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Glutathione peroxidase-1 is required for self-renewal of murine embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells are pluripotent cells that are capable of giving rise to any type of cells in the body and possess unlimited self-renewal potential. However, the exact regulatory mechanisms that govern the self-renewal ability of ES cells remain elusive. To understand the immediate early events during ES cell differentiation, we performed a proteomics study and analyzed the proteomic difference in murine ES cells before and after a 6-h spontaneous differentiation. We found that the expression level of glutathione peroxidase-1 (GPx-1), an antioxidant enzyme, is dramatically decreased upon the differentiation. Both knockdown of GPx-1 expression with shRNA and inhibiting GPx-1 activity by inhibitor led to the differentiation of ES cells. Furthermore, we showed that during early differentiation, the quick degradation of GPx-1 was mediated by proteasome. Thus, our data indicated that GPx-1 is a key regulator of self-renewal of murine embryonic stem cells.

1. Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts [1]. The defining hallmarks of ES cells are the ability to give rise to all cell types within the body and the unlimited self-renewal potential [2]. This unlimited self-renewal capacity allows ES cells to maintain the undifferentiated state during dividing [2,3]. Defects in self-renewal mechanisms result in severe developmental problems. The elucidation of the self-renewal process and the underlying mechanisms provides the fundamental insights into development, aging and cancers [3]. It is believed that the pluripotency and the unlimited self-renewal potential of ES cells are under both cell-intrinsic and extrinsic control [4–7], however, the exact regulating mechanisms are not fully understood.

Recently, redox homeostasis has been recognized to play important roles in maintaining the self-renewal of ES cells [8]. ES cells have highly efficient machinery for antioxidant defense to maintain the redox homeostasis [9]. Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, are generated as a byproduct by mitochondria during aerobic metabolism [8–10], and these species can lead to DNA damage, senescence and cells death by causing oxidative damage to DNA and proteins [10]. GPx-1 is one of the antioxidant enzymes that modulate the overall redox homeostasis by reducing hydrogen peroxide to water, and therefore eliminating the toxic effects of ROS [10]. Recent studies have suggested the essential role of GPx-1 in many physiological and pathological procedures [11–13], but the role of GPx-1 in modulating ES cell self-renewal still remains elusive.

Much effort has been devoted to identifying potential regulators of ES cell self-renewal by comparing ES cells with their differentiated counterparts, however, the immediate early events upon ES cell differentiation are still largely unknown [14–17]. Here, by performing a differential proteomics study, we analyzed murine ES cells and the spontaneously differentiated cells, which were differentiated by withdrawing leukemia inhibitory factor (LIF), serum and feeder cells for 6 h, and we found that the expression level of GPx-1 was dramatically decreased upon the differentiation of ES cells. Both knockdown of GPx-1 expression and inhibition of GPx-1 activity resulted in the differentiation of ES cells. We further showed that during the early differentiation of ES cells, proteasome mediated the quick degradation of GPx-1. Thus, our data indicated that GPx-1 is required for self-renewal of murine embryonic stem cells.

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2. Materials and methods

2.1. Embryonic stem cell culture

Murine ES cells (SCRC-1002; American Type Culture Collection, Manassas, VA) were cultured in DMEM medium (Gibco), supplemented with 15% fetal bovine serum (Hyclon), 1000 U/ml LIF (Chemicon), 2 mM L-glutamine (Macgene), 1 mM sodium pyruvate (Gibco), 1% MEM nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Sigma) and 1% penicillin–streptomycin (Macgene) on mouse embryonic fibroblast (MEF) feeder layers treated previously with mitomycin C (Sigma, M4287).

2.2. Differentiation of murine ES cells

Prior to differentiation, ES cells were disassociated by trypsin, the cell suspension was plated on a cultural dish for 30 min at 37 °C to remove the feeder cells. The purified cells were then cultured with ES cell culturing medium without LIF and serum in a new dish.

To form embryoid body (EB), murine ES cell aggregates were cultured in Ultra Low Attachment Plates (Corning Costar, 3471) with ES cell culturing medium lacking sodium pyruvate and LIF.

2.3. Protein identification by label-free LC–MS analysis

Murine ES cells were spontaneously differentiated. Proteins from undifferentiated and differentiated murine ES cells were extracted and digested as described previously [18]. The peptide mixture was acidified by formic acid for followed MS analysis. The label-free LC–MS analysis was performed as described by Shen et al. [19].

