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Expression and function of a novel isoform of Sox5 in malignant B cells

Shanique K.E. Edwards^{1,2}, Anand Desai¹, Yan Liu¹, Carissa R. Moore¹, and Ping Xie^{1,3}

¹Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, New Jersey 08854

²Graduate Program in Molecular Biosciences, Rutgers University, Piscataway, New Jersey 08854

³Member, Rutgers Cancer Institute of New Jersey

Abstract

Using a mouse model with the tumor suppressor TRAF3 deleted from B cells, we identified Sox5 as a gene strikingly up-regulated in B lymphomas. Sox5 proteins were not detected in normal or premalignant TRAF3^{-/-} B cells even after treatment with B cell stimuli. The Sox5 expressed in TRAF3^{-/-} B lymphomas represents a novel isoform of Sox5, and was localized in the nucleus of malignant B cells. Overexpression of Sox5 inhibited cell cycle progression, and up-regulated the protein levels of p27 and β -catenin in human multiple myeloma cells. Together, our findings indicate that Sox5 regulates the proliferation of malignant B cells.

Keywords

Sox5; B lymphoma; multiple myeloma; TRAF3; p27; β -catenin

1. Introduction

Identification and validation of new genetic risk factors are imperative for a better understanding of B lymphomagenesis and for the development of new therapeutic strategies. TRAF3 was recently identified as a novel tumor suppressor in human non-Hodgkin lymphoma, including splenic marginal zone lymphoma, B cell chronic lymphocytic leukemia, mantle cell lymphoma, as well as multiple myeloma (MM) and Waldenström's macroglobulinemia [1-5]. Using a new mouse model with the TRAF3 gene specifically deleted in B cells (B-TRAF3^{-/-} mice), we recently demonstrated that TRAF3 deletion causes prolonged survival of mature B cells, which eventually leads to B lymphoma development at the age of 9 – 18 months [6,7]. The long latency of B lymphoma development observed in B-TRAF3^{-/-} mice suggest that additional oncogenic alterations are required for B

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Address correspondence to: Dr. Ping Xie, Department of Cell Biology and Neuroscience, Rutgers University, 604 Allison Road, Nelson Labs Room B336, Piscataway, New Jersey 08854. Phone: (732) 445-0802; Fax: (732) 445-1794; xiep@rci.rutgers.edu.

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lymphomagenesis. To delineate such oncogenic alterations in TRAF3^{-/-}B lymphomas, we performed a microarray analysis and identified Sox5 as a gene strikingly up-regulated in B lymphomas spontaneously developed in different individual B-TRAF3^{-/-}mice.

Sox5 is a member of the Sox family of transcription factors that contain a highly conserved sex-determining region (Sry)-related high-mobility-group (HMG) box, which mediates the binding to the minor groove of target DNA sequences [8]. Sox5 together with Sox6 and Sox13 constitute group D of Sox (SoxD) genes, which are expressed into several isoforms by alternative splicing [8]. As a consequence, SoxD proteins exist in long and short isoforms. Only the long isoforms contain a characteristic N-terminal domain, including a leucine zipper, coiled-coil domains and a glutamine-rich region (Q-box), which allows them to homo-dimerize or hetero-dimerize with other SoxD proteins [8]. The short isoform of Sox5 (S-Sox5) is expressed in the testis, brain, and lung, and likely plays a specialized role in testis development and ciliogenesis [9,10]. In contrast, the long isoform of Sox5 (L-Sox5) is expressed in multiple tissues, including the cartilage, heart, brain, kidney, lung, and skeletal muscle [11-14]. It has been shown that L-Sox5 plays important roles in regulating diverse processes of embryonic development and cell fate determination, including chondrogenesis [15], notochord and joint development [16,17], neural crest generation [18], oligodendrogenesis [19], melanogenesis [20], lung development [14], and the sequential generation of cortical neurons [21,22]. Sox5 proteins achieve these developmental roles by modulating cell proliferation, survival, differentiation, or terminal maturation in different cell lineages [8].

