國立臺灣大學生命科學院生命科學系

博士論文

Department of Life Science

College of Life Science

National Taiwan University

Doctoral Dissertation

鈣網蛋白下游調控因子–岩藻糖轉移酵素調控膀胱癌轉移機制之研究

Fucosyltransferase-1 as a Downstream Effector of Calreticulin in Regulating the Metastatic Behavior of Bladder Cancer Cells

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中華民國 103 年 06 月

June, 2014

國立臺灣大學博士學位論文
口試委員會審定書

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本論文係呂易謙君（學號 F96B41001）在國立臺灣大學生命科學學系所完成之博士學位論文，於民國一零三年六月十一日承下列考試委員審查通過及口試及格，特此證明

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致謝

碩博七年的研究生涯即將告一段落，在確定畢業的這一刻，心底的激動是無法言喻，回首近十年的研究歲月，記憶裡曾經痛苦掙扎的感覺早已雲淡風輕，留下的只有突破困境的鮮明畫面。 感謝一路上曾經幫助過我的每一位，因為您的支持鼓勵，讓我能有信心堅持到最後，在此獻上最誠摯的謝意。

完成夢想的路上，首先最要感謝的是造就我成功的重要推手-李心予老師。回想當初大學剛畢業，非本科系的我完全沒有操作實驗的經驗，李老師卻願意秉持著教育家的精神，給予我學習的機會。 在整個研究期間裡，提供我專業的指導及充分的信任，並且不斷地鼓勵提醒我，身為一個科學家，最重要的不僅是技術，還要擁有獨立思考的能力。 我從懵懂的新生蛻變成可以獨力完成論文的博士，李老師絕對功不可沒! 另外，很榮幸可以邀請張正琪老師、陳炯年醫師、許文明醫師以及朱家瑩老師擔任我的口試委員，並在整個過程當中，給予我的建議與肯定，讓我的論文更臻完善。

在這段超過我人生經歷四分之一的日子，研究室就像是我第二個家，很慶幸在這裡結識的各位都是如此地有趣又優秀，使我的研究生涯充滿了歡笑。 特別感謝已畢業的學長姐亭恩、小黃和齊倫，帶領我學習做研究紮實的基礎外，同時品嚐收穫的喜悅;此外，所有曾經一起打拼奮鬥的大家: 沛翊、柏堅、元勵、小六、怜慧、Milky、仔仔、芷歆、耆豪、季霖、傳恩、翠華、岳謙、予農、子晴、居正、士閎、貫淀、亞軒、徐董、小孟、偉民、義程、亞軒、心怡、雅然、龍五…等，不論是和你們激辯討論或是吃喝玩樂，都帶給我許多快樂又充實的時光，未來想起這段愉快的回憶，相信我仍會打從心底發出會心一笑。

最後，最感謝的就是默默在背後支持我的家人們，完成博士班的學業並不是個一蹴可幾的目標，過程中遇到挫折的時刻總多過於成功，箇中的辛酸無法與外人道，很感謝在我心灰意冷的時候，爸媽和老公都陪伴在我身邊，即使簡單一句「加油」也能讓我感到安心不孤單。 在我完成夢想之際，我願把這份榮耀與你們共享，謝謝!

中文摘要

膀胱癌是國人相當常見之腫瘤，鈣網酪蛋白 calreticulin 為一多功能蛋白並參與細胞內多種機制，臨床上 calreticulin 已被使用來檢測膀胱癌。於本研究中，我們成功構築表達低量鈣網酪蛋白之穩定膀胱癌細胞株(J82 CRT-RNAi)顯示降低鈣網酪蛋白之表達會抑制膀胱癌細胞株之移行，並對細胞附著於基質之能力有重要之影響；反之，表達大量鈣網酪蛋白會促進膀胱癌細胞之移行與貼覆基質之能力。更重要的是，降低鈣網酪蛋白之表達亦會抑制膀胱癌細胞株於裸鼠中形成腫瘤，並明顯降低肺臟及肝臟之轉移，這些結果證明了鈣網酪蛋白可能是影響膀胱癌細胞癌化之重要調控因子。藉由核酸晶片比較 J82 CRT-RNAi 細胞株與未轉染之細胞間基因表達之差異後，我們發現岩藻醣轉移酵素 FUT-1 為其中表達差異性極大的一種蛋白質。岩藻醣化作用為細胞常見之蛋白質修飾，報告指出許多病症與高度岩藻醣化極具相關性。FUT-1 為細胞內負責蛋白質醣基修飾的重要酵素，本研究結果顯示 CRT 透過調控整合蛋白 β1-integrin 由 FUT1 所修飾之岩藻醣化作用，進而影響細胞附著能力。透過大量表達岩藻醣轉移酵素可增加細胞整合蛋白 β1-integrin 之岩藻醣化程度並有效提升膀胱癌細胞貼附能力。 我們的研究結果更進一步證實，整合蛋白 β1-integrin 之岩藻醣化作用影響細胞貼附能力是經由調控其整合蛋白本身之活性。 此外，我們也進一步釐清 J82 CRT-RNAi 細胞株之 FUT-1 表達降低之原因，是由於 FUT1 RNA 穩定性下降之結果。根據本研究成果之驗證 CRT 透過穩定 FUT1 mRNA，進而提升

β1-integrin 岩藻醣化作用藉此促進其活性，影響膀胱癌細胞轉移之能力。

關鍵字: 膀胱癌、細胞貼附、癌症轉移、鈣網蛋白、岩藻醣轉移酵素、整合蛋白活性

Abstract

Bladder cancer is a common urothelial cancer. Through proteomic approaches, calreticulin (CRT) was identified and proposed as a urinary marker for bladder cancer. CRT is a multifunctional molecular chaperone that regulates various cellular functions such as Ca^{2+} homeostasis and cell adhesion. CRT was reported to be overexpressed in various cancers; however, the mechanisms of CRT in bladder tumor development remain unclear. To clarify the roles of CRT in bladder cancer, J82 bladder cancer cells stably overexpressed or knockdown of CRT were generated to investigate the physiological effects of CRT on bladder tumors. Compared to the transfected control vector cells, the knockdown of CRT suppressed cell proliferation, migration, and attachment; on the contrary, overexpression of CRT enhanced cell migration and attachment. Most importantly, we observed that tumors derived from J82 CRT-RNAi cells were significantly smaller and had fewer metastatic sites in the lung and liver *in vivo* than did transfected control vector cells. To further investigate the precise mechanism of tumor metastasis regulated by CRT, we used DNA array to identify fucosyltransferase-1 (FUT1) as a gene regulated by CRT expression levels. CRT regulated cell adhesion through α1,2-linked fucosylation on β1-integrin and this modification was catalyzed by FUT1. To clarify FUT1 roles in bladder cancer, we transfected the human FUT1 gene into CRT-RNAi stable cell lines. FUT1 overexpression in CRT-RNAi cells resulted in increased levels of β1-integrin

fucosylation and rescued cell adhesion to type-I collagen. Treatment with Ulex europaeus agglutinin I (UEA-1), a lectin recognizes FUT1-modified glycosylation structures, did not affect cell adhesion. In contrast, a FUT1-specific fucosidase diminished the activation of β1-integrin. These results indicated that α1,2-fucosylation on β1-integrin were not involved in the integrin-collagen interaction but promoted β1-integrin activation. In addition, we demonstrated that CRT regulated FUT1 mRNA degradation in 3´-untranslated region (3´-UTR). In conclusion, our findings suggested that CRT stabilized FUT1 mRNA, thereby leading to increase in fucosylation of β1-integrin. Furthermore, increased fucosylation levels activate β1-integrin rather than directly modifying the integrin binding sites.

Keywords: Bladder cancer, Cell adhesion, Metastasis, Calreticulin, Fucosyltransferase, β1-integrin activation.

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Chapter I.

Introduction

1. Fundamental information on calreticulin

1.1 Protein structure

Calreticulin (CRT) is a 46 KDa multifunctional protein in the endoplasmic reticulum (ER) and is highly conserved in diverse species. It is synthesized with a cleavable signal sequence at the N-terminus and an ER KDEL (Lys-Asp -Glu-Leu) retrieval signal at the C-terminus. Structural predictions of CRT show that the protein is composed of three domains, including N-, P-, and C-domain [1].

N-domain

The N-terminal region of CRT is a global domain containing eight antiparallel β-strands [2]. This domain can interact with α-integrins [3] and DNA-binding sites of the steroid receptor [4]. The disulfide bond formed by cysteine residues in the N-domain can interact with the P-domain to form the important chaperone function of calreticulin [5].

P-domain

The proline-rich P-domain contains two sets of three repeated amino acid sequences (repeat A: PXXIXDPDAXKPEDWDE and repeat B: GXWXPPXI-XNPXYX) [6]. These repeat A and B sequences form the lectin-like chaperone structures which are responsible for the protein-folding function of CRT. Moreover, the P-domain of CRT is also a high affinity but low capacity Ca^{2+}-binding region [7,

8].

C-domain

The C-domain of CRT is highly acidic region which is important for Ca^{2+}-buffering functions. It binds to Ca^{2+} with high capacity and low affinity [9]. It is known that Ca^{2+} binding to this region plays a critical role in the interaction with other chaperone proteins in the ER [1, 10].

1.2 Location of CRT

Since there is a KDEL sequence for retrieval in the ER at the C-terminal of CRT, it is not surprising that the most frequent distribution of this protein is within the ER lumen. However, evidences from other reports demonstrate that CRT is also expressed in the cytosol [11] and on the cell surface [12]. Some studies have showed that cytoplasmic CRT can interact with the cytoplasmic tail of α-integrin through the KxGFFFKR sequences [13, 14]. In addition, cell surface calreticulin is associated with phagocytic uptake and immunogenicity of cells [15]. The evidence provides more insights into why CRT is a multifunctional protein that regulates various physical and pathological events in cells.

2. Biological functions of calreticulin

CRT has previously been proposed to participate in various physiological and pathological processes in cells. The two major functions of CRT inside the ER are protein chaperoning and regulation of Ca^{2+} homeostasis. Furthermore, accumulated studies have indicated that non-ER CRT also regulates some important biological functions including cell adhesion, gene expression and RNA stability.

2.1 Protein chaperone

The ER is the important organelle for synthesis, folding, and transportation of secretory proteins. These functions are carried out by molecular chaperones which facilitate correct protein folding and assembly. CRT is one of the best-characterized lectin-like ER chaperons for many proteins [16-19]. Recent studies indicated that CRT is involved in the quality-control process during protein synthesis, including integrins, surface receptors, and transporters [1].

2.2 Calcium homeostasis

Ca^{2+} is mainly stored in the ER lumen and is a universal signaling molecule affecting many developmental and cellular processes in the cytosol [20]. Numerous studies have indicated that Ca^{2+}-binding chaperones influence Ca^{2+} storage capacity in the ER lumen [21]. CRT is considered to be an intracellular Ca^{2+} regulator since it

contains two Ca^{2+} -binding sites in the P-domain (high-affinity, low-capacity) and C-domain (low-affinity, high-capacity) [7, 22]. Higher levels of CRT lead to increase levels of intracellular stored Ca^{2+} [23, 24]. In contrast, CRT-deficient cells have showed lower capacity for Ca^{2+} storage in the ER lumen [9].

2.3 Cell adhesion

The concept that CRT might be involved in cell adhesion is based on the regulation of focal contact via multiple mechanisms [25, 26]. It is clear that the extracellular matrix (ECM) molecules are important for focal contact formation. Several studies have elucidated that the alteration of CRT levels affect cell adhesion on variant ECM [27-29]. Papp *et al.* implicated that calreticulin plays a role in the control of cell adhesiveness through regulation of fibronectin expressions and matrix deposition. These effects are mediated via Ca^{2+}-dependent effect of CRT on c-Src activity [30]. In addition, previous studies have revealed that CRT-mediated cell adhesion might be modulated by interaction between CRT and integrins by binding to the cytoplasmic KXGFFKR motif of the integrin α-subunit [3, 31, 32]. These studies have provided evidence that CRT plays a critical role in cellular adhesiveness.

2.4 RNA stability

In 2002, Nickenig *et al.* first identified CRT as a novel mRNA binding protein that destabilizes type I angiotensin II receptor mRNA by binding to AU-rich region in 3′-UTR [33]. Moreover, Totary-Jain *et al*. reported that CRT also binds to specific element in 3′-UTR of glucose transporter-1 mRNA and destabilizes the mRNA under high-glucose conditions [34]. These results identified a new function of CRT as a trans-acting factor that regulates mRNA stability.

3. Calreticulin in cancer

3.1 Clinical outcome of calreticulin levels

The correlation between CRT expression levels and tumorigenesis has been extensively studied in various cancers and most reports have indicated that tumor tissues express significant increases in the levels of CRT compared to normal tissues [35]. These clinicopathological significances for CRT in different cancers are summarized in Table 1. It has been reported that CRT is a novel independent prognosis marker in neuroblastoma [36]. Although higher level of CRT predicted a better survival rate in patients with advanced-stage in neuroblastoma, other studies have demonstrated that the CRT expression level was positively correlated with stage and lymph node metastasis in gastric cancer [37], breast cancer [38], and ovarian cancer [39]. In addition, patients with higher CRT levels had a poor survival rate in

pancreatic cancer and esophageal squamous cell carcinoma [40, 41]. Some studies also revealed CRT expression levels to be significantly up-regulated in oral cancer [42], breast ductal carcinoma [43, 44], colorectal cancer [45], prostate cancer [46], and vaginal carcinoma [47]. Furthermore, not only do CRT levels increase in bladder cancer tissues [48], but urinary CRT might also be a useful biomarker for diagnosis of bladder urothelial cancer. Kageyame *et al.* implicated that the concentration of urinary CRT has a tendency to increase in high stage and high grade tumors [49, 50]. These results indicate that CRT expression might play a crucial role in cancer progression.

3.2 Functions of calreticulin in the immune system

An important role for CRT exposed on the cell surface, which is relevant for destruction of cancer cells, is via induction of the immune response [15]. Results from several laboratories have mentioned that cell-surface CRT facilitates the phagocytic uptake of apoptotic and cancer cells [51-53]. Obeid *et al.* demonstrated that drug treatments (anthracyclins) caused tumor cell to expose a surface pro-phagocytic protein, CRT, which induced immunogenic cell death [54]. Additionally, suppression of CRT by siRNA inhibited the anthracyclin-induced phagocytosis by dendritic cells and destroyed their immunogenicity in mice [52]. It is becoming clear that surface exposure of CRT is required for phagocytosis on dying tumor cells. CRT expressed on

the cell surface is considered as an “eat-me” signal for multiple human cancers. However, this pro-phagocytic function of CRT was disrupted by an anti-phagocytic signal CD47 [51]. It has been previously described that an anti-phagocytic signal CD47 was increased with high amounts of CRT on cancer cell surfaces to avoid phagocytosis by the immune system [53]. Therefore, interruption of the ability of CD47 by anti-CD47 antibodies might have a therapeutic effect to enhance cancer cell phagocytic uptake [15]. Taken together, these results indicate that CRT-mediated immune mechanisms might be an important feature for developing new anticancer therapy.

3.3 Functions of calreticulin in cancer cell proliferation

Caner formation is characterized by rapidly proliferating of mutated cell. Many studies have elucidated that manipulation of CRT levels had profound effects on tumor cell proliferation in diverse cell types. First, in pancreatic cells, overexpressed CRT enhanced cell growth; in contrast, knockdown of CRT had the opposite effect on cell growth [40]. Second, Chen *et al.* showed that the higher levels of CRT increased cell proliferation and migration and modulated several molecules related to cancer metastasis and angiogenesis, such as connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), and placenta growth factor (PlGF) [37].

In addition, depletion of CRT caused cell cycle arrest at the G0/G1 phase which resulted in significantly suppressed growth rate, colony-formation capacity, and anchorage-independent growth in oral cancer cell [42]. Although many reports have indicated that CRT has a positive effect on cell growth, other studies have provided different viewpoints on this issue. A recent study demonstrated that prostate cancer cells with higher CRT levels produced fewer colonies as well as inhibition of tumor growth both *in vitro* and *in vivo* [55]. Moreover, vasostatin, a fragment of CRT, is considered to be an antiangiogenic factor and inhibits VEGF-induced endothelial cell proliferation [56]. According to these studies, whether CRT promotes or suppresses cell proliferation depends on different cell types.

3.4 Functions of calreticulin in cell migration and adhesion

Metastasis is a critical event for caner progression. This mechanism involves many processes, including cell adhesion, migration, and invasion. Previous studies have revealed that overexpressed CRT contributes to cancer metastasis in gastric, pancreatic, prostate, and ovarian cancers [37, 39, 40, 57]. The possible mechanisms for CRT-mediated cell migration or adhesion have been well surveyed. One suggested mechanism is that CRT is one of the few cytoplasmic proteins that directly interact with integrin α-subunits [58, 59]. Integrins are important molecules which interact

with ECM to regulate cell adhesion, migration, and cancer metastasis. In 1995, Coppolino *et al.* have indicated that the interaction between integrin α2β1 and CRT can be stimulated by integrin activation [60]. They further used the PC-3 prostate cancer cell line as a model to demonstrate that the interaction between integrins and CRT is modulated by phosphorylation and dephosphorylation status [61]. A recent study also attempted to clarify that integrin-dependent cell adhesion on fibronectin was apparently affected when CRT is overexpressed in epithelial-mesenchymal transition (EMT)-like cells [62].

Other mechanisms have also proposed that CRT modulates cell adhesion and migration through focal-contact dependent manners [25]. This theory is further supported by different levels of CRT can influence ECM expressions [30]. Manipulation of CRT expression in Mouse L fibroblasts has had a profoundly effect on fibronectins synthesis. These effects might be due to regulation of c-Src activity [30]. Cells with higher levels of CRT exhibited increased adhesiveness ability, which is relevant for the calmodulin /calmodulin-dependent kinase II pathway [63]. Moreover, CRT has been reported as a positive regulator for another important focal contact molecule, vinculin. Up-regulation of CRT enhanced cell adhesiveness and cell spreading, while knockdown of CRT showed inverse effects in L fibroblast cells [29]. Furthermore, cell surface CRT interacted with thrombospondin to modulate focal

adhesion disassembly through the PI3-kinase pathway [64]. Evidence from these studies have suggested that CRT plays a critical role in cell adhesion and migration via various mechanisms.

4. Functions of fucosylation in cancer biology

4.1 Fucosylation in cancer

Fucosylation is a common type of post-translational protein glycosylation, which regulates various physiological and pathological events in cells. This process is catalyzed by at least 11 types of fucosyltransferases (FUTs), which transfer a fucose (Fuc) residue from GDP-Fuc to oligosaccharides through α1,2-, α1,3/4-, or α1,6-linkages [65]. Aberrant expression of fucosylated haptoglobin has been reported in various tumor tissues, particularly during advanced cancers stages [66-68]. In papillary carcinoma, higher FUT8 levels are significantly correlated with tumor size and lymph node metastasis [69]. In addition, fucosylated haptoglobin is considered to be a novel biomarker for pancreatic cancer detection [70]. These results suggest that fucosylation may be a key event in regulating cancer development and progression.

4.2 Fucosyltransferase 1 (FUT1)

FUT1 is a common type of FUTs and is involved in catalyzing the addition of

fucose in α1,2-linkage to galactose. It has been reported that higher levels of Lewis Y antigen, catalyzed by FUT1 and FUT4, are found in over 60% of human epithelial carcinomas [71-73]. Moreover, studies suggest that in certain cancer cells, tumor growth and metastasis are regulated by changes in FUT1 levels [74, 75]. These results indicated that FUT1-regulated fucosylation is closely associated with tumor progression.

4.3 Glycan on integrins regulates its functions

Integrins are a family of receptors comprising α- and β-subunits. They are involved in cell-cell and cell-matrix interactions and regulate key physiological processes such as cell migration, adhesion, and cancer metastasis. Accumulated evidence indicates that integrin function is regulated by glycosylation [76, 77]. Overexpression of N-acetylglucosaminyltransferase V (GnT-V), which increases β1,6-branching of N-linked glycan, enhanced α5β1 integrin-mediated cell migration and invasion [78], whereas an increase in N-acetylglucosamine (GlcNAc) bisecting-linkage on N-glycan by N-acetylglucosaminyltransferase III (GnT-III) inhibited α5β1 integrin-mediated cell spreading and migration [79]. Furthermore, FUT8 catalyzed core fucosylation (α1,6-fucosylation) on α5β1 integrins and

stimulated cell migration on laminin 5 [80]. However, the consequences of FUT1 expression and its role on integrins have not been surveyed thus far.

5. Bladder cancer

In the US, bladder cancer was among the top ten most frequently diagnosed cancers in 2009 [81]. The most common type of bladder cancer is transitional cell carcinoma (TCC), a malignant tumor which grows from the epithelium of the bladder. It was reported that around 30% of patients presented with a muscle-invasive metastasis at the time of diagnosis, and that 85% of these patients would die from the disease within 2 years if left untreated [82]. This means that metastasis is one of the leading causes of cancer-related death for patients with bladder cancer. Metastasis is a critical event in cancer and is associated with significant reduction in patient survival rates. Tumor cells detach, invade, and disseminate to other sites and form secondary tumors during metastasis. Each step involves many molecules including cell-adhesion molecules, matrix degradation enzymes, and various growth factors [83]. However, the gene that regulates metastasis in bladder cancer is still unclear.

6. Rationale

As we mentioned above, CRT plays a critical role in regulating diverse cellular functions and cancer development processes. Furthermore, higher levels of CRT are found in urinary cancer tissues and urinary CRT can be used as a biomarker for bladder cancer detection. However, the precise mechanism by which CRT modulated bladder cancer progression is still poorly understood. Thus, in this study, **we attempt to investigate the roles of CRT on bladder cancer progression.**

In order to evaluate the metastatic behavior of bladder tumor, we used an aggressive bladder cancer cell line J82 to investigate the roles of CRT on bladder cancer progression [84]. We first established CRT overexpressed and knockdown stable cell lines in J82 cells to clarify the role of CRT in bladder cancer. Moreover, DNA microarray was used to identify CRT-regulated gene to further investigate the detail mechanism of how CRT modulated bladder cancer progression.

Chapter II.

Materials and Methods

Cell culture

The J82 human bladder cancer cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) under a humidified atmosphere of 95% air and 5% CO_2 at 37 ℃. For the subcultures, cells were trypsinized with 0.05% EDTA-trypsin.

Construction of inducible cell lines

To set up a tetracycline-inducible CRT system, the pEYFP-N1 was subcloned into pcDNA5/TO (Invitrogen) by Kpn I and Not I to generate pcDNA5/TO-YFP ($^{\#}$5y) used as control. CRT was amplified from HEK293 cDNA and subcloned into pcDNA5/TO-YFP to generate pcDNA/TO-ss-YFP-CRT ($^{\#}$5yCRT) by the following primers.

ss-foward-Nhe I: 5’-CCGCTAGCTTGATGCTGCTATCCGTGCCGCTGC-3’,

ss-reverse-Hind III: 5’-CCAAGCTTGGCGACGGCCAGGCCGAGGA-3’.

YFP-foward-Hind III: 5’-CCAAGCTTGTGAGCAAGGGCGAGGAGCT,

YFP- reverse-Kpn I: 5’-AAGGTACCCTTGTACAGCTCGTCCATGC-3’.

CRT-foward-Kpn I: 5’-AAGGTACCGAGCCCGCCGTCTACTTCAA-3’,

CRT- reverse-Xba I: 5’- AATCTAGACTACAGCTCGTCCTTGGCCTGG-3’

The coding region sequence of FUT1 (NM_000148) gene was purchased from OriGene (MD, USA). The lentival vectors for cDNA expression, including pLKO_AS2.puro and pLKO_AS2.zeo were obtained from the National RNAi Core Facility Platform, Academia Sinica, and Taipei, Taiwan. EGFP template was purchased from pEGFP-C1 (Clontech, CA, USA). PCR was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., MA, USA). FUT1 was ligated into pLKO_AS2.puro between Nhe I and Bsr GI sites to build pLKO_AS2.puro-FUT1. EGFP was subcloned into pLKO_AS2.zeo between Nhe I and Eco RI sites to generate pLKO_AS2.zeo-EGFP. The following primers were used for FUT1 PCR reactions:

Fut1-forward-Nhe I: 5'- AAGCTAGCATGTGGCTCCGGAGCCATCGTCAG-3'.

Fut1-reverse-Bsr GI: 5'- GGTGTACATCAAGGCTTAGCCAATGTCCAGAG-3'.

Transfection and selection of stable cell lines

For transfection experiments, J82 cells were plated 2 x 10^5 cells/well in six-well plates, and two μg of plasmid DNA for CRT overexpression ($^{\#}$5y and $^{\#}$5yCRT) or knockdown (pCR3.1 and CRT-RNAi) was transfected into cells using 10 μl of Lipofectamine TM2000, Invitrgen (Camarillo, CA). For single-cell selection, the transfected J82 cells were passaged at a 1:10 dilution in 10cm culture plate at two days after

transfection, and the stable cells were picked from a single colony using cloning rings. Medium with antibiotics (200ug/ml Hygromycin for $^{\#}$5y and $^{\#}$5yCRT inducible clones and 700μg/ml G418 for PCR 3.1 and CRT-RNAi stable clones) was replaced every 2 or 3 days. Two months later, antibiotic-resistant single clones were selected and amplified to test CRT mRNA and protein expressions. The inducible YFP, ss-YFP-CRT cell lines ($^{\#}$5y and $^{\#}$5yCRT) were pre-treated with 1 μg/ml tetracycline for 48 hours to perform the experiments.

For generation of overexpressed FUT1 stable cell lines, J82 CRT-RNAi cells were first infected with pLKO_AS2.zeo-EGFP and selected with 250 μg/ml zeocin to generate J82 CRT-RNAi-GFP cells. J82 CRT-RNAi-GFP cells were then infected with pLKO_AS2.puro or pLKO_AS2.puro-FUT1 to generate J82 CRT-RNAi-GFP Control (#R-EGFP) and J82 CRT-RNAi-GFP FUT1 (#R-FUT1) cell lines. The stably-infected cells were selected using 500 μg/ml G418, 250 μg/ml zeocin and 2 μg/ml puromycin.

Image analysis

For analysis of cell shape, morphological images of J82 cells were thresholded and outlined by using Metamorph software. Changes in cell shape were assessed by a shape factor ($sf = 4A/p^2$) calculated from the area and the perimeter of the delineated

object. Value close to "1" represents a flat object, whereas a value close to "0" represents a circular object.

Immunoblotting

Cells were plated at 2 x 10^5 cells/well in 6-well plates overnight. For harvesting cell lysates, cells were washed with cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris (pH 7.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecylsulfate (SDS, pH 7.4)) containing protease inhibitors. Equal amounts of each sample (30 μg) were boiled with 6x sample buffer for 5 min and resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked by 5% bovine serum albumin (BSA) in TBSt (10 mM Tris, 150 mM NaCl, and 0.1% Tween-20; pH 7.4) at room temperature for 2 h. Blocked membranes were incubated with the following primary antibodies: CRT, Upstate Biotechnology (Lake Placid, NY) and Santa Cruz (Santa Cruz, CA); paxilln [pY118], Cell Signaling (Danvers, MA); FAK [pY397], Invitrogen (Camarillo, CA); FAK, paxillin, c-Src, and actin, Santa Cruz (Santa Cruz, CA); and Src[pY418], Biosource, (Camarillo, CA); β1-integrin, BD Transduction Laboratories (USA; 1:2000) & GeneTex (Hsinchu, Taiwan; 1:2000); CRT, Upstate (NY, USA; 1:5000);

biotin-labeled UEA-1, Vector Laboratories (CA, USA; 1:5000), and FUT1 and FUBP1, Santa Cruz (CA, USA; 1:2000) for 4℃ overnight. Membranes were washed, and then incubated with horseradish peroxidase (HRP)-conjugated or streptavidin HRP-conjugated secondary antibodies (1:5000) for 1 h. Immunoreactive bands were quantified with TotalLabV2.01 software.

Lectin pull-down assay

Detection of glycoproteins decorated with terminal galactose was achieved by lectin pull-down assays using biotinylated UEA-1 or Lotus tetragonolobus lectin (LTL) (Vector Laboratories, CA, USA). Briefly, 500 mg of total cell lysates were incubated with biotinylated UEA-1. Following 16 h at 4℃, streptavidin agarose beads were added and incubated for an additional 6 h. The pulled down proteins were then subjected to Western blot analysis.

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA were isolate by the TRIzol reagent following the manufacturer's instructions. Reverse-transcription (RT)-PCR was carried out using ReverTra Ace reverse transcriptase. Real-time PCR was performed using the iCycle iQ Real-time detection system (Bio-Rad, Hercules, CA) with the DNA double-strand specific

SYBR Green I dye for detection. Cycling condition was 95 ℃ for 15 min, followed by 40 cycles of 95 ℃ foe 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. For quantification, the target gene was normalized to GAPDH, an internal control gene. Primer sequences were: GAPDH (F-5'-ggtggtctcctctgacttcaac and R-5'-tctctcttcctcttgtgctcttg), CRT (F-5'-aagttctacggtgacgaggag; R-5'-gtcgatgttctgctcatgtttc), FUT1-CDS (F-5′-aagttctacggtgacgaggag-3′ and R-5′-gtcgatgttctgctcatgtttc-3′), FUT1-3′UTR (F-5′-cgtgctcattgctaaccactgtc-3′ and R-5′-tcgtgctcctgcctggatct-3′), EGFP (F-5′-gtgagcaagggcgaggag-3′ and R-5′-cgtaggtcagggtggtca-3′), DsRed (F-5′-gagggcttcaagtgggag-3′ and R-5′-catagtcttcttctgcattacgg-3′), and FBP1 (F-5′-tgggaccatacaaccctgcacct-3′ and R-5′- agctggatcaggagcctg-3′).