2.4. 2-dimensional electrophoresis (2-DE) and MALDI-TOF/TOF-MS analysis

Protein extracts of undifferentiated and differentiated murine ES cells were separated by 2-DE as described previously [20]. The 2-DE images were captured using ImageScanner (Amersham Pharmacia Biotech). Thirty-two spots representing significantly altered between undifferentiated and differentiated cells proteins were analyzed by ultraflex III MALDI-TOF/TOF-MS (Bruker). Peptide mass fingerprinting (PMF) data was searched on local Mascot v2.1 against the non-redundant protein database NCBInr and the MS/MS ion database search was conducted using Mascot (http://www.matrixscience.com).

2.5. RNA interference

Two RNA interference sequences for knockdown GPx-1, 5′-AGAAACCCTGCTGTCCCAG-3′ and 5′-GTTTGAGAAGTGCGAAGTG-3′ were sub-cloned into pLKO lentiviral vectors. 24 and 48 h after co-transfection of 293T cells with lentiviral packaging plasmids, the viral particles were harvested. The virus were concentrated by ultracentrifugation for 1.5 h at 25,000g and resuspended in D-PBS (Hyclon) before incubation with murine ES cells. At 36 h post-infection, ES cells were harvested for protein expression analysis and cell morphological observation.

2.6. Protein extraction and Western blotting

Cells were washed with PBS before being collected and lysed in lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40 and protease inhibitor cocktail (Roche, 04693124001)). Cell debris was removed by centrifugation at 12,000g, for 10 min at 4 °C. Protein concentrations were determined using Bradford Dye (Bio-Rad, Hercules, CA). Total protein was separated by SDS–PAGE and transferred to Hybond-P PVDF membrane (GE Healthcare, Piscataway, NJ). Specific antibodies used for Western blotting are GPx-1 (Abcam, ab108427), Oct4 (Santa cruz, sc5279 and α-tubulin (Sigma, T5168).

2.7. Alkaline phosphatase staining

The differentiation phenotype of murine ES cells was evaluated by determining alkaline phosphatase (ALP) activity using ALP staining kit (Millipore, SCR004) according to the manufacturer's instruction. Briefly, cells in 24-well plates were fixed by 4% paraformaldehyde for 1–2 min and washed with Rinse Buffer. After wash, 0.5 ml staining solution was added to each well and incubated in dark at room temperature for 15 min. Aspirate staining solution and rinse the wells with Rinse Buffer again, the cells were then photographed.

2.8. Flow cytometry

The ES cells were disassociated by trypsin and then resuspended with 10% FBS in PBS. After 30 min blocking, cells were washed and incubated with SSEA-1 antibody (Santa cruz, sc21702-PE) or the control IgG for 30 min at room temperature. After wash with 1% FBS in PBS for 3 times, cells were resuspended in 0.5 ml PBS and analyzed on flow cytometer.

2.9. RT-PCR

Total RNA was extracted with TRIzol according to the manufacturer's guidelines (Invitrogen). CYP-26 mRNA was measured by RT-PCR with a Prime Script RT Master Mix kit (Takara) and the primers, 5′-TTCTGCAGATGAAGCGCAGG-3′ (forward) and 5′-TTTC GCTGCTTGTGCGAGGA-3′ (reverse). GAPDH was used as a control, the forward and reverse primers were 5′-CGACTTCAACAGCAA CTCCCACTCTTCC-3′ and 5′-TGGGTGGTCCAGGGTTTCTTACTCCTT-3′ respectively. The RT-PCR reactions (30 cycles) were migrated on a 1% agarose gel and stained with ethidium bromide.

3. Results

3.1. Proteomic identification of altered expression of proteins upon murine ES cell differentiation

In order to understand the immediate early events during ES cell differentiation, we sought to identify the proteomic difference before and after ES cell differentiation. Especially, the down-regulated proteins during differentiation may represent the potentially essential regulators for ES cells self-renewal. We first let the murine ES cells differentiate spontaneously by withdrawing LIF, serum and feeder cells. At 6 h post differentiation when the morphological change could be observed, the early differentiated cells together with their undifferentiated counterpart were collected and subjected to a label-free LC–MS analysis (Fig. 1A). Proteins that are uniquely expressed in undifferentiated cells and in differentiated cells were listed (Table 1). We then used 2-DE to analyze independent samples (Fig. 1A). The down-regulated proteins after differentiation were arrowed (Fig. 1B), and these protein spots were subjected to in-gel digested by trypsin, and the resulting peptides were analyzed by MALDI-TOF/TOF MS. GPx-1 was identified to be a down-regulated protein after differentiation, which is consistent with the data listed in Table 1 (Table 1 and Fig. 1C).