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Hepatic differentiation of human pluripotent stem cells in miniaturized format suitable for high-throughput screen

Arnaud Carpentier *,[1], Ila Nimgaonkar, Virginia Chu, Yuchen Xia, Zongyi Hu, T. Jake Liang *

Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD 20892, USA

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ABSTRACT

The establishment of protocols to differentiate human pluripotent stem cells (hPSCs) including embryonic (ESC) and induced pluripotent (iPSC) stem cells into functional hepatocyte-like cells (HLCs) creates new opportunities to study liver metabolism, genetic diseases and infection of hepatotropic viruses (hepatitis B and C viruses) in the context of specific genetic background. While supporting efficient differentiation to HLCs, the published protocols are limited in terms of differentiation into fully mature hepatocytes and in a smaller-well format. This limitation handicaps the application of these cells to high-throughput assays. Here we describe a protocol allowing efficient and consistent hepatic differentiation of hPSCs in 384-well plates into functional hepatocyte-like cells, which remain differentiated for more than 3 weeks. This protocol affords the unique opportunity to miniaturize the hPSC-based differentiation technology and facilitates screening for molecules in modulating liver differentiation, metabolism, genetic network, and response to infection or other external stimuli.

1. Introduction

Hepatic differentiation of patient-derived induced pluripotent stem cells (iPSCs) constitutes a unique *in vitro* approach to analyze hepatic biology in the context of the genetic background of the patient, and could also hold the key to the large production of hepatocytes for autologous transplantation. Several protocols of hepatic differentiation of human hPSCs have been described recently (Basma et al., 2009; Si Tayeb et al., 2010; Sullivan et al., 2010). They rely on a multi-step process in which the treated cells go through a definitive endoderm stage induced by treatment of colony-type culture of hiPSC with Activin A, followed by hepatic specification to commit the cells toward the hepatic lineage. Finally cells are matured *in vitro* to obtain albumin-positive cells, which structurally look like mature hepatocytes. Functional assays demonstrate that these hepatocytes-like cells (HLCs) also secrete albumin, metabolize urea, recapitulate lipid metabolism, and express various isoforms of the cytochrome *p*450.

These differentiated HLCs retain a strong fetal and immature phenotype, as demonstrated by the persistence of alpha-fetoprotein (AFP) and diminished hepatic functions when compared to primary adult hepatocytes (PHHs). Strategies to improve the differentiation protocol are being explored. For example, treatment of differentiated cells with small-molecule compounds may hold the key to improving hepatic maturation or functionality (Shan et al., 2013). However, in order to screen for small molecules, the differentiation protocol needs to be miniaturized to 384-well plates.

Thus far, the efficient differentiation of hPSCs has relied on a preliminary colony-type culture of hPSCs, directly treated to produce hepatocytes without passage at any stage of the hepatic differentiation (Hannan et al., 2013; Mallanna and Duncan, 2013). Using this approach, efficient differentiation of hPSC-derived HLCs is usually limited to 6-, 12- or 24-well plates, curtailing the usefulness of this approach in generating cell populations suitable for high-throughput assay (Reviewed in Schwartz et al., 2014). Some published protocols suggested passing definitive endoderm (DE) cells (Hay et al., 2008; Agarwal et al., 2008; Liu et al., 2010; Duan et al., 2010), to allow a more homogeneous and flatter cell population (Inamura et al., 2011), but none of them took advantage of this approach to miniaturize the format of plates. Furthermore the HLCs tend to lose their differentiated phenotype with time without special culturing condition (Khetani and Bhatia, 2008). Here we describe a protocol allowing efficient and consistent differentiation of hESC and hiPSC into functional hepatocyte-like cells in 96- and 384-well plates. We also describe a new HLC culturing condition, allowing maintenance of SC-derived HLCs for more than 3 weeks in these high-throughput formats, with a gradual improvement of their hepatic phenotype and functions.

2. Results

2.1. Limitations of current differentiation protocol

Our previously published protocol (Carpentier et al., 2014), adapted from Basma et al. (Basma et al., 2009) described efficient differentiation

* Corresponding authors.
E-mail addresses: arnaud.carpentier@twincore.de (A. Carpentier), jakel@bdg10.niddk.nih.gov (T.J. Liang).
[1] Current address: Twincore, Institute for Experimental Virology, Hannover, Germany.

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of hESCs or hiPSCs into HLCs in 6 or 12-well plates. This protocol allowed us to obtain HLCs with up to 80% of hepatic markers (AFP, ALB and/or AAT)-positive cells, but exhibited several limitations in terms of homogeneity of the HLCs population and miniaturization to smaller-well format (Fig. 1). First hPSCs were routinely maintained in a colony-type culture and passaged in aggregates. The definitive endoderm (DE) induction was thus performed on 70–80% confluent large SC colonies treated with 100 ng/ml of Activin A and bFGF in the presence of increasing concentrations of FCS (Basma et al., 2009). While allowing up to 60–80% of cells expressing SOX17 and FOXA2 after 3 days of treatment in the best case, this protocol was hampered by a high variability in efficiency of DE induction, because of variability within the lots of Activin A. Moreover, it usually led to a non-homogeneous induction of SOX17/FOXA2-positive DE cells. Importantly, OCT4-positive cells within the DE cell population were still observed at the end of the DE induction stage.

