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ORIGINAL CONTRIBUTION

Epigallocatechin-3-gallate (EGCG) downregulates EGF-induced MMP-9 in breast cancer cells: involvement of integrin receptor $\alpha5\beta1$ in the process

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Abstract
Purpose Epidermal growth factor receptor (EGFR/ErbB1) is a transmembrane protein with tyrosine kinase activity activated mainly by ligand, EGF. Matrix metalloproteinases (MMPs) are a family of proteinases that catalyses the destruction of ECM, among which MMP-9 has important role in tumor cell invasion. Secretion of MMP-9 is stimulated by a variety of factors, EGFR being significant. Epigallocatechin-3-gallate (EGCG) is a major polyphenol of green tea that inhibits cell proliferation and invasion. Here, we study the effect of EGFR alone and in collaboration with fibronectin on the status of MMP-9 in human breast cancer cell MDA-MB-231 and its molecular mechanism; study the role of EGCG on the induced MMP-9; and elucidate the signaling molecules involved in the process.
Methods We performed zymography, immunoblots, real-time RT-PCR, cell adhesion assay, siRNA studies, and electrophoretic mobility shift assay to demonstrate the findings.
Result EGF induces MMP-9 activity and expression; FAK, PI3 K, and ERK are mainly involved in the process. EGF also causes the transactivation of MMP-9 gene by increasing the DNA binding activity of the transcription factors. EGCG downregulates EGF-induced MMP-9 expression by inhibiting the involved regulatory kinases. EGF collaborates with fibronectin to create a synergistic response, and EGCG inhibits the synergistic response in MDA-MB-231.
Conclusion The study demonstrates the requirement of cross talk between cell matrix adhesion molecules and growth factor receptors to improve biological responses and shows FAK/ERK as the pivotal point of this convergence in human breast carcinoma cell line MDA-MB-231. We also establish EGCG as the potential anti-tumor agent in human breast carcinoma.

Keywords Epidermal growth factor receptor · MMP-9 · Fibronectin · EGCG · Breast cancer · Signaling

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Introduction

Epidermal growth factor receptor (EGFR/ErbB1) is a transmembrane protein with intrinsic protein tyrosine kinase activity that is activated by ligand binding, most important being EGF. Activation of EGF/EGFR has been correlated as an integral contributor of various tumor types including breast cancer [1, 2]. EGFR activation involves homo- and heterodimerization with other EGFR family members [3], recruitment of various signaling proteins, and the activation of a number of different downstream signaling pathways [4] that enhance tumor growth and invasion [5].

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that localizes with integrins at focal contact sites, [6] is a critical intermediate mediator of signaling between tumor cells and their extracellular matrix [7]. Increased FAK autophosphorylation strongly correlates with the acquisition of an invasive phenotype of tumor cells [8] and occurs in response to many stimuli including integrin engagement and growth factor-dependant activation [9]. There is evidence to suggest that autophosphorylation of EGFR involves FAK-dependant signaling [10].

EGFR has been reported to activate PI3 K and ERK, which in turn modulates the activation of NFkB and AP-1 respectively [11]. However, additional signaling inputs through extracellular matrix–integrin interactions also play important roles in modulating EGF-stimulated cell responses [12].

Integrins are a family of heterodimeric transmembrane proteins that serve as receptors for ECM proteins. Among the ECM components, FN has been recognized as the key element in promoting cell adhesion and migration. The coordinated cellular responses to ECM attachment through integrins have been shown to induce tumor cell metastasis [13] by activating signaling pathways involving FAK, PI3 K, ERK etc. [14]. Thus, many of the signaling pathways activated by EGFR are also activated by integrin receptor–ligand interaction. There are several reports of cross talk between integrins and receptor tyrosine kinase (RTK) pathways [14–16]. Although increasing number of responses of integrin synergism with EGFR is being described at the biological level, but the possible intermediates and the mechanism of such synergies are still unclear for various tumor types, including breast cancer. Some studies have indicated the involvement of FAK and ERK in such cross talk [15].

Matrix metalloproteinases (MMPs) are a family of neutral proteinases that catalyse the destruction of extracellular matrix (ECM). Among the MMPs identified, MMP-9 (gelatinase B) appears to have an important role in tumor cell invasion [17]. Secretion of MMP-9 can be stimulated by a variety of factors, EGFR being the most important RTK with regard to MMP expression [18]. Earlier report from our laboratory has shown that EGF induces MMP-9 expression in MDA-MB-231 cells by involvement of PI-3 K and NF-kB signaling pathways [19]. There are not many reports regarding the effect of cross talk between integrin and EGFR signaling on the status of MMP-9 in human breast cancer. Since breast cancer develops slowly and requires multiple events, the use of dietary chemopreventive agents is an important option for the treatment of this disease.

Epigallocatechin-3-gallate (EGCG) is a major bioactive polyphenol present in green tea with respect to inhibiting cell proliferation and invasion [20]. Liang et al. [21] discovered that EGCG binds to and inhibits the tyrosine kinase activity of EGFR in human A431 epidermoid carcinoma cells. EGCG has also been shown to inhibit the activation of ERK and also to inhibit RTK phosphorylation by inhibiting PI-3 K and NF-kB signaling pathways [22]. Reports reveal that EGCG blocks tumor promoter-induced MMP-9 expression via suppression of MAPK and AP-1 activation in human gastric AGS cells [23] and represses MMP-9 expression and NF-κB localization, thereby inhibiting lung carcinoma 95-D cell invasion [24]. The present study aims to study the effect of EGFR alone and in collaboration with FN on the status of MMP-9 in human breast cancer cell line MDA-MB-231 and to study the signaling molecules involved in the cross talk; study the role of EGCG on the induced MMP-9; and elucidate the signaling molecules involved in the process, in human breast cancer cell line, MDA-MB-231.

Materials and methods

Materials

Minimal essential medium (MEM), fetal bovine serum (FBS), fibronectin (440 kDa), Protease Inhibitor Cocktail Tablets (complete, mini, EDTA-free) and Protein G agarose were purchased from Roche, Germany. Gelatin Sepharose 4B beads was purchased from GE Healthcare Bio-Sciences AB, Sweden. All primary antibodies, secondary antibodies, substrates, FAK siRNA, and negative control siRNA were purchased from Santa Cruz (USA). Epidermal growth factor (EGF), EGCG (95% pure), and SYBR Green JumpStart^{TM} Taq Readymix^{TM} were purchased from Sigma–Aldrich, USA. PCR primers and AP1, NF-κB, Sp1 probes were synthesized by Operon, Germany. RNAqueous 4 PCR (Total RNA isolation kit) and Retroscript (RT-PCR Kit) were purchased from Ambion, USA. T4 polynucleotide kinase was purchased from Promega (USA). ERK inhibitor (PD 98059), PI-3 K inhibitor (LY 294002), p38 inhibitor (SB 203580), and MEK inhibitor (U0126) were purchased from Promega, WI. NF-κB inhibitor (BAY-11-7085) was purchased from Alexis Biochemicals, Switzerland. Lipofectamine^{TM} 2000 was purchased from Invitrogen Life Technologies (USA).

Methods

Cell culture

MDA-MB-231 (human breast cancer cell line) cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown and maintained in MEM containing 10% FBS in a 5% CO_2 incubator at 37 °C.

Treatment of cells

MDA-MB-231 cells (300,000 cells/ml) were treated with different concentrations of EGF (2.5, 5, 10 ng/ml) and EGCG (10, 20 μM) at different time points (2-, 4-, 8-h EGF; 24- and 48-h EGCG). All further experiments were performed from MDA-MB-231 cells (300,000 cells/ml) grown in absence or presence of 10 ng/ml EGF for 8 h and/or 20 μM EGCG for 48 h in SFCM. In case of

experiments with fibronectin treatment, MDA-MB-231 cells (300,000 cells/ml) were grown in absence or presence of 20 μg/ml fibronectin for 2 h.

Gelatin zymography

MDA-MB-231 cells (300,000 cells/ml) were grown in absence and presence of EGCG in required concentrations and time periods, followed by treatment with EGF in serum-free culture medium (SFCM); the cells were grown in absence (C) and presence of 1 μg/ml anti-α5 antibody (E) for 1 h, and then, both the sets were treated with 10 ng/ml EGF for 8 h; the cells were grown in absence (control) and presence of PD 98059, LY 294002, SB 203580, U0126 at a concentration of 50 μM for 1 h and BAY-11-7085 at a concentration of 10 μM for 24 h; then, both the control and experimental sets were treated with 10 ng/ml EGF for 8 h in SFCM. Gelatin zymography was performed as previously described [25].