Cell cycle analysis and Proliferation assay

Cells were starved overnight then treated with cultured medium (DMEM with 10% FBS) for 10 h. Cells were trypsinized and re-suspended in 1 ml PBS. Propidium iodide (PI) was added to the suspensions and then incubated in a 37 ℃ water bath for 20 min in the dark. PI-stained cells were analyzed by flow cytometry, and the percentages of different phases were automatically analyzed by Partec FloMax software. To estimate cell proliferation rate, cells were plated in triplicates at 2500 cells/well in 96-well plates. Ten μl of 5mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,

5-diphenyltetrazolium bromide (MTT) was added into each well at 24, 48, and 72 hours after plating. After incubation for 4 hours at 37℃, MTT-containing medium was removed. MTT metabolic products were subsequently dissolved in 100 μl DMSO for 20 min at 37℃, followed by measurement of absorbance at 550 nm.

Cell migration assay

Migration rates of different cell lines were assayed in a modified Boyden's chamber (Neuro Probe, Gaithersburg, MD, USA). Cells suspended in 50 μl (1.5×10^4 cells/well) were loaded in the upper chamber. DMEM and FBS were loaded in the lower chamber as the negative control and chemoattractant, respectively. After 4 h, migrating cells were fixed and quantified by a colorimetric measurement using crystal violet staining with TotalLab v2.01 software.

Cell attachment assay

The 96-well culture plates were coated with 10 μg/ml collagen I and incubated at 37 ℃ for 30 min followed by a PBS wash. To determine the effects of various functional blocking antibodies or lectin on cell adhesion, suspensions of J82 cells suspension was pre-incubated with anti-integrin antibody (1μg/ml for α1, α2, α3, αV, β3, αVβ3 and 0.25μg/ml for β1; Millipore, USA) for 1 h or lectin (Vector Laboratories, USA)

for 30 min. Cells (5 x 10^4 cells/100 μl) were seeded in the well and incubated at 37 ℃ for 20 min. After removing the medium and non-attached cells, wells were washed with PBS, and 0.1% crystal violet was added for 10 min. The plate was gently washed with PBS three times. Then 10% acetic acid was added for 20 min, and the plate was read at 550 nm.

Apoptosis detection assay

Cells were starved overnight and treated with 5 μg/ml actinomycin D, Sigma (Missouri, USA) as positive control for twenty-four hours. Cell apoptosis rate was detected by using FITC Anneixn-V apoptosis detection kit I, BD (San Diego, CA). Cells were trypsinized and re-suspended in 100 μl 1xbinding buffer. 5μl FITC annexin-V and PI were added to the suspensions and then incubated for 20 min at RT in the dark. Annexin V-stained cells were analyzed by flow cytometry. To measure end-stage apoptosis, TUNEL labeling was performed in tumor sections of the two groups using a DNA fragmentation detection kit, Calbiochem (Gibbstown, USA) following manufacturer's instructions. Each section had been chose for 5 images, and TUNEL positive cells were quantified under microscope by high power field (200X). For a positive control, sections were incubated in DNase I before addition of equilibration buffer, while DDW was used instead of TdT reaction mix in the negative

control.

Subcutaneous injection model

For models of bladder cancer to measure tumorigenicity, 8-week-old male BALB/c nude mice (n=10; National Defense Medical Center, Taipei, Taiwan) were housed in pathogen-free conditions and acclimatized for 1~2 weeks. Mice were randomized to one of two groups and were subcutaneously injected with vector control cells (n=5) and CRT-RNAi cells (n=5) in the inguinal region. Cells (10^7) were suspended in PBS and Matrigel in a 1:1 (v/v) ratio. Tumors were measured every 3 days (width^2 x length x 0.5). Mice were sacrificed after 45 days, and subcutaneous tumors were surgically excised.

Tail vein injection model

For the metastasis model, 8-week-old male SCID mice (n=12; BioLASCO, Taipei, Taiwan) were used for tail vein injection. Cells (10^6) suspended in PBS were injected through the tail vein. After 40 days, the lung and liver were resected and fixed in 3.7% formalin for paraffin embedding in preparation for immunohistochemiical analysis. The percentage of the lung metastasis area was calculated as the metastatic lung area/total lung area.

Microarray analysis

Total RNA from control and CRT-RNAi cells were isolate by the TRIzol reagent and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA). The cRNA was fragmented and then hybridized on the Human OneArray™ microarray (HOA 4.3; Phalanx Biotech, Palo Alto, CA) containing 30,968 human gene probes. Arrays were scanned by Microarray Scanner GenePix 4000B (Axon Instruments, Union City, CA), and fluorescence intensities were measured by GenePix Pro, version 6.0 (Molecular Devices). The raw data were preprocessed by log2 transformation and global LOWESS normalization.

Determination of integrin activation

Cells were trypsinized and re-suspended in 100 μl 1xPBS containing α1,2 -fucosidase (BioLabs, MA, USA; 1:100) at 37℃. After 30 min, cells were stained with PE conjugated anti-HUTS-21 antibody (BD Pharmingen, CA, USA) and analyzed by flow cytometry.

Determination of mRNA stability

J82 cells were treated with 5μg/ml actinomycin D (Act-D) (Sigma, Missouri, USA) for indicated time points. The FUT1 mRNA was detected by real-time PCR.

RNA Electrophoretic Mobility Shift Assay

The synthesized biotinylated FUT1-ARE and mutant ARE (mARE) probes (MDBio) were incubated with CRT recombinant protein (Abnova) for 10 min at 4℃ in REMSA buffer containing 2 μg tRNA to a final volume of 20 μl. Samples were analyzed on a native PAGE (4% polyacrylamide:bisacrylamide, 37.5:1). Following electrophoresis, the gel was transferred to Nylon membrane and the RNA-protein complexes were detected by chemiluminescence following manufacturer's instructions, Thermo (Rockford, USA).

RNA immunoprecipitation

Synthetic biotinylated FUT1 ARE and mARE probes were incubated with whole cell lysate (500 ug) containing streptavidin mag sepharose (GE Healthcare) at 4℃ overnight. The pulled down RNA-protein complexes were analyzed by western-blotting with anti-CRT antibody.

Statistical analysis

Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference (LSD) test (StatView, Abacus Concept, Berkeley, CA, USA). Each result was obtained from at least three

independent experiments and a value of $p<0.05$ was considered statistically significant.

Chapter III.

Results

Generation of stable cell lines

In order to clarify the effects of CRT in bladder cancer cells, we attempted to select CRT overexpression and knockdown stable cell lines. J82 bladder cancer cells were transfected with a CRT overexpressed plasmid and a CRT-RNAi plasmid, and then selected by respective antibiotics. After selection by G418 over 2 months, CRT-RNAi cells were generated and CRT knockdown efficiency was confirmed with a real-time PCR and Western blotting to detect mRNA (Fig. 1B) and protein (Fig. 1C) expression levels compared to the transfected control vector cells (PCR 3.1). However, during the stable cell line selection, constitutive overexpression of CRT resulted in cell spreading (Fig. 1E) followed by cell death within two weeks. Therefore, an inducible-CRT bladder cancer cell line was generated by tetracycline-regulated gene system. Cells were treated with 1 μg/ml tetracycline 48 hours to induce CRT mRNA and protein expressions (Fig. 1B and 1D). Cell morphology was shown in Figure 1A, and the shape factors calculated by MetaMorph software were not significantly different between PCR 3.1 and CRT-RNAi or $^{\#}$5y and $^{\#}$5yCRT cells (Fig. 1F).

Cell proliferation rate is lower in CRT knockdown cells

To understand how CRT regulates cell behavior, we used functional assays to investigate the physiological effects of CRT expression in bladder cancer cells. Their

cell cycle was analyzed by flow cytometry. In these assays, the CRT-RNAi group showed more cells arrested in the G1 phase (41%) compared to the control group (30%) which indicated that CRT knockdown suppressed cell proliferation (Fig. 2A). Nevertheless, our results demonstrated that there was no significant difference between $^{\#}$5y and $^{\#}$5yCRT (Fig. 2A). Cell proliferation rate was also assessed by MTT assays over several time points after plating. The MTT reading for CRT-RNAi cells were significantly lower than those from control cells (PCR 3.1) at 24, 48, and 72 hours (Fig.2B, left panel). However, no significant difference was observed between CRT overexpressed cells and vector-transfected cells (Fig.2B, right panel). In order to estimate if the decreasing numbers in CRT-RNAi group due to apoptosis, we used annexin-V/PI staining to determine the apoptotic cells. PCR 3.1 and CRT-RNAi cells were starved for 24 hours. Actinomycin-D (act-D), which is a potent inducer for apoptosis in several cell lines [85], was used as positive control. As shown in Fig. 2C, the percentage of annexin-V positive cells were less than 3% in both PCR 3.1 and CRT-RNAi cells, which indicated that knockdown of CRT did not induce apoptosis in J82 cells. These results justified that down-regulation of CRT inhibited bladder cancer cell proliferation rather than induction of apoptosis.

Alteration of CRT levels affects cell attachment and migration

We also used cell migration and attachment assays to explore the impact of CRT levels in J82 cancer cells. Equal numbers of cells were seeded on a collagen-coated plate for 20 min. Results showed that the cell's attachment ability was suppressed in CRT-RNAi cells compared to transfected vector control cells (Fig. 3A, lanes 1 and 2). On the contrary, CRT over-expression enhanced cell attachment significantly than vector control cells (Fig. 3A, lanes 3 and 4). In order to determine if the effects of CRT are universal to bladder cancer, we used another bladder cancer cell line to perform our experiments. Down-regulation of CRT in T24 bladder cancer cells also suppressed cell attachment to type-I collagen (Fig. 3C), suggesting that the effects of CRT we observed are not only specific to J82 cell line. Furthermore, by using the modified Boyden chamber method, J82 cells with low CRT expression had lower migration capacities than control cells (Fig. 3B, lanes 1 and 2), and cells with higher CRT expression showed an opposite effect (Fig. 3B, lanes 3 and 4). Consequently, these results demonstrated a positive correlation between CRT expression levels and cell attachment and migration in bladder cancer cells.

Phosphorylation levels of FAK and paxillin are lower in CRT knockdown cells

Papp et al. reported that altering CRT expression in fibroblasts can affect cell

motility and adhesion through regulating c-Src and FAK activity [63, 86]. Paxillin is one molecule of the focal adhesion complex which regulates the cell adhesion mechanism. Based on those previous studies, we used Western blotting to analyze the downstream signaling pathway affected by CRT in our system. Phosphorylation levels of Src [pY418], FAK [pY397], and paxillin [pY118] were also examined. As shown in Fig. 4, the phosphorylation levels of FAK (-40%) and paxillin (-40%) decreased in CRT knockdown cells, while no difference in Src phosphorylation was observed.

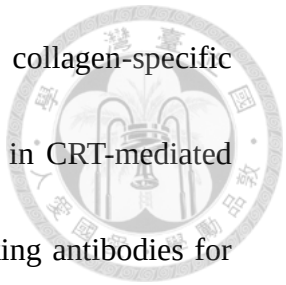
Knockdown of CRT inhibits tumor growth in vivo

Stably transfected cells were subcutaneously injected into nude mice to determine the effects of CRT on tumor growth. Tumor volumes were measured every 3 days. Compared to tumors of control mice, those of mice injected with CRT-RNAi were significantly smaller after 21 days. The mean volume of tumors of CRT-RNAi clones was suppressed 60% more than was that of control clones at day 35 (Fig. 5A). Moreover, we further determined the end-stage apoptosis in the injection of cells from nude mice. Tumor sections from both groups were analyzed by TUNEL assays. The number of TUNEL-positive cells in control group was no significantly increase in comparison to CRT-RNAi group (Fig. 5B). These results are consistent with our observations in vitro.

Knockdown of CRT inhibits metastasis of bladder cancer cells

In addition, we further confirmed that the metastatic capacity was lower in the CRT-RNAi subcutaneous-injection model. After sacrificing the mice, we found metastatic nodules in the liver of control mice but not in CRT-RNAi-injected mice (Fig. 6A). These results strongly suggest that expression levels of CRT are closely related to the metastatic behavior of bladder cancer cells. To further confirm this hypothesis, we performed a tail-vein injection experiment, which is a specific model used to investigate cancer cell metastasis. Liver metastasis was observed to be at 40% (2/5) in the control group and at 0% (0/7) in the CRT-RNAi group. In comparison, 100% (5/5) of control cells and 71% (5/7) of CRT-RNAi-injected mice developed lung metastasis. Although lung metastasis was observed in both groups, the percentage of metastatic areas was much higher in the control group (Fig. 6B & 6C). From the two mice models, we can conclude that vector control cells induced tumor formation and visible liver and lung metastasis, whereas CRT-RNAi cells formed smaller tumors and fewer metastatic nodules in the liver and lungs. These results are summarized in Table 2.

β1-integrin participated in CRT-mediated cell adhesion in J82 human bladder cancer cells



Since integrins play a crucial role in cell adhesion, we used collagen-specific integrins investigator kit to identify the type of integrins involved in CRT-mediated changes in cell adhesion. As shown in Figure 7A, functional blocking antibodies for α2- or β1-integrin inhibited the adhesion in vector control cells but not in CRT-RNAi cells. Blocking other integrins did not result in any significant differences in cell adhesion between control and CRT-RNAi cells. These suggested that α2- and β1-integrin might be involved in CRT-mediated adhesion on type I collagen. However, the level of total β1-integrin showed no difference between control and CRT-RNAi cells (Figure 7B). Subsequently, we used DNA arrays to compare the gene expression profiles between J82 control and CRT-RNAi cells and identified many candidate genes whose expression was likely to be CRT-regulated (Tables 3 & 4). Among these, FUT1 was significantly suppressed in J82 CRT-RNAi cells. Because recent studies have shown that changes in glycosylation of β1-integrin modulates its function [87], we performed a lectin pull-down assay using either lectin LTL or UEA-1, which are known to bind to α1,2-linkage oligosaccharides through a reaction catalyzed by FUT1. The pull-down samples were subjected to immuno-blotting against antibodies specific for β1-integrin. The fucosylation levels of β1-integrin were profoundly decreased in CRT-RNAi cells (Figure 7C). In addition, we also performed glycomics analysis in our stable cell lines. J82 cells showed different UEA-1-positive glycoprotein

expression patterns in western blot when we knockdown of CRT (Figure 7D). These results suggested that CRT mediates cell adhesion through post-translational modification of β1-integrin in bladder cancer.

FUT1 overexpression in CRT-knockdown cells enhanced cell adhesion

Fucosylation is one of the common post-translational protein modifications. It has been shown that transfection of FUT1 gene into RMG-1 cells enhanced cell adhesion [88]. To further examine the role of FUT1 on CRT-mediated changes in cell adhesion, the FUT1 gene was stably transfected into CRT-RNAi cells to generate J82 #R-FUT1 cells. FUT1 overexpression in J82 #R-FUT1 cells was confirmed by real-time PCR (Figure 8A). To investigate the effect of FUT1 on the changes β1-integrin fucosylation, we pulled down UEA-1 lectin and probed for β1-integrin as described in the previous section. Binding of β1-integrin to UEA-1 lectin was significantly increased in J82 #R-FUT1 cells compared to control vector transfected cells (Figure 8B). Furthermore, as shown in Figure 8C, reintroduction of FUT1 restored cell adhesion in CRT-RNAi stable cells. These results suggested that CRT modulated cell adhesion in bladder cancer by regulating β1-integrin fucosylation through FUT1.

Fucosylation status of β1-integrin affected integrin activation but not association between integrin to collagen

Since the fucosylation status of β1-integrin could influence cell adhesion, we hypothesized that α1, 2-linked fucosylated glycan on β1-integrin might participate in β1-integrin and type I collagen interaction. To assess the potential inhibitory effects of UEA-1, we tested J82 cells in a cell adhesion assay presence of UEA-1 lectin. As shown in Figure 9A, treatment with UEA-1 did not show any effect on adhesion even at higher dosages. These results prompted us to inspect the activation status of β1-integrin. Several studies have revealed that variation in glycosylation patterns of β1-integrin has an influence on its functions [79, 89, 90]. To verify the effect of fucosylation levels on β1-integrin activity, the active forms of β1-integrin were detected by flow cytometry. We found that active integrins were significantly lower in CRT-RNAi cells (13.0±2.6) than in control cells (22.9±3.9) (Figure 9B). These results suggested that β1-integrin fucosylation modified integrin activity. To further confirm this hypothesis, cells were treated with a FUT1-specific α1, 2-fucosidase. Both the control and CRT-RNAi cells showed reduced β1-integrin activity upon fucosidase treatment (Figure 9B). Taken together, these findings suggested that β1-integrin fucosylation affected J82 cell adherent ability by activating β1-integrin rather than directly modifying the integrin binding sites. The data for down-regulation of α1,2

-fucosylation after fucosidase treatment was shown in figure 9C.

CRT-knockdown suppressed FUT 1 expression by regulating its mRNA stability through AU-rich element

In order to verify how CRT affected FUT1 expression, real-time PCR and western blot analyses were performed in our stable cell lines. The results showed that CRT down-regulation attenuated the expression of FUT-1 protein and mRNA level of 3′untranslated region (UTR), but not the mRNA levels of coding region (Figure 10). It is known that certain specific sequences in 3′-UTR of mRNA can influence the RNA stability and protein expression [91]. These reports prompted us to investigate whether CRT regulates FUT1 expression levels by affecting mRNA stability rather than transcription. Act-D time course experiment was performed to determine the degradation rate of FUT1 mRNA. We found that FUT1 mRNA was significantly less stable with the CRT-knockdown in J82 cells (Figure 11A). These results strongly suggested that CRT down-regulation affected the mRNA stability of FUT1.

Numerous studies have shown that sequences rich in adenosine and uridine nucleotides, called AU-rich element (ARE), in 3′-UTR are important sequences for mRNA decay [92-94]. FUT-1 gene contains ARE sequence in its 3′-UTR. J82 control and CRT-RNAi cells were transfected with cmv-driven EGFP reporter fused to FUT1

3′-UTR containing wild-type, mutated, or deleted ARE as shown in Figure 11B. The EF1α-driven DsRed was used as an internal control. EGFP mRNA expression was measured by real-time PCR after 48 hours of transfection. Control cells showed a lower EGFP/DsRed ratio when ARE sequences from FUT1 3′-UTR were mutated or deleted (Figure 11C, left panel). However, no significant differences were observed between the full lengths, and ARE mutation, or deletion constructs in CRT knockdown cells (Figure 11C, right panel). Consequently, these results indicated that CRT influenced the FUT1 mRNA stability through ARE sequence.

Identification of far upstream element binding protein 1 as FUT1 mRNA binding protein

The proteins which bind to ARE, called ARE-binding proteins, play an important role in regulation of mRNA stability [92]. CRT was reported previously as an ARE-BP that regulates mRNA degradation in various cell types [33, 34]. We carried out the RNA electrophoretic mobility-shift assay (REMSA) using biotin-labeled probe and recombinant CRT to assess the CRT-FUT1 ARE interaction in vitro. No specific bands with higher molecular weight were observed (Figure 12A). We next incubated whole cell extracts with the biotin labeled probes and the bound proteins were pulled down with streptavidin. As shown in Figure 12B, CRT did not co-precipitate with

ARE or mARE probes. These results indicated that CRT did not bind to ARE in FUT1 3′-UTR.

To identify the proteins that bind to FUT1 ARE, the synthetic probe was incubated with whole cell lysate. We performed an RNA pull-down assay and the RNA-protein complex was separated on 8% SDS-PAGE. In all three independent experiments, we consistently noticed a band near 75 KD, which was suppressed in mARE smaples (Figure 12C). Using mass spectrometry we identify this ARE binding protein as far upstream element binding protein 1 (FBP1). Our results confirmed that both the protein and mRNA levels of FBP1 were reduced in CRT knockdown cells (Figure 12D & 12E). Consequently, these results suggested that CRT affected FUT1 mRNA stability through FBP1 regulation, which binds to the ARE sequence in FUT1 3′-UTR.

Chapter IV.

Discussion

CRT is well established as a multifunctional protein that regulates cell behavior [1]. In recent studies, it was shown that CRT is located in the cytosol and extracellular environments and on cell surfaces to modulate diverse physiological and pathological effects [95]. Many researchers reported that in some cancers, CRT is upregulated in tumor tissues more often than in normal tissues. Kageyama et al. also identified that CRT levels are higher in urothelial tissues of bladder cancer patients, and that urinary CRT can act as a biomarker for bladder cancer detection [49]. In this study, we surveyed the ability of CRT knockdown bladder cancer cells to suppress cell proliferation, migration, and adhesion. We further proved that *in vivo* down-expression of CRT diminishes bladder cancer tumorigenesis and metastasis.

ER is one of important organelles for protein folding. Some studies revealed that abnormal expression of CRT, an ER-resident chaperon, would trigger unfolded protein response (UPR) which result in ER stress [96, 97]. In the present study, over-expressing CRT in bladder cancer caused morphological changes and cell death whthin two weeks. These changes are consistent with a previous study showing that albumin-induced ER stress caused a conversion to the spindle-like morphology in tubular cells [98]. Therefore, we surmised that constitutively high level of CRT expreession might disturb the ER stress regulation and lead to cells to exhibit an abnormal morphology.

The N-terminal fragment of CRT, vasostatin, is an antiangiogenic factor and inhibits endothelial cell proliferation [99, 100]. This protein was implied to be an inhibitor of lung tumor growth and metastasis in different mouse models [101]. However, there is no current study exploring the effects of vasostatin in bladder cancer. Previous studies have revealed that CRT affects nuclear localization and activities of p53, which subsequently leads to apoptosis and cell cycle arrest [102, 103]. In this study, inducible CRT expression in J82 cells by tetracycline-regulated gene system did not enhance cell proliferation. We surmised that it might be due to the short induction time (only 48 hours) for J82 cells to enhance CRT expression transiently, which might not have profound effects on cell proliferation. Nevertheless, there is a decreased cell proliferation rate in the J82 CRT-RNAi cell line which has been down-regulated of CRT stably. This correlates with another study demonstrating that exogenous addition of CRT stimulates human keratinocyte and fibroblast proliferation [104]. In addition, overexpression of CRT in gastric cancer enhances cell proliferation and upregulates the expression and secretion of the well-known proangiogenic factors, VEGF and PlGF [37]. This evidence is consistent with our results showing that knockdown of CRT inhibited tumor growth *in vivo*. Moreover, neuroendocrine cells overexpressing vasostatin are also known to enhance malignant behaviors in a nude mice model [105]. Therefore, we speculated that the effects of

CRT on cell proliferation and tumorigenesis may be via regulation of the VEGF signaling pathway.

Cells adhere to extracellular matrix is regulated by various Ca^{2+}-relative pathways. The roles of CRT in cell adhesion have been extensively studied. Different expression levels of CRT affect cell adhesiveness, motility, and spread in various cell types [26, 29, 106, 107]. In this study, we demonstrated that up-regulated CRT expression increased cell attachment to type-I collagen. Furthermore, we also verified that down-regulation of CRT suppressed both J82 and T24 bladder cancer cells attachment to type-I collagen. These results suggested that CRT plays an important role in regulation of bladder cancer adhesion, and this effect may not be a specific event in J82 cells. A previous study demonstrated that CRT interacts with integrins by binding to the cytoplasmic KXGFFKR motif of the integrin α-subunit [3]. Several studies mentioned that CRT is co-localized with the $\alpha_3\beta_1$ integrin via the N-domain, and surface CRT interacts with the collagen receptors, $\alpha_2\beta_1$ integrin and glycoprotein VI, in human platelets [108, 109]. Furthermore, active integrins increase the association between CRT and $\alpha_2\beta_1$ integrin [60]. Abnormal cell migration is a typical characteristic of cancer metastasis, and integrins, an important modulator for cell adhesion and migration, are involved in this process [110]. Although CRT is proposed to play a suppressive role in prostate cancer growth and metastasis [111], it was also

shown that mRNA and protein expressions of CRT are elevated in highly metastatic prostate cancer cell lines [57]. This indicates a possible correlation between CRT and metastasis. According to these references, we hypothesized that our observation of downregulated CRT diminishing cell migration, adhesion, and metastatic abilities in bladder cancer cells might have been due to the reduced interaction between CRT and integrins.

Protein tyrosine phosphorylation is a major event for the cell adhesive mechanism [112, 113]. Cell adhesiveness decreases in CRT-underexpressing mouse L fibroblasts which contain higher levels of active c-Src [86]. However, the results of our experiments demonstrated that c-Src activity is unaltered, which led us to think that the relationship between CRT and c-Src activation may differ depending on the type of cell. Although there was no difference in c-Src phosphorylation, we observed decreases in phosphorylation levels of other crucial focal contact proteins such as FAK and paxillin. FAK is a cytoplasmic non-receptor tyrosine kinase identified as the downstream signaling molecule of integrin-dependent cell adhesion. It is also known that the c-terminal of FAK is the binding site for the integrin-mediated protein, paxillin [114]. A previous study showed that breast cancer cells with high metastatic capacity have increased mRNA expression and paxillin protein levels [115]. In addition, FAK was also proven to be a key signaling factor in tumor initiation,

angiogenesis, and metastasis [116]. This evidence supports our data which demonstrated that decreased CRT expression in bladder cancer inhibits cancer metastasis through regulating FAK and paxillin phosphorylation.

CRT regulates various cell behaviors which suggest that it may also play a part in cancer development. Much clinical research has revealed a correlation between CRT expression levels and clinicopathologic factors in different cancers. CRT is highly expressed in gastric tumor tissues and significantly correlated with tumor serosal invasion, lymph node metastasis, and microvessel density [37]. Moreover, CRT is upregulated in bladder tumor tissues, and the concentration of urinary CRT has a tendency to increase in histological grade and pathological T stage in bladder cancer [49, 50]. If a bladder cancer patient is diagnosed with advanced-stage (T2 to T4) tumors, it means that the cancer cells possess a higher invasive ability to grow into the muscle layer and can even spread to areas outside the bladder which increases the incidence of tumor metastasis. These results provide strong evidence which supports the phenomenon of bladder cancer cells that express lower levels of CRT having diminished tumor growth and metastasis in mice models. Therefore, we concluded that CRT might play an important role in bladder cancer metastasis.

RNA stability is an important control point for gene expression. It has been reported that interaction between specific sequences (*cis*-acting elements) in 3′-UTR

of mRNA and specialized RNA-binding proteins (*trans*-actin factor) regulate mRNA degradation [117]. Base on the characteristics of *cis*-acting elements, there are three classes of mRNA, and the most common type is AU-rich element (ARE) identified in 3′-UTR [91, 94, 118, 119]. A number of studies have shown that certain metastasis-associated genes with AREs are over-expressed in cancers [120-122]. Previously, we have reported that CRT levels regulate metastasis of bladder cancer cells [27]. Here, we showed that CRT-knockdown suppressed FUT1 mRNA degradation in J82 cells. The ARE database (http://brp.kfshrc.edu.sa/ARED/) screens classified FUT1 as class II cluster I of the ARE-mRNA. In our fluorescence reporter experiments, mutations or deletions in FUT1 3′-UTR-ARE sequence resulted in a significant decrease in EGFP levels in control cells. These results are consistent with previous reports that ARE sequence is important for regulating mRNA stability [118, 123]. Consequently, we suggested that CRT may modulate metastasis of bladder cancer by influencing FUT1 mRNA stability in ARE region.

Protein factors that bind to AREs are key regulators for mRNA stability. Many ARE binding proteins (ARE-BPs), including AUF1, HuR, and Tristetraprolin (TTP), have been well studied [92]. Numerous studies have reported that these ARE-BPs bind to AU-rich region and influence the stability of certain inflammatory and tumor-associated genes, including *c-myc*, *c-fos*, GM-CSF, TNF-α, and VEGF

[124-126]. CRT is a multifunctional protein that participates in various cell processes. In 2002, Nickenig *et al.* first indicated CRT as a novel mRNA binding protein that destabilizes type I angiotensin II receptor mRNA by binding to AU-rich region in 3′-UTR [33]. Moreover, Totary-Jain *et al*. reported that CRT also binds to specific element in 3′-UTR of glucose transporter-1 mRNA and destabilizes the mRNA under high-glucose conditions [34]. In this study, the fluorescence signal of mARE or dARE was suppressed only in control cells but not in CRT-RNAi cells, suggesting that CRT levels influence FUT1 mRNA stability. However, we did not notice any direct association between CRT and FUT1 ARE directly in J82 cells. On the other hand, we identified FBP1 as the protein that interacts with FUT1 ARE probe. Recent studies indicated that FBP1 is an RNA-binding protein that participates in mRNA translation and/or stabilization [127-129]. In our studies, FBP1 levels were significantly decreased in CRT-knockdown cells. Taken together, our results suggested that CRT can stabilize FUT1 mRNA through FBP1, which binds to FUT1 ARE in 3′-UTR.

Fucosylation regulated cancer cell tumorigenesis and metastasis has been extensively studied [65]. It has been reported that FUT1/FUT4-knockdown by siRNA significantly diminishes cell proliferation and tumor growth both in *vitro* and in *vivo* [74]. FUT1 overexpression restored cell adhesion in CRT-RNAi cells, suggesting that FUT1-related fucosylation affects cell adhesion and subsequent metastasis.

