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AN ANIMAL-FREE FULLY DEFINED WORKFLOW FOR HUMAN INDUCED PLURIPOTENT STEM CELL RESEARCH

Poster # 305

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INTRODUCTION

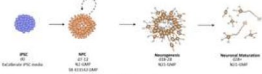
Human induced pluripotent stem cells (iPSC) have great potential for cellular therapies but are also critical for *ex vivo* models for studying development, disease mechanisms and performing drug discovery. However, many iPSC workflows involve undefined culture surfaces and cell medium with lot-to-lot variability and animal components that can limit data reproducibility or hamper transition to the clinic. It is critical to overcome these obstacles by creating an animal-free and fully defined workflow that ensures validity, reproducibility of results and easier compliance with clinical cGMP standards. In this work, we devised and screened multiple human vitronectin (hVN) constructs for their capacity to support iPSC attachment and proliferation. We also developed an animal component-free ExCellerate™ iPSC Expansion Medium to support the proliferation and growth of iPSC. Human iPSC grown with these constructs and the media retained their pluripotency by successfully differentiating to multiple cell types including germ layer lineages. Finally, we created animal-free differentiation processes for neurons and dopaminergic (DA) progenitors using the aforementioned hVN construct, iPSC media and animal-free supplements. These results demonstrate the feasibility of a standardized fully defined, animal-free, and reproducible process for iPSC research and clinical translation.

METHODS

iPSC Maintenance: iPSC were maintained in ExCellerate™ iPSC Expansion Medium (Catalog # CCM036) and passaged at 70% confluence with Versene™. Viability counts were obtained using the trypan blue exclusion method and a hemocytometer for each passage. Cells were plated on glass coverslips or 6 well plates coated with Recombinant Human Vitronectin (VN FL, Catalog # 2308-VN), hVN constructs, or Cultrex™ UltiMatrix (Catalog # BME001). G-band analysis was performed by WiCell.

Flow Cytometry: iPSCs were dissociated using Accutase™ and stained for stemness markers using H/M Pluripotent Stem Cell Multi-Color Flow Cytometry Kit (Catalog # FMC001). cTNT1 antibody was conjugated to Alexafluor 647 in-house. Cells were then processed with BD LSRFortessa and data analyzed in FlowJo.

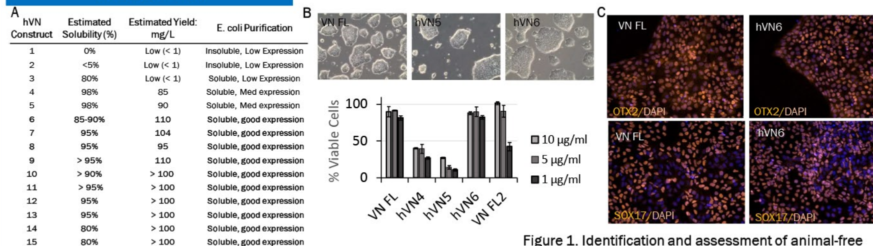
Cell Differentiation: iPSC were differentiated to various lineages using StemXVivo™ Ectoderm (Catalog # SC031B), Endoderm (Catalog # SC019B), Hepatocyte (Catalog # SC033), and Cardiomyocyte (Catalog # SC032B) differentiation kits. ICC quantification of various markers was performed using Perkin Elmer Operetta. Animal-free neuronal differentiation process is described in the following figure:



Animal-free dopaminergic progenitor differentiation is obtained using a modified version of the process above with Sonic Hedgehog (Catalog # 1845 GMP) and LDN193189 (TB6053-RMU)

Immunocytochemistry: Cells were fixed with 4% PFA and permeabilized with 0.3% Triton-X-100. All primary antibodies are from R&D Systems: Oct-3/4 (Catalog # AF1759), Tra-1-60 (Catalog # MAB4770), SOX17 (Catalog # AF1924), Otx2 (Catalog # AF1979), cTNT1 (Catalog # MAB1874), SerpinA1 (Catalog# AF1268), HNF4A (Catalog # PP-H1415), Cytokeratin18 (Catalog # MAB7619), Albumin (Catalog # MAB1455), Nurr1 (Catalog # AF2156), FOXA2 (Catalog # AF2400). Cells were then stained with appropriate NorthernLights™ secondary antibodies (Catalog # NL001, NL003) and DAPI.

RESULTS



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hVN Construct	Estimated Solubility (%)	Estimated Yield: mg/L	E. coli Purification
1	0%	Low (< 1)	Insoluble, Low Expression
2	<5%	Low (< 1)	Insoluble, Low Expression
3	80%	Low (< 1)	Soluble, Low Expression
4	98%	85	Soluble, Med expression
5	98%	90	Soluble, Med expression
6	85-90%	110	Soluble, good expression
7	95%	104	Soluble, good expression
8	95%	95	Soluble, good expression
9	> 95%	110	Soluble, good expression
10	> 90%	> 100	Soluble, good expression
11	> 95%	> 100	Soluble, good expression
12	95%	> 100	Soluble, good expression
13	95%	> 100	Soluble, good expression
14	80%	> 100	Soluble, good expression
15	80%	> 100	Soluble, good expression

Figure 1. Identification and assessment of animal-free hVN constructs. A) 15 different hVN constructs were modeled, expressed and screened. hVN6 was selected for best performance B) Brightfield image of iPSC colonies on different hVN constructs and full length VN (VN FL) along with cell viability at different coating concentrations. C) ICC images of iPSC differentiated to endo and ectoderm on VN FL and hVN6
D) Flow cytometry data of stemness marker expression from iPSC grown on hVN6 for over 5 passages.

