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RLIM interacts with Smurf2 and promotes TGF-β induced U2OS cell migration

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ABSTRACT

TGF-β (transforming growth factor-β), a pleiotropic cytokine that regulates diverse cellular processes, has been suggested to play critical roles in cell proliferation, migration, and carcinogenesis. Here we found a novel E3 ubiquitin ligase RLIM which can directly bind to Smurf2, enhancing TGF-β responsiveness in osteosarcoma U2OS cells. We constructed a U2OS cell line stably over-expressing RLIM and demonstrated that RLIM promoted TGF-β-driven migration of U2OS cells as tested by wound healing assay. Our results indicated that RLIM is an important positive regulator in TGF-β signaling pathway and cell migration.

1. Introduction

TGF-β (transforming growth factor-β) belongs to a superfamily containing more than 30 structurally related cytokines. Based on the differences in ligand structures, activation process and transduction components, this superfamily can be divided into two subfamilies, TGF-β subfamily and BMP subfamily. While the former contains TGF-β, activin and Nodal, the latter includes BMPs (bone morphogenetic proteins), GDFs (Growth differentiation factors) and AMH (anti-mullerian hormone) [1]. Cytokines in TGF-β superfamily bind to type II and type I serine/threonine kinase receptors, and transmit signals through Smad proteins [1,2]. TGF-β signaling is tightly regulated at multiple levels, and its deregulation was found to be associated with several human diseases, including cancer development. Smad is regulated by ubiquitin-proteasomal degradation. And the E3 ligases, Smad ubiquitination-related factor 1 (Smurf1) and Smurf2 have been implicated in Smad degradation [3]. In addition, using Smad proteins as adaptors, Smurf1/2 regulates TGF-β signaling via targeting other signaling molecules, including the TGF-β receptor complex and the transcriptional repressor, SnoN [4]. Thus, by controlling the level of Smads as well as its upstream receptors, Smurf1/2 provides a fine control of the signal output [5].

TGF-β maintains tissue homeostasis and prevents preliminary tumors from progressing into malignancy by regulating not only cellular proliferation, differentiation, survival, and adhesion, but also the cellular microenvironment [6,7]. However, as genetically unstable entities, cancer cells could avoid or even could alternate the suppressive influence of the TGF-β pathway. In this case, pathological forms of TGF-β signaling promote tumor growth and evasion of immune surveillance, resulting in cancer cell metastasis and propagation [6,7].

RLIM (RING finger LIM domain-binding protein) was originally identified as an E3 ubiquitin ligase capable of targeting CLIM for proteasome-dependent degradation, thereby inhibiting developmental LIM homeodomain activity [8,9]. RLIM also enhances transcriptional activation of endogenous estrogen receptor alpha (ERalpha) target genes. Since signaling pathways controlled by ERalpha are profoundly implicated in mammary ontogenesis, RLIM may play an important role during the development of human cancer.

However, whether and how RLIM affects TGF-β signaling during carcinogenesis has not been determined so far. In this study, we found that RLIM up-regulated TGF-β signaling by TGF-β inducible luciferase reporter assays. We also confirmed that RLIM interacted with TGF-β signaling inhibitor Smurf2 *in vivo* and *in vitro*. Moreover, we demonstrated that RLIM promoted TGF-β-driven migration of U2OS cells. In sum, our results showed that RLIM is an important positive regulator in TGF-β pathway and cell migration.

2. Materials and methods

2.1. Cell culture, plasmids and transfection

Osteosarcoma U2OS cells were obtained from the American Type Culture Collection and maintained in McCoy's 5A medium with 10% FBS. Myc-Smurf2 was kindly provided by Dr. Kohei Miyazono (University of Tokyo, Japan) and subcloned into pGEX-4T-2 vector to add GST tag. cDNA of RLIM was introduced into

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pCMV-HA vector (Clontech) and was then cloned into pCMV-Myc vector and pET28a vector (Clontech). Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions.

2.2. Immunoprecipitation and immunoblotting

Cells were lysed with 1× cell lysis buffer (cell signaling) and centrifuged. The supernatant was precleared by protein A/G beads (Sigma, USA), then incubated with indicated antibody and protein A/G beads overnight, all at 4 °C. Pellets were washed 5 times with lysis buffer and resuspended in sample buffer. Immunoprecipitates were subjected to 12% SDS–PAGE and proteins were transferred to nitrocellulose membranes (GE Healthcare, USA) in 190 mA for 2 h at 4 °C. The membrane was blocked for nonspecific binding in Tris-buffered saline (TBS pH 7.4) containing 5% non-fat milk and 0.1% Tween-20 for 1 h, washed twice in TBS, and incubated with primary and following secondary antibody for 2 and 1 h, respectively, at room temperature. Afterward, the proteins of interest were visualized using ECL chemiluminescence system (Santa Cruz Biotechnology, USA).