ELISA of MMP-9 in serum-free culture medium (SFCM)

MDA-MB-231 cells (300,000 cells/ml) were grown in absence (control) and presence of the signaling inhibitors in the aforementioned dose and time point, and then both the control and experimental sets were treated with 10 ng/ml EGF for 8 h in SFCM. ELISA was performed with anti-MMP-9 antibody as described before [26].

Cell adhesion assay

MDA-MB-231 cells (300,000 cells/ml) were grown in absence (control), presence of 10 ng/ml EGF for 8 h (+EGF), treatment of 20 μM EGCG for 48 h followed by 10 ng/ml EGF for 8 h (+EGF + EGCG). The microtitre plate wells were coated with fibronectin (5 μg/ml, 10 and 20 μg/ml fibronectin) in triplicate. Cell adhesion assay of both control and experimental sets were performed as described earlier [25].

Semi-quantitative RT-PCR

RNA was extracted from MDA-MB-231 (1×10^6 cells/ml) cells grown in absence or with treatment of EGF, EGCG, and FN at their respective time points and concentrations. Cells were washed and total RNA was extracted as previously discussed [25]. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers were used as control to normalize for mRNA integrity and equal loading. The primer sequences and PCR cycles/conditions for each primer are given below.

Quantitative real-time RT-PCR

Real-time quantitative RT-PCR using relative quantitation by the comparative C_T method was used to determine mRNA expression. Two microlitres of cDNA was subjected to real-time quantitative RT-PCR using SYBR Green as a fluorescent reporter using the SYBR Green JumpStart™ Taq Readymix™ (Sigma, USA). The specific gene primers (MMP-9, FAK, α5, β1 and the internal control gene G3PDH) were amplified in separate reaction tubes using the protocol previously described [25]. The ΔC_T value being inversely proportional to the mRNA expression of the samples. The reaction conditions and the primer sequences are given below. The PCR cycles in all cases were started with Taq activation at 94 °C for 5 min and followed by final extension of 72 °C for 7 min. *Primer sequences, PCR cycles and conditions: hMMP-9:* 5′-CGCTACCACCTCGAACTTTG-3′(forward), 5′-GCCATTCACGTCGTCCTTAT-3′(94 °C-30 s, 56 °C-30 s, 72 °C-90 s); *hFAK:*5′-GCGCTGGCTGGAAAAAGAGGAA-3′(forward), 5′TCGGTGG GTGCTGGCTGGTAGG-3′(reverse 94 °C-30 s, 60 °C-30 s, 72 °C-90 s); *α5:*5′-CATTTCCGAGTCTGGG CCAA-3′(forward),5′-GGAGGCTTGAGCTGAGCTT-3′(reverse); *β1:*5′-TGTTCAGTGCAGAG CCTTCA-3′ (forward), 5′-CCTCATACTTC GGATTGACC-3′ (reverse; 94 °C-30 s, 58 °C-30 s, 72 °C-90 s); and *G3PDH:*5′-CGGAGTCAACGGATTTGGTCGTAT-3′(forward), 5′-AGCCTTCTCCAT GGTG GTGAAGAC-3′(reverse).

Western blot analysis of MMP-9, EGFR, p-EGFR, FAK, p-FAK, ERK, p-ERK, PI-3 K, p-PI-3 K, and NFκB

MDA-MB-231 cells (300,000 cells/ml) were treated with above-mentioned concentrations of EGF, EGCG, and FN for respective time periods. In case of MMP-9, SFCM was collected and gelatinase was extracted from it by Gelatin Sepharose beads and then eluted at 37 °C for 30 min. The cells were first treated with PI3 K inhibitor prior to EGF treatment and then the cells were collected for immunoblot with ERK antibody, which is shown in Fig. 3f. The cells (and SFCM elute in case of MMP-9) were collected; cells were extracted and equal amount of protein (100 μg) was subjected to western blot analysis with anti-MMP-9, anti-EGFR, anti-p-EGFR, anti-FAK, anti-p-FAK, anti-ERK, anti-p-ERK, anti-PI-3 K, anti-p-PI-3 K, anti-NFκB, and anti-Actin antibodies (1:1,000 dilution) following previously described method [25].

Electromobility shift assay (EMSA)

MDA-MB-231 cells (300,000 cells/ml) were grown in absence (C), presence of 10 ng/ml EGF for 8 h (1) and in

presence of both EGF and EGCG (20 μM) for 48 h (2) in SFCM. Cells were collected and nuclear protein was extracted as described before [26]. The probes of the double-stranded oligonucleotides for NF-κB, Sp1, and AP1 were based on human MMP-9 promoter sequence as follows: NF-κB (5′-TGG AAT TCC CAG), Sp1 (5′-CCT TCC GCC CCC), and AP1 (5′-CCT GAG TCA GCA). The complementary oligonucleotides were annealed and probed as described earlier [26]. Five micrograms of nuclear protein from control and treated cells were incubated with ^{32}P-labeled oligonucleotide probes as described earlier [26]. After binding, the protein–DNA complexes were electrophoresed on a native 5% polyacrylamide gel using 0.5× TBE buffer. Each gel was then dried and subjected to autoradiography at −80 °C.

Focal adhesion kinase small interfering RNA (siRNA) treatment

MDA-MB-231 cells were seeded and grown to 50% confluence. For the transfection process, FAK siRNA and negative control siRNA were transfected using Lipofectamine™ 2000 following the manufacturer's protocol and the method described earlier [26]. After transfection, the cells were exposed to EGF (10 ng/ml) for 8 h and later collected for real-time RT-PCR assays for FAK and MMP-9 in the process described above.

Quantification of the results

Bands of zymography, western blots, and RT-PCR were quantitated using Image J Launcher (version 1.4.3.67).

Statistical analysis

All experiments were repeated at least three times. All data collected from gelatin zymography, western blot, real-time RT-PCR, and EMSA were expressed as mean ± SD. The data presented in some figures are from a representative experiment, which was qualitatively similar in the replicate sets. Statistical significance was determined by Student's *t* test (two-tailed) comparison between two groups of data sets. $P < 0.05$ (marked as *, $P < 0.001$ is marked as **) was considered significant.

Results

EGF induces MMP-9 gelatinolytic activity, mRNA and protein expression in MDA-MB-231 cells

Figure 1a and b shows that culture of MDA-MB-231 cells in presence of EGF showed an appreciable increase in MMP-9 gelatinolytic activity in SFCM in a dose- and time-dependant manner, respectively, with 16-fold induction of MMP-9 at 10 ng/ml EGF treatment for 8 h. EGF treatment of 10 ng/ml for 8 h also stimulated MMP-9 mRNA expression to 20-fold as determined by RT-PCR (Fig. 1e) and was confirmed by real-time RT-PCR (Fig. 1f). EGF treatment at 10 ng/ml for 8 h appreciably (20-fold) induced MMP-9 protein expression in both serum-free culture medium (SFCM; Fig. 1 g, Lane 1) and whole-cell extract (WCE; Fig. 1g, Lane 1) in MDA-MB-231 cells when compared to untreated cells (Lane C) as determined by western blot analysis.

EGCG downregulates the EGF-induced MMP-9 gelatinolytic activity and expression in a dose- and time-dependant manner

The zymographic analysis showed that EGCG downregulates the EGF-induced MMP-9 gelatinolytic activity in a dose (Fig. 1c)- and time (Fig. 1d)-dependant manner, and 12-fold inhibition was observed with 20 μM EGCG treatment for 48 h. EGCG treatment (20 μM, 48 h) also appreciably inhibited the mRNA level of MMP-9 as seen by RT-PCR (Fig. 1e, Lane 2) and confirmed by real-time RT-PCRRT-PCR (Fig. 1f. +EGF + EGCG) and the protein level of MMP-9 both in the WCE (Fig. 1 g. upper panel, Lane 2) and in the SFCM (Fig. 1g. lower panel, Lane 2) as determined by western blot analysis.

PI-3 K, ERK, NF-κB, and FAK are involved in EGF-mediated MMP-9 stimulation

The zymographic analysis of Fig. 2a shows that EGF-induced MMP-9 gelatinolytic activity considerably decreases upon treatment of the cells with PI-3 K inhibitor (LY 294002), ERK inhibitor (PD 98059), and NF-κB inhibitor (BAY-11-7085), whereas no appreciable effect was observed upon treatment with MEK inhibitor (U0126) and p38 inhibitor (SB 203580). Treatment with LY 294002, PD 98059, and BAY-11-7085 also reduced the stimulatory effect of EGF on MMP-9 mRNA expression as observed by real-time RT-PCR (Fig. 2b) and decreased the EGF-induced protein expression of MMP-9 as observed by ELISA (Fig. 2c). FAK siRNA blocked endogenous FAK mRNA expression (Fig. 2d). EGF-mediated MMP-9 mRNA expression (Fig. 2e) was significantly inhibited in FAK siRNA-treated MDA-MB-231 cells.

