penguin 600CL digital camera (Pixera Corporation, Los Gatos, CA). Digital images in Figure 5 were sized and balanced for brightness and contrast using Photoshop® version 7.0.1 (Adobe Systems, San Jose, CA); no additional digital image manipulation was performed.

Cardiomyocyte Differentiation

H7 hESCs were differentiated on the Corning Synthemax-T Surface for approximately 25 to 30 days using a direct differentiation protocol described by Laflamme, M.A. et al. (8). Briefly, undifferentiated H7 hESCs were cultured on Synthemax-T Surface in serum replacement medium (SRM: KnockOut™ DMEM + 20% KnockOut SR, 1 mM L-glutamine, 1% NEAA, 0.1 mM 2-ME, 80 ng/mL hrbFGF, 0.5 ng/mL hrTGFβ1) for one week (adaptation phase) prior to sub-cultivation onto Synthemax-T Surface at the density of 100,000 cell/cm². Cells were re-fed daily with SRM for 6 days. Cardiac differentiation was induced by sequential exposure of cells to 100 ng/mL of activin A for 24 hours, followed by 10 ng/mL BMP4 for 4 days in RPMI-B27 medium. Cells were re-fed every 2 to 3 days with RPMI-B27 medium without growth factors for additional 2 to 3 weeks. Widespread spontaneous beating activity was typically observed by 12 days post-activin A induction. Cardiomyocyte differentiation was assessed by looking for specific markers (α-actinin, Nkx2.5) using immunofluorescent staining.

Results and Discussion

Corning® Synthemax-T™ Surface Supports Self-renewal of hESCs for >10 Serial Passages in Xeno-free, Defined Medium

Historically, the viability of a culture system to support hESC growth has been evaluated by determining whether or not the culture system can support >5 serial passages on the cell line. Therefore, the Corning Synthemax-T Surface was evaluated with H7 hESCs by serial passaging as described in the Methods section above. Figure 1 demonstrates expansion of H7 hESCs for 12 serial passages on Corning Synthemax-T Surface and compares the results to one of the feeder-free culture systems that incorporates the use of a Matrigel™ biological surface. Cells passages on the Corning Synthemax-T Surface demonstrated very stable doubling time on average of 41 ± 5 hours, indicative of a consistent culture environment. Cell doubling time for cultures passaged in parallel on Matrigel coated surface exhibited much higher variability (average doubling time of 50 ± 12 hours), probably due in part to the inherent variability of this surface coated with a biological material.

In order to evaluate whether or not serial passage on the Corning Synthemax-T Surface supported the propagation of hESCs in their undifferentiated state, the cells were evaluated for the expression of the hESC phenotypic markers, Oct4 and SSEA4. Oct4 is a nuclear transcription factor and SSEA4 and TRA-1-60 are surface markers, which are typically expressed in undifferentiated hESCs. During 11 serial passages on the Corning Synthemax-T Surface, H7 cells retained high expression levels of hESC phenotypic markers Oct4, TRA-1-60 and SSEA4, as shown by flow cytometry analysis for Oct4 marker (Figure 2) and immunofluorescent staining for Oct4, TRA-1-60 and SSEA4 (Figure 3), respectively. These results confirm the undifferentiated status of H7 hESCs after serial passaging on the Corning Synthemax-T Surface in chemically defined medium.

Typically, when culturing hESC lines, karyotypic analyses are frequently recommended to ensure there is no acquisition of gross chromosomal abnormalities. Therefore, the karyotype for the H7 hESCs was evaluated. Importantly, no genetic abnormalities were associated with hESC propagation on Corning Synthemax-T Surface, and cells retained normal karyotype after 10 passages on Corning Synthemax-T Surface (Figure 4).