Interestingly, corroborating our finding that Sox5 is strikingly up-regulated in TRAF3^{-/-}mouse B lymphomas, up-regulation of SOX5 mRNA has also been identified in human memory B cells [23], in clonal B cells of patients with hepatitis C virus (HCV)-associated B cell lymphoproliferative disorders mixed cryoglobulinemia [24], and in human patients with follicular lymphoma [25]. However, the role of Sox5 in B cell physiology and pathology remains unclear. The present study aimed to address this gap in knowledge. Specifically, the objectives of this study are: (1) to determine the expression of Sox5 in TRAF3^{-/-}B lymphocytes and B lymphomas; (2) to identify which isoform of Sox5 is expressed in TRAF3^{-/-}B lymphomas; (3) to elucidate the function and mechanism of Sox5 in B cell malignancies.

2. Materials and Methods

2.1. Mice and cell lines

TRAF3^{flox/flox}CD19^{+/-}Cre(B-TRAF3^{-/-}) and TRAF3^{flox/flox}(littermate control, LMC) mice were generated as previously described [6]. All mice were kept in specific pathogen-free conditions in the Animal Facility at Rutgers University, and were used in accordance with NIH guidelines and under an animal protocol (Protocol # 08-048) approved by the Animal Care and Use Committee of Rutgers University. Human MM cell lines 8226 (contains biallelic TRAF3 deletions) and LP1 (contains inactivating TRAF3 frameshift mutations) were generously provided by Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ), and were cultured as previously described [3]. Mouse splenic B lymphocytes were prepared as previously described [6].

2.2. Antibodies and reagents

Rabbit Sox5 antibody (Ab) was purchased from Abcam (Cambridge, MA). Rabbit Abs against p27, p21, cyclin D1, cyclin D2, c-Myc, Bcl-xL, Mcl1, and phosphorylated or total β -catenin or Akt were from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit Abs against TRAF1, p53 and HDAC1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin Ab was from Chemicon (Temecula, CA). HRP-labeled secondary Abs and

affinity-purified (Fab')₂ goat anti-mouse IgM (μ -chain specific, anti-BCR) Abs were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hamster anti-mouse CD40 Abs were obtained from eBioscience (San Diego, CA). DNA oligonucleotide primers and CpG oligonucleotide 2084 (T*C*C*T*G*A*C*G*T*T*G*A*A*G*T; * denotes phosphorothioate bond) were obtained from Integrated DNA Technologies (Coralville, IA). Lipopolysaccharides (LPS) and propidium iodide (PI) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Tissue culture supplements including stock solutions of sodium pyruvate, L-glutamine, and non-essential amino acids and Hepes (pH 7.55) were from Invitrogen (Carlsbad, CA). Pfu UltraII was purchased from Agilent (Santa Clara, CA).

2.3. Taqman assay of Sox5 mRNA expression

Total cellular RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA (cDNA) was prepared from RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR of Sox5 was performed using TaqMan Gene Assay Kit (Applied Biosystems) as previously described [26].

2.4. Protein extraction and immunoblot analysis

Total protein lysates, cytosolic and nuclear extracts were prepared as previously described [6]. Immunoblot analysis was performed using various antibodies as described [27]. Images of immunoblots were acquired using a low-light imaging system (LAS-4000 mini, FUJIFILM Medical Systems USA, Inc., Stamford, CT).

2.5. Cloning of the full-length cDNA of the Sox5 gene from TRAF3^{-/-}mouse B lymphomas

Total cellular RNA was prepared from B lymphomas spontaneously developed in four individual B-TRAF3^{-/-} mice, and the corresponding cDNA samples were used as templates to clone the Sox5 coding sequences as detailed in the Supplementary Materials and Methods.

