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A COMPREHENSIVE CULTURE SYSTEM RECAPITULATING *IN VIVO* LIVER DEVELOPMENT

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ABSTRACT

Human embryonic stem cells (hESCs) hold a potential as an unlimited source to generate hepatocytes to be used in cell therapy and toxicity screening. However, a more rigorous understanding of the instructive signals that govern the hESCs into the hepatocyte lineage is in demand. We herein have studied some of the approaches that improve the *in vitro* hepatic protocols that are ultimately aimed at obtaining functional hepatocytes. Differentiation of definitive endoderm (DE) is a pre-requisite for its derivative organ, the liver. Therefore, signalling governing DE fate has been the focus of the research in this field. In this study, we have demonstrated that suppression of PI3K singling pathway enhances the Activin-induced DE differentiation via mesendoderm formation in chemically defined conditions, hence improving hepatocyte generation from enriched DE. On the other hand, achieving functional maturation of hepatocytes remains one of the key challenges for hESC–derived hepatocytes, as they lose their *in vivo* niche when growing in monolayer. Thus, recreating the 3-dimensional (3D) niche *in vitro* is the key step needed to improve the maturation of these cells. Our findings underscore the feasibility of 3D-induced hepatic function, and reveal the behaviour of hepatocyte differentiation of hESCs in 3D culture and using more controlled differentiation stages than in previous work by embryoid body formation. Thus, we anticipate that these findings will bring us closer to the ultimate goal of efficient generation of hepatocytes from hESCs for both clinical and biomedical applications.

1.0 Introduction

Human embryonic stem cells (hESCs) possess 2 unique properties (1) pluripotency and (2) self-renewal, and therefore, hold great promise for biomedical application and regenerative medicine. *In vitro* differentiation of hESCs is a vital tool to generate unlimited human hepatocytes. To date, several multi-step protocols have been established to generate hepatocyte-like cells from undifferentiated hESCs via definitive endoderm (DE) formation. However, hESC derived-hepatocytes in these systems exhibit some immature characteristics, thus it remains a challenge on how to further improve hepatic differentiation. In addition, the molecular mechanisms regulating the differentiation are still ambiguous, making *in vitro* differentiation a difficult task. We aim to improve the differentiation of hESCs to functional hepatocytes by employing 2 strategies; (1) modulating signalling pathways to control and enhance DE and developing a 3-dimensional (3D) culture system to improve the functionality of hESC-derived hepatocytes.

2.0 Results

We have found that suppression of the PI3K signalling using the LY 294002 inhibitor (LY) during hESC differentiation significantly improves Activin A (AA)-induced DE generation, which subsequently augments hepatocyte production. Further mechanistic interrogation of this

phenomenon has revealed that dual treatment of hESCs with AALY enhances the Activin downstream signalling, Smad2/3 phosphorylation and their nuclear translocation with Smad4.

Liver development *in vivo* is regulated by cell–cell contacts in a 3D environment and the absences of this may account for, at least partly, some of the immature features of hESC-derived hepatocytes. We found that the DE cell derived from hESCs cultured in alginate-based 3D culture system produced hepatocytes with higher functionality compared to of those on monolayer culture conditions as assessed by hepatic gene expression and functionality tests.

3.0 Discussion and Conclusion

These findings suggest that suppression of PI3K/Akt modulates Nodal/Activin, the most important signalling involved in mesendoderm and DE cell fate specification, therefore improved DE differentiation of hESCs. Subsequently, 3D culture microenvironment enhanced hepatic differentiation and functionality of hESCs-DE. Collectively, this study has demonstrated a significant cornerstone in the strategies to improve hepatic differentiation of hESCs by addressing the molecular signalling and micro-niche cues that govern hepatocyte lineage commitment. Hence, this will pave the way for the use of these hepatocytes in future regenerative therapies and biomedical applications.

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