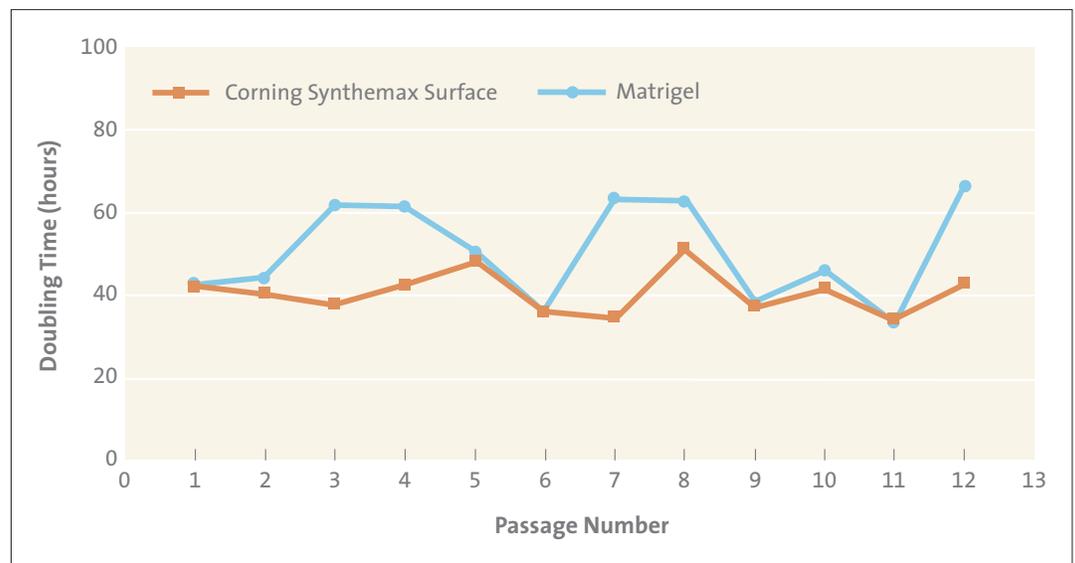


Figure 1. Long-term culture of H7 hESCs on Corning Synthemax-T Surface in defined, xeno-free medium. Doubling time of H7 cells over the course of 12 serial passages on Matrigel and Corning Synthemax-T Surface. Average doubling time for Corning Synthemax-T Surface was 41 ± 5 hours, for Matrigel surface 50 ± 12 hours.

These results suggest that H7 hESC can be successfully propagated on Corning® Synthemax™-T Surface for multiple passages in xeno-free, defined medium, while retaining important hESC characteristics such as stable proliferation rate, phenotypic marker profile and normal karyotype.

hESCs Retain Pluripotency After 10 Serial Passages on Corning Synthemax-T Surface

A fundamental property of hESCs is their ability to differentiate into cells of all three germ layers. Maintaining this property is a critical parameter when evaluating the use of new culture conditions for hESC culture. Therefore, we examined whether H7 hESCs maintained on Corning Synthemax-T Surface retained their ability to differentiate into cells of all three germ layers using an *in vivo* differentiation assay (teratoma formation in mice). Figure 5

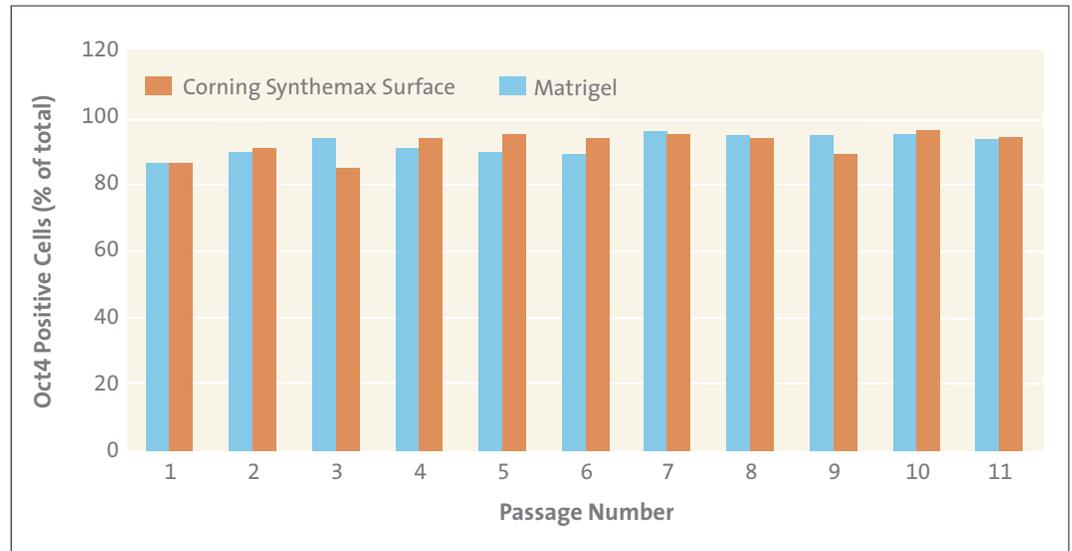


Figure 2. Flow cytometry analysis of Oct4 phenotypic marker expression in H7 cells during 11 serial passages on Corning Synthemax-T Surface.