2.6. Generation of lentiviral Sox5 expression vectors

The Sox5 coding cDNA sequence cloned from TRAF3^{-/-} mouse B lymphomas (Sox5-BLM) and the L-Sox5 cDNA expressed in other tissues (purchased from Open BioSystems, Pittsburgh, PA) were subcloned into the lentiviral expression vector pUB-eGFP-Thy1.1 [28] (kindly provided by Dr. Zhibin Chen, the University of Miami, Miami, FL) by replacing the eGFP coding sequence with the Sox5 coding sequences, respectively. For subcloning into pUB-eGFP-Thy1.1 digested with BamHI and XbaI, the BamHI and XbaI restriction enzyme sites were added to flank the Sox5-BLM or L-Sox5 coding sequence by PCR using primers mSox5 5' BamHI and mSox5-R-XbaI (sequences detailed in Supplementary Table 1). Each lentiviral vector was verified by DNA sequencing.

2.7. Lentiviral packaging and transduction of human multiple myeloma cells

Lentiviruses of Sox5 expression vectors were packaged and titered as previously described [28,29]. Human multiple myeloma cells were transduced with the packaged lentiviruses at a MOI of 1:5 (cell:virus) in the presence of 8 μ g/ml polybrene [28]. At 96 hours post transduction, the transduction efficiency of cells was analyzed by Thy1.1 immunofluorescence staining and flow cytometry. Transduced cells were subsequently analyzed for Sox5 protein expression. Cell cycle distribution of transduced cells was determined by propidium iodide (PI) staining followed by flow cytometry as previously described [6,30].

2.8. Immunostaining and confocal imaging

Immunofluorescence staining of Sox5 and confocal imaging were performed as described in the Supplementary Materials and Methods.

2.9. Statistics

For cell cycle analysis experiments, statistical significance was assessed by Student *t* test. *P* values less than 0.05 are considered significant.

3. Results

3.1. Striking up-regulation of Sox5 in TRAF3^{-/-}B lymphomas

To delineate secondary oncogenic alterations in TRAF3^{-/-} mouse B lymphomas, we performed a microarray analysis (Edwards *et al.*, manuscript in preparation) and identified Sox5 as a strikingly up-regulated gene. We first verified the transcriptional up-regulation of Sox5 in splenic B lymphomas and ascites spontaneously developed in 6 different individual B-TRAF3^{-/-} mice using TaqMan gene expression assay (Fig. 1A). We also verified the up-regulation of Sox5 at the protein level using Western blot analysis (Fig. 1B). Interestingly, only the long isoform of the Sox5 protein (MW: ~80 kDa), but not the short isoform (MW: ~48 kDa), was detected and up-regulated in TRAF3^{-/-} B lymphomas.

We next investigated the potential involvement of Sox5 up-regulation in the survival, proliferation and activation of B lymphocytes. Splenic B cells were purified from LMC and tumor-free young B-TRAF3^{-/-} mice (age: 10-12 weeks), and then stimulated with a variety of B cell stimuli. These include agonistic anti-CD40 Abs, LPS (TLR4 agonist), anti-B cell receptor (BCR) crosslinking Abs, and CpG2084 (TLR9 agonist), alone or in combination. We found that the transcript of Sox5 was modestly up-regulated by the combined treatment with CpG and CD40 in premalignant TRAF3^{-/-} B cells, but not induced in LMC B cells or by other treatment (Fig. 1C). Interestingly, Sox5 proteins were not detectable in normal LMC or premalignant TRAF3^{-/-} B cells after treatment with any examined B cell stimuli, although TRAF1 proteins were potently induced by these stimuli (Fig. 1D). Thus, Sox5 protein was only up-regulated and detected in TRAF3^{-/-} B lymphoma cells.

