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WW domain-containing oxidoreductase is involved in upregulation of matrix metalloproteinase 9 by Epstein–Barr virus latent membrane protein 2A

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ABSTRACT

WW domain-containing oxidoreductase (WOX1) participates in tumor suppression and many other biologic functions, but its molecular and functional interactions with viral proteins remain largely unknown. This study reveals that WOX1 is physically associated with latent membrane protein 2A (LMP2A), an oncoprotein of Epstein–Barr virus. The molecular interaction involves the tyrosine residue 33 of WOX1 and the proline-rich motifs of LMP2A. Interestingly, endogenous WOX1 is required for some LMP2A-triggered, cancer-promoting effects, including activation of extracellular signal-regulated kinase-1/2, upregulation of matrix metalloproteinase 9 (MMP9) and promotion of cell invasion. Upon knockdown of endogenous WOX1, LMP2A-triggered MMP9 induction is restored by exogenous wild-type WOX1, but not by a WOX1 mutant defective in LMP2A binding. These results indicate that, through interaction with LMP2A, WOX1 is involved in MMP9 induction, suggesting a novel role of WOX1 in Epstein–Barr virus-associated cancer progression.

1. Introduction

WW domain-containing oxidoreductase, designated WOX1, WWOX or FOR, is encoded by a putative tumor suppressor gene located at a chromosomal fragile site [1,2]. The role of WOX1 in tumor suppression is generally supported by its functions of inducing apoptosis and sequestering some oncoproteins [1–3]. Recent studies reveal that WOX1 also participates in many other biologic functions, including postnatal survival, normal bone metabolism, epidermal and neuronal differentiation, and responses to stress [4–6]. The diverse roles of WOX1 depend on cellular context and its binding partners, and most of the functions require protein–protein interaction through the first WW domain of WOX1 [1,3]. Within this domain, the tyrosine residue 33 of WOX1 has been shown to be critical for binding to partner proteins [7,8]. The WW domain prefers interaction with a proline-rich motif with the consensus amino acid sequence "PPXY", which is present in many WOX1-binding proteins [1].

Latent membrane protein 2A (LMP2A) of Epstein–Barr virus (EBV) is frequently expressed in EBV-associated human malignancies including nasopharyngeal carcinoma (NPC) [9,10]. Acting as a viral oncoprotein, LMP2A triggers multiple cellular signaling pathways and thus affects gene expression and biologic functions of host cells. Relevant to NPC pathogenesis, LMP2A promotes transformation, migration, invasion and a poorly differentiated phenotype of epithelial cells [9–11]. Most of these effects are attributed to the amino-terminal intracellular domain of LMP2A. This domain contains two PPPPY (PY) motifs, which have been found responsible for activation of oncogenic β-catenin signaling and inhibition of epithelial cell differentiation [12,13]. Our recent study indicates that the PY motifs of LMP2A are also critical for upregulation of matrix metalloproteinase (MMP)-9 and promotion of MMP9-dependent cell invasion [10]. In NPC cells, the PY motifs contribute to activation of extracellular signal-regulated kinase (ERK)-1/2 and a downstream transcription factor Fra-1, thus resulting in induction of MMP9 expression [10]. Nevertheless, the mediators

Abbreviations: EBV, Epstein–Barr virus; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; LMP2A, latent membrane protein 2A; MMP, matrix metalloproteinase; NPC, nasopharyngeal carcinoma; siRNA, small interfering RNA; WOX1, WW domain-containing oxidoreductase.

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linking the PY motifs of LMP2A to induction of the ERK1/2-Fra-1-MMP9 pathway have not been identified.

Thus far, molecular and functional interactions between WOX1 and viral proteins remain largely unknown. WOX1 is only documented once in the literature regarding its functional antagonism with a viral oncoprotein Tax [14]. Considering that EBV LMP2A has PY motifs that match with the WOX1-binding consensus sequence and trigger some oncogenic effects, we speculated that WOX1 may be associated with LMP2A physically and functionally. The present study demonstrated WOX1-LMP2A interaction and found that WOX1 is involved in LMP2A-induced MMP9 expression and cell invasion, suggesting a novel role of WOX1 in EBV-associated cancers.

