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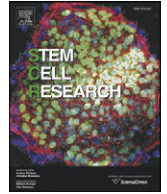

The Forkhead box transcription factor FOXM1 is required for the maintenance of cell proliferation and protection against oxidative stress in human embryonic stem cells

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ABSTRACT

Human embryonic stem cells (hESCs) exhibit unique cell cycle structure, self-renewal and pluripotency. The Forkhead box transcription factor M1 (FOXM1) is critically required for the maintenance of pluripotency in mouse embryonic stem cells and mouse embryonal carcinoma cells, but its role in hESCs remains unclear. Here, we show that FOXM1 expression was enriched in undifferentiated hESCs and was regulated in a cell cycle-dependent manner with peak levels detected at the G2/M phase. Expression of *FOXM1* did not correlate with *OCT4* and *NANOG* during in vitro differentiation of hESCs. Importantly, knockdown of FOXM1 expression led to aberrant cell cycle distribution with impairment in mitotic progression but showed no profound effect on the undifferentiated state. Interestingly, FOXM1 depletion sensitized hESCs to oxidative stress. Moreover, genome-wide analysis of FOXM1 targets by ChIP-seq identified genes important for M phase including *CCNB1* and *CDK1*, which were subsequently confirmed by ChIP and RNA interference analyses. Further peak set comparison against a differentiating hESC line and a cancer cell line revealed a substantial difference in the genomic binding profile of FOXM1 in hESCs. Taken together, our findings provide the first evidence to support FOXM1 as an important regulator of cell cycle progression and defense against oxidative stress in hESCs.

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1. Introduction

Human embryonic stem cells (hESCs) are undifferentiated cells derived from the inner cell mass of human blastocysts in the pre-implantation stage (Thomson et al., 1998; Trounson, 2006; Pera and Tam, 2010). The hESCs are characterized by the capacity to generate any cell type of all three germ layers (pluripotency) and to grow indefinitely in an undifferentiated state (self-renewal) (Pera and Tam, 2010; Young, 2011). They also have a uniquely short cell cycle with an abbreviated G1 phase (Becker et al., 2006). Thanks to these remarkable properties, hESCs hold great promise for the development of tissue replacement therapy and provide a model system for the study of early embryonic development and lineage specification (Trounson, 2006).

The molecular control of pluripotency and self-renewal in hESCs is attributed to an interactive network of transcription factors. OCT4, NANOG and SOX2 are uniquely expressed in pluripotent cells to orchestrate the transcriptional regulation of pluripotency by collaboratively activating the transcription of one another, constituting an autoregulatory circuitry (Boyer et al., 2005; Young, 2011). These three factors are responsible for driving the expression of genes essential to pluripotency and self-renewal (Boyer et al., 2005; Young, 2011). Regulation of this complex transcriptional network in stem cells deserves further analysis to fully understand the molecular basis of the initiation and maintenance of pluripotency/self-renewal and its pliability.

FOX transcription factors display a vast diversity of biological functions, including cell proliferation, metabolism, apoptosis and differentiation (Myatt and Lam, 2007). Recent studies revealed the involvement of FOX factors in the regulation of self-renewal and pluripotency in embryonic stem (ES) cells. In particular, downregulation of FOXD3 in hESCs was shown to disrupt self-renewal and lead to cell

Abbreviations: hESCs, human embryonic stem cells; ES cells, embryonic stem cells; CDK, cyclin-dependent kinases; EC cells, embryonal carcinoma cells; EB, embryoid bodies; PI, propidium iodide; ROS, reactive oxygen species; GO, gene ontology; RPE, retinal pigment epithelium; RA, retinoic acid; bFGF, basic fibroblast growth factor 2; CM, conditioned medium; DE, definitive endoderm; AVBF medium, medium based on Stemline II Hematopoietic Stem Cell expansion medium supplemented with BMP4; hFF, human foreskin fibroblast; PH3, phosphorylation of histone H3 at Ser10.

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differentiation towards the endoderm and mesoderm lineages (Arduini and Brivanlou, 2012), whereas FOXO1 was found to be essential for the regulation of hESC pluripotency via the direct transcriptional activation of *OCT4* and *SOX2* (Zhang et al., 2011).

The proliferation-associated FOX factor FOXM1 plays important roles in the regulation of cell proliferation, metastasis, apoptosis and DNA damage repair (Wierstra, 2013b). Studies using various cell models have shown that FOXM1 is essential for proper cell cycle progression by regulating the G1/S and G2/M transitions and the execution of the mitotic program (Laoukili et al., 2005; Wang et al., 2005; Wonsey and Follettie, 2005; Laoukili et al., 2007). FOXM1 activates the expression of the cell cycle genes *CCNB1*, *CCNB2*, *CDC25B* and *PLK1*, which in turn leads to the activation of cyclin-dependent kinases (CDKs), thereby propelling cells through different cell cycle phases (Leung et al., 2001; Wang et al., 2002, 2005; Wonsey and Follettie, 2005).

Recent reports have indicated the functional significance of FOXM1 in pluripotent cells. FOXM1 is required for the maintenance of pluripotency in mouse embryonal carcinoma (EC) cells, via direct regulation of *Oct4* transcription (Xie et al., 2010). More importantly, FOXM1 is important for maintaining pluripotency in mouse ES cells as a downstream target of the LIF/STAT3 signaling pathway, thereby stimulating the expression of pluripotent genes (Tan et al., 2014). Depleting FOXM1 in mouse ES cells led to a rapid loss of pluripotency as well as a decreased rate of cell proliferation (Tan et al., 2014). A recent study in human EC cells provided evidence that FOXM1 is required for *OCT4* expression, underscoring the potential functional role of FOXM1 in the context of human pluripotent stem cells (Chen et al., 2015). However, a detailed study of the expression and function of FOXM1 in hESCs is still lacking.