EGF induces the phosphorylation of its receptor EGFR and signaling kinases like FAK, PI-3 K, ERK

Immunoblot analysis revealed that EGF treatment did not cause any appreciable change in the expression of EGFR

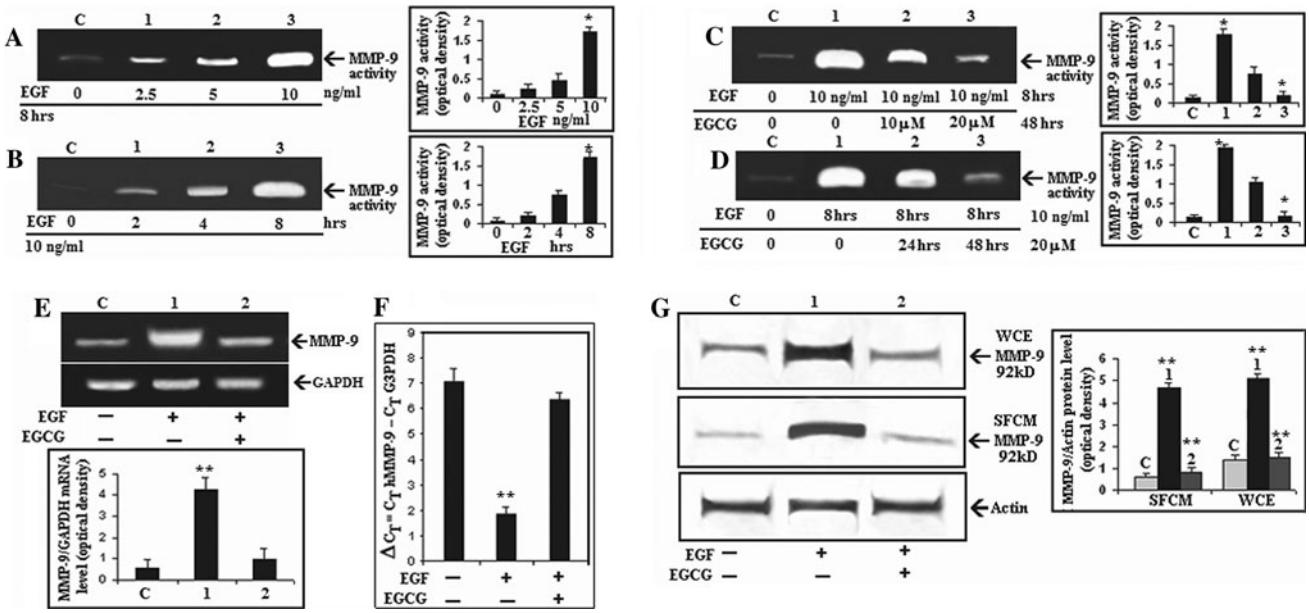

Fig. 1 EGF increases and EGCG downregulates the gelatinolytic activity and expression of MMP-9: MDA-MB-231 cells (300,000 cells/ml) were grown in serum-free culture medium (SFCM) in absence (*C*) and in presence of 2.5 ng/ml (*1*), 5 ng/ml (*2*), and 10 ng/ml (*3*) EGF, 8 h (**a**); in absence (*C*) and in presence of 10 ng/ml EGF for 2 h (*1*), 4 h (*2*), and 8 h (*3*) (**b**); in absence (*C*) and in presence of 10 ng/ml EGF for 8 h in all sets along with 0 (*1*), 10 μM (*2*), and 20 μM (*3*) EGCG for 48 h (**c**); in absence (*C*) and in presence of 10 ng/ml EGF for 8 h in all sets along with 0 (*1*), 24 h (*2*), and 48 h (*3*) of 20 μM EGCG treatment (**d**). The gelatinases in all cases were separated from SFCM by mixing Gelatin Sepharose 4B beads and subjected to gelatin zymography. For mRNA and protein analysis, MDA-MB-231 cells (300,000 cells/ml) were grown in absence (*C*) and in presence of 10 ng/ml EGF for 8 h (*1*) and in presence of 10 ng/ml EGF for 8 h along with 20 μM EGCG for 48 h (*2*) in SFCM. Total RNA was extracted from control and experimental MDA-MB-231 cells (1×10^6 cells). **e** 2 steps RT-PCR was done with equal amounts of total RNA, using specific primers for PCR (MMP 9). GAPDH primers were used to confirm equal loading. **f** 2 μl of cDNA was subjected to real-time quantitative *RT-PCR* with SYBR Green as a fluorescent reporter. Relative levels of expression of MMP-9 and the control G3PDH in control and experimental sets were measured by quantitative real-time *RT-PCR* by calculating the C_T value. The calibrator used in our experiments is the control EGF or EGCG untreated (*C*) MDA-MB-231 cells, and the samples are the EGF (*1*)- and EGF + EGCG (*2*)-treated cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples. For immunoblot, the culture supernatants (SFCM—**g**) were collected and the gelatinases were separated from SFCM using Gelatin Sepharose 4B beads shaking for overnight at 4 °C. In case of whole-cell extract (WCE—**g**), cells were collected; extracted and equal protein (100 μg) was subjected to western blot analysis with anti-MMP-9 antibody (1:1,000 dilution for 1½ hrs at 37 °C). Actin was used as internal control and done in parallel to all blots

(upper panel, Fig. 3a, Lane 1), FAK (upper panel, Fig. 3b, Lane 1), PI-3 K (upper panel, Fig. 3c, Lane 1), or ERK (upper panel, Fig. 3c, Lane 1) but considerably increased the level of p-EGFR (lower panel, Fig. 3a, Lane 1), p-FAK (lower panel, Fig. 3b, Lane 1), p-PI-3 K (lower panel, Fig. 3c, Lane 1), and p-ERK (lower panel, Fig. 3d, Lane 1), when compared to control (Lane C).

EGCG inhibits EGF-mediated activation of EGFR, FAK, ERK, and PI3 K

Immunoblot analysis revealed that EGCG does not appreciably affect the expression level of EGFR (upper panel, Fig. 3a, Lane 2), FAK (upper panel, Fig. 3b, Lane 2), PI-3 K (upper panel, Fig. 3c, Lane 2), or ERK (upper panel, Fig. 3d, Lane 2) but appreciably inhibits the EGF-induced expression of p-EGFR (lower panel, Fig. 3a, Lane 2), p-FAK (lower panel, Fig. 3b, Lane 2), p-PI-3 K (lower panel, Fig. 3c, Lane 2), and p-ERK (lower panel, Fig. 3d, Lane 2).

Inhibition of PI-3 K signaling pathway does not cause any appreciable change in the EGF-induced activation of ERK

Immunoblot analysis revealed that inhibition of PI-3 K signaling pathway by LY 294002 (Fig. 3f, Lane 2) prior to treatment with EGF did not cause any appreciable change in EGF-induced ERK activation (Fig. 3f, Lane 1).

EGCG inhibits EGF-induced expression of major transcription factor NFκB and EGF-mediated enhanced DNA binding activity of NFκB and Ap-1 to the MMP-9 promoter

Immunoblot assay showed that EGF treatment appreciably induced the expression of NFκB (Fig. 3e. Lane 1) when compared to untreated control set (Fig. 3f. Lane C) in MDA-MB-231. The EGF-induced NFκB expression was appreciably reduced by EGCG treatment (Fig. 3e. Lane 2).