2. Materials and methods

2.1. Plasmids, siRNAs and transfection

Two plasmids, pSG5-LMP2A expressing wild-type LMP2A and pSG5-LMP2A(ΔPY) expressing LMP2A with mutations at both PY motifs (from PPPPY into AAAAA), have been used in our previous study [10]. The plasmid pFlag-WOX1, which expresses Flag-tagged wild-type WOX1, was constructed by using a StrataClone N-terminal FLAG vector system (Stratagene). We adapted pFlag-WOX1 by using a QuikChange II XL site-directed mutagenesis kit (Stratagene) to generate the plasmid pFlag-WOX1(Y33R), where the codon of amino acid residue 33 of WOX1 is mutated from tyrosine into arginine. The plasmids expressing small interfering RNA (siRNA)-resistant wild-type WOX1 and mutated WOX1, designated WOX1-R(WT) and WOX1-R(Y33R), respectively, were also generated by using the mutagenesis approach. Their siRNA-targeted nucleic acid sequence 5′-GGTCTGGGAGGGATGTACTTCAACA-3′ was changed into 5′-GGCCTCGGAGGGATGTATTTTAATA-3′, without altering the amino acid residues. Two anti-WOX1 siRNAs (siW-1: 5′-GGUCUGGGAGGGAUGUACUUCAACA-3′ and siW-2: 5′-GCCCUCACCAGAAGCUCAGAGCGAA-3′) and a control siRNA with comparable GC content were purchased from Invitrogen. HONE-1, an EBV-negative epithelial cell line derived from NPC, was cultured in RPMI 1640 medium with 10% fetal bovine serum (Hyclone Laboratories).

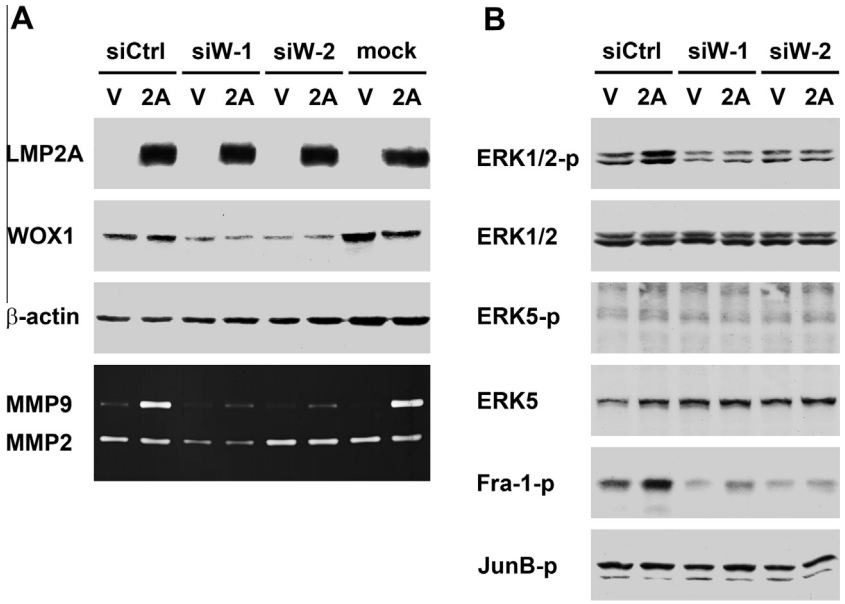

Fig. 1. WOX1 is required for the LMP2A-induced ERK1/2-Fra-1-MMP9 pathway. HONE-1 cells were transfected with a vector plasmid (V) or an LMP2A-expressing plasmid (2A), in combination with a mock control, a control siRNA (siCtrl), or WOX1-targeted siRNAs (siW-1 and siW-2). At 36 h posttransfection, cells and culture supernatants were harvested for the following experiments. (A) LMP2A, endogenous WOX1 and β-actin in the cell lysates were examined by using an immunoblotting assay. MMP9 and MMP2 proetins in the cell culture supernatants were detected by using a gelatin zymography assay. (B) An immunoblotting assay was performed to detect phosphorylated/activated forms of ERK1/2 (ERK1/2-p), ERK5 (ERK5-p), Fra-1 (Fra-1-p), and JunB (JunB-p) and total ERK1/2 and ERK5.

