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Ubiquitin C-terminal hydrolase-L3 regulates EMT process and cancer metastasis in prostate cell lines

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ABSTRACT

Ubiquitin C-terminal hydrolase-L3 (UCH-L3) is among the deubiquitinating enzymes (DUBs) that cleave ubiquitin (Ub) from Ub precursors or protein substrates. Many DUBs have been shown to participate in cancer progression in various tissues. However, the mechanism and role of UCH-L3 in carcinogenesis has largely been unknown until recently. Here we investigated the implication of UCH-L3 in prostate cancer progression. Interestingly, UCH-L3 is upregulated in normal or non-metastatic prostate cancer cells and is downregulated in metastatic prostate cancer cell lines. Notably, knockdown of UCH-L3 in normal prostate cell line RWPE1 promotes epithelial-to-mesenchymal transition (EMT), an important process for cancer cell invasion and metastasis. The induction of EMT by UCH-L3 knockdown results in an increase of cell migration and invasion. Yet, to the contrary, overexpression of UCH-L3 in highly metastatic prostate cancer cell line PC3 reverses EMT but the active site mutant UCH-L3 did not. Collectively, our findings identify UCH-L3 as a novel EMT regulator in prostate cells and highlight UCH-L3 as a potential therapeutic target for preventing metastatic prostate cancer.

1. Introduction

Deubiquitinating enzymes (DUBs) remove ubiquitin (Ub) from protein substrates and Ub precursor proteins and function as cellular regulators implicated in numerous biological pathways such as cell growth and differentiation, development, chromosome structure and transcriptional regulation, and many diseases including cancer [1,2]. DUBs are subdivided into five subfamilies: the ubiquitin-specific proteases (UBPs), the ubiquitin C-terminal hydrolases (UCHs), the ovarian tumor proteases (OTUs), the Josephin or Machado-Joseph disease protein domain proteases (MJDs), and the Jab1/MPN domain-associated metalloisopeptidases [1,2]. The UCH enzymes prefer to cleave Ub from the relatively small protein substrates and have a core catalytic domain (~230 amino acid) consisting of conserved Cys, His, and Asp residues [3]. There are four members in UCH family: UCH-L1, UCH-L3, UCH-L5, and BAP1. Among them, UCH-L1 and UCH-L3 are most closely related members; 53% amino acid identity and significant structural similarity [4]. However, there are many distinct biochemical characteristics between them. In addition to the Ub cleavage activity, UCH-L1 possesses Ub ligase activity depending on its dimerization [5,6]. UCH-L1 associates with monoubiquitin and stabilizes Ub in neurons [7]. Conversely, UCH-L3 possesses neither dimerization nor Ub ligase activity. A unique feature of UCH-L3 is that this enzyme has dual hydrolase specificity towards Ub and Nedd8 [8]. UCH-L3 is expressed ubiquitously in all tissues but the expression of UCH-L1 is restricted to the testes, ovaries, and neurons [4,6,9].

The implication of UCH-L1 in cancer progression has been suggested in many tissues [10] and we have already reported that UCH-L1 promotes cancer cell invasion and metastasis through EMT induction in prostate cancer cells [11]. The possible involvement of UCH-L3 in carcinogenesis has been suggested in some reports. Upregulation of UCH-L3 expression was observed in breast cancer tissue and cervical carcinoma [12,13]. However, the detailed mechanism and role of UCH-L3 in oncogenesis has not been elucidated.

