C-MYC Controlled *TIP110* Protein Expression Regulates *OCT4* mRNA Splicing in Human Embryonic Stem Cells

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We reported previously that HIV-1 the Tat-interacting protein of 110 kDa (*TIP110*; *P110(NRB)/SART3/p110*) is important in regulation of hematopoiesis, and in maintaining pluripotent factor (*NANOG*, *OCT4*, and *SOX2*) expression in and pluripotency of human embryonic stem cells (hESCs). Here we show that *TIP110* expression in hESCs line H9 and embryonal carcinoma cell line NT-2 is regulated by *C-MYC* expression in ESCs through an E box present in the *TIP110* promoter region. Through up- and down- modulation of expression, *TIP110* induces *OCT4* exon 1a inclusion and exon 1b skipping in our *OCT4* minigene model. Thus, *TIP110* expression in ESCs regulates alternative splicing of *OCT4*, an event required for pluripotency of ESCs.

Introduction

EMBRYONIC STEM CELLS (ESCs), derived from the inner cell mass of blastocyst-stage embryos, can self-renew and differentiate along 3 germ layer lineages [1,2]. The undifferentiated state is maintained by actions of transcription factors (TF). The POU domain TF *OCT4* (also known as *OCT3*, *OCT3/4*, and *POU5F1*), located on chromosome 6p21.3, is a core component controlling self-renewal and maintenance of the undifferentiated state of ESCs [3,4]. *OCT4* is also an essential factor for generation of induced pluripotent stem (iPS) cells [5]. The *OCT4* gene can potentially encode 2 different spliced variants, *OCT4A* and *OCT4B*. Both isoforms share identical POU DNA-binding and C-terminal trans-activation domains, but they differ at their N termini. Alternative splicing of *OCT4* is important in that *OCT4A* is highly expressed in pluripotent ESCs that self-renew and maintain the undifferentiated state of early pluripotent cells, while *OCT4B* can be detected in various nonpluripotent cell types and cannot sustain self-renewal and pluripotency of ESCs [6,7]. Takeda et al. first characterized human *OCT4* cDNA, and amplified *OCT4A* and *OCT4B* cDNA sequences [8]. They analyzed the *OCT4* sequence of exon–intron organization and identified *OCT4A* with exon 1 of the 447 base pair and *OCT4B* with exon 1b of the 344 base pair, as well as detailed splice donor and splice acceptor sites [8]. However, it is not known how *OCT4A* and *OCT4B* isoform splicing is regulated.

Although the pluripotent state of ESCs is controlled through certain levels of core TFs, such as *OCT4*, *SOX2*, and *NANOG*, *C-MYC* is also essential for maintaining a pluripotent state [9]. The oncoprotein *C-MYC* is a master regulator for cell proliferation, is virtually undetectable in quiescent

cells, and its expression is rapidly induced as cells enter the G1 phase of the cell cycle in response to stimulation. Expression of *C-MYC* is transient and directly related to the proliferative potential of cells, and subsequently, the abundance of *C-MYC* decreases gradually to a low steady-state level, where it remains for as long as the cells continue to proliferate [10,11]. *C-MYC* forms a heterodimer with the bHLH/Zip protein Max that binds to the E-box motif (cagctg) containing genes to activate its target gene transcription [12].

We hypothesized that *TIP110* expression is modulated through *C-MYC* and herein demonstrate that the oncogenic TF *C-MYC* upregulates transcription of the RNA binding protein *TIP110* through interaction with the *TIP110* E-box in the *TIP110* promoter, thus ensuring high-level *Tip110* expression in proliferating hESCs. We further show that *TIP110* regulates *OCT4* alternative splicing in hESCs.