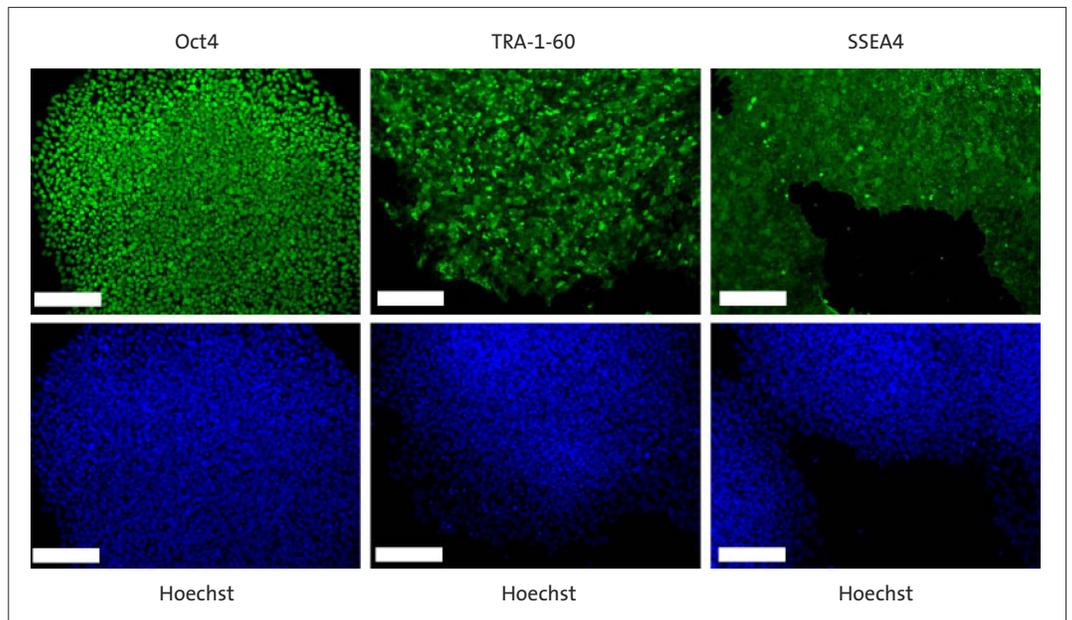


Figure 3. Immunofluorescent staining of phenotypic markers in H7 cells after 10 serial passages on Corning Synthemax-T Surface. Indirect immunofluorescent staining of H7 cells for Oct4, TRA-1-60 and SSEA4 markers after 10 serial passages on Corning Synthemax-T Surface. Hoechst nuclear staining was used to show the entire cell population. Scale bar: 100 μM.

shows positive staining for representative tissues from all three germ layers (secretory epithelium, bone and melanocytes) in teratomas developed after injection of mice with H7 cells cultured on Corning® Synthemax-T™ Surface and Matrigel™ coated surface for 10 serial passages. This data confirms retention of pluripotency of H7 cells after long-term culture on Corning Synthemax-T Surface in defined medium (Figure 5).

Differentiation of H7 hESC into Cardiomyocytes After Long-term Culture on Corning Synthemax-T Surface

For therapeutic applications of hESC it is highly desirable to have defined culture conditions for both the expansion and differentiation phase of therapeutic cell production. Therefore, the H7 hESCs passages and expanded on the Corning Synthemax-T Surface sequentially exposed to the appropriate cytokines as described in Methods to stimulate differentiation into cardiomyocytes. As demonstrated by immunofluorescent staining in Figure 6, Corning Synthemax-T Surface supports differentiation of H7 hESCs into functional cardiomyocytes, expressing cardiomyocytes specific markers α -actinin and Nkx2.5. Additional quantitative assessment of differentiation was performed by flow cytometry analysis of α -actinin-positive cell fraction. This analysis confirmed up to 83% of cells differentiated on Corning Synthemax-T Surface express α -actinin marker, suggesting high purity of cardiomyocytes differentiation on Corning Synthemax-T Surface (data not shown). These results suggest that Corning Synthemax-T Surface can support directed differentiation of H7 cells into functional cardiomyocytes, therefore providing a synthetic surface solution for both expansion and differentiation phases of hESC culture (Figure 6).