3.2. A novel isoform of Sox5 was expressed in TRAF3^{-/-} B lymphomas

Three different variants of mouse L-Sox5 transcripts have been reported in the literature and GenBank databases [10-12]. To identify which isoform of Sox5 was expressed in TRAF3^{-/-} mouse B lymphomas, we cloned the full-length Sox5 coding cDNA from B lymphomas of 4 different individual B-TRAF3^{-/-} mice using reverse transcription and PCR as described in the Supplementary Materials and Methods (Supplementary Tables 1, 2 and 3). Surprisingly, our sequencing data revealed that the Sox5 cDNA cloned from TRAF3^{-/-} mouse B lymphomas represents a novel isoform of mouse Sox5 (Sox5-BLM), which is distinct from previously reported mouse Sox5 isoforms (Fig. 2). We thus submitted the sequence of Sox5-BLM to GenBank database (accession number: KF793916). Sox5-BLM contains a 35 amino acid (aa) deletion in the N-terminal region in front of the leucine zipper domain. Although a similar 35 aa deletion is also present in Sox5 variant 3 (Sox5-V3), the latter has an additional deletion of 49 aa between the first and the second coiled-coil domains. Examination of the exon and intron structure of the mouse Sox5 gene revealed that this novel isoform, Sox5-BLM, is likely generated by alternative splicing (Supplementary Fig. 1).

To further determine whether other known Sox5 transcript variants were present in TRAF3^{-/-} B lymphomas, we designed multiple pairs of PCR primers flanking the alternative splice sites of Sox5 isoforms (Supplementary Materials and Methods, and Supplementary

Table 1). We did not detect any transcript expression of L-Sox5, Sox5-V2, or S-Sox5 by PCR (Supplementary Tables 2 and 4). Interestingly, we observed low level of expression of the Sox5-V3 transcript in TRAF3^{-/-} mouse B lymphomas (Supplementary Table 4). Thus, our results demonstrated that although Sox5-V3 transcript is also present, the novel isoform (Sox5-BLM) is the predominant transcript expressed in TRAF3^{-/-} mouse B lymphomas.

To generate research tools for transduction of human B cell lines, we constructed lentiviral expression vectors using the Sox5-BLM cDNA cloned from TRAF3^{-/-} mouse B lymphomas and the L-Sox5 cDNA expressed in other tissues, respectively. We use these vectors to transduce human patient-derived multiple myeloma cell lines 8226 and LP1 cells, which contain TRAF3 deletions or inactivating mutations and do not express endogenous Sox5 proteins (Supplementary Fig. 2). Our flow cytometric data demonstrated that the lentiviral transduction efficiency was > 80% in human multiple myeloma cells (Fig. 3A). We found that the cloned Sox5-BLM was expressed into a protein of ~80 kDa, a size identical to that detected in primary TRAF3^{-/-} mouse B lymphomas but smaller than that of L-Sox5 (Fig. 3B). Thus, the transduction data further verified that the novel isoform, Sox5-BLM, is the predominant isoform expressed in TRAF3^{-/-} mouse B lymphomas.

3.3. Overexpression of Sox5-BLM inhibited cell cycle progression in transduced human multiple myeloma cells

To explore the functional roles of Sox5 in the survival and proliferation of malignant B cells, we analyzed the cell cycle distribution of human multiple myeloma 8226 and LP1 cells transduced with lentiviral expression vectors of Sox5-BLM or L-Sox5 using PI staining and FACS analysis. Cells transduced with an empty lentiviral expression vector (pUB-Thy1.1) were used as control in these experiments. We found that overexpression of either Sox5-BLM or L-Sox5 resulted in a significant decrease in the proliferating cell population (S/G2/M phase: 2n < DNA content < 4n) and an increase in the apoptotic cell population (DNA content < 2n) of transduced 8226 or LP1 cells (Fig. 3C and 3D, and data not shown). These results indicate that Sox5 plays a role in regulating cell cycle progression in malignant B cells.

3.4. Sox5-BLM was localized in the nucleus of TRAF3^{-/-} mouse B lymphomas and transduced human multiple myeloma cells

Previous studies have shown that Sox5 is localized in the nucleus of chondrocytes, neurons, and oligodendrocytes [8]. To verify whether this is case and to elucidate where Sox5 exerts its roles in regulating cell cycle progression in malignant B cells, we examined the subcellular localization of Sox5 using a biochemical fractionation method. Cytosolic and nuclear extracts were prepared from mouse splenic B cells, TRAF3^{-/-} mouse B lymphomas, or transduced human multiple myeloma cells. We observed that Sox5-BLM protein was primarily localized in the nucleus of TRAF3^{-/-} mouse B lymphomas (Fig. 4A). Similarly, both Sox5-BLM and L-Sox5 were also predominantly localized in the nucleus of transduced human multiple myeloma 8226 and LP1 cells (Fig. 4B and data not shown). We also used an immunostaining method to verify the predominant nuclear localization of Sox5-BLM and L-Sox5 in transduced human multiple myeloma cells (Supplementary Fig. 3). These data suggest that Sox5 may function as a transcription factor or transcriptional regulator in malignant B cells.

