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Research Article

Anacardic acid inhibits gelatinases through the regulation of Spry2, MMP-14, EMMPRIN and RECK

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ABSTRACT

Earlier studies from our laboratory have identified Anacardic acid (AA) as a potent inhibitor of gelatinases (MMP-2 and 9), which are over-expressed in a wide variety of cancers (Omanakuttan et al., 2012). Disruption of the finely tuned matrix metalloproteinase (MMP) activator/inhibitor balance plays a decisive role in determining the fate of the cell. The present study demonstrates for the first time, that in addition to regulating the expression as well as activity of gelatinases, AA also inhibits the expression of its endogenous activators like MMP-14 and Extracellular Matrix MetalloProteinase Inducer (EMMPRIN) and induces the expression of its endogenous inhibitor, REversion-inducing Cysteine-rich protein with Kazal motifs (RECK). In addition to modulating gelatinases, AA also inhibits the expression of various components of the Epidermal Growth Factor (EGF) pathway like EGF, Protein Kinase B (Akt) and Mitogen-activated protein kinases (MAPK). Furthermore, AA also activates the expression of Sprouty 2 (Spry2), a negative regulator of EGF pathway, and silencing Spry2 results in up-regulation of expression of gelatinases as well as MMP-14. The present study thus elucidates a novel mechanism of action of AA and provides a strong basis for utilizing this molecule as a template for cancer therapeutics.

1. Introduction

Matrix metalloproteinases (MMPs) play a predominant role in degrading the extracellular matrix (ECM) during several normal and pathological processes. Along with the degradation of the ECM, MMPs also participate in the regulation of tumor growth by helping in the release of several factors essential for cell proliferation [1]. Among the different MMPs, gelatinases are considered as potential biomarkers for cancer therapy since they are over expressed in majority of the cancers and play a prominent role in promoting cancer progression. While MMP-9 levels were seen to be elevated in patients with breast [2], pancreatic [3] and lung cancer [4,5], elevated levels of MMP-2 found in incidences of pancreatic cancer [6,7]. Significantly high levels of MMP-2 and MMP-9 were found in patients with colorectal cancers and adenomatous polyps [8]. Additionally, along with MMP-2 and MMP-9, the expression of MMP-14 was also found to be elevated in patients with ovarian cancer [9–11] and aggressive brain tumors [12–15].

MMPs are regulated at the level of gene transcription, pro-enzyme activation and enzymatic activity. At the level of gene transcription, MMPs are regulated by signaling pathways like the Epidermal Growth Factor (EGF) pathway, which is implicated in several cancers [16–21]. The activation of the EGF pathway enhances the production of proteolytic enzymes like MMP-2, MMP-9 and MMP-14, which in turn cleave ligands that bind to the EGFR and activates the cascade, thereby resulting in an increase in the migratory potential of the cells [22–25]. Increased levels of EGFR have been observed in a variety of human cancers [26] and are usually associated with metastasis and a greater likelihood of tumor recurrence with poor patient survival [27]. Activated EGFR further stimulates a repertoire of effector pathways like the PI3K/AKT/mTOR and Raf/MEK/ERK, which play a significant role in cancer cell survival. The PI3K/AKT/mTOR pathway is up-regulated in human tumors mainly due to the activation of EGFR and/or Ras proteins, loss of or mutational activation of PI3K (Phosphoinositide 3 kinase) or AKT [28]. PTEN antagonizes AKT function and is found to be repressed in most of the tumors, thereby serving as a marker for advanced neoplastic disease [29]. Phosphorylation of EGFR further activates the ERK cascade, comprised of the Ras, Raf, MEK and ERK kinases, which also play a significant role in promoting tumor growth [30]. The primary function of ERK signaling is to promote cell cycle progression, cell proliferation and

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resistance to apoptosis. Suppression of EGF-induced Erk activity results in down regulation of the expression of several MMPs [17].

Several positive and negative feedback loops help in regulation of the EGF pathway. Among them, Spry, a family comprising of four structurally related proteins, plays a prominent role as negative-feedback regulators of the EGF signaling pathway [31]. Spry2 has been shown to bind to the phosphorylated EGFR and prevent the association of the adaptor proteins required for activation of the pathway, effectively blocking the downstream signaling [31]. Spry2 has also been shown to activate PTEN, inhibit AKT and thereby downregulate the EGF cascade [32]. Additionally, Spry2 is also known to interact with different components of the Ras/MAPK pathway to inhibit Ras and MAP kinase activation. Furthermore, over expression of Spry1, Spry2 and Spry4 was also shown to inhibit the migration and proliferation of various cancer cells [33–35].

MMPs, like many other enzymes, are secreted as pro-enzymes which require activation by cleaving the N-terminal pro-domain [36]. This process is facilitated by the interaction of pro-MMP-2 with the membrane-bound MT1-MMP also known as MMP-14 [37] and Tissue Inhibitor of Metalloproteinases (TIMPs) [38]. The formation of this tri-molecular complex plays a significant role in activation of pro-MMP-2. The N-terminal of TIMP-2 binds to MMP-14 and inhibits it, allowing the C-terminal of TIMP-2 to interact with the hemopexin domain of pro-MMP-2, juxtaposing it to MMP-14 that is anchored to the membrane. This pro-MMP-2 is then cleaved and activated by an adjacent MMP-14 molecule present on the membrane [38–40]. However, the activation of proMMP-2 also occurs independent of TIMP-2 [41,42].

Additionally, MMPs are also regulated at the level of their enzymatic activity by several endogenous activators and inhibitors like EMMPRIN and RECK. EMMPRIN is a cell surface glycoprotein that contains two extracellular domains (I and II, where domain-I has been reported to form homodimers, that stimulates the expression of MMPs [43]. Several MMPs like MMP-1, MMP-2 and MMP-14 are also known to cleave and activate EMMPRIN [44–46]. MMP-14 cleaves EMMPRIN and releases a small 22-kDa fragment from tumor cells, which is enhanced by treatment of the cells with phorbol 12-myristate 13-acetate (PMA), an activator of MMP-9, and inhibited in the presence of TIMP-2, an endogenous inhibitor of MMPs [47]. EMMPRIN is also known to stimulate the production and activation of MMPs independent of TIMPs [48,49]. EMMPRIN mediates tumor invasion, metastasis, and angiogenesis by stimulating extracellular matrix remodelling through the induction of these MMPs. Furthermore, RECK, a glycoprotein containing a serine protease inhibitor-like domain also plays an important role in regulation of MMP-2, MMP-9 and MMP-14, either by direct inhibition of protease activity or preventing their release from the cell [50]. In addition to direct inhibition of MMP-2 activity, RECK also plays a prominent role in inhibition of MMP-14 that is required to process and activate pro-MMP-2 [51]. Over-expression of RECK in fibroblasts is known to effectively block invasion and metastasis of malignant tumor cells [52] by functionally antagonizing MMP-9, MMP-2 and MMP-14 [51]. A positive correlation was observed between the expression of RECK in several tumor tissues and the survival of patients [53–58].

Although our earlier studies have suggested that , isolated from cashew nut shell liquid regulates the gelatinase activity, the mechanism by which AA inhibits MMP-2 and MMP-9 is not clearly understood. The present study demonstrates that AA inhibits both the expression as well as the activity of MMP-2 and MMP-9 from fibrosarcoma cells, HT1080. The study further elucidates the novel mechanism by means of which AA regulates MMP-2 and MMP-9 by inhibiting their endogenous activators like MMP-14 and EMMPRIN while activating RECK, the endogenous inhibitor. Additionally, the study also demonstrates for the first time, the unique role of Spry2, the negative modulator of the EGF pathway, in regulating gelatinases. AA up-regulates the expression of Spry2, and silencing Spry2 activates both the expression as well as the activity of MMP-2 and MMP-9. The present study thus illustrates a novel regulation of MMP-2 and MMP-9, enzymes that are prognostic biomarkers in a wide variety of cancers.