2.3. Transcriptional response assay

TGF-β-inducible luciferase reporter assay was conducted in U2OS cell that was transiently transfected with the reporter construct (CAGA-Luc), pCMV-galactosidase construct and the indicated expression constructs or with the empty vector alone. To induce the luciferase reporter, cells were treated overnight with 100 pM of TGF-β. Luciferase activity in cell lysates was measured using the luciferase assay system in a Berthold Lumat LB 9507 luminometer (Promega). Luciferase activity was normalized to galactosidase activity as an internal control. Each assay was performed in triplicate and results were confirmed by at least three individually repeating experiments.

2.4. Immunofluorescence

U2OS cell was transfected with GFP-RLIM and Myc-Smurf2, respectively. Thirty-six hours afterward, cells were fixed in 4% paraformaldehyde for 10 min. The fixed cells were treated with 0.2%Triton X-100 for 5 min and blocked in 10% horse serum plus 1% BSA (Amersham Biosciences, Piscataway, NJ) at room temperature for 1 h. The cells were incubated overnight with anti-Myc antibody diluting in 1% BSA at 4 °C. All the washing and diluting buffer was 1× PBS. After being washed 3 times in TBS containing 0.1% Tween-20, cells were incubated with Rhodamine Red-conjugated anti-mouse second antibody diluted in 1% BSA for 1 h. The cells were stained with DAPI for their nucleus. Fluorescent images were captured using Olympus Inverted Microscope System.

2.5. Establishment of the stably transfected U2OS cells

U2OS cells were separately transfected with pcDNA3.1-RLIM and/or pcDNA3.1 plasmids. Twenty-four hours after transfection, cells were trypsinized and diluted by 1/5, 1/10 and 1/20, respectively, into 100-mm dishes, and then subjected to 250 ng/ml G418 (Invitrogen, Carlsbad, USA) for 2 weeks. After G418 screening, the resisted colonies were separately trypsinized and transferred to 24-well plate for expansion. The expression of RLIM protein in different stable cell lines was determined by Western-blotting.

2.6. Cell migration assay

Stable RLIM-expressing or empty vector-bearing U2OS cells were plated in 6-well plates and cultured to confluence of 98%. Cells were scraped with a p200 tip (0 h), transferred to low serum and treated as described. Migrated cells were counted in five individual fields taken at each indicated time points and averaged.

3. Results

3.1. RLIM enhanced TGF-β signaling

We analyzed the effect of RLIM on TGF-β dependent transcription activation by TGF-β responsive promoter–reporter constructs CAGA-Luc. It was revealed that wild-type RLM enhanced the transcriptional activity induced by TGF-β (Fig. 1). The luciferase reporter assay showed that the TGF-β responsive promoter (containing CAGA box) is highly active in TGF-β treated cells and inactive in the cells absence of TGF-β treatment (Fig. 1). We found that the reporter activity reduced to about 25% by cotransfection with Smurf2 expression vector even in the presence of exogenous TGF-β (Fig. 1 Lane 1, 4, 6). Interestingly, reporter gene activity is greatly restored when RLIM co-transfected with Smurf2 (Fig. 1 Lane 5, 7). Collaborative interaction between Smurf2 and Smad7 results in inhibition of TGF-β signaling by degradation of the TGF-β receptors and R-Smads/Co-Smad proteins [10]. In this study, we demonstrated that RLIM interacted with Smurf2 (Section 3.2). Therefore, RLIM protein might affect TGF-β signal transduction by inhibiting interactions between Smad7 and Smurf2.

