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Determination of the Myogenic Potential of Human Embryonic Stem Cell-Derived Mesenchymal Stem Cells

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University Scholar Program

University Scholar Thesis

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Abstract

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Human embryonic stem cells (hESCs) have the potential to differentiate to all adult somatic cells. This property makes hESCs a very promising area of research for the treatment of disorders in which specific cell populations need to be restored. Despite this potential, research that focuses on producing mesodermally derived cell populations from hESCs is decidedly limited, notwithstanding the prevalence of disorders involving mesodermal tissues for which treatment options are limited. Skeletal muscle myoblasts are derivatives of mesodermal cells and are characterized by the expression of the MyoD gene. These cells are difficult to obtain from hESCs in a reproducible and efficient manner. Recent developments in the field have showed some success in obtaining myogenic cells from hESCs through a mesenchymal stem cell (MSC)-like intermediate population. MSCs, which are an adult stem cell population typically derived from the bone marrow, are capable of generating multiple cell types including skeletal muscle. The aim of this study was to develop an efficient method that derives myoblasts from an MSC-like intermediate. To accomplish this goal, we first set out to isolate and expand the MSC-like intermediate from hESCs differentiated in vitro. Difficulties in reproducing published cell-differentiation methodologies, which represent a significant and familiar challenge in hESC research, are highlighted in this report.

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Introduction

I. Human Embryonic Stem Cells

Human embryonic stem cells (hESCs) are a pluripotent cells with the potential to generate all the tissues of the body (Trounson et al, 2006). While ES cells have been utilized for years in mouse, it was not until 1998 that Thomson *et al.* described the first successful isolation and cultivation of hESCs (Thomson, et al., 1998). HESCs are derived from cells isolated from the inner cell mass (ICM) of the preimplantation blastocyst-stage embryo. Following isolation, the ICM cells are plated onto a monolayer of irradiated mouse embryonic fibroblast (MEFs) in media supplemented with basic fibroblast growth factor (FGF-2). The MEFs and FGF-2 play a critical role in maintaining the ICM cells in a pluripotent state (Thomson, et al., 1998). After multiple passages under these conditions, the ICM cells take on a characteristic hESC phenotype, forming monolayer colonies of cells (Fig. 1)

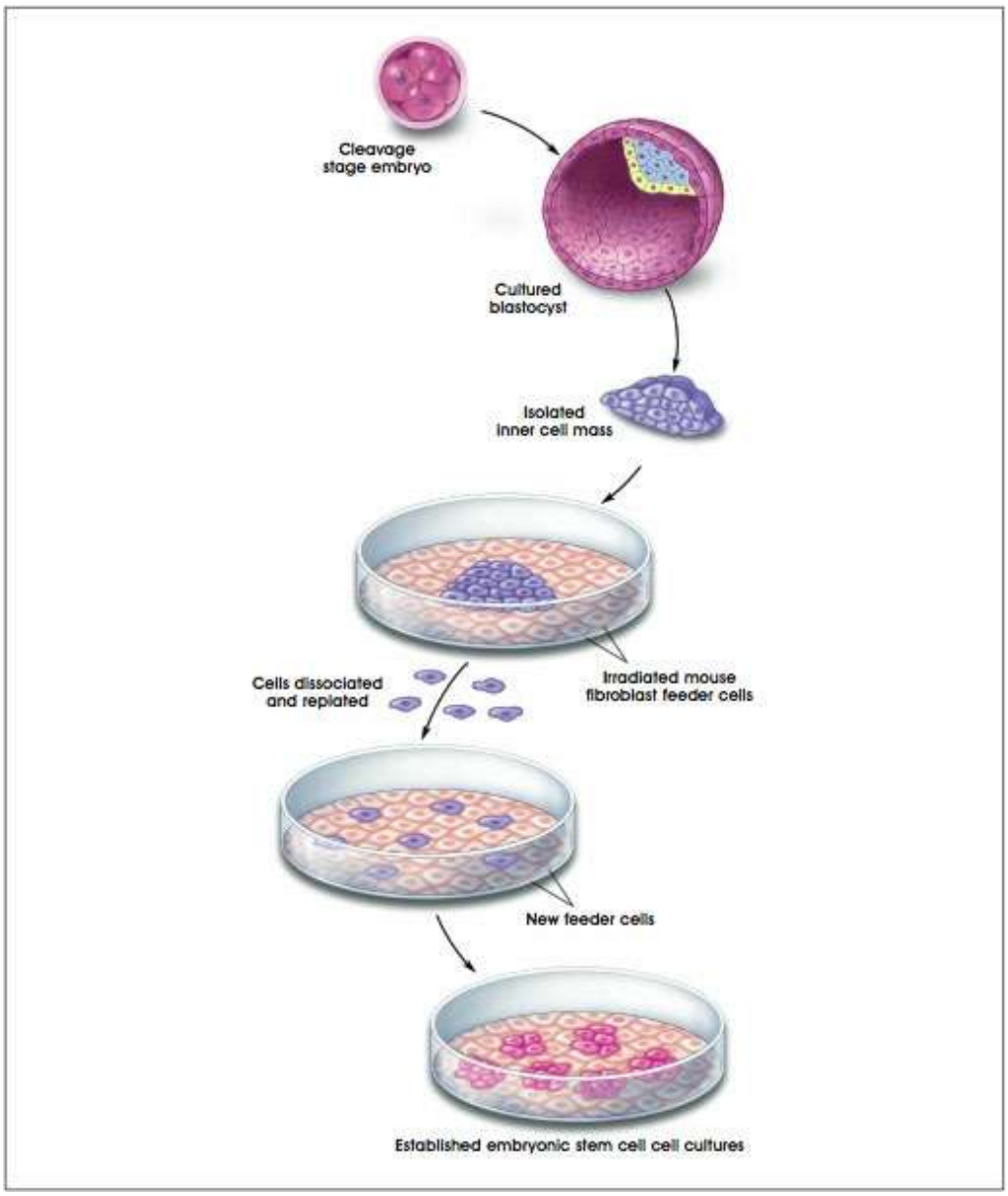

Figure 1. Diagram of the process of deriving human embryonic stem cells from the ICM of blastocysts (Winslow & Duckwall, 2001).

Upon derivation of a new hESC line from an embryo, the cells are characterized to verify their pluripotency. The undifferentiated state of a hESC line is characterized by the expression of a number of different genes, including cell surface markers such as the glycolipid antigens SSEA-3, -4, the keratin sulfate antigens TRA-1-60 and TRA-1-81 (Ohtsuka & Dalton, 2008), and by transcription factors that play an important role in

maintaining the undifferentiated state such as Oct4, Nanog, and Sox2 (Johnson et al, 2006). Because the expression of only a single marker is insufficient to denote an undifferentiated hESC population, the expression pattern of this set of genes is taken together to characterize a specific hESC line.

To confirm pluripotency, cells are shown to be able to differentiate into nearly all cell types. This can be accomplished by two means. HESCs can be differentiated as embryoid bodies (EBs). Specifically, colonies are isolated from the tissue culture plate and grown as units in suspension. These culture conditions cause the hESC cells within the colonies to spontaneously aggregate to form spheres. EBs recapitulate the earliest stages of embryonic development, in that the three germ layers, endoderm, mesoderm, and ectoderm, are formed. Because EBs generate all three germ layers, they serve as an important tool for testing the differentiation potential of hESCs (Hwang et al, 2008).

The second pluripotency test utilized in hESCs is teratoma formation. When hESCs are injected into immunodeficient mice they form tumors known as teratomas. These teratomas contain randomly differentiated cells of a number of different lineages, again serving the important purpose of testing the differentiation potential of the cells (Thomson et al, 1998).

The ultimate test of true pluripotency is the ability of an ESC line to generate a complete organism. This is accomplished by injecting ESCs into a developing embryo, allowing the embryo to grow to form an adult organism. If the ESCs are contained in the germline of the developing embryo then the resulting progeny will be entirely derived from the ESC line used in the study. This procedure is routinely used with mouse ESCs,

but cannot be used for hESCs due to a number of ethical dilemmas. As a result, hESCs can never be shown to be truly pluripotent.

In the twelve years since the derivation of the first hESC lines significant progress has been made in developing differentiation strategies for the generation of specific lineages. HESCs have been shown to readily generate ectodermal lineages, consistent with the observation that the ectoderm appears to be a developmental default pathway. Furthermore, neurectodermal differentiation, in particular, has proven to be to be generated efficiently and is thus, considered to possess significant therapeutic potential (Reubinoff et al, 2001). Endodermal lineages have also been derived from hESCs, as evidenced by the generation of two clinically important cell types: liver hepatocytes (Rambhatla et al, 2003) and insulin-producing β cells (Segev et al, 2004). Unfortunately, the *in vitro* generation of the majority of mesodermal lineages have proven to be much more challenging. In addition, the derivation of skeletal muscle from hESCs, the mesodermal cell type of interest in this study, has been particularly difficult to accomplish. In fact, only a few groups have claimed to be able to differentiate hESCs into skeletal muscle. The *in vitro* differentiation protocols reported by one of these groups is examined closely in this study (Barberi et al, 2007).

II. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are an adult stem cell population that was originally isolated from the adult bone marrow and are characterized by their ability to adhere to tissue culture plastics. This ability to adhere to tissue culture plates facilitates the purification of MSCs away from the hematopoietic stem cells (HSCs) that make up a

large portion of the cells of the bone marrow, because HSCs do not adhere to plastic (Friedenstein et al, 1978). Importantly, MSCs that were derived in this manner were shown to be able to differentiate into mesenchymal (bone associated) cell types.. Specifically, MSCs are defined by their ability to differentiate into adipogenic, chondrogenic, and osteogenic cell lineages (Pittenger et al, 1999), and, to a lesser extent, skeletal muscle (Drost et al, 2009). The ability of these cells to generate such a wide variety of cell types highlights their therapeutic potential..

Unfortunately, the clinical applications of MSCs is limited by the fact that MSCs are difficult to isolate in an efficient manner from the bone marrow and are only capable of limited expansion *in vitro*. It is believed that these limitations can potentially be addressed by deriving the MSCs from hESCs instead of the adult bone marrow. In fact, multiple studies have recently demonstrated the ability to generate cells from hESCs that have an MSC-like phenotype and potential (Lian et al, 2007; Olivier et al, 2006; Trivedi & Hematti, 2007).

MSCs are typically identified by an array of cell surface markers that are consistently expressed in MSCs. Again, no single marker can be utilized to identify MSCs, but taken together, their expression pattern can be used to define specific cell types. The most consistent and well-defined MSC marker profile is CD73-, CD105-, and CD90-positivity, as well as CD34- and CD45-negativity (Dominici et al, 2006). HESC-derived MSCs expressing this profile of markers have been successfully used to derive chondrogenic, adipogenic, and osteogenic cell types. Importantly, HESCs offer the benefit that they can be expanded indefinitely in culture, thereby offering a possible solution to the limited proliferation capacity of bone marrow-derived MSCs.

A recent application of this strategic approach demonstrated the potential of hESC-derived MSCs to differentiate into skeletal muscle myoblasts (Barberi et al, 2007). Although this is a well-characterized property of bone marrow-derived MSCs, it was the first report of hESC-derived MSCs. The methodology used by the Barberi group is shown in Fig. 2.

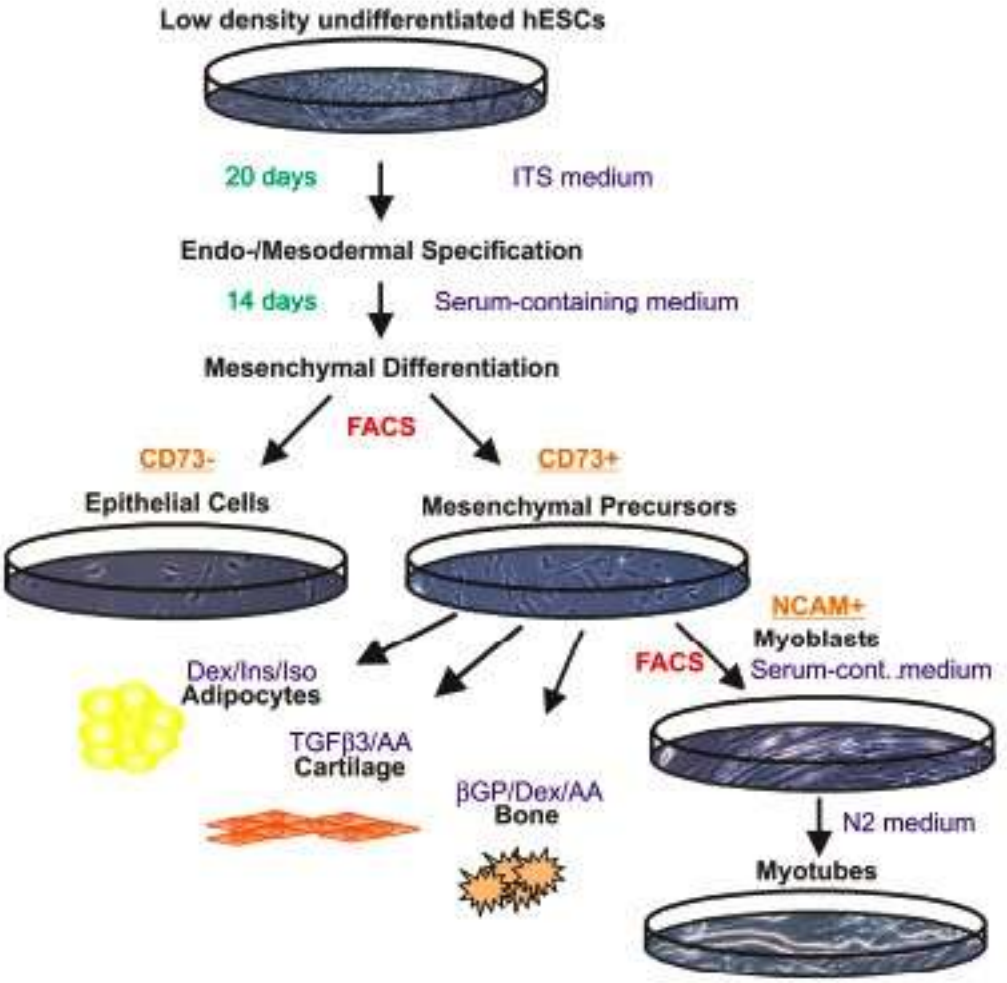

Figure 2. Flow chart of the methodology described by Barberi *et al.* (2007)

Because the Barberi protocol produced myoblasts at a very low efficiency and required long *in vitro* cell culture conditions (37-38 days), the goal of this study was to

modify this protocol with the aim of dramatically increasing the efficiency at which skeletal muscle progenitors are obtained.

To facilitate the identification of MSC-like cells derived from hESCs, we use the cell markers CD73 and CD105. CD73 is the Ecto-5'-nucleotidase gene, which functions as a purine salvage-pathway enzyme located on the cell's surface. It catalyzes the extracellular dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding nucleosides. Although CD73 is expressed in a number of cell types, particularly lymphocytes, it is consistently expressed in MSCs (Hansen et al, 1995). CD105 is the endoglin receptor, a part of the transforming growth factor-β receptor complex (TGF-βR). It is expressed in vascular cells and a number of blood cell types, and again is a found to be consistently expressed in MSCs (Pierelli et al, 2001).

III. Skeletal Muscle Development & Regeneration

Skeletal muscle begins developing in the embryo primarily from transient condensations of paraxial mesoderm that border either side of the neural tube known as somites. The dermomyotome is generated within the developing somites, and gives rise to the dermis and the skeletal muscle of the trunk and the limbs (Parker et al, 2003). A family of genes of known as the Myogenic Regulatory Factors (MRFs) plays an important role in controlling myogenic differentiation. Within this group of genes are the MyoD and Myf-5 genes. Simultaneous knockout of these two genes gives rise to animals devoid of skeletal muscle myoblasts, demonstrating that these genes are necessary for skeletal muscle lineage determination (Chen & Goldhamer, 1999).

In adults, skeletal muscle is normally a relatively stable tissue. Following injury-, exercise- or disease-induced damage muscle tissue demonstrates an extraordinary capacity for regeneration. The muscle stem cell population, known as satellite cells, mediates this regenerative process. Satellite cells remain quiescent under the basal lamina of adult muscle fibers until damage-induced activation. Upon activation, these cells migrate from their niche, upregulate members of the MRF gene family, such as MyoD, proliferate, and differentiate to repair the damaged muscle (Fig. 3; Hawke & Garry, 2001).