2. Materials and methods

2.1. Cell culture

The cell lines used for the study were HT1080 and MDA-MB-231, obtained from National Centre for Cell Science, Pune, Maharashtra, India. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (v/v), 1% penicillin, 1% streptomycin, 0.1% amphotericin B (SigmaAldrich, St. Louis, MO).

2.2. Cellular studies

HT1080 cells were seeded in a 24-well plate and on reaching confluency, treated with different concentrations of AA. After 24 h, the conditioned media was collected, centrifuged to avoid cellular debris, mixed with 4X sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, and 0.00625% (w/v) bromphenol blue) and loaded for electrophoresis on a 10% SDS-polyacrylamide gel containing gelatin for zymography studies. All the experiments were performed in triplicates.

2.3. MTT assay

MTT assay was performed according to the protocol described earlier [59]. The cells were plated at a density of 5,000/well in a 96-well plate and incubated overnight, after which they were treated with different concentrations of AA for 24 h. Cell death was assayed by addition of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). The absorbance was read at 590 nm and 620 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Results were calculated from three independent experiments.

2.4. Gelatin zymography

The zymography assay was performed according to the protocol described by Ratnikov et al. [60]. Gelatin substrate gels were prepared by incorporating gelatin (2 mg/ml) into 10% polyacrylamide gel containing 0.4% SDS. Electrophoresis was carried out under non-reducing conditions at 120 V for 150 min. The gels were washed in 2.5% Triton X-100 (v/v) for 30 min and then incubated overnight at 37 °C in developing buffer containing 50 mM Tris–HCl, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2% (v/v) Brij-35. Digestion bands were quantified using BIORAD image analyzer systems.

2.5. Gelatin degradation assay

The assay was carried out according to the protocol described previously [61]. MDA-MB-231cells, that are known to secrete significant amount of MMP-9, were treated with different concentrations of AA for 24 h at 37 °C. After treatments, the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in phosphate-buffered saline (PBS) for 20 min, mounted using ProLong® Gold Antifade reagent (Molecular probes, P-36931) and observed using IX71 Inverted microscope (Olympus).

2.6. cDNA synthesis and real-time PCR analysis

Total RNA was isolated from different treatments with Trizol. RNA was converted to cDNA using Super Script® III First-Strand Synthesis kit (Invitrogen). 1 μg cDNA was used for real-time PCR. Real-time PCR

analysis was performed using primers specific for MMP-2, MMP-9, MMP-14, TIMP-2, EGFR, PTEN and Spry2. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the internal control. SYBR Green (Biorad) was used as the fluorescent dye for real-time detection of PCR products.

2.7. Western blotting

Western blot analysis was performed according to the protocol described by Gierschik et al. [62]. Cell lysates were prepared in Laemmli sample buffer and the proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane overnight

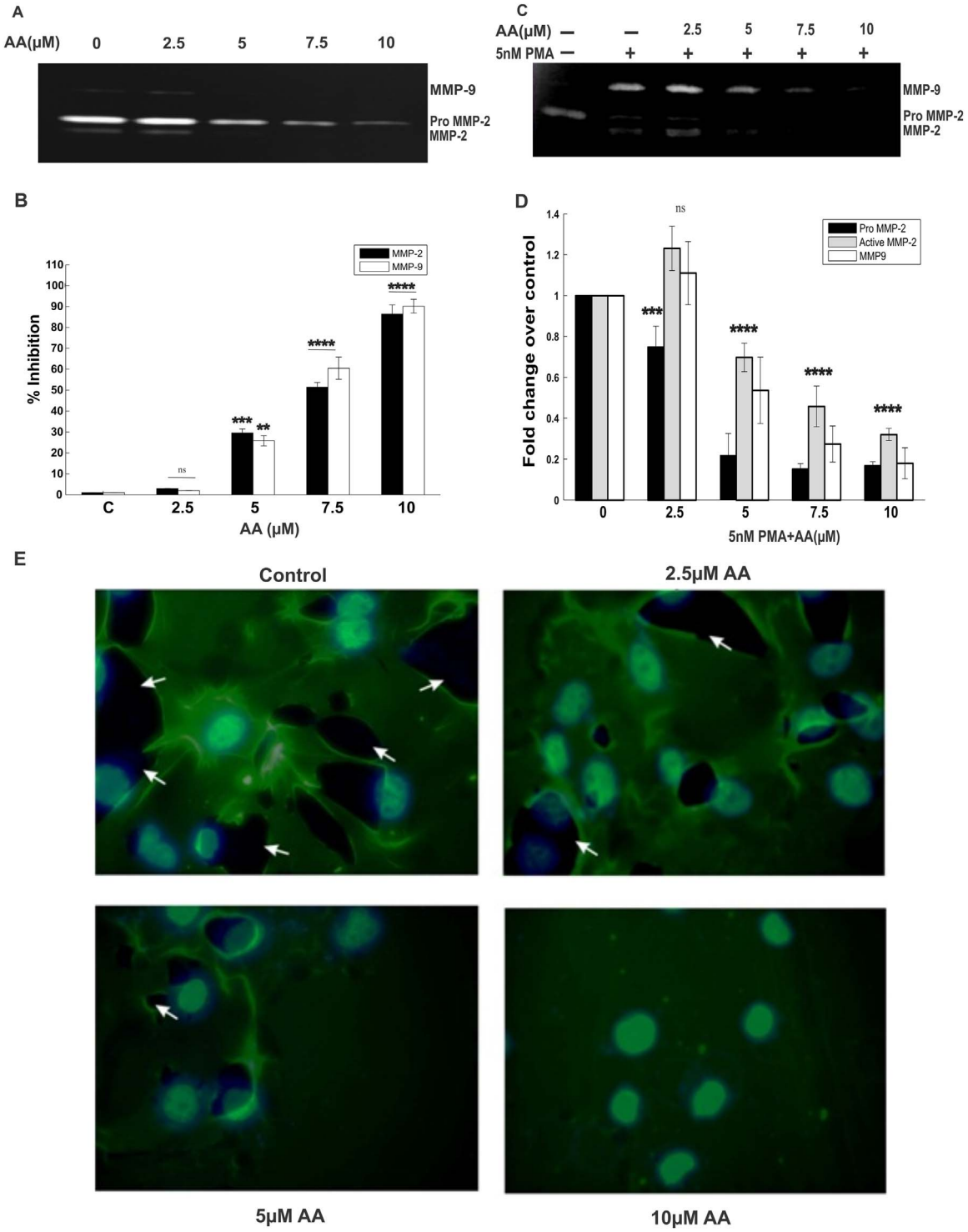

Fig. 1. Dose-dependent regulation of gelatinase activity by AA. (A) Zymogram showing gelatinase activity of conditioned media from HT1080 cells treated with 0.5% DMSO (lane 1), 2.5 μM, 5μM, 7.5 μM and 10 μM AA (lanes 2–5 respectively) for 24 h. (B) A representative plot of percentage inhibition observed in the zymogram. (C) Zymogram showing gelatinase activity of the conditioned media from HT1080 cells treated with 5 nM PMA (lane 2) in combination with 2.5μM, 5μM, 7.5μM, 10μM AA (lanes 3–6 respectively) for 24 h. (D) A representative plot of percentage inhibition observed in the zymogram. (E) Gelatin degradation assay demonstrating the reduction in degradation of the fluorescent gelatin with increasing concentrations of AA for 24 h. Arrows indicate areas containing degraded gelatin. Each bar represents the Mean ± S.E. of triplicate determinations from three independent experiments. ****, $P < 0.0001$; ***, $P < 0.001$; ** $P < 0.01$ (One-Way Analysis of Variance with Dunnett's Multiple-Comparison Post-Test).

at 20 V at 4 °C. Membranes were blocked in PBS containing 5% (w/v) non-fat dry milk with 0.1% Tween 20. The following primary antibodies were used: Akt, p44/42 MAPK, beta-actin, EGFR, MMP-14, MMP-2, MMP-9 (9272, 4060, 4370, 4695 and 4970 from cell signaling Technology respectively), EMMPRIN (Invitrogen, 345600) and RECK (sc-373929). The blot was developed using Super Signal West Dura Extended Duration Substrate (34076, Thermo Scientific).