3.2. RLIM interacted with Smurf2 in vivo and in vitro

Immunofluorescence was applied to test the interaction between RLIM and Smurf2. U2OS cells were transfected with GFP-tagged RLIM and Myc-tagged Smurf2. Thirty-six hours after transfection, cells were treated as describe in Section 2. Fluorescent images showed that most proportions of RLIM and Smurf2 co-localized in the nucleus of U2OS cells (Fig. 2A). Furthermore, co-immunoprecipitation was used to confirm the interaction between RLIM and Smurf2. Expression plasmids encoding HA-RLIM and Myc-Smurf2 were transiently transfected into HEK293T cells,

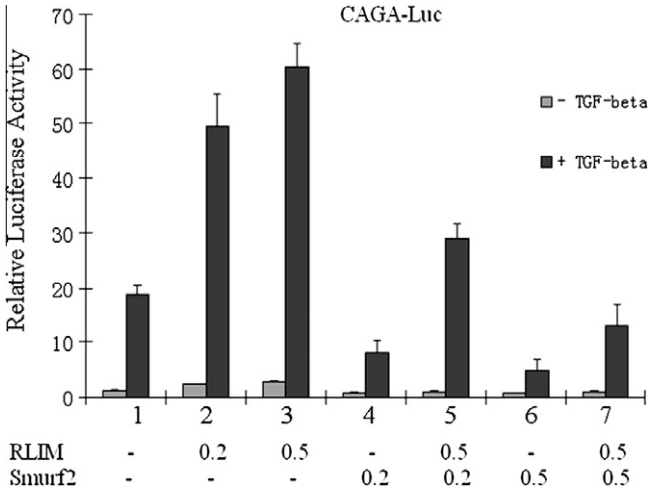

Fig. 1. RLIM enhances transcriptional activities of TGF-β signaling. U2OS cells were transiently transfected with 0.1 μg CAGA-Luc construct along with varying concentrations of RLIM and Smurf2 as indicated. Cells were incubated overnight in the absence or presence of 100 pM TGF-β and the relative luciferase activity was measured. Luciferase activity was expressed as the mean ± SD of triplicates from a representative experiment.

