Biochemical and Biophysical Research Communications 401 (2010) 480–486

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Chemical inhibition of sulfation accelerates neural differentiation of mouse embryonic stem cells and human induced pluripotent stem cells

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ARTICLE INFO

Article history:
Received 12 September 2010
Available online 25 September 2010

Keywords:
Chlorate
Sulfation
Neural differentiation
Mouse embryonic stem cell
Human induced pluripotent stem cell

ABSTRACT

Pluripotency of embryonic stem cells (ESCs) is maintained by the balancing of several signaling pathways, such as Wnt, BMP, and FGF, and differentiation of ESCs into a specific lineage is induced by the disruption of this balance. Sulfated glycans are considered to play important roles in lineage choice of ESC differentiation by regulating several signalings. We examined whether reduction of sulfation by treatment with the chemical inhibitor chlorate can affect differentiation of ESCs. Chlorate treatment inhibited mesodermal differentiation of mouse ESCs, and then induced ectodermal differentiation and accelerated further neural differentiation. This could be explained by the finding that several signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) or inhibition of neural differentiation (Wnt and BMP) were inhibited in chlorate-treated embryoid bodies, presumably due to reduced sulfation on heparan sulfate and chondroitin sulfate. Furthermore, neural differentiation of human induced pluripotent stem cells (hiPSCs) was also accelerated by chlorate treatment. We propose that chlorate could be used to induce efficient neural differentiation of hiPSCs instead of specific signaling inhibitors, such as Noggin.

1. Introduction

Embryonic stem cells (ESCs) [1–3] are promising tools for biotechnology and possess key features that should allow their exploitation in the development of cell replacement therapies [4]. Extrinsic signaling pathways are key mechanisms for determining ESC cell fate, and sulfated glycans, such as heparan sulfate (HS), are well known regulators of signal transduction [5]. HS chains are present abundantly on the cell surface of undifferentiated mouse ESCs (mESCs) and functional roles of HS chains have been demonstrated [6,7]. Thus, the modification of sulfated glycans is an attractive approach for developing methods to regulate ESC differentiation.

Sulfated glycans are synthesized in the Golgi as follows. The high energy sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which is required as a substrate for sulfation, is synthesized in the cytosol and nucleus by PAPS synthetase [8], and subsequently is translocated via the PAPS transporter (PAPST) into the Golgi [9–13], where it is used by sulfotransferases to sulfate glycans. Recently, we have demonstrated that the reduction of sulfation by knockdown of *PAPST* using vector-based RNA interference (RNAi) promotes neurogenesis of mESCs [13]. However, the rapid, simple, and safety method for modification of sulfated glycans instead of gene transfer is desired particularly for application of human ESCs (hESCs) to regenerative medicine.

Chlorate inhibits ATP sulfurylase activity of PAPS synthetases by competing with sulfate ions in binding to ATP-sulfurylase resulting in reduction of PAPS [14]. Thus, inhibition of PAPS synthesis by chlorate leads to reduction of sulfation on several sulfated proteins, glycoproteins, glycolipids, and proteoglycans [15,16].

Since induced pluripotent stem cells (iPSCs) were reported [17], the application of human iPSCs (hiPSCs) to regenerative medicine has been expected. In particular, neural induction of hiPSCs is an important research tool for several neural diseases and has been applied to cell replacement therapies. Therefore, methods of efficient and rapid neural induction are required [18]. In this study, we examined whether neural differentiation of hiPSCs in addition to mESCs was enhanced by the chemical down-regulation of sulfation with chlorate.

2. Materials and methods

2.1. Cell culture

R1 [19] and E14TG2a [20] mESC lines were maintained on mouse embryonic fibroblasts (MEFs) inactivated with 10 μg/ml

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doi:10.1016/j.bbrc.2010.09.085