Materials and Methods

Cell culture and cell transfections

The hESC line, H9, was cultured in the knockout Dulbecco's modified Eagle's medium (DMEM): F12, supplied with 20% serum replacement (KSR; Invitrogen) and the basic fibroblast growth factor (bFGF; Invitrogen) on mouse embryonic feeders mitotically inactivated with mitomycin C as described [13]. The human embryonal carcinoma cell line NTero-2 cl.D1(NT-2; CRL-1973) was purchased from American Tissue Culture Collection (ATCC) and maintained in the DMEM supplemented with 10% fetal calf serum at 37°C in 5% CO₂. Human cord blood was collected and used according to institutional guidelines. CD34 cells were purified

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within 24 h of collection using immunomagnetic selection (Miltenyi Biotec). CD34⁺ cells (>93% pure) were cultured in 10% FBS with the cytokine combination of: 100 ng/mL of stem cell factor, 100 ng/mL FLT3 ligand, and 20 ng/mL of thrombopoietin (SFT; R&D Systems). Detailed purification and culture of human cord blood CD34⁺ cells were as described [14]. The K562 cell line was purchased from American Tissue Culture Collection (ATCC, CCL-243) and maintained in the RPMI medium supplemented with 10% fetal calf serum at 37°C in 5% CO₂. NT-2 cells were transfected by using Lipofectamine 2000 (Invitrogen). H9 cells were transfected by using the Amaxa Human Stem cell Nucleofactor Kit (Lonza, Cat. No. VPH-5002) and Lipofectamine LTX and Plus reagent (Invitrogen, Cat No. 15338).

Constructs and primers

pCSC.Tip110GFP and pCSC.c-mycGFP, as well as RNAi for *TIP110* and *C-MYC* were as described [15]. The *OCT4* minigene was constructed by cloning human *OCT4* genomic DNA (Genebank: Z11900) from 60-1130, named minigene 1, which includes *OCT4* exon 1a, and from 3864 to 4710, named minigene 2, which includes exon 1b/2. Minigene1 primers were Oct4-60 5'-taccgagctcgatctaacaggcagctt-3' and Oct4-1130 5'-ctggactagtggatctcaccggcagtt-3'; minigene 2 primers were Oct4-3864 5'-ccgtcgacaggtgttctcgaggccagggtctc-3' and Oct4-4710 5'-ccctcgagccagtgtggaagcaatgga-3'. Minigene1 was inserted into pcDNA3.1 (Invitrogen; Cat. No. K4800-01). Minigene 2 was cloned into the cloning site right after minigene 1 on the same construct.

RNA isolation, semiquantitative and real-time polymerase chain reaction analysis

Total cellular RNAs were isolated using an Invitrogen TRIzol RNA isolation kit according to the manufacturer's instructions. Before RNA precipitation, RNA is extracted one more time with Acid-phenol:chloroform (PH:4.5, with IAA 125:24:1) to remove traces of DNA. One to 20 nanograms of total RNA were used for reverse transcription and polymerase chain reaction (PCR) in a one-step semiquantitative reverse transcription polymerase chain reaction (RT-PCR) reaction (Roche, Cat.No. 11939832001). Primers for *OCT4a*, *OCT4b*, *C-MYC*, and the internal control β -actin gene have been described [15,16]. The same c-myc primers were applied for one tube RT-PCR and q-PCR, which could detect both endogenous and transfected c-myc gene. The one tube RT-PCR was set at 50°C for 30 min and 95°C for 2 min for reverse transcription, followed by a total of 30 cycles of PCR. Five ng total RNA is used for detection *OCT4* from the *OCT* minigene-transfected NT-2 or hESC cells, while 20 ng total RNA is used for detection of endogenous *OCT4* genes in NT-2 cells and hESCs. Aliquots of PCR products were analyzed on a 1.2% agarose gel. For quantitative real-time PCR, reverse transcription was performed by using a Superscript III kit with random hexamer primers. Q-PCR was performed by using SYBR Green PCR Master Mix reagents (Invitrogen).

His-tagged Tip110 protein purification and western blot

6 $\times$ his-tagged *TIP110* expression plasmid was transfected into 293T cells. The cells were harvested at day 3 after transfection.

After washing with cold PBS, the cells were resuspended in a washing buffer (50 mM Tris.HCl, pH 8.0, 30 mM NaCl, 10% glycerol, 1% NP-400) with protease inhibitors and placed on ice for 20 min. The resuspended cells were sonicated, and then centrifuged for 15 min at 4°C. The supernatant was incubated with the Ni-beads column (Qiagen, Cat: 31014). The Tip110 protein was eluted with 250 mM imidazole in 1 $\times$ Buffer E (20 mM Hepes-HCl, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, and 1 mM DTT) and frozen in aliquots at -80°C. The Tip110 antibody used for western blot has been described before [15]; the antibody used for western Blot analysis is anti-c-myc (Santa Cruz; Cat: SC-789).