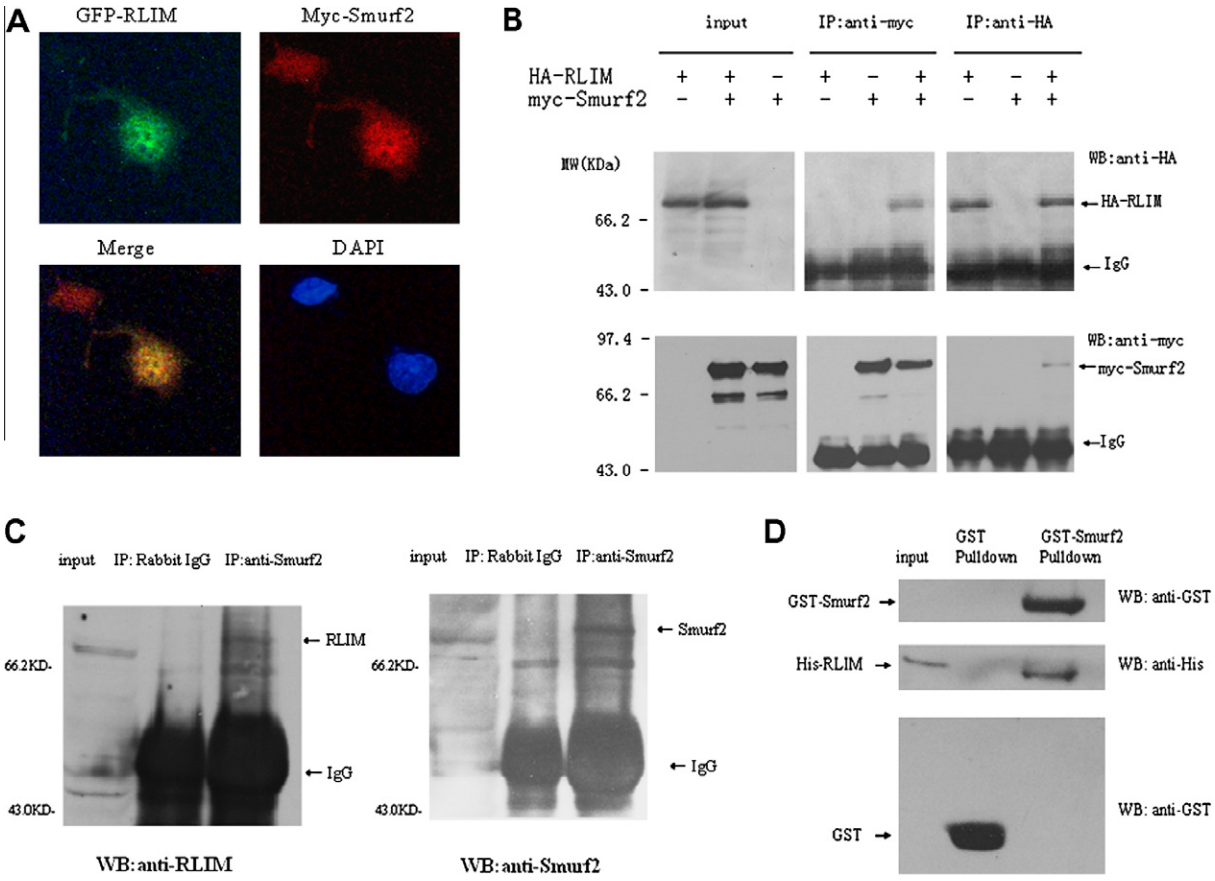

Fig. 2. RLIM interacts with Smurf2 *in vivo* and *in vitro*. (A) U2OS cells were transfected with GFP-RLIM and Myc-Smurf2, respectively. Thirty-six hours later, cells were fixed and subjected to indirect immunofluorescence analysis. (B) 293T cells were transfected with Myc-Smurf2 and HA-RLIM as indicated. Cell lysates were prepared and subjected to IP 36 h afterward with HA or Myc antibodies, respectively. The immunoprecipitates were analyzed by Western-blotting with anti-HA and anti-Myc antibodies, respectively. (C) Co-immunoprecipitation of endogenous RLIM and Smurf2 proteins in MG132 treated U2OS cells. (D) Bacterially expressed GST protein, GST fusion protein with Smurf2 and His-tagged fusion protein with RLIM were incubated and bound to glutathione-Sepharose beads as indicated. Bound His-RLIM, GST-Smurf2 were detected by Western-blotting with anti-His and anti-GST antibodies, respectively.

respectively. Thirty-six hours after transfection, cells were lysed and co-immunoprecipitation was carried out. As shown in Fig. 2B, Myc antibody successfully immunoprecipitated HA-RLIM. In a reciprocal experiment, antibody against HA was able to pull down Myc-Smurf2. These results revealed that Smurf2 and RLIM interact with each other *in vivo*.

Then we determined the interaction between Smurf2 and RLIM at the endogenous level. U2OS cells were first treated with 20 μM MG132 for 4 h. Endogenous Smurf2 was immunoprecipitated from cell lysate then, and we found the endogenous RLIM was detectable in the Smurf2 immunoprecipitates (Fig. 2C). Furthermore, an *in vitro* GST pulldown assay was also applied to demonstrate that purified GST-Smurf2 protein and His-RLIM protein directly interacted with each other (Fig. 2D).

3.3. RLIM promotes TGF-β induced migration of U2OS cells

Since RLIM could up-regulate TGF-β signaling, we sought to study the biologic effects of RLIM over-expression on the cells' response to TGF-β (Fig. 3). We used U2OS cells stably reconstituted with expression vectors encoding HA-tagged RLIM or the control vector. As shown in Fig. 3A, the band at approximately 70 kDa was corresponding to HA-tagged RLIM protein. This band could be detected in U2OS-RLIM cells but not in U2OS-vector cells (U2OS Null) by Western-blotting with the β-actin as the internal control. To examine if expression of RLIM conferred migratory properties to cells receiving TGF-β treatment, we used a wound healing assay in which cells are induced to migrate into a wound created by scratching confluent cultures with a pipette tip. After 30 h of TGF-β treatment, control U2OS cells had migrated slightly, while RLIM over-expressing cells almost invaded the wound completely (Fig. 3B). TGF-β treatment of U2OS cells over-expressing RLIM also caused a strikingly different phenotype compared with the control cells, as cells shed their cuboidal epithelial shape and acquired a more mesenchymal phenotype (Fig. 3C).

4. Discussion

RLIM is indentified as an important protein involved in X chromosome inactivation [11–13], embryogenesis [14] and neural tube development [15]. It is also reported that RLIM modulated telomere length homeostasis through the proteolysis of TRF1 [16].

Recently, RLIM's effects in cancer have been studied. RLIM and CLIM colocalize and interact with ERA (estrogen receptor alpha) in primary human breast tumors [17]. CLIM enhances transcriptional activity of LIM-HDs, whereas it inhibits transcriptional activation mediated by ERA on most target genes. In turn, RLIM inhibits transcriptional activity of LIM-HDs but enhances transcriptional activation of endogenous ERA target genes [17]. Since ERA exerts oncogenic effect by stimulating the proliferation of breast cancer cells through the activation of target genes, RLIM might also be a promoter in the breast carcinogenesis.

