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Intestinal Epithelial Cancer Cell Anoikis Resistance: EGFR-Mediated Sustained Activation of Src Overrides Fak-Dependent Signaling to MEK/Erk and/or PI3-K/Akt-1

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ABSTRACT

Herein, we investigated the survival roles of Fak, Src, MEK/Erk, and PI3-K/Akt-1 in intestinal epithelial cancer cells (HCT116, HT29, and T84), in comparison to undifferentiated and differentiated intestinal epithelial cells (IECs). We report that: (1) cancer cells display striking anoikis resistance, as opposed to undifferentiated/differentiated IECs; (2) under anoikis conditions and consequent Fak down-activation, cancer cells nevertheless exhibit sustained Fak–Src interactions and Src/MEK/Erk activation, unlike undifferentiated/differentiated IECs; however, HCT116 and HT29 cells exhibit a PI3-K/Akt-1 down-activation, as undifferentiated/differentiated IECs, whereas T84 cells do not; (3) cancer cells require MEK/Erk for survival, as differentiated (but not undifferentiated) IECs; however, T84 cells do not require Fak and HCT116 cells do not require PI3-K/Akt-1, in contrast to the other cells studied; (4) Src acts as a cornerstone in Fak-mediated signaling to MEK/Erk and PI3-K/Akt-1 in T84 cells, as in undifferentiated IECs, whereas PI3-K/Akt-1 is Src-independent in HCT116, HT29 cells, as in differentiated IECs; and (5) EGFR activity inhibition abrogates anoikis resistance in cancer cells through a loss of Fak–Src interactions and down-activation of Src/MEK/Erk (T84, HCT116, HT29 cells) and PI3-K/Akt-1 (T84 cells). Hence, despite distinctions in signaling behavior not necessarily related to undifferentiated or differentiated IECs, intestinal epithelial cancer cells commonly display an EGFR-mediated sustained activation of Src under anoikis conditions. Furthermore, such sustained Src activation confers anoikis resistance at least in part through a consequent sustenance of Fak–Src interactions and MEK/Erk activation, thus not only overriding Fak-mediated signaling to MEK/Erk and/or PI3-K/Akt-1, but also the requirement of Fak and/or PI3-K/Akt-1 for survival. J. Cell. Biochem. 107: 639–654, 2009.

KEY WORDS: Akt; ANOIKIS; CANCER; COLON; EGFR; ENTEROCYTE; Erk; Fak; IEC; PI3-K; Src; SURVIVAL

Normal epithelial cells undergo apoptosis by a process termed "anoikis" whenever a disruption, or loss, of integrin-mediated cell adhesion occurs [Frisch and Screaton, 2001; Grossmann, 2002; Stupack and Cheresh, 2002; Martin and Vuori, 2004; Reddig and Juliano, 2005; Vachon, 2006]. Integrin-mediated cell adhesion is not only largely responsible for the establishment of a physical link between the extracellular matrix and the cytoskeleton, but also prevents the activation of the common anoikis pathway while at the same time driving the stimulation of various survival-promoting pathways [Frisch and Screaton, 2001; Grossmann, 2002; Stupack and Cheresh, 2002; Martin and Vuori, 2004; Reddig and Juliano, 2005; Mitra and Schlaepfer, 2006; Vachon, 2006; Moschos et al., 2007]. To this effect, survival-promoting cell adhesion signaling often implicates Fak ($p125^{Fak}$), Src ($p60^{Src}$), the MEK1, -2/Erk-1, -2 ($p42^{Erk2}$/$p44^{Erk1}$) pathway, and/or the PI3-K/Akt ($p57^{Akt}$) pathway, depending on the cell and tissue context [Grossmann, 2002; Stupack and Cheresh, 2002; Frame, 2004; Martin and Vuori, 2004; Altomare and Testa, 2005; Reddig

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and Juliano, 2005; Mitra and Schlaepfer, 2006; Vachon, 2006; Berrier and Yamada, 2007; Shaul and Seger, 2007]. In intestinal epithelial cells (IECs), the regulation of survival and anoikis is furthermore subjected to differentiation state-specific control mechanisms [Ménard et al., 2005; Vachon, 2006]. For instance, undifferentiated IECs exhibit a susceptibility to apoptosis/anoikis that is distinct from differentiated ones through: (i) their expression of cell death regulators [e.g., Bcl-2 homologs; Vachon et al., 2000; Gauthier et al., 2001; Ménard et al., 2005; Vachon, 2006]; (ii) selective roles of specific integrins and Fak in the suppression of anoikis [Gauthier et al., 2001; Harnois et al., 2004]; (iii) distinct roles and requirements of the PI3-K/Akt-1 and MEK/Erk pathways in their survival [Gauthier et al., 2001; Dufour et al., 2004; Harnois et al., 2004; Vachon et al., 2002]; and (iv) selective functions of Src in the integrin/Fak-mediated engagement of the MEK/Erk and PI3-K/Akt-1 pathways for the suppression of anoikis [Bouchard et al., 2007, 2008].

In contrast to normal cells, cancer cells display an anchorage-independence for growth in addition to exhibiting anoikis resistance [Liotta and Kohn, 2004; Mitra and Schlaepfer, 2006; Moschos et al., 2007]—and intestinal epithelial (colon) cancer cells are no exception to this rule [Windham et al., 2002; Ménard et al., 2005; Le Tourneau et al., 2007]. However, the signaling bases, which underlie anoikis resistance in colon cancer cells, remain poorly understood. For instance, it has been reported that negating Fak activity alone is not sufficient to abrogate anoikis resistance in colon cancer cells, instead requiring a concomitant inhibition of Src [Golubovskaya et al., 2003]. Although high Src activity has been shown to drive anoikis resistance in transformed rat IECs and human colon cancer cells [Rosen et al., 2001; Loza Coll et al., 2002, 2005; Windham et al., 2002; Golubovskaya et al., 2003], some studies have reported that Src does so through the PI3-K/Akt pathway only [Windham et al., 2002; Golubovskaya et al., 2003], through the MEK/Erk pathway only [Loza Coll et al., 2002], or through both pathways [Loza Coll et al., 2005]. Conversely, it has been reported that the expression of a constitutive active MEK1 mutant confers anchorage-independent growth in rat IECs [Boucher et al., 2004]. In light of our previous studies which have shown that Src acts as a cornerstone for the Fak-mediated engagement of MEK/Erk and PI3-K/Akt-1 in undifferentiated human IECs, whereas PI3-K/Akt-1 becomes Src-independent (but remains Fak-dependent) in differentiated ones [Bouchard et al., 2007, 2008], and considering that IEC survival and anoikis are subjected to differentiation state-distinct mechanisms, the question therefore remains open as to the roles enacted by Fak, Src, MEK/Erk, and PI3-K/Akt-1 in the survival of human intestinal epithelial cancer cells—including whether the signaling behavior of such "dedifferentiated" [Zweibaum and Chantret, 1989] cells is akin to undifferentiated or differentiated IECs.

Another aspect of anoikis resistance in cancer cells, including colon cancer ones, is the role played by an autocrine-driven EGF/TGF-α receptor (EGFR) activity in this process [Henson and Gibson, 2006; Normanno et al., 2006]. Human intestinal epithelial cancer cells can produce abundant EGF/TGF-α [Anzano et al., 1989; Ciardiello et al., 1991; Howell et al., 1998; McCole et al., 2001; Uribe et al., 2002; Thomson et al., 2003]. In addition, EGFR is frequently expressed in epithelial cancers, including colon cancer [Mao et al., 1997; Karnes et al., 1998; Danielsen and Maihle, 2002; Thomson et al., 2003; Henson and Gibson, 2006; Normanno et al., 2006]. To this effect, exogenous TGF-α was shown to protect rat IECs against anoikis [Rosen et al., 2001], whereas the inhibition of EGFR has been reported to induce apoptosis in human colon cancer cells [Karnes et al., 1998; Wang et al., 1998].

Consequently, we investigated the anoikis susceptibility and cell survival signaling bases implicated in three "dedifferentiated" intestinal epithelial (colon) cancer cells which are good-to-high expressors of EGF/TGF-α (HCT116, HT29, and T84), in comparison to the "enterocyte-like" Caco-2/15 colon cancer-derived cells (in their undifferentiated and differentiated states) and two cell models of normal undifferentiated (human intestinal epithelial crypt, HIEC) and differentiated (pure culture differentiated enterocytes, PCDE) human IECs. Herein, we report that despite distinctions in signaling behavior not necessarily related to undifferentiated or differentiated IECs, intestinal epithelial cancer cells which are good-to-high expressors of EGF/TGF-α commonly display an EGFR-mediated sustained activation of Src under anoikis conditions. Furthermore, such sustained Src activation confers anoikis resistance at least in part through a consequent sustenance of Fak–Src interactions and MEK/Erk activation, thus not only overriding Fak-mediated signaling to MEK/Erk and/or PI3-K/Akt-1, but also the requirement of Fak and/or PI3-K/Akt-1 for survival.