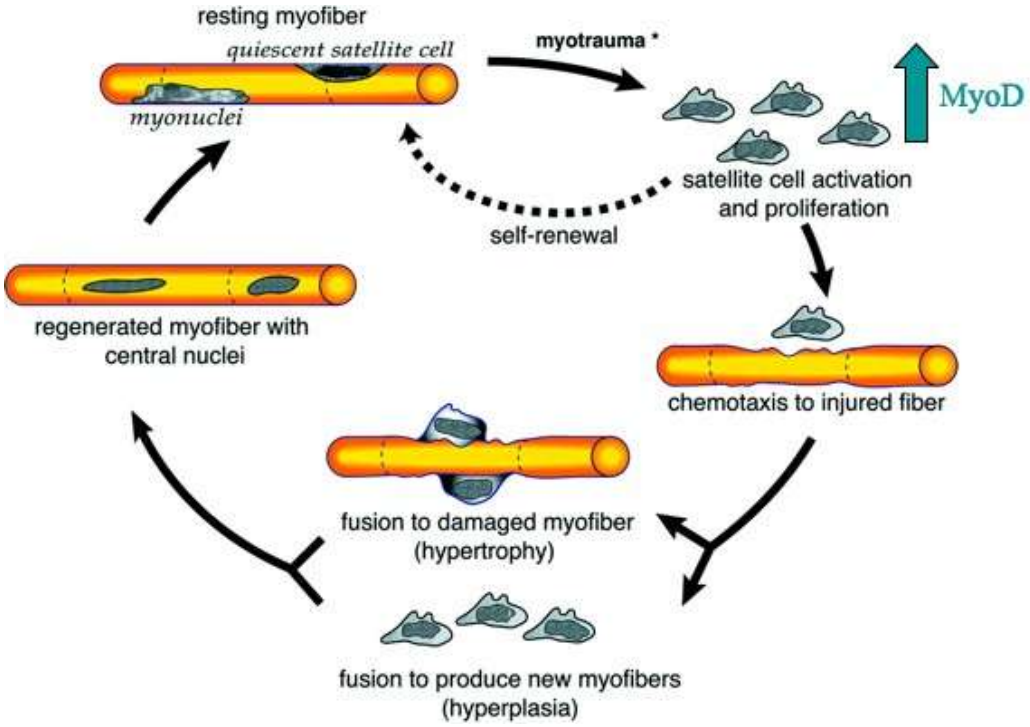

Figure 3. Muscle regeneration pathway (Hawke & Garry, 2001)

HESCs offer the potential to derive myogenic precursors with the ability to repopulate the satellite cell pool. As a possible therapeutic tool, repopulation of the satellite cell population could mean a long-term or even a permanent cure to chronic muscular disorders.

Materials & Methods

I. hESC Culture

All experiments utilize H9 (WA-09, 7D) hESCs of passage 48-60. Cells were maintained on matrigel-coated dishes and fed on a daily basis with mouse embryonic fibroblast (MEF)-conditioned media (KO-CM). Unconditioned media consisted of Knockout DMEM/F12, 10% Knockout Serum Replacement, 1% L-glutamine, 1% non-essential amino acids, and 4ng/ml bFGF. Media was conditioned by culturing mitotically inactivated, irradiated MEFs in T75 flasks with 18ml of unconditioned media for 1 day, after which the media was collected and stored at -80˚C until used. When used for feeding hESCs the stored media was thawed, and 4ng/ml of bFGF was added to make up the final KO-CM. HESCs were mechanically passaged every 4-6 days.

II. Gene Expression Analysis

For RT-PCR analysis, total RNA was extracted from cell samples using the Qiagen RNeasy kit and treated with RNase-free DNase I. Primers were designed to be intron-spanning so that amplification off genomic DNA produced bands sizes that were readily distinguished from those amplified off of mRNA-derived cDNA. PCR annealing temperatures were optimized prior to experimental use.

III. Lentiviral Transductions

Lentivirus for cell transduction studies was prepared by transfecting 293FT cells with the reporter construct and RRE, REV, and VSV-G helper plasmids. SuperFect®

reagent was used for the transfection of the plasmids. Virus-containing media was collected off of the transfected cells once a day for two days. The two viral collections were pooled together at the end of the second day and concentrated together using an ultracentrifuge at 25,000rpm for 90 minutes. The concentrated virus was then used to transduce cells by adding 200µL of the virus to the media for 8-12 hours.

IV. FACS Isolation

The FACS experiment was carried out by first washing the experimental cells with phosphate buffered saline (PBS) followed by trypsinization of the cells for 3min. The trypsin was inactivated using serum-containing media, the cells were washed with PBS, and stained with either mouse anti-human CD73 monoclonal antibody (BDPharmingen) or purified mouse IgG1, κ Isotype control. The cells were then stained with the secondary, goat anti-mouse AlexaFluor 633 antibody (Invitrogen). The cells were then treated with propidium iodide and filtered through a 100µm mesh prior to cell sorting to remove clumps. Cells were sorted and CD73+ and CD73- cells were collected and plated on gelatin-coated dishes.

V. MSC Differentiation

HESCs were trypsinized for 5 minutes and plated at a density of 1000 cells/cm^2 onto fibronectin-coated plates (denoted as day -4 experimentally) and cultured in the MEF-Conditioned media for 4 days at which point the hESC colonies contained about 50 cells. At this point (Day 0), experimental cells were transferred to ITS media for 21 days. ITS media consists of DMEM/F12 (Invitrogen), 1% Insulin-Transferrin-Selenium (ITS)

(Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), and 2.438g/L $NaHCO_3$ (Fisher). After the 21 days in ITS media, the cells were transferred to MEM media for an additional 7 days. MEM media consists of Alpha MEM (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2mM L-glutamine (Invitrogen), and 1% Penicillin/Streptomycin.

Results

I. Lentiviral reporter constructs

As described in the introduction, the aim of this study was to significantly increase the efficiency at which skeletal muscle progenitors can be generated from hESCs. In order to achieve this goal we designed a lentiviral reporter system that would allow for the identification of cells that had committed to the skeletal muscle lineage. Commitment to myogenesis was identified by MyoD expression, a critical muscle determination gene described earlier. MyoD expression was monitored through the use of the reporter construct containing the MyoD promoter and core enhancer regions (denoted as 258/-2.5) driving expression of Cerulean Fluorescent Protein (CFP). Because the MyoD promoter and core enhancer regions should reflect normal MyoD expression, cells marked by cerulean fluorescence should also express MyoD..

The construct also contained the constitutively active human elongation factor-1α (EF1α) promoter. EF1α is a highly expressed housekeeping gene that is involved in protein synthesis and expressed in all tissue types (Knudsen *et al,* 1993). In the lentiviral reporter construct the EF1α promoter drives the expression of the mCherry Fluorescent Protein and a Puromycin resistance gene (Puromycin is an antibiotic that kills both most eukaryotic and prokaryotic cells); with the mCherry and Puromycin resistance genes being separated by the self-cleaving T2a peptide sequence. Thus, this portion of the construct functions with EF1α constitutively driving expression of the mCherry-T2a-Puromycin protein. Following translation of the fusion protein, the T2a amino acid sequence self-cleaves to generate separate mCherry and Puromycin gene products.

The constitutive expression of the puromyosin gene product facilitates the purification of transduced cells through their ability to grow in media containing Puromycin. Additionally, the constitutive mCherry-expression is important for future *in vivo* studies Specifically, the mCherry-expression would allow for the easy identification of cell of hESC origin and allow us to identify the fate of the transplanted cells. The bicistronic reporter construct is depicted in Fig. 4.