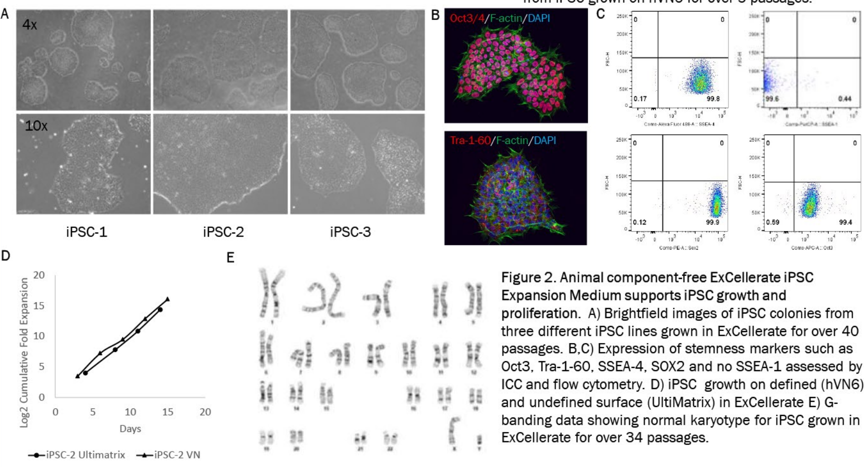

Figure 2. Animal component-free ExCellerate iPSC Expansion Medium supports iPSC growth and proliferation. A) Brightfield images of iPSC colonies from three different iPSC lines grown in ExCellerate for over 40 passages. B,C) Expression of stemness markers such as Oct3, Tra-1-60, SSEA-4, SOX2 and no SSEA-1 assessed by ICC and flow cytometry. D) iPSC growth on defined (hVN6) and undefined surface (UltiMatrix) in ExCellerate E) G-banding data showing normal karyotype for iPSC grown in ExCellerate for over 34 passages.

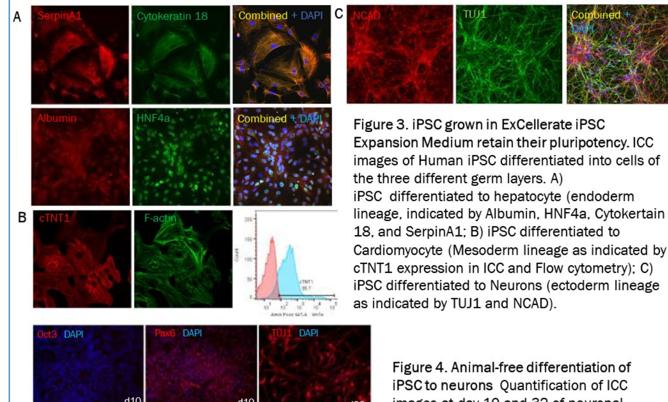

Figure 3. iPSC grown in ExCellerate iPSC Expansion Medium retain their pluripotency. ICC images of Human iPSC differentiated into cells of the three different germ layers. A) iPSC differentiated to hepatocyte (endoderm lineage, indicated by Albumin, HNF4a, Cytokertain 18, and SerpinA1; B) iPSC differentiated to Cardiomyocyte (Mesoderm lineage as indicated by cTNT1 expression in ICC and Flow cytometry); C) iPSC differentiated to Neurons (ectoderm lineage as indicated by TUJ1 and NCAD).

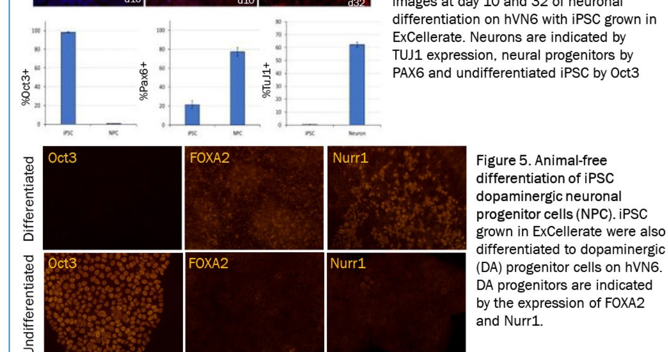

Figure 4. Animal-free differentiation of iPSC to neurons Quantification of ICC images at day 10 and 32 of neuronal differentiation on hVN6 with iPSC grown in ExCellerate. Neurons are indicated by TUJ1 expression, neural progenitors by PAX6 and undifferentiated iPSC by Oct3

Figure 5. Animal-free differentiation of iPSC dopaminergic neuronal progenitor cells (NPC). iPSC grown in ExCellerate were also differentiated to dopaminergic (DA) progenitor cells on hVN6. DA progenitors are indicated by the expression of FOXA2 and Nurr1.

CONCLUSION

A complete animal-free workflow was successfully created to expand human iPSC and differentiate them into neurons (including dopaminergic progenitors). These results serve as the basis for additional defined animal-free development processes that can improve studies' reproducibility, eliminate animal contaminants and facilitate translational and clinical research.

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