MATERIALS AND METHODS

MATERIALS

Specific antibodies directed against $p125^{Fak}$, the phosphotyrosine397 activated form of $p125^{Fak}$ ($^{pY397}p125^{Fak}$), the Src-phosphorylated tyrosine 576 and 577 residues of $p125^{Fak}$ ($^{pY576/577}p125^{Fak}$), $p57^{Akt-1}$, the phosphoserine473 activated form of $p57^{Akt-1}$ ($^{pS473}p57^{Akt-1}$), $p42^{Erk2}/p44^{Erk1}$, the doubly phosphorylated (phosphothreonine202 and phosphotyrosine204) activated forms of $p42^{Erk2}/p44^{Erk1}$ ($^{p(T202,Y204)}p42^{Erk2}/p44^{Erk1}$), $p60^{Src}$, and the phosphotyrosine418 activated form of $p60^{Src}$ ($^{pY418}p60^{Src}$) were used as described previously [Gauthier et al., 2001; Dufour et al., 2004; Bouchard et al., 2007, 2008] and were purchased from Cell Signaling Technology (Beverly, MA) and/or Upstate Biotechnology (Lake Placid, NY). Also used were antibodies directed to $p170^{EGFR}$ (Ab610016; BD Biosciences, Mississauga, ON) and the phosphotyrosine1173 activated form of $p170^{EGFR}$ ($^{pY1173}p170^{EGFR}$; mAb53A5; Cell Signaling Technology). All other materials were purchased from Sigma (Oakville, ON, Canada) and/or Fischer Scientific (St. Laurent, QC, Canada), except where otherwise specified.

CELL CULTURE

Two normal, non-transformed and non-immortalized human IEC models were used in this study, one representing proliferating/undifferentiated crypt cells and the other representing non-proliferating/differentiated villus cells:

(A) The HIEC cell strain model consists of pure, proliferating undifferentiated crypt cells which do not undergo enterocytic differentiation [Pageot et al., 2000]. These cells were cultured

and used as described previously [Gauthier et al., 2001; Harnois et al., 2004; Bouchard et al., 2008].

(B) The PCDE cell model consists of primary cultures of pure, freshly isolated, fully differentiated (and thus non-proliferating) monolayers of villus enterocytes [Pageot et al., 2000]. These cells were cultured/maintained and used as described previously [Gauthier et al., 2001; Vachon et al., 2002].

Four human colon cancer cell lines were used in the present study, each with varying polarizing/differentiating capabilities and with EGF/TGF-α autocrine expression/secretion properties:

(A) The enterocyte-like Caco-2/15 cells undertake, under normal culture conditions (i.e., with glucose and 10% serum), a full morphological and functional enterocytic differentiation process which takes place spontaneously once confluence (0 day post-confluence, 0PC) has been reached and which is completed after 25–30PC, thus resulting in a monolayer of PCDE-like cells [Zweibaum and Chantret, 1989; Vachon and Beaulieu, 1992; Pageot et al., 2000]. Caco-2/15 cells are considered very poor expressors of EGF/TGF-α [Beaulieu and Quaroni, 1991; Cross and Quaroni, 1991]. These cells were cultured/maintained as previously described [Vachon and Beaulieu, 1992; Gauthier et al., 2001; Dufour et al., 2004; Bouchard et al., 2007] and used as either proliferating/undifferentiated (−2PC) or non-proliferating/fully differentiated (30PC).

(B) The T84 cells, under normal culture conditions, form a monolayer of polarized intestinal epithelial cells upon reaching confluence without, however, being able to undergo enterocytic differentiation [Zweibaum and Chantret, 1989]. T84 cells are considered as good expressors of EGF/TGF-α [McCole et al., 2001; Uribe et al., 2002]. These cells were cultured as Caco-2/15 cells and used as (newly confluent) monolayers of non-proliferating/polarized cells.

(C) The HT29 cells under normal culture conditions grow in multi-layers and do not undergo polarization or enterocytic differentiation, although they can be induced/adapted to do so in absence of glucose [Zweibaum and Chantret, 1989]. HT29 cells are considered as strong expressors of EGF/TGF-α [Anzano et al., 1989; Wang et al., 1998]. These cells were cultured as Caco-2/15 cells and used as proliferating/non-differentiating (newly confluent) cells.

(D) The HCT116 cells under normal culture conditions grow in multi-layers and are incapable of polarization or enterocytic differentiation [Zweibaum and Chantret, 1989]. HCT116 cells are considered as high expressors of EGF/TGF-α [Ciardiello et al., 1991; Howell et al., 1998; Wang et al., 1998]. These cells were cultured as Caco-2/15 cells and used as proliferating/non-differentiating (newly confluent) cells.

The above-mentioned cell models were separated into three groups according to their cancerous origin (or not), their state of differentiation in culture, and/or incapacity to differentiate under normal culture conditions, namely undifferentiated IECs (HIEC, Caco-2/15, −2PC), differentiated IECs (PCDE, Caco-2/15, 30PC), and intestinal epithelial cancer cells (T84, HT29, HCT116).

For experiments, all cell cultures were maintained 0–24 h in medium without serum (controls) or with (i) 1 μM cytochalasin D (CD; Sigma), which has been shown to inhibit Fak at this concentration range without affecting actin filament polymerization [Wary et al., 2005]; (ii) 30 μM Ly294002 (Calbiochem, San Diego, CA), for the inhibition of PI3-K; (iii) 20 μM PD98059 (Calbiochem), for the inhibition of MEK1/-2; (iv) 20 μM PP2 (Calbiochem), for the inhibition of Src; or (v) 25 μM PD153035 (Calbiochem), for the inhibition of the tyrosine kinase activity of EGFR. The working concentrations of the inhibitors used were determined previously with dose–response assays (not shown). It is noteworthy that control cultures included exposure to the same solvent as that used for inhibitors and showed no significant differences with cultures maintained in serum-free medium only (not shown). Cells were also kept in suspension (''Susp.'') for 0–24 h by either seeding freshly trypsinized cells onto poly-2-hydroxyethyl methacrylate (polyHEMA)-coated dishes, or by detaching intact monolayers of differentiated cells by gentle flushing underneath the monolayer with serum-free medium, as already described [Gauthier et al., 2001; Vachon et al., 2002; Bouchard et al., 2007].

CASPASE-ACTIVATED DNAse (CAD)-MEDIATED DNA LADDERING ASSAYS

DNA was isolated and the visualization of CAD-mediated internucleosomal DNA fragmentation (DNA laddering) was performed as already described [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008].

IN SITU TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TDT)-MEDIATED dUTP NICK-END LABELING (ISEL) ASSAYS

Coverslip-grown/maintained cell cultures were processed and ISEL was then carried out as previously described [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008]. Evaluation of ISEL-positive cells was performed as described elsewhere [Vachon et al., 2000, 2002; Gauthier et al., 2001].

WESTERN BLOTTING (WB)

Cell cultures were lysed in sample buffer and processed as described previously [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008]. Proteins were resolved by SDS–PAGE, electrotransferred, and probed as already described [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008]. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) method (Amersham/Pharmacia, Baie D'Urfé, QC, Canada), according to the manufacturer's instructions. Band intensities were scanned and semi-quantified with Scion Image (Scion, Frederick, MD), as already described [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008].

IMMUNOPRECIPITATION (IP) AND RELATIVE KINASE ACTIVATION ASSAYS

Cell cultures were lysed in cold IP buffer and processed for IP as described previously [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008]. Immunoprecipitates were solubilized in sample buffer, resolved by SDS–PAGE,

and probed by WB (see above). Relative kinase activation analyses were performed as already described [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Bouchard et al., 2007, 2008]. Typically, immunoreactive bands were semi-quantified and the relative activated levels of kinases were established with the ratios phosphorylated kinase/total kinase, which in turn were compared to control cultures $\times$ 100 (expressed as "% of control"). Relative phosphorylated levels of proteins were similarly established.

