ARTICLE
Journal of Cellular Biochemistry 116:2494–2503 (2015)

Journal of Cellular Biochemistry

Urocortin Attenuates TGFβ1-Induced Snail1 and Slug Expressions: Inhibitory Role of Smad7 in Smad2/3 Signaling in Breast Cancer Cells

Lai Jin, Chao Zhu, Xiaofei Wang, Chuanhua Li, Chunxuan Cao, Jie Yuan, and Shengnan Li*

Department of Pharmacology, Jiangsu Provincial key Lab of Cardiovascular Diseases and Molecular Intervention, Nanjing Medical University, Nanjing 210029, China

ABSTRACT

Corticortropin-releasing hormone (CRH) family are multifunctional endocrine-factors that regulate proliferation, apoptosis, and migration of various types of cancer cells. Deregulation of the transforming growth factor β1(TGFβ1) signal transduction promotes aggressive metastatic properties in late-stage breast cancers. We previously have demonstrated in breast cancer cell line that CRH suppressed TGFβ1-induced Epithelial-Mesenchymal Transition (EMT) via induction of E-cadherin. Our present data in MCF-7 and MDA-MB-231 cells showed that Urocortin (Ucn, a member of CRH family) inhibited TGFβ1 signaling by reducing Smad2/3 activation and subsequent nuclear translocation through increasing Smad7 expression, leading to downregulation of Snail1 and Slug, the two EMT promoters. We further found that Antalarmin (CRH receptor type 1, CRHR1 antagonist) and Antisauvagine-30 (CRH receptor type2, CRHR2 antagonist) abrogated the effects of Ucn on TGFβ1 signaling, implying that both active CRHR1 and CRHR2 participate in Ucn-repressed TGFβ1 signaling. Our findings, for the fist time, identify Ucn as a potential mediator that inhibits oncogenic signaling by TGFβ1 and suggest that activating CRHR1 and R2 may prove effective in diminishing breast cancer progression stimulated by TGFβ1. J. Cell. Biochem. 116: 2494–2503, 2015.

KEY WORDS: SLUG; SMAD2/3; SMAD7; SNAIL1; TGFβ1; UROCORTIN

Transforming growth factor β1 (TGFβ1) is a secreted multi-functional peptide that regulates cell proliferation, differentiation, cell motility, and apoptosis [Wakefield and Roberts, 2002; Roberts et al., 2003]. In normal mammary epithelial cells, TGFβ1 acts as a tumor suppressor but during breast cancer progression, it induces cell proliferation, invasion, and migration in part through the stimulation of Epithelial-Mesenchymal Transition (EMT) [Bierie and Moses, 2006; Leivonen and Kahari, 2007; Wendt and Schiemann, 2009]. Indeed, TGFβ1, a key mediator of EMT, initiates the phosphorylation of Smad2 and/or Smad3 which oligomerize with Smad4 and translocate to the nucleus to adjust gene transcription, including increase of Snail1 and Slug expressions [Derynck et al., 1998; Zavadil and Bottinger, 2005]. Snail1 and Slug are currently considered to repress E-cadherin as negative transcriptional regulators, which drive EMT, and EMT is related molecularly with many of some new players such as Smad family [Huber et al., 2005]. Smad6 and Smad7 are inhibitory Smads which block Smad2/3 activation, then inhibiting TGFβ1 signaling [Hayashi et al., 1997; Zhang et al., 2007].

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide that plays a key role in adjusting the basal and stress-activated hypothalamic-pituitary-adrenal axis (HPA) [Bonfiglio et al., 2011]. The effects of CRH and its homologue Urocortin (Ucn) are mediated through the two subtypes receptors, CRH receptor

Abbreviations: Anta, antalarmin; Anti, antisauvagine-30; Ast, astressin; CRH, corticortropin-releasing hormone; CRHR1, CRH receptor type 1; CRHR2, CRH receptor type 2; EMT, epithelial-mesenchymal transition; Realtime PCR, realtime polymerase chain reaction; siRNA, short-interfering RNA; TGFβ1, transforming growth factor β1; Ucn, urocortin.

Grant sponsor: Natural Science Foundation of China; Grant numbers: 81302866, 91129711; Grant sponsor: Priority Academic Program; Grant sponsor: Science and Technology Development; Grant number: 2012NJMU014; Grant sponsor: Priority Academic Program Development of Jiangsu Higher Education Institutions; Grant number: JX10131801145.

*Correspondence to: Dr. Shengnan Li, Department of Pharmacology, Nanjing Medical University, Nanjing, 210029, China.
E-mail: snli@njmu.edu.cn

Manuscript Received: 13 April 2015; Manuscript Accepted: 14 April 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 2 July 2015
DOI 10.1002/jcb.25194 • © 2015 Wiley Periodicals, Inc.

type 1 (CRHR1) and 2 (CRHR2), which belong to G-protein-coupled receptors (GPCRs) and play different roles in central and periphery systems [Perrin and Vale, 1999]. CRH is a high-affinity ligand for CRHR1 and Ucn binds with similar affinities to both receptors [Bonfiglio et al., 2011]. During past few years, CRH family peptides were found in a series of endocrine related-cancer tissues, such as breast cancer [Ciocca et al., 1990] and prostate cancer [Jin et al., 2011]. It is reported that CRH/Ucn in cancer cells regulates cellular proliferation [Graziani et al., 2007; Arranz et al., 2010], apoptosis [Chatzaki et al., 2006; Jin et al., 2012], and migration [Yang et al., 2007; Jin et al., 2014]. Our previous data demonstrated that CRH inhibited TGFβ1-induced Epithelial-Mesenchymal Transition (EMT) of breast cancer cells via CRHR1 and R2, in which Snail1 and Twist-repressed E-cadherin expression were involved.

To investigate the profound mechanism of CRH family peptides on TGFβ1 signaling in breast cancer cells, Smad2/3 activation and Snail1 and Slug expressions (EMT transcriptional factor) were detected in MCF-7 and MDA-MB-231 cells. Further, Ucn and three kinds of antagonists (selective R1and R2 antagonist, unselective antagonist) were selected to get insight into the precise effect of each receptor. Our data showed that Ucn attenuated TGFβ1-induced Smad2/3 phosphorylation and nuclear translocation through Smad7 upregulation, which restrained Snail1 and Twist expressions. This study implied that activation of both CRHR1 and R2 opposed the effects of TGFβ1 via a classic non-genomic mechanism.

