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Hepatocyte-like cells derived from human embryonic stem cells specifically via definitive endoderm and a progenitor stage

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ABSTRACT

Human embryonic stem cells offer a potential unlimited supply for functional hepatocytes, since they can differentiate into hepatocyte-like cells displaying a characteristic hepatic morphology and expressing various hepatic markers. These cells could be used in various applications such as studies of drug metabolism and hepatotoxicity, which however, would require a significant expression of drug metabolizing enzymes. To derive these cells we use a stepwise differentiation protocol where growth- and maturation factors are added. The first phase involves the formation of definitive endoderm. Next, these cells are treated with factors known to promote the induction and proliferation towards hepatic progenitor cell types. In the last phase the cells are terminally differentiated and maturated into functional hepatocyte-like cells. The cultures were characterized by analysis of endodermal or hepatic markers and compared to cultures derived without induction via definitive endoderm. Hepatic functions such as urea secretion, glycogen storage, indocyanine green uptake and secretion, and cytochrome P450-expression and activity were evaluated.

The DE-Hep showed a hepatocyte morphology with sub-organized cells and exhibited many liver-functions including transporter activity and capacity to metabolize drugs specific for important cytochrome P450 sub-families. This represents an important step in differentiation of hESC into functional hepatocytes.

1. Introduction

Pluripotent human stem cells are expected to change the accessibility to a variety of human cell types. The possibility to propagate pluripotent human embryonic stem cells (hESC) and subsequently differentiate them into the desired target cell types will provide a stable and virtually unlimited supply of cells for a range of applications *in vivo* and *in vitro*.

The liver is the major organ for metabolism and detoxification in the human body, and therefore huge efforts have been undertaken in order to identify a reliable source of functional hepatocyte-like cell types for *in vitro* testing. Up to now the complexity and function of the liver are not reflected by any available *in vitro* cell type or cellular system. The availability of human primary liver cells is limited and furthermore these cells lose their normal phenotype and functional properties (typically within 24 h) when used *in vitro*. Transformed hepatic cell lines contain very low levels of drug metabolizing enzymes and have expression profiles of other important proteins substantially different from the native hepatocyte *in vivo*. Thus, many tests e.g. drug metabolism and toxicity are still performed using animal material, even though liver metabolism is known to be species-specific. Due to this, difficulties to predict liver toxicity in another species than the one tested remain present. Therefore early prediction of human liver toxicity liabilities is of principal importance when selecting compounds to enter clinical trials. Accordingly there is an urgent need for a model system that mimics human liver cells and that is able to predict effects of candidate molecules in the development of new drugs or chemicals. Regarding both availability and physiological relevance human pluripotent stem cells may serve as an ideal renewable source of functional human hepatocytes. When hESC have been placed in a proper environment, certain hepatic characteristics have been observed 2–4 weeks after differentiation. The present investigation is based on the fact that definitive endoderm (DE) cells give rise to endodermal organs and consequently to e.g. hepatic cell types. Early endoderm development is not well understood. Fate

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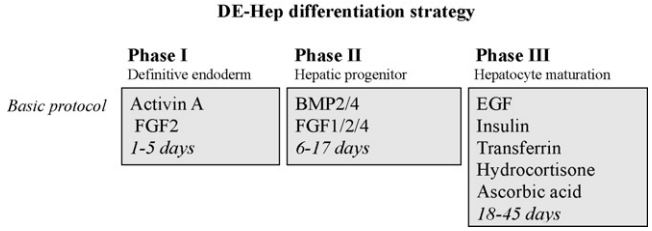

Fig. 1. Schematic overview of the differentiation strategy into definitive endoderm derived hepatocytes (DE-Hep). The differentiation protocol is divided into three main phases. The starting material consists of undifferentiated hESC cultured on MEF. In phase I the hESC are induced into definitive endoderm (DE). In phase II the DE is induced to early liver endoderm or liver progenitor cells. During phase III the DE-Hep cultures are maturated.

mapping studies of cultured mouse embryos (Lawson et al., 1986, 1991; Lawson and Pedersen, 1987) have revealed that DE begins to form at the embryonic days 6–6.5 (E6–6.5) and that by the end of gastrulation (E7.5), some labeled cells only give rise to endodermal derivatives. Other fate mapping studies (Lawson et al., 1991; Tremblay and Zaret, 2005) suggested that the first endodermal cells that migrate through the primitive streak (PS) at E6.5 are fated to become liver, ventral pancreas, lungs and stomach.

A complication in the study of endoderm is that mammals generate extraembryonic endoderm (ExE). ExE arises at the blastocyst stage and eventually forms two subpopulations: visceral endoderm and parietal endoderm. These cells share the expression of many genes with DE (cells that give rise to the endodermal organs), including the transcription factors Sox17 (Kanai-Azuma et al., 2002), HNF3β and HNF4α (Belo et al., 1997; Sasaki and Hogan, 1993). D'Amour et al. (2005, 2006) have developed a protocol for deriving DE from hESC. Previous studies have identified cells with some hepatocyte-like characteristics, e.g. cytochrome P450 (CYP) activity and ability to store glycogen, in differentiated hESC cultures (Rambhatla et al., 2003; Cai et al., 2007; Duan et al., 2007; Agarwal et al., 2008). So far the cells generated have not shown the metabolic qualities necessary for potentially replacing traditional liver systems in terms of drug transporter expression and specific CYP expression patterns needed for industrial applications.