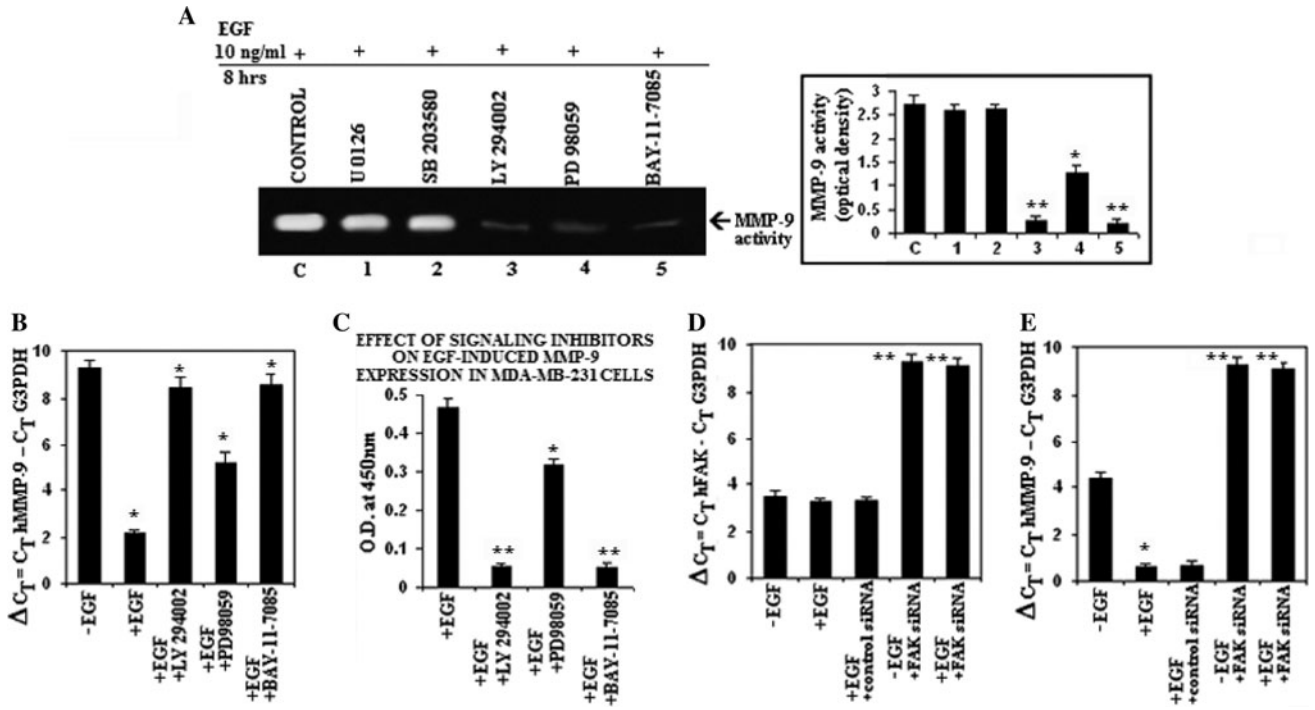

Fig. 2 Inhibition of PI3 K, ERK, and FAK abrogates the EGF-induced MMP-9 activity and expression: **a** MDA-MB-231 cells (300,000 cells/ml) were grown in absence (*Lane C*) and presence of MEK inhibitor (U0126) (*Lane 1*), p38 inhibitor (SB 203580) (*Lane 2*), PI-3 K inhibitor (LY 294002) (*Lane 3*), and ERK inhibitor (PD 98059) (*Lane 4*) at a concentration of 50 μM for 1 h and NF-κB inhibitor (BAY-11-7085) (*lane 5*) at a concentration of 10 μM for 24 h; then both the control and experimental sets were treated with EGF (10 μg/ml) for 8 h in SFCM. The culture supernatants were collected, and gelatin zymography was performed by using a 7.5% SDS–PAGE co-polymerized with 0.1% gelatin. **b** Total RNA was extracted from control, EGF-treated, and inhibitor-treated MDA-MB-231 cells (1×10^6 cells). 2 μl of cDNA from each sample was subjected to real-time quantitative RT-PCR as before using specific primer for MMP-9. Relative levels of expression of MMP-9 and the control G3PDH in control, EGF-treated (10 ng/ml for 8 h), and inhibitor (LY 294002, PD 98059, BAY-11-7085)-treated cells as measured by quantitative real-time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (−EGF) MDA-MB-231 cells and the samples are the EGF-treated (10 ng/ml, 8 h) (+EGF) and inhibitor-treated (+EGF + inhibitors) MDA-MB-231 cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples. **c** The culture supernatants (*SFCM*) were collected; the wells of an ELISA plate was coated with 50-μl culture SFCM of both control (with EGF) and experimental sets (with EGF and signaling inhibitors—+EGF + LY 294002, +EGF + PD 98059, +EGF + BAY-11-7085) (in triplicate) and kept at 4 °C overnight. ELISA was performed the next day with MMP-9 antibody (1:1,000 dilution). The OD was measured at 450 nm. Effect of blocking FAK signals on EGF-induced MMP-9 expression by quantitative real-time RT-PCR: MDA-MB-231 cells transfected with control and FAK siRNA (100nM each) for 48 h before treating the cells with 10 ng/ml EGF for 8 h. **d** Total RNA was extracted from control, EGF-treated and siRNA-treated MDA-MB-231 cells (1×10^6 cells). 2 μl of cDNA from each sample was subjected to real-time quantitative RT-PCR as before using specific primer for FAK (**d**) and MMP-9 (**e**). Relative levels of expression of FAK, MMP-9, and the control G3PDH in control, EGF-treated (10 ng/ml for 8 h) and siRNA (control siRNA and FAK siRNA)-treated MDA-MB-231 cells as measured by quantitative real-time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (-EGF) MDA-MB-231 cells and the samples are the EGF-treated (10 ng/ml, 8 h) (+EGF) and siRNA-treated (+EGF + control siRNA or +EGF + FAK siRNA) MDA-MB-231 cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples

EMSA revealed that treatment of MDA-MB-231 cells with EGF (10 ng/ml for 8 h) appreciably enhances the NF-κB (Fig. 4a, Lane 1) and AP-1 (Fig. 4b—Lane 1) DNA binding to nuclear protein when compared to untreated controls (Lane C); there was however no appreciable difference in the Sp1 DNA binding activity (Fig. 4c). EGCG treatment appreciably reduced the EGF-induced DNA binding activity of NF-κB (Fig. 4a, Lane 2) and AP-1 (Fig. 4b—Lane 2). Sp1 level remained almost the same.

Fibronectin can synergistically act with EGF to induce MMP-9 gelatinolytic activity, mRNA and protein expression in MDA-MB-231 cells

Figure 5a shows that culture of MDA-MB-231 cells in presence of fibronectin enhanced MMP-9 gelatinolytic activity in SFCM in a dose-dependant manner (5 μg/ml, Lane 1; 10 μg/ml, Lane 2; 20 μg/ml Lane 3) with appreciable induction of MMP-9 at 20 μg/ml FN treatment for 2 h (Lane 3). The MMP-9 gelatinolytic activity considerably