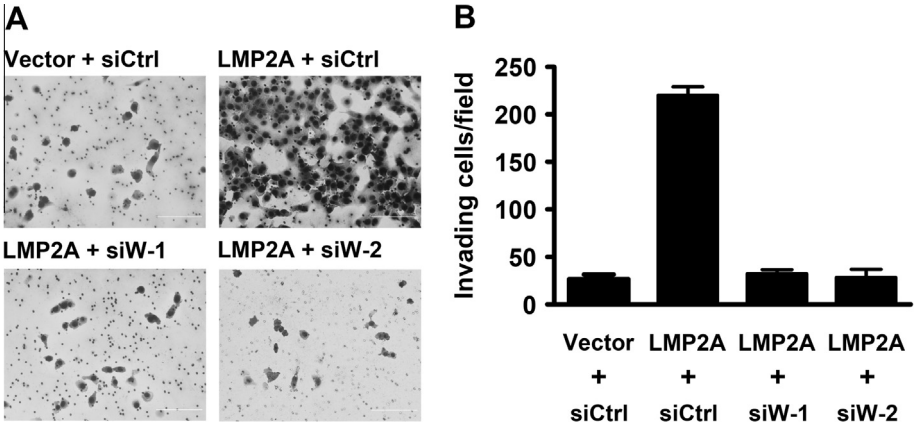

Fig. 2. WOX1 is required for LMP2A-induced cell invasion. HONE-1 cells were transfected with a vector plasmid or a plasmid expressing LMP2A, in combination with a control siRNA (siCtrl) or WOX1-targeted siRNAs (siW-1 and siW-2). Cell invasiveness through ECM was examined by using a Matrigel invasion assay. Shown are the invading cells under a microscope (A) and the average numbers of invading cells per microscopic field (B).

Transfection of the NPC cells with plasmid DNA and/or siRNA was performed by using Lipofectamine 2000 reagent (Invitrogen) as described previously [10].

2.2. Immunoblotting assay and gelatin zymography assay

Cells and their culture supernatants were harvested at 36 h posttransfection. Cell lysate preparation and the immunoblotting assay were performed as described previously [10,15]. Primary antibodies for detection of LMP2A and endogenous WOX1 were purchased from Santa Cruz Biotechnology; antibodies recognizing phosphorylated and total ERKs, phosphorylated Fra-1 and phosphorylated JunB were from Cell Signaling; an anti-Flag antibody was from Roche; an anti-β-actin antibody was from Chemicon. MMP9 and MMP2 proteins in cell culture supernatants were detected by using a gelatin zymopgraphy assay as described in our previous study [10,16].

2.3. Matrigel invasion assay

To test cell invasiveness in extracellular matrix (ECM), the cells transfected for 24 h were re-seeded onto the upper chamber of BD Biocoat Matrigel invasion chamber (Becton Dickinson) with serum-free medium, while 10% fetal bovine serum in the lower chamber was used as the chemoattractant. After incubation for additional 24 h, the cells remaining on the upside of the ECM-coated membrane were removed by swabbing and the cells invading to the underside were fixed with methanol for 20 min and stained with Giemsa dye (Merck) for 1 h. Numbers of stained cells in five randomly-chosen microscopic fields were counted and the cell invasiveness was expressed as average numbers of invading cells per field.

