EPS8 Facilitates Cellular Growth and Motility of Colon Cancer Cells by Increasing the Expression and Activity of Focal Adhesion Kinase

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In an attempt to study the role of Eps8 in human carcinogenesis, we observe that ectopic overexpression of Eps8 in SW480 cells (low Eps8 expression) increases cell proliferation. By contrast, expressing eps8 small interference RNA in SW620 and WiDr (high Eps8 expression) reduces their proliferation rate. Interestingly, attenuation of Eps8 decreases Src Pi-Tyr-416, Shc Pi-Tyr-317, and serum-induced FAK Pi-Tyr-397 and Pi-Tyr-861. Remarkably, by virtue of mammalian target of rapamycin/STAT3 Pi-Ser-727, Eps8 modulates FAK expression required for cell proliferation. Within 62% of colorectal tumor specimens examined, >2-fold enhancement of Eps8 as compared with their normal counterparts is observed, especially for those from the advanced stage. In agreement with the modulation of FAK by Eps8, the concomitant expression of these two proteins in tumor specimens is observed. Notably, Eps8 attenuation also impedes the motility of SW620 and WiDr cells, which can be rescued by ectopically expressed FAK. This finding discloses the indispensability of Eps8 and FAK in cell locomotion. These results provide a novel mechanism for Eps8-mediated FAK expression and activation in colon cancer cells.

The signal transduction of the epidermal growth factor receptor (EGFRT) is important for normal cell physiology (1, 2). During the search for novel EGFR substrates, Eps8 (EGFR pathway substrate number 8) as suggested by its designation was originally identified as a putative EGFR target devoid of phosphotyrosine-binding SH2 domain (3). Among its 97- and 68-kDa isoforms, only the former is well characterized and thus referred to as Eps8. Later, Eps8 also is the substrate for Src tyrosine kinase (4). In addition to tyrosyl phosphorylation, expression of Eps8 is also affected by Src activity (4, 5). Remarkably, its aberrant overexpression in murine fibroblasts can lead to cellular transformation (6), and of note, Eps8 overexpression contributes to Src-mediated transformation (7).

As an adaptor protein, Eps8 contains several structural features such as a split pleckstrin homology, a putative nuclear targeting sequence, a central SH3 domain, and several proline-rich regions. Although the split pleckstrin homology confers the ability of Eps8 to associate with plasma membrane in response to serum stimulation and conveys signals to ERK activation (6), Eps8 can also complex with Abi-1/E3b1 and RN-tre separately through its SH3 domain (8, 9). By interacting with Abi-1 or RN-tre, Eps8 integrates signals leading to actin cytoskeleton via Rac and receptor endocytosis via Rab5, respectively (10, 11). Recently, IRSp53 has been demonstrated as an Eps8-binding protein whose complex with Eps8 reinforces Rac activation and cell migration in fibrosarcoma cells (12). Besides, Eps8 is also identified as an actin capper, which is capable of regulating actin-based motility (13).

Focal adhesion kinase (FAK) is an intracellular tyrosine kinase localized prominently within focal adhesion (14, 15) and participates in a variety of integrin-elicited biological activities including cell adhesion, migration, growth, and survival (16, 17). Upon integrin engagement, FAK becomes activated and autophosphorylated at Tyr-397, which mediates the SH2-dependent binding of Src family tyrosine kinases. Through complex with FAK, Src mediates its phosphorylation at Tyr-407, -576/577, -861, and -925 (18, 19). Although Pi-Tyr-576/577 and Pi-Tyr-861 individually enhance FAK trans- and cis-phosphorylation activity (20), Pi-Tyr-925 provides the binding site for Grb2 SH2 domain and triggers Ras signaling (21, 22). In addition, FAK may interact with other signaling molecules, including phosphatidylinositol-3-OH kinase Cas, paxillin, Graf, and Asap1 resulting in cell adhesion and migration (16). Overexpression of FAK has been reported in many types of tumors and is strongly correlated with tumor invasiveness (23–25). However, its underlying mechanism is still vague.