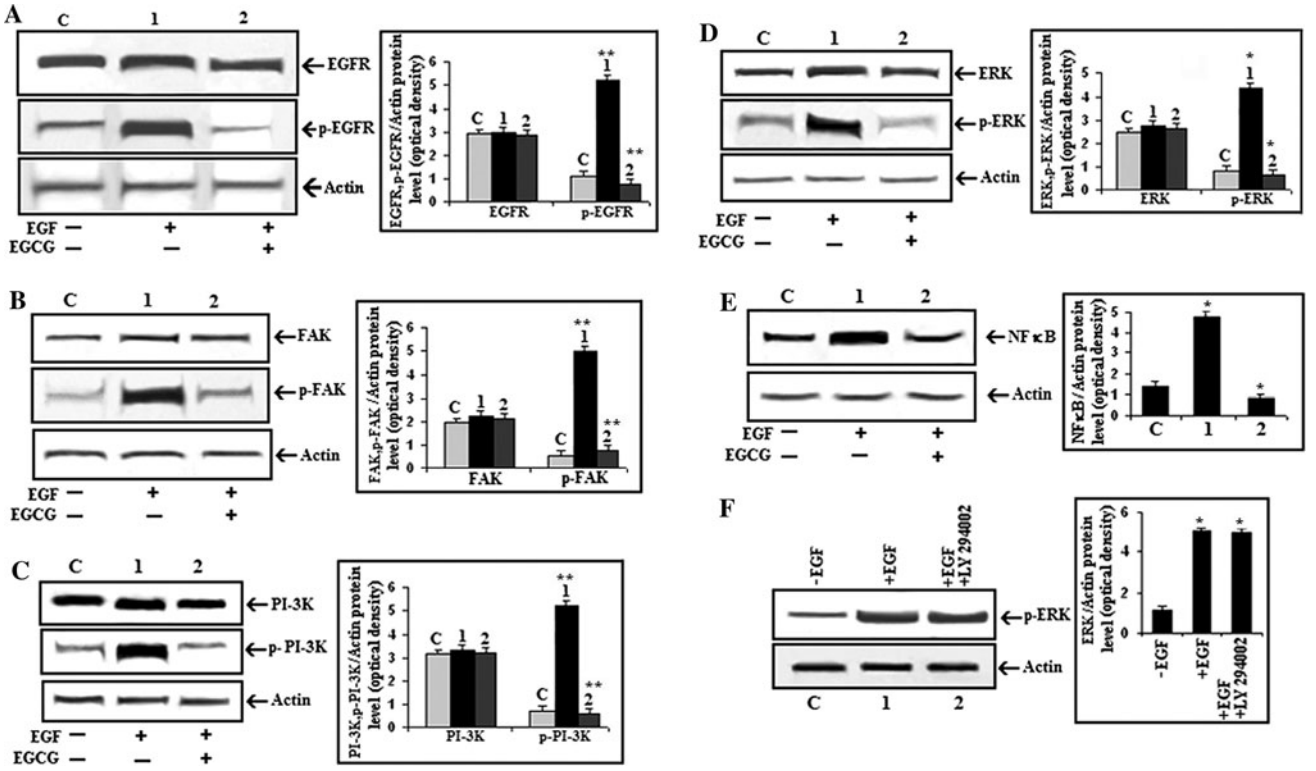

Fig. 3 EGF enhances and EGCG downregulates the level of p-EGFR, p-FAK, p-PI3 K, p-ERK, and NFκB: MDA-MB-231 cells were grown in absence (0), presence of 10 ng/ml EGF for 8 h (1) and the treatment of 10 ng/ml EGF for 8 h and 20 μM EGCG for 48 h (2) in SFCM. For **f**, the cells were treated with PI-3 K inhibitor (50 μM, 1 h) prior to treatment with EGF (10 ng/ml, 8 h). The cells were collected; extracted and equal amount of protein (100 μg) was subjected to western blot analysis with anti-EGFR (*upper panel*), anti-p-EGFR (*lower panel*) (**a**), anti-FAK (*upper panel*), anti-p-FAK (*lower panel*) (**b**), anti-PI-3 K (*upper panel*), anti-p-PI-3 K (*lower panel*) (**c**), anti-ERK (*upper panel*), anti-p-ERK (*lower panel*) (**d**), anti-NFκB (**e**), and anti-ERK (**f**) antibodies (1:1,000 dilution for 1½ hrs at 37 °C). Actin was used as internal control and done in parallel to all blots

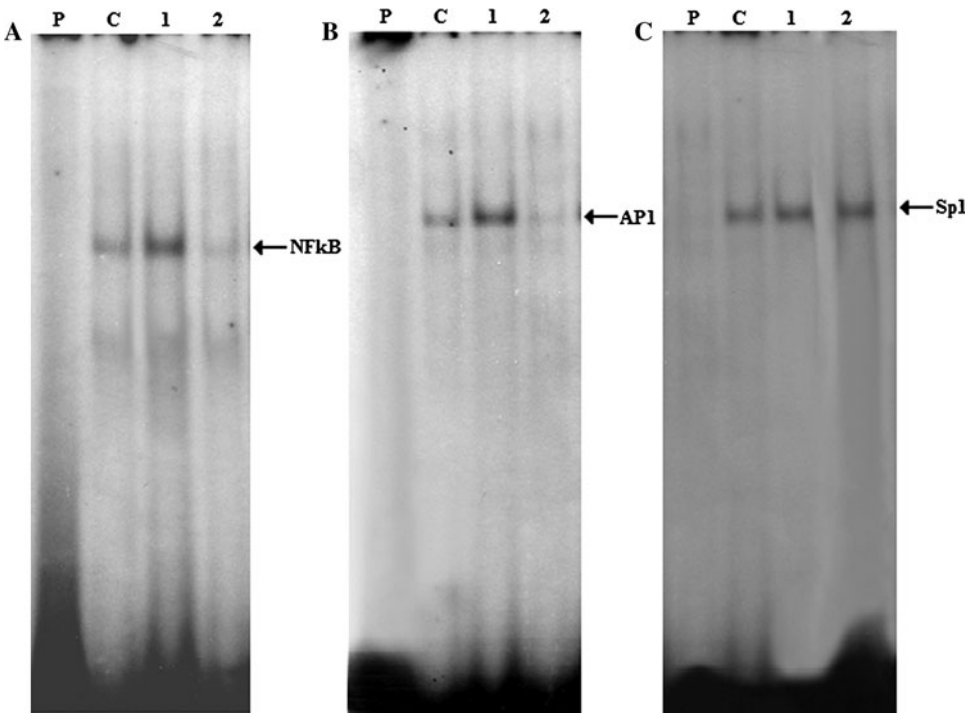

Fig. 4 EGF enhances and EGCG inhibits the DNA binding of NFκB, AP-1, and Sp1 protein in the MMP-9 promoter: Effect of EGF (10 ng/ml, 8 h) and EGCG (20 μM, 48 h) on NFκB (*panel* **a**), AP-1 (*panel* **b**) and Sp1 (*panel* **c**) binding activities. Oligonucleotides containing the NFκB, AP-1, and Sp1 sites were end-labeled with [γ^{-32}P] ATP and incubated with nuclear extracts (5 μg) from MDA-MB-231 cells grown in absence (*C*), presence of EGF (10 ng/ml, 8 h) (*1*) and treatment of EGF (10 ng/ml, 8 h) and EGCG (20 μM, 48 h) (*2*). *Lane P* in each case denotes the lane for free probe (without the nuclear protein)

increased when treated with both FN (20 μg/ml, 2 h) and EGF (10 ng/ml, 8 h) and the induction was more than when treated with either of the ligands alone (Lane 4). Fibronectin and EGF treatment also caused eightfold stimulation of MMP-9 mRNA expression (Fig. 5b. Lane 1), and the expression was more than the mRNA expression when treated with EGF alone (Lane C) as observed by RT-PCR (Fig. 5b) and confirmed by real-time RT-PCR (Fig. 5c). Fibronectin and EGF treatment caused about eightfold induction of MMP-9 protein expression in both serum-free culture medium (SFCM; Fig. 5d, Lane 1) and whole-cell extract (WCE; Fig. 5d, Lane 1) in MDA-MB-231 cells when compared to only EGF-treated cells (Lane C) as determined by western blot analysis.

EGCG downregulates the FN- and EGF-induced MMP-9 gelatinolytic activity and expression

The zymographic analysis of Fig. 5a showed that EGCG treatment of 20 μM for 48 h could cause about 16-fold downregulation of FN + EGF-induced MMP-9 gelatinolytic activity. EGCG treatment (20 μM, 48 h) also appreciably (up to 12-fold) inhibited the FN + EGF-induced mRNA level of MMP-9 as seen by RT-PCR (Fig. 5b, Lane 2) and confirmed by real-time RT-PCRRT-PCR (Fig. 5c, FN + EGF + EGCG) and also inhibited the FN + EGF-induced protein level of MMP-9 both in the WCE (Fig. 5d, upper panel, Lane 2) and in the SFCM (Fig. 5d, lower panel, Lane 2) as determined by western blot analysis.

Interrelation of integrin receptor $\alpha5\beta1$ and EGF and the effect of EGCG on $\alpha5\beta1$ in MDA-MB-231 cells

RT-PCR analysis revealed that the treatment of EGF only (10 ng/ml, 8 h; Fig. 6a, Lane 1) or with EGCG (20 μM, 48 h; Fig. 6a, Lane 2) did not significantly change the mRNA level of α5 (Fig. 6a, upper panel) or $\beta1$ (Fig. 6a, lower panel). It was confirmed by real-time-RT-PCR analysis (α5—Fig. 6b; $\beta1$ Fig. 6c). But when MDA-MB-231 cells were treated with anti-α5 antibody prior to treatment with EGF (10 ng/ml for 8 h), blockade of integrin α5 appreciably abrogated the EGF-induced MMP-9 gelatinolytic activity. EGF treatment (+EGF) increased the binding of MDA-MB-231 cells to ECM ligand fibronectin to up to 25% when compared to EGF untreated set (-EGF). When the cells were pretreated with anti-α5 antibody, prior to treatment with EGF, the binding of the cells to fibronectin was appreciably decreased (+EGF + α5) by about 40%. EGF treatment followed by EGCG treatment noticeably inhibited the EGF-induced binding of MDA-MB-231 cells to fibronectin (+EGF + EGCG) by about 70%.

Treatment of FN + EGF causes activation of EGF receptor, FAK, and ERK signaling pathway

Immunoblot analysis revealed that treatment of FN and EGF together caused a more pronounced phosphorylation of EGFR (Fig. 7a, lower panel, Lane 1) when compared to only EGF-treated set (Fig. 7a, lower panel, Lane C). FN + EGF treatment, however, did not cause any significant change in the expression of EGFR (Fig. 7a, upper panel). FN treatment along with EGF treatment did not cause any change in the expression of PI-3 K (Fig. 7b, panel c) or in the phosphorylation of PI-3 K (Fig. 7b, panel d). However, FN + EGF treatment causes a noticeable increase in the phosphorylation of FAK (eightfold; Fig. 7b, panel b) and ERK (Fig. 7b, panel f) but does not change the expression of either FAK (Fig. 7b, panel b) or ERK (Fig. 7b, panel e).