2.4. Immunoprecipitation assay

Cells were transfected with plasmids expressing Flag-WOX1 and LMP2A for 48 h and then lysed with RIPA buffer (Sigma) containing protease inhibitors (Roche). For each immunoprecipitation reaction, cell lysates (500 μg in 500 μl RIPA buffer) were incubated with 3 μg rabbit anti-Flag IgG (Cell Signaling) or a control rabbit antibody at 4 °C overnight, and then the immunocomplexes were precipitated with 50 μl magnetic Dynabeads conjugated with protein A (Invitrogen). After washing for three times with phosphate-buffered saline containing 0.01% NP-40, the immunoprecipitates were resolved in protein loading buffer and analyzed by the immunoblotting assay using primary mouse antibodies detecting Flag-WOX1 and LMP2A (Roche) and a secondary goat anti-mouse, light chain-specific antibody (Jackson).

3. Results

3.1. WOX1 is required for LMP2A-induced ERK1/2 activation, MMP9 expression and cell invasion

LMP2A upregulates an ERK1/2-Fra-1-MMP9 pathway in NPC cells through its PY motifs [10], which match with the putative WOX1-binding sequence "PPXY" [1]. Therefore, we first tested whether WOX1 is functionally involved in the LMP2A-induced effect. As the NPC cell line HONE-1 constitutively expressed WOX1 (Fig. 1A), we knocked down the endogenous WOX1 by using RNA interference. While a control siRNA did not interfere with LMP2A-mediated induction of MMP9, two WOX1-targted siRNAs largely reduced the MMP9 induction (Fig. 1A). Knockdown of WOX1 also inhibited phosphorylation of ERK1/2 and Fra-1 (Fig. 1B), indicating that, upstream of the MMP9 induction, WOX1 is required for LMP2A-triggered activation of the signaling event (ERK1/2) and the transcription factor (Fra-1). ERK5 and its downstream transcription factor JunB have been reported to upregulate MMP9 expression [17], but knockdown of WOX1 did not affect phosphorylation (i.e., activation) of ERK5 and JunB (Fig. 1B). Accordingly, WOX1 seems to be specifically involved in the ERK1/2-Fra-1-MMP9 pathway. On the other hand, ectopic WOX1 expression did not further increase LMP2A-induced MMP9