TRANSFECTIONS AND cDNA CONSTRUCTS

Coverslip-grown/maintained cell cultures were transfected transiently and then processed for ISEL assays, as already described [Vachon et al., 2002; Dufour et al., 2004; Harnois et al., 2004; Bouchard et al., 2007, 2008]. The cDNA constructs used were as follows: $^{WT}p125^{Fak}$, coding for wild-type Fak [Nolan et al., 1999]; $p45^{FRNK}$, coding for the dominant-negative, kinase domain-lacking isoform of Fak [FRNK, "Fak-related nonkinase"; Nolan et al., 1999]; $^{Y397F}p125^{Fak}$, coding for a dominant-negative, non-activable mutant of Fak [Nolan et al., 1999]; $^{WT}p57^{Akt\text{-}1}$, coding for wild-type Akt-1 [Rokudai et al., 2000]; $^{K179A/T308A/S473A}p57^{Akt\text{-}1}$, coding for a dominant-negative, non-activable triple mutant of Akt-1 [Rokudai et al., 2000]; $^{WT}p60^{Src}$ (Upstate Biotechnology), coding for wild-type Src; $^{K297R/Y529F}p60^{Src}$ (Upstate Biotechnology) coding for a dominant negative, non-repressible, kinase dead double mutant of Src; and $^{K297R}p60^{Src}$ (Upstate Biotechnology) coding for a dominant-negative, kinase dead mutant of Src. All constructs have been used previously as described [Vachon et al., 2002; Dufour et al., 2004; Harnois et al., 2004; Bouchard et al., 2007, 2008]. Fak constructs were generous gifts from J.T. Parsons (Health Science Center, University of Virginia, Charlottesville, VA). Transfection controls ("sham-transfected") consisted of transfections of the expression vectors without cDNA inserts.

DATA PROCESSING

Results and values shown represent mean $\pm$ SEM for at least three ($n \geq 3$) separate experiments and/or cultures. Statistically significant differences were determined with the Student's *t*-test. Data were compiled, analyzed, and processed with Excel (Microsoft, Redmond, WA) and Cricket Graph (Computer Associates, Islandia, NY). Except otherwise specified, images from blots, gels, and scans were processed with Vistascan (Umax Technologies, Fremont, CA), Photoshop (Adobe, San Jose, CA), and PowerPoint (Microsoft).

RESULTS

INTESTINAL EPITHELIAL CANCER CELLS DISPLAY MARKED ANOIKIS RESISTANCE

We first assessed the susceptibility or resistance to anoikis of the cells studied by establishing a 0–24 h time-course appearance of apoptosis/anoikis-associated CAD-mediated DNA laddering. As mentioned above, the cell models studied herein were separated into three groups: undifferentiated IECs (HIEC, Caco-2/15, −2PC), differentiated IECs (PCDE, Caco-2/15, 30PC), and intestinal epithelial cancer cells (T84, HT29, HCT116).

Internucleosomal DNA fragmentation in undifferentiated IECs was weakly discernible beginning at 8 h, in order to become intense by 24 h (Fig. 1, lanes 1–5; HIEC cells shown). Although similar kinetics of DNA laddering were observed in differentiated IECs, we found that internucleosomal DNA fragmentation became weakly discernible at 4 h, instead of 8 h for their undifferentiated counterparts, and furthermore displayed greater DNA laddering intensity at 24 h (Fig. 1, lanes 6–9; Caco-2/15, 30PC cells shown). We have previously established that differentiated IECs are more susceptible to anoikis than undifferentiated ones [Gauthier et al., 2001; Vachon et al., 2002; Vachon, 2006; Bouchard et al., 2007] and these results further confirm this. In sharp contrast, intestinal epithelial cancer cells displayed a marked anoikis resistance, as indicated by the emergence of weakly discernible internucleosomal DNA fragmentation by the 12 h time point and distinctively weaker DNA laddering than undifferentiated and differentiated IECs at the 24 h time point (Fig. 1, lanes 10–13; HCT116 cells shown).

Hence, with regards to anoikis susceptibility, these results confirm that undifferentiated Caco-2/15 cells behave as the normal HIEC cells, and that differentiated Caco-2/15 cells behave as the normal PCDE ones, despite their cancerous origin. However, all three intestinal epithelial cancer cells tested (T84, HT29, HCT116) displayed striking anoikis resistance.

CELL ADHESION/FAK SIGNALING TO MEK/ERK AND PI3-K/Akt-1 IN SURVIVAL OF INTESTINAL EPITHELIAL CANCER CELLS

To establish an overall signaling basis for the anoikis resistance displayed by intestinal epithelial cancer cells, the relative activation levels of Fak, Erk1/Erk2, and Akt-1 were analyzed at the 24 h time point following anoikis induction, in comparison to undifferentiated and differentiated IECs. As expected from our previous reports [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Harnois et al., 2004; Bouchard et al., 2007, 2008], the activation of Fak (Fig. 2A, $^{pY397}p125^{Fak}$, Susp.; B, open columns), Erk2 (Fig. 2A, $^{p(T202,Y204)}p42^{Erk2}$, Susp.; B, filled columns), and Akt-1

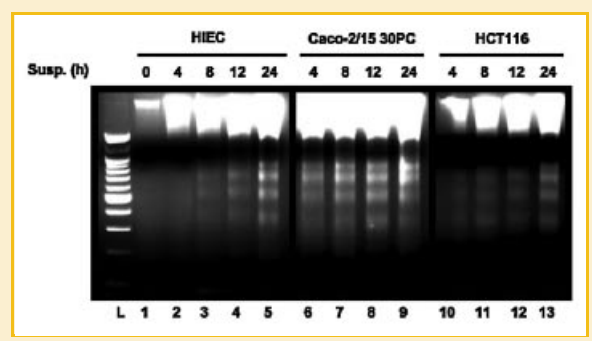

Fig. 1. Intestinal epithelial cancer cells display a marked resistance to anoikis. Representative DNA laddering assays from undifferentiated (HIEC; lanes 1–5) and differentiated (Caco-2/15, 30PC; lanes 6–9) IECs, as well as "dedifferentiated" intestinal epithelial cancer cells (HCT116; lanes 10–13), maintained 0–24 h in suspension (Susp.) in serum-free medium. DNA (20 μg/lane) was isolated, then separated by agarose gel electrophoresis, and stained with EtBr for the visualization of CAD-mediated internucleosomal DNA fragmentation ("DNA laddering"). Similar results were obtained with Caco-2/15, −2PC, PCDE and T84, HT29 cells as shown here for HIEC, Caco-2/15, 30PC, and HCT116 cells, respectively. L, 100-bp DNA size markers.