3.5. Overexpression of Sox5-BLM up-regulated the protein levels of p27 and β -catenin in transduced human multiple myeloma cells

To understand the mechanisms of Sox5-mediated regulation of cell cycle progression, we measured the protein levels of a number of cell cycle and survival regulators in human multiple myeloma 8226 and LP1 cells transduced with Sox5-BLM or L-Sox5. We found

that overexpression of either Sox5-BLM or L-Sox5 led to specific up-regulation of the protein levels of the cell cycle inhibitor p27, but not other cell cycle and survival regulators examined, including p21, cyclin D1, cyclin D2, cyclin B1, c-Myc, p53, Bcl-xL, Mcl1, Bcl2, Bim, and Bad (Fig. 4C and data not shown). In light of the evidence that Sox5 regulated p27 protein levels via the Akt or β -catenin pathway [18,31], we further examined the levels of phosphorylated and total Akt and β -catenin in transduced human multiple myeloma cells. We found that overexpression of either Sox5-BLM or L-Sox5 caused robust up-regulation of the total protein levels of β -catenin, but not Akt (Fig. 4C). Thus, Sox5-BLM and L-Sox5 appear to function equivalently in malignant B cells. Furthermore, our findings suggest that Sox5 inhibits cell cycle progression by up-regulating p27 and β -catenin protein levels in human multiple myeloma cells.

4. Discussion

Intragenic and complete deletions of the SOX5 gene, either inherited or *de novo*, have recently been identified as causal genetic alterations in human patients with developmental delay, intellectual disability, and behavior abnormality [32-34]. In contrast, amplification of the SOX5 gene has been reported in human prostate cancer [35] and testicular seminomas [36]. Additionally, up-regulation of SOX5 has been shown in several other human cancer types, including nasopharyngeal carcinoma [37] and melanoma [38]. Contradictory up-regulation [39] and down-regulation [31,40] of SOX5 expression have been documented in human glioma.

In the present study, we demonstrated the striking up-regulation of Sox5 expression in primary TRAF3^{-/-} mouse B lymphomas. Expression of Sox5 proteins was not detected in normal splenic B cells purified from LMC mice or premalignant B cells purified from tumor-free young B-TRAF3^{-/-} mice, even after treatment with a variety of stimuli of B cell survival, proliferation and activation. This suggests that aberrant up-regulation of Sox5 is associated with B lymphomagenesis. However, we did not detect SOX5 protein expression in human multiple myeloma cell lines 8226 and LP1, which contain bi-allelic deletions and frameshift mutations of the TRAF3 gene respectively. The striking up-regulation of Sox5 in TRAF3^{-/-} mouse B lymphomas together with the absence of SOX5 in human multiple myeloma cell lines with TRAF3 deletions/mutations prompted us to further examine SOX5 expression in other malignant murine and human B cells. We thus surveyed the gene expression omnibus (GEO) database and the public gene expression database of human cancers (<http://www.oncomine.org>), and found that SOX5 expression is also aberrantly and significantly up-regulated in a variety of other murine and human B cell malignancies. These include diffuse large B cell lymphoma developed in Brd2-transgenic mice (GEO accession number: GSE6136) [41], multiple myeloma developed in XBP-1 transgenic mice (GEO accession number: GSE6980) [42], human follicular lymphoma [43], and human hairy cell leukemia (a sub-type of chronic lymphoid leukemia of B lymphocytes) [44]. Hence, up-regulation of SOX5 appears to be frequently associated with B cell malignant transformation. How Sox5 is up-regulated during TRAF3^{-/-} B lymphomagenesis remains unclear. Using Taqman gene copy number assay, we found that the Sox5 gene copy number was not altered in TRAF3^{-/-} mouse B lymphomas (data not shown). Consistent with our observation, the SOX5 gene copy number is not changed in human multiple myeloma [45,46] and marginal zone lymphoma either [47]. Interestingly, up-regulation of SOX5 expression in a case of human follicular lymphoma is due to a chromosomal translocation t(X;12), which fuses the promoter region of the G-protein coupled purinergic receptor P2Y8 gene with the SOX5 coding sequence [25]. Future study is required to investigate whether similar chromosomal translocation involving Sox5 occurs in TRAF3^{-/-} mouse B lymphomas. Alternatively, transcriptional activation of Sox5 may be acquired by activation or up-regulation of its upstream transcription factors, inactivation or down-regulation of its