MATERIALS AND METHODS

CELLS AND REAGENTS

Human breast cancer cell lines MCF-7 and MDA-MB-231 cells (10–25 passages) were from the Institute of Biochemistry and Cell Biology, and incubated in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, at 37°C in a humidified atmosphere of 5% CO_2. Hoechst 33,285 (Beyotime) staining was performed to rule out mycoplasma contamination. Ucn, CRHR1/R2 antagonist Antalarmin (Anta)/Antisauvagine-30 (Anti-30), CRHRs antagonist Astressin (Ast), Phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma. TGFβ1 was from PeproTech. Cytoplasmic and Nuclear Protein Extraction Kit was from KeyGEN BioTECH. Lipofectamine™ 2,000 transfection reagent, Alexa Fluor 488 Goat Anti-Rabbit IgG (H + L) and RNA isolation kit (TRIzol) were acquired from Invitrogen (Carlsbad, California). Specific antibodies to Snail1, Slug, Histone, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were provided by BioWorld. Total Smad2/3, phospho-Smad2 (Ser4 65/467)/3(Ser423/425) antibodies were purchased from Cell Signaling Technologies. Smad7 antibody was from Abcam. SYBR green PCR Supermix with ROX was purchased from Bio-Rad (Hercules, CA). Small interfering RNA (siRNA) kit was from GenePharma.

CELL LYSATE EXTRACTION AND WESTERN BLOT ANALYSIS

Cultured cells after treatment were lysed and protein was extracted for western blot analysis for Snail1, Twist, Phospho-Smad2/3, Smad2/3, Smad7, GAPDH, and Histone as previously described [Jin et al., 2011, 2014]. Cytoplasmic and nuclear proteins were isolated using a Cytoplasmic and Nuclear Protein Extraction Kit (Fermatas, Canada) according to manufacturer's instructions. Briefly, cells were lysed in a buffer (with protease inhibitors, PMSF, and DTT) followed by vortex for 15 s and incubation at 4°C for 10 min. Lysates were then centrifuged at 16,000×*g* to remove the cytoplasmic fraction from nuclei. Cytoplasmic lysates were cleared by centrifugation at 16,000×*g* and nuclei were washed with nuclei washing buffer (with protease inhibitors, PMSF, and DTT), then resuspended in Nuclei Lysis Reagent [Jin et al., 2012]. The signals were visualized by the Chemiluminescence gel imaging system (SYNGENE, UK) and the ratio for each protein was determined by densitometry, using GEL-PRO analyzer 4 software (USA).

REAL-TIME PCR

Total RNA from cells was isolated with TRIzol (Invitrogen) and analyzed by real-time PCR with SYB green and Bio-Rad CFX Connect System (Bio-Rad). The primers were as follows: Snail1, CGCGCTCTTTCCTCGTCAG (forward), TCCCAGATGAGCATTGGCAG (reverse) [Dohadwala et al., 2006]; Slug, CATGCCTGTCATACCACAAC (forward), GGTGTCAGATGGAGGAGGG (reverse) [Hotz et al., 2007]; and GAPDH TGTTCGACAGTCAGCCGC (forward), GGTGTCTGAGCGATGTGGC (reverse) [Ghosh et al., 2010]. The housekeeping gene GAPDH was used as an internal standard and changes in other gene expressions were determined with the comparative CT (ΔΔCT) method.

SMALL INTERFERING RNA AND TRANSFECTION

Small interfering RNAs (siRNA) were introduced with 100 nmol/L using Lipofectamine™ 2,000 transfection reagent. The Smad7-specific sequences are as follows: Smad-homo-824 (siRNA1), GUGAAUCUUACGGGAAGAUTT (sense) and AUCUUCCCGUAAGAUUCACTT (sntisense); Smad-homo-1466 (siRNA2), CCGUGCAGAUCAGCUUUGUTT (sense) and ACAAAGCUGAUCUGCACGGTT (antisense); Smad-homo-1961 (siRNA3), GAGACAACGUGCUCUUUGUTT (sense) and ACAAAGAGCACGUUGUCUCTT (antisense). The sequence of negative control is sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and antisense: 5′-ACG UGA CAC GUU CGG AGA ATT-3′ (GenePharma). Western blot assays after 48 h posttransfections confirmed the knock-down efficiency and one of the sequences against Smad7 was chosen for subsequent experiment.

IMMUNOFLUORESCENCE

Cells were plated on coverslips and treated with Ucn or Ucn along with TGFβ1 for 24 h. Then the cells were fixed with 100% methanol for 30 min at room temperature, permeabilized with 0.5% Triton X-100 for 10 min, blocked with goat serum for 30 min at 37°C and incubated with rabbit anti-phospho-Smad2 (Ser465/467)/3(Ser423/425) (1:100) overnight at 4°C. After three washes, the cells were incubated with Alexa Fluor 488 Goat Anti-Rabbit IgG (H + L) for 30 min. DAPI was added to stain the nuclei and the slides were visualized at 40× magnification using LSM10 confocal microscope (Zeiss, Germany).

STATISTICAL ANALYSIS

The results were expressed as means ± S.E.M. Data was analyzed through GraphPad Prism 5.0 software by One-way ANOVA with Turkey's multiple comparison tests. $P < 0.05$ was considered to be of statistical significance. Each experiment was repeated for at least three times.

RESULTS

UCN ATTENUATED TGFB1-INDUCED SNAIL1 AND SLUG EXPRESSIONS VIA CRHR1 AND CRHR2

Our previous data demonstrated that CRH, homologous peptide of Ucn, decreased Snail1 expressions at both RNA and protein levels in MCF-7 and MDA-MB-231 cells [Jin et al., 2014]. Here, we investigated the effects of Ucn on Snail1 and Slug expressions under TGFβ1 treatment. As shown in Figure 1a, in the presence of Ucn and TGFβ1, Snail1 mRNA expression was drastically decreased compared to that with TGFβ1 only in MCF-7 cells. Similar results were obtained in Slug mRNA expression. In MDA-MB-231 cells, Ucn also exerted an inhibitory effect on TGFβ1-induced Snail1 and Slug expressions (Fig. 1b). To examine whether Ucn inhibits TGFβ1-induced Snail1 and Slug expressions via CRHR1 or CRHR2, we used Antalarmin (CRHR1 antagonist, Anta), Antisauvagine-30 (CRHR2 antagonist, Anti-30), and Astressin (unselective antagonist, Ast) for further exploration. In MCF-7 cells, western blot assay showed that TGFβ1 induced Snail1 and Slug protein expressions, which could be inhibited by Ucn, and pretreatment with Ast, Anta or Anti-30 almost abolished this Ucn effect (Fig. 1c). While in MDA-MB-231 cells, Ast and Anti-30 also abrogated the Ucn-mediated decrease in Snail1 and Slug expressions. However, Anta was unable to abolish the inhibitory effect of Ucn on Snail1 and Slug expressions because of CRHR1 deficiency (Fig. 1d) [Jin et al., 2014]. These findings suggest that Ucn interrupt TGFβ1-induced Snail1 and Slug expressions via both CRHR1 and CRHR2.