Nevertheless, a deficiency in GDP-mannose-4,6-dehydratase, resulting in loss of fucosylation, has been shown to cause colon cancer cell metastasis by escaping from tumor immune surveillance [130, 131]. Although this study contradicts our data, cumulative evidence suggest that tumor tissues expressed higher levels of fucosylation compare to normal tissues [132-134]. Furthermore, Numahata *et al.* revealed that sialyl Lewis X, a blood carbohydrate catalyzed by fucosyltransferase, is a predictor of invasive and metastatic outcome of bladder cancer [135]. These findings suggested a positive correlation between fucosylation and bladder cancer progression. Our results provided further evidence that FUT1 plays an important role in metastasis of bladder cancer.

Cell adhesion is one of the common features of caner metastasis. It is known that integrins are the main cell surface molecules that regulate cell-matrix interaction. Many studies revealed that changes in glycan modification on integrins affect its biological functions [136, 137]. A recent study showed that deletion of core fucosylation (FUT8) on α3β1-integrin suppressed cell migration on laminin-5 in MEFs [80]. In the present study, we found that FUT1 increased fucosylation on β1-integrin and promoted cell adhesion of bladder cancer cells on type-I collagen. Overexpression of FUT1 gene enhanced cell adhesion in CRT-knockdown cells. However, higher levels of FUT1 only partially restored cell adhesion in

CRT-knockdown cells. Consequently, our results provide evidence that FUT1-mediated fucosylation on β1-integrin is one of the important mechanisms for cell adhesion in J82 bladder cancer cells. In another study FUT1 has been shown to increase the total expression of α5β1-integrin and promote RMG-1 cell adhesion on fibronectin [88]. Nevertheless, based on our results, we suggest that these observations might due to increasing activity of β1-integrin. We also noticed a clear reduction in the activity of β1-integrin with reduction in fucosylation in our stable cell lines. Most importantly, cells treated with FUT1-specific α1,2-fucosidase effectively diminished integrin activity. Taken together, we suggested that CRT regulates cell adhesion in bladder cancer by regulating β1-integrin activity through FUT1-dependent fucosylation. Since previous studies only indicated changes in the glycosylation of integrins may affect its function on cell adhesion and migration, our results presented here provide further evidence showing the role of fucosylation on the activity of β1-integrin. It is known that there are 12 potential N-glycosylation sites on β1-integrin subunit [76]. Our stable cell lines can be excellent tools to identify the functional fucosylation sites on β1-integrin.

Chapter V.

Concluding remarks and future perspectives

Based on the clinical data published by Kageyama [49, 50], our results further clarify how CRT modulates the physiological mechanisms in bladder cancer progression. These findings, for the first time, provide evidence that altered CRT expression affects bladder cancer tumorigenesis and metastasis. Moreover, we further analyzed the mechanistic detail on CRT-dependent fucosylation and bladder cancer progression. Our findings demonstrated that CRT stabilizes FUT1 mRNA through affecting the ARE-BP, FBP1 levels. Therefore, the FUT1 activates β1-integrin by fucosylation, thereby enhancing bladder cancer adhesion and subsequent metastasis.

This study provides new insights into the biological function of α1,2-linked fucosylation on β1-integrin. Studies are underway to address the specific fucosylation modification site of β1-integrin. Our results presented here can provide a new strategy toward therapeutic development for bladder cancer. Schematic illustration summarizes the role of CRT in regulation of bladder cancer metastasis (Fig. 13).

Protein glycosylation is a common post-translational modification. Alteration in cellular glycosylation might disrupt cellular functions which causes several diseases. Our result showed that knockdown of CRT significantly suppressed fucosylated modification of β1-integrin, which might be a key reason to interrupt adhesion of J82 cells. In the future, we will attempt to investigate the detail mechanism by which FUT1-mediated β1-integrin activation.

First, this study indicated that there is a clear reduction in the activity of β1-integrin with reduction in fucosylation in our stable cell lines. Most importantly, cells treated with FUT1-specific α1,2-fucosidase effectively diminished integrin activity. Taken together, these results suggested that CRT regulates cell adhesion in bladder cancer by regulating β1-integrin activity through FUT1-dependent fucosylation. In order to clarify how fucosylation affects β1-integrin activity, we attempt to compare the protein structure by X-ray crystallography between β1-integrin with or without fucosylation. These results might provide evidences which active domain on β1-integrin would be exposed after fucosylation.

Moreover, it has been reported that there are twelve glycosylated sites on β1-integrin [76], therefore, we will attempt to profile the fucosylated site of integrin β1 by glycomic mass spectrometric analysis to compare the glycoform mapping between J82 control and CRT-knockdown cells. These results will provide information to design specific blocker by which may prevent bladder cancer adhesion and therefore subsequent metastasis.

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Table 1. Expression of CRT in different cancers

Cancer	CRT levels in tumor tissue	Clinical outcomes	Reference
Oral	increased	—	[42]
Esophagus	increased	↓survival (poor prognosis)	[41]
Breast	increased	↑metastasis, ↑invasion, ↓survival	[38, 43, 44]
Pancreas	increased	↑lymph node metastasis, ↑UICC stage, ↓survival	[40]
Gastric	increased	↑lymph node metastasis, ↑invasion, ↑microvessel density, ↓survival	[37]
Colon	increased	—	[45]
Bladder	increased	↑urinary CRT, ↑histological grade, ↑pathological T stage	[48-50]
Prostate	increased	—	[46]
Ovarian	increased	↑solid metastasis	[39]
Vagina	increased	—	[47]
Neuroblastoma	increased	↑differentiation , ↑survival	[36]

↑: increased; ↓: decreased

Table 2. Lung and liver metastatic incidence in CRT-stable cell lines.

PCR 3.1	1	2	3	4	5
Lung	¥++	+	+	+++	+
Liver	+	NT	+	NT	NT

CRT-RNAi	1	2	3	4	5	6	7
Lung	+	+	NT	+	+	+	NT
Liver	NT	NT	NT	NT	NT	NT	NT

¥ Metastatic Area: + <25%; ++ >25%; +++ >40%; NT: no tumor

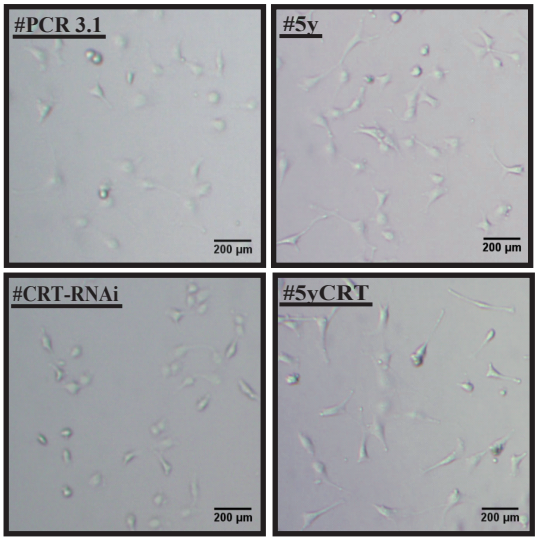
Table 3. Genes down-regulated by CRT

Gene Symbol	Description	CRT-RNAi/Control Ratio	
		fold	*P* value
FGFBP1	Fibroblast growth factor binding protein 1	-4.35	0.00
NNMT	Nicotinamide N-methyltransferase	-3.75	0.01
TES	Testis derived transcript (3 LIM domains)	-2.99	0.01
TESC	Tescalcin	-2.91	0.01
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-2.69	0.04
FUT1	**Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)**	**-2.46**	**0.01**
KIF21B	Kinesin family member 21B	-1.96	0.02
MAGED1	Melanoma antigen family D, 1	-1.86	0.00
ACSL5	Acyl-CoA synthetase long-chain family member 5	-1.85	0.02
NFIX	Nuclear factor I/X (CCAAT-binding transcription factor)	-1.77	0.03
CSPG5	Chondroitin sulfate proteoglycan 5 (neuroglycan C)	-1.71	0.04
IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	-1.71	0.02
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	-1.62	0.04
ALPP	Alkaline phosphatase, placental (Regan isozyme)	-1.60	0.00
CENTB1	Centaurin, beta 1	-1.54	0.01
EGR1	Early growth response 1	-1.42	0.00
HSPB8	Heat shock 22kDa protein 8	-1.41	0.05
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	-1.31	0.05
VEGFA	Vascular endothelial growth factor A	-1.16	0.02
TNFSF13	Tumor necrosis factor (ligand) superfamily, member 13	-1.11	0.04
SSTR2	Somatostatin receptor 2	-1.10	0.05
TM4SF19	Transmembrane 4 L six family member 19	-1.10	0.00
SCARA3	Scavenger receptor class A, member 3	-1.10	0.02
DDIT4	DNA-damage-inducible transcript 4	-1.09	0.01
PRSS35	Protease, serine, 35	-1.06	0.01
CSAG2	CSAG family, member 2	-1.05	0.01

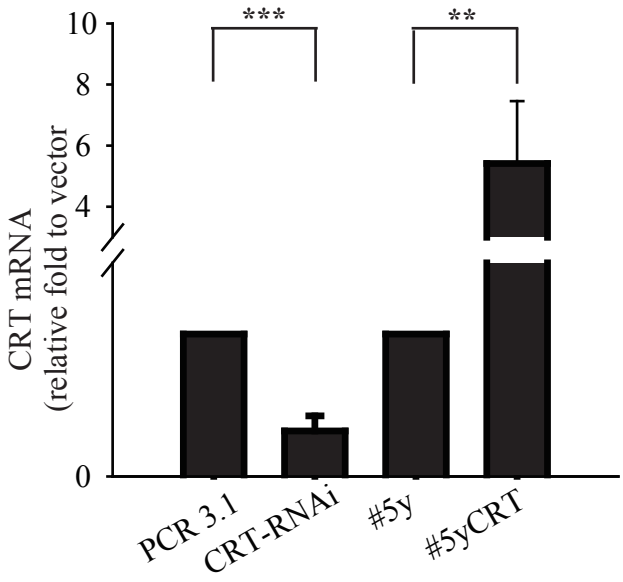
Table 4. Genes up-regulated by CRT

Gene Symbol	Description	CRT-RNAi/ Control Ratio	
		fold	*P* value
TRIP6	Thyroid hormone receptor interactor 6	5.94	0.00
DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	2.80	0.00
CSMD1	CUB and Sushi multiple domains 1	1.98	0.00
CASP8	Caspase 8, apoptosis-related cysteine peptidase	1.57	0.01
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	1.47	0.04
EPS8	Epidermal growth factor receptor pathway substrate 8	1.44	0.01
SERINC1	Serine incorporator 1	1.41	0.01
LOC165186	Similar to RIKEN cDNA 4632412N22 gene	1.40	0.02
FLJ41327	FLJ41327 protein	1.34	0.03
FLJ23861	Hypothetical protein FLJ23861	1.28	0.00
BZW1	Basic leucine zipper and W2 domains 1	1.26	0.05
NAT14	N-acetyltransferase 14	1.24	0.01
DALRD3	DALR anticodon binding domain containing 3	1.19	0.01
OXR1	Oxidation resistance 1	1.09	0.01
MAPK1	Mitogen-activated protein kinase 1	1.07	0.01
RBM13	RNA binding motif protein 13	1.05	0.02
PROSC	Proline synthetase co-transcribed homolog (bacterial)	1.05	0.03
NAP1L4	Nucleosome assembly protein 1-like 4	1.04	0.00
HDDC2	HD domain containing 2	1.02	0.04

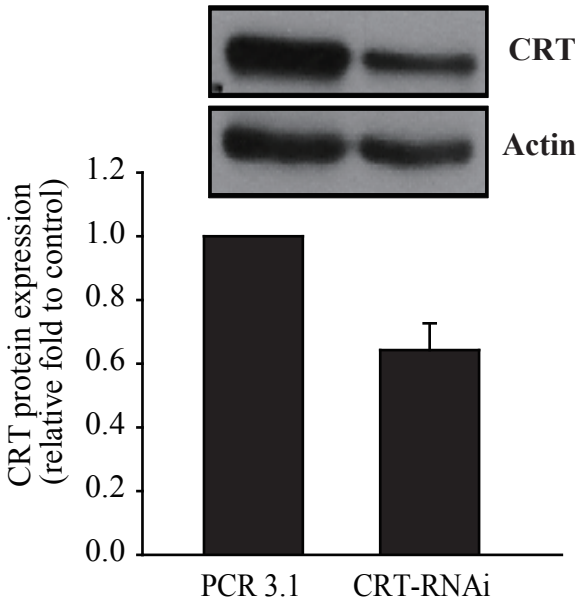
A.


#PCR 3.1
#5y
#CRT-RNAi
#5yCRT
200 μm

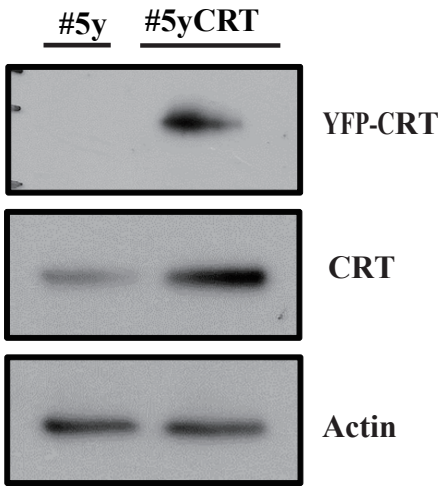
B.


***
**
10
8
6
4
0
CRT mRNA
(relative fold to vector
PCR 3.1
CRT-RNAi
#5y
#5yCRT

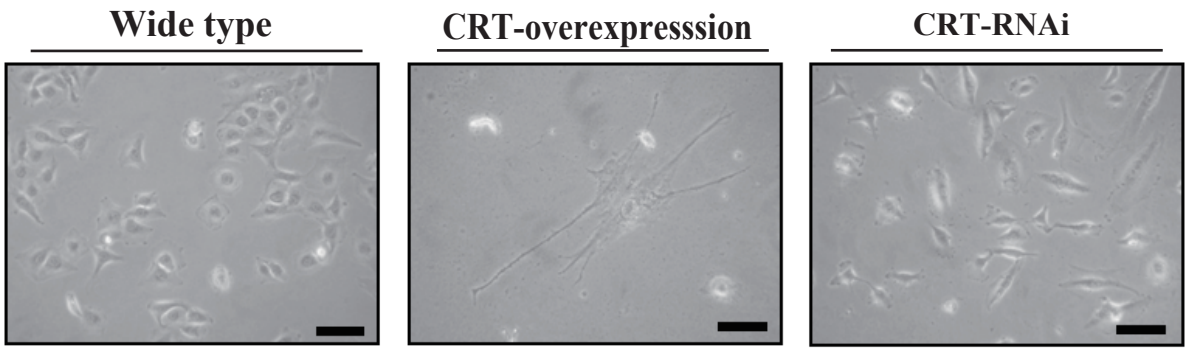
C.


CRT
Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CRT protein expression
(relative fold to control)
PCR 3.1
CRT-RNAi

D


#5y
#5yCRT
YFP-CRT
CRT
Actin

E.


Wide type
CRT-overexpresssion
CRT-RNAi

F.

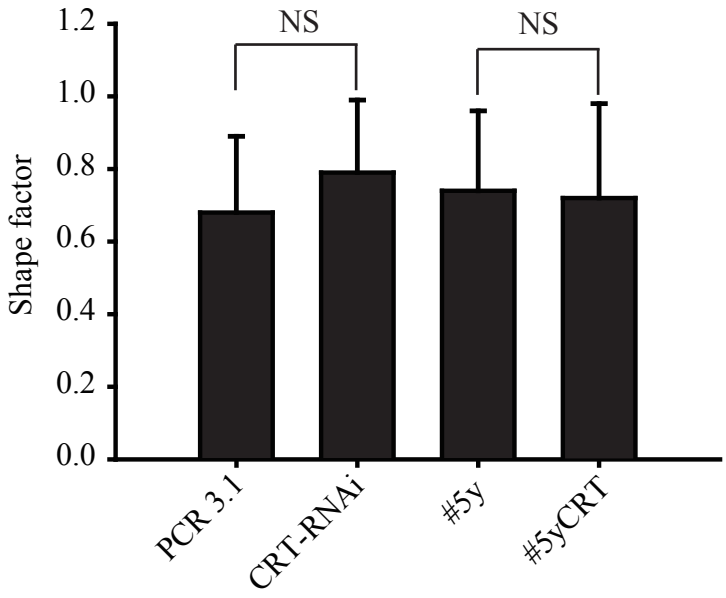
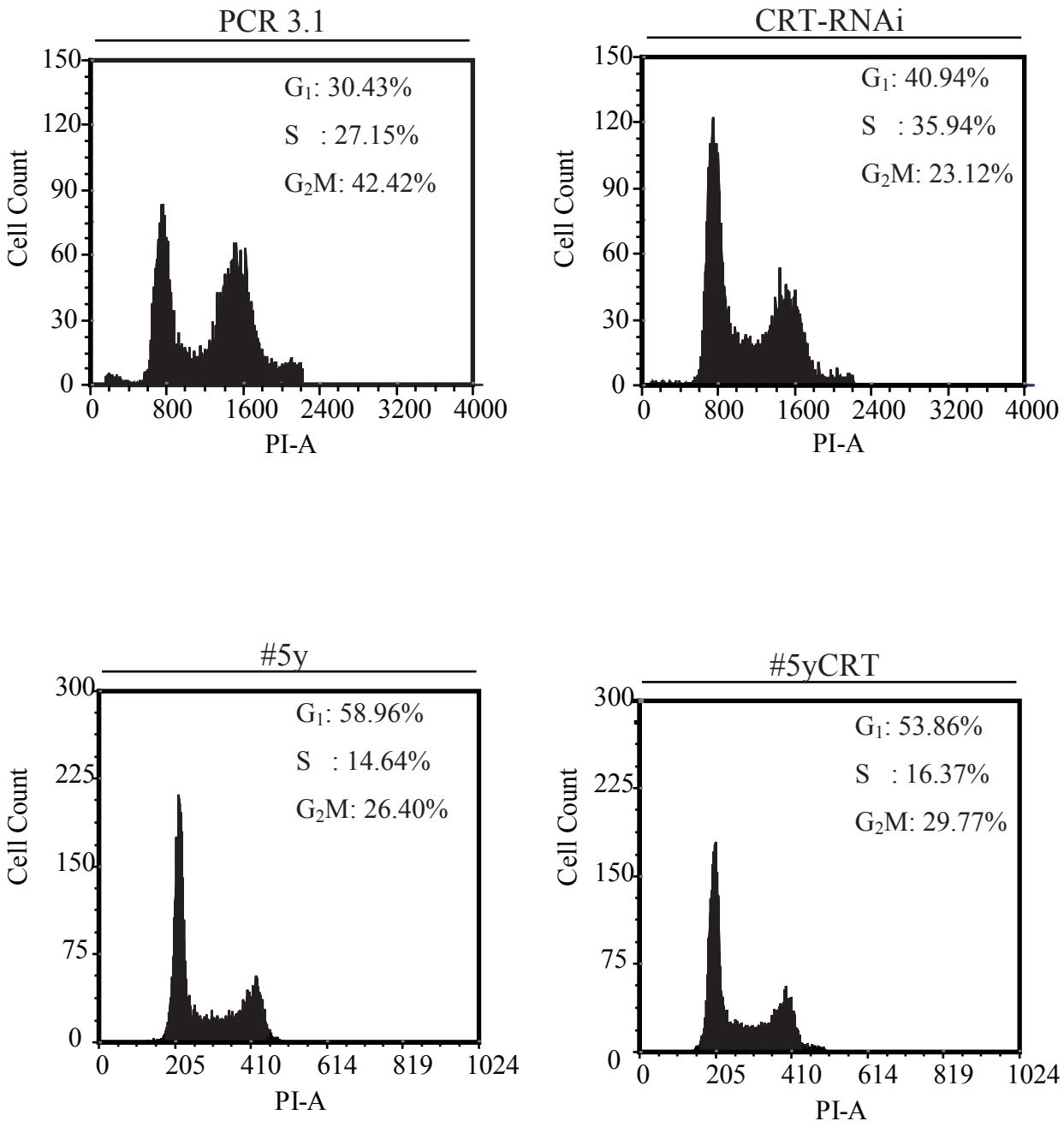

NS
NS
Shape factor
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PCR 3.1
CRT-RNAi
#5y
#5yCRT

Figure 1. J82 bladder cancer cells were stably transfected with calreticulin (CRT) overexpressing and RNAi plasmids. (A) Cell morphology of CRT in differentially expressed cell lines in serum-containing medium. #5y and #5yCRT cells were stimulated with 1 μg/ml tetracycline for 48h to induce CRT expression. Bar, 200 μm. (B) mRNA expressions were confirmed by real-time PCR in CRT knockdown and overexpression stable cell lines. CRT mRNA expression was normalized to the internal control, GAPDH. (C) Western blot analysis demonstrates protein expression in CRT-RNAi stable cell lines. The human β-actin level was used as a loading control. (D) An endogenous CRT protein band (about 60 KD) was both detected in #5y and #5yCRT cells. An exogenous YFP-CRT fusion protein (about 87 KD) was only detected in #5yCRT cells. All the results are repeated at least three independent experiments. (E) Cell morphology of CRT in differentially expressed cell lines. Compared to wild-type cells, J82 bladder cancer cells were spindle-shaped and differentiated when CRT was overexpressed. (F) Morphological changes in CRT knockdown and overexpression cells were assessed of the shape factor (sf = 4A/p2) by using Metamorph program. A shape factor of "1" represents a circular object, and a shape factor of "0" represents a flat object. Each bar of the histogram represents quantified results and are shown as the mean value ± SD. Statistical differences were compared to the control level (** $p<0.01$; *** $p<0.001$).

A.


PCR 3.1
G1: 30.43%
S : 27.15%
G2M: 42.42%
CRT-RNAi
G1: 40.94%
S : 35.94%
G2M: 23.12%
#5y
G1: 58.96%
S : 14.64%
G2M: 26.40%
#5yCRT
G1: 53.86%
S : 16.37%
G2M: 29.77%
Cell Count
PI-A

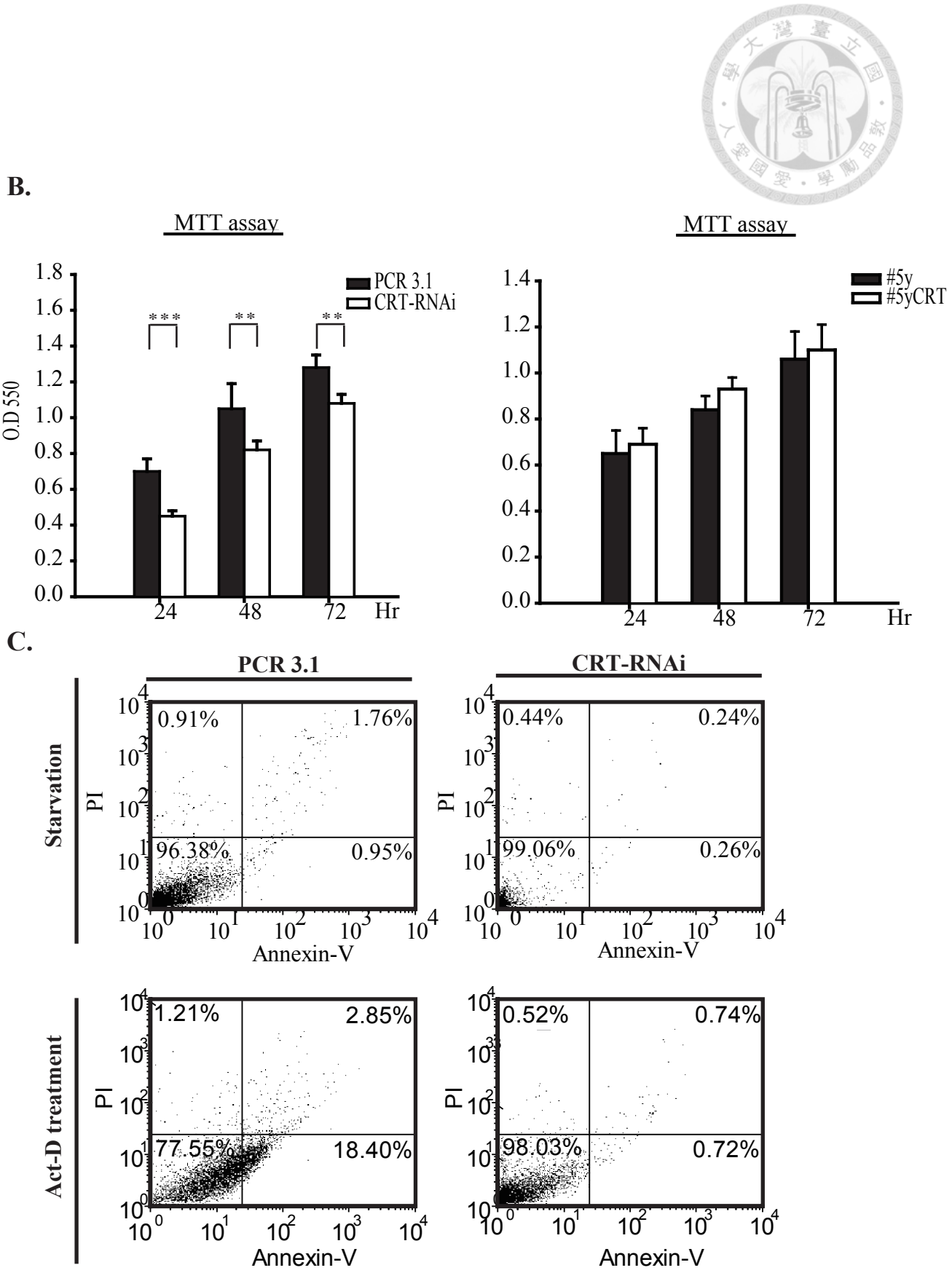
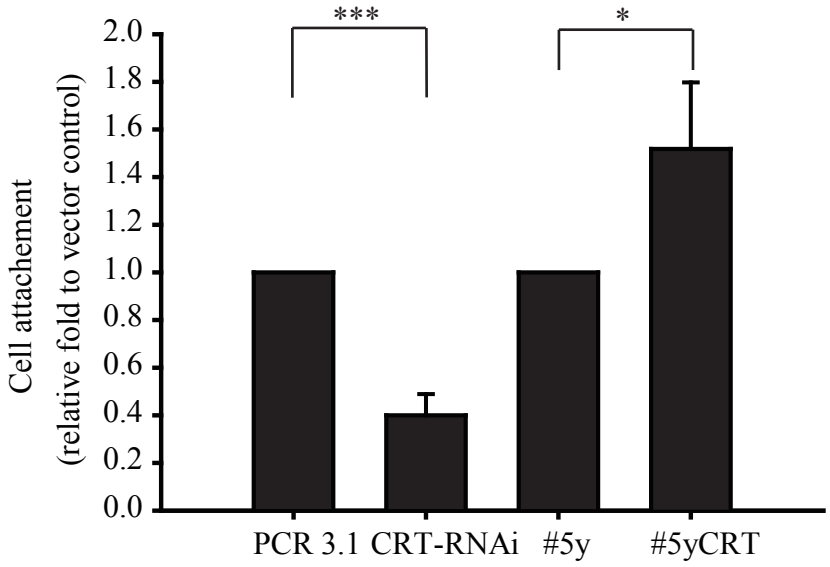

B.
MTT assay
O.D 550
PCR 3.1
CRT-RNAi
***
**
**
24
48
72
Hr
MTT assay
#5y
#5yCRT
24
48
72
Hr
C.
PCR 3.1
CRT-RNAi
Starvation
PI
Annexin-V
0.91%
1.76%
96.38%
0.95%
0.44%
0.24%
99.06%
0.26%
Act-D treatment
1.21%
2.85%
77.55%
18.40%
0.52%
0.74%
98.03%
0.72%

Figure 2. Knockdown of calreticulin (CRT) altered the bladder cancer cell proliferation rate. (A) The cell cycle was analyzed by flow cytometry. CRT-RNAi cells showed proportionally more cells in the G1 phase than the S and G2M phases compared to the vector control group (PCR 3.1). Cells induced CRT expression did not promote cell cycle transit. (B) Cell proliferation was determined by MTT assay. Cells (2500 cells/well) were seeded in 96-well plate. Cell numbers were determined by measuring the absorbance of 550 nm at 24, 48 and 72 hours after plating. (C) Apoptosis was evaluated after starvation for 24 hours, and stained with annexin-V. Treatment of act-D (5μg/ml) is used as positive control. The number showed the percentage of early apoptotic cells (lower right quadrant). These results were confirmed by at least three independent experiments (** $p<0.01$; *** $p<0.001$).

A.


***
*
Cell attachement
(relative fold to vector control)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PCR 3.1
CRT-RNAi
#5y
#5yCRT

B.