upstream transcriptional repressors, or alterations in epigenetic modifications during TRAF3^{-/-}B lymphomagenesis. Such mechanisms need to be further elucidated.

By cloning the coding sequence of the Sox5 cDNA expressed in TRAF3^{-/-}mouse B lymphomas, we found that it represents a novel isoform of Sox5, Sox5-BLM. Using subcellular fractionation, we found that this new isoform of Sox5 was localized in the nucleus of TRAF3^{-/-}mouse B lymphoma cells and transduced human multiple myeloma cells. Interestingly, in contrary to an oncogenic role as predicted by its striking up-regulation in TRAF3^{-/-}mouse B lymphomas, we found that overexpression of Sox5-BLM or L-Sox5 inhibited cell cycle progression in transduced human multiple myeloma cells. Furthermore, we found that overexpression of Sox5-BLM or L-Sox5 led to up-regulation of the cell cycle inhibitor p27 and another cell cycle regulator β -catenin in human multiple myeloma cells. These results suggest that Sox5 may play a tumor suppressive role in malignant B cells and that Sox5 expression may be up-regulated as a counteracting mechanism during B lymphomagenesis. Alternatively, it remains possible that Sox5 is oncogenic in B lymphomagenesis, but human multiple myeloma cells do not provide permissive cellular context to allow Sox5 to exert its transforming activity. Similar phenomena have been previously observed for the potent oncogene c-Myc [48-50]. When introduced into non-permissive cells, c-Myc induces cell cycle arrest, senescence, or apoptosis [48-50]. A third possibility is that murine Sox5 may not be able to interact with human SOX5-interacting factors in a similar way as with murine factors, or murine Sox5 may even act as dominant negative factor in the human system, thereby causing different functional consequence due to inter-species incompatibilities. In this regard, we noticed that the protein sequence of murine L-Sox5 is 97% (740/763 aa) but not 100% identical to that of human L-SOX5. Nonetheless, our findings suggest that Sox5 plays a role in regulating the proliferation of malignant B cells, which could be negative or positive depending on the cellular context.

Corroborating our findings, both oncogenic and tumor suppressive roles have been reported for SOX5. In nasopharyngeal carcinoma, up-regulation of SOX5 appears to promote tumor progression by down-regulating the expression of the tumor suppressor gene SPARC [37]. In contrast, Sox5 suppresses PDGFB-induced glioma development in Ink4a^{-/-}mice [31]. Interestingly, increased levels of p27 upon Sox5 expression were also observed in PDGFB-induced glioma of Ink4a^{-/-}mice and in chicken developing neurons [18,31]. However, overexpression of Sox5 results in a decrease in the levels of the dephosphorylated active form of β -catenin in chicken developing neurons [18], but an increase in the levels of β -catenin in human multiple myeloma cells (Fig. 4C). Other cell cycle regulators downstream of Sox5 identified in previous studies, including cyclin D1, cyclin D2, Akt, and Myc [18,31], are not changed by Sox5 overexpression in human multiple myeloma cells. Future studies are required to decipher how Sox5 up-regulates the protein levels of p27 and β -catenin in malignant B cells. Sox5 does not have a transactivation domain [8]. It has been shown that Sox5 may promote or repress gene expression by interacting with SoxE proteins (such as Sox9 and Sox10), by recruiting transcriptional repressors (such as CtBP and HDAC1), by competing with other Sox proteins (such as Sox4 and Sox11) for target sites, or indirectly by regulating chromatin architecture [8,51,52]. Regardless of the exact mechanisms, p27 and β -catenin appear to be common downstream targets of Sox5 in regulating cell cycle progression of neurons, glioma, and multiple myeloma.