2.8. Transfection

HT1080 cells were plated at 50–70% confluency in 6-well plates. Briefly, 5 µl of Polyethylenimine (PEI) was mixed thoroughly with 100 µl of serum-free media and incubated for 15 min at room temperature. hSPRY2 siRNA (25 nM) or mutant hSPRY2 siRNA (25 nM) was added to the above solution and incubated at room temperature for another 15 min. This was added to the cells after incubation for 30 min. After 36 h, the samples were checked for knockdown. hSPRY2-specific siRNA (5′-GAU CAG AUC AGA GCC AUC CGA AAC ACC-3′ (sense)/5′-GGU GUU UCG GAU GGC UCU GAU CUG AUC-3′ (antisense)), mutant hSPRY2 siRNA (5′-GGA GUU UCG CAU GGC UAU GAU CUG CUC-3′ (sense)/5′-GAG CAG AUC AUA GCC AUG CGA AAC UCC-3′ (anti-sense)).

2.9. Transwell cell migration assay

Cell migration assays were performed using Transwell chamber units (Corning, 6.5 mm; 8 µm pore size). HT1080 cells ($8*10^4$ cells) were treated with different concentrations of AA in 300 µl serum-free media and plated in the upper chamber. DMEM with 10% FBS was added to the lower chamber as a chemo-attractant. Cells were incubated at 37 °C and allowed to migrate through the chamber for 24 h. The non-migratory cells on the upper membrane surface of the insert were completely removed with a cotton tip, and the migratory cells attached to the membrane surface were fixed with 4% paraformaldehyde and stained with crystal violet solution (0.1%). After 24 h of incubation, non-migratory cells in the upper chamber were carefully removed with a cotton swab. The number of migrated cells was counted using IX71 Inverted microscope (Olympus) in five different fields. The experiments were performed in triplicates.

2.10. Transwell matrigel invasion assay

Cell invasion assay were performed using a 24-well Transwell chamber (Corning, 6.5 mm; 8 µm pore size). For invasion assay, the filter of the Transwell chamber was coated with Matrigel (20 µg/ml, Becton Dickinson Labware, USA) overnight at 4 °C. The invasion assay

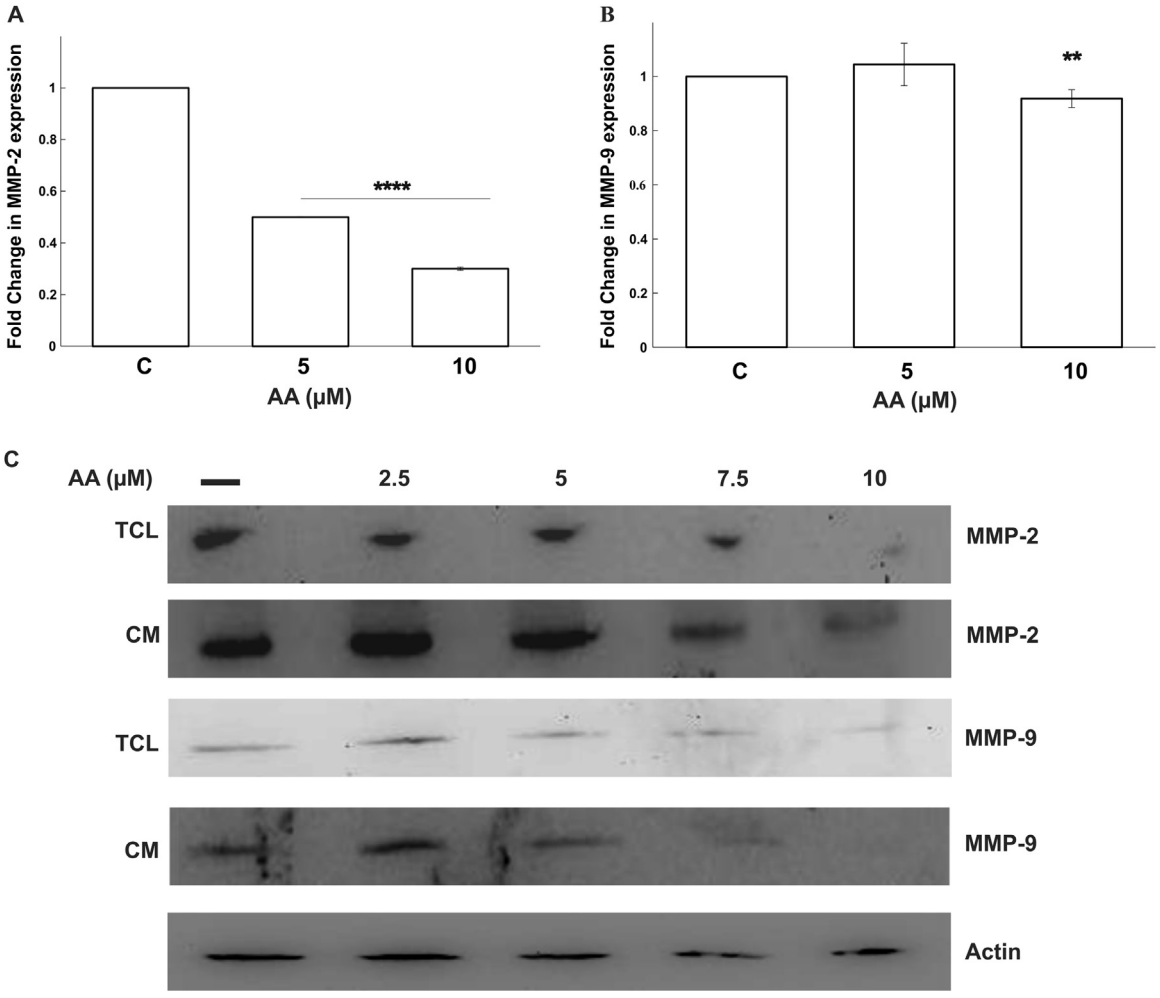

Fig. 2. Regulation of MMP-2 and MMP-9 expression by AA. (A) Real-time PCR analysis showing the effect of AA on MMP-2 expression in HT1080 cells treated with AA (5 µM and 10 µM) for 24 h. (B) Real-time PCR analysis showing the effect of AA on MMP-9 expression in HT1080 cells treated with AA (5 µM and 10µM) for 24 h. (C) Western blot analysis showing the effect of AA on MMP-2 and MMP-9 expression in HT1080 cells treated with 2.5 µM, 5 µM, 7.5 µM and 10 µM AA. TCL refers to the total cell lysate and CM refers to the conditioned media. TCL and CM were collected after 12 and 24 h of treatment respectively. The CM was concentrated using Trichloroacetic Acid (TCA) at a final concentration of 10%. Each bar represents the Mean ± S.E. of triplicate determinations from three independent experiments. ****, $P < 0.0001$; ** $P < 0.01$ (One-Way Analysis of Variance with Dunnett's Multiple-Comparison Post-Test).

was then performed using the protocol described for the migration assay.