As one of the ten leading cancers, colorectal cancer (CRC), is also one of the major causes of cancer mortality regardless of...
sex difference worldwide (26). Epidemiological studies indicate that the genetic defects in APC gene and genes involved in mismatch repair (i.e. hMSH2, hMLH1, and hPMS2) constitute the majority of inherited CRC (27). Albeit no specific lesions have been identified in the remaining sporadic cases, activation of k-ras oncogene, inactivation of tumor suppressor genes, including APC, p53, and DCC, and elevated expression and/or activity of Src (28) and FAK (24) are reported to associate with the progression of colorectal adenoma to invasive carcinoma. Although augmented expression of FAK causes abnormal cell growth (29, 30), its implication in human CRC still needs to be corroborated.

Given that Eps8 is an important player in proliferation and motility, its role in human epithelium-derived cancers is still unclear. In this study, we demonstrated the contribution of Eps8 in human colon cancer. Concurrent with its pivotal role in both cell proliferation and motility in colonocytes, preferentially increased Eps8 in the advanced stage of human CRC specimens was detected. Consistent with the concomitant enhancement of FAK and Eps8 observed in tumor specimen, we demonstrated that Eps8-induced FAK expression could be mediated through mTOR/STAT3 Pi-Ser-727. In addition, Eps8 also elevated the activity of Src, and increased serum-dependent FAK activity. Because forced expression of FAK in Eps8 knockdown cells restored cellular growth and motility, we concluded that Eps8-elicited mitogenesis and locomotion was partly attributable to FAK. Collectively, we not only indicated the involvement of Eps8 in colon tumor formation and progression but also revealed its effect on the expression and activity of FAK.

EXPERIMENTAL PROCEDURES

Cells and Lysate Preparation—Five human colon cancer cell lines (i.e. SW480, SW620, LS174T, WiDr, and HCT116) were used in this study. Cultured cells were lysed in modified radio-immune precipitation assay buffer as described previously (4), whereas clinical specimens and tumors recovered from cancer cell-inoculated mice were prepared as described before (31).

Antibodies and Immunoblotting—Antibodies against Eps8 (C-Eps8) or FAK (CTF3) were described previously (4, 20). The primary antibodies used were actin, Eps8, FAK (A-17), Myc (9E10), and STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA); Paxillin, Cas, and Shc (BD Transduction Laboratories); Pi-Y576 FAK and Pi-Y861 FAK (BIOSOURCE International); Pi-Y397 FAK, Src (GD11), Pi-Y416 Src, and Pi-Y317 Shc (Upstate); and Pi-Y527 Src, Pi-Y705 STAT3, Pi-Y727 STAT3, Pi-T389 p70 S6K, p70 S6K, Pi-S2448 mTOR, mTOR, Pi-Y473 Akt, Akt, and cyclin D1 (Cell Signaling Technology, Beverly, MD). Western immunobLOTS were performed as described pre-
Previously (6). The intensity of each detected band in clinical specimens was quantified by densitometry. For semiquantitation and comparison of the amount of the proteins of interest among the collected specimens, various amounts (0.5, 1, and 2) of lysate of a specific patient with tremendous amounts of Src, Eps8, and FAK were included as a reference. After normalization, the relative expression of interested proteins in various samples can be determined.

**Tumorigenicity**—Approximately 10^7 cells in 0.1 ml of sterile phosphate-buffered saline were injected subcutaneously into the hip of 4- to 6-week-old male nude mice (BALB/c-nu). Mice were checked every 2 or 3 days after 1-week inoculation, and the tumor formed was measured as described previously (6, 32).

After 3–4 weeks, mice were sacrificed, and the tumors were excised and weighed. The mouse experiments were performed according to the ethical guidelines for laboratory animal use and approved by the Institutional Animal Study Committee.