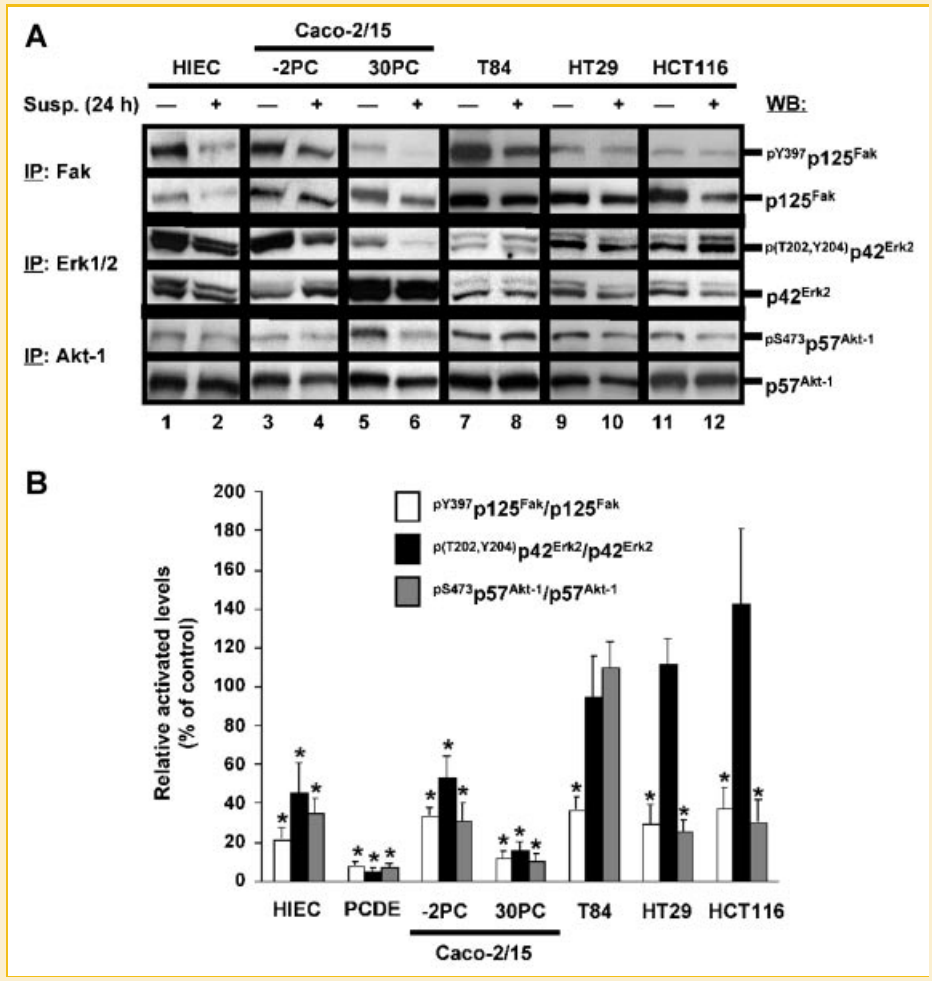

Fig. 2. Impact of anoikis on the activation of Fak, Erk1/Erk2, and Akt-1 in intestinal epithelial cancer cells. A: Representative Western blot analyses of Fak, Erk1/Erk2, and Akt-1 activation from undifferentiated (HIEC, Caco-2/15, −2PC; lanes 1–2 and 3–4, respectively) and differentiated (Caco-2/15, 30PC; lanes 5–6) IECs, as well as intestinal epithelial cancer cells (T84, HT29, HCT116; lanes 7–8, 9–10, and 11–12, respectively), which were maintained 24 h in serum-free medium (controls; lanes 1, 3, 5, 7, 9, 11) or kept in suspension (Susp.; lanes 2, 4, 6, 8, 10, 12). Cells were lysed and Fak, Erk1/Erk2, and Akt-1 were immunoprecipitated (IP) with specific antibodies. Total proteins from immunoprecipitates (50 μg/well) were separated by SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed (WB) with specific antibodies for the detection of the activated phosphorylated forms of Fak ($^{pY397}p125^{Fak}$), Erk1/Erk2 ($^{p(T202,Y204)}p42^{Erk2}$), and Akt-1 ($^{pS473}p57^{Akt-1}$). Membranes were thereafter reprobed with the antibodies used for IP in order to detect the Fak ($p125^{Fak}$), Erk1/Erk2 ($p42^{Erk2}$), and Akt-1 ($p57^{Akt-1}$) proteins. B: Same as in (A), except that PCDE were also analyzed and immunoreactive bands were semi-quantified. The relative activated levels of Fak, Erk-2, and Akt-1 were thereafter established with the ratios $^{pY397}p125^{Fak}/p125^{Fak}$ (open columns), $^{p(T202,Y204)}p42^{Erk2}/p42^{Erk2}$ (filled columns), and $^{pS473}p57^{Akt-1}/p57^{Akt-1}$ (gray columns), which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.005 \leq P \leq 0.05$) differences with control cultures are indicated by asterisk (*).

(Fig. 2A, $^{pS473}p57^{Akt-1}$, Susp.; B, gray columns) were found significantly lowered in both undifferentiated and differentiated IECs, as compared to their respective control cultures. Fak was found likewise significantly down-activated in intestinal epithelial cancer cells (Fig. 2A, $^{pY397}p125^{Fak}$, Susp.; B, open columns), despite their displayed resistance to anoikis (see above). However, T84 cells exhibited a sustained activation of both Erk2 (Fig. 2A, $^{p(T202,Y204)}p42^{Erk2}$, Susp.; B, filled columns) and Akt-1 (Fig. 2A, $^{pS473}p57^{Akt-1}$, Susp.; B, gray columns), whereas HT29 and HCT116 cells also exhibited sustained activated levels of Erk2 (Fig. 2A, $^{p(T202,Y204)}p42^{Erk2}$, Susp.; B, filled columns) but a significant down-activation of Akt-1 (Fig. 2A, $^{pS473}p57^{Akt-1}$, Susp.; B, gray columns).

We then verified whether the MEK/Erk and/or PI3-K/Akt-1 pathways are dependent (at least in part) on cell adhesion/Fak-mediated signaling, by inhibiting Fak in adhering cell cultures. We have previously shown that Fak is required for the survival of both undifferentiated and differentiated IECs, and that the MEK/Erk and PI3-K/Akt-1 pathways are engaged by cell adhesion/Fak-mediated signaling regardless of the state of IEC differentiation [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Harnois et al., 2004; Bouchard et al., 2007, 2008]. Hence, the inhibition herein of Fak with 1 μM CD induced apoptosis (Fig. 3A, open columns), as well as its own down-activation and those of Erk2 and Akt-1 (not shown), in both undifferentiated and differentiated IECs. Although such inhibition likewise caused a significant down-activation of Fak

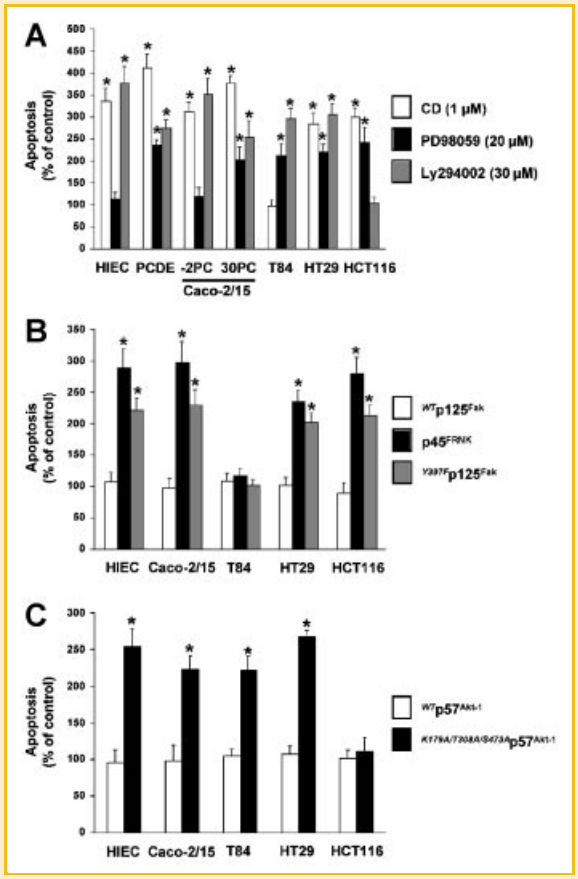

Fig. 3. Roles of Fak, MEK/Erk, and PI3-K/Akt-1 in the survival of intestinal epithelial cancer cells. A: Undifferentiated (HIEC, Caco-2/15, −2PC) and differentiated (PCDE, Caco-2/15, 30PC) IECs, as well as "dedifferentiated" intestinal epithelial cancer cells (T84, HT29, HCT116), were maintained 24 h in serum-free medium (control) with the addition of 1 μM cytochalasin D (CD; open columns), 20 μM PD98059 (filled columns), or 30 μM Ly294002 (gray columns). ISEL was then performed to establish the apoptotic indices, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control cultures are indicated by asterisk (*). B: HIEC, Caco-2/15, T84, HT29, and HCT116 cells were transfected with either one of the following cDNA constructs: wild-type Fak ($^{WT}p125^{Fak}$, open columns), FRNK, a dominant-negative, kinase domain-lacking isoform of Fak ($p45^{FRNK}$, filled columns) or a dominant negative, non-activable mutant of Fak ($^{Y397F}p125^{Fak}$, gray columns). Sham-transfected (empty expression vectors) cells were used as controls. Cells were thereafter kept in serum-free medium for 24 h. ISEL was then performed to establish the apoptotic indices, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control cultures are indicated by asterisk (*). C: Same as in (B), except that the following cDNA constructs were used: wild-type Akt-1 ($^{WT}p57^{Akt-1}$, open columns), or a dominant-negative, non-activable triple mutant of Akt-1 ($^{K179A/T308A/S473A}p57^{Akt-1}$, filled columns).

in all three intestinal epithelial cancer cells studied (not shown), apoptosis induction was observed in HCT116 and HT29 cells but not in T84 cells (Fig. 3A, open columns). These results were confirmed with the forced expression of $p45^{FRNK}$ (Fig. 3B, filled columns) and $^{Y397F}p125^{Fak}$ (Fig. 3B, gray columns), which both induced significant apoptosis as compared to the forced expression of $^{WT}p125^{Fak}$ (Fig. 3B, open columns) or empty expression vectors, in all cells studied except for T84 cells. Furthermore, Fak inhibition in intestinal epithelial cancer cells resulted in distinct impacts on the activation of the MEK/Erk and PI3-K/Akt-1 pathways. Indeed, while both Erk2 and Akt-1 were significantly down-activated in HT29 and HCT116 (not shown), therefore differing somewhat from what is observed when they are kept in suspension (see above), neither pathway was affected significantly in T84 cells (not shown) in accordance to when they are under anoikis conditions (see above).

Hence, these results altogether indicate that intestinal epithelial cancer cells show alterations in the cell adhesion/Fak-mediated engagement of the MEK/Erk and PI3-K/Akt-1 pathways, as compared to undifferentiated and differentiated IECs. Furthermore, intestinal epithelial cancer cells exhibit distinctive signaling behavior to this effect not only depending on whether they are adhering or under anoikis conditions, but also among themselves, including with respect to the requirement of Fak for their survival. Nevertheless, these cells commonly display a sustained activation of MEK/Erk under anoikis conditions.