Cancer metastasis is the spread of tumor cells from its original place to distant sites of the body and is the primary cause of death in cancer patients. This process involves local invasion and migration of tumor cells, intravasation, survival in the bloodstream, and extravasation into distant organs and survival at the secondary sites [14]. The initiation of cancer metastasis is characterized by increased motility and invasiveness of cancer cells. By undergoing physiological changes such as epithelial-to-mesenchymal transition (EMT), tumor cells acquire invasive abilities [15]. EMT is a

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process that generally immotile epithelial cells convert to a motile mesenchymal phenotype. Acquisition of invasiveness by EMT has been implicated in tumor progression and metastasis. A remarkable molecular event of EMT is the down-regulation of E-cadherin. E-cadherin is a cell–cell adhesion molecule and is highly expressed in epithelial cells. Loss of E-cadherin expression or function is associated with cancer progression and metastasis [16,17]. There are several transcription factors that respond to EMT inducing signals and repress E-cadherin expression. These factors including Snail, Slug, and Twist [18,19] induce EMT and function as master regulators of the EMT process. In addition to these transcription factors, matrixmetalloproteases (MMPs) play their roles in EMT induction. The loss of E-cadherin during tumor progression is also mediated by proteolytic degradation by MMPs [20].

In this report, we explore the novel function of UCH-L3 in prostate cell invasion and metastasis. We found that UCH-L3 is differentially expressed in various prostate cell lines. UCH-L3 is expressed in normal and non-metastatic prostate cells and is greatly down-regulated in metastatic prostate cancer cell lines. Knockdown of UCH-L3 in the normal prostate cell line RWPE1 induces morphological and molecular events of EMT. Further, induction of EMT by UCH-L3 knockdown results in the increase of cell migration and invasion. Contrarily, overexpression of UCH-L3 in the highly metastatic prostate cell line PC3 reverses EMT depending on the deubiquitinating activity of UCH-L3. Taken together, our findings identify UCH-L3 as a novel EMT regulator in prostate cells and highlight UCH-L3 as a potential therapeutic target for preventing metastatic prostate cancer.

2. Materials and methods

2.1. Cell lines and cell culture

RWPE1 cells were maintained in Keratinocyte-Serum Free Medium supplemented with 12.5 mg/L bovine pituitary extract (BPE) and 1.25 μg/L EGF. PC3 cells were maintained in DMEM supplemented with 10% FBS.

2.2. Antibodies and immunoblotting

Antibodies were purchased from the manufacturers as follows: anti-UCH-L3 antibody (Abcam), anti-E-cadherin (BD Transduction Laboratories), anti-vimentin (Santa Cruz), anti-β-catenin (Santa Cruz), anti-β-actin (Sigma–Aldrich).

2.3. Generation of stable cell lines

The target sequence of the small hairpin RNA for UCH-L3 is 5′-GAAGTTTATGGAGCGCGAC-3′. The primers containing the short hairpin RNA sequence targeting the UCH-L3 were annealed and cloned into a pMSCVpuro vector. UCH-L3 or UCH-L3 C95S was also cloned into a pMSCVpuro vector. The resulting vectors were co-transfected with VSV-G and MLV vectors into HEK293 cell lines. The retroviruses were mixed with polybrene (8 μg/ml) and added into RWPE1 or PC3. Positive clones were then selected in puromycin (5 μg/ml) 48 h after infection.

2.4. Real time RT-PCR

The abundance of mRNA was detected by real-time quantitative RT-PCR using the ABI prism 7300 system and SYBR Green. The mRNA quantity of the specific gene was calculated using the $\Delta\Delta C_t$ method and normalized to the GAPDH. All measurements were performed in triplicate. The sequences of the primers were as described previously [11].

2.5. Wounding healing assay

Cells were seeded in six-well dishes at a density of 1×10^5 cells per well. The cell monolayers were scraped with a sterile yellow micropipette tip to create a denuded area of constant width. The wound closure was monitored and photographed at indicated times after wounding.

2.6. Transwell migration assay and in vitro invasion assay

Migration assays were performed using uncoated polycarbonate membranes with 8 μm pores in 24-well microchemotaxis chambers and 24-well Matrigel invasion chamber assay plate for *in vitro* invasion assay. 2×10^4 cells were loaded for migration assay and 2.5×10^4 cells were loaded for invasion assay. K-SFM with BPE and EGF supplemented with 15% FBS for RWPE1 and DMEM medium containing 15% FBS for PC3 was added to the bottom chamber as a chemoattractant. After incubation for 22 h, cells that had migrated to the lower surface of the filter were fixed and stained. Cells were counted in 9 random fields per insert.

