GMP-GRADE CARDIOMYOCYTE DIFFERENTIATION MEDIA SYSTEM FOR PLURIPOTENT STEM CELLS IN BASIC AND TRANSLATIONAL RESEARCH

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ABSTRACT

Objective: Current protocols for differentiating pluripotent stem cells (PSCs) have led to heterogeneous results, varying purity levels, and long lead times for generation of cardiomyocytes. We hypothesized that a simplified and rapid cardiomyocyte differentiation media system can be developed in a scalable workflow to enable generation of large numbers of consistent, spontaneously active cardiomyocytes that could be used in basic and translational research.

Methods: High quality PSCs were maintained under xenofree, feeder-free culture conditions. At time of passaging, PSC were dissociated with 0.5 mM EDTA, seeded on 1:100 Geltrex®-coated surface as small clusters at ~0.5 to 1 x 10^5/well of a 12-well plate and maintained for four days under serum-free condition. After reaching target confluence of ~30 to 70%, an induction media was added for two days followed by addition of a second induction media for two days. After the induction step, the media was replaced with maintenance media and re-fed every other day for up to five weeks. PSC-derived cardiomyocytes were analyzed by morphology, gene expression, flow cytometry, immunocytochemistry and multi-electrode array (MEA).

Results: We observed individual beating cells as early as Day 6 and contracting syncytia by Day 8. An over 100-fold increase in cell number was noted from the time of plating to generation of contracting syncytia of cardiomyocytes. Quantitative flow cytometry detected populations of troponin T type 2 (TNNT2)-immunoreactive cells that reached as high as 96.6%. Number of TNNT2-positive cells dropped by 20% when induced at 90% versus 60% confluency. PCR studies confirmed expression of mesoderm (T, MIXL1, MESP1), cardiac mesoderm (ISL1, GATA4, MEF2C) and mature cardiomyocyte genes (NKX2.5, TNNT2, MYH6). Immunocytochemistry studies verified expression of cardiac markers NKX2.5, GATA4, MEF2C, TNNT2 and MYH6. Initial MEA studies corroborated the presence of electrically active cells.

Conclusions: We conclude that a simplified complete differentiation media system could serve as a standardized culture system for generating large numbers of consistent, spontaneously active cardiomyocytes for basic and translational research studies.

PSC DIFFERENTIATION WORKFLOW

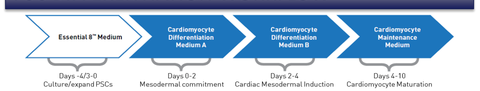


Figure 1: PSC Differentiation Workflow

The PSC Cardiomyocyte Differentiation kit consists of three ready-to-use medium manufactured under GMP conditions that are used in succession to induce undifferentiated PSC into differentiated cardiomyocytes.

In early studies, EDTA-dissociated PSC were used for routine passaging as well as seeding plates for subsequent differentiation, though confluency levels at time of differentiation varied. To minimize variation at time of seeding, PSC were dissociated with TrypLE™ into single cell suspension and plated at specific cell densities with Growth Supplement. This method was found to yield consistent, high purity cardiomyocytes.

After seeding, PSC were expanded in Essential 8™ media for three to four days (Day -4/-3 to Day 0). Upon reaching 30–70% confluence at Day 0, the PSC culture is treated with Cardiomyocyte Differentiation Medium A (CDM A) and cultured for two days. Subsequently, PSC culture is treated with Cardiomyocyte Differentiation Medium B (CDM B) and cultured for two days.

After being treated with CDM A and CDM B, PSC culture is refed every other day with Cardiomyocyte Maintenance Medium (CMM) for 10 days or longer. Contracting cardiomyocytes can appear as early as Day 6 with spontaneously contracting syncytium appearing around Day 8.

After reaching Day 10 or higher, these cells were analyzed through various methodologies including immunocytochemistry, flow cytometry, cellular imaging, electrophysiology, gene expression and RNA-Seq analysis. Some cardiomyocytes were cryopreserved and recovered for further analysis.