**Plasmid Construction and Cell Line Generation**—Plasmid pCMV-Myc-Eps8 was described previously (6). Plasmids pCMV-Myc-FAK and pCMV-Myc-FRNK were constructed by PCR amplification utilizing chicken fak or fnk cDNA (14, 33, 34) as a template, and the resulting DNA fragments were individually inserted into pCMV(Myc) followed by DNA sequence verification. The construction of eps8 siRNA expression construct, pS-hEps8 (5’-GAT CCC GCT GTG ATG CAT TCA TGC ATT CAA GAG ATG CAT GAA TGC ATC ACA

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**FIGURE 2. Eps8 promotes tumor growth of colon cancer cells in nude mice.** Equal numbers of cells (1 × 10^7) of SW480 and its derivative (i.e. Myc-Eps8-4) or SW620 and its derivatives (i.e. Ctrl-1, siRNA-1, and siRNA-2) were inoculated into nude mice (n = 6 in panel A and n = 5 in panel B). 1 week later, the size of each tumor was measured every 2 or 3 days and plotted as described under “Experimental Procedures” (bottom). Around 4 (left) or 3 (right) weeks later, the tumors were excised, photographed (top), and weighed (middle). The weights of the tumors excised are means ± S.D. in two independent experiments. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
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GCT TTT TTG GAA A-3′), was similarly carried out as described previously (7) according to human eps8 cDNA sequence (GenBank™ accession number: U12535).

To generate cells expressing eps8 siRNA (siRNA) or the nonspecific siRNA (Ctrl), SW620 and WiDr cells were transfected with DNA of pS-hEps8 or the negative control pSilencer plasmid (which contains sequences not present in human genome and provided by Ambion Inc.) by the Lipofectamine Plus method (Invitrogen) followed by hygromycin selection (7). To generate control cells (Vec) and cells expressing Myc-FAK and/or Myc-Eps8, SW480 cells or SW620 cells expressing eps8 siRNA were transfected with pBabe (puro) alone or pBabe (puro) plus pCMV-Myc-FAK and/or pCMV-Myc-Eps8 followed by puromycin selection. To generate cells transiently expressing vector or dominant negative STAT3 (i.e. STAT3F), SW620 cells were transfected with pBabe (puro) alone or pBabe (puro) and a plasmid encoding STAT3 Phe705 (35) followed by puromycin selection until they were used.

Reverse Transcriptase-PCR (RT-PCR)—The amount of fak and gapdh transcript was semiquanti-tated by RT-PCR as previously described (31). For the measurement of eps8 transcript, a pair of its specific primers (sense strand, 5′-AAG TGA AAG ACA CAA TGA ATG GTC-3′; antisense strand, 5′-AGG TGT TTC TTC CTG CTC ATG ATA CTG-3′) was used in addition to gapdh specific primers in the reaction mixture. The PCR reaction was carried out in the same condition as fak (31).

Wound Healing Assay—Conflu-ent cells were pretreated with 2 mM hydroxyurea for 24 h followed by scraping the cell layer with a 200-μl pipette tip to generate a wound. The dishes were incubated in fresh regular culture medium containing hydroxyurea (2 mM), and then 30 min later (0 h), three wounded areas in each dish were marked on the surface and photographed, and
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RESULTS

Eps8 Promotes Both Proliferation and Tumorigenesis in Human Colon Cancer Cells—Eps8 expression and cell proliferation of five human colon cancer cell lines (SW480, SW620, LS174T, WiDr, and HCT116) were analyzed. Notably, high Eps8 expressors (i.e. SW620, LS174T, and WiDr) tended to grow more rapidly than those with low Eps8 expression (i.e. SW480 and HCT116) (Fig. 1A). To verify that the amount of Eps8 did play a critical role in proliferation in colon cancer cells, plasmids expressing nonspecific siRNA or eps8-specific siRNA were introduced into SW620 and WiDr cells to obtain the corresponding Ctrl and siRNA cells. As shown in Fig. 1B, Eps8 attenuation resulted in a significant reduction in cellular growth in both cells. In contrast, ectopically expressed Eps8 in SW480 cells (Myc-Eps8-4 and Myc-Eps8-9) or Eps8-attenuated SW620 cells (siRNA-3) resulted in augmented proliferation (Figs. 1C and 4B). These results indicated the importance of Eps8 in mitogenesis of colon cancer cells. To corroborate the involvement of Eps8 in colon tumorigenesis, we observed that, relative to control, there was a significant (>50%) reduction of anchorage-independent growth in eps8 siRNA-expressing SW620 cells, and reintroduction of Eps8 rescued these defects (see Fig. 4, D and E). Consistently, Eps8 attenuation (or overexpression) can significantly reduce (or promote) the growth of tumors inoculated into nude mice (Fig. 2). However, we point out that the failure of SW480-derived Myc-Eps8-4 cells to elevate their in vivo tumorigenic ability to that of SW620 cells (Fig. 2) implicated that Eps8 was not the only determinant responsible for the low malignancy observed in SW480 cells.