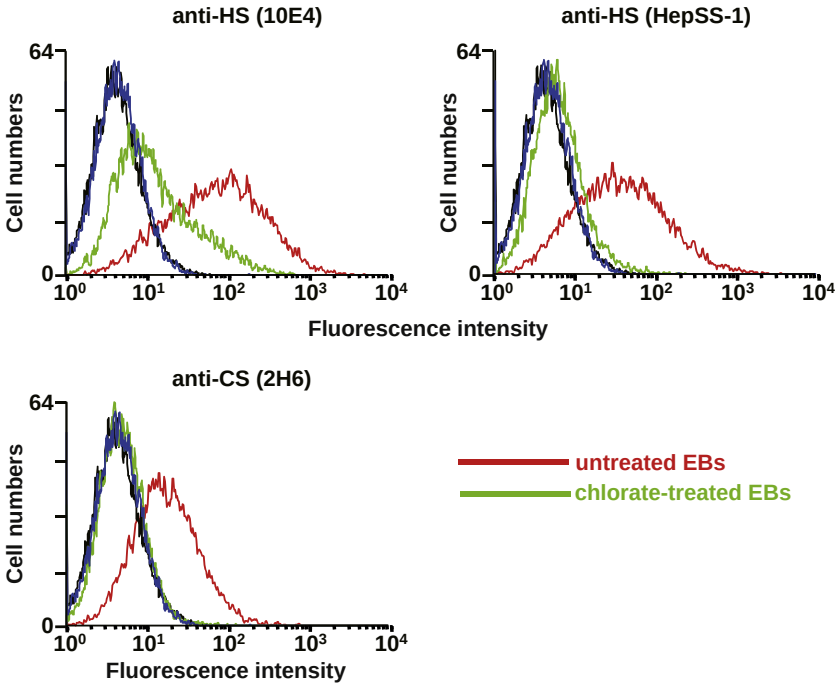

Fig. 1. Chlorate treatment reduces sulfation of HS and CS in EBs. FACS analysis of cells 3 days after EB formation using an anti-HS antibody (10E4 or HepSS-1) or an anti-CS antibody (2H6) (black and blue lines represent the IgM isotype control for untreated and chlorate-treated EBs, respectively). Chlorate treatment was performed for 24 h from 2 days after EB formation. Three independent experiments were performed and representative results are shown.

mitomycin C (Sigma) in ESC medium (DMEM supplemented with 15% FBS {Hyclone}, 1% penicillin/streptomycin {Gibco}, 0.1 mM 2-mercaptoethanol {Gibco}, and 0.1 mM non-essential amino acids {Gibco}) with 1000 U/ml LIF (Chemicon). hiPSC clones (MRC-hiPS_Fetch {NIHS0604} and MRC-hiPS_Tic {JCRB1331}) [21] were maintained on inactivated MEFs in iPSellon (Cell-Sight) with 10 ng/ml bFGF (Wako). All mESC experiments were performed using the R1 line and most results were confirmed using the E14TG2a line.

For embryoid body (EB) formation, mESCs or hiPSCs were transferred to low cell binding 60 mm dishes (Nunc) and cultured in ESC medium without LIF or iPSellon without bFGF, respectively. Before EB formation, hiPSCs were preplated on gelatin-coated dishes to remove feeder cells. For neuronal differentiation, 1 μM all-trans retinoic acid (RA) (Sigma) was added 4 days after EB formation. Then, 5 days after EB formation, EBs were plated onto PDL/laminin-coated 60 mm dishes (Becton Dickinson) in DMEM-F12 containing N2 supplement (Gibco).

To down-regulate sulfation, 50 mM chlorate (Sigma) was added from 2 days after EB formation throughout EB culture.

2.2. FACS analysis

FACS analysis was performed 3 days after EB formation. After EDTA treatment, the cell suspension was incubated with primary antibodies diluted in FACS buffer (0.5% bovine serum albumin and 0.1% sodium azide in PBS). After washing, the cell suspension was incubated with FITC-conjugated secondary antibody (Sigma) diluted in FACS buffer. Cell sorting and analysis were performed using a FACSAria Cell Sorter (Becton Dickinson). Primary antibodies were as follows: mouse IgM isotype control (Chemicon), the anti-HS antibody 10E4 (Seikagaku Corp.), the anti-HS antibody HepSS-1 (Seikagaku Corp.), the anti-chondroitin sulfate (CS) antibody 2H6 (Seikagaku Corp.).

2.3. Immunoblotting

Cells were lysed with lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitors). Isolation of nuclear extracts was performed as described previously [6]. Samples containing 5 μg of cell lysate or nuclear extract were separated by 10% SDS–PAGE and transferred onto PVDF membranes (Millipore). After blocking, the membranes were incubated with primary antibodies as follows: anti-ERK1/2 (Cell Signaling Technology), anti-phosphorylated ERK1/2 (Thr183/185; Cell Signaling Technology), anti-phosphorylated Smad1 (Ser463/465; Cell Signaling Technology), anti-β-actin (Sigma), anti-β-catenin (Cell Signaling Technology), anti-lamin B_1 (Zymed), and anti-βIII-tubulin (Chemicon). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology), washed and developed with ECL Plus reagents (GE Healthcare).

2.4. Immunostaining

After neural differentiation on PLL/laminin-coated glass chamber slides (Iwaki), cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. After washing and subsequent blocking, cells were stained with an anti-βIII-Tubulin antibody. After washing, cells were stained with an FITC-conjugated secondary antibody and counterstained with propidium iodide (PI). Immunofluorescence images were obtained using an LSM5Pascal confocal laser scanning microscope (Carl Zeiss).

2.5. Real-time PCR

Real-time PCR was performed as described previously [6]. For hiPSCs, FastStart Universal SYBR Green Master (Roche) was used. Primer sets and probes are listed in Supplementary Table 1.