In summary, we found that a novel isoform of Sox5, Sox5-BLM, was aberrantly expressed in B lymphomas spontaneously developed in different individual B-TRAF3^{-/-}mice. This new isoform of Sox5 was localized in the nucleus of TRAF3^{-/-}mouse B lymphomas and transduced human multiple myelomas, and able to inhibit the proliferation of human multiple myeloma cells by up-regulating the protein levels of p27 and β -catenin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Up-regulation of Sox5 expression in TRAF3^{-/-} mouse B lymphomas. (A) Quantitative real time PCR analyses of the Sox5 transcript. Total cellular RNA was prepared from splenocytes of LMC mice, or splenic B lymphomas and ascites of diseased B-TRAF3^{-/-} mice. Real time PCR was performed using TaqMan primers and probes (FAM-labeled) specific for mouse Sox5. Each reaction also included the probe (VIC-labeled) and primers for β -actin, which served as endogenous control. Relative mRNA expression levels of the Sox5 gene were analyzed using the Sequencing Detection Software (Applied Biosystems) and the comparative Ct ($\Delta\Delta$ Ct) method. Graphs depict the results of two independent experiments with duplicate reactions in each experiment (mean $\pm$ S.D.). (B) Western blot analysis of the Sox5 protein. Total cellular proteins were prepared from purified LMC splenic B cells or splenic B lymphomas or ascites of different individual B-TRAF3^{-/-} mice. Proteins were immunoblotted for Sox5, followed by actin. (C and D) Potential regulation of the expression of the Sox5 gene in response to B cell stimuli. Splenic B cells were purified from 10- to 12- week-old LMC and tumor-free B-TRAF3^{-/-} mice. Purified B cells were cultured *ex vivo* in the absence or presence of stimuli of B cell survival, proliferation, and activation. B cell stimuli examined include: 2 μ g/ml anti-CD40, 20 μ g/ml LPS, 1 μ g/ml anti-BCR, and 100 nM CpG2084, alone or in combination. RNA and protein samples of primary TRAF3^{-/-} mouse B lymphomas (mouse ID: 7060-8 and 6983-2) were used as positive control of Sox5 mRNA and protein in these experiments. (C) Total cellular RNA was prepared at 6 hours after treatment, and analyzed for the Sox5 transcript level. Taqman assay of Sox5 was performed as described in (A). Graphs depict the results of two independent experiments with duplicate reactions in each experiment (mean $\pm$ S.D.). (D) Total cellular proteins were prepared at 24 hours after treatment, and immunoblotted for Sox5, followed by TRAF1 and actin. The TRAF1 blot was used as control of successful B cell stimulation, and the actin blot was used as loading control.

Figure 2. A novel isoform of Sox5 was expressed in TRAF3^{-/-} mouse B lymphomas
 The protein sequence encoded by the Sox5 cDNA cloned from TRAF3^{-/-} mouse B lymphomas (Sox5-BLM, 728 aa) is aligned with those of the long isoform of Sox5 (L-Sox5, 763 aa, accession no.: NM_011444.3), the short isoform of Sox5 (S-Sox5, 392 aa, accession no.: X65657.1), Sox5 variant 2 (Sox5 V2, 715 aa, accession no.: NM_001113559.2), and Sox5 variant 3 (Sox5 V3, 679 aa, accession no.: NM_001243163.1). Similar to Sox5 variant 3, Sox5-BLM contains a 35 aa deletion in the N-terminal region in front of the leucine zipper domain. However, Sox5 variant 3, but not Sox5-BLM, has an additional 49 aa deletion between the two coiled-coiled domains. The first coiled-coil domain (1st CC) is indicated with a solid black box, the second coiled-coil domain (2nd CC) is indicated with a dotted black box, the high mobility box (HMG) domain is marked with a dashed black box, the leucine zipper (LZ) domain is shown in a dashed grey box, and the glutamine-rich domain (Q box) is highlighted with a solid grey box.