EGCG downregulates FN + EGF-mediated activation of EGFR, FAK, and ERK

Immunoblot analysis revealed that EGCG treatment appreciably (20-fold) inhibits the FN- and EGF-induced upregulation of p-EGFR (Fig. 7a, lower panel, Lane 2), but did not cause any noticeable change in the expression of EGFR (Fig. 7a. upper panel, Lane 2). EGCG treatment also downregulates the FN- and EGF-mediated stimulation of p-FAK (Fig. 7b, panel b, Lane 2) and p-ERK (Fig. 7b, panel f, Lane 2) by 16- and 20-fold, respectively, but did not change the expression of either FAK (Fig. 7b, panel a, Lane 2) or ERK (Fig. 7b, panel e, Lane 2). EGCG did not affect the status of PI-3 K (Fig. 7b, panel c, Lane 2) or of p-PI-3 K (Fig. 7b, panel d, Lane 2).

Discussion

In the present study, we demonstrate that EGF treatment of 10 ng/ml for 8 h enhances the gelatinolytic activity, mRNA level, and protein expression level of MMP-9 in MDA-MB-231 cells. This result is in conformity with earlier evidences which report that EGF stimulates the secretion of pro-MMP-9 in other cell lines [27, 28]. Our study shows that EGCG downregulates the gelatinolytic activity of EGF-induced MMP-9 in a dose- and time-dependant manner. EGCG also abrogated the EGF-induced mRNA and protein expression levels of MMP-9 in MDA-MB-231 cells. Previous studies have shown that EGCG, a major biologically active component of green tea, inhibited the activation of the EGFR and thereby inhibited multiple downstream signaling pathways in HNSCC cell lines [29]. One of the main downstream effecter of EGFR activation is MMP-9 stimulation that leads to increased tumor growth

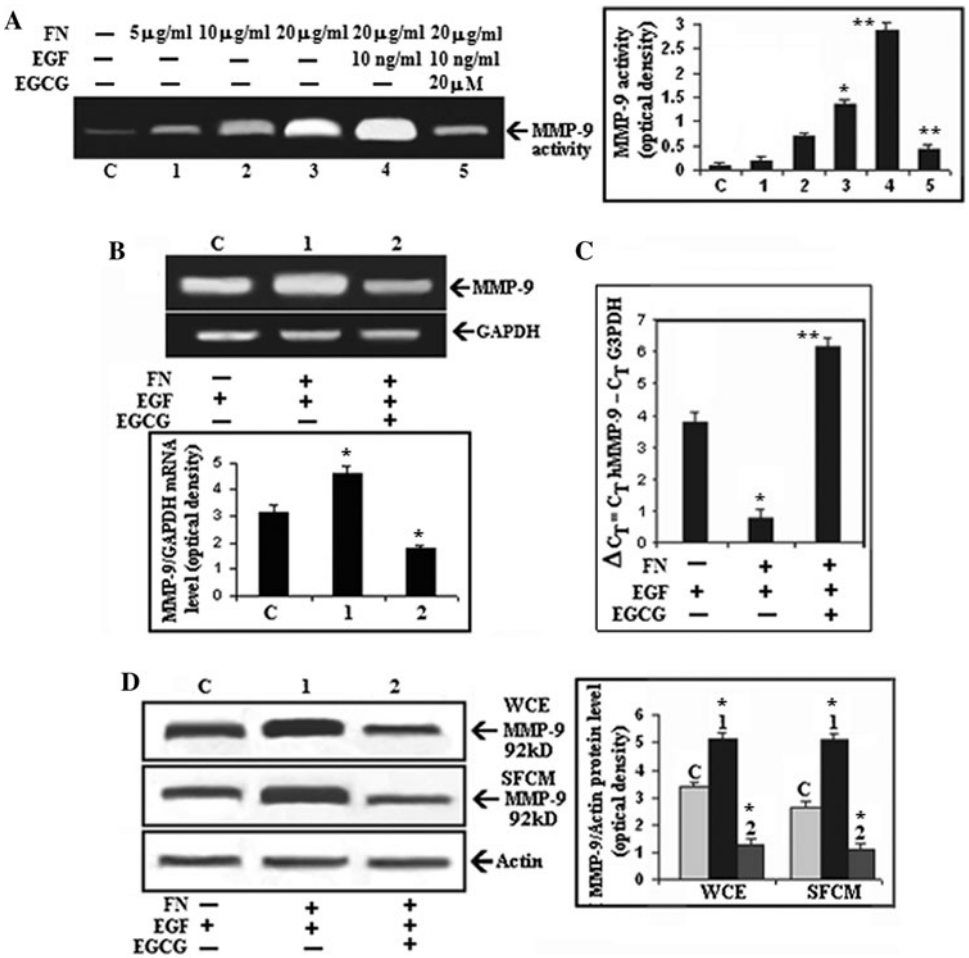

Fig. 5 EGF and fibronectin synergistically increase the expression and activity of MMP-9 in MDA-MB-231 cells, and EGCG decreases the induced MMP-9: MDA-MB-231 cells (300,000 cells/ml) were grown in serum-free culture medium (SFCM) in absence (*C*), in presence of 5 μg/ml FN, 2 h (*1*), 10 μg/ml FN, 2 h (*2*), 20 μg/ml FN, 2 h (*3*), 20 μg/ml FN, 2 h and 10 ng/ml EGF, 8 h (*4*), 20 μg/ml FN, 2 h + 10 ng/ml EGF, 8 h + 20 μM EGCG, 48 h (*5*) (**a**). The SFCM was collected and subjected to gelatin zymography. For mRNA and protein analysis, MDA-MB-231 cells (300,000 cells/ml) were grown in presence of 10 ng/ml EGF for 8 h (*C*), in presence of 10 ng/ml EGF for 8 h along with 20 μg/ml FN for 2 h (*1*), and in presence of 10 ng/ml EGF for 8 h + 20 μg/ml FN for 2 h + 20 μM EGCG for 48 h (*2*) in SFCM. Total RNA was extracted from control and experimental MDA-MB-231 cells (1×10^6 cells). **b** Two-step RT-PCR was done with equal amounts of total RNA, using specific primers for PCR (MMP 9). GAPDH primers were used to confirm equal loading. **c** 2 μl of cDNA was subjected to real-time quantitative RT-PCR with MMP-9 and GAPDH primer as above. The calibrator used in our experiments is the EGF-treated (*C*) MDA-MB-231 cells and the samples are the EGF + FN (*1*) and EGF + FN + EGCG (*2*) treated cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples. For immunoblot, the culture supernatants (SFCM—**d**) were collected and the gelatinases were separated from SFCM using Gelatin Sepharose 4B beads shaking for overnight at 4 °C. In case of whole-cell extract (WCE—**d**), cells were collected; extracted and equal protein (100 μg) was subjected to western blot analysis with anti-MMP-9 antibody (1:1,000 dilution for 1½ hrs at 37 °C). Actin was used as internal control and done in parallel to all blots

and progression [2]. So, EGCG downregulates the EGF-induced MMP-9 expression and activity in breast cancer cell line MDA-MB-231.

Protein expression study revealed that EGF treatment activates EGFR itself and also induces the phosphorylation and hence activation of FAK, ERK, and PI-3 K signaling pathways. EGF treatment also caused an upregulation in the NFκB protein expression level in MDA-MB-231. Earlier evidences strongly suggest the pivotal role of these signaling molecules in EGF-induced EGFR signaling activation and the activation of downstream signaling pathways like FAK, ERK, PI-3 K, and NFκB [30]. EGF causes the phosphorylation of FAK at the Tyr 397 residue and subsequent binding of FAK to SH2 containing proteins including Src kinase and the 85-kDa subunit of PI-3 K. The present study revealed that abrogation of the PI3 K signaling pathway did not cause any appreciable change in the EGF-mediated upregulation of p-ERK. Hence, it may be said that PI-3 K and ERK signaling pathways work independently to generate EGF-mediated effecter responses.