2.11. Far-western blot

Far-Western blot is based on the technique of Western blot, and is employed to detect in vitro protein-protein interactions. Prey proteins are spotted on the membrane at a concentration of 1 μg/ml. The blot is overlaid with purified bait proteins (3 μg/ml) overnight after the blocking and washing steps. The blot is incubated with 1° antibody against the bait protein. The bait proteins are detected only if they form a complex with the prey protein. The blot was developed using Super Signal West Dura Extended Duration Substrate (34076, Thermo Scientific).

2.12. Adhesion assay

Cells (8×10^5 cells/well) were seeded in a 96-well plate coated with matrigel overnight at 4 °C in the presence of different concentrations of AA for 90 min at 37 °C. The cells that were not attached were removed

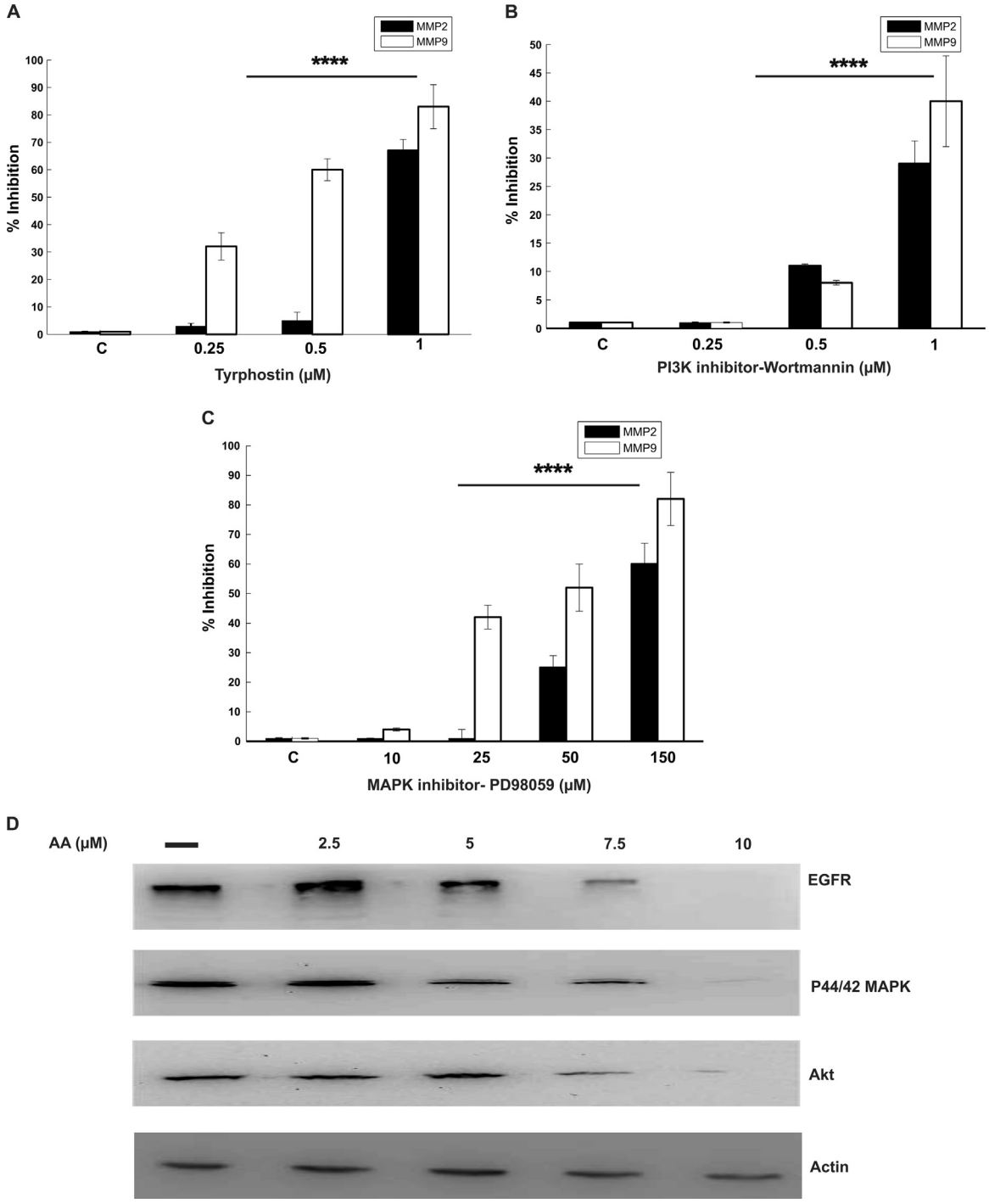

Fig. 3. Regulation of gelatinase activity by EGF pathway. A representative plot of percentage inhibition observed in the zymogram on treatment for 24 h with increasing concentrations of (A) Tyrphostin (0.25–1 μM), (B) Wortmannin (0.25–1 μM), (C) PD98059 (10 μM to150 μM), (D) Western blot analysis showing the effect of AA on different components of the EGF pathway in HT1080 cells treated with 2.5 μM, 5 μM, 7.5 μM, 10μM AA for 24 h. Each bar represents the Mean ± S.E. of triplicate determinations from three independent experiments. ****, $P < 0.0001$ (One-Way Analysis of Variance with Dunnett's Multiple-Comparison Post-Test).

by washing with PBS. Attached cells were incubated in a media containing 1 mg/ml MTT for 3 h and the O.D. was measured using a microplate reader at 490 nm (BioTek Instruments, Winooski, VT).

2.13. Statistical analysis

Statistical analysis was conducted using Prism (GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed using either Student's *t*-test or one-way analysis of variance. All values are expressed as the mean ± S.E.M. from three independent experiments.

3. Results

3.1. Regulation of MMP-2 and MMP-9 activity by AA

The fibrosarcoma cell line, HT1080, was used to study the regulation of MMP-2 and MMP-9 by AA. activity of both MMP-2 and MMP-9 in a dose-dependent manner (Fig. 1A, B). Since HT1080 cells show reduced expression of MMP-9, PMA was used to induce the basal activity of MMP-9. Cells were treated with the indicated concentrations of AA, in combination with 5 nM PMA for 24 h. As shown in Fig. 1C, treatment with PMA for 24 h significantly induced MMP-9 secretion in HT1080 cells by several foldand partially converted the MMP-2 pro-enzyme to its active form. A 3.5 fold increase in MMP-2 activity and 23