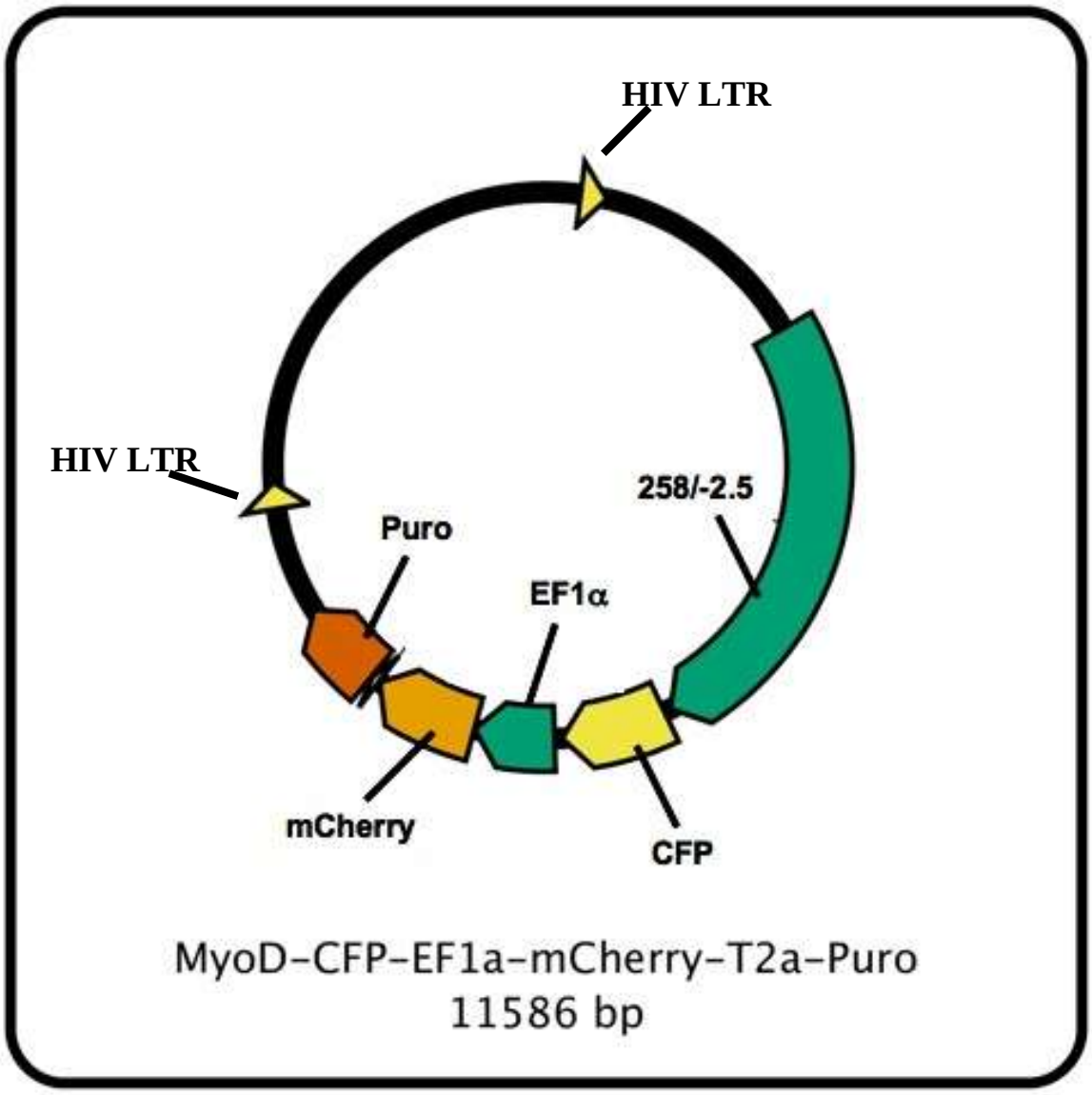

Figure 4. Map of the bicistronic lentiviral construct.

The lentiviral construct diagrammed in Fig. 4 denotes two "HIV LTR" sequences bordering the reporter sequences. These LTRs enable the genomic integration of the sequence residing between them.

The MyoD-CFP-EF1α-mCherry lentiviral construct was used to transduce H9 hESCs. The efficiency of the transduction was low, with only a few successfully transduced cells (marked by mCherry fluorescence) per colony. These cells were allowed to expand until there were small clusters mCherry+ cells. The cells were then cultured in media containing Puromycin. After a few days, the puromycin selection was complete and only transduced cells remained in culture.

While using the MyoD-CFP-EF1α-mCherry reporter construct in our cell differentiation studies, it was observed that cells lost their mCherry fluorescence upon differentiation (Fig. 5Bi & 5Bii). This disappointing finding suggested that the exogenous lentiviral EF1α promoter was either being silenced upon differentiation or that the EF1α promoter is not active in all lineages. This finding negated the usefulness of the reporter construct as a constitutive reporter.

Further characterization of the MyoD-CFP-EF1α-mCherry reporter construct also revealed problems with the MyoD-CFP portion of the reporter. Because we did not have access to a true positive control (MyoD+ cells derived from hESCs) we had to analyze the efficacy of the MyoD reporter using other cell types. This was accomplished using C2C12 cells, which are a line of immortalized mouse myoblast cells. These cells should express MyoD, and moreover, the 258/-2.5 sequence (human MyoD promoter and Core Enhancer region) used in the MyoD-CFP-EF1α-mCherry reporter construct is known to be constitutively expressed in mouse cells (data not shown). However, when C2C12 cells

were transduced with the MyoD-CFP-EF1α-mCherry reporter construct, no CFP fluorescence was observed (Fig. 5). These data demonstrated that the MyoD-CFP-EF1α-mCherry construct was ineffective as both a constitutive reporter and a marker of MyoD expression. It is likely that the failure of the 258/-2.5 sequence to report on MyoD expression is caused by the close proximity of the strong EF1α and MyoD promoters. Because the MyoD-CFP-EF1α-mCherry construct could not be utilized for its primary purpose, we were forced to redesign the expression construct.

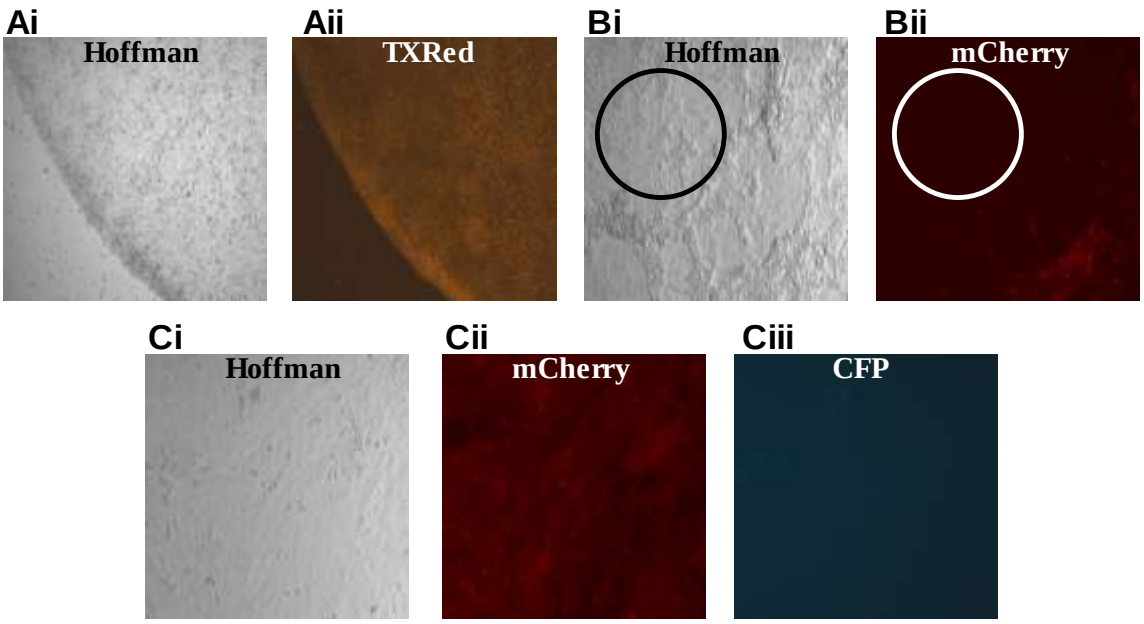

Figure 5. Characterization of the MyoD-CFP-EF1α-mCherry construct. (Ai,ii) Images of undifferentiated, transduced H9 hESCs. (Bi,ii) Images of serums-differentiated transduced hESCs. Circled spaces demonstrate cells that have stopped expressing mCherry fluorescent protein from the EF1α promoter (Ci,ii,iii) Images of transduced C2C12 cells. (10X images).

Because generation of a new lentiviral reporter system was critical for the successful identification of hESC-derived MyoD+ cells, we decided to utilize a two-vector system in order to avoid the problems associated with a bicistronic reporter described above. One lentiviral construct would serves as a constitutive reporter that

maintains its activity in all differentiated lineages, while the second construct will successfully recapitulate MyoD expression.

To solve the problems associated with the silencing of the EF1α promoter, we decided to design the new constitutive construct using the CAG promoter instead of the EF1α promoter. The CAG promoter is a very strong promoter that contains a modified chicken β-actin promoter and enhancer elements of the cytomegalovirus (CMV) that has shown promise for use in ESCs (Pfeifer et al, 2002). To develop this construct, the CAG promoter was excised from a pCAGGS vector and cloned into our lentiviral vector backbone. The backbone has a multiple cloning site (MCS) preceding the mCherry-T2a-Puro sequence, similar to the construct diagrammed in Fig. 4. Because the CAG promoter sequence was inserted into the MCS of the lentiviral vector by blunt-end ligation, the correct orientation of the CAG promoter was verified. The final CAG-mCherry construct can be visualized in Fig. 6.