3. Results

3.1. UCH-L3 is downregulated in metastatic prostate cancer cell lines

To address the role of UCH-L3 in prostate cancer progression, we assessed the expression of UCH-L3 in various prostate cancer cell lines. The protein level of UCH-L3 was high in RWPE1 (benign tumor) and RWPE2 (tumorigenic but non-metastatic) cells, but UCH-L3 was negligibly expressed in PC3 (androgen-insensitive, highly metastatic) and DU145 (androgen-insensitive, metastatic cancer) cells (Fig. 1A). However, there was no big difference in the level of UCH-L3 mRNA among these cell lines (Fig. 1B). Though the regulatory mechanism of UCH-L3 protein has not been elucidated yet, we assume the possibility of posttranslational regulation that explains the discordance between the protein and message level of UCH-L3. We have already shown that UCH-L1, a close relative of UCH-L3, induces EMT and promotes metastasis in prostate cancer cells [11]. Based on these results, we speculate that UCH-L3 might have a differential role in EMT progression which is important for metastasis in prostate cancer cells.

3.2. Knockdown of UCH-L3 in RWPE1 induces EMT

To verify the involvement of UCH-L3 in EMT process and metastasis, we generated UCH-L3 knockdown RWPE1 cells by expression of shRNA specific to UCH-L3. We found that UCH-L3 knockdown cells grow in a more scattered fashion and cell–cell interaction was decreased when compared to the mock or MSCV vector expressing cells (Fig. 2A). To further determine whether the morphological changes of UCH-L3 knockdown cells are linked to the EMT process, we examined the expression of epithelial markers

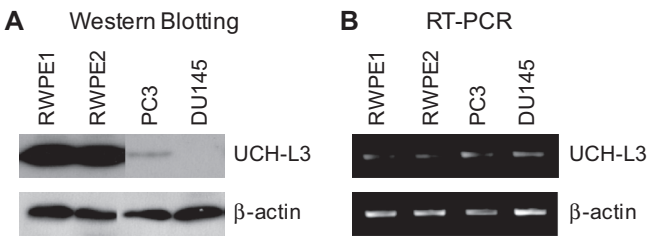

Fig. 1. UCH-L3 is downregulated in the highly metastatic prostate cancer cell lines. (A) The expression of UCH-L3 was analyzed by Western blotting in various prostate cancer cells. β-Actin was used as a loading control. (B) The message level of UCH-L3 was analyzed by semi-quantitative RT-PCR.