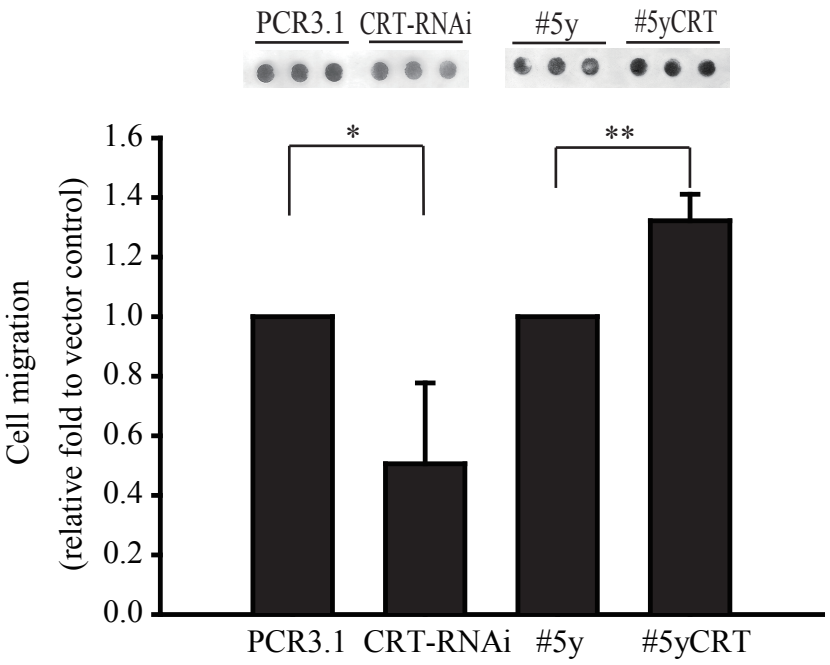
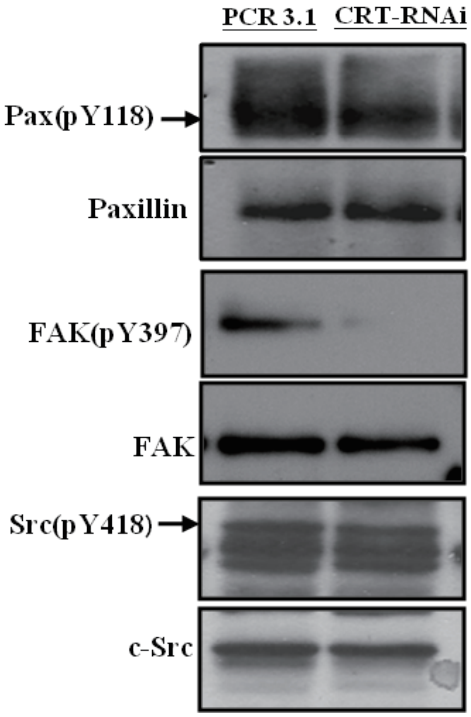
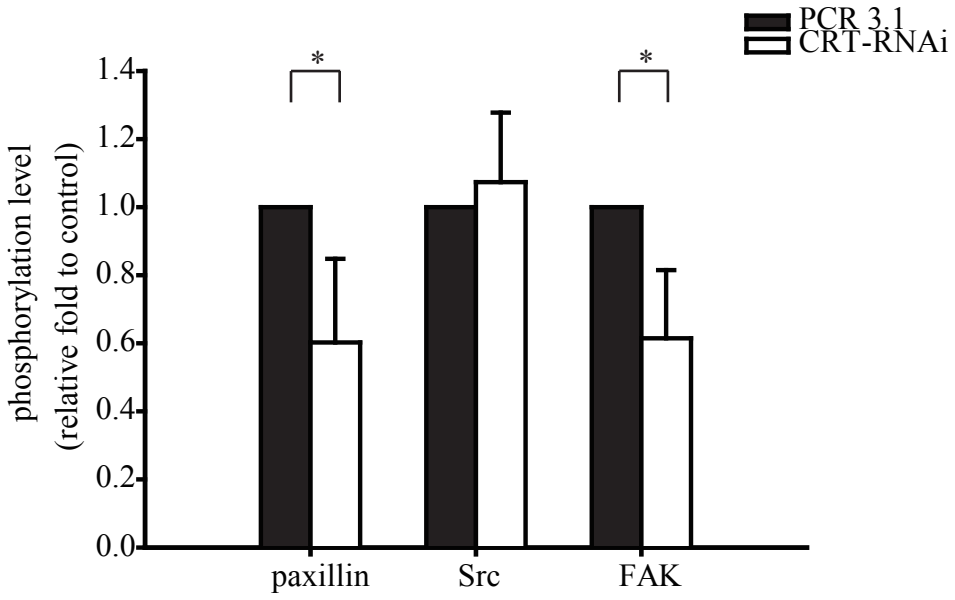

PCR3.1
CRT-RNAi
#5y
#5yCRT
*
**
Cell migration
(relative fold to vector control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PCR3.1
CRT-RNAi
#5y
#5yCRT

Figure 3. Cell adhesion and migratory ability were regulated by CRT expression levels. (A) Cells (5 x 10^4 cells/100µl) were seeded in 96-well collagen-coated (10 µg/ml) plates for 20 min. Compared to transfected control vector cells, CRT-RNAi cells adhered poorly to type-I collagen. Cells with higher CRT levels showed a stimulation of attachment to type-I collagen. (B) Cells (1.5 x 10^4 cells/well) were loaded into the upper chamber of a modified Boyden's chamber, and FBS was loaded into the lower chamber as a chemoattractant for cells. After 4 h, CRT-RNAi cells had migrated significantly slower than control (PCR 3.1) cells, and $^{\#}$5yCRT cells exhibited an opposite effect than its control ($^{\#}$5y) cells. (C) T24 Cells (5 x 105 cells/ml) were seeded in 96-well collagen-coated (10 µg/ml) plates for 20 min. Compared to transfected control vector cells, CRT-RNAi cells adhered poorly to type-I collagen. One of three independent experiments, performed in triplicate, is shown. Each bar of the histogram represents quantified results and is shown as the mean value ± SD. Statistical differences were compared to the control level (* p<0.05; ** p<0.01; *** p<0.001).

A.


PCR 3.1
CRT-RNAi
Pax(pY118)
Paxillin
FAK(pY397)
FAK
Src(pY418)
c-Src

B.


PCR 3.1
CRT-RNAi
phosphorylation level
(relative fold to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
paxillin
Src
FAK

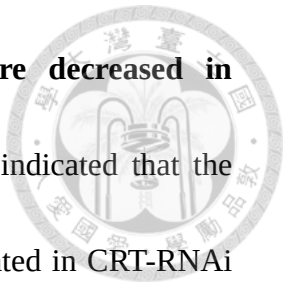
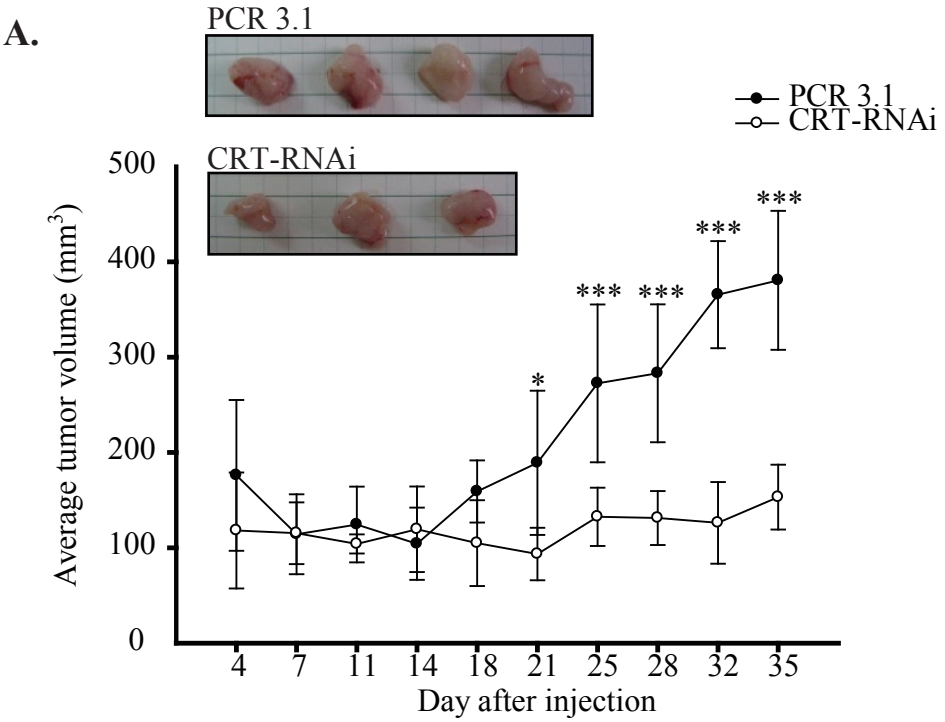


Figure 4. Phosphorylation levels of FAK and paxillin were decreased in calreticulin (CRT) knockdown cells. (A) Western blot analysis indicated that the phosphorylation levels of paxillin and FAK were more downregulated in CRT-RNAi cells than in control cells. The proportion of phosphorylated Src was not altered in CRT-RNAi cells. (B) Quantitative results of the phosphorylated ratios of paxillin, FAK, and Src were normalized by each total protein expressions. One of three independent experiments was shown. Each bar of the histogram represents quantified results and is shown as the mean value ± SD. Statistical differences were compared to the control level. (* $p<0.05$; NS, no significant difference)


A.
PCR 3.1
CRT-RNAi
PCR 3.1
CRT-RNAi
Average tumor volume (mm3)
500
400
300
200
100
0
*
***
***
***
***
4
7
11
14
18
21
25
28
32
35
Day after injection

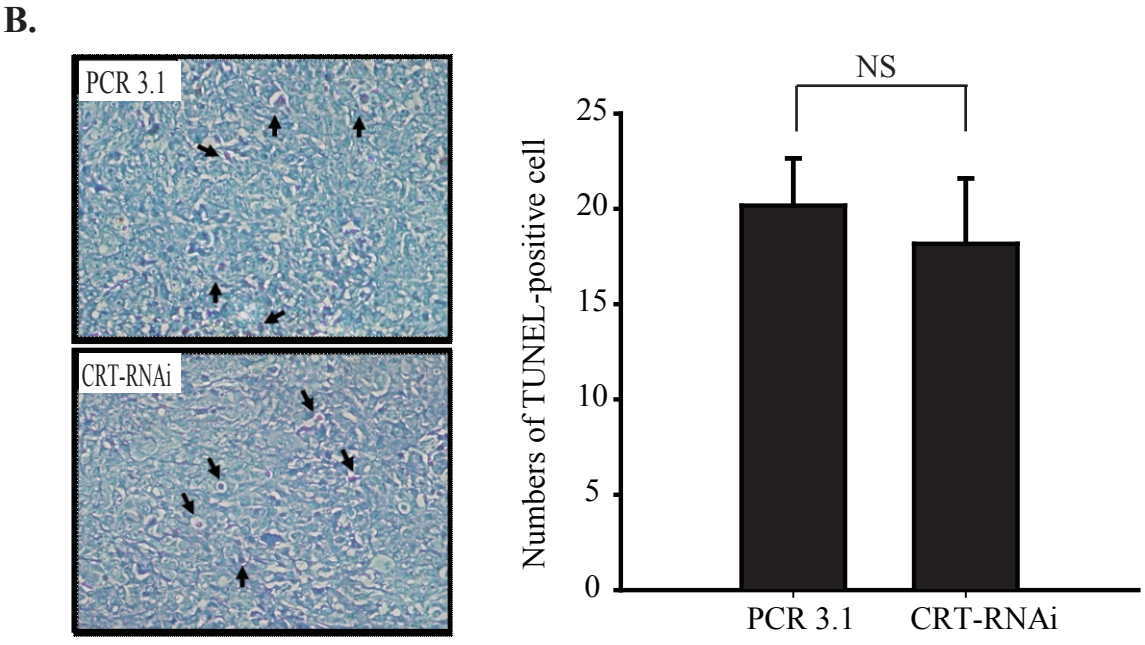
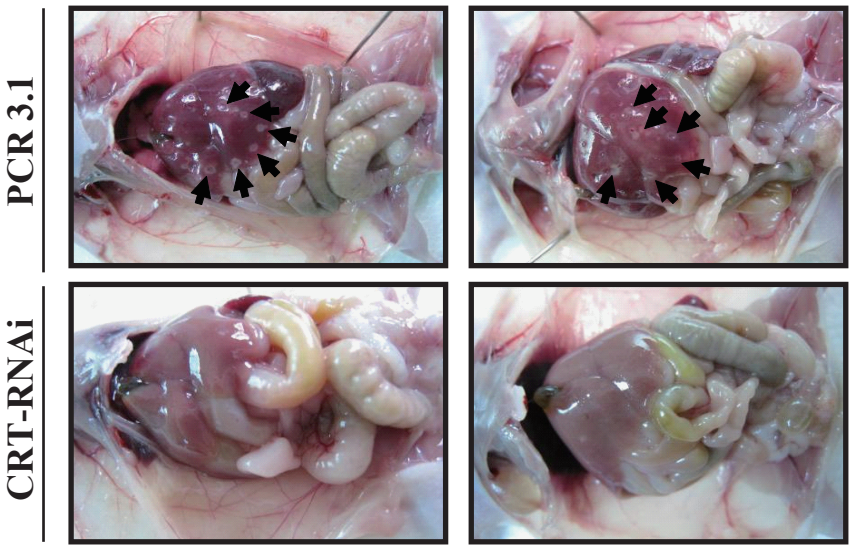
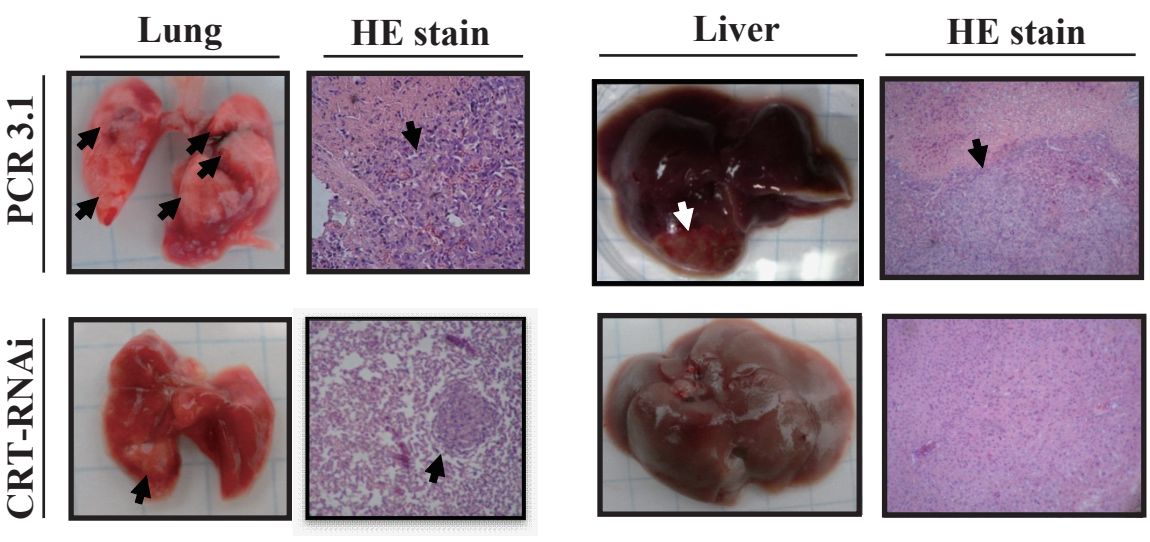

B.
PCR 3.1
CRT-RNAi
NS
Numbers of TUNEL-positive cell
25
20
15
10
5
0
PCR 3.1
CRT-RNAi

Figure 5. Knockdown of calreticulin (CRT) diminishes tumor formation in nude mice. (A) Mice were subcutaneously injected with vector control cells (closed circles) or CRT-RNAi cells (open circles), and tumor sizes were measured over time. After 20 days, tumor sizes significantly differed between the two groups. Knockdown of CRT suppressed tumorigenesis in bladder cancer cells. (B) Determination of TUNEL-positive cells for injection of cells from mice (high power field, 200X). The arrows indicate the positive cells. There is no significant difference between the two groups. Each bar of the histogram represents quantified results of the TUNEL-positive/field and is shown as the mean value ± SD. (* $p<0.05$, *** $p<0.001$).

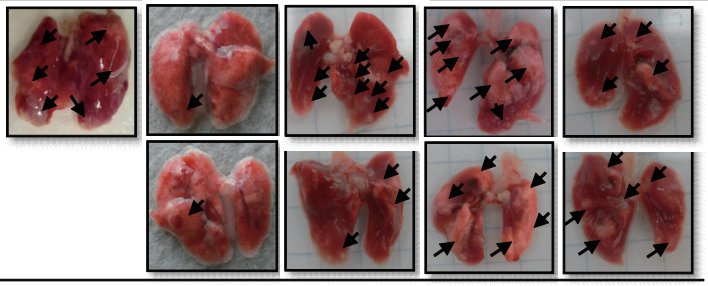
A.

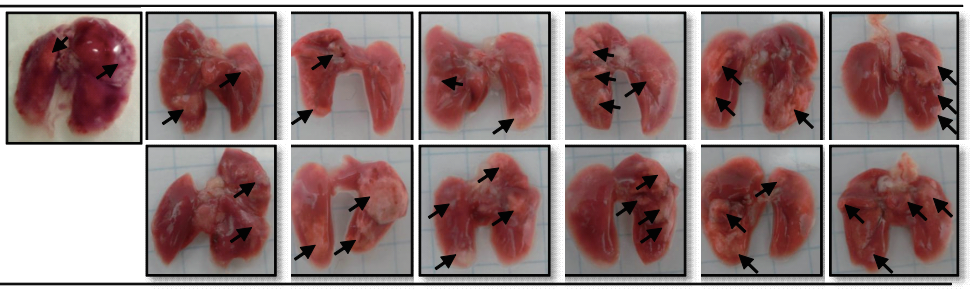


B.

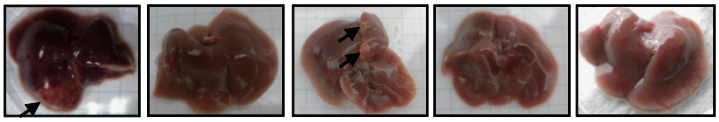


C.

Control	1	2	3	4	5
					
Metastatic percentage (%)	27.1	1.9	5.9	42.3	12.6

CRT-RNAi	1	2	3	4	5	6	7
							
Metastatic percentage (%)	10.0	7.8	24.8	7.6	16.8	21.6	20.1

D.

Control	1	2	3	4	5
					

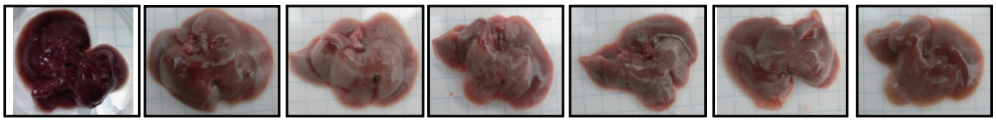
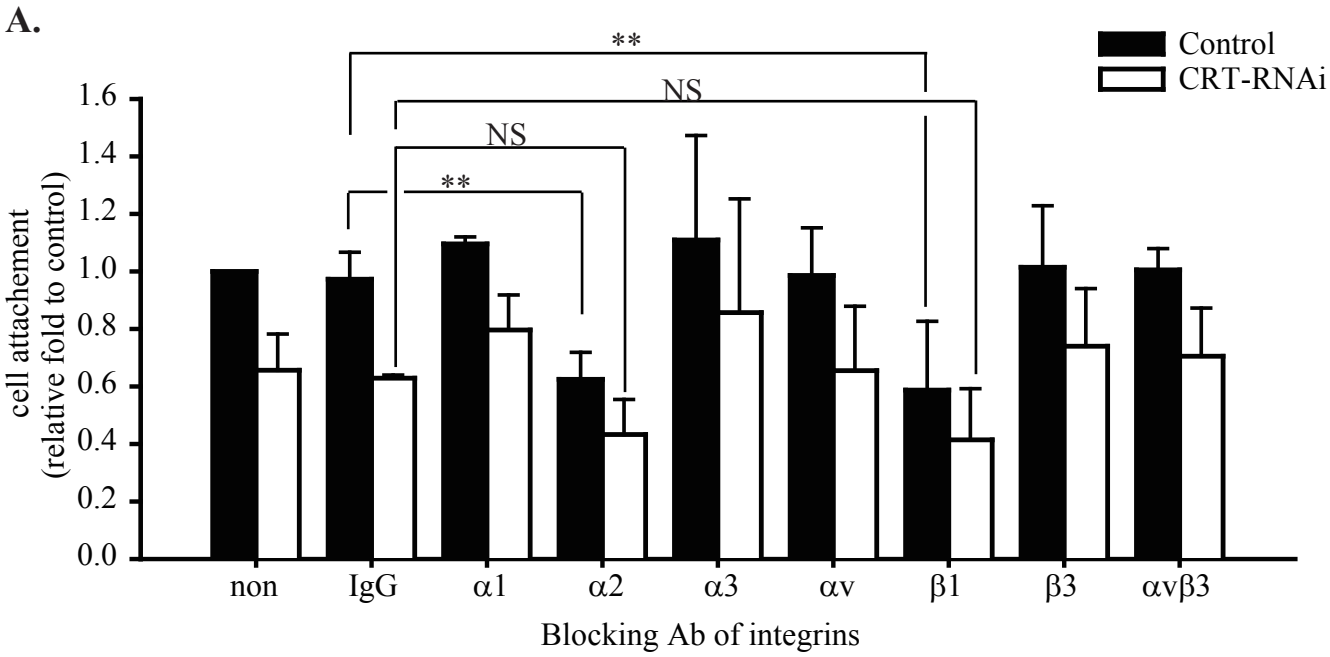
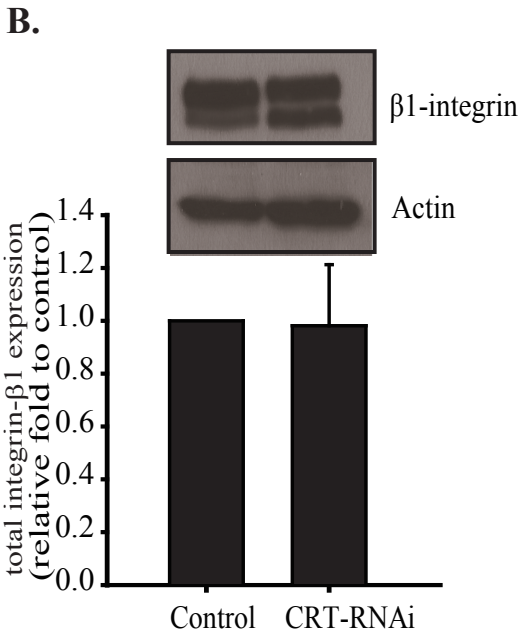
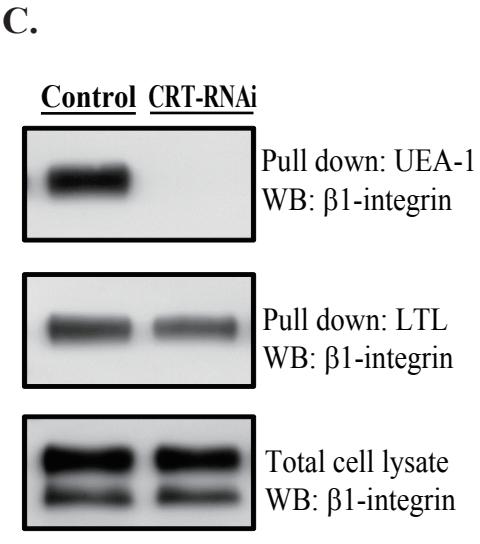
CRT-RNAi	1	2	3	4	5	6	7
							

Figure 6. Knockdown of calreticulin (CRT) inhibits metastasis of bladder cancer cells. (A) Nude mice were subcutaneously injected with 10^7 cells and sacrificed after 45 days. There were obvious metastatic nodules in the liver of the control group (shown by arrows) but not in the CRT-RNAi group. (B) Lung and liver metastasis were performed by an intravenous injection. In total, 10^7 cells (control and CRT-RNAi) were injected into the tail vein of SCID mice (n=5 and n=7, respectively). After 40 days, both groups had lung metastases, but the area of lung metastasis was higher in the control group. Liver metastasis was only observed in control mice. (C) Percentage of lung and liver metastatic areas. Both groups had lung metastases, and the metastatic percentages are shown. The arrows indicate the metastatic area of the lung. (D) Liver metastasis was only observed in the transfected vector control group, but none was shown in the CRT-RNAi group.


A.
Control
CRT-RNAi
**
NS
NS
**
cell attachement
(relative fold to control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
non
IgG
α1
α2
α3
αv
β1
β3
αvβ3
Blocking Ab of integrins


B.
β1-integrin
Actin
total integrin-β1 expression
(relative fold to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CRT-RNAi


C.
Control
CRT-RNAi
Pull down: UEA-1
WB: β1-integrin
Pull down: LTL
WB: β1-integrin
Total cell lysate
WB: β1-integrin

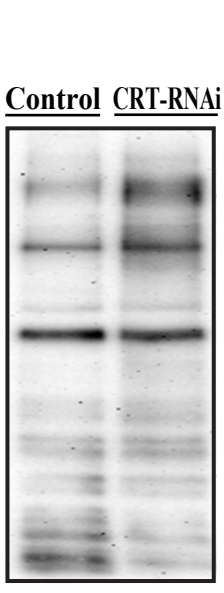
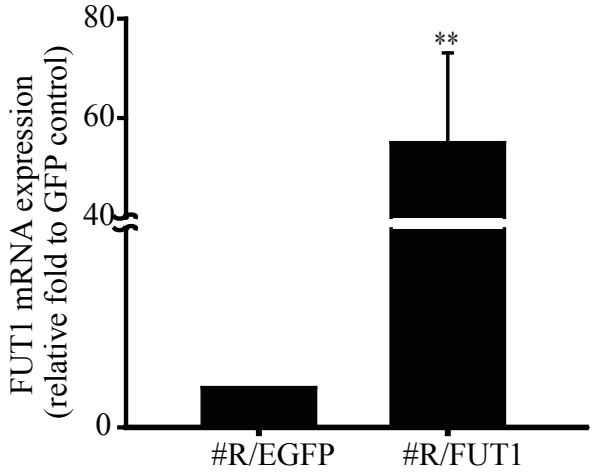
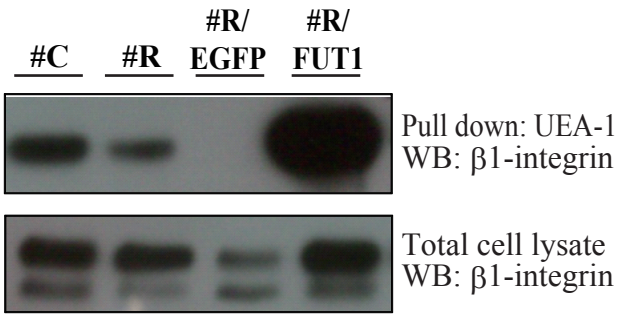

D.
Control
CRT-RNAi

Figure 7. Integrin α2β1 was involved in CRT-mediated cell adhesion in J82 bladder cancer cells. (A) To analyze the role of integrins in J82 cell adhesion, cells were pre-incubated with normal mouse IgG (M-IgG) or functional blocking antibodies at 37℃ for 1hr. $5x10^4$ cells were seed in a 96-well collagen-coated plate (10μg/ml) for 20 min. (B) Western blot (WB) analysis showing no changes in the total β1-integrin expression levels between the control and CRT-knockdown cells. The human β-actin was used as a loading control. (C) FUT1-modified glycosylation on β1-integrin in J82 stable cell lines. Cell lysates were pulled down with UEA-1 or LTL followed by WB with anti-β1-integrin antibody. (D) WB analysis showing the glycomic differences between the control and CRT-knockdown cells. Cell lysates were separated on 10% SDS-PAGE and probed with biotin-labeled UEA-1. Representative data from three independent experiments was shown. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared to the control level (** $p < 0.01$; *** $p < 0.001$).

A.


80
60
40
0
**
FUT1 mRNA expression
(relative fold to GFP control)
#R/EGFP
#R/FUT1

B.


#C
#R
#R/
EGFP
#R/
FUT1
Pull down: UEA-1
WB: β1-integrin
Total cell lysate
WB: β1-integrin

C.

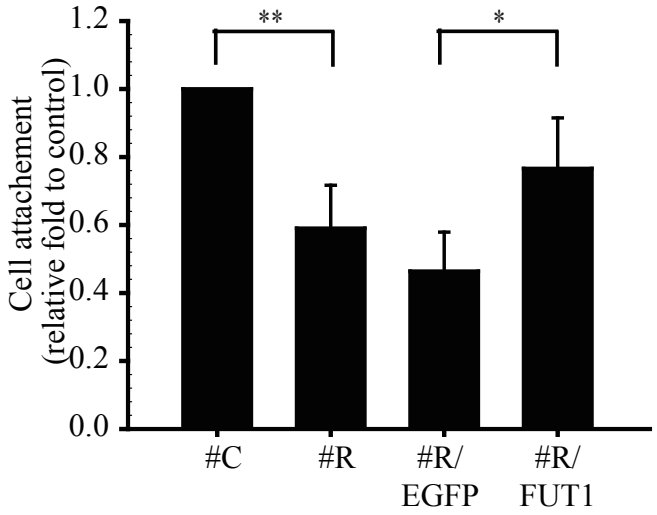
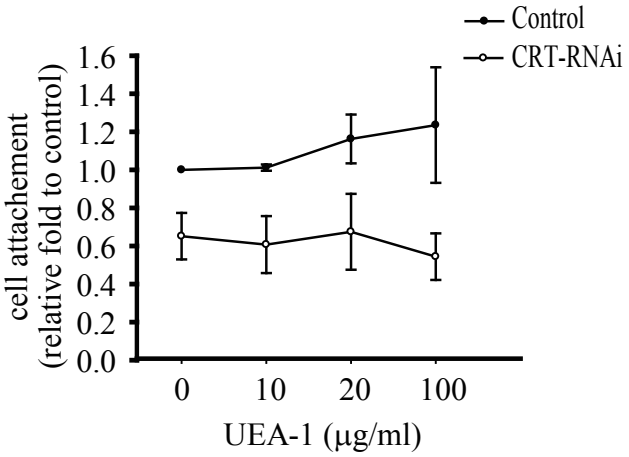

1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Cell attachement
(relative fold to control)
#C
#R
#R/
EGFP
#R/
FUT1

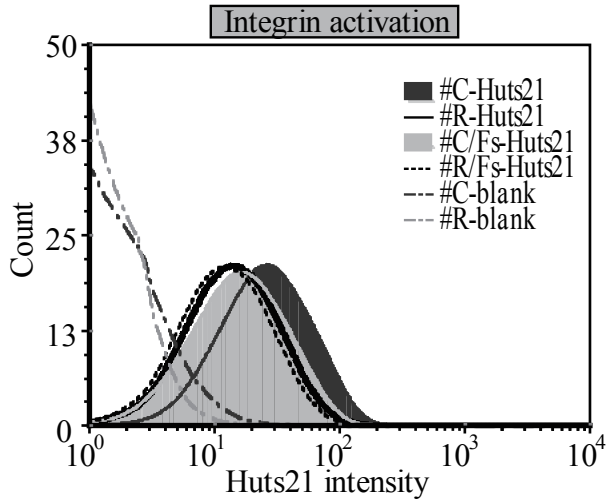
Figure 8. FUT1 overexpression rescued cell adhesion in CRT-knockdown cells. Reintroduction of FUT1 in J82 CRT-RNAi cells. CRT-knockdown stable cells were infected with the control (EGFP) or FUT1 plasmid to generate FUT1-overexpressed stable cell lines. (A) mRNA expression was confirmed by real-time PCR in FUT1 overexpression stable cell lines. GAPDH was used as an internal control. (B) Lectin pull-down assay was performed to assess whether overexpressed FUT1 could enhance glycosylation of β1-integrin. Cell lysates were pulled down with UEA-1 followed by WB with anti-β1-integrin antibody. Representative data from three independent experiments was shown. (C) Effects of FUT1 on CRT-mediated cell adhesion were performed by adhesion experiment. Cells ($5x10^4$cells/100μl) were seeded in 96-well collagen-coated (10μg/ml) plates for 20 min. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared to the vector control level (* $p < 0.05$; ** $p < 0.01$). #C, control cells; #R, CRT-RNAi cells; #R-EGFP, CRT-knockdown cells infected with EGFP; #R-FUT1, CRT-knockdown cells infected with FUT1/EGFP.