UCN REPRESSED TGFB1-SMAD2/3 SIGNALING PATHWAY VIA CRHR1 AND CRHR2

As Smad proteins are key transducers in TGFβ1 signaling, it is possible that Ucn inhibits TGFβ1 signaling by blocking the phosphorylation of Smad2/3 to translocate into the nucleus. Therefore, we performed western blot assay to detect the activity of Smad2/3 in the presence of Ucn. As shown in Figure 2a, TGFβ1 treatment alone increased time-dependent phosphorylation of Smad2/3 peaking at 45 min. Ucn treatment offset this TGFβ1-induced Smad2/3 phosphorylation in both MCF-7 and MDA-MB-231 cells. In addition, the inhibitory effect of Ucn on TGFβ1-induced Smad2/3 activity could be blocked by Ast and Anti-30 in both cells. The selective CRFR1 antagonist, Anta, abolished this Ucn effect only in MCF-7 cells, consistent with our report that there is no CRFR1 expression in MDA-MB-231 cells (Fig. 2b). These results indicate that both CRHR1 and CRFR2 are involved in the Ucn suppression of TGFβ1-induced Smad2/3 phosphorylation. This is supported by the finding that Knockdown of endogenous Smad2/3 signaling pathway plays an anti-metastatic role in breast cancer cells [Tian et al., 2003; Zhang et al., 2013].

UCN INHIBITED NUCLEUS TRANSLOCATION OF PHOSPHO-SMAD2/3 BY TGFB1

The transcription factor Smad2/3 is the downstream modulator of TGFβ1 signaling and active Smad2/3 enters into the nucleus to regulate the transcription of target genes. Smad2/3 is a key signaling molecule for induction of EMT by TGFβ1 [Padua and Massague, 2009]. To determine whether Ucn could restrain nucleus accumulation of active Smad2/3 induced by TGFβ1, the cellular distribution of phospho-Smad2/3 was examined by western blot and Immunofluorescence analyses. As shown in Figure 3a, there was an increased phospho-Smad2/3 expression in both cytoplasm and nucleus after TGFβ1 treatment and Ucn attenuated this effect, especially the expression of phospho-Smad2/3 in nucleus. Results in Figure 3b showed that nuclear phospho-Smad2/3 staining was intense in TGFβ1-stimulated cells and very faint in Ucn and TGFβ1-treated cells. The present findings demonstrated that Ucn inhibited TGFβ1-induced nuclear accumulation of phosphor-Smad2/3.

UCN BLUNTED TGFB1-INDUCED SMAD2/3 PHOSPHORYLATION THROUGH INCREASING THE PROTEIN LEVEL OF SMAD7

Smad7 functions as an inhibitory Smad by mediating the proteasome degradation of TGFβ receptor type 1 and subsequently impeding Smad2/3 phosphorylation, thereby mitigating TGFβ1 signaling [Kavsak et al., 2000; Hsu et al., 2013]. We therefore examined whether Ucn-inhibited TGFβ1 signaling was associated with Smad7 expression. Based on the data of Smad2/3 activation by TGFβ1 in 2 h, we detected the Smad7 expression also in this time range. As shown in Figure 4a, after TGFβ1 treatment, there was no obvious change for Smad7 protein expression. And Ucn time-dependently up-regulated Smad7 expression, peaking at 30 min in MCF-7 and 60 min in MDA-MB-231 cells. In addition, the effect of Ucn on Smad7 expression was in part attenuated by Anta or Anti-30, and completely blocked by Ast (Fig. 4b). In order to explore whether Smad7 could mediate the effect of Ucn on Smad2/3 phophorylation by TGFβ1, we knocked Smad7 down by 61.6% and 80.2% using siRNA in MCF-7 and MDA-MB-231 cells (Fig. 4c, siRNA1 and siRNA2). As seen in Figure 4d, after Smad7 siRNA knockdown, Ucn lost the ability to attenuate Smad2/3 phosphorylation by TGFβ1. Taken together, the results reveal that Ucn inhibits Smad2/3-TGFβ1 signaling mainly through Smad7 in a short period of time.

DISCUSSION

To the best of our knowledge, this is the first study showing that Ucn weakens the classic TGFβ1-Smad2/3 signaling pathway through augmenting Smad7 protein level via CRHR1 and R2. Our previous study demonstrated that engagement of CRHR1 and R2 activated by CRH restrained TGFβ1-induced EMT through up-regulating E-cadherin expression, a key regulator of cell-to-cell conjunction [Jin et al., 2014]. It is generally believed that active Smad2/3 formed heteromeric complexes with Smad4 and translocated into nucleus, where they could interact with DNA-binding factors, including the EMT-inducing factors such as Snail1 and Slug in order to regulate responsive genes such as E-cadherin [Padua and Massague, 2009; Tsuji et al., 2009]. The present study demonstrated that after prior TGFβ1 stimulation, Ucn quickly increased the level of Smad7 within an hour, which attenuated Smad2/3 phosphorylation. Furthermore, Ucn decreased the expressions of Snail1 and Slug, the Smad2/3 co-regulator of EMT gene. Based on the previous research and our results above, it is reasonable to postulate that Ucn interferes TGFβ1-induced EMT through Smad7, Smad2/3, Snail1, and Slug signaling pathway.