In this investigation several cell lines have been induced by growth factors to generate hepatocyte-like cells via definitive endoderm (DE-Hep). The cells were subsequently cultured under conditions known to support the development of liver cell lineages (Hamazaki et al., 2001; Hamazaki and Terada, 2003). Our strategy involves three distinct important steps in liver development. The study shows that the hepatocyte-like cell population obtained via DE has many similarities with human adult hepatocytes, including capacity to metabolize several specific pharmaceutical compounds.

2. Methods

2.1. Culture of hESC

The hESC lines SA001, SA002, SA002.5 and SA167 (Cellartis AB, Göteborg, Sweden, http://www.cellartis.com) were derived, cultured and characterized as previously described (Heins et al., 2004, 2006).

2.2. Culture of DE-Hep

Differentiation of hESC into DE-Hep cells was carried out as described in Fig. 1. Before initiating differentiation, hESC were washed in PBS+/+ (Invitrogen/Gibco). During differentiation into DE (phase I) the fetal calf serum concentration was 0% the first day and 0.2% for the following days. During the first day of differentiation into DE recombinant human Activin A (100 ng/ml, R&D) was supplemented to the medium, the following days of DE differentiation (phase I) Activin A (100 ng/ml) and sometimes FGF2 (4 ng/ml) or Wnt3a (50 ng/ml) was added to the medium. Phase II was carried out either in RPMI or DMEM (both from Invitrogen/Gibco). Different growth factors were added to the media. In phase II, RPMI advanced medium supplemented with PEST (1%) (Invitrogen/Gibco) and Glutamax (1%) (Invitrogen–Sigma) was used as a basic medium. BMP4 (100 ng/ml, PromoKine) and FGF2 (4 ng/ml) were added to the medium to differentiate the DE into hepatic progenitors. In some experiments in phase II the medium was supplemented with FGF1 (100 ng/ml, PromoKine), FGF2 (5 ng/ml, R&D), BMP2 (50 ng/ml, PromoKine), BMP4 (200 ng/ml) and 0.2% FBS (Gibco), with medium change every second day. At phase III hepatocyte maturation was carried out in HCM (Cambrex) with dexamethasone (Dex) (0.1 μM, Calbiochem), oncostatin M (OSM) (10 ng/ml, PromoKine), HGF (20 ng/ml, PromoKine) and SingleQuots (Lonza). The medium was changed every second day.

2.3. Culture of controls and tissue material

As controls we used intrinsic differentiated hESC as well as mouse embryonic feeder cells (MEF), HepG2 cells, human primary hepatocytes, and human liver samples. Intrinsic differentiated hepatocyte-like cells that we use as control in this study were derived according to Söderdahl et al. (2007) and Ek et al. (2007). hESC were allowed to differentiate for 18–45 days in VitroHES™ supplemented with 4 ng/ml FGF2. MEFs were derived and cultured as previously described (Hogan et al., 1994). HepG2 cells (HB-8065, American Type Culture Collection) were cultured as previously described (Butura et al., 2004). HepG2 cells (HB-8065, American Type Culture Collection, Manassas, VA) were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin–streptomycin, 1% sodium pyruvate and 1% non-essential amino acids (all from Invitrogen) and collected by trypsinization when 80–90% confluence were reached. Platable cryopreserved human primary hepatocytes (In Vitro Technologies) were cultured according to the manufacturer instructions. In short, hepatocytes were thawed at 37 °C and propagated at 0.3 million cells per ml and well in *InVitroGRO CP* medium (In Vitro Technologies) at collagen I-coated 24-well plates. After 24–48 h *in vitro* the hepatocytes were fixed and subjected to immunofluorescence stainings. After 24–120 h *in vitro* the hepatocytes were harvested for activity assays and mRNA. All cultures were kept at 37 °C, 5% CO_2 and 90–95% humidity. Human liver samples were obtained from Sahlgrenska Hospital (Göteborg, Sweden) and originated from patients undergoing liver resection. All tissues were obtained through qualified medical staff, with donor consent and with the approval of the Local Ethics Committee at Sahlgrenska Hospital.

2.4. Immunocytochemical methods and antibodies

Cells in culture were fixed in 4% (w/v) paraformaldehyde (PFA) for 15 min, and washed in PBS. For visualizing extracellular proteins cells were blocked for 30 min in 5% FBS in PBS, for intracellular protein staining cells were blocked and permeabilized for 30 min in 5% FBS in 0.1% TritonX-100 in PBS. The primary antibodies were incubated in 1% FBS in PBS overnight at 4 °C and the secondary antibodies were diluted in 1% FBS in PBS for 1 h at room temperature (RT). For CYP-stainings the cells were incubated with secondary antibodies in PBS for 3 h at RT. All washes were performed in PBS. To visualize the nucleus cells were incubated with DAPI at 0.05 mg/ml for 5 min at RT and mounted in DAKO Cytomation mounting medium. Primary antibodies used were rabbit anti-albumin (1:500, DAKO Cytomation), rabbit anti-α_1-antitrypsin (1:200, DAKO Cytomation), mouse IgG2a αFP (1:500,