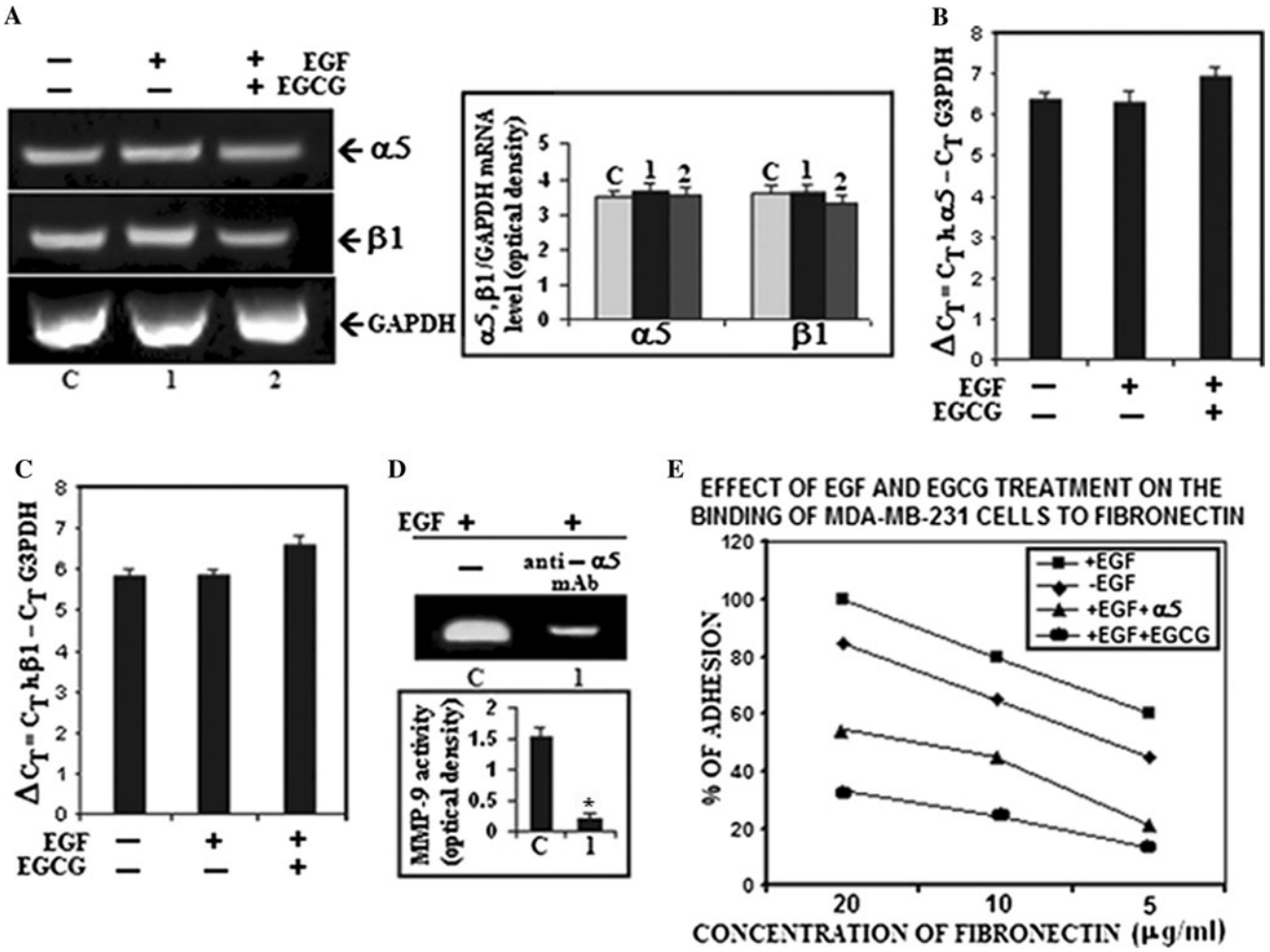

Fig. 6 EGF treatment does not affect the gene expression of α5 and β1: MDA-MB-231 cells (300,000 cells/ml) were grown in absence (*C*), in presence of 10 ng/ml EGF, 8 h (*1*), and in presence of 10 ng/ml EGF and 20 μM EGCG for 48 h (*2*) in SFCM. Total RNA was extracted from control and treated MDA-MB-231 cells (1×10^6 cells). Two-step RT-PCR was done with equal amounts of total RNA, using specific primers for PCR (α5 (*upper panel*) and β1 (*lower panel*) **a**). GAPDH primers were used to confirm equal loading. 2 μl of cDNA was subjected to real-time quantitative RT-PCR as before. Relative levels of expression of α5 (**b**) and β1 (**c**) and the control G3PDH in control and experimental MDA-MB-231 cells were measured by quantitative real-time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (-EGF-EGCG) MDA-MB-231 cells and the samples are the EGF-treated (+EGF) and EGF- and EGCG-treated (+EGF + EGCG) MDA-MB-231 cells. In the given graph, the C_T value is inversely proportional to the mRNA expression of the samples Abrogation of integrin α5 inhibits the EGF-induced MMP-9 activity in MDA-MB-231 cells: **d** MDA-MB-231 cells (300,000 cells/ml) were grown in absence (*C*) and presence of 1 μg/ml anti-α5 antibody (*E*) for 1 h, and then both the sets were treated with 10 ng/ml EGF for 8 h. The gelatinases in all cases were separated from SFCM by mixing Gelatin Sepharose 4B beads and subjected to gelatin zymography. EGF increases and EGCG inhibits the binding of MDA-MB-231 cells to ECM ligand fibronectin: **e** MDA-MB-231 cells were grown in absence (−EGF), presence of 10 ng/ml EGF, 8 h (+EGF), presence of 10 ng/ml EGF, 8 h and 1 μg/ml α5 antibody (+EGF + α5), and presence of 10 ng/ml EGF, 8 h and 20 μM EGCG for 48 h (+EGF + EGCG). The microtitre plate wells were coated with fibronectin (5, 10, and 20 μg/ml fibronectin) in triplicate. Cell adhesion assay of both control and experimental sets was preformed

ERK has already been shown to be an important signaling molecule that lies downstream of EGFR [31]. Inhibitor assay further demonstrated the involvement of ERK, PI-3 K, and NFκB in the EGF-induced MMP-9 function, as abrogation of these signaling molecules by specific chemical inhibitors considerably reduced EGF-induced MMP-9 gelatinolytic activity, mRNA level, and protein expression in MDA-MB-231 cells. Our results are in good agreement with the earlier evidences which suggest that multiple signaling pathways including ERK, PI-3 K, and NFκB are essential signals for the activation of MMP-9 by growth factors of the EGF family of proteins [32, 33].

FAK siRNA studies revealed that abrogation of endogenous FAK noticeably reduced EGF-induced MMP-9 mRNA level. This strongly indicates the critical role of FAK in EGF-induced MMP-9 function. This is in conformity with

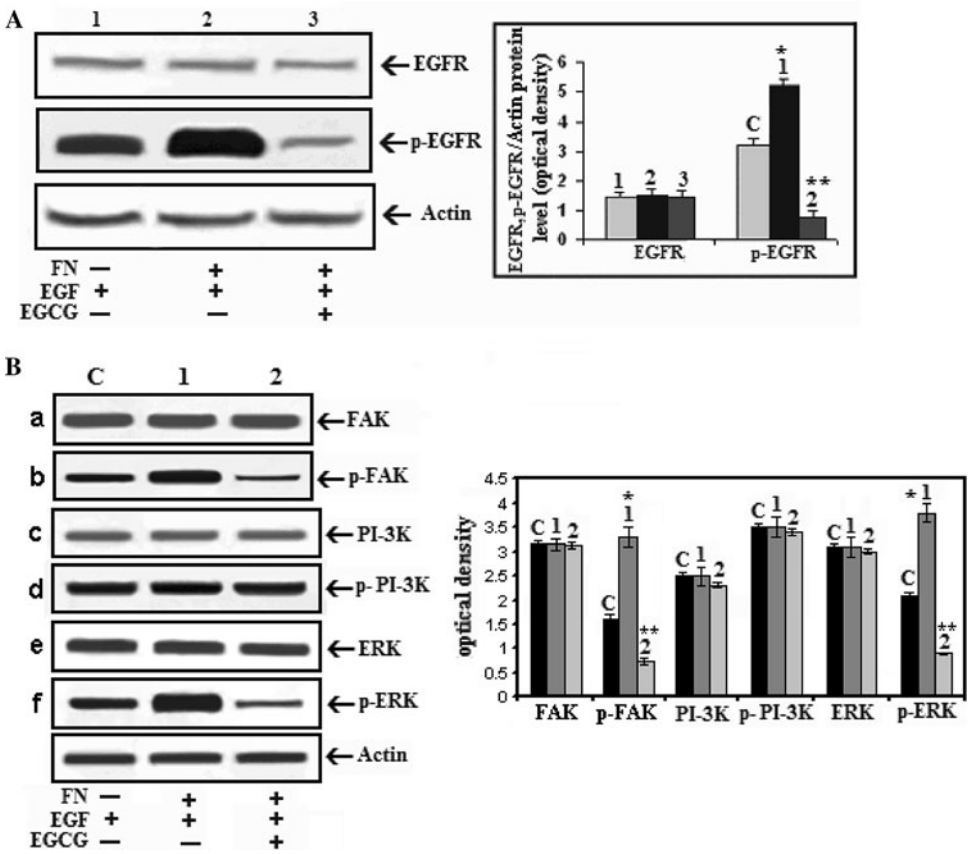

Fig. 7 EGF and fibronectin synergistically activate ERGF, FAK, and ERK but do not have any effect on PI3 K; EGCG inhibits the induced effect: MDA-MB-231 cells (300,000 cells/ml) were grown in presence of 10 ng/ml EGF for 8 h (*C*), in presence of 10 ng/ml EGF for 8 h along with 20 μg/ml FN for 2 h (*1*), and in presence of 10 ng/ml EGF for 8 h + 20 μg/ml FN for 2 h + 20 μM EGCG for 48 h (*2*) in SFCM. The cells were collected; extracted and equal amount of protein (100 μg) was subjected to western blot analysis with anti-EGFR (*upper panel*), anti-p-EGFR (*lower panel*) (**a**), anti-FAK (**b**, *lower panel* **a**), anti-p-FAK (**b**, *panel* **b**), anti-PI-3 K (**b**, *panel* **c**), anti-p-PI-3 K (**b**, *panel* **d**), anti-ERK (**b**, *panel* **e**), anti-p-ERK (**b**, *panel* **f**) antibodies (1:1,000 dilution for 1½ hrs at 37 °C). Actin was used as internal control and done in parallel to all blots. The accompanying graphs in all cases represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means ± SEM of three experiments