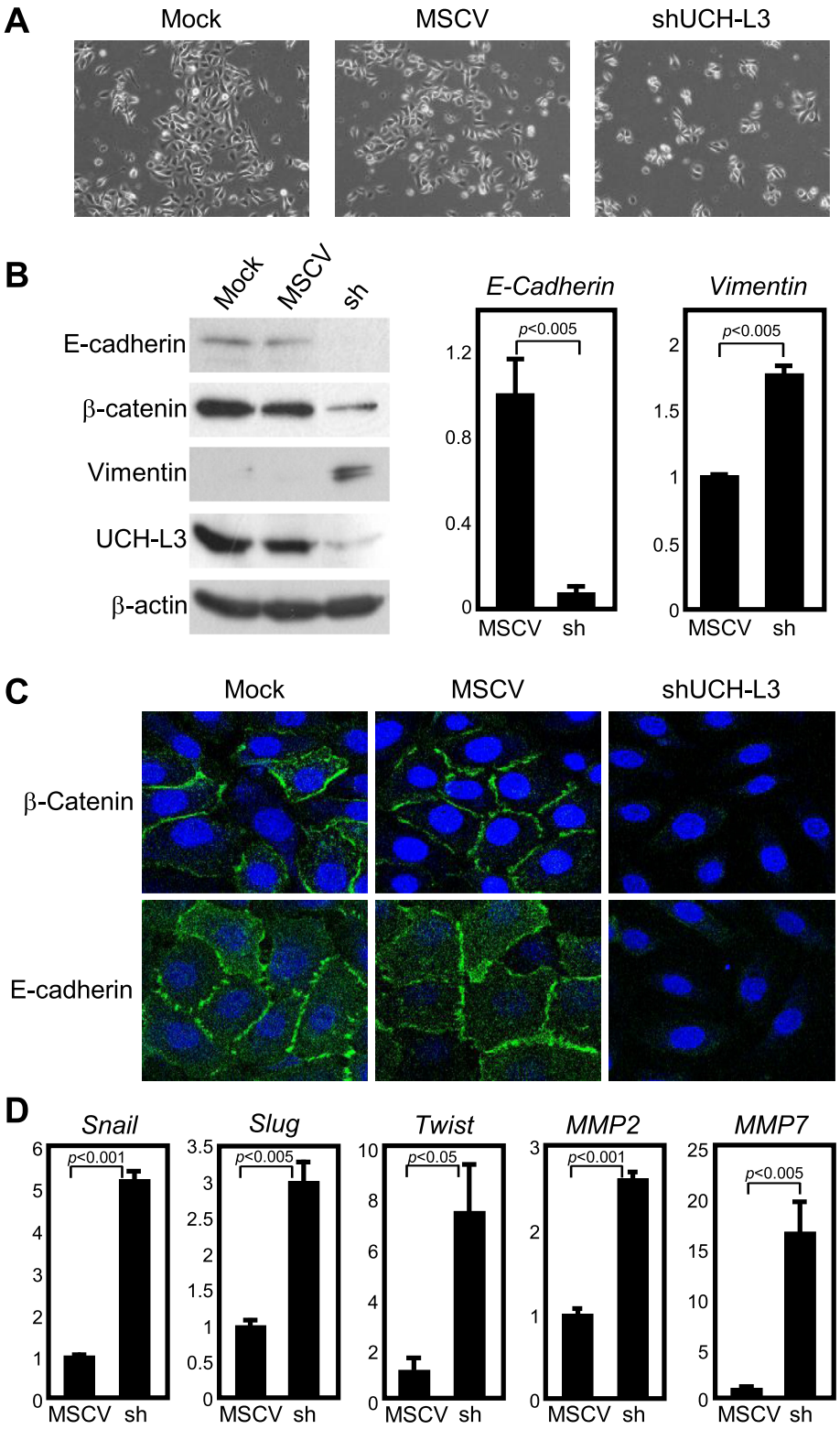

Fig. 2. Induction of EMT by UCH-L3 knockdown in RWPE1 cells. (A) Morphologic changes induced by knockdown of UCH-L3 in RWPE1. (B) Expression of epithelial proteins, including E-cadherin, β-catenin and mesenchymal protein vimentin was examined by immunoblotting in the RWPE1 control and shUCH-L3 RWPE1 cells. β-Actin was used as a loading control. The message level of E-cadherin and vimentin in stable cells was analyzed by real-time quantitative RT-PCR. Values are expressed as mean ± SD of three independent experiments, and the *p* value is shown from a Student's *T*-test analysis. (C) Immunofluorescent staining of E-cadherin and β-catenin in RWPE1 stable cell lines. The green signal represents the staining of the corresponding protein and the blue signal represents nuclear DNA staining by DAPI. (D) Real-time quantitative RT-PCR analysis of *Snail*, *Slug*, *Twist*, *MMP2*, and *MMP7* in RWPE1 stable cells. Values are expressed as mean ± SD of three independent experiments, and the *p* value is shown from a Student's *T*-test analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

E-cadherin and β-catenin, as well as the mesenchymal marker vimentin. As shown in Fig. 2B, knockdown of UCH-L3 induces the molecular alterations of EMT. E-cadherin and β-catenin decreased and vimentin increased in UCH-L3 knockdown cells both at the protein and mRNA levels. The membrane localization of adherens junction proteins such as E-cadherin and β-catenin indicates their biological functions. Thus, we performed immunofluorescent staining for these proteins on the stable cells. E-cadherin and β-catenin had disappeared from the cell membrane in UCH-L3 knockdown cells (Fig. 2C). According to these data, we suggest that UCH-L3 is involved in the inhibition of EMT process in prostate cells.