Eps8 Increased the Activation of Src and Serum-induced FAK Pi-Tyr-397 and Pi-Tyr-861—Mounting evidence indicated that occupied SH3 and/or SH2 domains of Src result in its activation. Because Eps8 interacts with Src SH3 in vitro (4), its overexpression might activate Src. To verify this point, the activity of Src in Eps8-attenuated SW620 cells was determined. As depicted in Fig. 3A, diminished Eps8 reduced the activity of Src, which was reflected by its decreased autophosphorylation on Tyr-416. In addition, ectopically expressed Eps8 restored its activation. Consistently, increased Src Pi-Tyr-527 (a negative regulator) and Shc Pi-Tyr-317 (a Src substrate) were detected in eps8 siRNA cells (Fig. 3A). Furthermore, a significant reduction of serum-induced FAK activity (as reflected by Pi-Tyr-397 and Pi-Tyr-861) was detected in Eps8 knockdown cells as compared with control (Fig. 3, B and D). Similar results were also observed in WiDr cells (data not shown). To further verify this result, we performed a rescue experiment. Suppressed serum-induced FAK Pi-Tyr-397 and Pi-Tyr-861 was observed in siRNA-3 and siRNA-3/Vec cells, which could be recovered by the restored expression of Eps8 in siRNA-3 cells (Fig. 3, C and E). These findings indicated that Eps8 was able to increase Src activity and phosphorylation of FAK Tyr-397 and Tyr-861. It is worthy to note that, in addition to tyrosyl phosphorylation, the amount of FAK per se was also decreased in Eps8-attenuated cells (Fig. 3, B and C).

Eps8 Modulates FAK Expression Required for Proliferation and Anchorage-independent Growth of Colorectal Cancer Cells—The intimate correlation of Eps8 and FAK observed in Fig. 3 (B and C) and the concomitant expression of these two proteins detected in Fig. 1A raised a possibility that the expression of FAK might be modulated by Eps8 or vice versa. To clarify this issue, the levels of FAK in high (SW620 and WiDr) and low (SW480) Eps8-expressing cells were examined. As shown in Fig. 4A, diminished Eps8 in SW620 cells markedly suppressed the expression of FAK but not that of Cas and paxillin. This attenuated Eps8-caused FAK reduction was also observed in WiDr cells. By contrast, Eps8 overexpression in SW480 cells could significantly augment FAK expression. Intriguingly, enhanced FAK did not affect the amount of Eps8 (Figs. 4C and 5). Because FAK has been implicated in proliferation and its expression was suppressed in cells with diminished Eps8 that exhibited a slower growth rate, we speculated that it was involved in Eps8-mediated proliferation. To answer this question, we measured bromodeoxyuridine incorporation in SW620 and WiDr cells harboring vector alone or DNA construct encoding dominant negative FAK (i.e. FRNK). We observed that FRNK could greatly suppress cell proliferation (data not shown). This confirmed the role of FAK in the mitogenic pathway of colon cancer cells. Furthermore, ectopically expressed FAK could alleviate the suppression of mitogenesis and colony-forming ability in soft agar of Eps8-attenuated SW620 cells (Fig. 4, C and D). Remarkably, both the number and size of colonies were increased in response to FAK overexpression (Fig. 4, D and E). To examine whether FAK overexpression alone could elicit cell growth of colon cancer cells in soft agar, SW480-based Eps8, or FAK single overexpressors as well as Eps8/FAK double overexpressors were generated. As demonstrated in Fig. 5, overexpression of Eps8, but not FAK, increased the ability of SW480 to form colonies in soft agar. In addition, a significantly increased number of colonies was observed in Eps8-attenuated cells. These findings indicated that Eps8 was able to increase Src activity and phosphorylation of FAK Tyr-397 and Tyr-861. It is worthy to note that, in addition to tyrosyl phosphorylation, the amount of FAK per se was also decreased in Eps8-attenuated cells (Fig. 3, B and C).
detected in cells overexpressing Eps8 and FAK both. Thus, by up-regulating FAK, Eps8 could promote proliferation- and anchorage-independent growth in colon cancer cells.