earlier evidences that demonstrate the role of FAK as an important signaling intermediate in EGF-dependant upregulation of MMP-9 expression in human follicular thyroid carcinoma [2]. EGF-mediated upregulation of MMP-9 may be thus dependant partially upon the regulation via FAK signaling pathway in MDA-MB-231 cells as also seen in other cancer cell lines [31, 34]. Western blot analysis revealed that EGCG treatment appreciably reduced the EGF-induced EGFR activation. There are earlier evidences which show that EGCG inhibits the activation of EGFR. The present study also shows that EGCG successfully abrogates the EGF-induced phosphorylation of FAK, ERK, and PI-3 K. Thus, we may say that in MDA-MB-231 cell line, EGCG effectively blocks the activation of EGFR and consequently blocks the activation of multiple downstream signaling molecules FAK, ERK, and PI-3 K. EGCG also reduced the protein expression level of EGF-induced NFκB. EGCG has earlier been shown to inhibit NFκB level in human colon carcinoma cells [31]. Earlier reports have also shown that NFκB activation is in close cross talk with the activation of ERK signaling pathway, so EGCG might either directly inhibit EGF-induced NFκB expression or might do so by downregulating ERK activation.

A major target of ERK is AP-1 and Sp1, important transcription factors that play vital role in growth and progression of various tumor types [35]. Our study reveals that EGF increases the promoter binding activity of NFκB, AP-1 transcription factors to the MMP-9 promoter. This is in agreement with earlier evidences which suggest that increased binding of the transcription factors like AP-1 and NFκB to the MMP-9 promoter might be the possible cause of MMP-9 induction by EGF [35]. EGCG treatment was shown to cause a noticeable downregulation of the EGF-induced binding of NFκB and AP-1 transcription factors to the MMP-9 promoter. Thus, in the present communication, we show that EGF treatment enhances the activation of

EGFR and some of the downstream signaling kinases like FAK, ERK, and PI-3 K, which work in concert to induce the transcription factor binding activity to the MMP-9 promoter, thus causing an appreciable upregulation of MMP-9 expression and activity in MDA-MB-231 cells. We also show that EGCG downregulates the EGF-induced activation of EGFR, downstream signaling kinases, and consequently also downregulates EGF-induced MMP-9.

In the present study, we showed that FN treatment causes a dose-dependent induction of MMP-9 activity, and when cells were treated with EGF and FN together, a synergistic effect was observed. The treatment of FN + EGF treatment caused an induction of MMP-9 activity, MMP-9 mRNA and protein level (both SFCM and cell extract) more than observed with the treatment of either FN or EGF alone. EGCG treatment appreciably downregulates the EGF- and FN-induced MMP-9 activity and expression. Hence, the downstream effect of EGF and FN treatment together is more effective than the effect of either ligand alone. EGCG causes an inhibitory effect on this effecter response. We wanted to demonstrate the signaling molecules involved in the synergistic pathway of FN and EGF together.

We investigated the effect of EGF on the status of integrin receptor $\alpha 5$ and $\beta 1$. mRNA analysis revealed that EGF treatment did not appreciably change the mRNA expression of $\alpha 5$ or $\beta 1$ but caused a noticeable upregulation of the binding of MDA-MB-231 cells to ECM ligand fibronectin. Thus, EGF has a synergistic effect on the $\alpha 5\beta 1$-induced functional responses but EGF is not essential in integrin expression. Growth factors like EGF that activate RTKs like EGFR can regulate integrin-mediated events such as cell adhesion, cell spreading, and cell migration through alterations in integrin binding and activation [36]. Treatment of MDA-MB-231 cells with $\alpha 5$ monoclonal antibody appreciably reduced the EGF-induced binding of cells to ECM ligand fibronectin and also the EGF-induced gelatinolytic activity of MMP-9. Hence, $\alpha 5$ integrin receptor is an essential component in EGF-mediated cell attachment and other effecter functions. This is in agreement with earlier studies demonstrating the necessity of integrin-induced signaling for the EGF-induced responses. EGCG was shown to inhibit the EGF-induced adhesion of MDA-MB-231 cells to ECM ligand fibronectin. EGCG treatment, however, did not alter the mRNA level of $\alpha 5\beta 1$ integrin receptor. Hence, EGCG did not have any effect on the expression level of the receptors itself but had inhibitory effect on the binding of MDA-MB-231 cells to the ECM ligand fibronectin.

Here, we observed that the treatment of FN and EGF together causes appreciably more phosphorylation of EGFR, FAK, and ERK than that of either EGF of FN treatment alone, but does not have much effect on phosphorylation of PI-3 K. The cytoplasmic domains of the main integrin receptors of FN, $\alpha 5$, and $\beta 1$ are short and devoid of any enzymatic activity. So, it is likely that interactions with transuding molecules have to take place in order to promote intracellular signaling. An important candidate is p125FAK; it has also been proposed that phosphorylated FAK interacts with adaptor molecules leading to ERK activation [37]. So we have demonstrated the possibility that integrins receptor $\alpha 5\beta 1$ and EGF might function co-operatively through FAK $\rightarrow$ ERK activation in MDA-MB-231 cells. There are earlier evidences that suggest FAK and ERK as the focal point of signaling cross talk between integrin receptors and growth factor receptors [15], and our study also demonstrates FAK and ERK as the main molecules involved in the cross talk.

Immunoblot analysis showed that EGCG noticeably reduced EGF- and FN-induced p-EGFR level. EGCG also abrogates the p-FAK level and p-ERK level induced by EGF and FN treatment but does not cause any appreciable change in the p-PI-3 K level post EGF + FN treatment. So, EGCG successfully suppresses the activation of EGFR which was synergistically induced by EGF and FN treatment. The present findings suggesting the inhibitory role of EGCG on the synergistic effects of EGF and FN treatment together might be a novel finding in MDA-MB-231 cells. In the present system, we have shown that EGCG plays a very important role in decreasing the co-operative effect of EGF and FN on FAK and ERK activation in a highly metastatic breast cancer cell line, MDA-MB-231. Earlier studies have shown the inhibitory effect of EGCG on the EGF-mediated responses and on integrin-mediated responses alone [38], but in the present study we demonstrate first the inhibitory effect of EGCG on the synergistic signaling responses of integrin and growth factor receptors.

Conclusion

In the present study, we demonstrate that EGF induces MMP-9 activity in MDA-MB-231 cells by the activation of multiple signaling pathways like FAK, PI3 K, and ERK. We also demonstrate that PI3 K and ERK function separately and independently of each other in EGF-mediated downstream responses. EGF treatment was shown to induce the promoter binding activity of NF-κB, AP1 but not Sp1. Green tea polyphenol, EGCG, reduced EGF-mediated responses at all levels and hence appreciably reduced EGF-induced MMP-9 activity and expression. We also show that integrin-mediated signaling is necessary for EGF-mediated responses and integrins collaborate with EGF for phosphorylation and enhanced the activation of tyrosine kinases like FAK and ERK. FAK and ERK were demonstrated to be the convergence point of signal

integration. EGCG was shown for the first time to inhibit even the co-operative responses of integrin and growth factor receptors. So the study demonstrates the requirement of cross talk between cell matrix adhesion molecules and growth factor receptors to improve biological responses and shows FAK/ERK as the pivotal point of this convergence in human breast carcinoma cell line MDA-MB-231. We also establish EGCG as the potential anti-tumor agent in human breast carcinoma.

Conflict of interests The authors declare that they have no conflict of interests.

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