A.



B.

	Geometric Mean	
	Control	CRT-RNAi
fucosidase -	22.9 ± 3.9	13.0 ± 2.6
fucosidase +	15.5 ± 3.6 *	9.8 ± 1.4 NS



C.

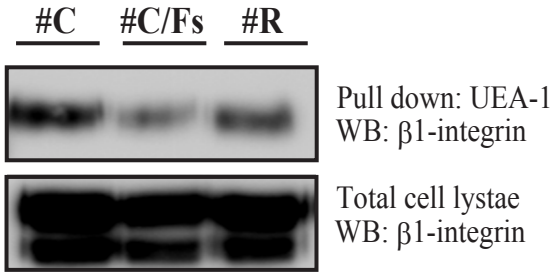
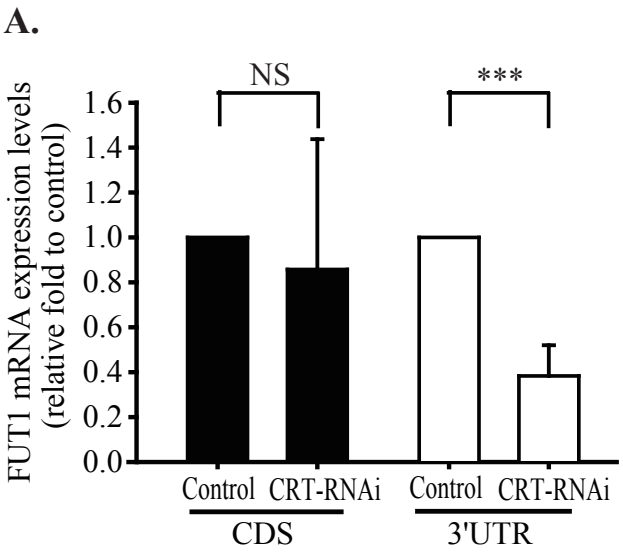


Figure 9. The activation of β1-integrin decreased by reduction in α1,2-linked fucosylation. (A) To evaluate the involvement of α1,2-linked glycan in integrin-collagen interaction, cells were pre-incubated with varying concentration of UEA-1 for 30 min . $5x10^4$ cells were seeded in 96-well collagen-coated plates (10μg/ml) for 20 min. Each point of the histogram represents quantified results and is shown as the mean ± SD. (B) The activity of β1-integrin was analyzed by flow cytometry. Cells were trypsinized and incubated with PE-conjugated anti-HUTS21 antibody (active form of β1-integrin) for 20 min. Cells were also pre-treated with α1,2-fucosidase for 30 min before staining with anti-HUTS21 antibody to further analyze the effect of α1,2-linked fucosylation on β1-integrin activity. (C) FUT1-modified glycosylation on β1-integrin after treatment with α1,2-fucosidase. J82 control cells were pre-treated with α1,2-fucosidase before lysed with NP-40 lysis buffer. Cell lysates were pulled down with UEA-1 or LTL followed by WB with anti-β1-integrin antibody. Representative data from three independent experiments was shown. The quantified results are shown as the geometric mean± SD. Statistical differences were compared to no-fucosidase group (* $p < 0.05$; NS: not significant). Fs, cells were treated with α1,2-fucosidase.


A.
NS
***
FUT1 mRNA expression levels
(relative fold to control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CRT-RNAi
Control
CRT-RNAi
CDS
3'UTR

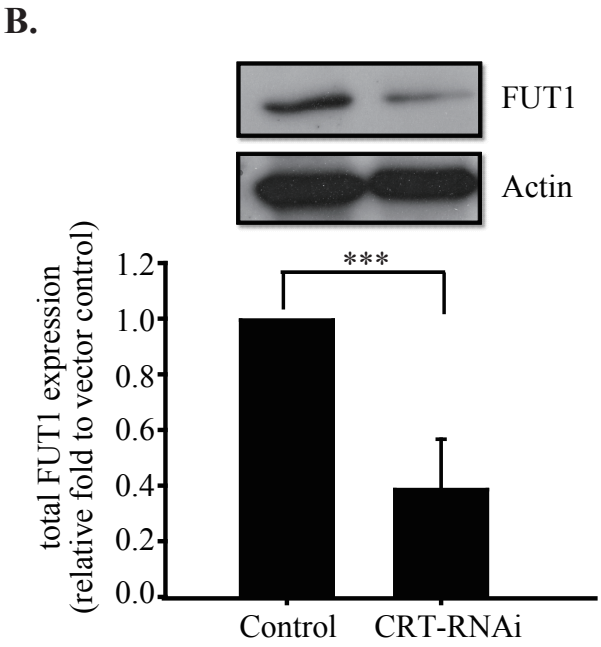
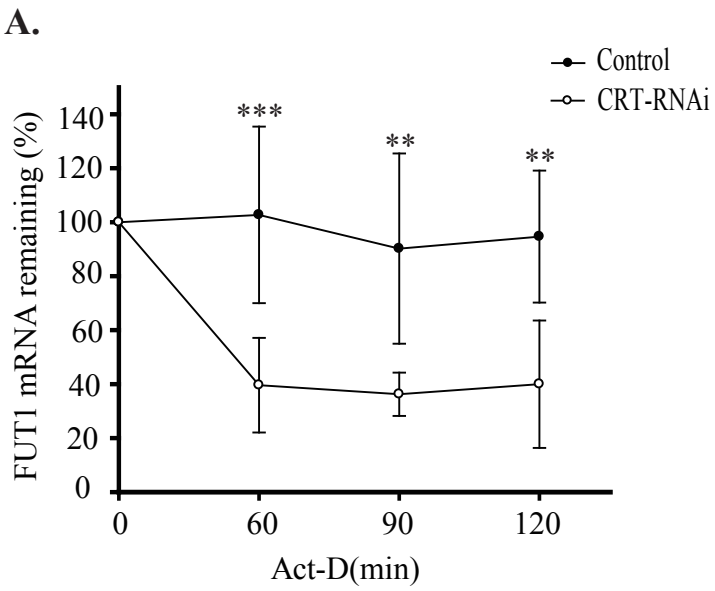
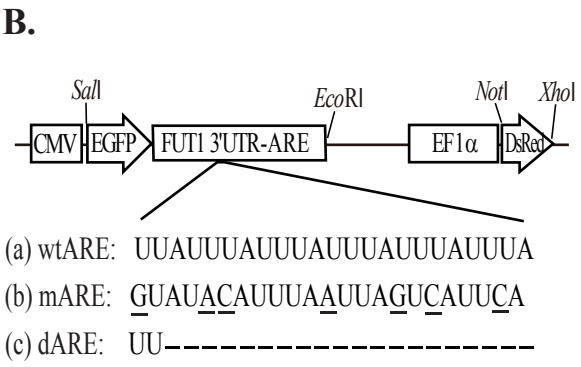
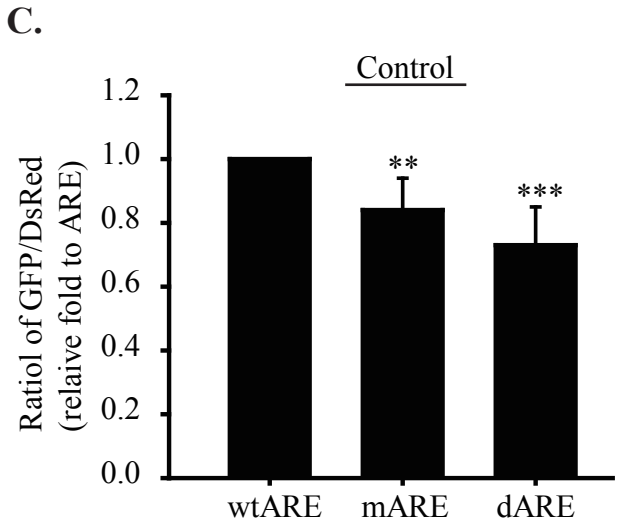

B.
FUT1
Actin
***
total FUT1 expression
(relative fold to vector control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CRT-RNAi

Figure 10. Calreticulin-knockdown inhibited the FUT1 protein and 3′-UTR mRNA expression levels but not the mRNA of coding region. (A) FUT1 mRNA levels were detected by real-time PCR in J82 control and CRT-knockdown cells. mRNA expression was normalized to the internal control, GAPDH. CDS, coding region sequence; 3′-UTR, 3′- untranslated region. (B) Western blot analysis demonstrates protein expression in J82 stable cell lines. The human β-actin level was used as a loading control. Each bar of the histogram represents quantified results and shown as the mean ± SD. Statistical differences were compared to the control level (NS, no significant; *** $p < 0.001$).


A.
Control
CRT-RNAi
FUT1 mRNA remaining (%)
140
120
100
80
60
40
20
0
***
**
**
0
60
90
120
Act-D(min)


B.
SalI
EcoRI
NotI
XhoI
CMV
EGFP
FUT1 3'UTR-ARE
EF1α
DsRed
(a) wtARE: UUAUUUAUUUAUUUAUUUAUUUA
(b) mARE: GUAUACAUUUAAUUAGUCAUUCA
(c) dARE: UU----------


C.
Control
Ratiol of GFP/DsRed
(relaive fold to ARE)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
wtARE
mARE
dARE

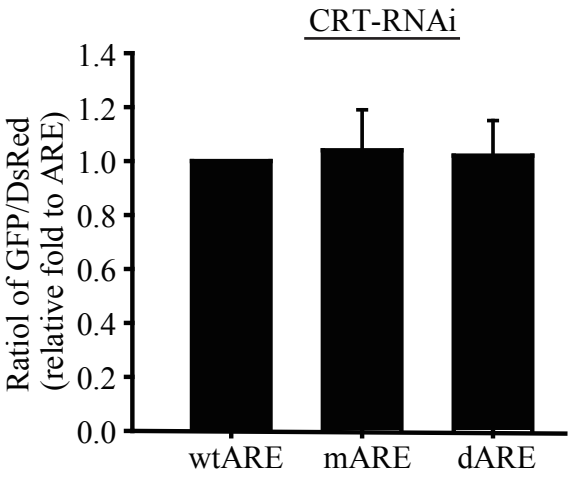
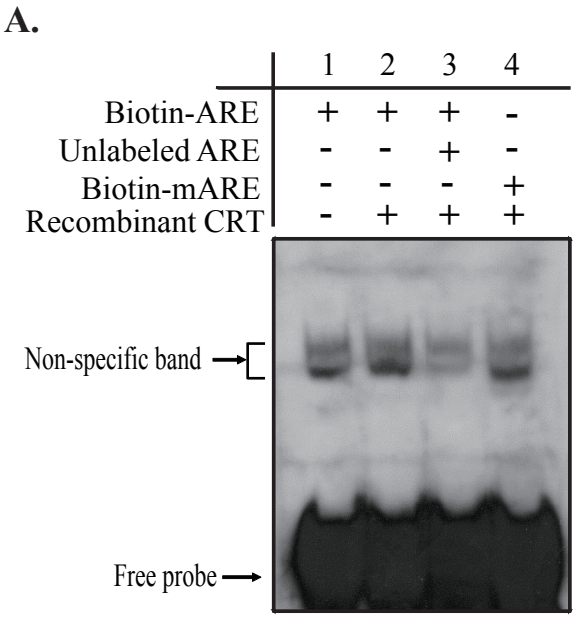
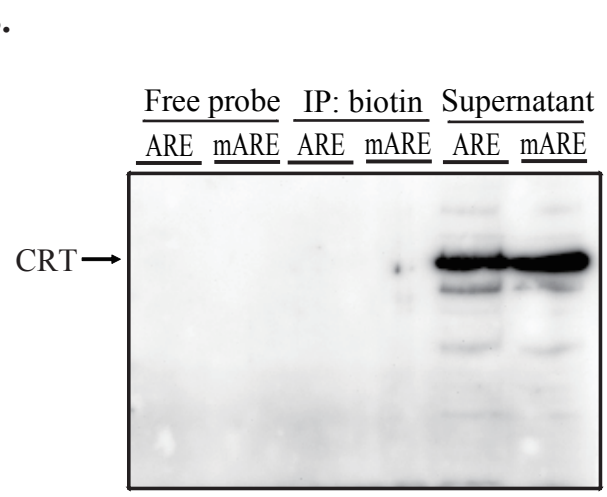
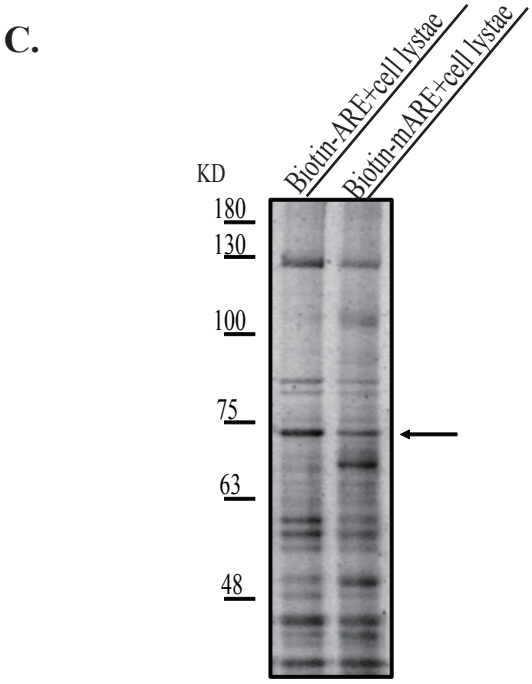
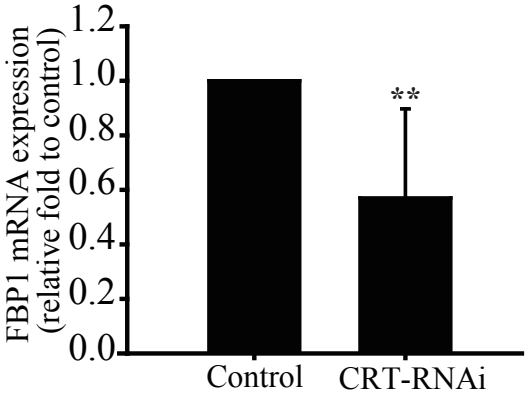

CRT-RNAi
Ratiol of GFP/DsRed
(relative fold to ARE)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
wtARE
mARE
dARE

Figure 11. Calreticulin-knockdown destabilized FUT1 mRNA through AU-rich element. (A) Cells were treated with 5 μg/ml actinomycin-D for indicated time points. FUT1 mRNA was assessed by real-time PCR and normalized to the internal control, GAPDH. (B) Schematic diagram of reporter constructs containing EF1α-driven DsRed and CMV-driven EGFP fused to various ARE sequences. (C) Cells were transfected with the plasmids shown in Fig 5B. Total RNA was harvested after 24h of transfection. Each bar of the histogram represents the ratio of EGFP to the internal transfected control, DsRed. The quantified results were shown as the mean v± SD. Statistical differences were compared to the control level (** $p < 0.01$; *** $p<0.001$). ARE, AU-rich element; wild-type ARE (wtARE); mutated ARE (mARE); deleted ARE (dARE).


A.
1 2 3 4
Biotin-ARE + + + -
Unlabeled ARE - - + -
Biotin-mARE - - - +
Recombinant CRT - + + +
Non-specific band
Free probe


B.
Free probe
IP: biotin
Supernatant
ARE mARE ARE mARE ARE mARE
CRT


C.
Biotin-ARE+cell lysate
Biotin-mARE+cell lysate
KD
180
130
100
75
63
48


D.
FBP1 mRNA expression
(relative fold to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
CRT-RNAi

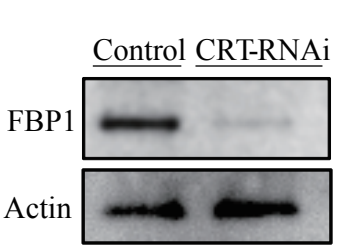

E.
Control CRT-RNAi
FBP1
Actin

Figure 12. Calreticulin-knockdown suppressed far upstream element binding protein 1 that binds to ARE in FUT1 3′-UTR. (A) Biotinylated RNA probes (ARE or mARE) were incubate with recombinant CRT. The RNA-protein complex was separated by native PAGE and detected using chemiluminescence. (B) Whole cell lysates were incubated with biotinylated RNA probes (ARE or mARE). The RNA-protein complex was pulled-down by streptavidin beads and analyzed using Western blot with anti-CRT antibody. (C) Identification of FBP1 as the FUT1 ARE binding protein. Biotinylated RNA probes (ARE or mARE) were incubated with whole cell lysate and the RNA-protein complex was pulled down by streptavidin beads. Arrow indicates the putative binding protein. (D) Real-time PCR was performed to detect FBP1 expression levels in J82 control and CRT-RNAi cells. mRNA expression was normalized to the internal control, GAPDH. (E) Western blot analysis demonstrates protein expression in J82 stable cell lines. The human β-actin was used as a loading control. Each bar of the histogram represents quantified results mean ± SD. Statistical differences were compared to the control level (** $p < 0.01$). FBP1, Far upstream element-binding protein 1.

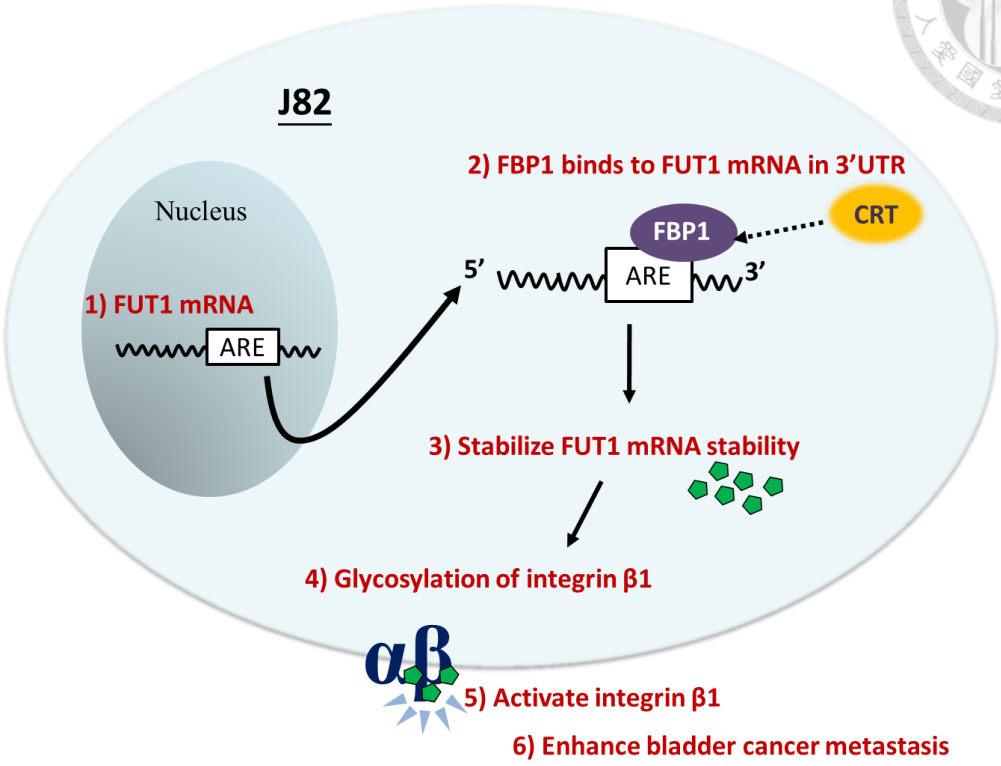


Figure 13. Schematic illustration summarizes the role of CRT in regulation of bladder cancer metastasis.

The American Journal of Pathology, Vol. 179, No. 3, September 2011

DOI: 10.1016/j.ajpath.2011.05.015

Tumorigenesis and Neoplastic Progression

Changes in Tumor Growth and Metastatic Capacities of J82 Human Bladder Cancer Cells Suppressed by Down-Regulation of Calreticulin Expression

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Bladder cancer is a common urothelial cancer. Through proteomic approaches, calreticulin (CRT) was identified and proposed as a urinary marker for bladder cancer. CRT is a multifunctional molecular chaperone that regulates various cellular functions such as Ca^{2+} homeostasis and cell adhesion. CRT is overexpressed in various cancers, but its mechanism of action in the development of bladder tumors remains unclear. We generated J82 bladder cancer cells lines that either stably overexpressed or knocked down CRT to investigate the physiological effects of CRT on bladder tumors. Compared with the transfected control vector cells, the knockdown of CRT suppressed cell proliferation, migration, and attachment, whereas overexpression of CRT enhanced cell migration and attachment. We further demonstrated that the phosphorylation status of focal adhesion kinase and paxillin, important regulators of the focal adhesion complex, was also regulated in these cells. In contrast, phosphorylation of Src, a protein tyrosine kinase reported to be affected by CRT, was not significantly different between the control and CRT-RNAi groups. Most importantly, we observed that tumors derived from J82 CRT-RNAi cells were significantly smaller and had fewer metastatic sites in the lung and liver *in vivo* than did transfected control vector cells. In conclusion, our results suggest that alteration of CRT expression levels might affect bladder cancer progression *in vitro* and *in vivo*. *(Am J Pathol 2011, 179:1425–1433; DOI: 10.1016/j.ajpath.2011.05.015)*

In the United States, bladder cancer was among the top 10 most frequently diagnosed cancers in 2009.[1] The most common type of bladder cancer is transitional cell carcinoma, a malignant tumor that grows from the epithelium of the bladder. Approximately 30% of patients present with a muscle-invasive metastasis at the time of diagnosis, and 85% of these patients will die of the disease within 2 years if left untreated.[2] This means that metastasis is one of the leading causes of cancer-related death for patients with bladder cancer. Tumor cells detach, invade, and disseminate to other sites and form secondary tumors during metastasis. Each step involves many molecules, including cell-adhesion molecules, matrix degradation enzymes, and various growth factors.[3] However, the gene that regulates metastasis in bladder cancer is still unclear.

Calreticulin (CRT) is a 46-kDa multifunctional molecular chaperone protein in the endoplasmic reticulum (ER) that is conserved in various species.[4] CRT functions have been implicated in Ca^{2+} homeostasis, signaling transduction, gene expression, and glycoprotein folding.[5–7]

Supported by the Frontier and Innovative Research Division of National Taiwan University (98R0318) and by grants (NSC97-2311-B-002-002-MY3 and NSC99-2120-M-002-004) from the National Science Council of the ROC (H.L.).

Accepted for publication May 12, 2011.

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Supplemental material for this article can be found at *http://ajp.amjpathol.org* or at doi: 10.1016/j.ajpath.2011.05.015.

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Many studies elucidated that the alteration of CRT levels affects cell adhesion ability.[8–10] Cells with overexpressed CRT have increased adhesiveness; in contrast, knockdown of CRT suppresses adhesion.[11–13] Moreover, it was also reported that activation of matrix metalloproteinase 2, which plays an important role in cancer invasion and metastasis, increases as a result of CRT overexpression in rhabdomyosarcoma cells.[14]

In some cancers, tumor tissues express higher levels of CRT compared with normal tissues. CRT expression is significantly up-regulated in hepatoma, prostate, colon, and vaginal cancers.[15–18] Furthermore, not only do CRT levels increase in urinary cancer tissues, but they also act as a biomarker for bladder cancer.[19,20] In addition to being overexpressed in tumor tissues, CRT also regulates cancer cell behavior. Chen et al[21] showed that the overexpression of CRT increased cell proliferation and migration and modulated several molecules related to cancer metastasis and angiogenesis, such as connective tissue growth factor, vascular endothelial growth factor, and placenta growth factor.[21] These results indicate that CRT expression might play a crucial role in cancer progression.

Although higher levels of CRT are found in urinary cancer tissues and urinary CRT can be used as a biomarker for detection of bladder cancer, the mechanism of CRT in bladder cancer is still poorly understood. To evaluate the metastatic behavior of bladder tumor, we used an aggressive bladder cancer cell line J82 to investigate the roles of CRT on progression of bladder cancer.[22] In this study, we established CRT overexpressed and knockdown stable cell lines in J82 cells to clarify the role of CRT in bladder cancer. We demonstrated that knockdown of CRT suppressed cell proliferation, attachment, and migration in bladder cancer; contrarily, overexpression of CRT enhanced cell attachment and migration. Furthermore, we showed that CRT downexpression in bladder cancer diminished tumor formation and metastasis *in vivo*. Our results provide evidence that CRT plays an important role in progression of bladder cancer.

Materials and Methods

Cell Culture

The J82 human bladder cancer cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For the subcultures, cells were trypsinized with 0.05% EDTA-trypsin.

Construction of Inducible Cell Lines

To set up a tetracycline-inducible CRT system, the pEYFP-N1 was subcloned into pcDNA5/TO (Invitrogen, Camarillo, CA) by KpnI and NotI to generate pcDNA5/TO-YFP (#5y) used as control. CRT was amplified from HEK293 cDNA and subcloned into pcDNA5/TO-YFP to generate pcDNA/TO-ss-YFP-CRT (#5yCRT) by the following primers: ss-forward-NheI, 5′-CCGCTAGCTTGATGCTGCTATCCGTGCCGCTGC-3′, ss-reverse-HindIII, 5′-CCAAGCTTGGCGACGGCCAGGCCGAGGA-3′; YFP-forward-HindIII, 5′-CCAAGCTTGTGAGCAAGGGCGAGGAGCT-3′, YFP-reverse-KpnI, 5′-AAGGTACCCTTGTACAGCTCGTCCATGC-3′; CRT-forward-KpnI, 5′-AAGGTACCGAGCCCGCCGTCTACTTCAA-3′, and CRT-reverse-XbaI, 5′-AATCTAGACTACAGCTCGTCCTTGGCCTGG-3′.

Transfection and Selection of Stable Cell Lines

For transfection experiments, J82 cells were plated 2×10^5 cells/well in 6-well plates, and 2 μg of plasmid DNA for CRT overexpression (#5y and #5yCRT) or knockdown (pCR3.1 and CRT-RNAi) was transfected into cells with the use of 10 μL of Lipofectamine TM2000, (Invitrogen). For single-cell selection, the transfected J82 cells were passaged at a 1:10 dilution in 10-cm culture plate at 2 days after transfection, and the stable cells were picked from a single colony with the use of cloning rings. Medium with antibiotics (200 μg/mL Hygromycin for #5y and #5yCRT inducible clones and 700 μg/mL G418 for pCR3.1 and CRT-RNAi stable clones) was replaced every 2 or 3 days. Two months later, antibiotic-resistant single clones were selected and amplified to test CRT mRNA and protein expressions. The inducible YFP, ss-YFP-CRT cell lines (#5y and #5yCRT) were pretreated with 1 μg/mL tetracycline for 48 hours to perform the experiments.

Image Analysis

For analysis of cell shape, morphologic images of J82 cells were thresholded and outlined with the use of MetaMorph software (Sunnyvale, CA). Changes in cell shape were assessed by a shape factor ($sf = 4A/p^2$) calculated from the area and the perimeter of the delineated object. Value close to "1" represents a flat object, whereas a value close to "0" represents a circular object.

Immunoblotting

Cells were plated at 2×10^5 cells/well in six-well plates overnight. For harvesting cell lysates, cells were washed with cold PBS and lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.0), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS (pH 7.4)] containing protease inhibitors. Equal amounts of each sample (30 μg) were boiled with 6× sample buffer for 5 minutes and resolved by 10% SDS-PAGE. After electrophoresis, the gel was transferred to a polyvinylidene difluoride membrane. Membranes were blocked by 5% bovine serum albumin in 10 mmol/L Tris, 150 mmol/L NaCl, and 0.1% Tween-20 (pH 7.4) at room temperature for 2 hours. Blocked membranes were incubated with the following primary antibodies: CRT, Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA); paxilln (pY118), Cell Signaling (Danvers, MA); focal adhesion kinase (FAK; pY397), Invitrogen; FAK, paxillin, c-Src, and actin, Santa Cruz Biotechnology; and Src (pY418), Biosource, for 2 hours or 4°C overnight. Membranes were washed and then incubated with horseradish peroxidase–conjugated second-

ary antibodies (1: 5000) for 1 hour. Immunoreactive bands were quantified with TotalLab software version 2.01 (Durham, NC).