Eps8 Forms a Complex with FAK and Regulates Cell Migration—Co-immunoprecipitation experiments conducted in SW620 cells revealed the presence of Eps8 and FAK in the

FIGURE 4. Eps8 modulates the expression of FAK that is involved in proliferation of colon cancer cells. A, lysates (100 μg) of indicated cells were resolved by SDS-PAGE and immunoblotted with antibodies as indicated. B, SW620, Ctrl-1, siRNA-3, and siRNA-3 bearing vector (Vec) or Myc-Eps8 (Eps8) were described above. C, the generation of siRNA-1 cells (left) or siRNA-3 cells (right) harboring vector alone (Vec) or plasmid encoding Myc-FAK (FAK) were described under “Experimental Procedures.” The expression of Eps8, FAK, and actin in these cells was determined by Western immunoblotting (top). Exogenous Myc-Eps8 and -FAK are marked with an asterisk and an arrow, respectively. For proliferation (bottom), see Fig. 1 D, for colony formation in soft agar, 3 × 10^4 cells of each cell line were applied in the top agar at the beginning, and the number of colonies (>8 cells) formed after 2-week incubation was counted and plotted. ***, p < 0.001. The colonies of the indicated cells were photographed (×40), and the representatives are shown (E).
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same immunocomplex (Fig. 6A and supplemental Fig. S1, A and B). This was further confirmed by their partial co-localization in the punctate structures observed in confocal microscope (data not shown). Given that Eps8 is an actin capper and is able to increase Src and FAK activity, therefore, its involvement in colonocyte motility is speculated. Indeed, Eps8 attenuation resulted in decreased cell locomotion, and ectopically expressed FAK could rescue this effect (Fig. 6B). Because DNA synthesis was completely inhibited by hydroxyurea (2 mM), a ribonucleotide reductase inhibitor, present in the medium, the elevated cell migration could not be ascribed to the increased potential of cell proliferation (supplemental Fig. S1C). These data corroborate the importance of Eps8 in colonocyte motility, and FAK is a pivotal player in this process.

Concomitant Overexpression of Eps8, FAK, and Src in the Advanced Stage of Human Colorectal Tumors—To determine the importance of Eps8 in human CRC, a total number of 76 tumor specimens derived from primary colon carcinomas along with their normal counterparts were analyzed by Western immunoblotting. Aberrant Eps8 overexpression was defined as the ratio of its expression in paired tumor (T) and normal mucosa (N) was equal to or greater than 2 (T/N ≥ 2), and 62% of the specimens analyzed exhibited Eps8 overexpression. Remarkably, statistical analysis of fold induction of Eps8 in advanced stages (i.e. Duke Stages C and D; n = 37) was significantly greater than that in early stages (i.e. Duke Stages A and B; n = 39) by Mann-Whitney test (3.1 ± 2.0 versus 2.3 ± 1.7; p = 0.024). Interestingly, simultaneous up-regulation of Eps8, FAK, and Src within these specimens was observed, and to corroborate this correlation, their protein expression in 36 randomly selected tumor specimens among the 76 patients was semi-quantified. Fig. 7A is a representative Western immunoblot with 12 tumor specimens analyzed. For accurate measurement, the specific specimen (Pt. 1, Fig. 7A), which exhibited a significant amount of all these three proteins, was chosen as a reference. After normalization with actin, the relative expression of Eps8, Src, and FAK in each individual sample could be derived (the value of the referenced band was assumed to be 100). The mean values ± S.D. for Eps8, Src, and FAK in these tumor specimens were 36.0 ± 27.3, 26.9 ± 18.1, and 34.4 ± 32.0, respectively. These three proteins were positively correlated as indicated by Spearman rank correlation (Fig. 7B). This result strengthened the association between Eps8 and FAK in colorectal tumors.