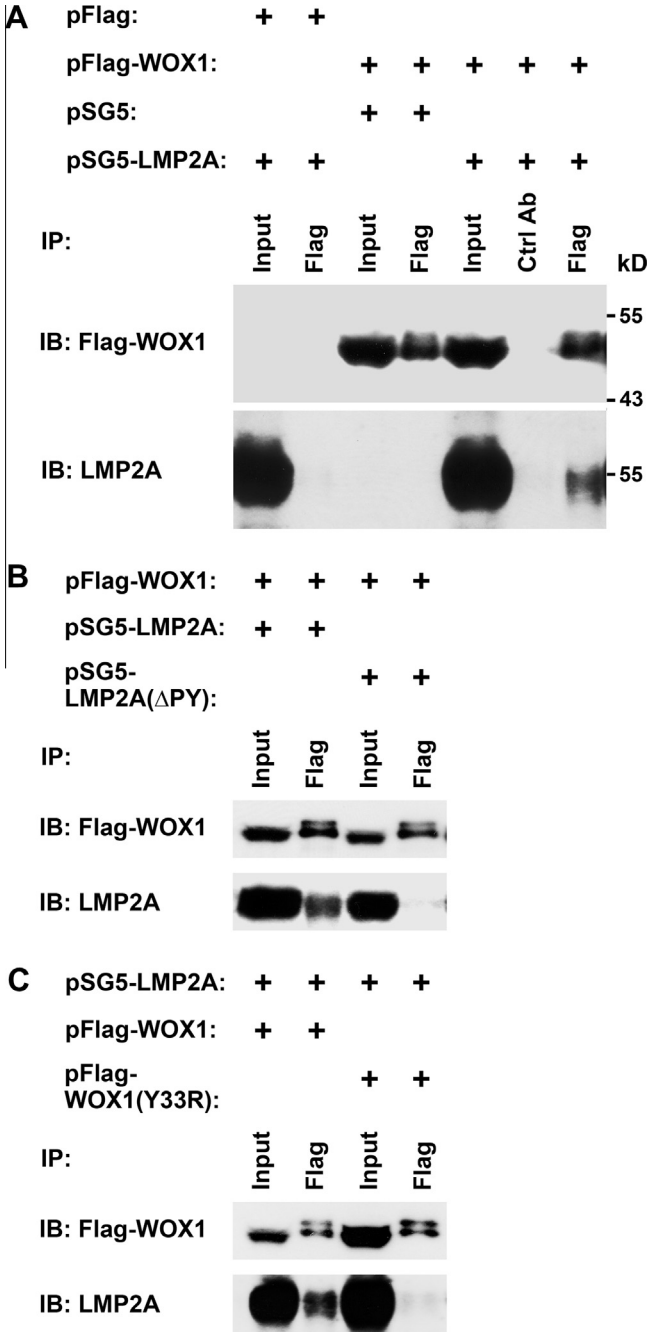

Fig. 3. WOX1 physically interacts with LMP2A. HONE-1 cells were transfected with vector plasmids (pFlag and pSG5) or expression plasmids [pFlag-WOX1, pFlag-WOX1(Y33R), pSG5-LMP2A and pSG5-LMP2A(ΔPY)] as indicated. At 48 h post-transfection, cells were harvested for an immunoprecipitation assay (IP) by using an anti-Flag antibody (Flag) or a control antibody (Ctrl Ab). The immunoprecipitates were examined by using an immunoblotting assay (IB) for detecting Flag-WOX1 and LMP2A. (A) LMP2A was coimmunoprecipitated with Flag-WOX1. (B) Association of LMP2A with Flag-WOX1 was largely lost when PY motifs of LMP2A were disrupted (ΔPY). (C) The WOX1-LMP2A association was also largely diminished when the tyrosine residue 33 of Flag-WOX1 was mutated into arginine (Y33R).

expression (data not shown), suggesting that endogenous WOX1 is sufficient to mediate LMP2A's effect.

LMP2A enhances invasiveness of NPC cells in ECM through MMP9 [10], so we further tested the biologic significance of endogenous WOX1 in LMP2A-induced cell invasion. In a Matrigel invasion assay, knockdown of WOX1 inhibited the cell invasiveness induced by LMP2A (Fig. 2), supporting that WOX1 contributes to the proinvasive effect of LMP2A. Together, these results indicate that WOX1 is essential for LMP2A-induced ERK1/2 activation and MMP9 expression, thus participating in promotion of cell invasion.

3.2. WOX1 is physically associated with LMP2A

Next we examined molecular binding between WOX1 and LMP2A by using coimmunoprecipitation. Owing to unavailability of a workable antibody for immunoprecipitation of endogenous WOX1, we used an anti-Flag antibody to test binding of Flag-tagged WOX1 to LMP2A. For the cells expressing both proteins, LMP2A was coimmunoprecipitated with Flag-WOX1 (Fig. 3A). It seemed that not all LMP2A was associated with Flag-WOX1, which may be attributed to the presence of endogenous WOX1 or other WW domain-containing cellular proteins distracting LMP2A binding. As PY motif is the putative WOX1-binding site [1], WOX1-LMP2A association was abolished when both PY motifs of LMP2A were mutated (Fig. 3B). As the tyrosine residue 33 in the first WW domain of WOX1 is critical for WOX1 binding to partner proteins [7,8], mutation of this tyrosine residue also largely diminished the association between WOX1 and LMP2A (Fig. 3C). Therefore, WOX1 is physically associated with LMP2A, which requires the tyrosine residue 33 of WOX1and the PY motifs of LMP2A.