Figure 3. Overexpression of Sox5-BLM inhibited cell cycle progression in human multiple myeloma cells

Human multiple myeloma cell line 8226 cells were transduced with lentiviral expression vectors of Sox5-BLM (pUB-Sox5-BLM) or L-Sox5 (pUB-L-Sox5). Cells transduced with an empty lentiviral expression vector (pUB-Thy1.1) were used as control in these experiments. **(A)** Transduction efficiency analyzed by Thy1.1 staining and flow cytometry. Gated population (Thy1.1+) indicates cells that were successfully transduced with the lentiviral expression vector. **(B)** Expression of the transduced Sox5 proteins analyzed by Western blot analysis. Total cellular proteins were prepared at 4 days post transduction, and then immunoblotted for Sox5, followed by actin. Molecular weights of the Sox5 proteins expressed in transduced 8226 cells were compared to those expressed in primary TRAF3^{-/-} mouse B lymphomas (mouse ID: 115-6 and 7060-8). Actin blot was used as loading control. **(C)** Representative FACS histograms of cell cycle analysis. Cell cycle distribution of transduced 8226 cells was analyzed by PI staining and flow cytometry at 5 days post transduction. Gated populations indicate apoptotic cells (sub-G0: DNA content < 2n) and proliferating cells (S/G2/M phase: 2n < DNA content < 4n). **(D)** Statistical analysis of the results of PI staining. The graph depicts the results of 3 independent experiments

(mean $\pm$ S.D.). P values were analyzed using Student's *t*test, and are indicated above bar graphs by asterisks. *, significantly different from pUB-Thy1.1 control (*t*test, $p < 0.05$). Similar results were obtained in another human multiple myeloma cell line LP1 cells.

Figure 4. Predominant nuclear localization and signaling pathways of Sox5-BLM in malignant B cells

(A) Nuclear localization of endogenous Sox5 in primary TRAF3^{-/-} mouse B lymphomas. Splenic B cells were purified from LMC (samples 1 to 3) or tumor-free young B-TRAF3^{-/-} mice (samples 4 to 6), or splenic B lymphomas of diseased B-TRAF3^{-/-} mice (samples 7 to 8). (B) Nuclear localization of transduced Sox5 in human multiple myeloma cells. Human multiple myeloma cell line 8226 cells were transduced with lentiviral expression vectors of Sox5 (pUB-Sox5-BLM or pUB-L-Sox5) or a control lentiviral expression vector (pUB-Thy1.1). Cytosolic and nuclear extracts were fractionated using a biochemical method, and immunoblotted for Sox5, followed by HDAC1 and actin. The actin blot was used as loading control for cytosolic proteins, and the HDAC1 blot was used as loading control for nuclear proteins. (C) Up-regulation of p27 and β-catenin protein levels by Sox5 overexpression in human multiple myeloma cells. Human multiple myeloma cell line 8226 cells were transduced with lentiviral expression vectors of Sox5 (pUB-Sox5-BLM or pUB-L-Sox5) or a control lentiviral expression vector (pUB-Thy1.1). Total cellular lysates were prepared and immunoblotted for p27, p21, cyclin D1, cyclin D2, phosphorylated β-catenin (Phospho-β-catenin), β-catenin, phosphorylated-Akt (Phospho-Akt), Akt, c-Myc, Bcl-xL, and Mcl1, followed by Sox5 and actin. The Sox5 blot was used as control of Sox5 transduction, and the actin blot was used as loading control. Results shown are representative of two independent experiments, and similar results were also observed in another human multiple myeloma cell line LP1 cells.