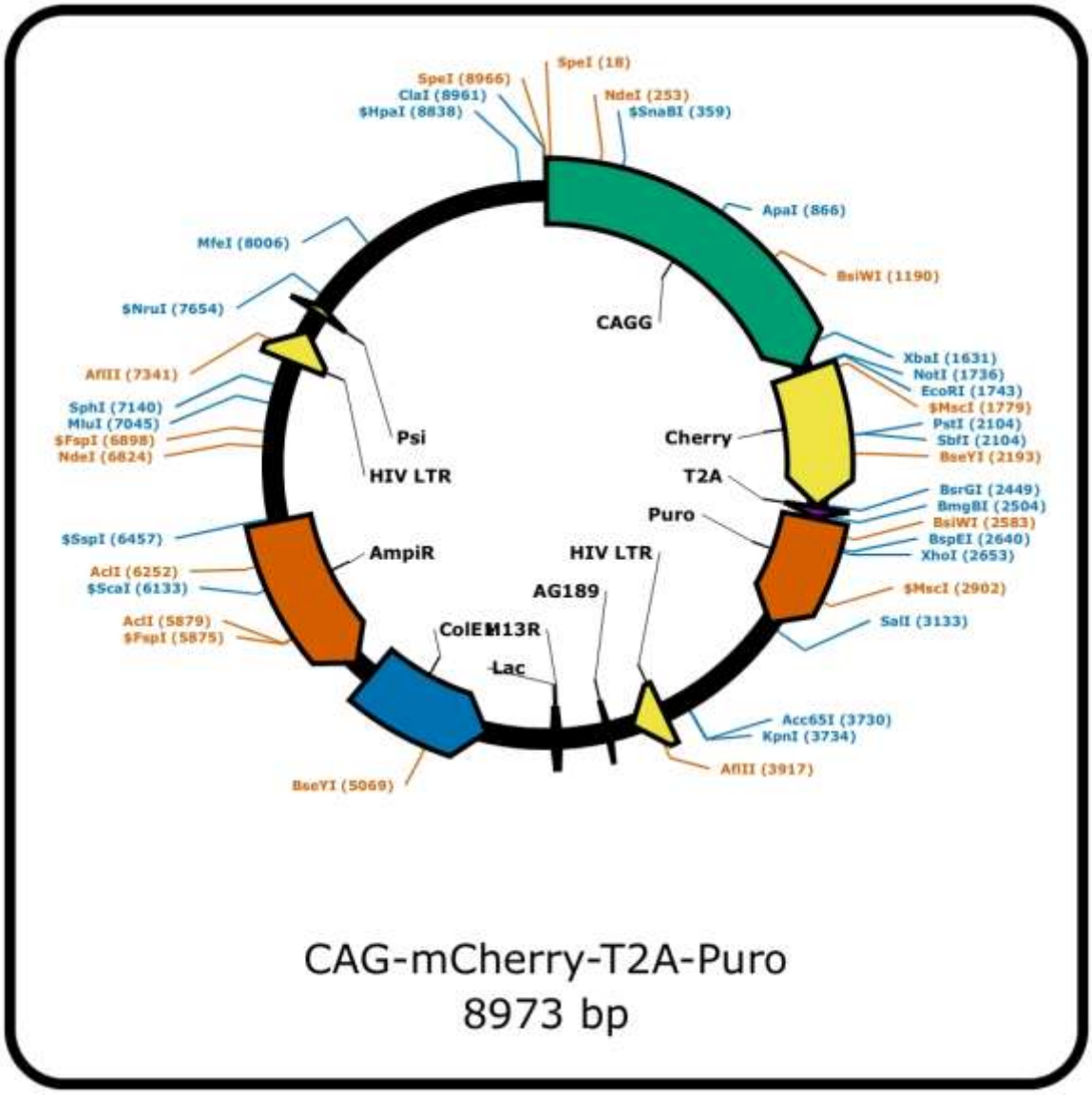

Figure 6. Map of the redesigned constitutive reporter construct. Restriction sites with two cut sites are denoted by blue and single cut restriction sites are in orange.

To facilitate full characterization of the new constitutive reporter construct, it was transduced into H9 hESCs as well as Detroit and Rhabdomyosarcoma (RD) cell lines. Additionally, transduced hESCs were differentiated in both serum-containing and ITS medium in order to ensure that continual expression of mCherry. The Detroit and RD cell lines were selected to ensure that the CAG promoter maintains expression in multiple cell types. Detroit cells are a human primary fibroblast line and RD cells are a human skeletal

muscle tumor cell line that constitutively expresses MyoD protein. As illustrated in Fig.7, the CAG-mCherry construct maintained mCherry expression throughout hESC differentiation, as well as in Detroit and RD cells.

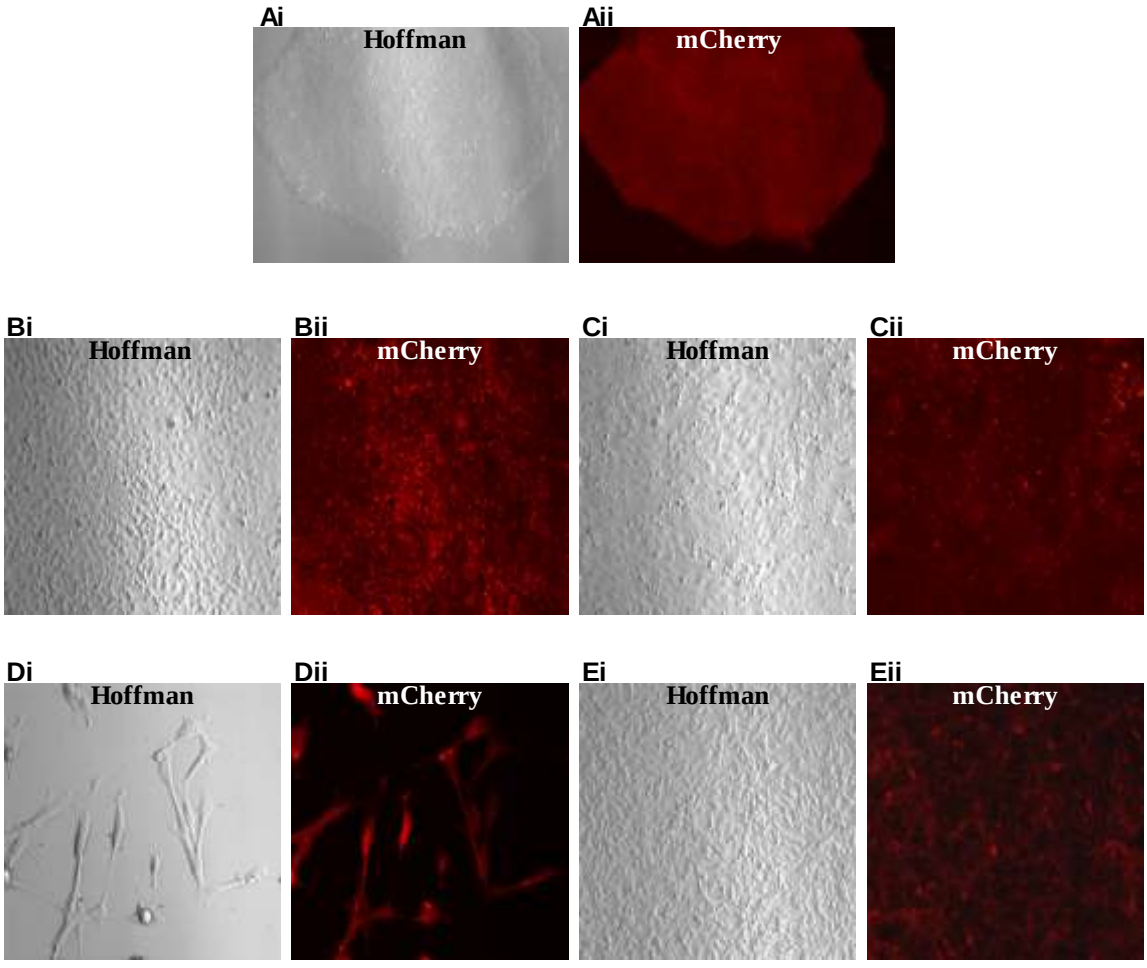

Figure 7. Characterization of the CAG-mCherry constitutive reporter construct. (Ai,ii) Undifferentiated transduced H9 hESCs. (Bi,ii) Images of transduced hESCs differentiated in serum-containing media. (Ci,ii) Images of transduced hESCs differentiated in ITS-containing media. (Di,ii) Images of transduced Detroit cells. (Ei,ii) Images of transduced RD cells (10X images).

Because the CAG-mCherry expression was found to be active throughout differentiation and in a variety of cell types, the newly designed constitutive reporter will allow us to identify cells of hESC origin in future *in vivo* studies; a feature that

was absent in the EF1α-mCherry portion of the original bicistronic construct. Cells transduced with the newly designed constitutive construct will also need to be transduced with a separate MyoD reporter construct that is still under development. Although the use of two lentiviral constructs is not ideal because of the increased chance that a construct will integrate into a coding region of the host genome, the new strategy will allow us to avoid the transcriptional interference caused by proximal promoters. Although the new MyoD reporter system was not prepared in time for use in the studies described in this report, it will be available for use in future experiments.

II. Differentiation of hESCs into an MSC-like cell type

As described in the introduction, the study published by Barberi *et al.* (2007) is a promising protocol for the derivation skeletal muscle from hESCs. The study described the generation of skeletal myoblasts from a hESC-derived MSC-like intermediate cell population. The MSC-like phenotype was defined by the expression of cell surface markers characteristic of bone marrow-derived MSCs: CD166, CD105, CD44, and most importantly CD73. The CD73 marker was used to isolate the putative hESC-derived MSCs by FACS. The MSC-like nature of the hESC-derived cells was further demonstrated by their ability to undergo adipogenic, chondrogenic, and osteogenic differentiation.

As discussed previously, the Barberi protocol was suboptimal because it required prolonged culture conditions and yielded skeletal myoblasts at a very low efficiency. The lentiviral reporter construct described above was designed to improve these liabilities. Unfortunately, after a number of attempts to replicate this protocol we failed to even

duplicate the first step, which required the plating of hESCs at a low-density onto gelatin-coated dishes (a substrate to which undifferentiated hESCs do not typically attach).