PSC DIFFERENTIATION OPTIMIZATION

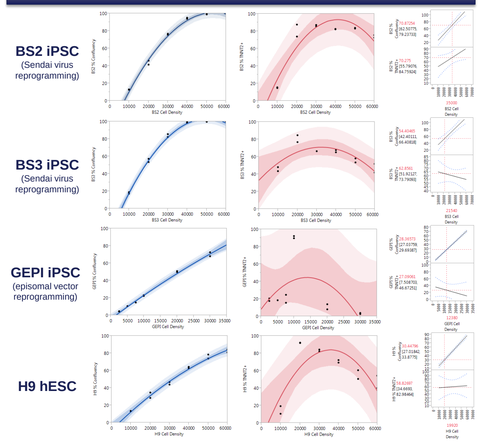


Figure 2: Seeding Density, Confluency, % TNNT2 & iPSC lines

Seeding density is crucial for optimal PSC cardiomyocyte differentiation. TrypLE™-dissociated PSC lines were used for set up of these studies. For two CytoTune®-derived lines, BS2 iPSC was observed to be promiscuous at higher density while differentiation of BS3 iPSC was optimal only at a specific density. For GIBCO episomal iPSC (GEPI), it was also found to be optimal at a specific density. For hESC, H9 was observed to be promiscuous at various densities. JMP® Profiler tool identified optimal seeding density for efficient differentiation of different PSC lines.

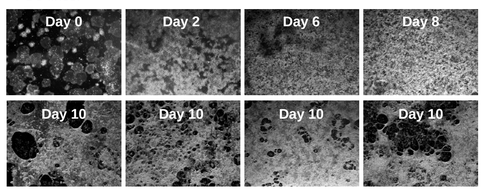


Figure 3: Morphology

Representative dark phase contrast images of H9 undergoing cardiac differentiation over time. Differing morphologies were observed as early as Day 2 and contracting cardiomyocytes observed as early as Day 6.

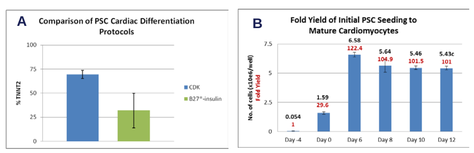


Figure 4: Comparison of Methods and Cardiomyocyte Yield

A. Differentiated over a period of 17 days, flow cytometry revealed that PSC Cardiomyocyte Differentiation kit (CDK) is more efficient than a popular "homebrew" protocol based on B27® minus insulin protocol (3). **B.** Starting with an initial seeding population of H9 and performing duplicate cell counts on specific days over time, these cells can expand and differentiate into mature cardiomyocytes up to 100 fold yield.

CHARACTERIZATION

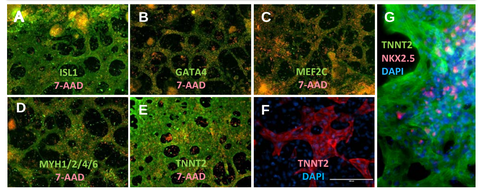


Figure 5: Immunocytochemical Staining of Differentiated Cardiomyocytes

A-E: ISL1, GATA4, MEF2C, pan-MYH, & TNNT2 immunostaining of Day 15 H9-derived cardiomyocytes. F: TNNT2 immunostaining of Day 15 GEPI iPSC-derived cardiomyocytes. G: TNNT2 & NKX2.5 immunostaining of GEPI iPSC-derived cardiomyocytes..