Before inducing hepatic specification, we reported that it is preferable to pass the DE cells. Indeed in our hands, direct hepatic specification on confluent DE cells led to overcrowded cell population affecting both the maturation and visualization of the differentiated cells. When passaged, our DE cells exhibited the same limitations as colony-type hPSCs and could not be re-seeded at a single cell level. To allow reproducible and consistent survival of the cells, DE cells had to be passed in a similar way to colony-type hPSCs: re-suspending and re-plating aggregates of approximately 20–50 DE aggregated cells. Treatment with high concentration of HGF and DMSO then induced proliferation of

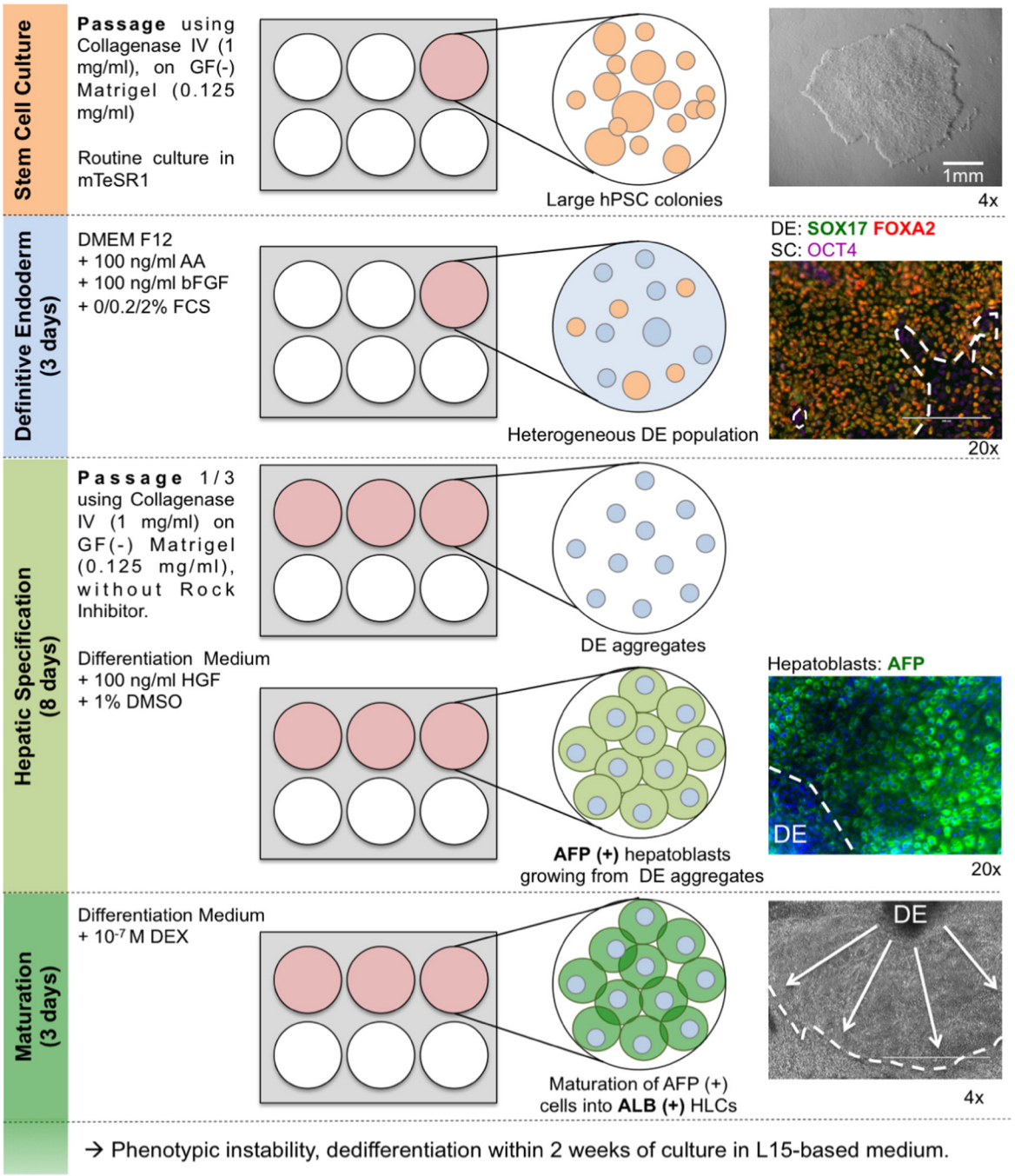

Fig. 1. Detailed previous protocol with its main limitations. Colony-type cultures of hPSCs were differentiated into definitive endoderm population. DE cells were then passed in aggregates, seeded on Matrigel, before undergoing hepatic specification and maturation. One well of a 6-well plate of hPSCs would usually produces 3 wells of a 6-well plate of heterogeneous AFP(+) ALB(±) HLCs growing around DE-derived aggregates. Our detailed improved protocol can be found in Fig. 7.