ROLES OF MEK/Erk AND PI3-K/Akt-1 IN SURVIVAL OF INTESTINAL EPITHELIAL CANCER CELLS

To further understand such signaling behavior in intestinal epithelial cancer cells, we verified whether the MEK/Erk and/or PI3-K/Akt-1 pathways are basically required for their survival by inhibiting these pathways in adhering cell cultures—again, as compared to undifferentiated and differentiated IECs.

As in the case of Fak, our previous studies have shown that the PI3-K/Akt-1 pathway is required for the survival of both undifferentiated and differentiated IECs [Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Harnois et al., 2004; Bouchard et al., 2007, 2008]. To this effect, the inhibition herein of PI3-K activity with 30 μM Ly294002 induced apoptosis (Fig. 3A, gray columns) and a down-activation of Akt-1 (not shown) in undifferentiated and differentiated IECs. Although such inhibition likewise caused a significant down-activation of Akt-1 in all three intestinal epithelial cancer cells analyzed (not shown), apoptosis induction was observed in T84 and HT29 cells, but not in HCT116 cells (Fig. 3A, gray columns). These results were confirmed with the forced expression of $^{K179A/T308A/S473A}p57^{Akt-1}$ (Fig. 3C, filled columns), which induced significant apoptosis as compared to the forced expression of $^{WT}p57^{Akt-1}$ (Fig. 3C, open columns) or empty expression vectors, in all cells studied except for HCT116 cells.

Our previous studies have established that the MEK/Erk pathway is required for the survival of differentiated IECs, but not of undifferentiated ones [Gauthier et al., 2001; Harnois et al., 2004; Bouchard et al., 2007, 2008]. Accordingly, the inhibition herein of MEK activity with 20 μM PD98059 resulted in significant apoptosis (Fig. 3A, filled columns) and down-activation of Erk1/Erk2 (not shown) in differentiated IECs. Although such inhibition likewise caused a significant down-activation of Erk1/Erk2 in undifferentiated IECs (not shown), no induction of apoptosis was observed (Fig. 3A, filled columns). The same inhibition of MEK in all three intestinal epithelial cancer cells studied resulted in a similar induction of apoptosis (Fig. 3A, filled columns), as well as a down-activation of Erk1/Erk2 (not shown), as observed for differentiated

IECs. In addition, the inhibition of MEK sensitized these cells to anoikis (not shown; see for Src below). Wild-type and dominant-negative mutants of MEK-1/-2 were not available at the time these studies were performed.

In any event, these results indicate that intestinal epithelial cancer cells show distinctive requirements for the MEK/Erk and/or PI3-K/Akt-1 pathways in their survival, not necessarily corresponding to those of undifferentiated or differentiated IECs. In addition, such distinctive requirements of the MEK/Erk and/or PI3-K/Akt-1 pathways for survival are not only altered depending on whether they are adhering or under anoikis conditions, but also among themselves—including with respect to the requirement of PI3-K/Akt-1 for their survival.

ROLES OF Src IN SURVIVAL OF INTESTINAL EPITHELIAL CANCER CELLS

Considering the established implication of Src in the cell adhesion/Fak-mediated survival signaling in IECs [Bhattacharya et al., 2006; Bouchard et al., 2007, 2008], we analyzed the relative activation levels of Src in intestinal epithelial cancer cells at the 24 h time point following anoikis induction, in comparison to undifferentiated and differentiated IECs. As expected [Bouchard et al., 2007, 2008], the activation of Src was found significantly lowered (Fig. 4A, $^{pY418}p60^{Src}$; Susp.; B, filled columns) in both undifferentiated and differentiated IECs, as compared to their respective control cultures. However, the three intestinal epithelial cancer cells studied exhibited a sustained activation of Src (Fig. 4A, $^{pY418}p60^{Src}$; Susp.; B, filled columns)—despite a down-activation of Fak [see again Fig. 2A; Bouchard et al., 2007, 2008].

We then verified whether such sustained activation of Src under anoikis conditions nevertheless implicated a sustained participation in (disrupted) cell adhesion/Fak signaling. To do so, the Src-mediated relative phosphorylation levels of Fak on residues Y576/577 were analyzed, such phosphorylation being indicative not only of Src activity but of Fak–Src interactions as well [Playford and Schaller, 2004; Mitra and Schlaepfer, 2006]. As expected from the above observations and from our previous studies [Bouchard et al., 2007, 2008], the Y576/577 phosphorylation of Fak was found significantly lowered (Fig. 5A, $^{pY576/577}p125^{Fak}$; Susp.; B, filled columns) in both undifferentiated and differentiated IECs, as compared to their respective control cultures. However, the three intestinal epithelial cancer cells studied exhibited no significant

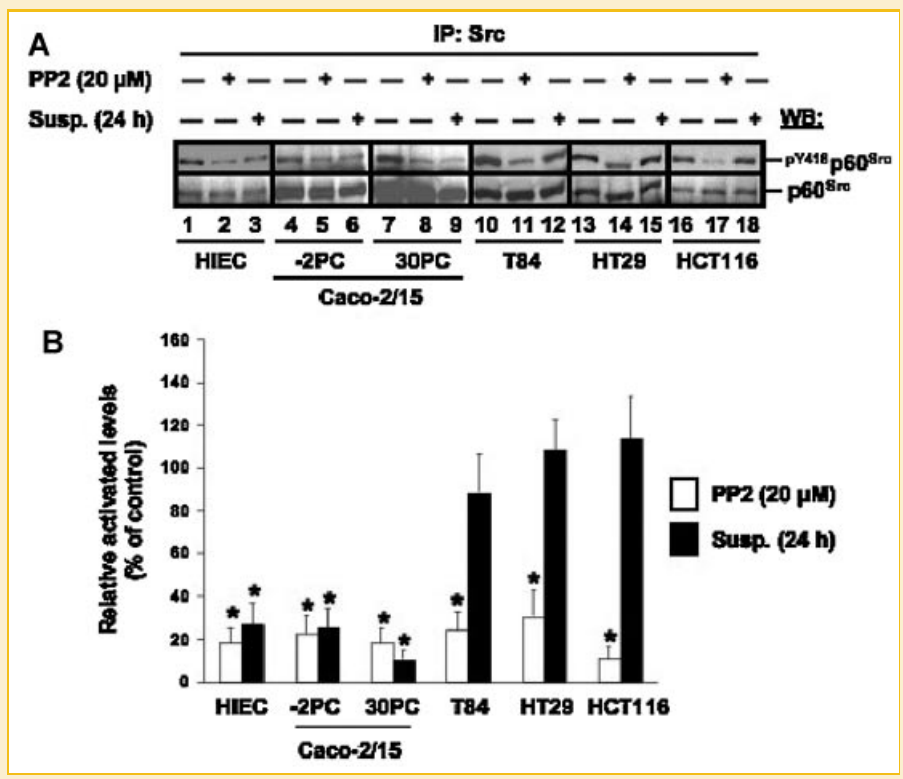

Fig. 4. Impact of anoikis on the activation of Src in intestinal epithelial cancer cells. A: Representative Western blot analyses of Src activation from undifferentiated (HIEC, Caco-2/15, −2PC; lanes 1–3 and 4–6, respectively) and differentiated (Caco-2/15, 30PC; lanes 7–9) IECs, as well as intestinal epithelial cancer cells (T84, HT29, HCT116; lanes 10–12, 13–15, and 16–18, respectively), which were maintained 24 h in serum-free medium (controls; lanes 1, 4, 7, 10, 13, 16) with the addition of 20 μM PP2 (lanes 2, 5, 8, 11, 14, 17), or kept in suspension (Susp.; lanes 3, 6, 9, 12, 15, 18). Cells were lysed and Src was immunoprecipitated (IP) with specific antibodies. Total proteins from immunoprecipitates (50 μg/well) were separated by SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed (WB) with specific antibodies for the detection of the activated phosphorylated form of Src ($^{pY418}p60^{Src}$). Membranes were thereafter reprobed with the antibodies used for IP in order to detect the Src ($p60^{Src}$) protein. B: Same as in (A), except that immunoreactive bands from PP2 (open columns) and suspension (Susp., filled columns) treatments were semi-quantified and the relative activated levels of Src were thereafter established with the ratios $^{pY418}p60^{Src}/p60^{Src}$, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.005 \leq P \leq 0.05$) differences with control cultures are indicated by asterisk (*).