Although our inability to duplicate the initial steps of the Barberi protocol prevented the use of the original cell differentiation protocol, the Barberi group recently published a modified protocol (Stavropoulos et al, 2009). Interestingly, their new protocol modified the first step of the cell differentiation protocol. Specifically, the new protocol utilized fibronectin-coated dishes rather than gelatin for the initial low-density hESC plating step. Importantly, we confirmed that hESCs readily attached to fibronectin-coated plates.

As described in the methods section, the MSC differentiation protocol entails a 3-4 day hESC attachment step, a 21 day culture step in ITS media, and finally a 7 day culture step in MEM media. At this point (day 28) the Barberi group reported the first signs of myogenic cells, as evidenced by MyoD expression. Because the generation of MyoD+ myoblasts from hESCs is our primary goal, we screened day 28 cultures for the presence of MyoD+ cells. The morphology and gene expression signature of the day 28 cells are shown in Fig. 8 and Fig. 9, respectively.

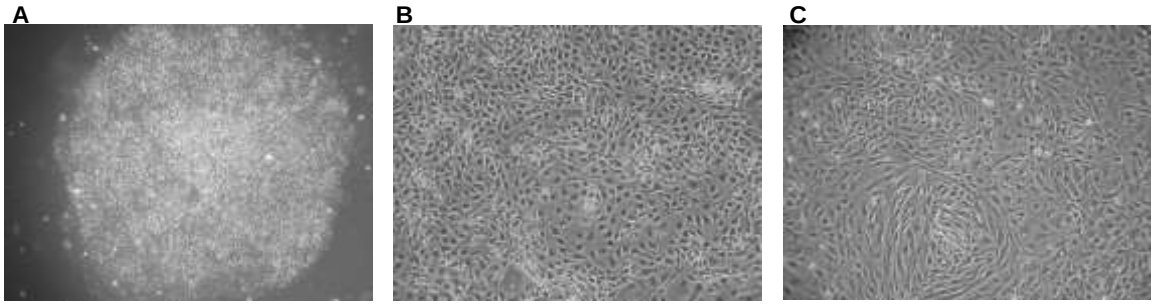

Figure 8. Morphology of experimental cells treated according to the Barberi protocol. (A) Image of undifferentiated H9 hESCs (4X Hoffman image). (B) Cells after 21 days of culture in ITS media (10X Hoffman image). (C) Cells after 21 days in ITS media followed by an additional 7 days of culture in MEM media (10X Hoffman image).

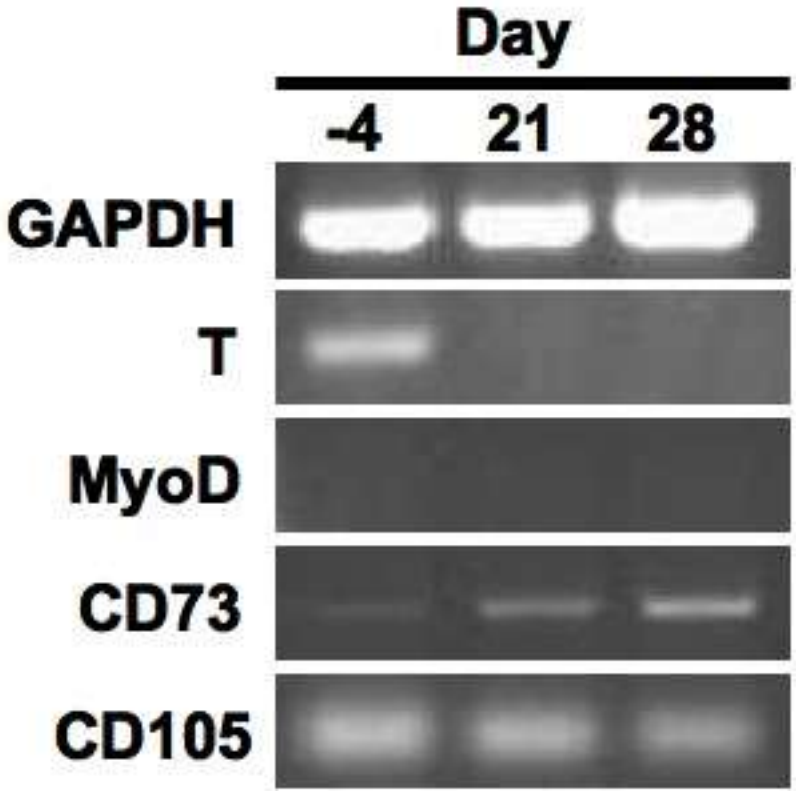

Figure 9. RT-PCR of experimental cells after 28 days of culture according to the Barberi protocol (1µg of RNA was used).

A clear change in cell morphology is evident in hESCs differentiated according to the methods described by the modified Barberi protocol (Fig. 8). However, contrary to the results reported by Barberi *et al.*, the RT-PCR results shown in Fig. 9 demonstrate that MyoD expression is not detected by Day 28 cells. This is a significant finding because it means that we cannot use the lentiviral reporter system to isolate MyoD+ cells at an earlier stage of the protocol.

The gene expression analysis depicted in Fig. 9 demonstrates an upregulation of the MSC-markers CD73 and CD105, at each time point examined. Surprisingly, CD73 expression was also observed in undifferentiated hESCs. This observation suggests that either hESCs express very low levels of CD73, or that a fraction of hESCs spontaneously differentiate into an MSC-like cell type (this would also explain the expression of CD105 in undifferentiated hESCs).

Taken together, these data suggest that while we were not able to get myogenic cells utilizing this protocol, it does appear that the protocol works for obtaining MSCs from hESCs. Since muscle differentiation is a well-known potential of bone marrow-derived MSCs, it is possible that these cells may have the same ability.

III. Determination of the Myogenic Potential of hESC-derived MSCs

Due to the fact that we were unable to obtain MyoD+ cells by the time point Barberi *et al.* described, we wished to see if we could achieve myogenic differentiation from MSC derived by the protocol. Specifically, the modified Barberi protocol was utilized in conjunction with CD73 FACS analysis, allowing us to isolate the CD73+,

putative MSC-like population. Experimental cells were prepared for FACS isolation of the CD73+ at Day 28 of the protocol. The FACS parameter can be seen in Fig. 10.