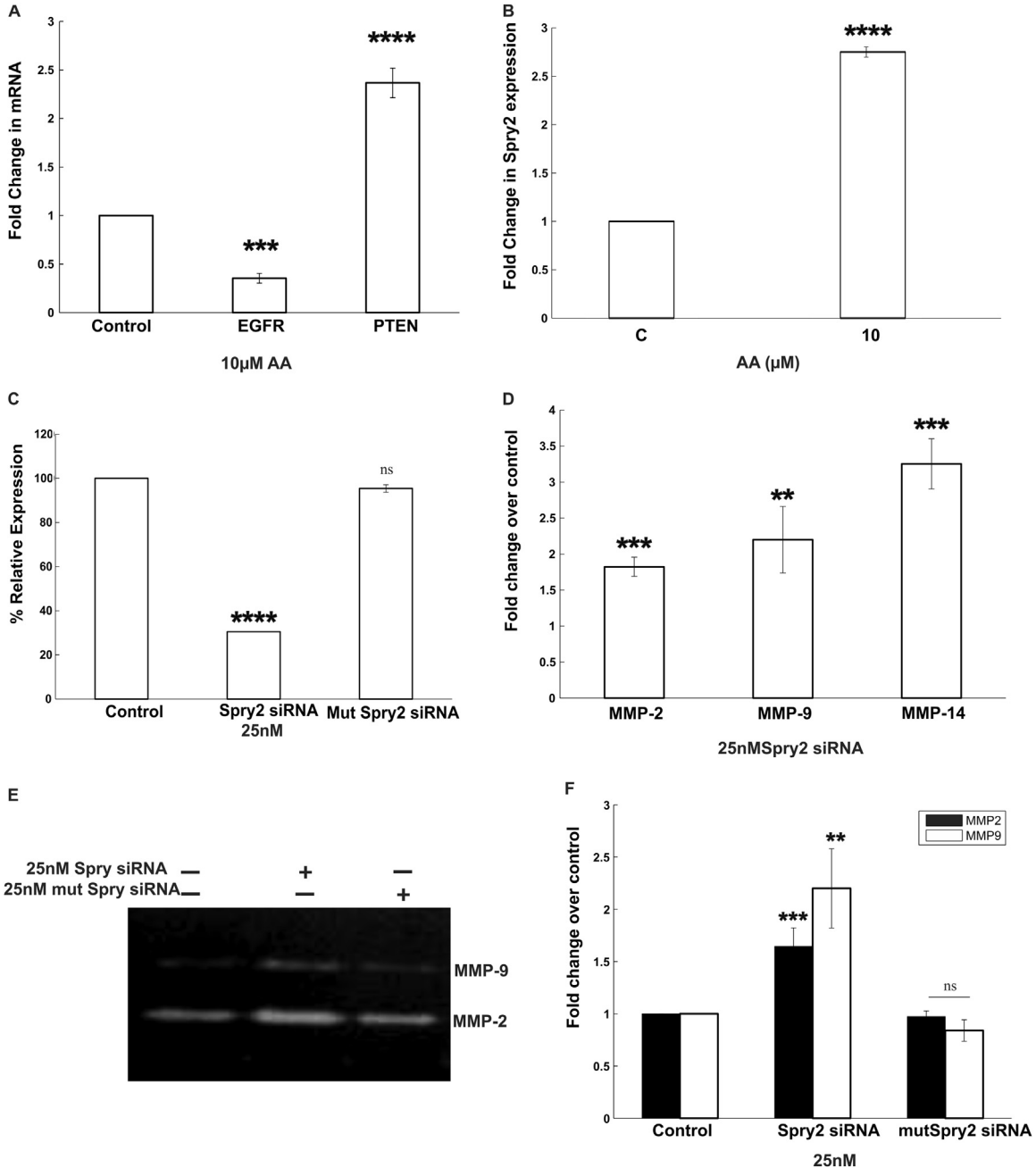

Fig. 4. Regulation of Spry2 expression by AA. (A) Real-time PCR analysis showing the effect of 10 μM AA on the expression of EGFR, PTEN and (B) Spry2. (C) Effect on Spry2 mRNA expression after transfection with 25 nM Spry2 siRNA and 25 nM mut Spry2 siRNA for 36 h. (D) Effect on MMP-2, MMP-9 and MMP-14 mRNA expression after transfection with 25 nM for 36 h. (E) Zymogram showing gelatinase activity of conditioned media from HT1080 cells transfected with 25 nM Spry2 siRNA (lane 2), 25 nM mut Spry2 siRNA (lane 3) for 36 h. (F) Representative plot of percentage inhibition observed in the zymogram. Each bar represents the Mean ± S.E. of triplicate determinations from three independent experiments. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$ (One-Way Analysis of Variance with Dunnett's Multiple-Comparison Post-Test and *t*-test).

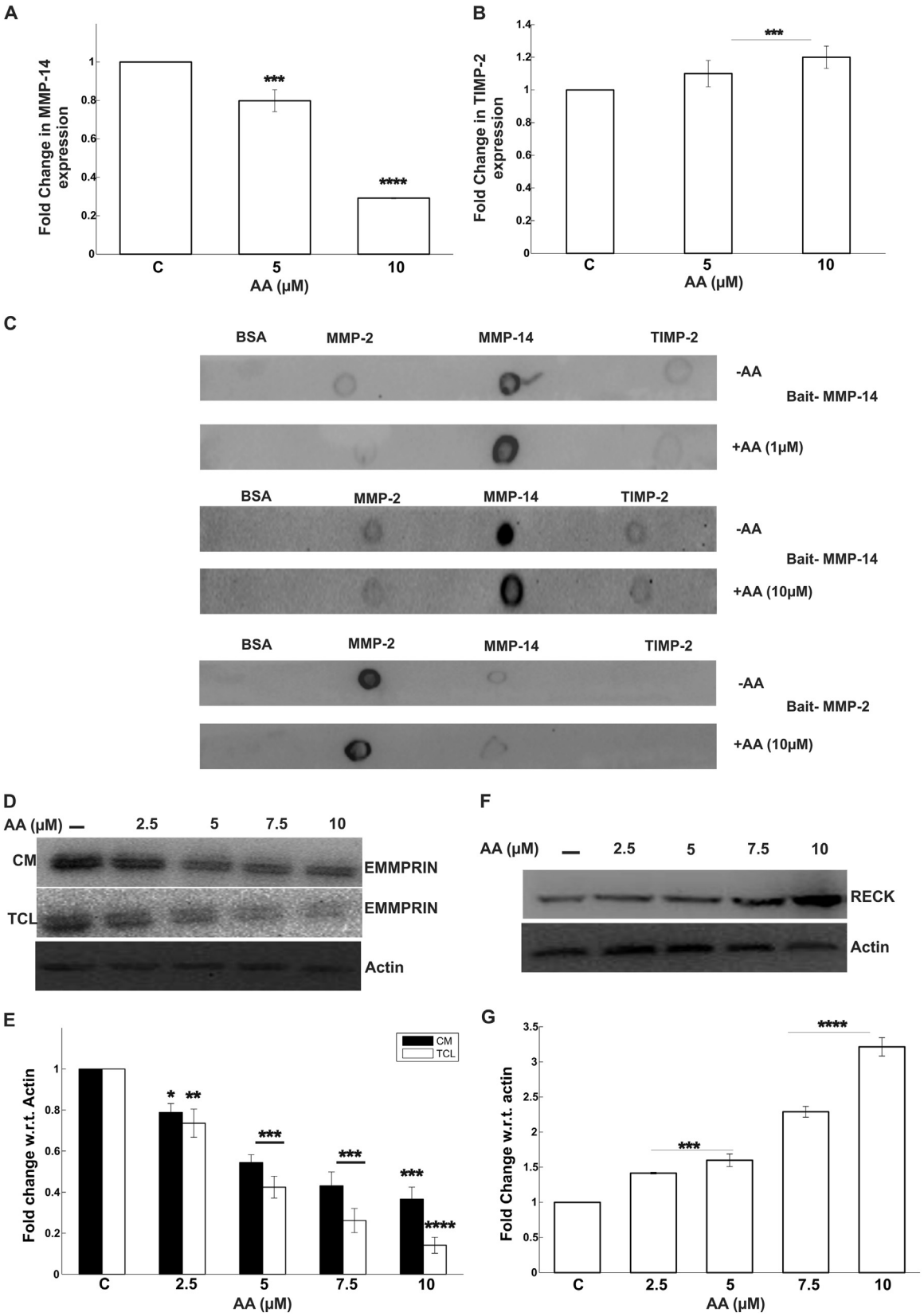

Fig. 5. Regulation of MMP-14, TIMP-2, EMMPRIN and RECK expression by AA. (A) Real-time PCR analysis showing the effect of AA (5μM and 10 μM) on MMP-14 and (B) TIMP-2 expression. (C) Far-Western blot analysis showing the interaction between MMP-14 and MMP-2/TIMP-2 with MMP-14/MMP-2 as bait in the presence and absence of AA. (D) Western blot analysis showing the effect of AA on EMMPRIN expression in TCL and CM of cells treated with AA (2.5μM, 5μM, 7.5μM, 10 μM) for 24 h. TCL refers to the total cell lysate and CM refers to the conditioned media. (E) Quantitative analysis of the blot. (F) Western blot analysis showing the effect of AA on RECK expression in TCL of cells treated with AA (2.5 μM, 5 μM, 7.5μM, 10 μM) for 24 h. (G) Quantitative analysis of the blot. TCL and CM were collected after 24 h of treatment with AA. The CM was concentrated using Trichloroacetic Acid (TCA) at a final concentration of 10%. Each bar represents the Mean ± S.E. of triplicate determinations from three independent experiments. ****, $P < 0.0001$; ***, $P < 0.001$; ** $P < 0.01$; *, $P < 0.01$(One-Way Analysis of Variance with Dunnett's Multiple-Comparison Post-Test).