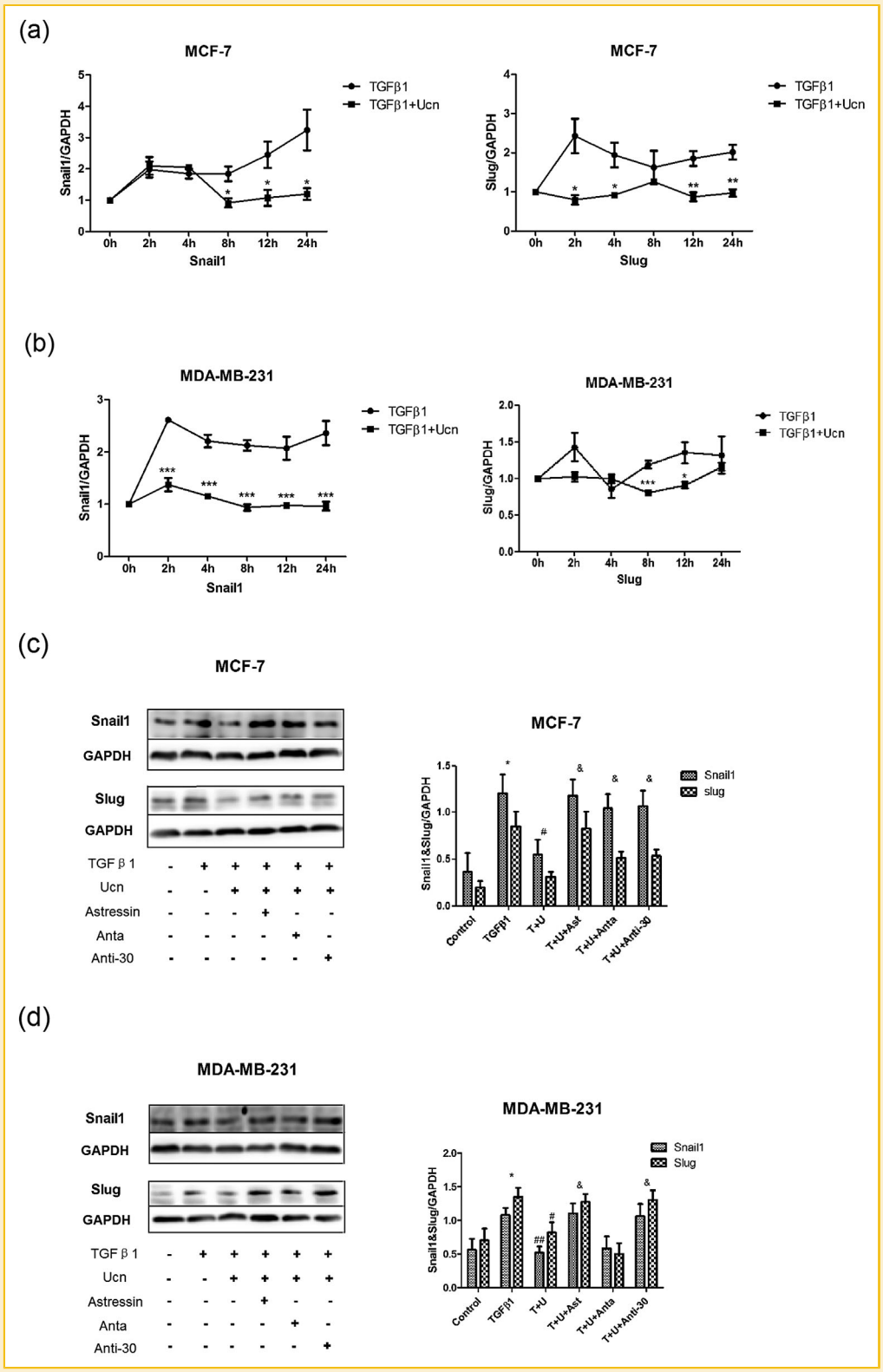

Fig. 1. Ucn attenuated TGFβ1-induced Snail1 and Slug expressions via CRHR1 and CRHR2 in both MCF-7 and MDA-MB-231 cells. (a and b) under the stimulation of TGFβ1 (T), Ucn (U) blocked the mRNA levels of Snail1 and Slug in time-dependent manners. (c and d) effects of CRHR1 antagonist, Antalarmin (Anta), CRHR2 antagonist, Antisauvagine-30 (Anti-30), and CRHRs antagonist, Astressin (Ast) on protein levels of Snail1 and Slug under the condition of TGFβ1 and Ucn treatment. Experiments were done for five times and a representative experiment was shown. Data were expressed as mean ± S.E.M. of five independent experiments and the statistical graph was on the right side. $^{*}P < 0.05$, versus 0h/control; $^{**}P < 0.01$, versus 0h/control; $^{***}P < 0.001$, versus 0h/control; $^{\#}P < 0.05$, versus TGFβ1 treatment; $^{\#\#}P < 0.01$, versus TGFβ1 treatment; $^{\$}P < 0.05$, versus TGFβ1 + Ucn treatment.