Eps8 Modulates Phosphorylation on mTOR Ser-2448 and STAT3 Ser-727, Which Is Required for FAK Expression in SW620 Cells—Because Eps8 regulated the expression of FAK, we further demonstrated the reduction of its transcript in eps8 siRNA cells (Fig. 8A). The concomitant suppression of both eps8 and fak transcripts and the reduction of FAK in actinomycin D-treated SW620 cells (Fig. 8B) implied that Eps8 could regulate FAK at least at the transcription level. STAT3 is a latent transcription factor whose activity is modulated by Pi-Tyr-705 and Pi-Ser-727. Although the former is required for STAT3 dimerization, translocation into nucleus, and DNA binding, the later is essential for full STAT3 activation (36, 37). Because STAT3 is constitutively activated in Src-transformed cells (38) and expression of dominant-negative STAT3 abrogates Src-induced transformation (39, 40), we wondered whether it participates in Eps8-mediated FAK expression in colon cancer cells. As exhibited in Fig. 8C, Eps8 attenuation resulted in diminished STAT3 Pi-Ser-727, whereas Pi-Tyr-705 was unaltered in SW620 cells. Consistently, cyclin D1 (a STAT3 target gene (41)) and Pi-Ser2448 on mTOR (a kinase responsible for Pi-Ser-727 on STAT3 (37)) were also significantly reduced in Eps8 knockdown SW620 cells (Fig. 8C). Because Akt (42) and p70 S6K (43) have been indicated as an upstream and downstream target of mTOR, respectively, we further examined their activity by analyzing Akt Pi-Ser-473 and S6K Pi-Thr-389. Intriguingly, the reduced Akt Pi-Ser-473 and S6K Pi-Thr-389 in Eps8-attenuated SW620 cells implicated the involvement of mTOR in Eps8-mediated FAK induction (Fig. 8C). To further demonstrate the importance of STAT3 in the regulation of FAK expression, SW620 cells harboring vector alone (Vec) or plasmid encoding dominant negative STAT3 (STAT3F) were generated. As shown in Fig. 8D, the protein expression of FAK as well as cyclin D1 was greatly reduced in STAT3F cells relative to control Vec cells. In contrast, the amount of paxillin and actin was unchanged. RT-PCR experi-
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DISCUSSION

In this study, we hypothesize that the high level of Eps8 (Fig. 1A) enhances cell proliferation and motility, indicators of tumor progression, in colon cancer cells via Src and FAK proteins. Results of Eps8 attenuation by siRNA (Figs. 1B, 2, 3, 4A, and 8A) in SW620 and WiDr colon cancer cells, overexpression of exogenous Eps8 (Figs. 1C, 2A, and 4A) in SW480 (a colon cancer cell line with extremely low endogenous Eps8), and reintroduction of Eps8 into an Eps8-deprived SW620-derived cell line (Figs. 3A and 4B) have clearly demonstrated that the change of FAK level and Src Pi-Tyr-416 was dependent on the level of Eps8. Consistently, Shc Pi-Tyr-317, the Src-mediated event, was also inhibited in response to

FIGURE 6. Eps8 is present in a complex with FAK and promotes cell motility of colon cancer cells. A, SW620 cell lysate (2 mg) was immunoprecipitated with indicated antibody. The presence of FAK and Eps8 in FAK (left) or Eps8 (right) immunoprecipitates was revealed by FAK or Eps8 Western immunoblot. IgG: nonspecific rabbit IgG; PI: pre-immunized rabbit serum. B, indicated confluent cells were wounded with a pipette tip. After wounding, culture medium was replaced with fresh medium containing hydroxyurea (2 mM), and wound closure was monitored by microscopy (×40). A representative micrograph for each condition is demonstrated, taken immediately (0 h) or 48 h after wounding (top). Cell motility (bottom) was determined as described under "Experimental Procedures." ***, p < 0.001.