In vitro splicing assay

The *OCT4* minigene, which contains a T7 promoter was in vitro transcribed into pre-mRNA by using a 5 $\times$ MEGascript + T7 kit (Ambion; Cat: AMB 1334-5) at 30°C for 3 h. After transcription, the DNA template was digested by adding DNase I from the kit. In vitro splicing was performed in 40 μ L splicing reactions, which contained a 4 μ L 10 $\times$ SP buffer (5 mM ATP, 0.2 M creatine phosphate, 16 mM MgCl₂), a 20 μ L NT-2 cell extract, and a 5- μ L pre-mRNA probe. A 20 μ L reaction was taken out right after the reaction was set up as input, and the other 20 μ L reaction was incubated at 30°C for 3 h. RNA from both the input and splicing reactions were extracted from Trizol and resuspended in a 50 μ L buffer. One microliter out of the 50 μ L total RNA was used for quantitative RT-PCR.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed by using a ChIP assay kit (Upstate Biotechnology). Briefly, Hela cells or NT2 cells in 10-cm dishes were plated at 60% confluency and allowed to grow until 80% confluency over 40 h. Cells (10⁷) were crosslinked and lysed. Chromatin was sheared to 300- to 900-bp fragments. Normalized inputs were incubated with the 4- μ g anti-c-Myc antibody (Santa Cruz; Cat: SC-789) at 4°C overnight. IgG (Rabbit anti-mouse IgG, (MP Biomedical Cat#55436) was used as a negative control. The RNA polymerase II antibody (the RNA polymerase II 8WG monoclonal antibody, Covance Cat#MMS-126R) was used as a positive control (primers: 5'-agatgaacaggtgtccaaact-3' and 5'-aggttacggcagttgtctctc-3'). Immunoprecipitated DNA was amplified by PCR using *TIP110* promoter primers, which span the -412- to -250-bp region flanking the putative E-box (5'-) in the human *TIP110* promoter (primers: 5'-gcgtattaatgtaattact-3' and 5'-atccatttcccagattctc-3').

Preparing nuclear extract and DNA electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed with the ChIP assay kit (Upstate Biotechnology) using the protocol recommended by the manufacturer. Briefly, Hela cells and NT2 cells were cultured and nuclear extracts were prepared as described in the protocol. Double-stranded E-box probes (5'-aacccgaaaactcagctgtatttaacgtct-3' and 5'-agacgttaaatacagctgagttttccggtt-3') or E-box mutant probes (5'-aacccgaaaactcccgggtatttaacgtct-3' and 5'-agacgttaaataccggggagttttccggtt-3') were labeled with biotin at the 3' end. The DNA binding reaction was performed by incubation of the nuclear extract protein (20 μ g) with a biotin-labeled probe for 30 min at room

temperature. The nonbiotin-labeled probe or the C-MYC antibody were used for competition. Complexes were analyzed by electrophoresis through a 6% acrylamide nondenaturing gel. Biotin-labeled probes were transferred to Amersham Hibond-N+ (GE Healthcare) and detected by using the LightShift Chemiluminescent EMSA kit (Thermo Scientific) according to the manufacturer's instructions.