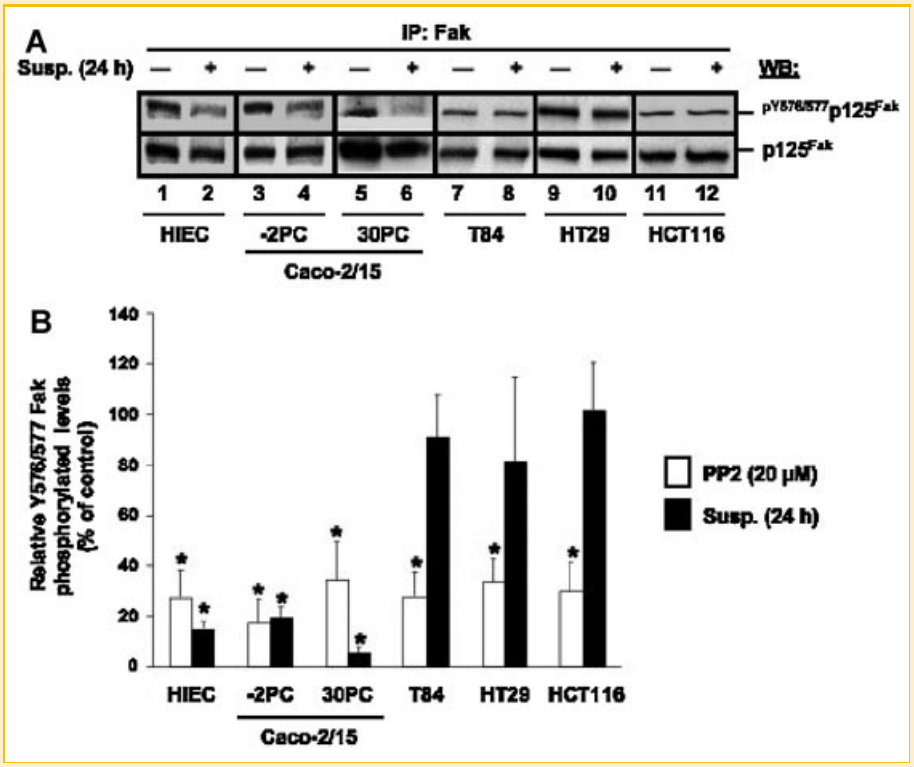

Fig. 5. Impact of anoikis on Fak–Src interactions in intestinal epithelial cancer cells. A: Representative Western blot analyses of the Src-driven phosphorylation of Fak on residues Y576/577 from undifferentiated (HIEC, Caco-2/15, −2PC; lanes 1–2 and 3–4, respectively) and differentiated (Caco-2/15, 30PC; lanes 5–6) IECs, as well as intestinal epithelial cancer cells (T84, HT29, HCT116; lanes 7–8, 9–10, and 11–12, respectively), which were maintained 24 h in serum-free medium (controls; lanes 1, 3, 5, 7, 9, 11) or kept in suspension (Susp.; lanes 2, 4, 6, 8, 10, 12). Cells were lysed and Fak was immunoprecipitated (IP) with specific antibodies. Total proteins from immunoprecipitates (50 μg/well) were separated by SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed (WB) with specific antibodies for the detection of the Src-mediated phosphorylated form of Fak ($^{pY576/577}p125^{Fak}$). Membranes were thereafter reprobed with the antibodies used for IP in order to detect the Fak ($p125^{Fak}$) protein. B: Same as in (A), except that cells were also treated 24 h in serum-free medium with 20 μM PP2. In addition, immunoreactive bands from PP2 (open columns) and suspension (Susp., filled columns) treatments were semi-quantified and the relative Y576/577 phosphorylated levels of Fak were thereafter established with the ratios $^{pY576/577}p125^{Fak}/p125^{Fak}$, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.005 \leq P \leq 0.05$) differences with control cultures are indicated by asterisk (*).

changes in $^{pY576/577}p125^{Fak}$ levels (Fig. 5A, $^{pY576/577}p125^{Fak}$; Susp.; B, filled columns).

The requirement of Src in the survival of intestinal epithelial cancer cells was thereafter analyzed in adhering cell cultures. As expected [Bouchard et al., 2007, 2008], the inhibition of Src activity with 20 μM PP2 induced apoptosis (Fig. 6A), as well as its own down-activation (Fig. 4A, $^{pY418}p60^{Src}$; PP2; B, open columns) and consequent drop of $^{pY576/577}p125^{Fak}$ levels (Fig. 5B, open columns), in both undifferentiated and differentiated IECs. Similarly, such inhibition of Src induced apoptosis (Fig. 6A,C, lane 2 vs. 1), as well as its own down-activation (Fig. 4A, $^{pY418}p60^{Src}$; PP2; B, open columns) and consequent drop of $^{pY576/577}p125^{Fak}$ levels (Fig. 5B, open columns), in all three intestinal epithelial cancer cells studied. These results were confirmed with the forced expression of $^{K297R}p60^{Src}$ (Fig. 6B, gray columns) and $^{K297R/Y529F}p60^{Src}$ (Fig. 6B, filled columns), which both induced significant apoptosis as compared to the forced expression of $^{WT}p60^{Src}$ (Fig. 6B, open columns) or empty expression vectors, in all cells studied herein.

Lastly, we verified whether the noted sustained activation of Src in intestinal epithelial cancer cells contributed in their observed resistance to anoikis. As shown in Figure 6C (lane 4 vs. 3) with HT29 cells as example, the inhibition of Src activity indeed sensitized these cells to anoikis—as in the case when the MEK/Erk pathway is inhibited (see above).

Hence, these results indicate that intestinal epithelial cancer cells show similar requirements for Src in their cell adhesion/Fak-mediated survival as those of undifferentiated or differentiated IECs. However, under anoikis conditions, they commonly exhibit a sustained activation of Src and a consequent sustained Fak–Src interaction, which contribute significantly in their displayed resistance to anoikis.

ROLES OF Src IN CELL ADHESION/Fak SIGNALING TO MEK/Erk AND PI3-K/Akt-1 IN SURVIVAL OF INTESTINAL EPITHELIAL CANCER CELLS

To further understand the implication of the sustenance of Src activation under anoikis conditions in intestinal epithelial cancer cells, especially in light of the previous observations herein with regards to the roles of cell adhesion/Fak-mediated signaling to MEK/Erk and PI3-K/Akt-1, as well as their distinctive requirements for these two pathways in their survival, we verified whether the MEK/

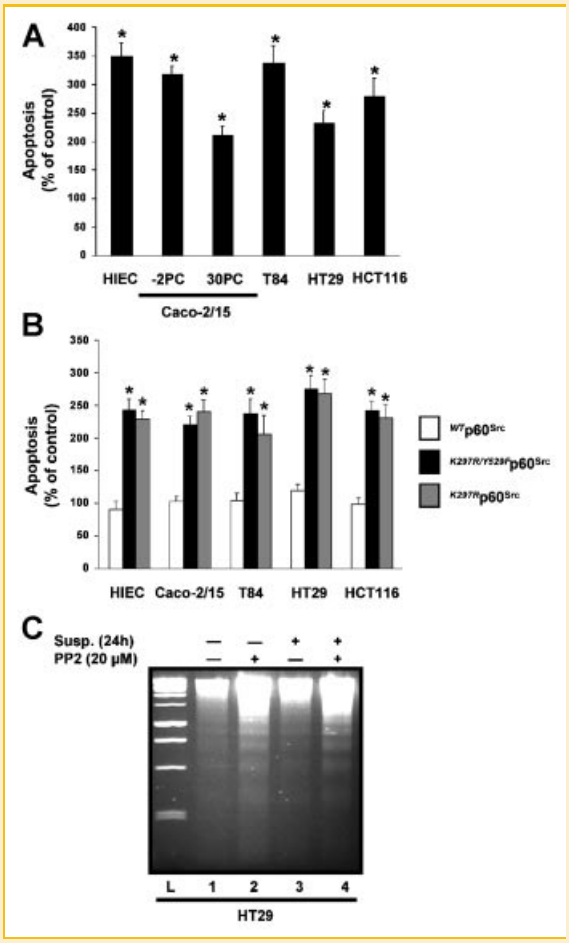

Fig. 6. Roles of Src in the survival of intestinal epithelial cancer cells. A: Undifferentiated (HIEC, Caco-2/15, −2PC) and differentiated (PCDE, Caco-2/15, 30PC) IECs, as well as "dedifferentiated" intestinal epithelial cancer cells (T84, HT29, HCT116), were maintained 24 h in serum-free medium (control) with the addition of 20 μM PP2. ISEL was then performed to establish the apoptotic indices, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control cultures are indicated by asterisk (*). B: HIEC, Caco-2/15, T84, HT29, and HCT116 cells were transfected with either one of the following cDNA constructs: wild-type Src ($^{WT}p60^{Src}$, open columns), a dominant-negative, non-repressible, kinase dead double mutant of Src ($^{K297R/Y529F}p60^{Src}$, filled columns), or a dominant-negative, kinase dead mutant of Src ($^{K297R}p60^{Src}$, gray columns). Sham-transfected (empty expression vectors) cells were used as controls. Cells were thereafter kept in serum-free medium for 24 h. ISEL was then performed to establish the apoptotic indices, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control cultures are indicated by asterisk (*). C: Representative DNA laddering assays from "dedifferentiated" intestinal epithelial cancer cells HT29 maintained in serum-free medium (control; lane 1) with 20 μM PP2 (lanes 2 and 4), and/or kept in suspension (Susp.; lanes 3 and 4). DNA (20 μg/lane) was isolated, then separated by agarose gel electrophoresis, and stained with EtBr for the visualization of CAD-mediated internucleosomal DNA fragmentation ("DNA laddering"). Similar results were obtained with T84 and HCT116 cells. L, 100-bp DNA size markers.