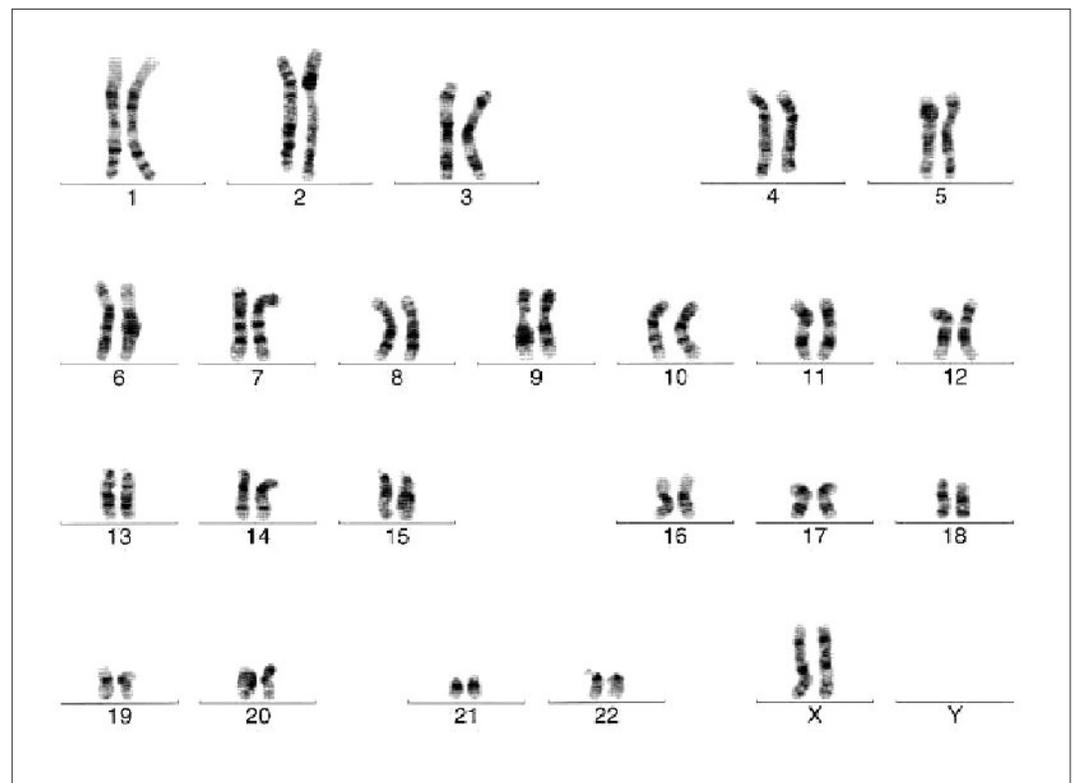


Figure 4. H7 cells retained normal karyotype after long-term culture on Corning Synthemax-T Surface. Cytogenetic analysis revealed normal karyotype for H7 cells cultured on Corning Synthemax-T Surface for 10 serial passages.

Conclusion

H7 hESCs were successfully cultured for >10 serial passages on Corning® Synthemax-T™ Surface using chemically defined, xeno-free medium. Throughout this long-term culture on Corning Synthemax-T Surface, cells demonstrated not only a stable doubling time of 41 ± 5 hours, but also a high level of expression of hESC phenotypic markers (Oct4, SSEA4 and TRA-1-60) and normal karyotype. Cell pluripotency was confirmed by teratoma formation in mice. In addition, Corning Synthemax-T Surface supported successful directed differentiation of H7 cells into functional cardiomyocytes, proving suitability of Corning Synthemax-T Surface for both expansion and differentiation of hESC culture.

