HIGHLY EFFICIENT DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO LONG-TERM EXPANDABLE "MINI-GUT" ORGANOIDS

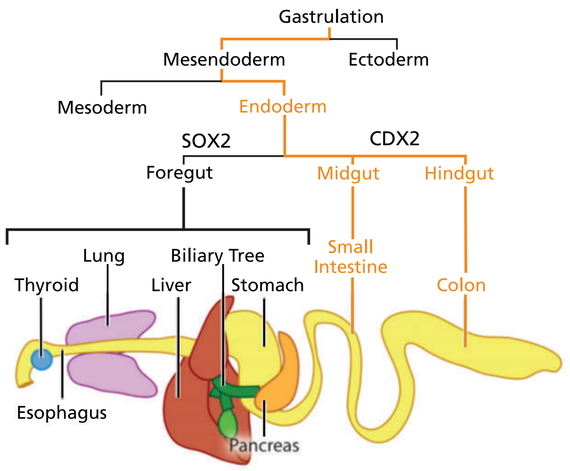
Philipp M. Kramer[1], Priscilla C. Soriano[1], Ryan K. Conder[1], Terry E. Thomas[1], Allen C. Eaves[1,2] and Sharon A. Louis[1]

[1]STEMCELL Technologies Inc., Vancouver, BC, Canada [2] Terry Fox Laboratory, BC Cancer Agency, Vancouver BC, Canada

Introduction

Human pluripotent stem cells (PSCs) are an important tool for disease modelling, drug screening and regenerative medicine. Recent studies have shown that hPSCs can be differentiated into tissue specific cell types by mimicking the signaling pathways in human development. Timing, duration and concentration of growth factors at specific stages of differentiation determine cell fate decision and lineage commitment. Recent work by Jason Spence, James Wells and colleagues have utilized these principles to develop a multi- stage (Spence *et al.*, Nature. 2011 Feb 3;470(7332):105-9), in vitro differentiation protocol for the generation of Human Intestinal Organoids (HIOs). Unfortunately, current state-of-the-art protocols are undefined since they rely on the use of FBS and conditioned medium, which contribute to inconsistent differentiation performance and is incompatible with using these hPSC derived intestinal cell types for future cell-replacement therapies.

To standardize the generation of HIOs, we developed STEMdiff™ Intestinal Organoid Kit, a specialized serum-free medium formulation that efficiently and reproducibly promotes differentiation of human PSCs through developmental stages of 1) definitive endoderm, 2) mid-/hindgut, and 3) small intestine **(Fig.1)**. Cells differentiated using this medium are capable of the robust formation of HIOs, composed of polarized intestinal epithelia patterned into villus-like structures and, a surrounding, niche factor-producing mesenchyme, both expressing key intestinal and mesenchymal marker genes, respectively. These organoids can be dissociated, passaged, expanded and maintained for multiple passages using STEMdiff™ Intestinal Organoid Growth Medium (OGM), which is part of the STEMdiff™ Intestinal Organoid Kit.



(Zorn & Wells, Annu Rev Cell Dev Biol. 2009; 25: 221–251)

FIGURE 1. Modified Schematic of Human Development and Endodermal Organogenesis
STEMdiff™ Intestinal Organoid Kit guides undifferentiated human PSCs efficiently through critical stages of mesendoderm to form definitive endoderm, which is subsequently patterned into posterior endoderm that gives rise to mid- and hindgut progenitors. These progenitors express the key transcription factor Caudal Type Homeobox Protein 2 (CDX2) and mature into small intestinal cells upon further differentiation.