Loss of E-cadherin expression is the hallmark of EMT process. EMT programs are mediated by many transcription factors and most EMT inducting transcription factors are transcriptional repressors that directly repress E-cadherin transcription [15]. The zinc-finger proteins Snail, Slug and the helix-loop-helix transcription factor Twist repress E-cadherin expression and induce EMT [18]. To check whether these EMT inducing transcription factors are regulated by UCH-L3, we assessed the message level of E-cadherin regulators in RWPE1 stable cells. We found that UCH-L3 knockdown in RWPE1 upregulates *Snail*, *Slug*, and *Twist* (Fig. 2D). In addition to the EMT inducing transcription factors, MMPs play important roles in tumor invasion and metastasis through digestion of the extracellular matrix. The loss of E-cadherin during tumor progression is also mediated by proteolytic degradation by MMPs such as MMP2 and MMP7 [20]. *MMP2* and *MMP7* were increased in UCH-L3 knockdown cells (Fig. 2D). These results indicate that UCH-L3 inhibits EMT by repression of EMT promoting genes such as *Snail*, *Slug*, *Twist*, and *MMPs* in normal prostate cell lines.

In our previous report, we found that UCH-L1 expression is very low in RWPE1 and overexpression of UCH-L1 induces EMT [11]. To verify UCH-L1 status in RWPE1, we examined the effects of the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-Aza-CdR) and histone deacetylase inhibitor trichostatin A (TSA) on UCH-L1 gene transcription. Compared with TSA or 5-Aza-CdR alone, the expression of UCH-L1 was significantly enhanced after treatment with the combination of TSA and 5-Aza-CdR (Fig. S1). These results mean that both DNA methylation and histone deacetylation are involved in the transcriptional repression of UCH-L1 in RWPE1. Next, we investigated whether the action of UCH-L3 knockdown on EMT is mediated through the regulation of UCH-L1 expression. We found that UCH-L1 is highly induced by UCH-L3 knockdown in RWPE1 (Fig. S2). Thus, it may be possible that the induction of EMT by UCH-L3 knockdown is, in part, mediated through the upregulation of UCH-L3.

We investigated where there is synergy effect on EMT by coexistence of UCH-L1 overexpression and UCH-L3 knockdown. We made stable cell lines with double genetic alterations in RWPE1, that is, UCH-L3 knockdown and UCH-L1 overexpression (shUCH-L3/UCH-L1). Compared to the UCH-L3 knockdown alone, there was no discernable increase of EMT phenotype in shUCH-L3/UCH-L1 stable cells (Fig. S3A). There was a little more decrease of E-cadherin in shUCH-L3/UCH-L1 than in shUCH-L3 cells (Fig. S3B). These results mean that both UCH-L3 knockdown and UCH-L1 overexpression regulates EMT in RWPE1 but they do not work synergistically.

3.3. UCH-L3 knockdown increases cell migration and invasion in RWPE1 cells

The EMT process is closely associated with the acquisition of migratory and invasive properties by tumor cells and tumor cells infiltrate surrounding tissues and ultimately metastasize to distant sites. To investigate whether the regulation of EMT by UCH-L3 contributes to the cell migration and invasiveness in prostate cells, we performed wound healing assays in RWPE1 stable cells. As shown in Fig. 3A, wound healing was increased in UCH-L3 knockdown stable cells. We confirmed the effect of UCH-L3 on cell motility by Transwell migration assay (Fig. 3B). UCH-L3-knockdown RWPE1 cells showed the increase of cell migration through the filter. We also assessed the effect of UCH-L3 on the ability of prostate cells to invade through matrigel using a quantitative *in vitro* invasion assay (Fig. 3C). The invasion through the matrigel was increased by UCH-L3 knockdown. As expected, UCH-L3 endowed inhibitory metastatic potential to normal prostate cell line RWPE1.