In this study, we investigated the role of FOXM1 in the regulation of pluripotency and cell proliferation in hESCs. We demonstrated that FOXM1 was expressed in undifferentiated hESCs in a cell cycle-dependent manner, with peak levels reached at the G2/M phase. We showed that FOXM1 depletion had subtle effects on the undifferentiated state of hESCs, but led to the downregulation of cell cycle genes and a delay in G2/M phase progression. Survival of hESCs under oxidative stress was also compromised when FOXM1 was depleted. Interestingly, genome-wide analysis of FOXM1 binding genomic sequences revealed FOXM1 targets and putative cooperative factors, which differ significantly from data previously reported in cancer cells. Our results highlight the important roles played by FOXM1 in maintaining the proliferation and survival of hESCs.

2. Materials and methods

2.1. Cell culture and differentiation

The human embryonic stem cell line VAL-3, obtained from the Spanish Stem Cell Bank (Valbuena et al., 2006), was cultured on plates coated with BD Matrigel™ hESC-qualified Matrix (BD Biosciences, USA) in mTeSR™1 Maintenance Medium (STEMCELL Technologies, Canada) at 37 °C in 5% CO_2 (Ludwig et al., 2006). Cell passage was performed by enzymatic dissociation using Accutase (Invitrogen), and culture media were supplemented with 10 μM ROCK inhibitor (Y-27632; Millipore) for the first day after cell seeding. Spontaneous differentiation was initiated by the formation of embryoid bodies (EB) from VAL-3 cells as previous described (Chen et al., 2012). Details of EB formation and other in vitro differentiation protocols are described in the Supplementary material (Extended methods).

2.2. FOXM1 knockdown

FOXM1 was depleted by RNA interference (RNAi) using short interfering RNA (siRNA). Cy3-labeled siRNA duplexes, of which two were against FOXM1 (FOXM1 siRNA #1: 5′-CUC UUC UCC CUC AGA UAU A-3′; FOXM1 siRNA #2: 5′-GGA AAU GCU UGU GAU UCA ACA-3′) and one was a non-specific siRNA control (Silencer® Cy3-labeled Negative Control No.1 siRNA), were purchased from Ambion® (Life Technologies) as in previous studies (Kong et al., 2013; Liu et al., 2013). Transfection of cells with siRNAs (25 nM) was performed with Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol.

2.3. Quantitative real-time PCR and immunoblot analyses

Real-Time quantitative PCR (qPCR) and immunoblot analyses were performed with the use of *mir*Vana™ PARIS™ Kit (Ambion®; Life Technologies) as previously described (Chen et al., 2012). FAM-labeled primers for genes of interest are listed in Supplementary Table 1. For details of antibodies and procedures, please see the Supplementary material (Extended methods).

2.4. Immunofluorescence

Cells were fixed with 4% paraformaldehyde (PFA), permeated with 0.1% Triton X-100 in PBST and then blocked with 3% BSA in PBST. After treatment with mouse anti-FOXM1 (ab55006; 1:100; Abcam), rabbit anti-OCT4 (H-134; 1:100; Santa Cruz), rabbit anti-phospho-histone H3 Ser 10 (PH3) (06–570; 1:500; Upstate) or mouse anti-SSEA-1 (MAB4301; 1:128; Millipore), cells were incubated with goat anti-rabbit IgG Alexa Fluor ® 488, goat anti-rabbit IgG Alexa Fluor ® 568, goat anti-mouse IgG Alexa Fluor ® 488 or goat anti-mouse IgG Alexa Fluor ® 568 (Invitrogen). Nuclear counterstaining was performed by incubation with 1:1000 Hoechst 33258 (Invitrogen), and images were taken using the confocal microscope LSM 700 (Carl Zeiss). For PH3 staining, the number of cells positive for PH3 was counted in at least four randomly selected fields at 100× magnification, comprising at least 1000 cells. Cells at different mitotic phases (prophase, prometaphase, or metaphase/anaphase/telophase) were judged based on the chromosome staining pattern as previously described (Neganova et al., 2014).

2.5. Flow cytometric analysis

Cell pellets containing 1×10^6 cells were collected and fixed with ice-cold 100% methanol. For cell cycle profiling, fixed cells were resuspended in Flow Staining Buffer containing 1:8333 Hoechst 33258 for DNA staining prior to flow cytometric analysis. Raw data obtained were analyzed with Modfit TM LT version 4.0 (Verity software). For bivariate flow cytometric analysis, fixed cells were blocked in 1% BSA, and then incubated with 1 μg mouse anti-FOXM1 (Abcam) (in 100 μl). Isotypic control was prepared by incubating cells with normal mouse IgG (Millipore) rather than FOXM1 antibody. Cells were then incubated with goat anti-mouse IgG Alexa Fluor ® 488 (Invitrogen) and resuspended in propidium iodide (PI) solution (0.5 mg/ml PI, 0.1 g RNase A, 0.5 g/ml Triton X-100). Cells were subjected to flow cytometric analysis in a BD LSR Fortessa Analyzer (BD Biosciences). Data were acquired by BD FACSDiva Software (BD Biosciences) and analyzed with FlowJo version 8.0 (FlowJo, LLC).

2.6. Cell proliferation assay

Cell growth after FOXM1 knockdown and/or hydrogen peroxide treatment were quantified by cell counting using the CyQUANT® NF Cell Proliferation Assay Kit (Life Technologies) according to the manufacturer's instruction. Details of the assay are given in Supplementary material (Extended methods).

2.7. ROS assay

To detect the cellular level of reactive oxygen species (ROS), assay using chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein

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