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Hypoxia efficiently induces differentiation of mouse embryonic stem cells into endodermal and hepatic progenitor cells

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ABSTRACT

Although ES cells have potential impact on cell-based therapy for liver diseases, their efficient differentiation into functional hepatocytes remains difficult. One possible approach to this issue is to control the culture environment to recapitulate the *in vivo* liver development. Given that the embryo is exposed to hypoxic condition in its early stage, hypoxic condition seems suitable for inducing early differentiation of ES cells. However, no studies have evaluated the effect of hypoxia on endodermal or early hepatic differentiation of ES cells. Here we investigated the potential role of hypoxia as a regulatory factor in endodermal and subsequent early hepatic differentiation of mouse ES cells. Under the stimulus of retinoic acid, a typical endodermal morphology emerged more dominantly under hypoxia (5% O_2) than normoxia. Accordingly, quantitative RT-PCR revealed significant increase in Foxa2 and GATA4, essential transcription factors for endoderm specification. In addition, hypoxia amplified the effects of hepatic induction factors FGF1, FGF4 and HGF, which was evidenced by dramatically increased ALB-GFP-positive cell fraction, as well as increased gene expression levels of ALB, AFP, and CK18. Furthermore, the hepatic progenitor cells induced under hypoxic condition were efficiently differentiated into mature hepatocytes with ALB secretion ability. We also found that the differentiating cells did not consume oxygen even under normoxia, suggesting that oxygen *per se* might have a negative effect on the normal endodermal and early hepatic differentiation of ES cells. These results will provide useful insight into efficient production of hepatic progenitor cells, and contribute to stem cell-based liver tissue engineering.

1. Introduction

An alternative cell source for adult hepatocytes has been essential for various applications in medicine. The only effective treatment for end-stage liver diseases is liver transplantation, but transplantation is limited due to donor shortage. Many alternative therapies have been developed and studied, including hepatocyte transplantation [1], extracorporeal bioartificial liver support devices [2], and heterotopic transplantation of engineered liver tissue [3]. In these studies, mature hepatocytes have been the main cell source. However, the use of a large amount of mature hepatocytes is constrained by availability in a clinical setting. Thus, many researchers have attempted to obtain functional hepatocytes from pluripotent stem cells including embryonic stem (ES) cells and induced pluripotent stem cells [4].

Although ES cells provide a valuable source of adult differentiated cells, their efficient differentiation into a specific cell lineage, especially to endoderm, has proven difficult to achieve [4,5]. To provide a source for cell-based therapy in liver diseases, a number of studies have attempted to generate ES cell-derived hepatocytes [4,5]. All these studies have used a temporal series of growth factor manipulations to mimic embryonic liver development. Although there have been tremendous improvements in this approach, the yield of obtained functional hepatocytes are still low. This is in part due to the low efficiency of the initial differentiation of ES cells into endodermal lineage. Thus, development of an efficient method for differentiating ES cells into endodermal lineage is strongly required.

One possible strategy to improve the differentiation efficiency is regulation of culture environment such as oxygen supply. During

Abbreviations: AFP, α-fetoprotein; ALB, albumin; CK18, cytokeratin 18; DAPI, 4′,6-diamidino-2-phenylindole; ES, embryonic stem; FGF, fibroblast growth factor; Foxa2, forkhead box A2; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; LIF, leukemia inhibitory factor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RA, retinoic acid.

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the early post-implantation period, embryos survive in a relatively anaerobic environment *in utero* and are vulnerable to a high oxygen tension [6]. Therefore, it is common in embryo culture to supply culture media with antioxidant compounds [7]. Furthermore, it has been revealed that ES cells occupy hypoxic niches and low oxygen regulates their differentiation [8]. These facts imply that oxygen tension is likely an important determinant of normal development. In fact, there are evidences indicating that physiological low oxygen tension induces early differentiation of mouse ES cells [9–11]. These evidences suggest that hypoxia provides a physiologically suitable environment that allows efficient differentiation of ES cells to specific lineages under a stimulation of appropriate combination of soluble factors. Regarding hepatic differentiation, however, there are no reports that have investigated the effect of hypoxic culture on differentiation efficiency of ES cells into hepatic progenitor cells.

In this study, we evaluated the potential role of hypoxia as a regulatory factor in endoderm differentiation of mouse ES cells and subsequent hepatic specification under appropriate stimuli of soluble factors.

2. Materials and methods

2.1. Culture of mouse ES cells

pALB-EGFP/ES cells, a J1 cell clone of 129X1/SvJ male origin [12], were cultured on STO feeder cells with leukemia inhibitory factor (LIF) (ESGRO; Millipore, Billerica, MA) (+) medium to maintain pluripotency [13]. Prior to endoderm induction, undifferentiated trypsinized ES cells were passaged twice onto STO-free gelatin-coated plates to remove STO cells.