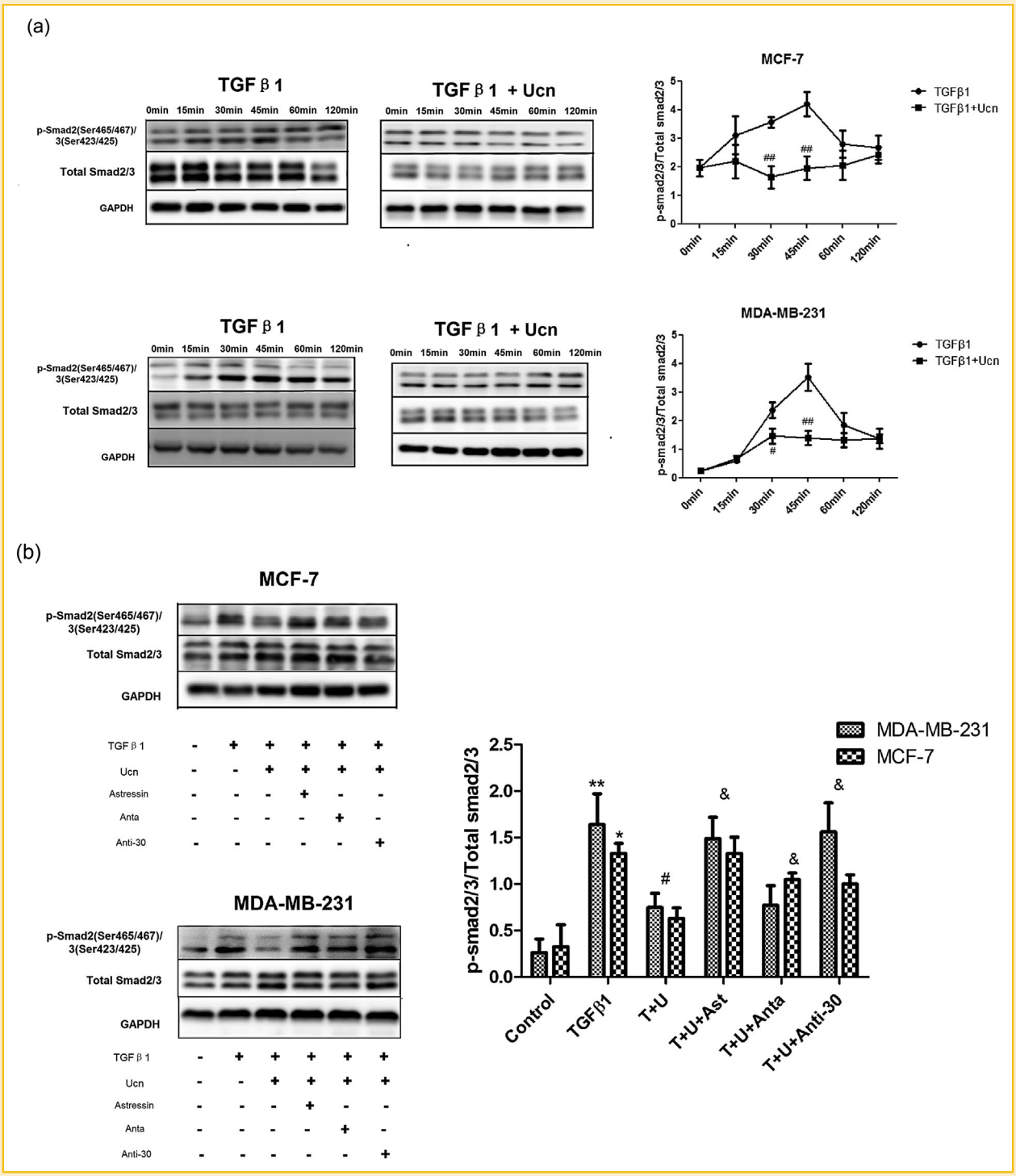

Fig. 2. Ucn abrogated TGFβ1-induced Smad2/3 phosphorylation via CRHR1 and R2 in MCF-7 and MDA-MB-231 cells. a, Ucn time-dependently inhibited TGFβ1-induced Smad2/3 activation at the sites of Ser465/467 and Ser423/425 in 120 min. (b) on the pretreatment of corresponding antagonist, Ucn lost the ability of inhibition in TGFβ1-induced Smad2/3 phosphorylation. Data were expressed as mean ± S.E.M. of five independent experiments and the statistical graph was on the right side. $^{*}P < 0.05$, versus 0h/control; $^{**}P < 0.01$, versus 0h/control; $^{\#}P < 0.05$, versus TGFβ1 treatment; $^{\#\#}P < 0.01$, versus TGFβ1 treatment; $^{\$}P < 0.05$, versus TGFβ1 + Ucn treatment.

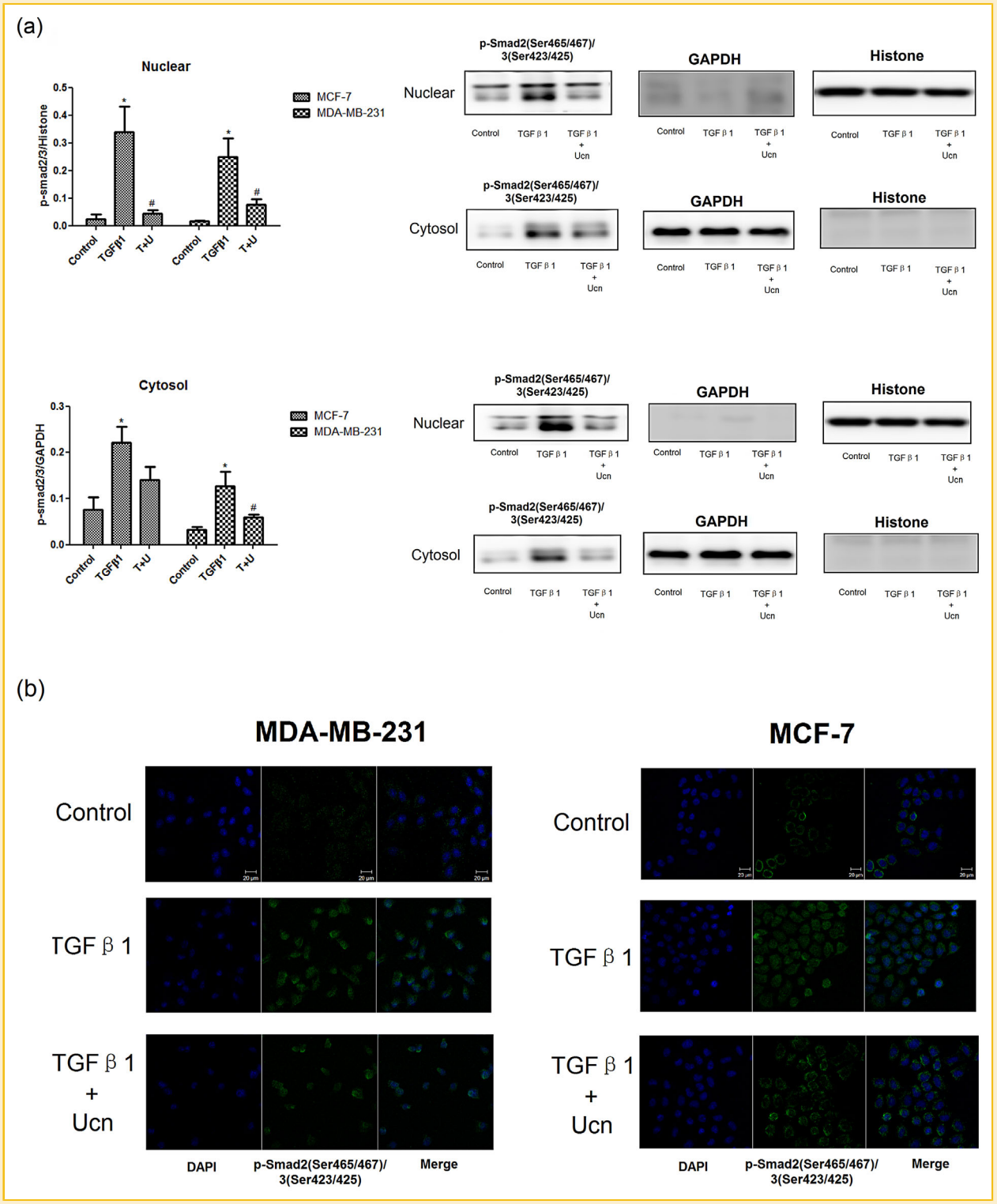

Fig. 3. Ucn inhibited TGFβ1-induced nucleus translocation of phospho-Smad2/3 in MCF-7 and MDA-MB-231 cells. (a) cytoplasmic and nuclear proteins were extracted separately from cells treated with TGFβ1 alone or along with Ucn for 24 h. (b) Immunofluorescence results of phospho-Smad2/3 location in cells (magnification 40×). Data were expressed as mean ± S.E.M. of five independent experiments and the statistical graph was on the left side. $^{*}P < 0.05$, versus control; $^{\#}P < 0.05$, versus TGFβ1 treatment.