fold increase in MMP-9 activity was seen in the presence of PMA (Fig. 1C). Treatment of HT1080 cells with AA suppressed PMA-induced MMP-2 and MMP-9 activity in a dose-dependent manner beginning from a concentration of 5 μM (Fig. 1D). To further confirm the results obtained from zymography, a gelatin matrix degradation assay was performed. Degradation of the fluorescent gelatin was studied at different concentrations of AA for 24 h. As seen in Fig. 1E, there is a inhibition in the degradation of the fluorescent gelatin on treatment with AA (5 and 10 μM), as compared to the untreated control (dark areas represent the degraded gelatin over the green background) (Fig. 1E).

In order to confirm that the reduced gelatinase activity was not due to cytotoxicity, MTT assay was performed. The cells were treated with different concentrations of AA for 24 h and the cell viability was measured. AA at concentrations lower than 10 μM did not have any cytotoxic effect on the cells, with 10–15% cytotoxicity being observed at 10 μM. In addition to cytotoxicity analysis, studies were also carried out to morphological changes in the cells upon treatment with AA over a range of concentrations from 2.5 to 10 μM (data not shown).

3.2. Regulation of MMP-2 and MMP-9 expression by AA

MMPs are highly regulated at the level of gene expression and synthesis, in addition to protein activation. Real-time PCR analysis was performed in order to determine whether the inhibition of MMP-2 and MMP-9 activity by AA resulted from the decreased expression at the mRNA level. Primers specific to MMP-2 and MMP-9 were used with GAPDH as the internal control. As indicated in Fig. 2A, treatment with AA decreased the expression of MMP-2 mRNA and MMP-9 mRNA in a dose-dependent manner (Fig. 2A and B).

Western blotting analysis was performed using total cell lysates (TCL) and conditioned media (CM) in order to determine the effect of AA at the level of. The cells were treated with different concentrations of AA for various time and both TCL and CM were subjected to Western blotting analysis. In addition to the decrease in the levels of mRNA observed in the presence of AA, there was a corresponding dose dependent inhibition of MMP-2 protein, in both the TCL and CM (Fig. 2C). However, in case of MMP 9, even though AA did not significantly affect the mRNA levels at the concentrations tested, there was a significant inhibition of MMP-9 protein expression present in TCL (12hrs) as well as CM (24hrs) (Fig. 2C).

3.3. Regulation of EGF pathway by AA

EGFR ligands like epidermal growth factor, amphiregulin, and transforming growth factor-α induces the expression of MMPs, thereby suggesting the role of EGF signaling pathway in contributing to the metastatic process by up-regulating gelatinases [63–66]. To understand the regulation of MMP-2 and MMP-9 by the EGF pathway, the cells were treated with different inhibitors of the pathway: (i) Tyrphostin AG 1478 a tyrosine kinase inhibitor specifically selective to EGFR, (ii) PD98059 mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) inhibitor and (iii) Wortmannin phosphatidylinositol-3 kinase (PI3-K) inhibitor, and their effect on MMP-2 and MMP-9 activity was studied using gelatin zymography. Interestingly, in the presence of these inhibitors, the activity of both MMP-2 and MMP-9 was inhibited, indicating that the EGF pathway plays a significant role in activation of these MMPs (Fig. 3A, B and C). Additionally, the effect of AA on different components of the EGF pathway, namely EGFR, PI3K and MAPK were also studied. Cells were treated with different concentrations of AA for 24 h followed by Western blot analyses which demonstrated that AA showed a significant inhibition of EGFR, in addition to its downstream components (Fig. 3D).

3.4. Role of Spry2 in regulation of gelatinases

The activity of Receptor Tyrosine Kinases (RTKs) like EGFR, is tightly regulated through the co-ordinated action of their regulators [67]. The Sprouty (Spry) proteins represent a major class of inhibitors of RTK-dependent signaling pathways [31]. They either interfere with the binding of the effector molecules to the activated receptor and block the EGF cascade [31] or can act downstream at the level of PI3K/Akt by stabilising PTEN [32] or Ras–ERK/MAPK signaling [68,69]. A decrease in Spry proteins, especially Spry2, has been implicated in a number of cancers, because of their role as inhibitors of RTK-mediated cell proliferation and migration. Treatment with AA decreased the expression of EGFR with a significant increase in the expression of PTEN mRNA (Fig. 4A). Since AA showed inhibition of MAPK and Akt with a significant induction of PTEN, its effect on Spry2 was studied. The cells were treated with AA at a concentration of 10 μM for 24 h, and real-time PCR analysis was carried out to study the effect of AA on Spry2. The results obtained clearly demonstrated that AA showed a significant up-regulation of Spry2 expression (Fig. 4B). To investigate the role of endogenous Spry2 in the regulation of MMP-2 and MMP-9 activity, a specific, small inhibitory RNA against hSpry2 (hSpry2 siRNA), was used to decrease endogenous expression of hSpry2. The control cells were either treated with PEI or transfected with equimolar amounts of mutant siRNA. When compared with the control (PEI or mutant siRNAs), transfection with hSpry2 siRNA markedly decreased (> 70%) the endogenous levels of hSpry2 (Fig. 4C). The effect of Spry2 on MMP-2, MMP-9 and MMP-14 expression was studied using RT-PCR analysis which showed a 1.82 fold, 3.25 fold and 2.2 fold increase in expression levels of MMP-2, MMP-14 and MMP-9 respectively, when Spry2 was silenced (Fig. 4D). Additionally, the effect of Spry2 on gelatinase activity was studied using zymography. In the absence of Spry2, a significant up-regulation of MMP-2 and MMP-9 activity was seen, which clearly confirms the role of Spry2 in regulation of both expression as well as the activity of MMPs (Fig. 4E and F).

3.5. AA inhibits MMP-2/9 by regulating the expression of its endogenous activators and inhibitors

In addition to the EGF pathway, the endogenous activators and inhibitors of gelatinases also provide a most effective means of regulation of MMP-2/9. Pro-MMP-2 binds to MMP-14 on the cell surface, using TIMP-2 as an adaptor and forming a tri-molecular complex, which plays a significant role in activation of pro-MMP-2. activation is usually mediated under conditions where MMP-14 levels are higher as compared to TIMP-2. Since the endogenous activator MMP-14, plays a prominent role in the conversion of pro-MMP-2 to its active form, studies were carried out to determine whether AA suppresses the expression of MMP-14 in HT1080 cells. In the present study, quantitativeRT-PCR demonstrated that the expression of mRNA coding for MMP-14 was reduced by ~80% in cells treated with10μM AA (Fig. 5A). However, AA did not have any effect on the expression of TIMP-2 (Fig. 5B). These results were further supported by Far-Western blot analysis which also demonstrated that AA did not affect the interaction of TIMP-2 with MMP-2 and MMP-14. As observed in Fig. 5C, at the concentrations tested, AA did not affect the interaction between MMP-14, MMP-2 and TIMP-2, when MMP-14 was used as bait. Similar results were also obtained when MMP-2 was used as bait, further supporting the fact that AA did not alter the interactions between MMP-2, MMP-14 as well as TIMP-2.