3.3. Upon knockdown of endogenous WOX1, the LMP2A-triggered effects are restored by exogenous wild-type WOX1, but not by a LMP2A-binding-defective WOX1 mutant

Next we tested, upon knockdown of endogenous WOX1, whether LMP2A-induced MMP9 expression can be restored by exogenous wild-type WOX1 or LMP2A-binding- defective WOX1 mutant. For this purpose, we constructed siRNA (siW-1)-resistant wild-type WOX1 and mutated WOX1 through site-directed mutagenesis without altering the amino acid sequence (Fig. 4A). In the absence of LMP2A, ectopic expression of WOX1 did not induce MMP9 expression, whether WOX1 was wild-type or mutated, and whether or not it was resistant to siRNA (Fig. 4B), indicating that WOX1 alone is not sufficient for MMP9 induction. Of note, when LMP2A was present and endogenous WOX1 was knocked down, siRNA-resistant wild-type WOX1 significantly restored ERK1/2 activation, Fra-1 phosphorylation and MMP9 production (Fig. 4C). By contrast, LMP2A-binding-defective WOX1, of which the tyrosine residue 33 was mutated, restored LMP2A's effect very weakly (Fig. 4C). This result supports that molecular interaction between WOX1 and LMP2A is required for activation of the ERK1/2-Fra-1-MMP9 pathway.

4. Discussion

This study reveals WOX1 as a new cellular factor that is associated with LMP2A and mediates functions of this EBV oncoprotein. Although PY motifs of LMP2A are critical for triggering some signaling pathways and oncogenic effects, the mediators bridging the PY motifs and the downstream effects remain largely unknown. A previous study shows that ΔNp63α interacts with the PY motifs and contributes to LMP2A-induced inhibition of epithe-