RNA Isolation and Real-Time PCR

Total RNA were isolate by the TRIzol reagent, following the manufacturer's instructions. RT-PCR was performed with ReverTra Ace reverse transcriptase. Real-time PCR was performed with the iCycle iQ real-time detection system (Bio-Rad, Hercules, CA) with the DNA double-strand specific SYBR Green I dye for detection. Cycling condition was 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. For quantification, the target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control gene. Primer sequences were forward 5′-GGTGGTCTCCTCTGACTTCAAC-3′ and reverse 5′-TCTCTCTTCCTCTTGTGCTCTTG-3′ for GAPDH and forward 5′-AAGTTCTACGGTGACGAGGAG-3′ and reverse 5′-GTCGATGTTCTGCTCATGTTTC-3′ for CRT.

Cell Cycle Analysis and Proliferation Assay

Cells were starved overnight and then treated with cultured medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) for 10 hours. Cells were trypsinized and resuspended in 1 mL of PBS. Propidium iodide was added to the suspensions and then incubated in a 37°C water bath for 20 minutes in the dark. Propidium iodide–stained cells were analyzed by flow cytometry, and the percentages of different phases were automatically analyzed by Partec FloMax software (Münster, Germany). To estimate cell proliferation rate, cells were plated in triplicates at 2500 cells/well in 96-well plates. Ten microliters of 5 mg/mL MTT was added to each well at 24, 48, and 72 hours after plating. After incubation for 4 hours at 37°C, MTT-containing medium was removed. MTT metabolic products were subsequently dissolved in 100 μL of dimethyl sulfoxide for 20 minutes at 37°C, followed by measurement of absorbance at 550 nm.

Cell Migration Assay

Migration rates of different cell lines were assayed in a modified Boyden's chamber (Neuro Probe, Gaithersburg, MD). Cells suspended in 50 μL (1.5×10^4 cells/well) were loaded in the upper chamber. Dulbecco's modified Eagle's medium and fetal bovine serum were loaded in the lower chamber as the negative control and chemoattractant, respectively. After 4 hours, migrating cells were fixed and quantified by a colorimetric measurement with the use of crystal violet staining with TotalLab v2.01 software.

Cell Attachment Assay

The 96-well culture plates were coated with 10 μg/mL collagen I and incubated at 37°C for 30 minutes followed by a PBS wash. Cells (5×10^4 cells/100 μL) were seeded in the well and incubated at 37°C for 20 minutes. After removing the medium and non-attached cells, wells were washed with PBS, and 0.1% crystal violet was added for 10 minutes. The plate was gently washed with PBS three times. Then 10% acetic acid was added for 20 minutes, and the plate was read at 550 nm.

Apoptosis Detection Assay

Cells were starved overnight and treated with 5 μg/mL actinomycin D (Sigma, St. Louis, MO) as positive control for 24 hours. Cell apoptosis rate was detected with the use of fluorescein isothiocyanate Annexin V apoptosis detection kit I (BD Pharmingen, San Diego, CA). Cells were trypsinized and resuspended in 100 μL of 1× binding buffer. Fluorescein isothiocyanate, Annexin V, and propidium iodide (5 μL) were added to the suspensions and then incubated for 20 minutes at room temperature in the dark. Annexin V–stained cells were analyzed by flow cytometry. To measure end-stage apoptosis, TUNEL labeling was performed in tumor sections of the two groups with the use of a DNA fragmentation detection kit (Calbiochem, Gibbstown, NJ), following manufacturer's instructions. Each section had been chosen for five images, and TUNEL-positive cells were quantified under microscope by high-power field (magnification, ×200). For a positive control, sections were incubated in DNase I before the addition of equilibration buffer, whereas double distilled water was used instead of terminal deoxynucleotide transferase reaction mix in the negative control.

Subcutaneous Injection Model

For models of bladder cancer to measure tumorigenicity, 8-week-old male BALB/c nude mice ($n = 10$; National Defense Medical Center, Taipei, Taiwan) were housed in pathogen-free conditions and acclimatized for 1 to ~2 weeks. Mice were randomized to one of two groups and were subcutaneously injected with vector control ($n = 5$) and CRT-RNAi ($n = 5$) cells in the inguinal region. Cells (1×10^7) were suspended in PBS and Matrigel in a 1:1 (v/v) ratio. Tumors were measured every 3 days (width^2 × length × 0.5). Mice were sacrificed after 45 days, and subcutaneous tumors were surgically excised.

Tail Vein Injection Model

For the metastasis model, 8-week-old male severe combined immunodeficient mice ($n = 12$; BioLASCO, Taipei, Taiwan) were used for tail vein injection. Cells (10^6) suspended in PBS were injected through the tail vein. After 40 days, the lungs and liver were resected and fixed in 3.7% formalin for paraffin embedding in preparation for immunohistochemical analysis. The percentage of the lung metastasis area was calculated as the metastatic lung area/total lung area.

Statistical Analysis

Data were statistically analyzed with one-way analysis of variance, followed by Fisher's protected least-significant difference test (StatView; Abacus Concept, Berkeley,

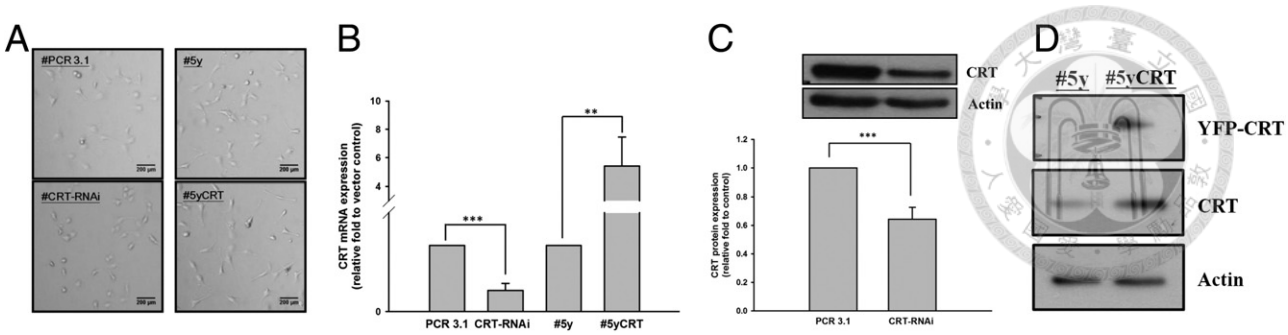

Figure 1. J82 bladder cancer cells were stably transfected with CRT overexpressing and RNAi plasmids. **A:** Cell morphology of CRT in differentially expressed cell lines in serum-containing medium. #5y and #5yCRT cells were stimulated with 1 μg/mL tetracycline for 48 hours to induce CRT expression. Scale bar = 200 μm. **B:** mRNA expressions were confirmed by real-time PCR in CRT knockdown and overexpression stable cell lines. CRT mRNA expression was normalized to the internal control, GAPDH. **C:** Western blot analysis shows protein expression in CRT-RNAi stable cell lines. The human β-actin level was used as a loading control. **D:** An endogenous CRT protein band (~60 kDa) was both detected in #5y and #5yCRT cells. An exogenous YFP-CRT fusion protein (~87 kDa) was only detected in #5yCRT cells. All of the results are repeated in at least three independent experiments. Each bar of the histogram represents quantified results and are shown as the mean ± SD. Statistical differences were compared with the control level (*$P < 0.01$, **$P < 0.001$).

CA). Each result was obtained from at least three independent experiments, and a value of $P < 0.05$ was considered statistically significant.

Results

Generation of Stable Cell Lines

To clarify the effects of CRT in bladder cancer cells, we attempted to select CRT overexpression and knockdown stable cell lines. J82 bladder cancer cells were transfected with a CRT overexpressed plasmid and a CRT-RNAi plasmid and then selected by respective antibiotics. After selection by G418 over 2 months, CRT-RNAi cells were generated, and CRT knockdown efficiency was confirmed with a real-time PCR and Western blotting to detect mRNA (Figure 1B) and protein (Figure 1C) expression levels compared with the transfected control vector cells (pCR3.1). However, during the stable cell line selection, constitutive overexpression of CRT resulted in cell spreading (see Supplemental Figure S1A at *http://ajp.amjpathol.org*) followed by cell death within 2 weeks. Therefore, an inducible-CRT bladder cancer cell line was generated by tetracycline-regulated gene system. Cells were treated with 1 μg/mL tetracycline for 48 hours to induce CRT mRNA and protein expressions (Figure 1, B and D). Cell morphology was shown in Figure 1A, and the shape factors calculated by MetaMorph software were not significantly different between pCR3.1 and CRT-RNAi or between #5y and #5yCRT cells (see Supplemental Figure S1B at *http://ajp.amjpathol.org*).

Cell Proliferation Rate Is Lower in CRT Knockdown Cells

To understand how CRT regulates cell behavior, we used functional assays to investigate the physiological effects of CRT expression in bladder cancer cells. Their cell cycle was analyzed by flow cytometry. In these assays, the CRT-RNAi group showed more cells arrested in the G_1 phase (41%) than the control group (30%), which indicated that CRT knockdown suppressed cell proliferation (Figure 2A, left panel). Nevertheless, our results showed that there was no significant difference between #5y and #5yCRT (Figure 2A, right panel). Cell proliferation rate was also assessed by MTT assays over several time points after plating. The MTT readings for CRT-RNAi cells were significantly lower than the MTT readings from control cells (pCR3.1) at 24, 48, and 72 hours (Figure 2B, left panel). However, no significant difference was observed between CRT overexpressed cells

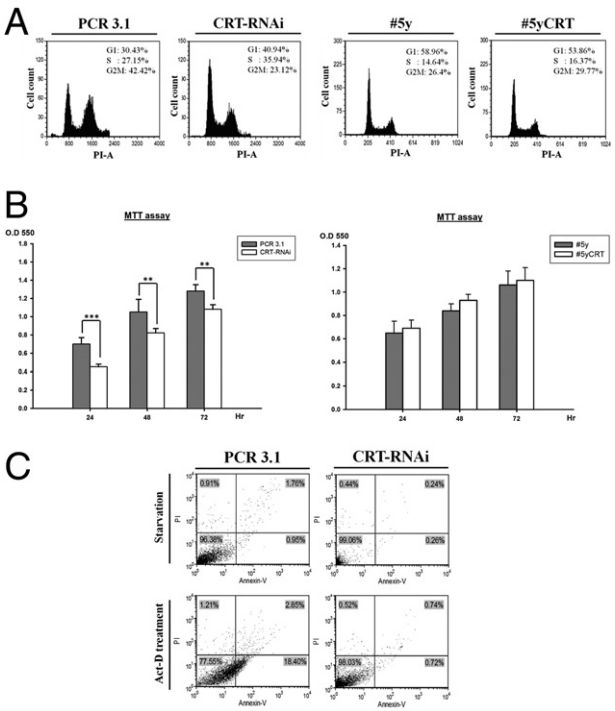

Figure 2. Knockdown of CRT altered the proliferation rate of bladder cancer cells. **A:** The cell cycle was analyzed by flow cytometry. **Left:** CRT-RNAi cells showed proportionally more cells in the G_1 phase than the S and G_2M phases compared with the vector control group (pCR3.1). **Right:** Cells that induced CRT expression did not promote cell cycle transit. **B:** Cell proliferation was determined by MTT assay. Cells (2500 cells/well) were seeded in 96-well plate. Cell numbers were determined by measuring the absorbance of 550 nm at 24, 48, and 72 hours after plating. **C:** Apoptosis was evaluated after starvation for 24 hours and stained with Annexin V. Treatment of actinomycin-D (act-D; 5 μg/mL) is used as positive control. The number showed the percentage of early apoptotic cells (**lower right quadrant**). These results were confirmed by at least three independent experiments (*$P < 0.01$, **$P < 0.001$).

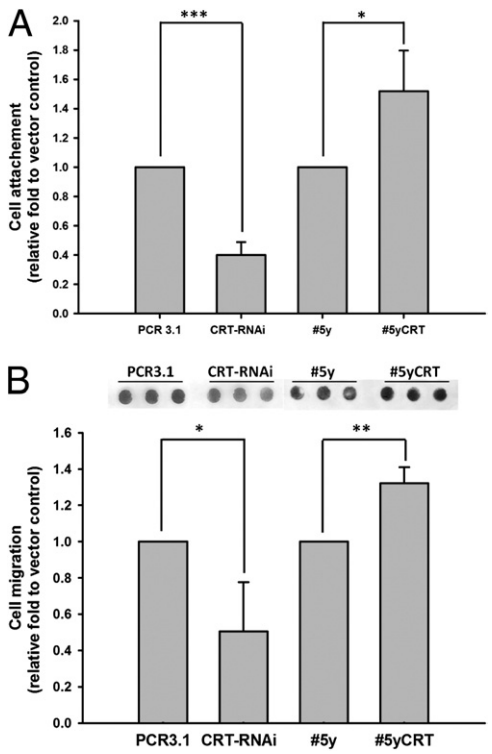

Figure 3. Cell adhesion and migratory ability were regulated by CRT expression levels. **A:** Cells (5×10^4 cells/100 μL) were seeded in 96-well collagen-coated (10 μg/mL) plates for 20 minutes. Compared with transfected control vector cells, CRT-RNAi cells adhered poorly to type-I collagen. Cells with higher CRT levels showed a stimulation of attachment to type-I collagen. **B:** Cells (1.5×10^4cells/well) were loaded into the upper chamber of a modified Boyden's chamber, and fetal bovine serum was loaded into the lower chamber as a chemoattractant for cells. After 4 hours, CRT-RNAi cells had migrated significantly slower than control (pCR3.1) cells, and #5yCRT cells exhibited an opposite effect than its control (#5y) cells. One of three independent experiments, performed in triplicate, is shown. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared with the control level (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

and vector-transfected cells (Figure 2B, right panel). To estimate whether the decreasing numbers in the CRT-RNAi group was because of apoptosis, we used Annexin V/propidium iodide staining to determine the apoptotic cells. pCR3.1 and CRT-RNAi cells were starved for 24 hours. Actinomycin-D, which is a potent inducer for apoptosis in several cell lines,[23] was used as positive control. As shown in Figure 2C, the percentage of Annexin V–positive cells were <3% in both pCR3.1 and CRT-RNAi cells, which indicated that knockdown of CRT did not induce apoptosis in J82 cells. These results justified that down-regulation of CRT inhibited proliferation of bladder cancer cells rather than induction of apoptosis.

Alteration of CRT Levels Affects Cell Attachment and Migration

We also used cell migration and attachment assays to explore the effect of CRT levels in J82 cancer cells. Equal numbers of cells were seeded on a collagen-coated plate for 20 minutes. Results showed that the attachment ability of the cell was suppressed in CRT-RNAi cells compared with transfected vector control cells (Figure 3A, bars 1 and 2). On the contrary, CRT overexpression enhanced cell attachment significantly than vector control cells (Figure 3A, bars 3 and 4). To determine whether the effects of CRT are universal to bladder cancer, we used another bladder cancer cell line to perform our experiments. Down-regulation of CRT in T24 bladder cancer cells also suppressed cell attachment to type-I collagen (see Supplemental Figure S2 at *http://ajp.amjpathol.org*), suggesting that the effects of CRT we observed are not only specific to J82 cell line. Furthermore, with the use of the modified Boyden chamber method, J82 cells with low CRT expression had lower migration capacities than control cells (Figure 3B, bars 1 and 2), and cells with higher CRT expression showed an opposite effect (Figure 3B, bars 3 and 4). Consequently, these results showed a positive correlation between CRT expression levels and cell attachment and migration in bladder cancer cells.

Phosphorylation Levels of FAK and Paxillin Are Lower in CRT Knockdown Cells

Papp and others[12,13] reported that altering CRT expression in fibroblasts can affect cell motility and adhesion through

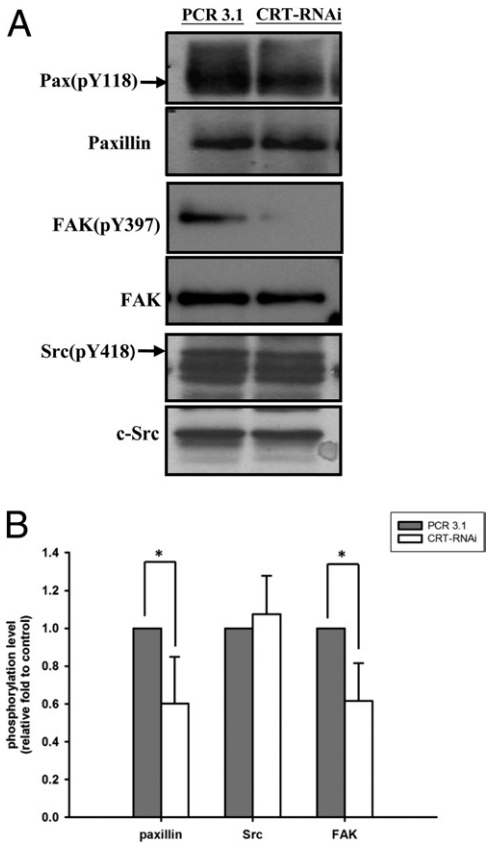

Figure 4. Phosphorylation levels of FAK and paxillin were decreased in CRT knockdown cells. **A:** Western blot analysis indicated that the phosphorylation levels of paxillin and FAK were more down-regulated in CRT-RNAi cells than in control cells. The proportion of phosphorylated Src was not altered in CRT-RNAi cells. **B:** Quantitative results of the phosphorylated ratios of paxillin, FAK, and Src were normalized by each total protein expressions. One of three independent experiments was shown. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared with the control level (*$P < 0.05$).

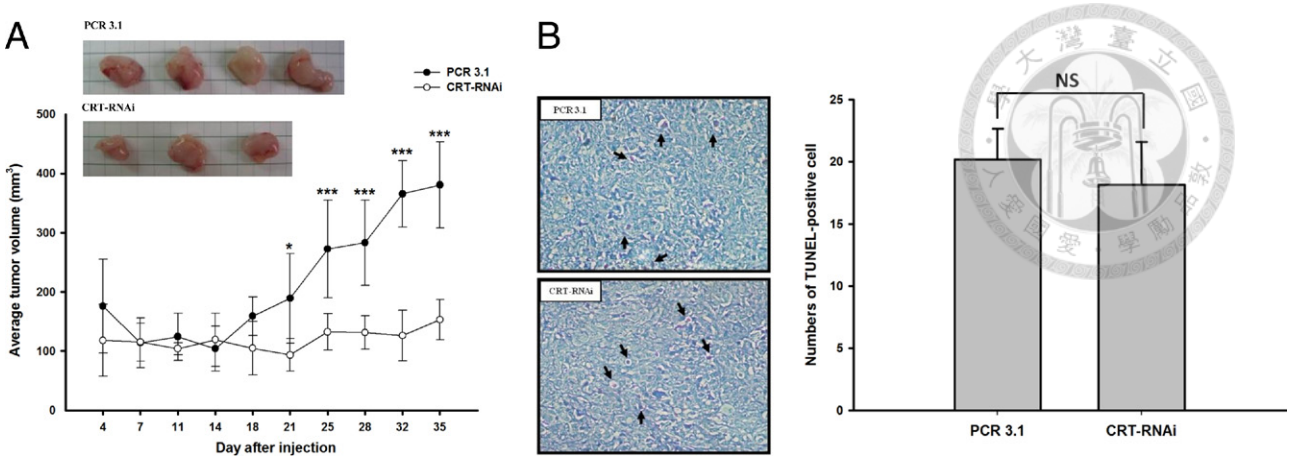

Figure 5. Knockdown of CRT diminishes tumor formation in nude mice. **A:** Mice were subcutaneously injected with vector control cells (closed circles) or CRT-RNAi cells (open circles), and tumor sizes were measured over time. After 20 days, tumor sizes significantly differed between the two groups. Knockdown of CRT suppressed tumorigenesis in bladder cancer cells. **B:** Determination of TUNEL-positive cells for injection of cells from mice (high power field, ×200). The **arrows** indicate the positive cells. No significant difference was observed between the two groups. Each bar of the histogram represents quantified results of the TUNEL-positive/field and is shown as the mean ± SD.

regulating c-Src and FAK activity. Paxillin is one molecule of the focal adhesion complex which regulates the cell adhesion mechanism. On the basis of those previous studies, we used Western blotting to analyze the downstream signaling pathway affected by CRT in our system. Phosphorylation levels of Src (pY418), FAK (pY397), and paxillin (pY118) were also examined. As shown in Figure 4, the phosphorylation levels of FAK (−40%) and paxillin (−40%) decreased in CRT knockdown cells, whereas no difference in Src phosphorylation was observed.

Knockdown of CRT Inhibits Tumor Growth in Vivo

Stably transfected cells were subcutaneously injected into nude mice to determine the effects of CRT on tumor growth. Tumor volumes were measured every 3 days. Compared with tumors of control mice, tumors of mice injected with CRT-RNAi were significantly smaller after 21 days. The mean volume of tumors of CRT-RNAi clones was suppressed 60% more than that of control clones at day 35 (Figure 5A). Moreover, we further determined the end-stage apoptosis in the injection of cells from nude mice. Tumor sections from both groups were analyzed by TUNEL assays. The number of TUNEL-positive cells in the control group was not significantly increased in comparison to the CRT-RNAi group (Figure 5B). These results are consistent with our observations *in vitro*.

Knockdown of CRT Inhibits Metastasis of Bladder Cancer Cells

In addition, we further confirmed that the metastatic capacity was lower in the CRT-RNAi subcutaneous injection model. After sacrificing the mice, we found metastatic nodules in the liver of control mice but not in CRT-RNAi-injected mice (Figure 6A). These results strongly suggest that expression levels of CRT are closely related to the metastatic behavior of bladder cancer cells. To further confirm this hypothesis, we performed a tail vein injection

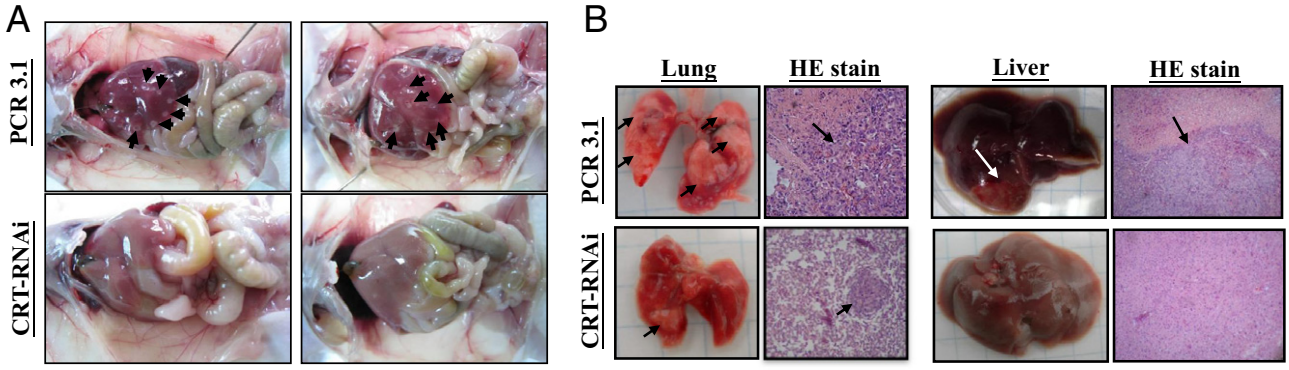

Figure 6. Knockdown of CRT inhibits metastasis of bladder cancer cells. **A:** Nude mice were subcutaneously injected with 10^7 cells and sacrificed after 45 days. There were obvious metastatic nodules in the liver of the control group (shown by **arrows**) but not in the CRT-RNAi group. **B:** Lung and liver metastases were performed by an intravenous injection. In total, 10^7 cells (control and CRT-RNAi) were injected into the tail vein of severe combined immunodeficient mice ($n = 5$ and $n = 7$, respectively). After 40 days, both groups had lung metastases, but the area of lung metastasis was higher in the control group. Liver metastasis was only observed in control mice.

Table 1. Lung and Liver Metastatic Incidence in CRT-Stable Cell Lines

	1	2	3	4	5	6	7
PCR 3.1							
Lung	++	+	+	+++	+		
Liver	+	NT	+	NT	NT		
CRT-RNAi							
Lung	+	+	NT	+	+	+	NT
Liver	NT	NT	NT	NT	NT	NT	NT

Metastatic area is indicated by the following +, <25%; ++, ≥25%; +++, >40%.
NT, no tumor.

experiment, which is a specific model used to investigate cancer cell metastasis. Liver metastasis was observed to be at 40% (2 of 5) in the control group and at 0% (0 of 7) in the CRT-RNAi group. In comparison, 100% (5 of 5) of control cells and 71% (5 of 7) of CRT-RNAi-injected mice developed lung metastasis. Although lung metastasis was observed in both groups, the percentage of metastatic areas was much higher in the control group (Figure 6B; see also Supplemental Figure S3 at *http://ajp.amjpathol.org*). From the two mice models, we can conclude that vector control cells induced tumor formation and visible liver and lung metastasis, whereas CRT-RNAi cells formed smaller tumors and fewer metastatic nodules in the liver and lungs. These results are summarized in Table 1.

Discussion

CRT is well established as a multifunctional protein that regulates cell behavior.[4] In recent studies, it was shown that CRT is located in the cytosol and extracellular environments and on cell surfaces to modulate diverse physiological and pathologic effects.[24] Many researchers reported that in some cancers CRT is up-regulated in tumor tissues more often than in normal tissues. Kageyama et al[19] also identified that CRT levels are higher in urothelial tissues of patients with bladder cancer, and that urinary CRT can act as a biomarker for detection of bladder cancer. In this study, we surveyed the ability of CRT knockdown bladder cancer cells to suppress cell proliferation, migration, and adhesion. We further proved that *in vivo* downexpression of CRT diminishes tumorigenesis and metastasis of bladder cancer.

ER is one of the important organelles for protein folding. Some studies showed that abnormal expression of CRT, an ER-resident chaperon, would trigger unfolded protein response that results in ER stress.[25,26] In the present study, overexpressing CRT in bladder cancer caused morphologic changes and cell death within 2 weeks. These changes are consistent with a previous study showing that albumin-induced ER stress caused a conversion to the spindle-like morphology in tubular cells.[27] Therefore, we surmised that constitutively high levels of CRT expression might disturb the ER stress regulation and lead to cells to exhibit an abnormal morphology.

The N-terminal fragment of CRT, vasostatin, is an anti-angiogenic factor and inhibits endothelial cell proliferation.[28,29] This protein was implied to be an inhibitor of lung tumor growth and metastasis in different mouse models.[30] However, there is no current study exploring the effects of vasostatin in bladder cancer. Previous studies have showed that CRT affects nuclear localization and activities of p53, which subsequently leads to apoptosis and cell-cycle arrest.[31,32] In this study, inducible CRT expression in J82 cells by tetracycline-regulated gene system did not enhance cell proliferation. We surmised that it might be because of the short induction time (only 48 hours) for J82 cells to enhance CRT expression transiently, which might not have profound effects on cell proliferation. Nevertheless, there is a decreased cell proliferation rate in the J82 CRT-RNAi cell line which has been down-regulated of CRT stably. This correlates with another study showing that exogenous addition of CRT stimulates human keratinocyte and fibroblast proliferation.[33] In addition, overexpression of CRT in gastric cancer enhances cell proliferation and up-regulates the expression and secretion of the well-known pro-angiogenic factors, vascular endothelial growth factor and placenta growth factor.[21] This evidence is consistent with our results showing that knockdown of CRT inhibited tumor growth *in vivo*. Moreover, neuroendocrine cells overexpressing vasostatin are also known to enhance malignant behaviors in a nude mice model.[34] Therefore, we speculated that the effects of CRT on cell proliferation and tumorigenesis may be via regulation of the vascular endothelial growth factor signaling pathway.

Cells that adhere to extracellular matrix are regulated by various Ca^{2+}-relative pathways. The roles of CRT in cell adhesion have been extensively studied. Different expression levels of CRT affect cell adhesiveness, motility, and spread in various cell types.[8,9,11,35] In this study, we demonstrated that up-regulated CRT expression increased cell attachment to type-I collagen. Furthermore, we also verified that down-regulation of CRT suppressed that attachment of both J82 and T24 bladder cancer cells to type-I collagen. These results suggested that CRT plays an important role in regulation of bladder cancer adhesion, and this effect may not be a specific event in J82 cells. A previous study showed that CRT interacts with integrins by binding to the cytoplasmic KXGFFKR motif of the integrin α-subunit.[36] Several studies mentioned that CRT is colocalized with the $\alpha_3\beta_1$ integrin via the N-domain, and surface CRT interacts with the collagen receptors, $\alpha_2\beta_1$ integrin and glycoprotein VI, in human platelets.[37,38] Furthermore, active integrins increase the association between CRT and $\alpha_2\beta_1$ integrin.[39] Abnormal cell migration is a typical characteristic of cancer metastasis, and integrins, an important modulator for cell adhesion and migration, are involved in this process.[40] Although CRT is proposed to play a suppressive role in growth and metastasis of prostate cancer,[41] it was also shown that mRNA and protein expressions of CRT are elevated in highly metastatic prostate cancer cell lines.[42] This indicates a possible correlation between CRT and metastasis. According to these references, we hypothesized that our observation of down-regulated CRT diminishing cell migration, adhesion, and metastatic abilities in bladder cancer cells might have been because of the reduced interaction between CRT and integrins.