In addition to MMP-14 and TIMP-2, EMMPRIN and RECK play a prominent role as key endogenous regulators of gelatinases. EMMPRIN is considered one the most well characterized cell surface regulators of MMPs. EMMPRIN is known to induce MMP-2, MMP-3, MMP-9, MMP-14 and MT2-MMP [70]. Since AA down-regulates MMP-2, MMP-9 and MMP-14, we were interested in determining the effect of AA on EMMPRIN. The results obtained clearly demonstrated

that AA down-regulated the expression of EMMPRIN in a dose-dependent manner, in both TCL and CM (Fig. 5D and E), suggesting that the inhibition of EMMPRIN by AA is, at least in part, responsible for the reduction of both expression and activity of MMP-2 and MMP-9.

RECK serves as an endogenous inhibitor of MMPs. It regulates the activity of MMP-2 by inhibiting both the catalytic and processing steps of proMMP-2 [71]. RECK is also associated with inhibition of MMP-2, MMP-9 and MMP-14 secretion [55]. Having studied the inhibition of MMP-2, MMP-9 and MMP-14 by AA, its effects on RECK was also

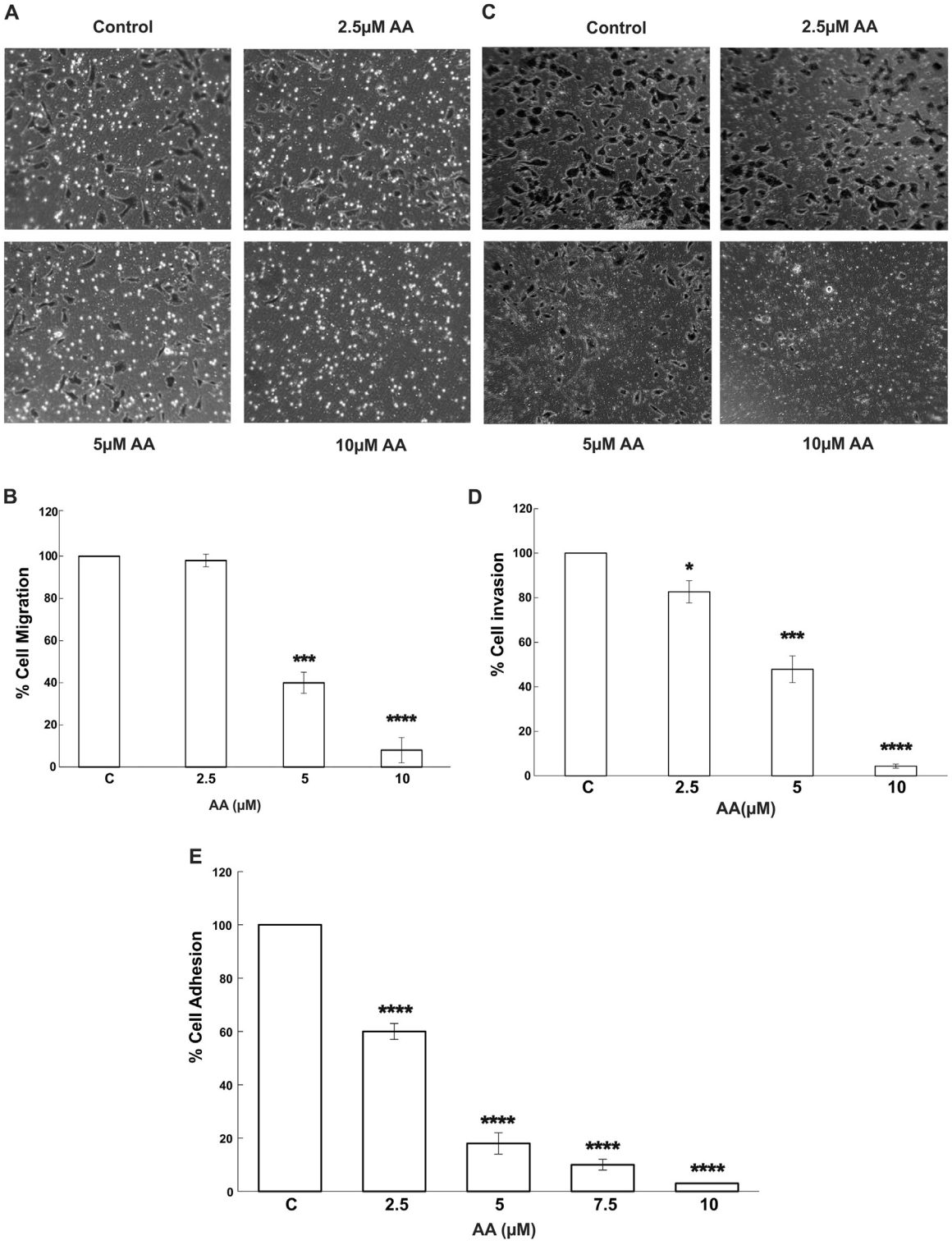

Fig. 6. Effect of AA on migration, invasion and adhesion of HT1080 cells. Transwell migration and invasion assay was carried out in the presence of AA. After 24 h of incubation with AA, the cells on the bottom side of the filter were fixed, stained with crystal violet and counted. (A) Cell migration in the presence of different concentrations of AA (B) Representative plot of the number of cells that migrated through the membrane. (C) Cell invasion in the presence of different concentrations of AA. (D) Representative plot of the number of cells that invaded through the membrane. (E) The attached cells after treatment for 90 min were stained using MTT and the number of attached cells was quantified by measuring the O.D. at 490 nm. Each bar represents the Mean ± S.E. of triplicate determinations from three independent experiments. ****, $P < 0.0001$; ***, $P < 0.001$; *, $P < 0.01$ (One-Way Analysis of Variance with Dunnett's Multiple-Comparison Post-Test).

analysed using Western blotting. The data obtained clearly demonstrated that RECK expression was significantly induced by AA in a dose-dependent manner (Fig. 5F and G), indicating that the up-regulation of RECK might also play a role in the inhibition of gelatinases by AA.

3.6. Effect of AA on cell migration, invasion and attachment

Metalloproteinase activity is strongly implicated in cell migration and control of ECM degradation. To investigate the role of AA in the migration of HT1080 cells, a transwell migration assay was performed. Incubation of HT1080 cells with AA reduced their migratory capacity in a dose dependent manner (Fig. 6A and B). AA at a concentration of 5 μM reduced cell migration by greater than 50% and up to 90% at 10 μM. MMP-9 has been known to promote migration and invasion in cancer cells [72,73]. To understand the role of AA in the invasion of HT1080 cells, the matrigel invasion assay was performed. Control cells were found to successfully penetrate the basement membrane-coated chambers. However, the number of cells that penetrated the membrane was reduced significantly in AA treated cells (Fig. 6C and D). Cellular adhesion is often dysregulated in cancers due to the metastatic capabilities possessed by tumor cells. AA inhibited the adhesion of HT1080 cells onto the Matrigel in a concentration-dependent manner compared with the untreated control (Fig. 6E).

4. Discussion

Aberrant expression of several MMPs is linked with various stages of cancer progression, which paved the path for the detailed study of their role in cancer and led to the development of Matrix Metalloproteinase Inhibitors (MMPIs). A co-ordinated secretion of MMPs, that is counter-balanced by their endogenous inhibitors, helps in maintaining tissue homeostasis in normal cells [74,75]. In cancer cells, this balance between MMPs and their regulators is lost, resulting in excessive degradation of ECM, thereby promoting the migration of these cancer cells to different sites. Among the different MMPs, gelatinases, which are upregulated in a wide variety of cancers, are considered to be promising targets for cancer therapy due to their ability to degrade majority of components of the ECM and promote metastasis.