Erk and/or PI3-K/Akt-1 pathways are dependent (at least in part) on Src by inhibiting its activity in adhering cell cultures. We have previously shown that Src performs differentiation state-selective roles in the cell adhesion/Fak-mediated signaling to the MEK/Erk and PI3-K/Akt-1 pathways in IECs, whereby Src acts as a cornerstone for the engagement of both pathways in undifferentiated cells, and whereas PI3-K/Akt-1 is Src-independent in differentiated ones [Bouchard et al., 2007, 2008]. To this effect, the inhibition of Src activity resulted in a significant down-activation of both Akt-1 (Fig. 7A, $^{pS473}p57^{Akt\text{-}1}$, PP2; B, open columns) and Erk2 (Fig. 7A, $^{p(T202,Y204)}p42^{Erk2}$, PP2; B, filled columns) in undifferentiated IECs, but caused a down-activation of Erk2 (Fig. 7A, $^{p(T202,Y204)}p42^{Erk2}$, PP2; B, filled columns) without affecting Akt-1 (Fig. 7A, $^{pS473}p57^{Akt\text{-}1}$, PP2; B, open columns) in differentiated ones. Such inhibition of Src in intestinal epithelial cancer cells produced effects, which were reflective of either undifferentiated or differentiated IECs. Hence, Src inhibition resulted in the down-activation of Erk2 but not Akt-1 in HT29 and HCT116 cells, whereas both were down-activated in T84 cells (Fig. 7A, $^{pS473}p57^{Akt\text{-}1}$, $^{p(T202,Y204)}p42^{Erk2}$, PP2; B, open and filled columns, respectively).

Therefore, these results indicate that intestinal epithelial cancer cells show distinctive roles of Src in the cell adhesion/Fak-mediated engagement of the MEK/Erk and/or PI3-K/Akt-1 pathways, corresponding to those of either undifferentiated or differentiated IECs. Furthermore, such distinctive roles constitute in large part the basis for their observed sustenance (or not) of the activation of these two pathways under anoikis conditions.

Src ACTIVATION SUSTENANCE IN INTESTINAL EPITHELIAL CANCER CELLS UNDER ANOIKIS CONDITIONS IS DRIVEN BY EGFR ACTIVITY

Because the intestinal epithelial cancer cells studied herein are good-to-high expressors of EGF/TGF-α (see Materials and Methods Section), and considering that Src is also well known for its engagement in growth factor receptor-mediated signaling [Frame, 2004; Playford and Schaller, 2004; Henson and Gibson, 2006], we verified whether EGFR activity is responsible for the observed sustained Src activation and consequent anoikis resistance in those cells.

The inhibition of EGFR activity with 25 μM PD153035 was found to induce apoptosis in all three intestinal epithelial cancer cells studied while adhering (Fig. 8A, lane 2 vs. 1; HT29 cells shown), as expected [Karnes et al., 1998; Giannopoulou et al., 2008]. By contrast, EGFR inhibition does not affect the survival of "enterocyte-like" Caco-2 cells [Giannopoulou et al., 2008], which happen to be very poor secretors of EGF/TGF-α (see Materials and Methods Section).

Incidentally, the inhibition of EGFR activity/activation with PD153035 sensitized all three intestinal epithelial cancer cells analyzed to anoikis (Fig. 8A, lane 4 vs. 3; HT29 cells shown). To this effect, the activation of EGFR was found to be sustained under anoikis conditions (Fig. 8B, $^{pY1173}p170^{EGFR}$, lane 2 vs. 1) and such sustained EGFR activation was severely diminished in presence of PD153035 (Fig. 8B, lane 3 vs. 2).

In addition, the inhibition of EGFR in all three intestinal epithelial cancer cells analyzed while adhering caused a significant down-activation of Src (Fig. 9A, $^{pY418}p60^{Src}$, PD153035; B, open columns). In this respect, the concomitant inhibition of EGFR while keeping all

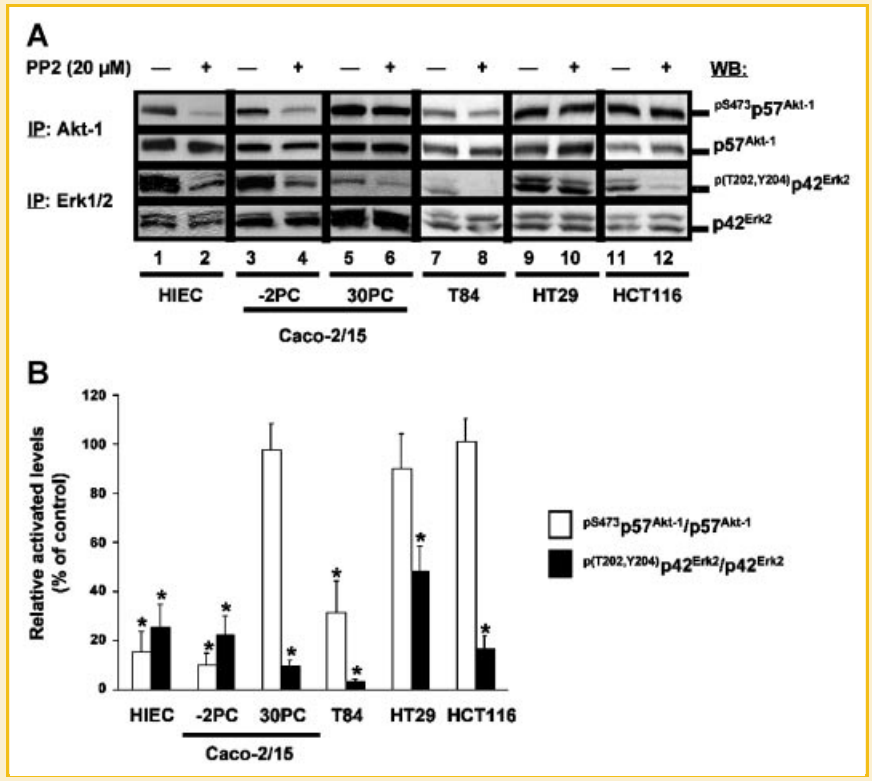

Fig. 7. Roles of Src in the cell adhesion/Fak-mediated engagement of the MEK/Eek and PI3-K/Akt-1 pathways in intestinal epithelial cancer cells. A: Representative Western blot analyses of Akt-1 and Erk1/Erk2 activation from undifferentiated (HIEC, Caco-2/15, −2PC; lanes 1–2 and 3–4, respectively) and differentiated (Caco-2/15, 30PC; lanes 5–6) IECs, as well as intestinal epithelial cancer cells (T84, HT29, HCT116; lanes 7–8, 9–10, and 11–12, respectively), which were maintained 24 h in serum-free medium (controls; lanes 1, 3, 5, 7, 9, 11) with 20 μM PP2 (lanes 2, 4, 6, 8, 10, 12). Cells were lysed and Erk1/Erk2 and Akt-1 were immunoprecipitated (IP) with specific antibodies. Total proteins from immunoprecipitates (50 μg/well) were separated by SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed (WB) with specific antibodies for the detection of the activated phosphorylated forms of Erk1/Erk2 ($^{p(T202,Y204)}p42^{Erk2}$) and Akt-1 ($^{pS473}p57^{Akt-1}$). Membranes were thereafter reprobed with the antibodies used for IP in order to detect the Erk1/Erk2 ($p42^{Erk2}$) and Akt-1 ($p57^{Akt-1}$) proteins. B: Same as in (A), except that immunoreactive bands were semi-quantified and the relative activated levels of Akt-1 and Erk1/Erk2 were thereafter established with the ratios $^{pS473}p57^{Akt-1}/p57^{Akt-1}$ (open columns) and $^{p(T202,Y204)}p42^{Erk2}/p42^{Erk2}$ (filled columns), which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.005 \leq P \leq 0.05$) differences with control cultures are indicated by asterisk (*).

three intestinal epithelial cancer cells in suspension likewise abrogated their sustenance of Src activation (Fig. 9A, $^{pY418}p60^{Src}$, Susp. + PD153035 vs. Susp.; B, gray vs. filled columns).