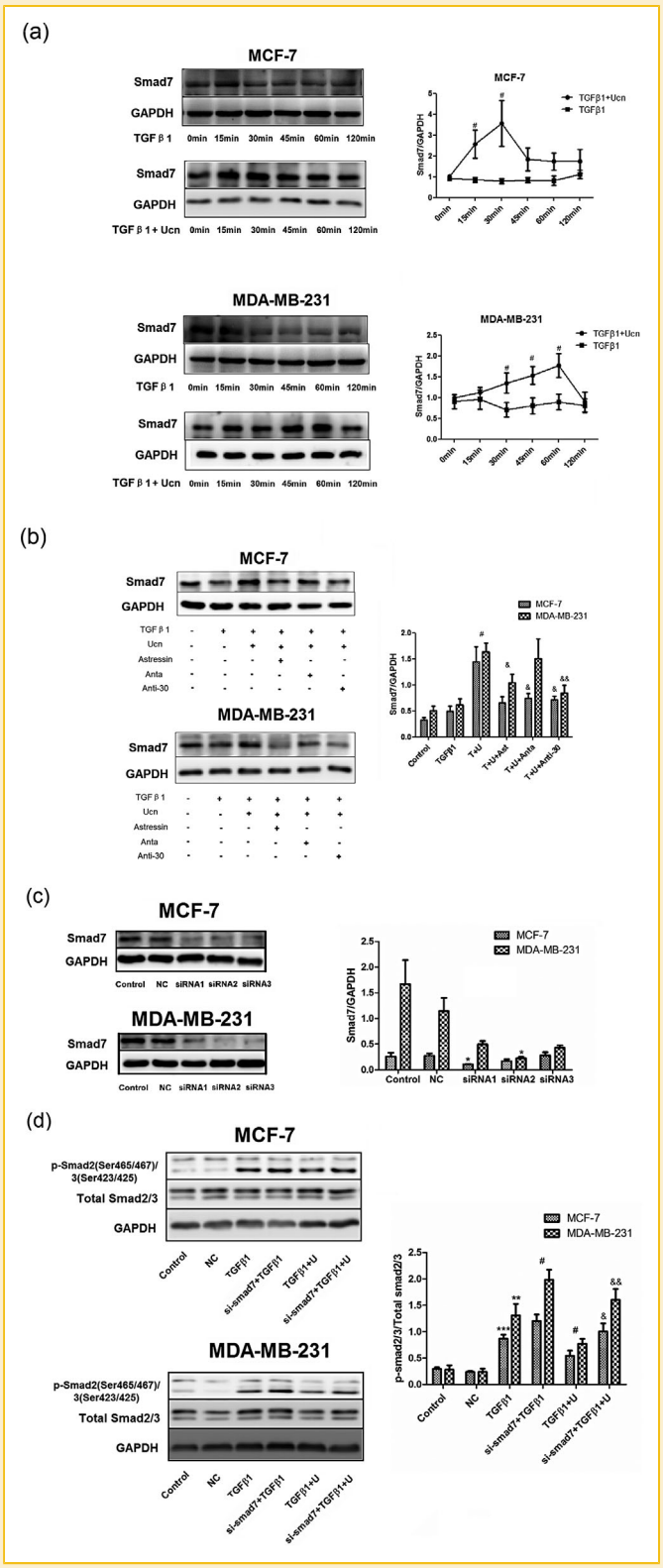

Fig. 4. Ucn increased the level of Smad7 protein expression. a, protein expressions of Smad7 in TGFβ1 alone or along with Ucn treatment. Briefly, the cells were treated with TGFβ1 for 0, 15, 30, 45, 60, and 120 min to detect Smad7 expression in 2 h. For Ucn and TGFβ1 group, the cells were treated with Ucn for 0, 15, 30, 45, 60, and 120 min immediately after TGFβ1 stimulation. (b) the effects of corresponding antagonists on Smad7 protein expressions. (c) western blot results of interference efficiency by siRNA. (d) phosphorylation of Smad2/3 after Smad7 knockdown. Data were expressed as mean ± S.E.M. of four independent experiments and the statistical graph was on the right side. $^{*}P < 0.05$, versus control; $^{**}P < 0.01$, versus control; $^{***}P < 0.001$, versus control; $^{\#}P < 0.05$, versus TGFβ1 treatment; $^{\$}P < 0.05$, versus TGFβ1 + Ucn treatment; $^{\$\$}P < 0.01$, versus TGFβ1 + Ucn treatment.

Breast cancer is the second leading cause of death due to cancer in women and the 5-year survival rate is only 27% once the primary tumor has metastasized [Jemal et al., 2008]. For this reason, it is vitally important to understand the processes of breast cancer invasion and metastasis. It has long been established that EMT is closely related to the occurrence of tumor metastasis as it makes tumor cells infiltrate the adjacent tissue, gain invasive properties, and ultimately form metastases at distal locations [Jakowlew, 2006; Lee et al., 2006]. TGFβ1-Smads signaling regulates EMT mainly through Smad2/3-dependent mechanism [Pardali and Moustakas, 2007; Ungefroren et al., 2011]. We previously found that CRH increased the expression of E-cadherin and decreased the expression of mesenchymal marker N-cadherin response to TGFβ1, suggesting CRH is involved in maintaining the epithelial status of cancer cells. The ability of CRH could result from inhibiting TGFβ1-Smad2/3 signaling pathway. Using Ucn, CRH family peptide, our recent research demonstrated that Ucn blocked normal TGFβ1-Smad2/3 signaling through Smad7 up-regulation, implying Ucn might inhibit Smad7 degradation to block Smad2/3 phosphorylation. It is reported that Smad7 in apoptosis-resistant MCF-7 cells markedly sensitized the cells to TRAIL-induced cell death [Hong et al., 2013]. In other in vitro models, Smad7 are demonstrated to potentiate cell apoptosis [Lallemand et al., 2001; Hong et al., 2007; Wang et al., 2013]. Our former research demonstrated that CRH and Ucn2 induced apoptosis of breast cancer cells via CRHR1 and R2 [Jin et al., 2012] and CRH promoted apoptosis of prostate cancer cells [Jin et al., 2011]. Hence, Ucn up-regulated Smad7 protein and thus be not only inhibitory for TGFβ1-induced EMT but also associated with apoptosis. Moreover, we found that EMT-related transcriptional promoters, Snail1 and Slug were differentially reduced in cells treated with TGFβ1 and Ucn, suggesting that Ucn impede EMT in response to TGFβ1 through Smad7-Smad2/3 activity and subsequent Snail1 and Slug increases.