Methods

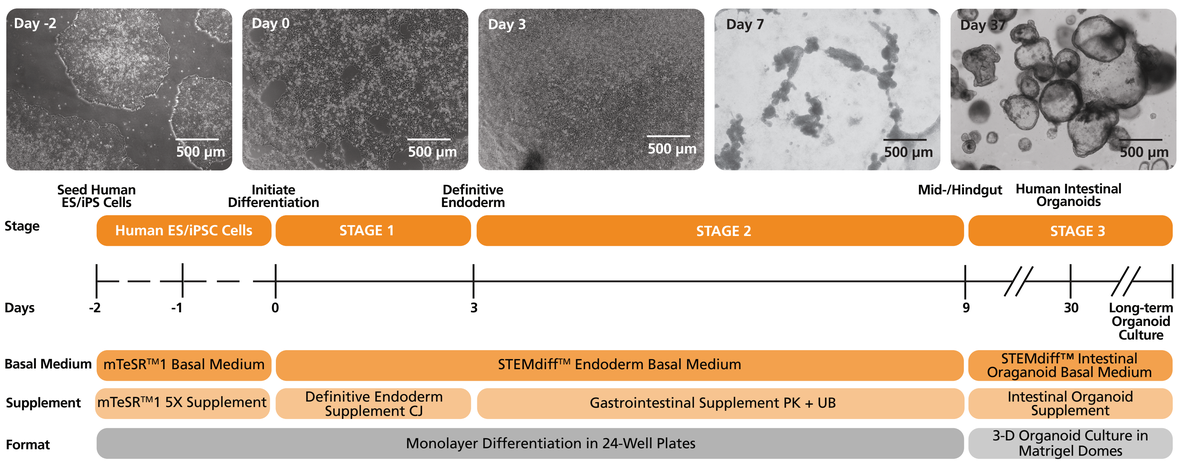


FIGURE 2. Protocol Schematic for STEMdiff™ Intestinal Organoid Kit and Overview of the Morphological Changes Over the Course of Differentiation
Human PSCs were seeded as single cells (~2 x 10^5/ well) with Y-27632 ROCK inhibitor or as small aggregates (50-200 µm in size) at low density (6,000 aggregates/well) in mTeSR™1 medium on Corning® Matrigel®-coated 24-well plates and allowed to attach overnight. 2-D monolayer cultures were maintained with daily mTeSR™1 medium changes until a near confluent monolayer (85-90%) was achieved. On day 0, the differentiation was initiated by replacing the medium to STEMdiff™ Intestinal Organoid stage 1 medium and daily medium changes were performed. On day 3, stage 1 medium was removed and replaced with complete STEMdiff™ Intestinal Organoid stage 2 differentiation medium. Between day 5 and day 9 of differentiation mid-/floating hindgut spheroids were harvested from the supernatant, embedded into Corning® Matrigel® and further differentiated using stage 3 STEMdiff™ Intestinal OGM. Seven to ten days post embedding HIOs were passaged and/or expanded until expression of intestinal markers were observed (~day 30). Beyond day 30, HIOs can be used for downstream applications, maintained in long-term culture or cryopreserved for future experiments.

Results

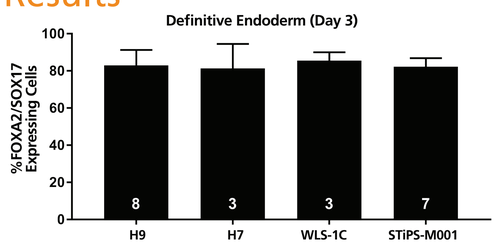


FIGURE 3: Efficient Differentiation to Definitive Endoderm in Multiple hES and hiPS Cell Lines
After 3 days of differentiation using STEMdiff™ Intestinal Organoid Kit, cells were analysed by flow cytometry for co-expression of definitive endoderm markers: SOX17 and FOXA2. Efficient and robust expression of these endodermal transcription factors are detected in multiple human ES (H9 and H7) and iPS (WLS-1C and STiPS-M001) cell lines (mean ± SD, n ≥ 3 as shown in the bar graph).

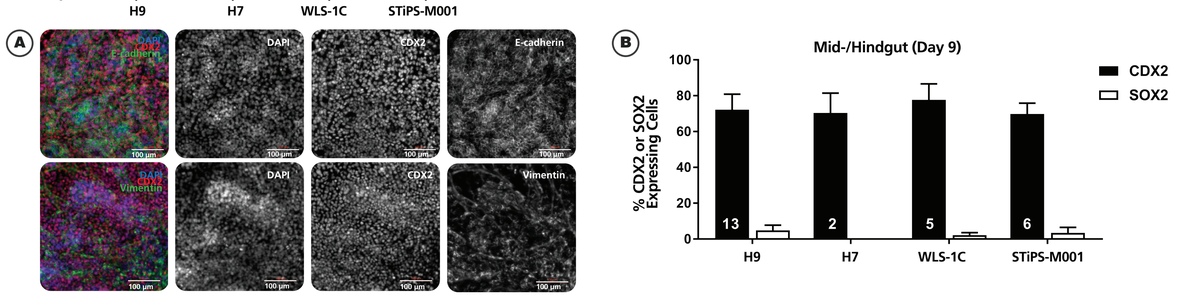


FIGURE 4. STEMdiff™ Intestinal Organoid Kit Efficiently Induces Mid-/Hindgut Marker CDX2 and Gives Rise to 3-D Spheroids
Post endoderm induction, cells were immediately exposed to STEMdiff™ Intestinal Organoid stage 2 medium. 24 hours of culture in mid-/hindgut inducing stage 2 medium resulted in significant morphological changes of the 2-D culture, the formation of epithelial ridges and 3-D mid-/hindgut spheroids. **(A)** 2-D monolayer cultures are highly enriched for mid-/hindgut marker CDX2, epithelial marker E-cadherin and vimentin expressing mesenchymal cells as assessed by immunocytochemistry staining. **(B)** Quantitative analysis of day 9 differentiated mid-/hindgut monolayers demonstrated by flow cytometry shows upregulation of the posterior endoderm marker CDX2 and absence of the anterior foregut marker SOX2 in multiple human ES (H9 and H7) and iPS (WLS-1C and STiPS-M001) cell lines (mean ± SD, n ≥ 2 as shown in the bar graph).