TGF-β regulates cell functions like cell growth and differentiation, and plays a key role in carcinogenesis [1]. Here, we first verified that RLIM was related to TGF-β signaling pathway. We showed that RLIM bound to Smurf2, a vital TGF-β pathway nega-

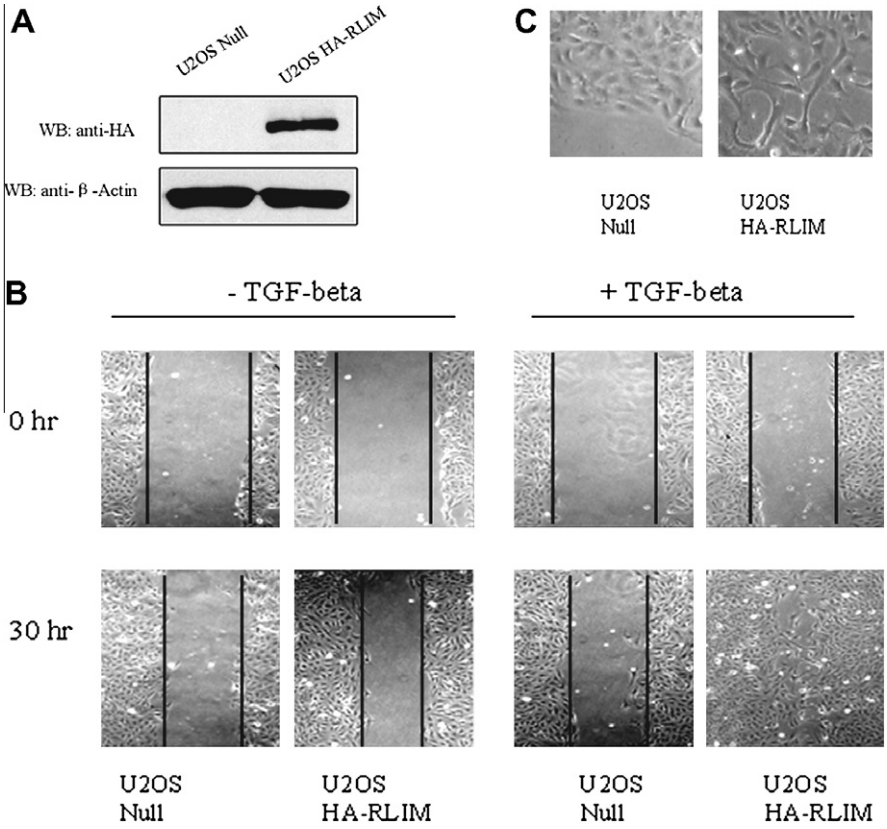

Fig. 3. RLM expression promotes TGF-β migratory responses. (A) The expression of RLIM protein in U2OS vector cells or U2OS HA-RLIM cells were confirmed by Western-blotting with the primary antibody of anti-HA and anti-β-actin. (B) Wound healing assays of U2OS cells showed the effect of RLIM on TGF-β-driven migration. (C) Effect of TGF-β1 (5 ng/ml for 30 h) on the morphology of U2OS cells.

tive regulator, thus activated TGF-β signaling. We also constructed a U2OS cell line stably expressing RLIM and demonstrated that RLIM promoted TGF-β driven migration of osteosarcoma U2OS cells through wound healing assay. Though RLIM could not directly increase the ubiquitination and degradation of Smurf2 (data not shown), our results showed that RLIM binds to Smurf2 and enhances TGF-β responsiveness of mammalian cells. This suggested that RLIM may possess similar cellular properties with R-Smads. It may also inhibit interactions between Smad7 and Smurf2 to prevent the degradation of TGF-β receptors.

It was reported that Smurf2 prevents migration of breast cancer cells. Knockdown of Smurf2 in human breast cancer MDA-MB-231 cells resulted in enhancement of cell migration *in vitro* and bone metastasis *in vivo* [18]. Furthermore, Smad2 and Smad3 induce tumor invasion and metastasis [19,20]. The interaction of RLIM and Smurf2 might be important because it restores TGF-β signaling and RLIM might function similarly to Smad2 and Smad3, especially in cancer cell metastasis. These abundant regulatory proteins, including growth factors and their receptors, adaptor proteins involved in signal transduction, transcription factors and cell cycle regulating proteins, are coordinated by balancing their synthesis and degradation. Identification of RLIM substrates will allow us to understand the explicit role of RLIM on tumorigenesis.

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