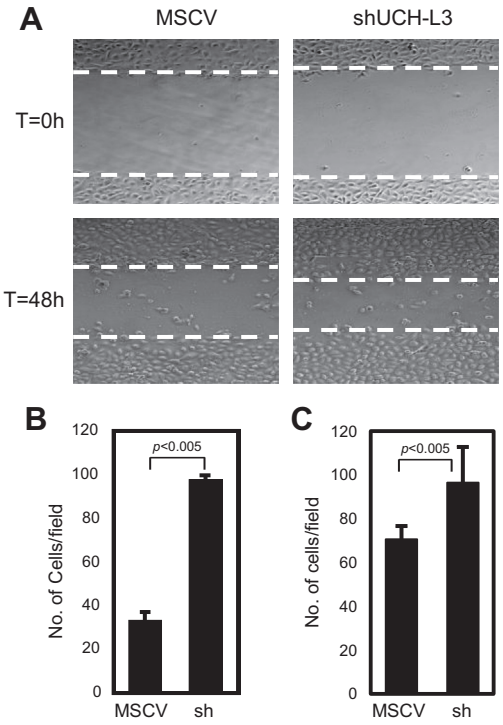

Fig. 3. UCH-L3 knockdown increases cell migration and invasion in RWPE1. (A) Wound healing assay of the RWPE1 stable cells. Each of the stable cell monolayers were scratched with a yellow micropipette tip and cell images were taken at the time points indicated. (B) Transwell migration assays of the RWPE1 stable cells. Same number of each stable cell was seeded on the transwell. After 22 h, the number of cells migrated to the lower chamber was counted. Values are expressed as mean ± SD of three independent experiments, and *p* value is shown from a Student's *T*-test analysis. (C) *In vitro* Matrigel invasion assays of the RWPE1 stable cells. Same amount of each stable cell was seeded on the matrigel coated chamber. After 22 h, the invaded cells on the lower surface of the membrane were counted. Values are expressed as mean ± SD of three independent experiments, and *p* value is shown from a Student's *T*-test analysis.

These results support the idea that UCH-L3 maintains the epithelial properties of normal prostate cells and keeps the normal prostate cells with low and inhibited migratory and invasive potential by inhibiting the EMT process.

3.4. Overexpression of UCH-L3 inhibits cell migration and invasion in PC3

To further investigate the inhibitory role of UCH-L3 on the EMT process in prostate cells, we generated UCH-L3 or an active site mutant form of UCH-L3 (UCH-L3 C95S), defective in Ub hydrolase activity, overexpressing PC3 stable cells. Firstly, we checked the molecular alterations of EMT markers in PC3 stable cells. As shown in Fig. 4A, E-cadherin increased but vimentin decreased in UCH-L3 overexpressing PC3 cells compared to mock or vector control cells.