2.2. Endoderm induction of ES cells and subsequent early hepatic differentiation

To initiate endoderm differentiation, trypsinized ES cells were plated onto 60-mm gelatin-coated dishes at 2.5×10^4 cells/plate, and treated for 3 days with ES cell culture medium containing reduced LIF (100 units/mL) and 10^{-7} M all-trans-retinoic acid (RA) (Sigma–Aldrich, St. Louis, MO) (Step 1). Hypoxic cultures were maintained at 5% O_2 in an O_2–CO_2 incubator (Hirasawa, Tokyo, Japan). The culture medium was changed every day.

On day 3 of Step 1, the cells were shifted to differentiation process toward hepatic cells under hypoxia or normoxia (Step 2). Trypsinized cells were plated onto gelatin-coated 6 well plates (IWAKI, Chiba, Japan) or gelatin-coated 35 mm glass-base dishes (IWAKI) at 2×10^4 cells/well (or dish), and treated for 7 days with ES cell culture medium containing 100 ng/mL fibroblast growth factor (FGF)1, 20 ng/mL FGF4 and 50 ng/mL hepatocyte growth factor (HGF) (PeproTech EC, London, UK). The culture medium was changed every day. Differentiated hepatic cells were identified as green fluorescent protein (GFP)-positive cells using confocal laser scanning microscopy (FV10i; Olympus, Tokyo, Japan). Hepatic differentiation efficiency for each observed colony was quantified as the ratio of GFP-positive area/total colony area using ImageJ software.

2.3. Maturation of hepatic progenitor cells

On day 7 of Step 2, the cells were passaged onto collagen I-coated 6 well plates at 2×10^5 cells/well. Then, the cells were cultured under normoxia for 5 days with ES cell culture medium containing 10 ng/mL oncostatin M (Wako, Osaka, Japan) and 10^{-6} M dexamethasone (Sigma–Aldrich), and the medium was changed every day (Step 3). Subsequently, the medium was changed to hepatocyte culture medium (HCM; Lonza, Walkersville, MD) containing 10^{-7} M dexamethasone, and cultured for 10 days (Step 4). The medium was changed every other day. The culture groups in which the cells were cultured under hypoxia and normoxia until Step 2 are denoted as "Hx → Nx" and "Nx → Nx", respectively.

2.4. Gene expression analysis

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) on day 3 of Step 1 and on day 7 of Step 2. Reverse transcription was performed with Superscript-III First-Strand Synthesis System using oligo(dT) primers (Invitrogen). Real-time quantitative polymerase chain reaction (qPCR) was performed using Applied Biosystems 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA). Reactions were carried out with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). The gene-specific primers are listed in Table 1. Each target gene was normalized to β-actin, and relative mRNA levels were then compared to normoxic samples in each experimental run. In albumin (ALB) gene expression analysis, we defined that the gene expression levels of non-detected samples as zero.

2.5. Immunocytochemistry

The cells on day 5 of Step 2 under hypoxia and normoxia were fixed in 4% paraformaldehyde (Wako). The samples were heated in 1:200 diluted ImmunoSaver (Nissin EM, Tokyo, Japan) at 75 °C for 15 min, and then treated with 0.2% Triton-X 100 for 15 min to permeabilize the cell membranes. After blocking with Blocking One solution (Nacalai Tesque, Kyoto, Japan), the cells were incubated with goat anti-AFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 for 1 h at room temperature. Thereafter, the cells were incubated with Alexa Fluor 594-conjugated donkey anti-goat IgG antibody (Invitrogen) at 1:500 for 1 h at room temperature. Nuclei were counterstained with Hoechst33342 (Dojindo, Kumamoto, Japan).

Table 1
Primers for qRT-PCR.

Gene symbol	Forward primer	Reverse primer
Foxa2	5′-CGAGAACGGCTGCTACCTG-3′	5′-GGTCTTCTTGCCTCCGCTAC-3′
GATA4	5′-TGTCCCAGACATTCAGTAC-3′	5′-GATTACGCGGTGATTATGTC-3′
GATA6	5′-TAGAAATGCTGAGGGTGAG-3′	5′-GAGGAAGTAGGAGTCATAGG-3′
AFP	5′-CTCAGCGAGGAGAAATGGTC-3′	5′-GGTGATGCATAGCCTCCTGT-3′
ALB	5′-AAGCTGAGACCTTCACCTTC-3′	5′-CAGCAGCCTTGCAACATGTA-3′
CK18	5′-CTTGCTGGAGGATGGAGAAG-3′	5′-GCCTCAGTGCCTCAGAACTC-3′
Oct3/4	5′-AGAACCTTCAGGAGATATGC-3′	5′-TCTTCTCGTTGGGAATACTC-3′
Sox2	5′-ACAAGGAAGGAGTTTATTCG-3′	5′-TTACCAACGATATCAACCTG-3′
HIF-1α	5′-GAATGGAACGGAGCAAAAGA-3′	5′-CTGCCTTGTATGGGAGCATT-3′
β-actin	5′-TCACCCACACTGTGCCCATCTACGA-3′	5′-CAGCGGAACCGCTCATTGCCAATGG-3′