Earlier studies in the laboratory [76] have shown the effect of AA on MMP-2 and MMP-9, which are known to play key roles in several pathological conditions. These studies demonstrated that AA directly inhibits the catalytic activity of MMP-2 with the carboxylate moiety functioning as a zinc-binding group, hydroxyl group binding to the backbone oxygen of Ala 192 and the lipophilic C15 side chain binding in the aliphatic S1′ tunnel [76]. However, the molecular mechanism by which AA inhibits these MMPs is not well understood. Therefore, the present study was designed to provide a better understanding of the complex mechanisms underlying the regulation of gelatinases by AA.

Studies using the highly metastatic cell line, HT1080, indicated a, dose-dependent inhibition of the transcript as well as the protein, of both MMP-2 and MMP-9 (Fig. 2A, B and C). Additionally, the gelatinase activity was also inhibited upon treatment with different concentrations of AA (Fig. 1A, B, C and D), which was further confirmed using gelatin degradation studies (Fig. 1E).

The EGF pathway is known to play a significant role in the regulation of MMPs and the expression of several MMPs including MMP-2 and MMP-14 was found to be impaired in EGFR (-/-) mouse fibroblasts when compared to their wild-type counterparts [17,20]. The significance of the EGF pathway in regulating gelatinase activity was further confirmed in the present study by using known inhibitors of the pathway and demonstrating that the activity of gelatinases was down-regulated in the presence of these inhibitors (Fig. 3A, B and C). Several positive and negative feedback loops help in the regulation of the EGF signaling pathway. Among the different regulators of this pathway, Spry2 plays a prominent role as a negative regulator [31,67]. The down-regulation of Spry2 in cancer confers a proliferative advantage for tumor cells by activation of the EGF pathway. Additionally, loss of Spry2 was also reported to promote carcinogenesis by enhancing ErbB trafficking and PI3K/AKT signaling [77]. Spry2 was known to exert its inhibitory role in the PI3K/AKT signaling pathway by enhancing the activation and stability of PTEN [32]. These observations were corroborated by our studies which demonstrated that treatment with AA resulted in a significant up-regulation of Spry2 (Fig. 4B), along with an increase in the expression of PTEN (Fig. 4A), which is known to play a prominent role in inhibiting Akt by dephosphorylating PIP3 to PIP2. In addition to Akt, Spry2 also inhibits the EGF pathway by binding to the phosphorylated EGFR and preventing the interaction of adaptor proteins with the receptor, thereby impeding the signaling cascade [31]. Furthermore, Spry2 is also known to prevent the phosphorylation and activation of MAPK by inhibiting the kinase activity of Raf [69,78,79]. The results from the present study clearly demonstrate the inhibition of Akt, MAPK and EGFR in a dose-dependent manner in the presence of AA (Fig. 3D). Since Spry2 modulates EGFR, Akt and MAPK, and AA enhances the expression of this upstream regulator (Fig. 4B), the inhibition of different components of the EGF pathway could be attributed to the up-regulation of Spry2 in the presence of AA. Furthermore, upon silencing Spry2 (Fig. 4C), a significant induction of expression of MMP-2, MMP-9 and MMP-14 was observed (Fig. 4D), along with increased activity of MMP-2 and MMP-9 (Fig. 4E and F), thereby suggesting a critical role for Spry2 in mediating the regulation of gelatinases by AA.

In addition to the transcriptional regulation of MMPs by the EGF pathway, MMPs are also controlled at the level of pro-enzyme activation and enzymatic activity. The process of pro-enzyme activation involves the post-translational regulation of pro-MMP-2 through its interaction with MMP-14 and TIMP-2 [38,40]. Studies performed to demonstrate the effect of AA on MMP-14 and TIMP-2 clearly shows that AA inhibited the expression of MMP-14 (Fig. 5A) without significantly affecting TIMP-2 (Fig. 5B). Furthermore, AA did not seem to effect the interaction between pro-MMP-2, MMP-14 and TIMP-2 (Fig. 5C) that form the tri-molecular complex required for the activation of pro-MMP-2 at the cell membrane.

In addition to the pro-enzyme activation, MMPs are also modulated at the level of activity, by endogenous regulators like EMMPRIN and RECK. Dysregulation of both EMMPRIN and RECK are considered to be prognostic biomarkers in several cancer [80–83]. In concurrence with these observations, a dose dependent inhibition of EMMPRIN

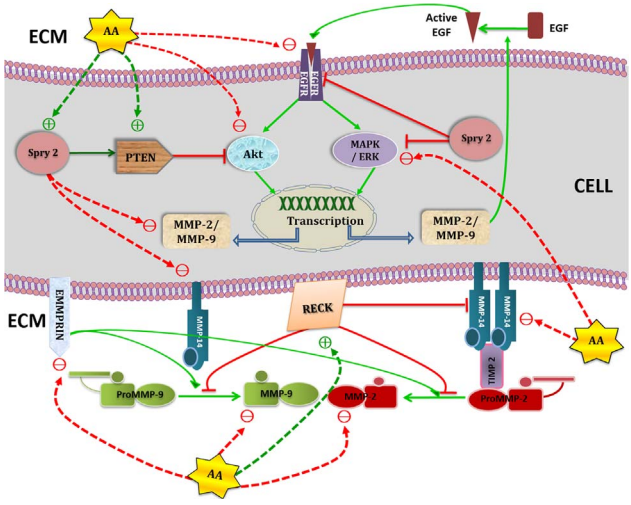

Fig. 7. Schematic representation of MMP regulation by AA. Several proteins involved in the inhibition of gelatinases are up regulated or down regulated by AA. Up regulated (→ +) - Spry2, RECK, PTEN, Down regulated (→−) - MMP-14, EMMPRIN, EGFR, Akt, MAPK (Green-Activation; Red-Inhibition).

(Fig. 5D, E) with a concomitant up-regulation of RECK (Fig. 5F, G) was seen in the presence of AA, suggesting that the modulation of these two regulators by AA responsible, at least in part, for the inhibition of MMP-2 and MMP-9. The knockdown experiments with Spry clearly indicated that there is an increase in the levels of MMP-14, along with the up-regulation of both activity and expression of MMP-2 and MMP-9 (Fig. 4D). Since MMP-14 is also known to be regulated by EMMPRIN and RECK, the endogenous regulators of gelatinases [84,85], the inhibition of EMMPRIN and up-regulation of RECK by AA could also be attributed to the increase in Spry2 seen in the presence of AA.

Additionally, decreased levels of the tumor suppressor Spry2, and the over expression of MMPs also resulted in increased migration and proliferation of various cancer cells [33–35] play a prominent role in promoting invasion and metastasis, which is the principal cause of death in cancer patients [14,86]. In accordance with these observations, we have clearly demonstrated that treatment of HT1080 cells with AA suppresses both migration and invasion, in a dose dependent manner (Fig. 6A, B, C, D).

In conclusion, the present study clearly elucidates a novel regulation of both the expression and activity of MMP-2 and −9, as well as their endogenous activators and inhibitors, mediated by anacardic acid. The study also suggests a pivotal role for Spry, the upstream regulator of the EGF cascade, in AA-mediated modulation of MMP-2 and −9, enzymes which are prognostic biomarkers in a wide variety of cancers, and demonstrates the complex interplay between the various players involved in this process (Fig. 7). Future studies can be focused on using anacardic acid as a template to design novel inhibitors for the regulation of gelatinases, providing an effective strategy for therapeutic modulation of a large number of disease states like cancer, where gelatinases and their effects have been implicated.

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