Results and Discussion

C-MYC upregulates TIP110 transcription and protein expression

We previously reported that *TIP110* was expressed in hematopoietic stem cells and hESC [13,15]. Since C-MYC was shown to activate the *TIP110* promoter by using a luciferase reporter system [15], we tested whether C-MYC expression correlated with *TIP110* expression. We analyzed C-MYC and *TIP110* expression from cord blood CD34⁺ cells at different stages. The *TIP110* expression pattern was similar to that of C-MYC (Fig. 1a). C-MYC and *TIP110* are not expressed in quiescent CD34⁺ cells (Fig. 1a, L1), but are expressed in cytokine-stimulated undifferentiated CD34⁺ cells (Fig. 1a, L2) as we previously reported [15], with decreased expression during further differentiation of CD34⁺ cells (Fig. 1a, L3). Thus, C-MYC expression correlates with *TIP110* expression in cord blood CD34⁺ cells. When the human leukemia cell line K562 was induced to differentiate toward the megakaryocyte lineage by PMA, expression of both *TIP110* and C-MYC was reduced (Fig. 1b). To test whether C-MYC and *TIP110* show the same expression pattern in hESCs, ESCs were cultured for maintenance of pluripotency and after differentiation. qRT-PCR showed that *TIP110* as well as C-MYC were expressed in hESCs (Fig. 1c, L1). hESCs were removed from feeder layers and bEGF for 3 days, to allow ESC differentiation. C-MYC expression levels were dramatically reduced (by 67%), along with a large reduction in expression of *TIP110* (85%) (Fig. 1c, L2), suggesting that *TIP110* might be under the control of C-MYC. To examine direct involvement of C-MYC over *Tip110* expression, we exogenously overexpressed C-MYC, or silenced C-MYC expression in NT-2 cells to test whether C-MYC regulates *TIP110* ex-

pression. There was a clear increase of *TIP110* expression with overexpression of C-MYC, and a reduction of *TIP110* expression with knockdown of C-MYC expression in NT-2 cells at both the mRNA (Fig. 2a) and protein (Fig. 2b) level. After overexpressing of C-MYC or silencing C-MYC expression in NT-2 cells, we analyzed the mRNA levels of stem cell markers for pluripotency, including *OCT4*, *SOX2*, and *NANOG* by qPCR. The results showed that the expression levels of *OCT4*, *SOX2*, and *NANOG* are increased with overexpression of C-MYC; the expression levels are reduced with silencing C-MYC expression (Fig. 2c).

Tip110 expression transactivated by C-MYC is through putative E-box binding sites located in the Tip110 promoter

Next, we searched for the elements through which C-MYC could regulate *TIP110* expression in the *TIP110* promoter region. Genomatrix programs were applied to predict binding sites of potential TFs within the *TIP110* promoter region (−687 to +1 bp). A putative E-box (CACGTG), a binding site for C-MYC TFs, was identified in the promoter of *TIP110* (Fig. 3a). To determine whether C-MYC could bind to this E box region of the *TIP110* promoter, we first performed EMSA experiments to identify C-MYC interactions with this specific E box on the *TIP110* promoter (Fig. 3b). Associations between the E box probe and nuclear extract from NT-2 cells were tested. One single complex was formed from the gel shift result (L1). The complex formation is competed by including the same, but unlabeled E box probe (L2–L5). The shifted bands were supershifted by including the C-MYC antibody in the binding reactions (L6–L9). When we mutate the E box sequence to destroy the E box, while still keeping other transcription sites intact, the association between the mutated E box probe and *TIP110* is no longer apparent (data not shown).

Next, we performed CHIP assay to determine if C-MYC localized to this *TIP110* E box region in NT-2 cells. Using ChIP analysis, we found that this putative binding site E box was bound by the endogenous C-MYC protein (Fig. 3c), confirming that the C-MYC protein interacts with the E box located in the *TIP110* promoter.

FIG. 1. *TIP110* and C-MYC share similar expression patterns. Semiquantitative RT-polymerase chain reaction (PCR) of: (a) Freshly isolated CD34⁺ cells (L1), CD34⁺ cells cultured in the Iscove's modified Dulbecco's medium (IMDM) with fetal bovine serum, stem cell factor, thrombopoietin, and Fms-related tyrosine kinase 3 ligand (FL) for 24 h (L2), or cells continued in culture for 8 days (L3); (b) K562 cells cultured in the RPMI medium (L1) or included with 20 nM phorbol 12-myristate 13-acetate for 5 days (L2). (c) ESC line (H9) cultured to yield undifferentiated human embryonic stem cells (hESCs) (L1) or differentiated hESC offspring under feeder-free conditions by withdrawing basic fibroblast growth factor in a medium for 3 days (L2). β -actin was used as a loading control. Data shown are from 1 of at least 3 reproducible experiments.