TGFβ1 is a potent regulator of EMT, which has been shown to promote metastasis of breast cancer in various mouse models [Kang et al., 2003; Katsuno et al., 2008]. Intracellular signaling of TGFβ1 is transduced principally by Smad proteins [Imamura et al., 2012]. Increasing evidence showed that blocking endogenous Smad signaling abrogated EMT response to TGFβ1 [Pardali and Moustakas, 2007]. TGFβ1-specific Smad proteins transcriptionally repressing *Id* genes released the E12/E47 basic helix-loop-helix factors, which could bind to the E-cadherin promoter and repress this gene [Kondo et al., 2004]. Consistent with this notion, our previous study found that CRH promoted E-cadherin expression to attenuate TGFβ1-induced EMT [Jin et al., 2014] and the present study profoundly demonstrated that Ucn, one of the CRH family peptide, blocked TGFβ1-Smad2/3 signaling pathway through increasing the level of Smad7, an efficiently inhibitory Smad in TGFβ1 signaling [Hanyu et al., 2001]. In addition, transcriptional repressors of E-cadherin gene, Snail1 and Slug were reported to be involved in the EMT response to TGFβ1 [Peinado et al., 2004]. TGFβ1 could induce expression of Slug in normal heart valval development and Snail1 via Smad3 in renal epithelia cells during EMT [Pardali and Moustakas, 2007]. Our present data demonstrate that TGFβ1 augmented Snail1 and Slug expressions and Ucn attenuated this effect in breast cancer cells. Taken together, it appears that Ucn restrains EMT response to TGFβ1 through classic Smad signals that lead to the dissolution of epithelial cell adhesion.

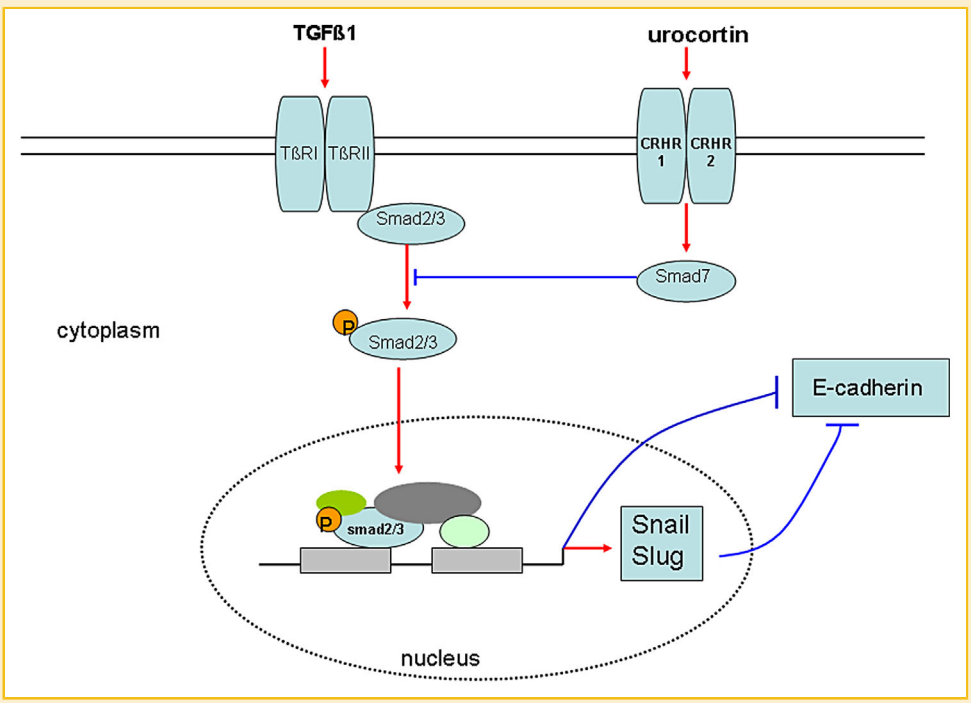

Fig. 5. Schematic diagram of signaling transduction pathway for this study.

CONCLUSION

In summary, our studies demonstrate that Ucn is a novel EMT inhibitor. On the one hand, Ucn signaled through CRHR1 and R2 to attenuate TGFβ1-induced Smad2/3 activation and subsequent nucleus translocation, in which the increased level of Smad7 was involved. On the other hand, activation of CRHR1 and R2 by Ucn obviously abolished the Snail1 and Slug up-regulations in response to TGFβ1. Under the condition of TGFβ1 stimulation, the transcriptional repressors of E-cadherin, Snail1 and Slug collaborated with TGFβ1-samds signaling pathway to induce EMT. Our current findings indicate that Ucn is a potential therapeutic agent for breast cancer and acts via increase of Smad7 level that inhibits the TGFβ1/Smad2/3/Snail1,Slug and EMT axes (Fig. 5).

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (nos. 81302866 and 91129711), also funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, Science and Technology Development Fund of Nanjing Medical University (2012NJMU014) and Priority Academic Program Development of Jiangsu Higher Education Institutions (JX10131801145).

REFERENCES

Arranz A, Venihaki M, Mol B, Androulidaki A, Dermitzaki E, Rassouli O, Ripoll J, Stathopoulos EN, Gomariz RP, Margioris AN, Tsatsanis C. 2010. The impact of stress on tumor growth: Peripheral CRF mediates tumor-promoting effects of stress. Mol Cancer 9:261.

Bierie B, Moses HL. 2006. Tumour microenvironment: TGFbeta: The molecular Jekyll and Hyde of cancer. Nat Rev Cancer 6:506–520.

Bonfiglio JJ, Inda C, Refojo D, Holsboer F, Arzt E, Silberstein S. 2011. The corticotropin-releasing hormone network and the hypothalamic-pituitary-adrenal axis: Molecular and cellular mechanisms involved. Neuroendocrinology 94:12–20.

Chatzaki E, Lambropoulou M, Constantinidis TC, Papadopoulos N, Tache Y, Minopoulos G, Grigoriadis DE. 2006. Corticotropin-releasing factor (CRF) receptor type 2 in the human stomach: protective biological role by inhibition of apoptosis. J Cell Physiol 209:905–911.

Ciocca DR, Puy LA, Fasoli LC, Tello O, Aznar JC, Gago FE, Papa SI, Sonego R. 1990. Corticotropin-releasing hormone, luteinizing hormone-releasing hormone, growth hormone-releasing hormone, and somatostatin-like immunoreactivities in biopsies from breast cancer patients. Breast Cancer Res Treat 15:175–184.

Derynck R, Zhang Y, Feng XH. 1998. Smads: Transcriptional activators of TGF-beta responses. Cell 95:737–740.

Dohadwala M, Yang SC, Luo J, Sharma S, Batra RK, Huang M, Lin Y, Goodglick L, Krysan K, Fishbein MC, Hong L, Lai C, Cameron RB, Gemmill RM, Drabkin HA, Dubinett SM. 2006. Cyclooxygenase-2-dependent regulation of E-cadherin: prostaglandin E(2) induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. Cancer Res 66:5338–5345.

Ghosh R, Lipson KL, Sargent KE, Mercurio AM, Hunt JS, Ron D, Urano F. 2010. Transcriptional regulation of VEGF-A by the unfolded protein response pathway. PLoS One 5:e 9575.

Graziani G, Tentori L, Muzi A, Vergati M, Tringali G, Pozzoli G, Navarra P. 2007. Evidence that corticotropin-releasing hormone inhibits cell growth of human breast cancer cells via the activation of CRH-R1 receptor subtype. Mol Cell Endocrinol 264:44–49.