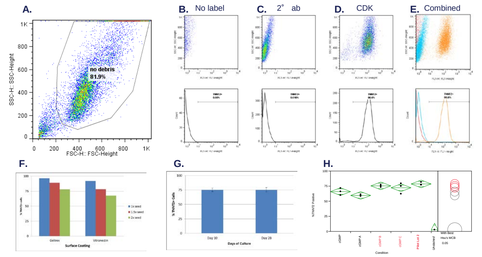


Figure 6: Troponin T Expression in Differentiated Cardiomyocytes

A-E. Flow cytometry gating strategy and expression of Troponin T (TNNT2) in differentiated H9 ESCs. Data following 10 days cardiac induction shown. CDK: Cardiac Differentiation. **F.** Efficient differentiation of H9 ESCs on Geltrex™ or vitronectin matrices. Effect of increased initial seeding densities is shown (1x-2x) on relative TNNT2 expression scale. **G.** Long-term culture of H9 ESCs. **H.** Multiple media lot testing.

CALCIUM IMAGING

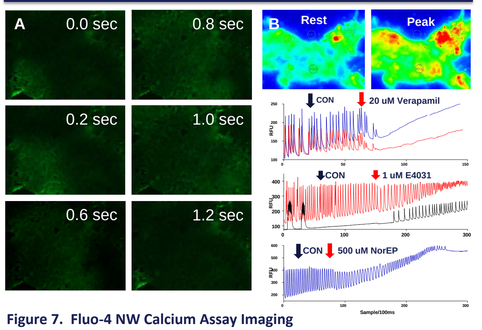


Figure 7. Fluo-4 NW Calcium Assay Imaging

A. H9-derived cardiomyocytes were labeled with the Fluo-4 Calcium Imaging Kit, exchanged with CMM, and imaged on EVOS® FL Auto at 4x magnification using LiteCam HD software capturing 30 frames per second.
B. Calcium imaging of cells imaged with Neuro-Backdrop quencher and no wash. Spontaneous calcium transients were imaged at 100 ms intervals with a Till Polychrome (FEI Systems) and regions of interest captured for plotting vs time with Vision™ Software. Carrier control or drug containing solution were added as indicated from a 10X stock in Live Cell Imaging Solution.

ELECTROPHYSIOLOGY

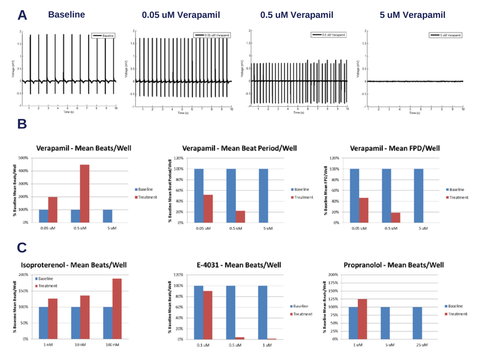


Figure 8: Baseline & Drug Treatment of Electrically Active Syncytium

Cardioactive compounds modulate PSC-derived cardiomyocyte beat rate. PSC-derived cardiomyocytes were plated at 4 x 10^4 cells/well on fibronectin-coated MEA wells and spontaneous action potential waveforms were averaged over a three minute period. A: Cardiomyocyte waveforms under baseline or Verapamil (L-type Ca2+ channel blocker) treatment conditions. B: Effect of Verapamil, on the mean beats rate/well, mean beat period/well and mean field duration potential (FPD)/well. C: Effect of isoproterenol (beta-adrenergic receptor agonist), E-4031 (hERG channel blocker) and propranolol (beta adrenergic receptor antagonist/blocker) on the spontaneous beat rate of H9-derived cardiomyocytes.