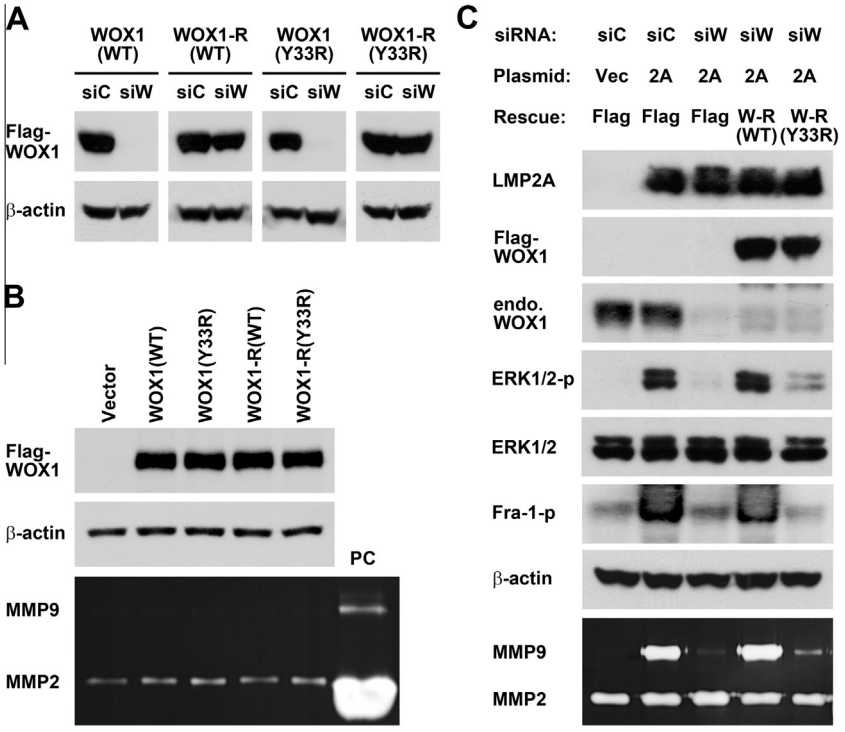

Fig. 4. Upon knockdown of endogenous WOX1, the LMP2A-induced ERK1/2-Fra-1-MMP9 pathway is restored by exogenous wild-type WOX1, but not by a LMP2A-binding-defective WOX1 mutant. Flag-tagged WOX1(WT) and WOX1(Y33R) are susceptible to knockdown by a WOX1-targeted siRNA (siW-1 in Fig.1), while WOX1-R(WT) and WOX1-R(Y33R) are their counterparts resistant to the siRNA. (A) HONE-1 cells were transfected with indicated expression plasmids in combination with a control siRNA (siC) or a WOX1-targeted siRNA (siW). (B) Cells were transfected with indicated plasmids (in the absence of siRNA and LMP2A). (C) Cells were cotransfected with indicated siRNAs and expression plasmids, in combination with the rescue plasmids expressing Flag, Flag-tagged wild-type WOX1 [W-R(WT)] or mutated WOX1 [W-R(Y33R)]. At 36 h posttransfection, cells and culture supernatants were harvested. Intracellular proteins were detected by using an immunoblotting assay, and MMP9 and MMP2 proteins in cell culture supernatant were detected by using a gelatin zymography assay. In panel B, a positive control (PC) for MMP9 and MMP2 was included.

lial cell differentiation [12]. PY motifs of LMP2A also recruit some WW domain-containing ubiquitin ligases that act as negative regulators of signal transduction [18,19]. Here we found that WOX1 is a WW domain-containing, PY motif-binding protein that functions as a positive mediator of the LMP2A-trggered ERK1/2-Fra-1-MMP9 pathway (Fig. 1). Since WOX1 can be recruited to membranous fractions and mediate signal transduction from certain membrane receptors [20–22], it may mediate ERK1/2 activation upon interacting with LMP2A. The detailed mechanism remains to be elucidated. Notably, effects of WOX1 on the ERK pathway are likely to be context-dependent, as WOX1 has been shown to downregulate ERK activation in T lymphocytes and neural cells [23,24].

This study provides new information about molecular and functional interactions between WOX1 and viral proteins. The functional antagonism between WOX1 and Tax, a viral oncoprotein of human T-cell leukemia virus type I, has been reported recently [14]. WOX1 suppresses Tax-induced canonical NF-κB signaling and tumorigenesis. To counteract WOX1's effect, Tax activates noncanonical NF-κB signaling to repress expression of WOX1. However, it is unknown whether WOX1 binds to any viral protein previously. In this study, we demonstrated physical association between WOX1 and EBV LMP2A (Fig. 3). PY motif-mutated LMP2A, which fails to interact with WOX1, cannot trigger the ERK1/2-Fra-1-MMP9 pathway [10]. Meanwhile, upon knockdown of endogenous WOX1, the LMP2A-induced effects can be restored by exogenous wild-type WOX1, but not by a LMP2A-binding-defective WOX1 mutant (Fig. 4). These results support that molecular interaction between WOX1 and LMP2A is functionally important for ERK1/2 activation and MMP9 induction.

Although many studies indicate that WOX1 functions as a tumor suppressor both *in vitro* and *in vivo* [1,2], some clues suggest that WOX1 may lose its tumor-suppressive effects or even promote cancer progression under certain circumstances. For example, while WOX1 expression is frequently reduced in tumor specimens and cancer cell lines, normal or even elevated expression of WOX1 protein has been reported in some other cases [25–27]. As NPC cells constitutively expressed WOX1 in our study, the tumor-suppressive effects of WOX1 may be inactivated, tolerated or altered therein. In line with this notion, we did not identify any proapoptotic or antiproliferative effect of WOX1 on NPC cells (data not shown). Intriguingly, although WOX1 suppresses attachment of ovarian cancer cells to ECM [28], it promotes migration of a breast cancer cell line through ECM and the bone metastasis of breast carcinoma shows increased expression of WOX1 [26,29]. Therefore, a metastasis-promoting effect of WOX1 may occur in a context-dependent manner. It is supported by our study showing that WOX1 is required for LMP2A-induced cell invasion (Fig. 2). We propose that LMP2A may adapt WOX1 for ERK1/2 activation, MMP9 induction and promotion of cell invasion, resulting in a novel role of WOX1 in EBV-associated pathogenesis. On the other hand, both LMP2A and WOX1 are also linked to regulation of keratinocyte differentiation and Wnt-β-catenin signaling [5,11,13,30], so we expect that WOX1-LMP2A interaction may be involved in additional biologic effects.

Acknowledgments

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