Hanyu A, Ishidou Y, Ebisawa T, Shimanuki T, Imamura T, Miyazono K. 2001. The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. J Cell Biol 155:1017–1027.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, Falb D. 1997. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell 89:1165–1173.

Hong S, Kim HY, Kim J, Ha HT, Kim YM, Bae E, Kim TH, Lee KC, Kim SJ. 2013. Smad7 protein induces interferon regulatory factor 1-dependent transcriptional activation of caspase 8 to restore tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. J Biol Chem 288:3560–3570.

Hong S, Lee C, Kim SJ. 2007. Smad7 sensitizes tumor necrosis factor induced apoptosis through the inhibition of antiapoptotic gene expression by suppressing activation of the nuclear factor-kappaB pathway. Cancer Res 67:9577–9583.

Hotz B, Arndt M, Dullat S, Bhargava S, Buhr HJ, Hotz HG. 2007. Epithelial to mesenchymal transition: Expression of the regulators snail, slug, and twist in pancreatic cancer. Clin Cancer Res 13:4769–4776.

Hsu HY, Lin TY, Hwang PA, Tseng LM, Chen RH, Tsao SM, Hsu J. 2013. Fucoidan induces changes in the epithelial to mesenchymal transition and decreases metastasis by enhancing ubiquitin-dependent TGFbeta receptor degradation in breast cancer. Carcinogenesis 34:874–884.

Huber MA, Kraut N, Beug H. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 17:548–558.

Imamura T, Hikita A, Inoue Y. 2012. The roles of TGF-beta signaling in carcinogenesis and breast cancer metastasis. Breast Cancer 19:118–124.

Jakowlew SB. 2006. Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev 25:435–457.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. 2008. Cancer statistics, 2008. CA Cancer J Clin 58:71–96.

Jin L, Chen C, Guo R, Wan R, Li S. 2012. Role of corticotropin-releasing hormone family peptides in androgen receptor and vitamin D receptor expression and translocation in human breast cancer MCF-7 cells. Eur J Pharmacol 684:27–35.

Jin L, Chen J, Li L, Li C, Chen C, Li S. 2014. CRH suppressed TGFbeta1-induced Epithelial-Mesenchymal transition via induction of E-cadherin in breast cancer cells. Cell Signal 26:757–765.

Jin L, Zhang Q, Guo R, Wang L, Wang J, Wan R, Zhang R, Xu Y, Li S. 2011. Different effects of corticotropin-releasing factor and urocortin 2 on apoptosis of prostate cancer cells in vitro. J Mol Endocrinol 47:219–227.

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J. 2003. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549.

Katsuno Y, Hanyu A, Kanda H, Ishikawa Y, Akiyama F, Iwase T, Ogata E, Ehata S, Miyazono K, Imamura T. 2008. Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. Oncogene 27:6322–6333.

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. 2000. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell 6:1365–1375.

Kondo M, Cubillo E, Tobiume K, Shirakihara T, Fukuda N, Suzuki H, Shimizu K, Takehara K, Cano A, Saitoh M, Miyazono K. 2004. A role for Id in the regulation of TGF-beta-induced epithelial-mesenchymal transdifferentiation. Cell Death Differ 11:1092–1101.

Lallemand F, Mazars A, Prunier C, Bertrand F, Kornprost M, Gallea S, Roman-Roman S, Cherqui G, Atfi A. 2001. Smad7 inhibits the survival nuclear factor kappaB and potentiates apoptosis in epithelial cells. Oncogene 20:879–884.

Lee JM, Dedhar S, Kalluri R, Thompson EW. 2006. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 172:973–981.

Leivonen SK, Kahari VM. 2007. Transforming growth factor-beta signaling in cancer invasion and metastasis. Int J Cancer 121:2119–2124.

Padua D, Massague J. 2009. Roles of TGFbeta in metastasis. Cell Res 19:89–102.

Pardali K, Moustakas A. 2007. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta 1775:21–62.

Peinado H, Portillo F, Cano A. 2004. Transcriptional regulation of cadherins during development and carcinogenesis. Int J Dev Biol 48:365–375.

Perrin MH, Vale WW. 1999. Corticotropin releasing factor receptors and their ligand family. Ann N Y Acad Sci 885:312–328.

Roberts AB, Russo A, Felici A, Flanders KC. 2003. Smad3: A key player in pathogenetic mechanisms dependent on TGF-beta. Ann N Y Acad Sci 995: 1–10.

Tian F, DaCosta Byfield S, Parks WT, Yoo S, Felici A, Tang B, Piek E, Wakefield LM, Roberts AB. 2003. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res 63:8284–8292.

Tsuji T, Ibaragi S, Hu GF. 2009. Epithelial-mesenchymal transition and cell cooperativity in metastasis. Cancer Res 69:7135–7139.

Ungefroren H, Groth S, Sebens S, Lehnert H, Gieseler F, Fandrich F. 2011. Differential roles of Smad2 and Smad3 in the regulation of TGF-beta 1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: Control by Rac1. Mol Cancer 10:67.

Wakefield LM, Roberts AB. 2002. TGF-beta signaling: Positive and negative effects on tumorigenesis. Curr Opin Genet Dev 12:22–29.

Wang J, Zhao J, Chu ES, Mok MT, Go MY, Man K, Heuchel R, Lan HY, Chang Z, Sung JJ, Yu J. 2013. Inhibitory role of Smad7 in hepatocarcinogenesis in mice and in vitro. J Pathol 230:441–452.

Wendt MK, Schiemann WP. 2009. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. Breast Cancer Res 11:R68.

Yang Y, Park H, Kim TS, Bang SI, Cho D. 2007. Enhancement of cell migration by corticotropin-releasing hormone through ERK1/2 pathway in murine melanoma cell line, B16F10. Exp Dermatol 16:22–27.

Zavadil J, Bottinger EP. 2005. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene 24:5764–5774.

Zhang L, Zhou F, Garcia de Vinuesa A, de Kruijf EM, Mesker WE, Hui L, Drabsch Y, Li Y, Bauer A, Rousseau A, Sheppard KA, Mickanin C, Kuppen PJ, Lu CX, Ten Dijke P. 2013. TRAF4 promotes TGF-beta receptor signaling and drives breast cancer metastasis. Mol Cell 51: 559–572.

Zhang S, Fei T, Zhang L, Zhang R, Chen F, Ning Y, Han Y, Feng XH, Meng A, Chen YG. 2007. Smad7 antagonizes transforming growth factor beta signaling in the nucleus by interfering with functional Smad-DNA complex formation. Mol Cell Biol 27:4488–4499.