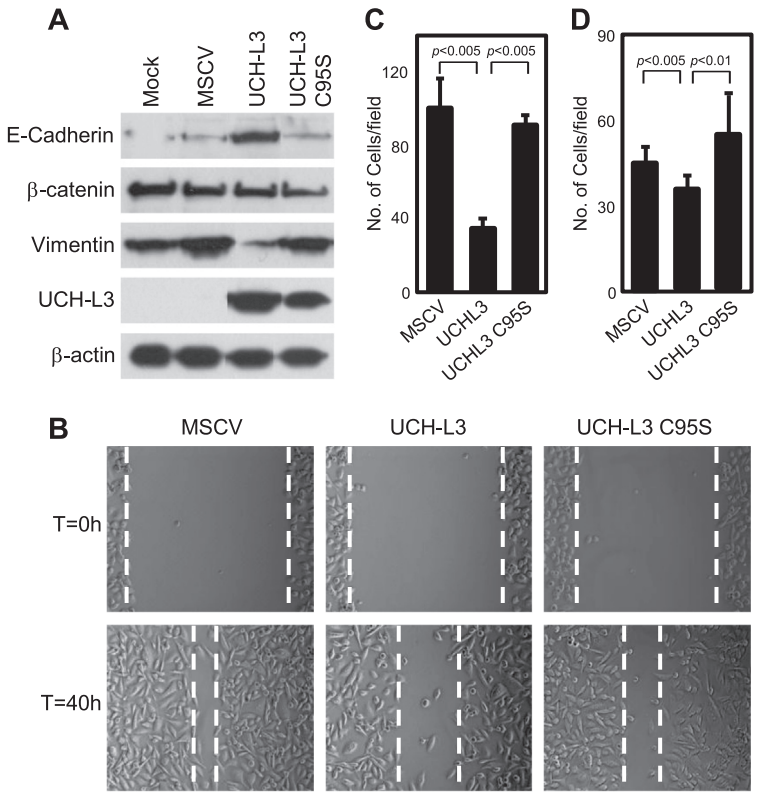

Fig. 4. UCH-L3 overexpression inhibits EMT in PC3 cells. (A) Expression of epithelial proteins, including E-cadherin, β-catenin and mesenchymal protein vimentin was examined by immunoblotting in the PC3 control and UCH-L3 or UCH-L3 C95S overexpressing PC3 stable cells. β-Actin was used as a loading control. (B) Wound healing assay of the PC3 stable cells. Each of the stable cell monolayers were scratched with a yellow micropipette tip and cell images were taken at the time points indicated. (C) Transwell migration assays of the PC3 stable cells. Same number of each stable cell was seeded on the transwell. After 22 h, the number of cells which had migrated to the lower chamber was counted. Values are expressed as mean ± SD of three independent experiments, and the *p* value is shown from a Student's *T*-test analysis. (D) *In vitro* Matrigel invasion assays of the PC3 stable cells. Same amount of each stable cell was seeded on the matrigel coated chamber. After 22 h, the invaded cells on the lower surface of the membrane were counted. Values are expressed as mean ± SD of three independent experiments, and the *p* value is shown from a Student's *T*-test analysis.

But there were no molecular alterations of EMT markers in UCH-L3 C95S transfectants. The alterations of the EMT marker expression induced by UCH-L3 overexpression in PC3 stable cells coincided with the change of migratory and invasive properties. As shown in Fig 4B, UCH-L3 inhibited the wound healing but active site mutant form of UCH-L3 did not. Also, we could confirm the inhibitory effect of UCH-L3 on the EMT process by migration and *in vitro* invasion assay (Fig. 4C and D). In highly metastatic prostate PC3 cells, UCH-L3 inhibits the cell migration and invasion but UCH-L3 C95S had no effects, suggesting that the hydrolase activity of UCH-L3 is required to inhibit the metastatic prostate cancer cell migration and invasion.

Taken together, we further conclude that EMT inhibitory property of UCH-L3 is largely conferred by the Ub hydrolase activity of UCH-L3 in prostate cells.

4. Discussion

In the present study, we explored the involvement of the deubiquitinating enzyme UCH-L3 in prostate cancer cell invasion and metastasis through EMT regulation. We found that UCH-L3 is downregulated in the highly metastatic prostate cell lines such as PC3 and DU145. To determine the involvement of UCH-L3 in prostate cell metastasis, we knockdowned UCH-L3 in the normal prostate cell line RWPE1. We found that knockdown of UCH-L3 induced EMT process. The cell–cell interaction was decreased and the molecular events of EMT were induced: downregulation of E-cadherin and upregulation of vimentin. The loss of membrane localization of adherens junction proteins E-cadherin and β-catenin were detected by immunofluorescence staining in UCH-L3 knockdown RWPE1 cells. Also, RT-PCR analysis showed that EMT promoting genes such as *Snail*, *Slug*, *Twist*, and *MMPs* were induced in UCH-L3 knockdown cells. The acquisition of increased motility and invasiveness was demonstrated by wound healing, migration, and *in vitro* invasion assays. Further, when UCH-L3 was overexpressed in PC3, where the endogenous UCH-L3 was negligibly detected, E-cadherin increased and vimentin decreased depending on the Ub hydrolase activity. The reverse of the EMT event caused by UCH-L3 overexpression in PC3 coincided with a decrease of cell migration and invasion.