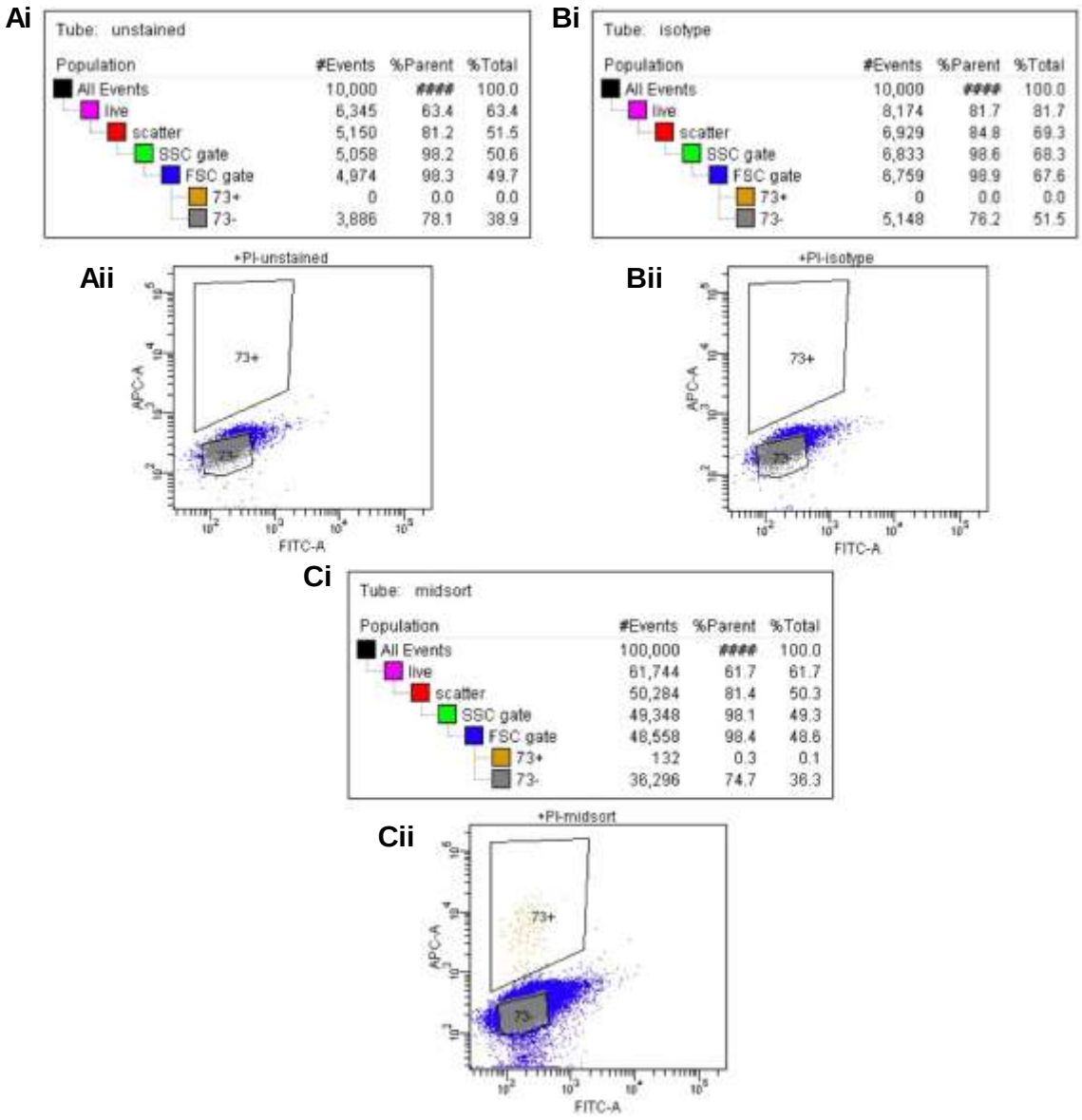

Figure 10. FACS experimental details. (Ai,ii) FACS table and gates for unstained cells. (Bi,ii) FACS table and gates for mouse anti-human IgG isotype control antibody. (Ci,ii) Total sort of cells stained with mouse anti-human CD73 antibody and with the AlexaFluor 633 goat anti-mouse secondary antibody.

The data diagrammed in Fig. 10 shows that after 28 days of culturing hESCs according to the modified Barberi protocol yielded only .1% of CD73+ cells. The

staining of cells is shown to be faithful as we did not see any false positives in either the unstained or isotype negative controls, although it is possible that there are CD73+ cells present in the population that remained unstained. The CD73+ and CD73- were collected and plated onto gelatin-coated dishes, the cell numbers are shown in Table 1.

	Cells Plated	**Cells Attached**	**Percentage Attached**	**Confluence Upon RNA-Extraction**
CD73+ Cells	544	44	8.1%	75-80%
CD73- Cells	1595	85	5.3%	50-55%

Table 1. The cell numbers following the CD73 FACS experiment.

The CD73+ and CD73- cell populations were cultured in MEM media for an additional 19 days (cells stopped proliferating before reaching confluency) as per Barberi *et al.'s* instructions for myogenic differentiation (Stavropoulos et al, 2009). As shown in Fig. 11, there is a clear morphological distinction between cultures of CD73+ and CD73- cells.

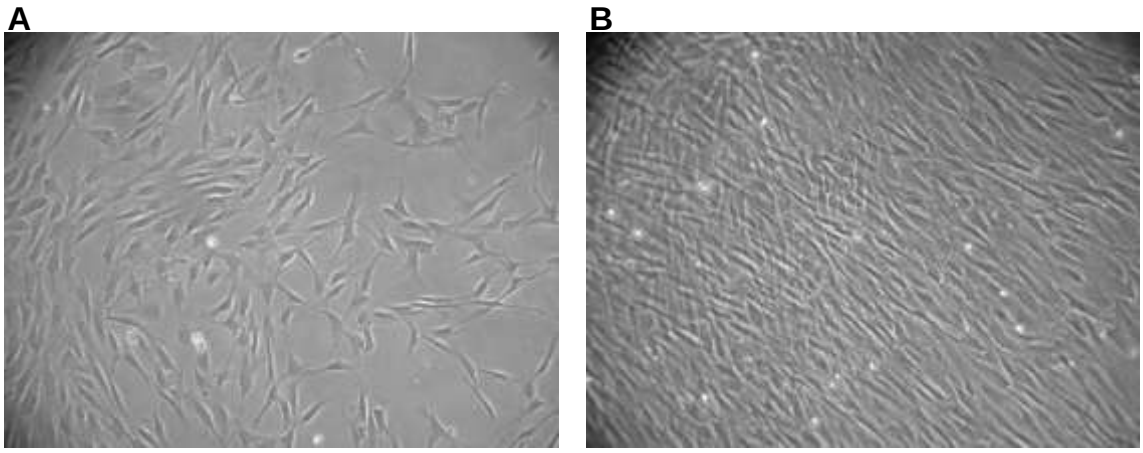

Figure 11. Morphology of FACS isolated cells following 19 days of expansion in MEM medium.

The CD73+ cells showed a higher rate of attachment and capacity for proliferation than the CD73- population (Table 1). Once the CD73+/- cells stopped proliferating, cells were harvested and RNA was extracted for gene expression analysis by RT-PCR. The analysis is demonstrated in Fig. 12.

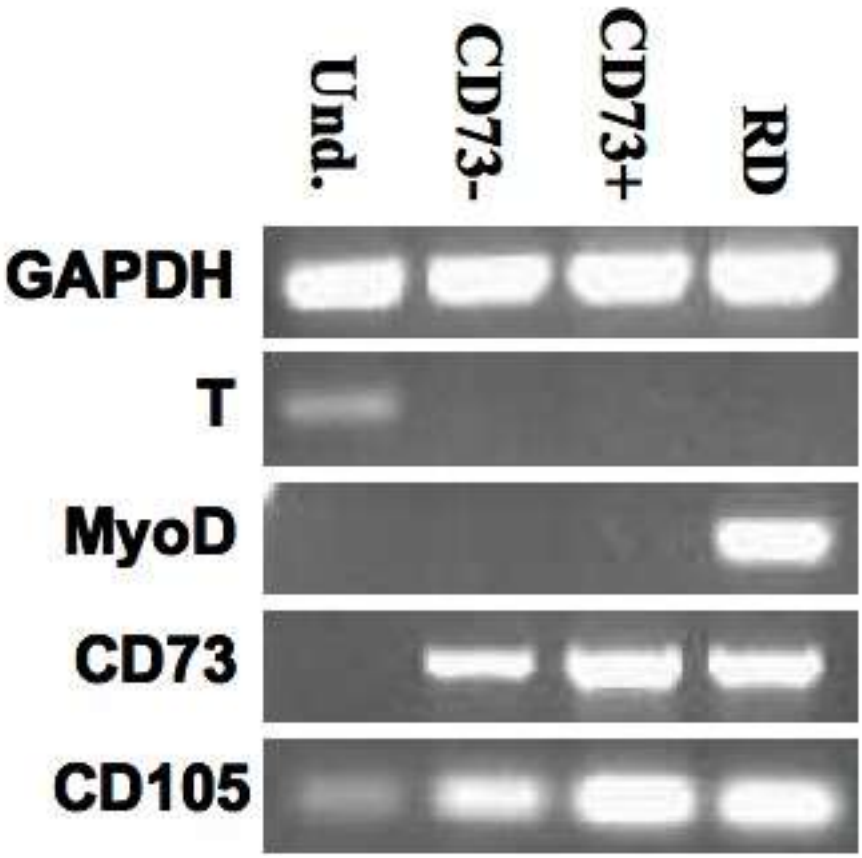

Figure 12. RT-PCR of experimental cells after FACS isolation of CD73+/- cells and 19 days of culture in MEM media (240ng of RNA; Und.-Undifferentiated H9 hESCs, RD-Rhabdomyosarcoma positive control)

Fig. 12 shows that the FACS purification of an MSC-like cell population failed to facilitate the isolation of MyoD+ cells. It should be noted that for terminal myogenic differentiation the Barberi group also included a sort for NCAM+ cells to select for myogenic cells within the MSC population, prior to differentiation. Despite this observation, we still expected to see MyoD expression without the NCAM sort as the Barberi group reported MyoD expression by day 28 of their protocol, a time point that proceeds our CD73 sort. The CD73+ population did have the appearance of a true MSC population, with robust CD73 and CD105 expression. Interestingly, the CD73- population showed expression of CD73 and CD105. This could be explained by the fact

that not all the CD73+ cells were labeled during the FACS isolation (discussed earlier) or that a portion of the CD73- cells had the potential to differentiate into an MSC-like population and did so in the MEM medium.

It is unfortunate that the methods published by the Barberi group have thus far proven to be very difficult to replicate. Our studies have shown no sign of myogenic potential in these cells. Despite this, the protocol does appear to have promise in producing MSC from hESCs, which may have the potential to form skeletal muscle if alternative approaches are taken.