FIGURE 7. Simultaneous overexpression of Eps8, FAK, and Src in colon tumor specimens. A, the protein expression of Eps8, FAK, Src, or actin in 12 colorectal tumor specimens was analyzed by Western immunoblotting with appropriate antibody and quantified by densitometer scanning. For further comparison, the lysate of patient 1 (Pt1, the one with the highest amount of Eps8, FAK, and Src) was chosen as a reference. ***, Spearman r = 0.88 (95% CI, 0.78-0.94; P < 0.0001), Spearman r = 0.63 (95% CI, 0.37-0.80; P < 0.0001), and Spearman r = 0.63 (95% CI, 0.37-0.80; P < 0.0001).
Eps8 attenuation. Furthermore, the reduction of mitogenesis and motility caused by Eps8 attenuation could be alleviated by ectopically expressed FAK (Figs. 4C, 4D, 4E, and 6B). These results indicate that FAK is an important Eps8 target. Indeed, compared with SW480-based cells overexpressing FAK or Eps8 alone, there was a significant increase in anchorage-independent growth in both SW480-expressing Eps8 and FAK (Fig. 5). Also, the correlation of Eps8 level with tumor stages and the concomitant expression of Eps8, FAK, and Src in colon tumor specimens (Fig. 7) further implied the contribution of Eps8-mediated signaling in human colon tumor development.

Overexpression and/or activation of Src and FAK have been attributed to increased cell proliferation, survival, and metastasis (16, 17). By checking their expression simultaneously, we identified hierarchy of mutual regulation among Eps8, Src, and FAK in colon tumor specimens (Fig. 7) further implied the contribution of Eps8-mediated signaling in human colon tumor development.

Albeit eps8 siRNA-expressing tumors were smaller than their counterparts, they still grew (Fig. 2). Interestingly, similar Eps8 levels were detected in tumors excised from mice inoculated with SW620 cells bearing control plasmid or plasmid encoding eps8 siRNA (supplemental Fig. S1E). These results indicated that there was a selective growth of cells that have lost the knockdown, and this might contribute to their continued growth. This finding further supported the importance of Eps8 in tumor growth in vivo.

It is well documented that Src activation can be achieved by its SH3 and/or SH2 occupation (45–47). Our observation of the in vitro association between Eps8 and Src SH3 (4) indicated that
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As an actin capper, Eps8 is able to regulate actin-based cell motility (13). By association with IRSp53, Eps8 enhances cell movement via up-regulation of Rac activity in fibrosarcoma cells (12). However, our failure to observe a significant reduction of serum- or EGF-induced Rac activation in Eps8-deprived SW620 or WiDr cells (data not shown) implied that an Rac-independent pathway might be involved in Eps8-mediated colonocyte migration. FAK, Shc, and its downstream MEK and ERK activation have been implicated in cell motility (48, 49); therefore, Eps8-provoked Src and FAK activation might also partly constitute the mechanism responsible for cancer cell locomotion (Fig. 6B).

In summary, we report that Eps8 is one of the significant players in the progression of colon cancer. Notably, Eps8 could modulate the expression of FAK via mTOR/STAT3, which confer the ability of cells to proliferate and migrate. Consistent with the concomitant expression of Eps8 and FAK in vitro was their simultaneous presence in colon tumor specimens. Because down-regulation of Eps8 could reduce proliferation and motility of colon cancer cells, searching for agents with Eps8 attenuation activity might open a new avenue for colon cancer treatment.

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