UCH-L3 and UCH-L1 are very close relatives with high amino acid identity and structural similarity [4]. In our previous report, we demonstrated that UCH-L1 promotes prostate cancer cell migration and invasion through EMT induction [11]. Put together with our present study, we suggest that UCH-L3 and UCH-L1 function differentially in the progression of prostate cancer cell invasion and metastasis through EMT regulation. Interestingly, we found that knockdown of UCH-L3 induces the expression of UCH-L1 which is repressed by epigenetic modification in RWPE1. To the extent of our knowledge, the EMT regulation between UCH-L3 knockdown and UCH-L1 overexpression are not identical. We can detect the increase of *Snail* by UCH-L3 knockdown (Fig. 2D), but there was no increase of *Snail* message by UCH-L1 overexpression (data not shown). Thus, we assume that the action of UCH-L3 knockdown on EMT regulation is, in part, mediated through the regulation of UCH-L1.

Reciprocal modulation of germ cell apoptosis by UCH-L1 and UCH-L3 has been reported. During spermatogenesis, the expression of these two isozymes was differentially regulated [21]. UCH-L1 was expressed in spermatogonia and Sertoli cells, whereas UCH-L3 was expressed highly in spermatocytes and spermatids. Examining UCH-L1-deficient gracile axonal dystrophy (*gad*) mutant mice and *Uchl3* knockout mice after cryptorchid injury, the differential regulatory function of UCH-L1 and UCH-L3 during testicular germ cell apoptosis has been investigated [22]. The testes of *gad* mice showed resistance to cryptorchid injury and there was a large increase in the anti-apoptotic proteins. On the contrary, cryptorchid testes of *Uchl3* knockout mice showed profound apoptotic germ cell loss and a slight increase of the apoptotic proteins was observed. The proapoptotic role of UCH-L1 during spermatogenesis had been further examined. The testes of *Uchl1* Tg mice induced apoptosis in primary spermatocytes [23]. These reports demonstrate the reciprocal functions of UCH-L3 and UCH-L1 with respect to the modulation of germ cell apoptosis [24]. UCH-L1 is highly expressed in neurons [9]. Further, the dysfunction of UCH-L1 contributes to the pathogenesis of Parkinson's disease (PD) and Alzheimer's disease (AD) [25,26]. Even though the function of UCH-L3 in neurons has not been elucidated to the extent of our knowledge, it may be plausible that there can be reciprocal or differential functions of UCH-L3 to the UCH-L1 in the pathogenesis of PD and AD.

It has been reported that UCH-L3 enhances osteoblast differentiation through the stabilization of Smad1 [27]. Unveiling the specific substrates of UCH-L3 in EMT regulation is a rate-limiting step to reveal the mechanisms and accurate functions of UCH-L3 in prostate cancer progression and metastasis. In our study, ablation of the UCH-L3 deubiquitinating activity did not inhibit EMT and it did not reduce cancer cell migration and invasion in PC3. Thus, determining the specific deubiquitinating targets of UCH-L3 will give many insights into the mechanisms of UCH-L3 in oncogenesis.

Many proteins implicated in the Ub pathways are related to tumor progression. In recent years, there have been many approaches to target the Ub system in cancer therapy [28]. From our present study, we highlight UCH-L3 as a novel EMT regulator in prostate cells. Thus, we believe that the inhibition of EMT by deubiquitinating enzyme UCH-L3 could be a new therapeutic target for improving the treatment efficiency of metastatic prostate cancer.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

H.M.S. and J.H.K. designed the research; H.M.S. and J.E.L. performed the research; H.M.S. and J.H.K. analyzed the data; and J.H.K. wrote the paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.144.

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