Discussion

Derivation of skeletal muscle from ESCs is an area of research that has shown little progress since the first hESCs were derived. Success has been made in mouse embryonic stem cells (mESCs) using a variety of strategies. Groups have successfully obtained skeletal muscle from mESCs by transducing with vector systems expressing MyoD in a transient (Craft et al, 2008) or inducible manner (Ozasa et al, 2007). Alternatively, mESCs have been induced to undergo myogenic differentiation by culturing the mESCs as EBs in FBS-containing media supplemented with spermine analogs (Sasaki et al, 2008) or with horse serum (Chang et al, 2009). Utilizing what is known from mESC research, which has been going on for decades, is the logical choice for directing hESC studies. Unfortunately, these differentiation strategies have not been able to be translated to hESCs. This problem of mESC research not fully applying to hESC research is most likely related to the fact that mESCs and hESCs exhibit largely different behavior. For example, mESCs require leukemia inhibitory factor (LIF) to maintain a pluripotent state while hESCs require FGF, bone morphogenic protein (BMP) is another cytokine important in maintaining the pluripotent state of mESCs, but in hESCs it is a potent inducer of differentiation, and furthermore, pluripotent mESCs and hESCs are identified by a number of different genes. These critical differences between mESCs and hESCs calls into question whether or not these two cell types actually represent cells of the same developmental stage and could potentially explain the inability to use mESC experimental protocols to achieve the same results with hESCs.

Due to the difficulties described above in translating mESC research to hESCs, hESCs studies have had to discover novel cellular differentiation strategies. In 2006 the first group published results showing the ability to generate cells expressing myogenic markers *in vitro* (Zheng et al, 2006). This study grew hESCs as EBs, plated the EB-differentiated cells, and cultured the outgrowths from the plated EBs in media supplemented with FBS, dexamethasone, ITS, and epidermal growth factor (EGF). This treatment produced cells expressing myogenic markers, including MyoD, after two weeks of culture. Despite the successes of this protocol, it had may short comings, including a very low efficiency of obtaining myogenic cells and an inability for the cells to undergo terminal differentiation and form myotubes and myofibers. This inability to obtain more maturely differentiated cells is a typical problem in ESC research, which frequently can generate more embryonic or fetal cell types, rather than the cells more characteristic of the adult organism. While our reporter system could potentially greatly increase the efficiency of this protocol, the limitations in cellular differentiation made undesirable for our purposes.

Very recently a publication came describing a new means for obtaining skeletal muscle from hESCs. This protocol generated myogenic cells by culturing as EBs and treating them serum-free media containing SB-431542, a small molecule inhibitor of the TGF-β/Activin/Nodal signaling pathway (Mahmood et al, 2010). As previously described, EB formation leads to the generation of endodermal, mesodermal, and ectodermal lineages. The study showed that SB-431542-mediated inhibition of TGF-β/Activin/Nodal signaling led to a decrease in the ability of the EBs to form endodermal cells types while a dramatic increase in the ability of the cells to form muscle cell types,

including skeletal muscle, at fairly high efficiency (52% of experimental cells expressed MyoD). This study is not only promising in its ability to produce skeletal muscle, but also in the information it provides about the nature of hESC differentiation. The fact skeletal muscle has proven so difficult to obtain from hESC, and yet are generated relatively easily and in abundance once endodermal differentiation is blocked suggests that difficulty in forming mesodermal cell types is a preferences of hESCs to differentiate into endodermal lineages. While our experiments did not include any analysis of endodermal differentiation, it is very possible that this is the lineage that cells progressed down, explaining our inability to generate myogenic cells Blocking endodermal with small molecule inhibitors, like the one used in this study, or by other may be critical in advancing hESCs research so that historically difficult to generate mesodermal cell types can be formed.

Unfortunately, at the beginning of this study the findings of Mahmood *et al.* were not yet published, and thus, Barberi's publication in 2007 regarding a protocol that supported the *in vitro* differentiation of skeletal myoblasts capable of terminal differentiation from hESCs (Barberi et al, 2007) was viewed as a major development. Although the cell differentiation protocol was inefficient and required a prolonged time in cell culture, the protocol did offer a simple, stepwise methodology for generating skeletal muscle. The original goal of this study was to modify the protocol provided by Barberi *et al.* using a lentiviral reporter system to enhance the ability to derive myoblasts from hESCs. Unfortunately, the original MyoD-CFP-EF1α-mCherry reporter we generated for this study had to be redesigned (Fig. 5) because of unacceptable limitations. While the modified reporter system may eventually facilitate the efficient isolation of myogenic

cells, our results suggest that that the Barberi protocol cannot be used for obtaining MyoD+ cells in a reproducible manner.

HESC research is beset with issues of reproducibility due to a lack of standardization in the field. Different labs utilize a variety of genetically diverse lines that may exhibit differences in potentiality or preferred lineages of differentiation. Additionally, hESCs are cultured under a variety of different culture conditions. For example, labs may culture their hESCs on feeder layers such as irradiated mouse embryonic fibroblasts (MEFs) or on irradiated human fibroblasts. There are also feeder-free culture systems, in which hESCs are cultured on matrigel or fibronectin and fed with MEF-conditioned media or chemically defined media known as mTeSR. It is reasonable to assume that culturing hESCs on other cell types or in different culture mediums could very well affect the potential of these cells. This is an important issue that could provide a potential explanation for our inability to replicate the results of the Barberi group. Specifically, Barberi *et al.* culture their cells on MEFs, whereas we wished to avoid contamination of our cells with mouse contaminants and therefore cultured our hESCs on matrigel with MEF conditioned media. The MEFs utilized by the Barberi group may have provided a lineage commitment that accounts for their ability to form myoblasts. This is the most probable cause for our inability to reproduce Barberi *et al.'s* results, as it is one of the few variables existing between their methods and ours that could potentially have a significant influence.

While MSC-like cells were derived using this protocol, the efficiency of their derivation was fairly low. While the Barberi group did not report the percentage of cells that they obtained that were CD73+, our percentage was only 0.1% (Fig. 10).

The results outlined in this report showed that CD73 is actually being expressed in undifferentiated hESCs (Fig. 12). As previously discussed, this suggests that there may be a small population of spontaneously differentiated MSCs within hESC colonies. This information could potentially be useful if MSCs could be efficiently derived directly from undifferentiated hESCs using a CD73 reporter system or other strategy.

We failed to detect MyoD expression at any point during differentiation. Although Barberi *et al.* reported the appearance of MyoD gene expression by Day 28 of their protocol, we could not reproduce this result, even after the expansion and differentiation of the CD73+ cells. However, it may be possible to obtain myogenic cells from the MSC-like cells using more established differentiation methods.

Future Directions

Immediate experimental goals would be to improve upon the Barberi group's methodology for obtaining myogenic cells from their CD73+ MSC-like cell population. Barberi *et al.* utilized αMEM media containing 10% fetal bovine serum to differentiate their hESC-derived MSCs into skeletal muscle. This is not a differentiation medium typically used to obtain skeletal muscle from MSCs. Bone marrow-derived MSCs, which have been studied for significantly longer than hESC-derived MSCs, are most typically differentiated into muscle *in vitro* using media supplemented with 20% FBS, 0.5% chicken embryo extract, and 10% horse serum (Pozzobon et al, 2008). Another study transduced bone marrow-derived MSCs using a retroviral system expressing Pax3 showed significant skeletal muscle differentiation (Gang et al, 2008). This protocol had the advantage over the Pozzobon study, in that they were capable of generating myogenic cells capable of terminal differentiation without the use of non-physiological agents such as 5-azacytidine. Using these strategy or possibly another, such as co-culture with C2C12 cells, it may be possible to successfully derive myogenic cells from the MSCs obtained by the Barberi protocol. If these techniques proved successful then the lentiviral reporter systems could be utilized in improving the efficiency of a new protocol.

For longer-term experimental design, the study published by Mahmood *et al.* offers important direction for developing future mesodermal differentiation strategies. One of the limitations of the Mahmood study was that it relied on EB formation for the initial stages of differentiation. As described previously, EB differentiation spontaneously generates all three germ layers. The difficulties in using such a strategy are that, even when a lineage inhibitor (like SB-431542) is used, the EBs generate a heterogeneous cell

population. For a protocol to be truly useful for clinical applications it would ideally allow for the directed, homogeneous differentiation of hESCs into the cell type of interest. This means that the best differentiation strategies would avoid random and heterogeneous differentiation steps such as those involved in EB formation. Therefore, future experiments should focus on utilizing the strategy of lineage restriction shown by Mahmood *et al.* to obtain skeletal myoblasts in a directed manner. Mimicking the signals known from embryonic development is the most logical approach to directing the differentiation of hESCs. By utilizing BMP-4, a known inducer of mesoderm in hESCs (Zhang et al, 2008), to generate a primitive mesodermal cell population followed by treatment with cytokines known to induce skeletal muscle development such as Wnt3a and Wnt7a (Parker et al, 2003). This strategy has been attempted previously in our lab, unsuccessfully, but with the lineage restriction provided by TGF-β/Activin/Nodal signaling pathway inhibition via the SB-431542 inhibitor could allow for the intended mesodermal differentiation due to the blocked default endodermal lineage.

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