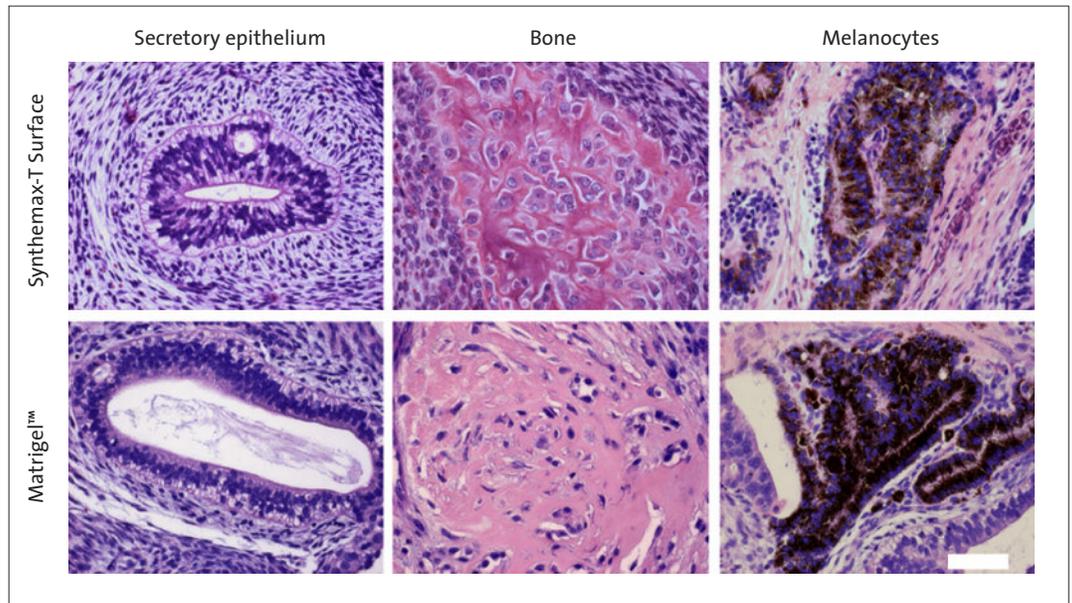


Figure 5. H7 cells retained pluripotency after long-term culture on Corning Synthemax-T Surface. Teratoma formation in mice after injection of H7 hESC expanded on Corning Synthemax-T Surface and Matrigel surfaces for 10 serial passages. All three germ layers, represented as secretory epithelium (endoderm), bone (mesoderm) and melanocytes (ectoderm), were demonstrated by H&E staining. Scale bar: 50 μm .

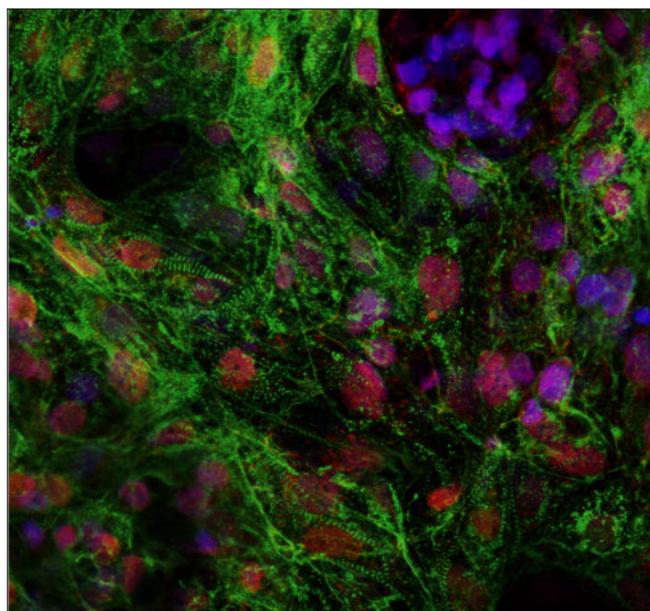


Figure 6. Directed differentiation of H7 hESCs into cardiomyocytes on Corning Synthemax-T Surface. Confocal fluorescent image of beating structures immunostained for cardiomyocyte-specific markers, Nkx2.5 (red), α -actinin (green).

References

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