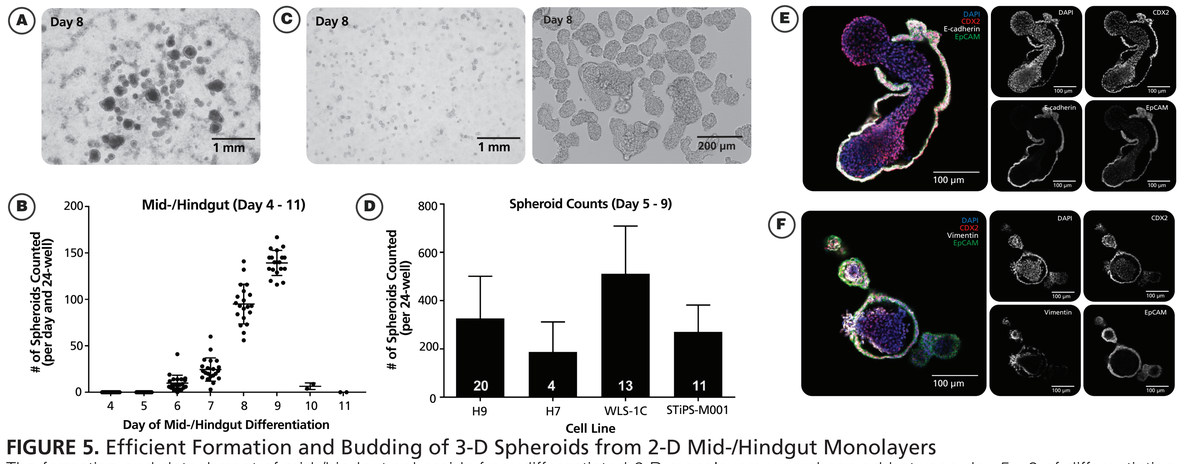


FIGURE 5. Efficient Formation and Budding of 3-D Spheroids from 2-D Mid-/Hindgut Monolayers
The formation and detachment of mid-/hindgut spheroids from differentiated 2-D monolayers was observed between day 5 - 9 of differentiation using STEMdiff™ Intestinal Organoid Kit. **(A)** Bright field microscopy of day 8 monolayer with floating hindgut spheroids and **(B)** manual quantification of detached spheroids over the course of differentiation (day 4 - 11) is shown using representative STiPS-M001 cells (mean ± SD, n = 1 with n ≥ 2 technical replicates). **(C)** Bright field images of organoid cultures at day 8 of WLS-1C differentiation were used to assess representative examples of budding formations of still attached hindgut spheroids (left) and released spheroids into the supernatant, which were transferred into an empty well for quantification (right). **(D)** All four tested cell lines were capable of forming mid-/hindgut spheroids with different budding frequencies due to the intrinsic variability of different hPSC lines (n for each cell line is indicated in the bar graph). **(E)** Immunofluorescence analysis of released mid-/hindgut spheroids after 9 days of differentiation. Spheroids are composed of a polarized epithelium expressing CDX2, E-cadherin and EpCAM, and **(F)** a mesenchyme expressing Vimentin and CDX2, but not EpCAM.

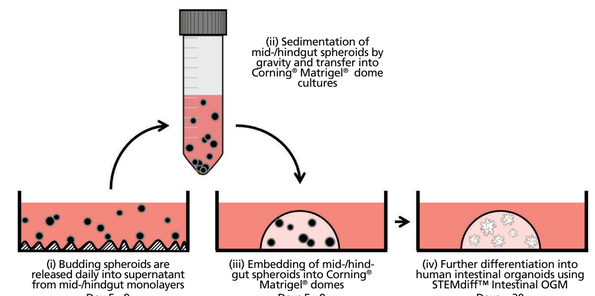


FIGURE 6. Schematic Showing the Morphological Changes During 2-D Monolayer Differentiation and the Procedure of **(i)** Spheroid Formation, Budding, **(ii)** Harvesting, **(iii)** Embedding into Corning® Matrigel® Domes and **(iv)** Further Differentiation into HIOs.