GENE EXPRESSION

Mesoderm Commitment (D0-D3)	Early Cardiomyocyte (D4-D6)	Late Cardiomyocyte (D7-D15)
T (brachyury)	ISL1	NKX2-5
MIXL1	GATA4	TNNT2
MESP1	MEF2C	MYH6

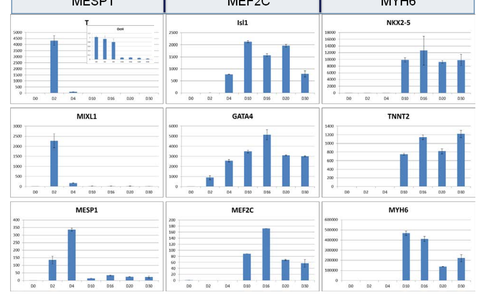
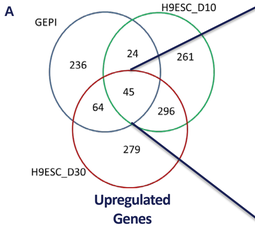


Figure 9: Gene expression profile at early, mid & late stage differentiation

TaqMan® qPCR analysis of differentiated (H9) ESCs from time of induction Day 0 to Day 30. Undifferentiated and differentiated cells were solubilized with Trizol®, RNA isolation by column purification, and cDNA generated for qPCR analysis. Relative gene expression for each time point is shown, normalized and presented as relative fold change to Day 0 control samples. Inset: D0 to Day 30 relative expression level of Oct4, a pluripotency marker.

RNA-Seq ANALYSIS



AEBP1	KAT2B	PRNP	
ASXL3	KLHL14	PTPRM	
CDH11	LBH	PYGO1	
CERCAM	LGI1	RASGEF1B	
COL4A1	LINC00963	RBFOX1	
COL5A2	MAGI2-AS3	RGL1	
COLGALT2	MALAT1	RIN2	
DKK3	MBNL2	SEMA6D	
ENG	MYRF	SLC1A4	
FAT4	NCALD	SMYD2	
FER1L4	NCAM1	TCP11L2	
FLRT2	PCDH17	THBS1	
GSTM3	PCDHGB6	ZBTB20	
IER5L	PCGF5	ZHX1	
IL6ST	PHC2	ZNF436	

AASS	CENPE	KCNS3	PRKX	
ADCYAP1R1	CHAF1B	KIF14	RAD51	
ARHGAP11A	COBL	KIF15	RAD54L	
ASNS	CORO2A	LDB2	RMI2	
ASPM	DEPDC1B	MCM10	RRM2	
B3GNT7	ECT2	MCM3	RTKN2	
BLM	FAM131B	MIS18BP1	SEMA6A	
BRCA1	FAM83F	MLK4	SEPHS1	
BRCA2	FANCD2	MTHFD2	SORL1	
CALB1	GLS2	MTL5	SPAG5	
CDC20	GPC4	NASP	TRIP13	
CDC25A	GPRIN2	NCAPG	TTF2	
CDC6	GYLTL1B	NLN	UNG	
CDCA2	HIST1H3E	PHC1	WDHD1	
CDH1	HMGB3	PIM2	WDR62	
CDK1	JARID2	PREX2		

Figure 10: RNA-Seq Differential Expression

A. We observed 369, 626 and 684 upregulated genes in GEPI_D73, H9ESCD10 and H9ESC_D30, respectively, at 0.01 FDR. 45 shared genes were found. B. We observed 202, 463 and 485 downregulated genes in GEPI_D73, H9ESCD10 and H9ESC_D30, respectively, at 0.01 FDR. 63 shared genes were found.

SUMMARY & CONCLUSIONS

- We have developed a simple and efficient GMP-grade media system for differentiation of human PSCs to contracting cardiomyocytes
- Both iPSC and ES lines have been shown to efficiently differentiate into cardiomyocytes
- iPSC derived using episomal and Sendai virus methods have been successfully differentiated
- Electrically coupled contracting syncytia and spontaneous calcium transients are observed as early as 6 days following cardiac differentiation.
- Initial RNAseq analysis reveals shared, overlapping and non-overlapping genes between hESC and iPSC lines at early and late stages of differentiation.

REFERENCES

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2. Priori et al J. Clin Invest 2013
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ACKNOWLEDGMENTS

Authors thank their colleagues for significant project support including David Piper, Rhonda Newman, Lauren Sangenario, Mike Hancock and Marian Piekarczyk.