Protein tyrosine phosphorylation is a major event for the cell adhesive mechanism.[43,44] Cell adhesiveness decreases in CRT-underexpressing mouse L fibroblasts that contain higher levels of active c-Src.[12] However, the results of our experiments showed that c-Src activity is unaltered, which led us to think that the relationship between CRT and c-Src activation may differ, depending on the type of cell. Although there was no difference in c-Src phosphorylation, we observed decreases in phosphorylation levels of other crucial focal contact proteins such as FAK and paxillin. FAK is a cytoplasmic non-receptor tyrosine kinase identified as the downstream signaling molecule of integrin-dependent cell adhesion. It is also known that the c-terminal of FAK is the binding site for the integrin-mediated protein, paxillin.[45] A previous study showed that breast cancer cells with high metastatic capacity have increased mRNA expression and paxillin protein levels.[46] In addition, FAK was also proven to be a key signaling factor in tumor initiation, angiogenesis, and metastasis.[47] This evidence supports our data which showed that decreased CRT expression in bladder cancer inhibits cancer metastasis through regulating FAK and paxillin phosphorylation.

CRT regulates various cell behaviors which suggests that it may also play a part in cancer development. Much clinical research has shown a correlation between CRT expression levels and clinicopathologic factors in different cancers. CRT is highly expressed in gastric tumor tissues and significantly correlated with tumor serosal invasion, lymph node metastasis, and microvessel density.[21] Moreover, CRT is up-regulated in bladder tumor tissues, and the concentration of urinary CRT has a tendency to increase in histologic grade and pathologic T stage in bladder cancer.[19,20] If a patient with bladder cancer is diagnosed with advanced-stage (T2 to T4) tumors, it means that the cancer cells possess a higher invasive ability to grow into the muscle layer and can even spread to areas outside the bladder which increases the incidence of tumor metastasis. These results provide strong evidence that supports the phenomenon of bladder cancer cells that express lower levels of CRT having diminished tumor growth and metastasis in mice models. Therefore, we concluded that CRT might play an important role in bladder cancer metastasis.

On the basis of the clinical data published by Kageyama et al,[19,20] our results further clarify how CRT modulates the physiological mechanisms in bladder cancer progression. These findings, for the first time, provide evidence that altered CRT expression affects bladder cancer tumorigenesis and metastasis. In the future, we will attempt to investigate detailed mechanisms of CRT-mediated adhesion in bladder cancer to optimize the research modality.

Acknowledgments

We thank Ching-Ling Lin for her excellent technical assistance and Carol Chang for critically reading this article.

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BJ Biomolecules

Biochem. J. (2014) **460**, 69–78 (Printed in Great Britain) doi:10.1042/BJ20131424

online data

Calreticulin activates β1 integrin via fucosylation by fucosyltransferase 1 in J82 human bladder cancer cells

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Fucosylation regulates various pathological events in cells. We reported that different levels of CRT (calreticulin) affect the cell adhesion and metastasis of bladder cancer. However, the precise mechanism of tumour metastasis regulated by CRT remains unclear. Using a DNA array, we identified *FUT1* (fucosyltransferase 1) as a gene regulated by CRT expression levels. CRT regulated cell adhesion through α1,2-linked fucosylation of β1 integrin and this modification was catalysed by FUT1. To clarify the roles for FUT1 in bladder cancer, we transfected the human *FUT1* gene into CRT-RNAi stable cell lines. FUT1 overexpression in CRT-RNAi cells resulted in increased levels of β1 integrin fucosylation and rescued cell adhesion to type-I collagen. Treatment with UEA-1 (*Ulex europaeus* agglutinin-1), a lectin that recognizes FUT1-modified glycosylation structures, did not affect cell adhesion. In contrast, a FUT1-specific fucosidase diminished the activation of β1 integrin. These results indicated that α1,2-fucosylation of β1 integrin was not involved in integrin–collagen interaction, but promoted β1 integrin activation. Moreover, we demonstrated that CRT regulated *FUT1* mRNA degradation at the 3′-UTR. In conclusion, the results of the present study suggest that CRT stabilized *FUT1* mRNA, thereby leading to an increase in fucosylation of β1 integrin. Furthermore, increased fucosylation levels activate β1 integrin, rather than directly modifying the integrin-binding sites.

Key words: activation, adhesion calreticulin, fucosylation, fucosyltransferase 1 (FUT1), integrin, RNA stability.

INTRODUCTION

Fucosylation is a common type of post-translational protein glycosylation, which regulates various physiological and pathological events in cells. This process is catalysed by at least 11 types of FUTs (fucosyltransferases), which transfer a fucose residue from GDP-fucose to oligosaccharides through α1,2-, α1,3/4- or α1,6-linkages [1]. Aberrant expression of fucosylated haptoglobin has been reported in various tumour tissues, particularly during the advanced stages of cancers [2–4]. In papillary carcinoma, higher FUT8 levels are significantly correlated with tumour size and lymph node metastasis [5]. In addition, fucosylated haptoglobin is considered to be a novel biomarker for pancreatic cancer detection [6]. These results suggest that fucosylation may be a key event in regulating cancer development and progression.

FUT1 is a common type of FUT and is involved in catalysing the addition of fucose via α1,2-linkage to galactose. It has been reported that higher levels of Lewis Y antigen, catalysed by FUT1 and FUT4, are found in over 60 % of human epithelial carcinomas [7–9]. Moreover, studies suggest that in certain cancer cells, tumour growth and metastasis are regulated by changes in FUT1 levels [10,11]. These results indicated that FUT1-regulated fucosylation is closely associated with tumour progression.

Integrins are a family of receptors comprising α- and β-subunits. They are involved in cell–cell and cell–matrix interactions and regulate key physiological processes such as cell migration and adhesion and cancer metastasis. Accumulated evidence indicates that integrin function is regulated by glycosylation [12,13]. Overexpression of GnT (*N*-acetylglucosaminyltransferase)-V, which increases β1,6-branching of N-linked glycan, enhanced $\alpha5\beta$1 integrin-mediated

Abbreviations: Act-D, actinomycin D; ARE, AU-rich element; ARE-BP, ARE-binding protein; CDS, coding sequence; CMV, cytomegalovirus; CRT, calreticulin; EF1α, elongation factor 1α; FBP1/FUBP1, far-upstream-binding protein 1; FUT, fucosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GnT, *N*-acetylglucosaminyltransferase; HRP, horseradish peroxidase; LTL, *Lotus tetragonolobus* lectin; mARE, mutant ARE; NP40, Nonidet P40; PE, phycoerythrin; REMSA, RNA EMSA; UEA-1, *Ulex europaeus* agglutinin-1.

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cell migration and invasion [14], whereas an increase in the GlcNAc (*N*-acetylglucosamine) bisecting linkage of N-glycan by GnT-III inhibited $\alpha5\beta1$ integrin-mediated cell spreading and migration [15]. Furthermore, FUT8-catalysed core fucosylation ($\alpha1,6$-fucosylation) of $\alpha5\beta1$ integrins stimulated cell migration via laminin 5 [16]. However, the consequences of FUT1 expression and its effect on integrins have not been surveyed so far.

CRT (calreticulin) is a multifunctional chaperon protein majorly localized to the ER (endoplasmic reticulum). It has been reported that CRT participates in a variety of important biological processes. Previously, CRT has been identified as an RNA-binding protein that regulates mRNA stability [17]. Our previous studies have shown that suppression of CRT levels in human bladder cancer cell line diminishes cell adhesion and migration, and subsequently inhibits cancer metastasis [18]. However, the precise mechanism by which CRT modulates the above-mentioned processes remains unclear.

In order to evaluate the metastatic mechanisms of bladder cancer, we generated stable CRT-knockdown cell lines and used DNA microarrays to identify CRT-regulated genes. Compared with control cells, FUT1 expression was significantly lower in CRT-knockdown (CRT-RNAi) cells. Since several studies have highlighted that CRT is essential for integrin-mediated cell adhesion [19,20], we hypothesized that CRT may affect FUT1 and regulate integrin functions as well as cell adhesion. In the present study, we have demonstrated that CRT-RNAi lead to significantly lower glycosylation of $\beta1$ integrins. Our results further indicated that changes in $\beta1$ integrin fucosylation affected integrin activity. Furthermore, we demonstrated that CRT regulated FUT1 expression levels by regulating its mRNA stability. In conclusion, we suggested that FUT1-modified fucosylation play an important role in $\beta1$ integrin activation and this process can enhance cancer metastasis of bladder cancer.

EXPERIMENTAL

Cell culture and generation of stably transfected cell lines

Details of the CRT-knockdown human bladder cancer cell line J82 (J82 CRT-RNAi) have been described previously [18]. J82 CRT-RNAi cells were infected with pLKO_AS2.zeo-EGFP and selected with 250 μg/ml zeocin to generate J82 CRT-RNAi–GFP cells. J82 CRT-RNAi–GFP cells were then infected with pLKO_AS2.puro or pLKO_AS2.puro-FUT1 to generate the J82 CRT-RNAi–GFP Control (#R-EGFP) and J82 CRT-RNAi–GFP FUT1 (#R-FUT1) cell lines. The stably infected cells were selected using 500 μg/ml G418, 250 μg/ml zeocin and 2 μg/ml puromycin. The J82 human bladder cancer cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS. For subcultures, cells were trypsinized with 0.05 % EDTA/trypsin.

Plasmid construction

The coding region sequence of the *FUT1* (NCBI Reference Sequence NM_000148) gene was purchased from OriGene. The lentival vectors for cDNA expression, including pLKO_AS2.puro and pLKO_AS2.zeo were obtained from the National RNAi Core Facility Platform, Academia Sinica, Taipei, Taiwan. EGFP template was purchased from pEGFP-C1 (Clontech). PCR was performed using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). FUT1 was ligated into pLKO_AS2.puro between the NheI and BsrGI sites to build pLKO_AS2.puro-FUT1. EGFP was subcloned into pLKO_AS2.zeo between the NheI and EcoRI sites to generate pLKO_AS2.zeo-EGFP. The following primers were used for FUT1 PCR: Fut1-forward-NheI, 5′-AAGCTAGCATGTGGCTCCGGAGCCATCGTCAG-3′ and Fut1-reverse-BsrGI, 5′-GGTGTACATCAAGGCTTAGCCAATGTCCAGAG-3′.

Cell adhesion assay

The 96-well culture plates were coated with 10 μg/ml type I collagen (Sigma) and incubated at 37 °C for 30 min followed by washing with PBS. The adhesion assay procedure was described previously [18]. To determine the effects of various functional blocking antibodies or lectin on cell adhesion, suspensions of J82 cells suspension was pre-incubated with an anti-integrin antibody (1 μg/ml for $\alpha1$, $\alpha2$, $\alpha3$, αV, $\beta3$ and αV$\beta3$ and 0.25 μg/ml for $\beta1$; Millipore) for 1 h or lectin (Vector Laboratories) for 30 min. Adhesion then was determined by using the adhesion assay as described previously [18].

Western blot analyses

The whole cell lysate were lysed with 1 % NP40 (Nonidet P40) lysis buffer and then incubated with the primary antibodies anti-$\beta1$ integrin (1:2000 dilution; BD Transduction Laboratories and GeneTex), anti-CRT (1:5000 dilution; Millipore), anti-(biotin-labelled UEA-1) (where UEA-1 is *Ulex europaeus* agglutinin-1; 1:5000 dilution; Vector Laboratories), and anti-FUT1 and anti-FUBP1 (far-upstream-binding protein 1; 1:2000 dilution; Santa Cruz Biotechnology) for 4 °C overnight. Membranes were washed and then incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies or HRP-conjugated streptavidin (1:5000 dilution) for 1 h. Immunoreactive bands were quantified using the Total Lab V2.01 software.

Lectin pull-down assay

Detection of glycoproteins decorated with terminal galactose was achieved by lectin pull-down assays using biotinylated UEA-1 or LTL (*Lotus tetragonolobus* lectin; Vector Laboratories). Briefly, 500 mg of total cell lysates were incubated with biotinylated UEA-1. Following a 16 h incubation at 4 °C, streptavidin–agarose beads were added and incubated for an additional 6 h. The pulled-down proteins were then subjected to Western blot analysis.

RNA isolation and real-time PCR

Total RNA was isolated using the TRIzol® reagent (Life Technologies) following the manufacturer's instructions. RT (reverse transcription)–PCR was carried out using ReverTra Ace reverse transcriptase (Toyobo). Real-time PCR was performed using the iCycle iQ real-time detection system with the dsDNA-specific SYBR Green I dye for detection (Bio-Rad Laboratories). For quantification, the target gene was normalized to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), an internal control gene. The primer sequences used were: GAPDH, 5′-GGTGGTCTCCTCTGACTTCAAC-3′ (forward) and 5′-TCTCTCTTCCTCTTGTGCTCTTG-3′ (reverse); FUT1-CDS (coding sequence), 5′-AAGTTCTACGGTGACGAGGAG-3′ (forward) and 5′-GTCGATGTTCTGCTCATGTTTC-3′ (reverse); FUT1-3′UTR, 5′-CGTGCTCATTGCTAACCACTGTC-3′ (forward) and 5′-TCGTGCTCCTGCCTGGATCT-3′ (reverse); EGFP, 5′-GTGAGCAAGGGCGAGGAG-3′ (forward) and 5′-CGTAGGTCAGGGTGGTCA-3′

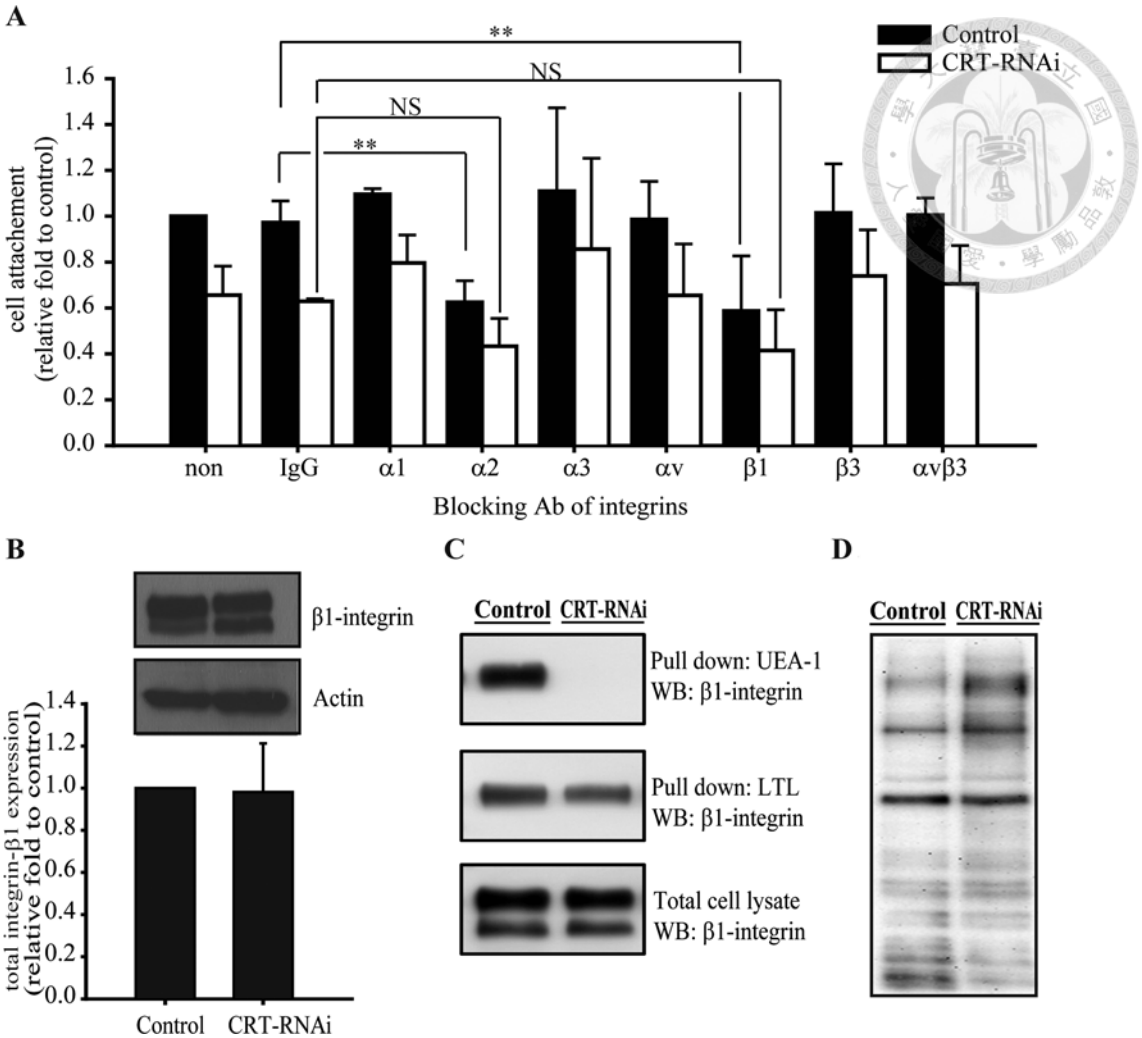

Figure 1 Integrin $\alpha 2\beta 1$ was involved in CRT-mediated cell adhesion in J82 bladder cancer cells

(**A**) To analyse the role of integrins in J82 cell adhesion, cells were pre-incubated with normal mouse IgG or functional blocking antibodies at 37°C for 1 h. Cells (5×10^4) were seeded in 96-well collagen-coated plates (10 μg/ml) for 20 min. NS, not significant. (**B**) Western blot analysis showing no changes in the total β1 integrin expression levels between the control and CRT-knockdown cells. Human β-actin was used as a loading control. (**C**) FUT1-modified glycosylation on β1 integrin in J82 stable cell lines. Cell lysates were pulled down with UEA-1 or LTL followed by Western blotting with an anti-(β1 integrin) antibody. (**D**) Western blot analysis showing the glycomic differences between the control and CRT-knockdown cells. Cell lysates were separated by SDS/PAGE (10% gel) and probed with biotin-labelled UEA-1. Representative data from three independent experiments are shown. (**A** and **B**) Results are means $\pm$ S.D. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control cells.

(reverse); DsRed 5′-GAGGGCTTCAAGTGGGAG-3′ (forward) and 5′-CATAGTCTTCTTCTGCATTACGG-3′ (reverse); and FBP1 5′- TGGGACCATACAACCCTGCACCT-3′ (forward) and 5′-AGCTGGATCAGGAGCCTG-3′ (reverse).

Microarray analysis

Total RNA from control and CRT-RNAi cells were isolated using the TRIzol® reagent and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The cRNA was fragmented and then hybridized to a Human OneArray™ microarray (HOA 4.3; Phalanx Biotech) containing 30968 human gene probes. Arrays were scanned by Microarray Scanner GenePix 4000B (Axon Instruments), and fluorescence intensities were measured by GenePix Pro version 6.0 (Molecular Devices). The raw data were preprocessed by $\log_2$ transformation and global LOWESS (locally weighted scatterplot smoothing) normalization.

Determination of integrin activation

Cells were trypsinized and re-suspended in 100 μl of PBS containing α1,2-fucosidase (1:100 dilution; BioLabs) at 37°C. After 30 min, cells were stained with PE (phycoerythrin)-conjugated anti-CD29 antibody (HUTS-21; BD Pharmingen) and analysed by flow cytometry.

Determination of mRNA stability

J82 cells were treated with 5 μg/ml Act-D (actinomycin D; Sigma) at the indicated time points. *FUT1* mRNA was detected by real-time PCR.

REMSA (RNA EMSA)

The synthesized biotinylated FUT1-ARE (AU-rich element) and mARE (mutant ARE) probes (MDBio) were incubated

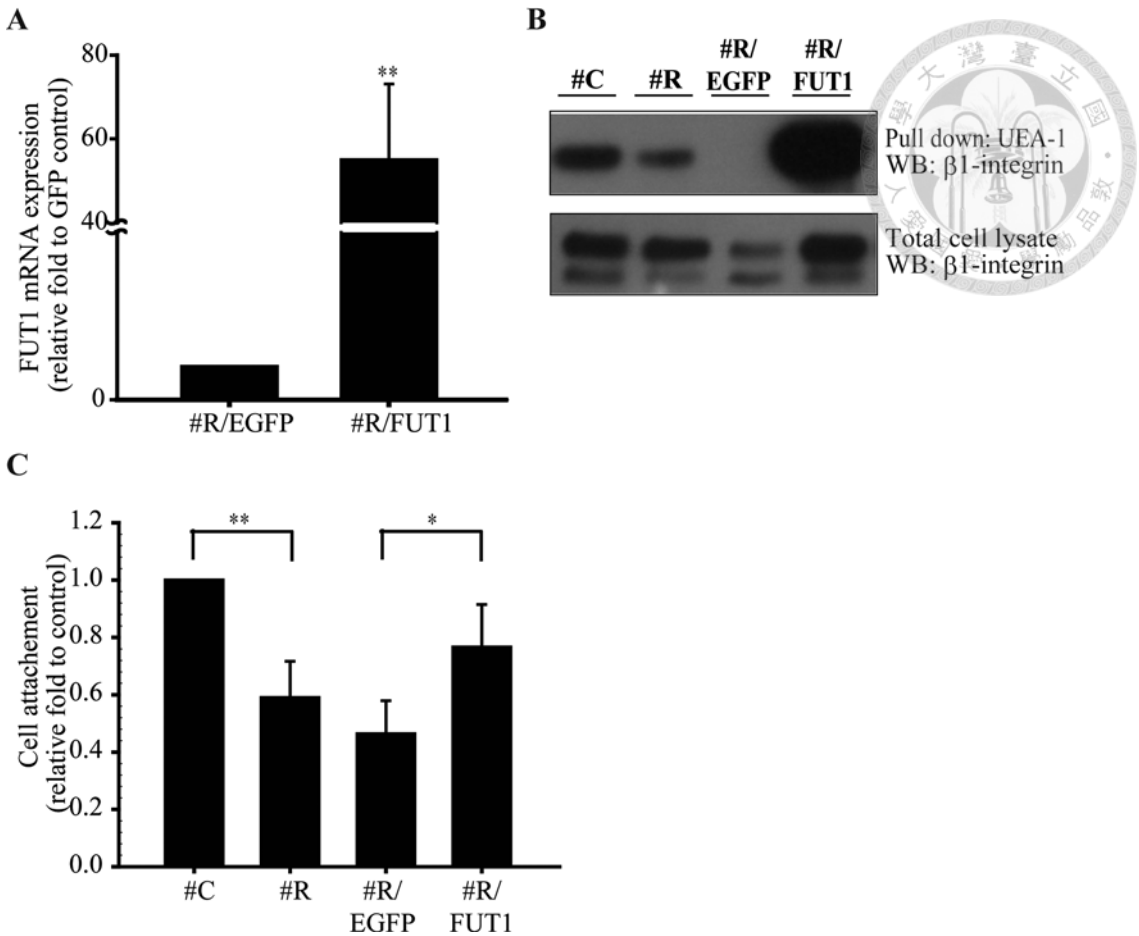

Figure 2 FUT1 overexpression rescued cell adhesion in CRT-knockdown cells

Reintroduction of FUT1 in J82 CRT-RNAi cells. CRT-knockdown stable cells were infected with the control (EGFP) or FUT1 plasmid to generate FUT1-overexpressed stable cell lines. (**A**) mRNA expression was confirmed by real-time PCR in FUT1-overexpressing stable cell lines. *GAPDH* was used as an internal control. (**B**) A lectin pull-down assay was performed to assess whether overexpressed FUT1 could enhance glycosylation of β1 integrin. Cell lysates were pulled down with UEA-1 followed by Western blotting with the anti-(β1 integrin) antibody. Representative data from three independent experiments are shown. (**C**) Effects of FUT1 on CRT-mediated cell adhesion were performed by adhesion experiment. Cells (5×10^4 cells/100 μl) were seeded in 96-well collagen-coated (10 μg/ml) plates for 20 min. (**A** and **C**) Results are means $\pm$ S.D. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the vector control level. #C, control cells; #R, CRT-RNAi cells; #R-EGFP, CRT-knockdown cells infected with EGFP; #R-FUT1, CRT-knockdown cells infected with FUT1–EGFP.

with recombinant CRT protein (Abnova) for 10 min at 4 °C in REMSA buffer (100 mM Hepes, 200 mM KCl, 10 mM $MgCl_2$ and 10 mM DTT) containing 2 μg of tRNA to a final volume of 20 μl. Samples were analysed by native PAGE [4 % polyacrylamide/bisacrylamide (37.5:1)]. Following electrophoresis, the gel was transferred on to a nylon membrane and the RNA–protein complexes were detected by chemiluminescence following the manufacturer's instructions (Thermo Scientific).

RNA immunoprecipitation

The synthetic biotinylated FUT1-ARE and mARE probes were incubated with whole cell lysate (500 μg) containing Streptavidin Mag Sepharose (GE Healthcare) at 4 °C overnight. The pulled-down RNA–protein complexes were analysed by Western blotting using the anti-CRT antibody.

Statistical analysis

Data were statistically analysed using one-way ANOVA followed by Fisher's protected LSD (least-significant difference) test (StatView; Abacus Concept). Each result was obtained from at least three independent experiments and $P < 0.05$ was considered statistically significant.

RESULTS

β1 Integrin participated in CRT-mediated cell adhesion in J82 human bladder cancer cells

It has been reported previously that down-regulation of CRT in J82 cells suppresses cell adhesion and metastatic behaviour [18]. Since integrins play a crucial role in cell adhesion, we used a collagen-specific integrins investigator kit to identify the type of integrins involved in CRT-mediated changes in cell adhesion. As shown in Figure 1(A), functional blocking antibodies for α2 or β1 integrin inhibited the adhesion in vector control cells, but not in CRT-RNAi cells. Blocking other integrins did not result in any significant differences in cell adhesion between control and CRT-RNAi cells. These suggested that α2 and β1 integrin might be involved in CRT-mediated adhesion on type I collagen. However, the level of total β1 integrin showed no difference between the control and CRT-RNAi cells

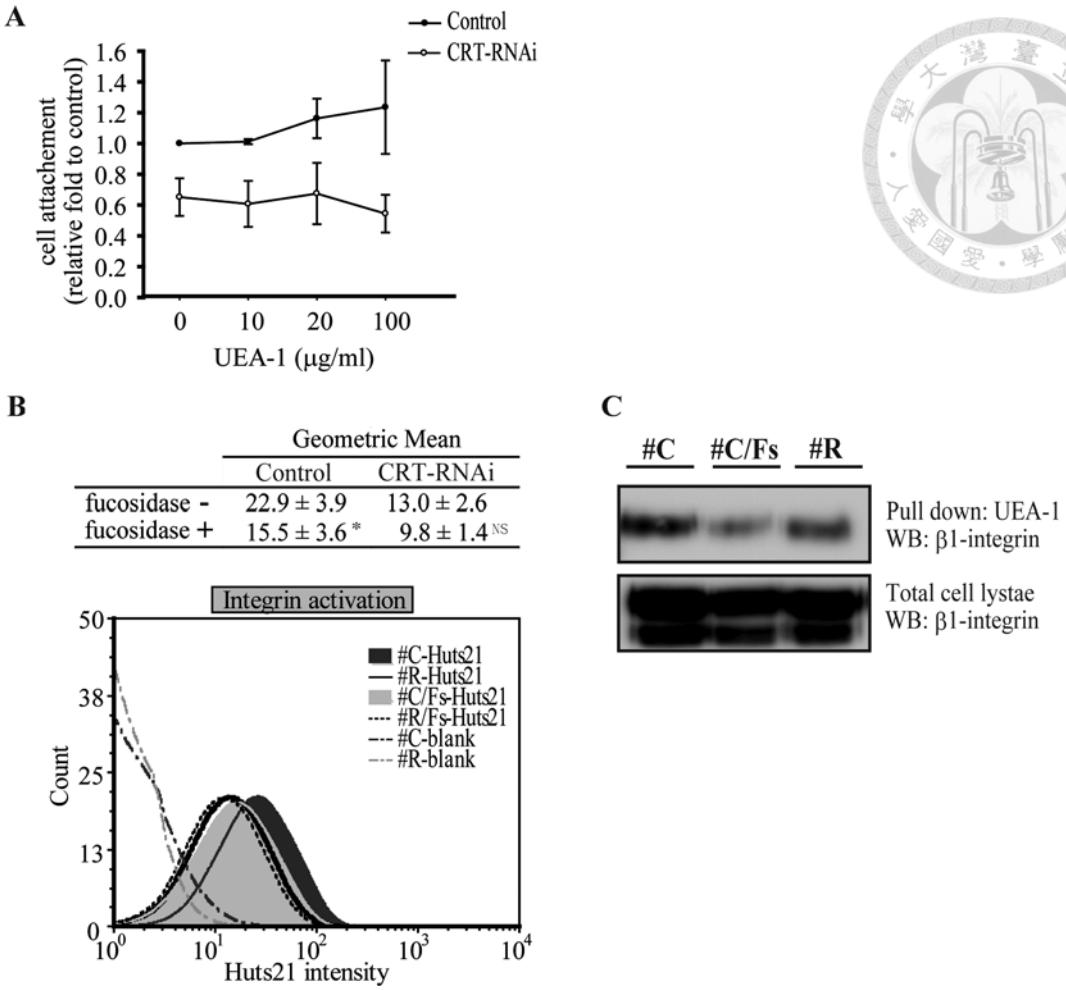

Figure 3 The activation of β1 integrin decreased by reduction in α1,2-linked fucosylation

(**A**) To evaluate the involvement of α1,2-linked glycan in integrin–collagen interaction, cells were pre-incubated with various concentrations of UEA-1 for 30 min. Cells (5×10^4) were seeded in 96-well collagen-coated plates (10 μg/ml) for 20 min. Results are means ± S.D. (**B**) The activity of β1 integrin was analysed by flow cytometry. Cells were trypsinized and incubated with PE-conjugated anti-CD29 antibody (HUTS21; active form of β1 integrin) for 20 min. Cells were also pre-treated with α1,2-fucosidase for 30 min before staining with the anti-CD29 antibody to further analyse the effect of α1,2-linked fucosylation on β1 integrin activity. Representative data from three independent experiments are shown. Results are means ± S.D. $^*P < 0.05$ compared with the no-fucosidase group. #C, control cells; Fs, cells were treated with α1,2-fucosidase; NS, not significant; #R, CRT-RNAi cells. (**C**) FUT1-modified glycosylation of β1 integrin after treatment with α1,2-fucosidase. J82 control cells were pre-treated with α1,2-fucosidase before being lysed with NP40 lysis buffer. Cell lysates were pulled down with UEA-1 or LTL followed by Western blotting with an anti-(β1 integrin) antibody.