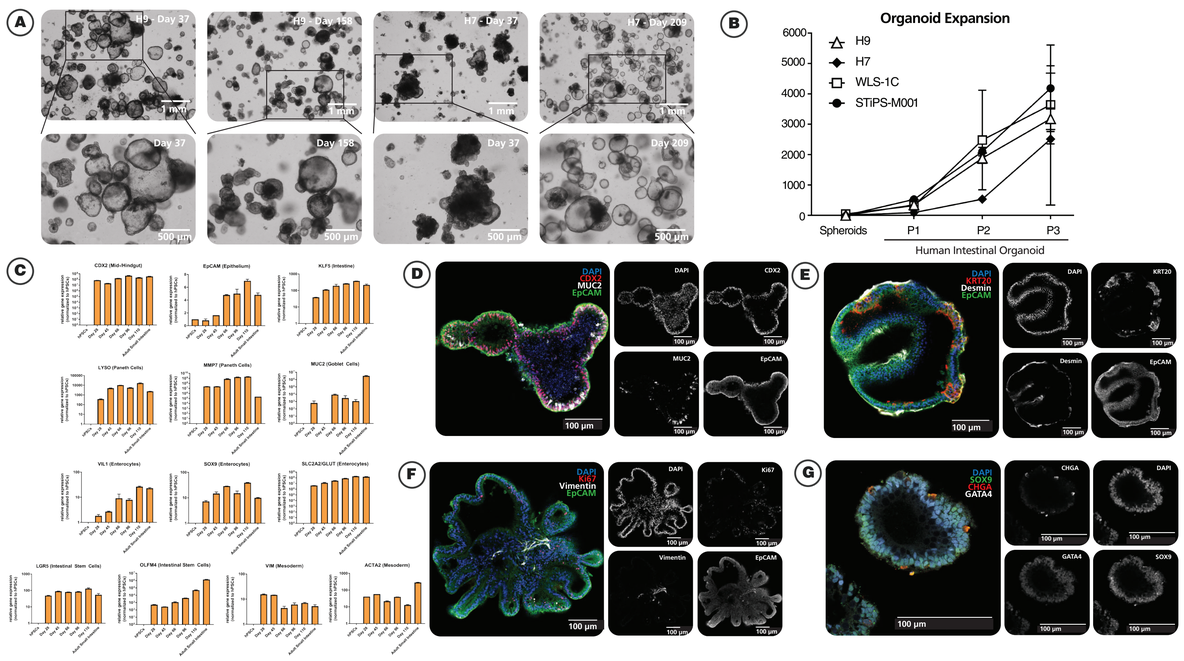


FIGURE 7. hPSC-Derived HIOs Expand Rapidly and Express Key Intestinal and Mesenchymal Markers
(A) hPSC-derived HIO cultures exhibit phenotypes of thick walled, polarized epithelial architecture with budding formations and cystic organoid morphology of thin walled epithelium surrounding a central, hollow lumen. Representative images from H9 and H7 ES cells are shown after 37 and ≥158 days of differentiation using STEMdiff™ Intestinal Organoid Kit. **(B)** HIOs derived from multiple human ES (H9 and H7) and iPS (WLS-1C and STiPS-M001) cell lines undergo rapid expansion when maintained in STEMdiff™ Intestinal Organoid Growth Medium (mean ± SD, n = 3). **(C)** HIOs at 28-110 days of differentiation expressed key intestinal epithelial and mesenchymal markers, as assessed by quantitative PCR. A commercial available mRNA sample of human adult small intestine was used as positive control. All samples were compared to undifferentiated hPSCs to calculate the relative gene expression (mean ± SD, n = 1). **(D-G)** Fluorescent immunohistochemistry was performed at day 193 of differentiation using H9 ES cells. HIOs express intestinal, goblet cell and enteroendocrine-specific markers CDX2, MUC2, EpCAM, KRT20, GATA4, SOX9, CHGA and mesenchymal markers desmin and vimentin. The presence of Ki67 confirms cell proliferation and presence of putative intestinal stem cells, which enable long-term organoid culture. HIOs maintained for >350 days were recently subjected to cryopreservation and successfully recovered (n = 3) and continued to grow in STEMdiff™ Intestinal OGM after 1 month in cryostasis.

Summary

- STEMdiff™ Intestinal Organoid Kit consists of a serum-free, media formulation for efficient and reproducible generation of human intestinal organoids within 30 days of differentiation
- HIOs generated with STEMdiff™ Intestinal Organoid Kit are composed of intestinal epithelium and a surrounding mesenchymal layer
- STEMdiff™ Intestinal Organoid Kit promotes successful generation of HIOs across multiple human ES and iPS cell lines compared to other published protocols
- PSC-derived HIOs can be expanded and maintained long-term (>11 month, n = 3) using STEMdiff™ Intestinal OGM and successfully subjected to cryopreservation