FIG. 2. C-MYC regulates *TIP110* expression. NT-2 cells were transduced with *c-myc* expression lentivirus and cells were harvested 3 days after cell transduction for analysis of *TIP110* expression via semi-quantitative RT-PCR (**a**), western blot (**b**). C-MYC, *TIP110*, *OCT4*, *SOX2*, and *NANOG* mRNA expression was analyzed through qPCR (**c**). β -actin was used to normalize q-PCR results. Data in panel **c** are expressed as mean $\pm$ SD of 3 independent experiments. $\blacklozenge P < 0.001$; $\blacksquare P < 0.05$ for the compared pair.

Tip110 regulates an alternative splicing of *OCT4*

Studies of others [17] and ourselves (unpublished data) showed that *TIP110* was involved in general splicing through interaction with U6 snRNA. The association of

TIP110 with splicing machinery and splicing factors suggests *TIP110* might have a regulatory role in alternative splicing. Our recent work [13] showed that *TIP110* functions in maintaining pluripotent factor *OCT4* expression in hESCs, and we had hypothesized that *TIP110* could play a role in

FIG. 3. C-MYC association in vivo/vitro with E box element in the *TIP110* promoter. (**a**) Schematic localization of specific primers/probes used for chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA); (**b**) EMSA was performed with biotin-labeled probes as indicated in (**a**). 20 μ g of nuclear extracts obtained from NT-2 cells transfected with C-MYC expression vector were used for the gel shift assays. Unlabeled probes were used in excess from 10 $\times$ to 100 $\times$ more for competition (lane 3–6). (**c**) CHIP assay is performed on NT-2 hESCs overexpressing C-MYC. Input DNA (L1); RNA polymerase II antibody as a positive control (L2); Rabbit IgG as negative control (L3); no antibody added as negative control (L4) and C-MYC antibody for bound or not bound fraction (L5) were amplified with the primers indicated in (**a**). *nonspecific binding; S: supershift; C: cmyc/probe complex; F: free probe.

alternative splicing of *OCT4*. To test our hypothesis that *TIP110* functions in *OCT4* alternative splicing, we first cloned a minigene for *OCT4* that includes exon 1a (minigene 1) and exon 1b/2 (minigene 2) (*OCT4* cDNA, GenBank: Z11900.1). Minigene I starts from gene 60 to 980 and includes exon 1a and its splicing donor, minigene II that starts from gene 3864 to gene 4710, which includes exon 1b/2, exon 1b splicing donor and its acceptor, and exon 2 and exon 2 splicing acceptor (Fig. 4a). We inserted minigene I and minigene II sequentially to pC.DNA3 vector cloning sites, which contain a CMV promoter. The product of the *OCT4* minigene undergoes 2 alternative splicing decisions to include either exon 1a or exon 2 (part of *OCT4A* product), or excluding exon 1a, but including exon 1b and exon 2 (part of the *OCT4B* product) (Fig. 4a). To test *TIP110* function in *OCT4* splicing, we co-transfected the *OCT4* minigene with *Tip110* expression or silencing constructs in NT-2 cells as well as hESCs. When the *TIP110* gene is overexpressed, the *OCT4* minigene is able to splice in the form of *OCT4A*; when *TIP110* gene expression is downregulated, the *OCT4* minigene is not able to splice in the form of *OCT4A*; while the *OCT4B* level is not changed (Fig. 4b). To validate the in vivo results that *TIP110* expres-

sion is able to splice the *OCT4A* form, we designed an in vitro splicing reaction. The *OCT4* minigene was in vitro transcribed into a pre-mRNA probe. The *OCT4* pre-mRNA probe was incubated with the NT-2 cell extract, the same amount of the NT-2 cell extract with the *TIP110* recombinant protein, and the same amount of *TIP110* siRNA NT-2 cell extract in the splicing reaction for 3 h. Instead of running a denature RNA gel for detecting unprocessed *OCT4* pre-mRNA, *OCT4A* and *OCT4B* isoforms due to the large sizes; total RNA was extracted at the end of the splicing reaction. 1/50 total RNA was used for quantitative RT-PCR to amplify unprocessed RNA, *OCT4A*, and *OCT4B* RNA. The results showed that *TIP110* caused more spliced *OCT4A*, but not *OCT4B*; while siRNA for *Tip110* caused less spliced *OCT4A*, but not *OCT4B*. Total transcript either decreased with *TIP110* or increased without *TIP110* presence accordingly (Fig. 4c). Both in vivo and in vitro results showed that *TIP110* is an important factor for *OCT4A* splicing. There is a slight increase in the stem cell marker *OCT4A*, *SOX2*, and *NANOG* levels with overexpression *TIP110*; however, the stem cell marker levels decreased with silent *TIP110* expression (Fig. 4d). This is consistent with our previous results from hESC