(Figure 1B). Subsequently, we used DNA arrays to compare the gene expression profiles between J82 control and CRT-RNAi cells and identified many candidate genes whose expression was likely to be CRT-regulated (Supplementary Tables S1 and S2 at http://www.biochemj.org/bj/460/bj4600069add.htm). Among these, FUT1 was significantly suppressed in J82 CRT-RNAi cells. Because previous studies have shown that changes in glycosylation of β1 integrin modulates its function [21], we performed a lectin pull-down assay using either LTL or UEA-1, which are known to bind to α1,2-linkage oligosaccharides through a reaction catalysed by FUT1. The pull-down samples were then subjected to immunoblotting against antibodies specific for β1 integrin. The fucosylation levels of β1 integrin were decreased profoundly in CRT-RNAi cells (Figure 1C). In addition, we also performed glycomics analysis in our stable cell lines. J82 cells had different UEA-1-positive glycoprotein expression patterns, as shown by Western blotting, when we knocked down CRT (Figure 1D). These results suggested that CRT mediates cell adhesion through post-translational modification of β1 integrin in bladder cancer.

FUT1 overexpression in CRT-knockdown cells enhanced cell adhesion

Fucosylation is one of the common post-translational protein modifications. It has been shown that transfection of *FUT1* into RMG-1 cells enhanced cell adhesion [22]. To further examine the role of FUT1 on CRT-mediated changes in cell adhesion, the *FUT1* gene was stably transfected into CRT-RNAi cells to generate J82 #R-FUT1 cells. FUT1 overexpression in J82 #R-FUT1 cells was confirmed by real-time PCR (Figure 2A). To investigate the effect of FUT1 on the changes in β1 integrin fucosylation, we pulled down the lectin UEA-1 and probed for β1 integrin, as described above. Binding of β1 integrin to UEA-1 lectin was increased significantly in J82 #R-FUT1 cells compared

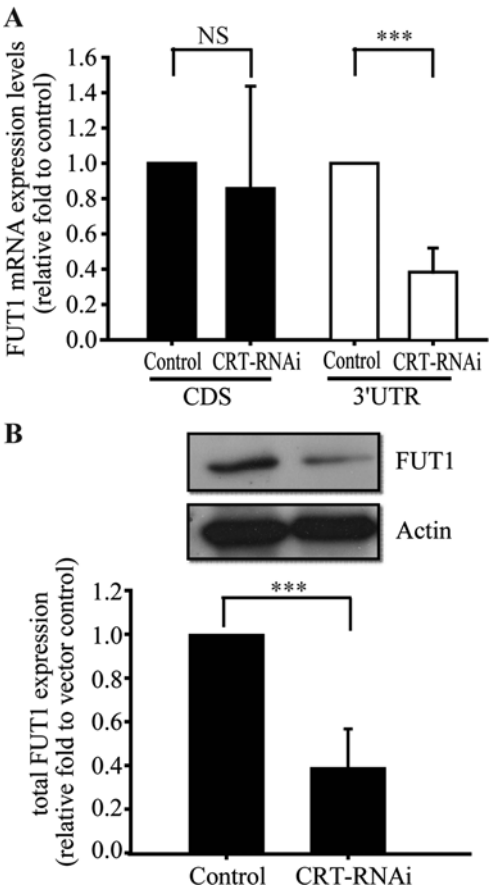

Figure 4 CRT knockdown inhibited the FUT1 protein and 3′-UTR mRNA expression levels, but not the mRNA of the CDS

(**A**) *FUT1* mRNA levels were detected by real-time PCR in J82 control and CRT-knockdown cells. mRNA expression was normalized to the internal control *GAPDH*. (**B**) Western blot analysis demonstrates protein expression in J82 stable cell lines. The human β-actin level was used as a loading control. Results are means $\pm$ S.D. ***$P < 0.001$ compared with the control level. NS, no significant.

with the control vector-transfected cells (Figure 2B). Furthermore, as shown in Figure 2(C), reintroduction of FUT1 restored cell adhesion in CRT-RNAi stable cells. These results suggested that CRT modulated cell adhesion in bladder cancer by regulating β1 integrin fucosylation through FUT1.

Fucosylation status of β1 integrin affected integrin activation, but not association between integrin and collagen

Since the fucosylation status of β1 integrin could influence cell adhesion, we hypothesized that α1,2-linked fucosylated glycan on β1 integrin might participate in β1 integrin and type I collagen interaction. To assess the potential inhibitory effects of UEA-1, we tested J82 cells in a cell adhesion assay in the presence of UEA-1 lectin. As shown in Figure 3(A), treatment with UEA-1 did not show any effect on adhesion even at higher dosages. These results prompted us to inspect the activation status of β1 integrin. Several studies have revealed that variation in the glycosylation patterns of β1 integrin has an influence on its functions [15,23,24]. To verify the effect of fucosylation levels on β1 integrin activity, the active forms of β1 integrin were detected by flow cytometry. We found that active integrins were significantly lower in CRT-RNAi cells (13.0 ± 2.6) than in control cells (22.9 ± 3.9) (Figure 3B). These results suggested that β1 integrin fucosylation modified integrin activity. To further confirm this hypothesis, cells were treated with a FUT1-specific α1,2-fucosidase. Both the control and CRT-RNAi cells showed reduced β1 integrin activity upon fucosidase treatment (Figure 3B). Taken together, these findings suggested that β1 integrin fucosylation affected the J82 cell adhesion ability by activating β1 integrin, rather than directly modifying the integrin-binding sites. The data for down-regulation of α1,2-fucosylation after fucosidase treatment are shown in Figure 3(C).

CRT knockdown suppressed FUT1 expression by regulating its mRNA stability through an ARE

In order to verify how CRT affected FUT1 expression, real-time PCR and Western blot analyses were performed in our stable cell lines. The results showed that CRT down-regulation attenuated the expression of FUT1 protein and mRNA levels of the 3′-UTR, but not the mRNA level of the CDS (Figure 4). It is known that certain specific sequences in 3′-UTR of mRNA can influence the RNA stability and protein expression [25]. These reports prompted us to investigate whether CRT regulates FUT1 expression levels by affecting mRNA stability rather than transcription. An Act-D time course experiment was performed to determine the degradation rate of *FUT1* mRNA. We found that *FUT1* mRNA was significantly less stable following CRT knockdown in J82 cells (Figure 5A). These results strongly suggested that CRT down-regulation affected the mRNA stability of FUT1.

Numerous studies have shown that in sequences rich in adenosine and uridine nucleotides, called AREs, the 3′-UTRs are important sequences for mRNA decay [26–28]. The *FUT1* gene contains an ARE sequence at its 3′-UTR. J82 control and CRT-RNAi cells were transfected with a CMV (cytomegalovirus)-driven EGFP reporter fused to wild-type, mutated or ARE-deleted FUT1 3′-UTR as shown in Figure 5(B). The EF1α (elongation factor 1α)-driven DsRed was used as an internal control. *EGFP* mRNA expression was measured by real-time PCR after 48 h of transfection. Control cells showed a lower EGFP/DsRed ratio when the ARE sequences from the FUT1 3′-UTR were mutated or deleted (Figure 5C, left-hand panel). However, no significant differences were observed between the wild-type, mutated or ARE-deleted FUT1 3′-UTR constructs in CRT-knockdown cells (Figure 5C, right-hand panel). Consequently, these results indicate that CRT influenced *FUT1* mRNA stability through the ARE sequence.

Identification of FBP1 as an *FUT1* mRNA-binding protein

The proteins which bind to AREs, called ARE-BPs (ARE-binding proteins), play an important role in the regulation of mRNA stability [26]. CRT was reported previously as an ARE-BP that regulates mRNA degradation in various cell types [29,30]. We carried out REMSAs using a biotin-labelled probe and recombinant CRT to assess CRT–FUT1 ARE interaction *in vitro*. No specific bands with a higher molecular mass than the biotin-labelled probes were observed (Figure 6A). We next incubated whole cell extracts with the biotin-labelled probes and the bound proteins were pulled down with streptavidin. As shown in Figure 6(B), CRT did not co-precipitate with the ARE or mARE probes. These results indicate that CRT did not bind to the ARE in the FUT1 3′-UTR.

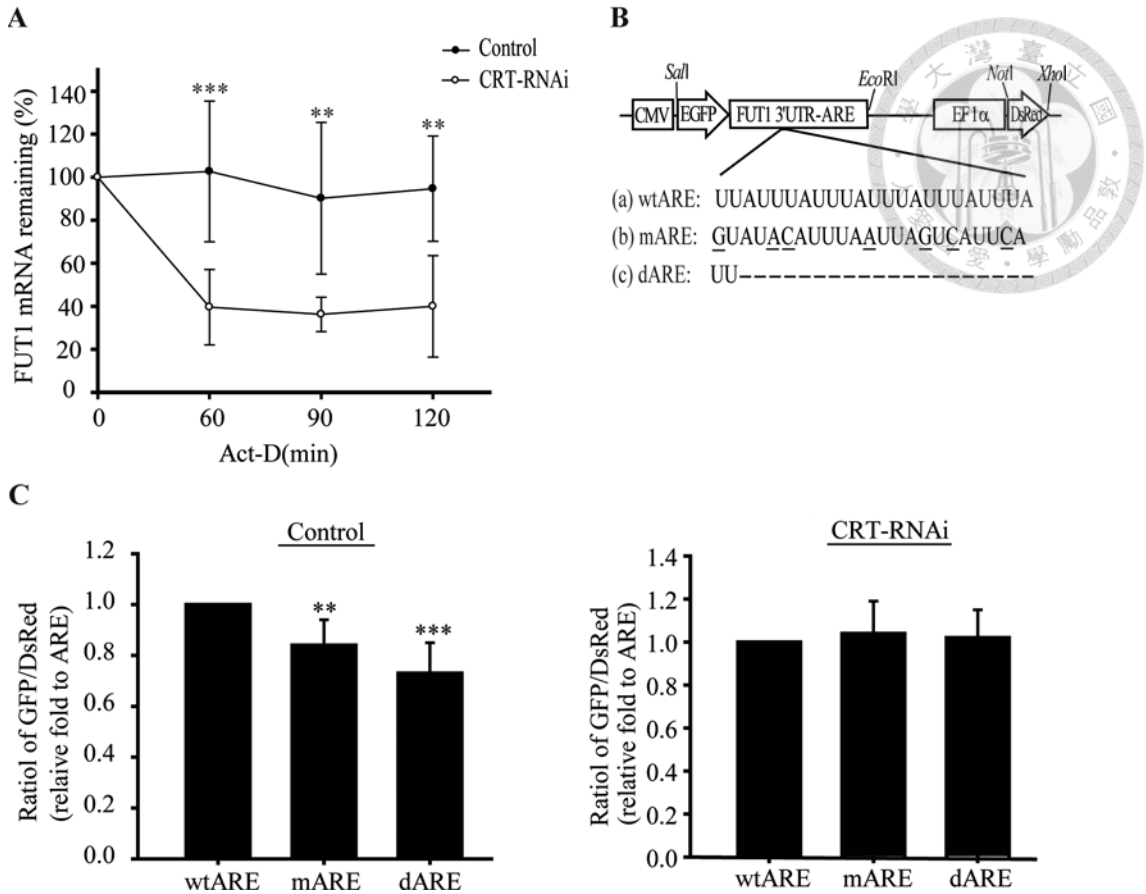

Figure 5 CRT-knockdown destabilized *FUT1* mRNA through the ARE

(**A**) Cells were treated with 5 μg/ml Act-D for the indicated time. *FUT1* mRNA was assessed by real-time PCR and normalized to the internal control *GAPDH*. (**B**) Schematic diagram of reporter constructs containing EF1α-driven DsRed and CMV-driven EGFP fused to various ARE sequences. (**C**) Cells were transfected with the plasmids shown in (**B**). Total RNA was harvested after 24 h of transfection. Results are the ratio of EGFP to the internal transfected control DsRed. Results are means ± S.D. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control level. dARE, deleted ARE; wtARE, wild-type ARE.

To identify the proteins that did bind to the FUT1 ARE, the synthetic probe was incubated with whole cell lysate. We performed an RNA pull-down assay and the RNA–protein complex was separated by SDS/PAGE (8 % gel). In all three independent experiments, we consistently noticed a band near 75 kDa, which was suppressed in the mARE samples (Figure 6C). Using MS we identified this ARE-binding protein as FBP1. Our results confirmed that both the protein and mRNA levels of FBP1 were reduced in CRT-knockdown cells (Figures 6D and 6E). Consequently, these results suggest that CRT affected *FUT1* mRNA stability through FBP1 regulation, which binds to the ARE sequence in the FUT1 3′-UTR.

DISCUSSION

RNA stability is an important control point for gene expression. It has been reported that interaction between specific sequences (*cis*-acting elements) in the 3′-UTR of mRNA and specialized RNA-binding proteins (*trans*-acting factor) regulate mRNA degradation [31]. On the basis of the characteristics of the *cis*-acting elements, there are three classes of mRNA, and the most common type identified in the 3′-UTR is the ARE [25,28,32,33]. A number of studies have shown that certain metastasis-associated genes with AREs are overexpressed in cancers [34–36]. Previously, we have reported that CRT levels regulate the metastasis of bladder cancer cells [18]. In the present study, we showed that CRT knockdown suppressed *FUT1* mRNA degradation in J82 cells. Screening using the ARE database (http://brp.kfshrc.edu.sa/ARED/) classified FUT1 as class II cluster I of the ARE mRNA. In our fluorescence reporter experiments, mutations or deletions in the FUT1 3′-UTR ARE sequence resulted in a significant decrease in EGFP levels in control cells. These results are consistent with previous reports that the ARE sequence is important for regulating mRNA stability [32,37]. Consequently, we suggested that CRT may modulate the metastasis of bladder cancer by influencing *FUT1* mRNA stability in the ARE region.

Protein factors that bind to AREs are key regulators for mRNA stability. Many ARE-BPs, including AUF1 (ARE RNA-binding protein 1), HuR and TTP (tristetraprolin) have been well studied [26]. Numerous studies have reported that these ARE-BPs bind to AU-rich regions and influence the stability of certain inflammatory and tumour-associated genes, including c-*Myc*, c-*Fos*, *GMCSF* (granulocyte/macrophage colony-stimulating factor), *TNFA* (tumour necrosis factor α) and *VEGF* (vascular endothelial growth factor) [38–40]. CRT is a multifunctional protein that participates in various cell processes. In 2002, Nickenig et al. [29] first indicated CRT as a novel mRNA-binding protein that destabilizes type I angiotensin II receptor mRNA by

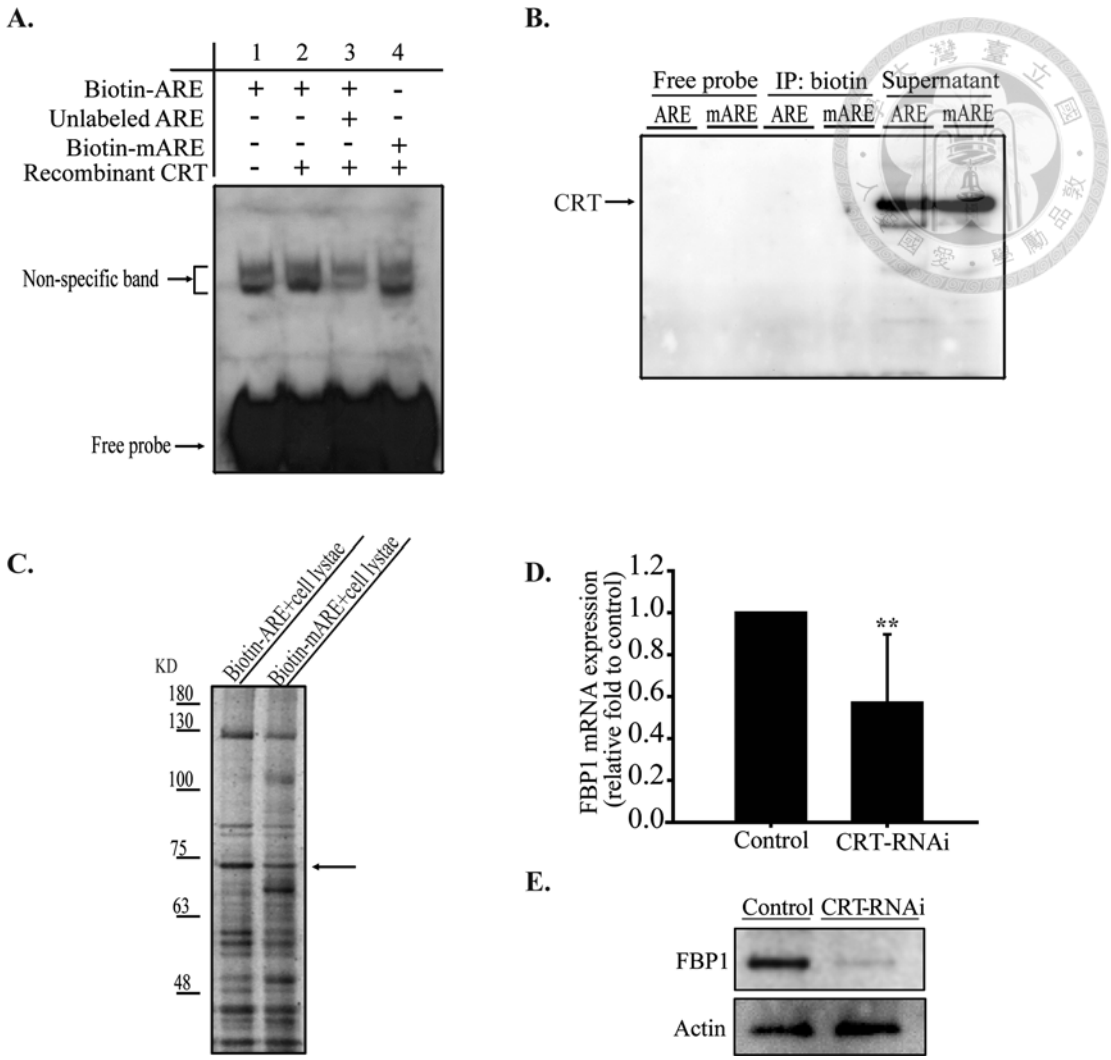

Figure 6 CRT knockdown suppressed FBP1 that binds to the ARE at the FUT1 3′-UTR

(**A**) Biotinylated RNA probes (ARE or mARE) were incubated with recombinant CRT. The RNA–protein complex was separated by native PAGE and detected using chemiluminescence. (**B**) Whole cell lysates were incubated with biotinylated RNA probes (ARE or mARE). The RNA–protein complex was pulled down by streptavidin beads and analysed using Western blotting with an anti-CRT antibody. IP, immunoprecipitation. (**C**) Identification of FBP1 as the FUT1 ARE-BP. Biotinylated RNA probes (ARE or mARE) were incubated with whole cell lysate and the RNA–protein complex was pulled down by streptavidin beads. Arrow indicates the putative binding protein. (**D**) Real-time PCR was performed to detect FBP1 expression levels in J82 control and CRT-RNAi cells. mRNA expression was normalized to the internal control *GAPDH*. Results are means ± S.D. $^{**}P < 0.01$ compared with the control level. (**E**) Western blot analysis demonstrating protein expression in J82 stable cell lines. Human β-actin was used as a loading control.

binding to the AU-rich region at the 3′-UTR. Moreover, Totary-Jain et al. [30] reported that CRT also binds to a specific element in the 3′-UTR of glucose transporter-1 mRNA and destabilizes the mRNA under high-glucose conditions. In the present study, the fluorescence signal of mARE or ARE-deleted FUT1 was suppressed in the control cells, but not in CRT-RNAi cells, suggesting that CRT levels influence *FUT1* mRNA stability. However, we did not notice any direct association between CRT and the FUT1 ARE directly in J82 cells. On the other hand, we identified FBP1 as the protein that interacts with an FUT1 ARE probe. Previous studies have indicated that FBP1 is an RNA-binding protein that participates in mRNA translation and/or stabilization [41–43]. In the present study, FBP1 levels were significantly decreased in CRT-knockdown cells. Taken together, our results suggested that CRT can stabilize *FUT1* mRNA through FBP1, which binds to the FUT1 ARE at the 3′-UTR.

Fucosylation-regulated cancer cell tumorigenesis and metastasis has been studied extensively [1]. It has been reported that FUT1/FUT4-knockdown by siRNA significantly diminishes cell proliferation and tumour growth both in *vitro* and in *vivo* [10]. FUT1 overexpression restored cell adhesion in CRT-RNAi cells, suggesting that FUT1-related fucosylation affects cell adhesion and subsequent metastasis. Nevertheless, a deficiency in GDP-mannose-4,6-dehydratase, resulting in a loss of fucosylation, has been shown to cause colon cancer cell metastasis by escaping from tumour immune surveillance [44,45]. Although those studies contradicts the results of the present study, cumulative evidence suggests that tumour tissues express higher levels of fucosylation compared with normal tissues [46–48]. Furthermore, Numahata et al. [49] showed that sialyl Lewis X, a blood carbohydrate catalysed by fucosyltransferase, is a predictor of an invasive and metastatic outcome of bladder cancer. These findings suggested a positive correlation between fucosylation and bladder cancer progression. Our results provided further evidence that FUT1 plays an important role in the metastasis of bladder cancer.

Cell adhesion is one of the common features of cancer metastasis. It is known that integrins are the main cell surface molecules that regulate cell–matrix interaction. Many studies have revealed that changes in glycan modification of integrins affect its biological function [50,51]. A previous study showed that deletion of core fucosylation (FUT8) on $\alpha 3\beta 1$ integrin suppressed cell migration on laminin 5 in MEFs (mouse embryonic fibroblasts) [16]. In the present study, we found that FUT1 increased fucosylation of β1 integrin and promoted the cell adhesion of bladder cancer cells with type I collagen. Overexpression of the *FUT1* gene enhanced cell adhesion in CRT-knockdown cells. However, higher levels of FUT1 only partially restored cell adhesion in CRT-knockdown cells. Consequently, our results provide evidence that FUT1-mediated fucosylation of β1 integrin is one of the important mechanisms for cell adhesion in J82 bladder cancer cells. In another study FUT1 has been shown to increase the total expression of $\alpha 5\beta 1$ integrin and promote RMG-1 cell adhesion on fibronectin [22]. Nevertheless, on the basis of the results of the present study, we suggest that these observations might due to increased activity of β1 integrin. We also noticed a clear reduction in the activity of β1 integrin with reduction in fucosylation in our stable cell lines. Most importantly, cells treated with FUT1-specific α1,2-fucosidase effectively diminished integrin activity. Taken together, our results suggest that CRT regulates cell adhesion in bladder cancer by regulating β1 integrin activity through FUT1-dependent fucosylation. Since previous studies only indicated that changes in the glycosylation of integrins may affect its cell adhesion and migration function, the results of the present study provide further evidence showing the role of fucosylation on the activity of β1 integrin. It is known that there are 12 potential N-glycosylation sites on the β1 integrin subunit [12]. Our stable cell lines can be excellent tools to identify the functional fucosylation sites on β1 integrin.

We have shown previously that alterations in CRT expression levels affect cell adhesion and the metastasis of bladder cancer. In the present study we further analysed the mechanistic details of CRT-dependent fucosylation and bladder cancer progression. Our findings demonstrate that CRT stabilizes *FUT1* mRNA through affecting the level of the FBP1 ARE-BP. Therefore FUT1 activates β1 integrin by fucosylation, thereby enhancing bladder cancer adhesion and subsequent metastasis. The present study provides new insights into the biological function of α1,2-linked fucosylation on β1 integrin. Studies are underway to address the specific fucosylation modification site of β1 integrin. The results of the present study can provide a new strategy towards therapeutic development for bladder cancer.

AUTHOR CONTRIBUTION

Hsinyu Lee and Cheng-Chi Chang designed the experiments. Yi-Chen Lu performed experiments, analysed the data and wrote the paper. All other authors performed experiments.

ACKNOWLEDGEMENT

We thank Enago (http://www.enago.tw) for a review of the English used in the present paper.

FUNDING

This work was supported by the Cutting-Edge Steering Research Project of the National Taiwan University [grant number NTU-CESRP-102R76263A], the National Science Council of Taiwan [grant number NSC 97-2311-B-002 -002 -MY3] and the National Health Research Institutes of Taiwan [grant number NHRI-EX101-10130BI (to H.L.)].

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Received 5 November 2013/21 February 2014; accepted 4 March 2014
Published as BJ Immediate Publication 4 March 2014, doi:10.1042/BJ20131424

Biochem. J. (2014) **460**, 69–78 (Printed in Great Britain) doi:10.1042/BJ20131424

online data

SUPPLEMENTARY ONLINE DATA

Calreticulin activates β1-integrin via fucosylation by fucosyltransferase 1 in J82 human bladder cancer cells

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Table S1 Genes down-regulated by CRT

Gene symbol	Description	CRT-RNAi/control ratio	
		Fold	*P* value
FGFBP1	Fibroblast growth factor-binding protein 1	−4.35	0
NNMT	Nicotinamide *N*-methyltransferase	−3.75	0.01
TES	Testis-derived transcript (three LIM domains)	−2.99	0.01
TESC	Tescalcin	−2.91	0.01
ID2	Inhibitor of DNA-binding 2, dominant-negative helix–loop–helix protein	−2.69	0.04
FUT1	Fucosyltransferase 1 (galactoside 2-α-L-fucosyltransferase, H blood group)	−2.46	0.01
KIF21B	Kinesin family member 21B	−1.96	0.02
MAGED1	Melanoma antigen family D, 1	−1.86	0
ACSL5	Acyl-CoA synthetase long-chain family member 5	−1.85	0.02
NFIX	Nuclear factor I/X (CCAAT-binding transcription factor)	−1.77	0.03
CSPG5	Chondroitin sulfate proteoglycan 5 (neuroglycan C)	−1.71	0.04
IGFBP2	Insulin-like growth factor-binding protein 2, 36 kDa	−1.71	0.02
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	−1.62	0.04
ALPP	Alkaline phosphatase, placental (Regan isozyme)	−1.6	0
CENTB1	Centaurin, β1	−1.54	0.01
EGR1	Early growth response 1	−1.42	0
HSPB8	Heat-shock 22 kDa protein 8	−1.41	0.05
ITGB1	Integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)	−1.31	0.05
VEGFA	Vascular endothelial growth factor A	−1.16	0.02
TNFSF13	Tumour necrosis factor (ligand) superfamily, member 13	−1.11	0.04
SSTR2	Somatostatin receptor 2	−1.1	0.05
TM4SF19	Transmembrane 4 L six family member 19	−1.1	0
SCARA3	Scavenger receptor class A, member 3	−1.1	0.02
DDIT4	DNA-damage-inducible transcript 4	−1.09	0.01
PRSS35	Protease, serine, 35	−1.06	0.01
CSAG2	CSAG family, member 2	−1.05	0.01

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Table S2 Genes up-regulated by CRT

Gene symbol	Description	CRT-RNAi/control ratio	
		Fold	*P* value
TRIP6	Thyroid hormone receptor interactor 6	5.94	0
DNAJC10	DnaJ (Hsp40) homologue, subfamily C, member 10	2.8	0
CSMD1	CUB and Sushi multiple domains 1	1.98	0
CASP8	Caspase 8, apoptosis-related cysteine peptidase	1.57	0.01
GPR37	G-protein-coupled receptor 37 (endothelin receptor type B-like)	1.47	0.04
EPS8	Epidermal growth factor receptor pathway substrate 8	1.44	0.01
SERINC1	Serine incorporator 1	1.41	0.01
LOC165186	Similar to RIKEN cDNA *4632412N22* gene	1.4	0.02
FLJ41327	FLJ41327 protein	1.34	0.03
FLJ23861	Hypothetical protein FLJ23861	1.28	0
BZW1	Basic leucine zipper and W2 domains 1	1.26	0.05
NAT14	*N*-acetyltransferase 14	1.24	0.01
DALRD3	DALR anticodon-binding domain-containing 3	1.19	0.01
OXR1	Oxidation resistance 1	1.09	0.01
MAPK1	Mitogen-activated protein kinase 1	1.07	0.01
RBM13	RNA-binding motif protein 13	1.05	0.02
PROSC	Proline synthetase co-transcribed homologue (bacterial)	1.05	0.03
NAP1L4	Nucleosome assembly protein 1-like 4	1.04	0
HDDC2	HD domain-containing 2	1.02	0.04

Received 5 November 2013/21 February 2014; accepted 4 March 2014
Published as BJ Immediate Publication 4 March 2014, doi:10.1042/BJ20131424