FIG. 4. Human *OCT4* genomic schematic organization, *OCT4* minigene construct, and RT-PCR analysis of *OCT4A* transcripts. **(a)** Two fragments of the *OCT4* genomic clones spanning *OCT* exon 1 and exon 2 were amplified by PCR, then they are cloned into pC.DNA3 vector multiple cloning sites (MCS), which could be spliced with either *OCT4A* and/or *OCT4B*. **(b)** Co-expression of plasmids for pC.DNA3 (control) and pC.DNA3TIP110; and shRNA (control) and shRNA.TIP110 with *OCT4* minigene in NT-2 cells and H9 hESCs. Cells were harvested 48 h after transfection for *OCT4A* and *OCT4B* mRNA expression by RT-PCR. *OCT4A*/*OCT4B* ratio was calculated through densitometry reading. β -actin was used as a loading control. **(c)** *OCT4* minigene was in vitro transcribed into pre-mRNA. The *OCT4* minigene transcribed pre-mRNAs were incubated with NT-2 cell extract (L1), NT-2 cell extract plus recombinant *TIP110* protein (L2), and NT-2 cell with siRNA for *TIP110* extract in splicing buffer for 0 hrs (input) or 3 hrs reaction at 30°C. RNAs were extracted from the input for quantitative RT-PCR of total transcript; quantitative RT-PCR for total transcript, *OCT4A*, and *OCT4B* from splicing reactions. **(d)** The stem cell markers (*OCT4*, *Nanog*, and *Sox2*) were analyzed in NT-2 cells by qPCR with overexpressing *TIP110* or silencing the expression. β -actin was used to normalize q-PCR results. Data in panel d are expressed as mean $\pm$ SD of 3 independent experiments. $\diamond P < 0.05$; $\blacksquare P < 0.001$ for the compared pair.

[13]. We further tested whether expression of *TIP110* could activate the *OCT4* promoter when we cotransfected the *OCT4* promoter-reporter gene and the *TIP110* expression vector. We did not detect *TIP110* activation of this *OCT4* promoter under these conditions (data not shown). This suggests that *TIP110* regulates *OCT4A* expression in hESCs through alternative splicing rather than by activation of this promoter.

Expression of *C-MYC* is generally high during early embryonic development, where it is required for ESCs pluripotency and reprogramming in addition to proliferation. *C-MYC* expression is low or undetectable in differentiated adult tissues, which is consistent with the virtual absence of cell proliferation. In this study, we demonstrated that *TIP110* expression in human CB CD34⁺ cells and hESCs is controlled by *C-MYC* expression. This explains expression of *TIP110* in these cells, which ensures *TIP110* functions in maintaining hematopoiesis and in maintaining pluripotent factor (*NANOG*, *OCT4*, and *SOX2*) expression in and pluripotency of hESCs.

We observed from our previous work that *TIP110* expression in ESCs is necessary for *OCT4A* expression. *OCT4A*, which is an alternatively spliced isoform of the *OCT4* gene, sustains ESC self-renewal. It is possible that other factors besides *TIP110* might also be important in determining *OCT4* alternative splicing, as our minigene could not produce the *OCT4A* isoform in Hela cells with *TIP110* expression (data not shown). Through our current demonstration that *Tip110* regulates expression of the *OCT4A* alternatively spliced isoform instead of the *OCT4B* isoform in our *OCT4* minigene model, we have enhanced understanding of the contribution of *TIP110* to pluripotency of hESCs.

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Author Disclosure Statement

No competing financial interests exist.

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