To confirm these results, we analyzed in turn the Y576/577 phosphorylation state of Fak. In all three intestinal epithelial cancer cells tested, the inhibition of EGFR under anoikis conditions abrogated their sustained Src-mediated $^{pY576/577}p125^{Fak}$ levels (Fig. 10A, $^{pY576/577}p125^{Fak}$, Susp. + PD153035 vs. Susp.; B, open columns, Susp. + PD153035 vs. Susp.), in a similar fashion as that observed in undifferentiated and differentiated IECs under anoikis conditions (see above).

We then verified the activation status of the MEK/Erk and PI3-K/Akt-1 pathways. In T84 cells, whereby Src acts as a cornerstone in the cell adhesion/Fak-mediated engagement of both pathways (see above), the inhibition of EGFR under anoikis conditions abrogated their sustained activation (Fig. 10A, $^{p(T202,Y204)}p42^{Erk2}$, $^{pS473}p57^{Akt-1}$, Susp. + PD153035 vs. Susp.; Fig. 10B, filled and gray columns, respectively, Susp. + PD153035 vs. Susp.). In HT29 and HCT116 cells, whereby MEK/Erk remains Src-dependent but not PI3-K/Akt-1 (see above), the inhibition of EGFR under anoikis conditions abrogated the sustenance of MEK/Erk activation (Fig. 10A, $^{p(T202,Y204)}p42^{Erk2}$, Susp. + PD153035 vs. Susp.; B, filled columns, Susp. + PD153035 vs. Susp.) while the previously observed down-activation of PI3-K/Akt-1 (see above) still occurred (Fig. 10A, $^{pS473}p57^{Akt-1}$, Susp. + PD153035 vs. Susp.; Fig. 10B, gray columns, Susp. + PD153035 vs. Susp.).

These data, altogether with the results reported in the previous sections, indicate that: (A) under anoikis conditions, an autocrine-driven EGFR-mediated sustained activation of Src is largely responsible for anoikis resistance in intestinal epithelial cancer cells; and (B) such illicit EGFR-driven sustained activation of Src confers anoikis resistance through a consequent sustenance of Fak–Src interactions and MEK/Erk activation, thus overriding not only Fak-mediated signaling to MEK/Erk and/or PI3-K/Akt-1, but also the requirement of Fak and/or PI3-K/Akt-1 in cell adhesion-driven survival (Fig. 11).

DISCUSSION

In the present study, we investigated the anoikis susceptibility and cell survival signaling bases implicated in three "dedifferentiated"

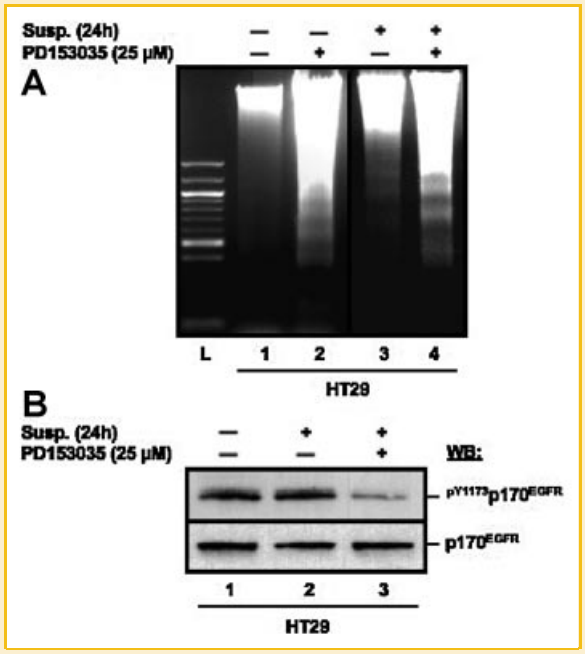

Fig. 8. Roles of EGFR activation/activity in survival and anoikis resistance of intestinal epithelial cancer cells. A: Representative DNA laddering assays from "dedifferentiated" intestinal epithelial cancer cells HT29 maintained in serum-free medium (control; lane 1) with 25 μM PD153035 (lanes 2 and 4), and/or kept in suspension (Susp.; lanes 3 and 4). DNA (20 μg/lane) was isolated, then separated by agarose gel electrophoresis and stained with EtBr for the visualization of CAD-mediated internucleosomal DNA fragmentation ("DNA laddering"). L, 100-bp DNA size markers. B: Representative Western blot analysis of EGFR activation/activity from HT29 cells maintained 24 h in serum-free medium (control; lane 1) with the addition of 25 μM PD153035 (lane 3), and/or kept in suspension (Susp.; lanes 2 and 3). Cells were lysed and total proteins (100 μg/well) were separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and then probed (WB) with specific antibodies for the detection of the activated phosphorylated form of EGFR ($^{pY1173}p170^{EGFR}$). Membranes were thereafter reprobed with specific antibodies for the detection of the EGFR ($p170^{EGFR}$) protein. A,B: Similar results were obtained with T84 and HCT116 cells.

intestinal epithelial (colon) cancer cells which are good-to-high expressors of EGF/TGF-α (HCT116, HT29, and T84), in comparison to undifferentiated (HIEC, Caco-2/15, −2PC) and differentiated (PCDE, Caco-2/15, 30PC) IECs. In the case of T84 cells specifically, we show that Src acts as a cornerstone for the cell adhesion/Fak-mediated engagement of MEK/Erk and PI3-K/Akt-1, as in undifferentiated IECs (Fig. 11A). However, T84 cells require both pathways for survival, as differentiated IECs do. In this context, an autocrine-driven EGFR activity under anoikis conditions allows for the sustenance of both pathways through Src, while maintaining Fak–Src interactions and thus bypassing altogether the requirement of Fak for survival (Fig. 11A). In the case of HT29 and HCT116 cells, MEK/Erk remains Src-dependent whereas PI3-K/Akt-1 does not (but remains Fak-dependent), as in differentiated IECs (Fig. 11B). While HT29 cells require Fak, MEK/Erk, and PI3-K/Akt-1 for their survival, like differentiated IECs, HCT116 do not require PI3-K/Akt-1. Nonetheless, in this context, an autocrine-driven EGFR activity under anoikis conditions allows for the sustenance of MEK/Erk through Src while maintaining Fak–Src interactions, thus bypassing the requirement of Fak and PI3-K/Akt-1 for survival (Fig. 11B). Hence, despite distinctions in signaling behavior not necessarily related to undifferentiated or differentiated IECs, "dedifferentiated" intestinal epithelial cancer cells commonly display an autocrine-driven, EGFR-mediated sustained activation of Src under anoikis conditions. Furthermore, such sustained Src activation confers anoikis resistance at least in part through a consequent sustenance of Fak–Src interactions and MEK/Erk activation, thus overriding not only Fak-mediated signaling to MEK/Erk and/or PI3-K/Akt-1, but also the requirement of Fak and/or PI3-K/Akt-1 in (integrin-mediated) cell adhesion-driven survival (Fig. 11).

The existence of extensive cross-talk between cell adhesion/Fak-mediated signaling and that of growth factor receptors with tyrosine kinase activity (RTKs) is well established [Grossmann, 2002; Stupack and Cheresh, 2002; Reddig and Juliano, 2005; Moschos et al., 2007]. In this respect, there is mounting evidence that illicit RTK signaling, through autocrine stimulation or mutations resulting in constitutive kinase activity, can contribute in conferring resistance to anoikis in cancer cells [Danielsen and Maihle, 2002; Liotta and Kohn, 2004; Playford and Schaller, 2004; Reddig and Juliano, 2005]. EGFR is frequently highly expressed and active (mainly through autocrine stimulation) in epithelial tumors, including colon cancers [Mao et al., 1997; Karnes et al., 1998; Danielsen and Maihle, 2002; Thomson et al., 2003; Henson and Gibson, 2006; Normanno et al., 2006]. While the stimulation of EGFR activity by exposure to exogenous TGF-α has been reported to protect normal rat IECs against anoikis [Rosen et al., 2001], a role for EGFR in suppressing anoikis in human IECs remained to be firmly established, especially when considering the signaling differences between rat and human with regards to IEC survival [Vachon, 2006; Bouchard et al., 2008]. To this effect, we have shown herein that an illicit, autocrine-driven EGFR activity does indeed confer anoikis resistance to human colon cancer cells, which are good-to-high expressors of EGF/TGF-α.

Our findings that EGFR-driven anoikis resistance goes through a sustenance of Src activation confirm a previous study which has shown that colon cancer cells, derived from metastases of the liver, exhibit high Src activity stimulated at least in part by EGFR [Mao et al., 1997]. In addition, our study corroborates previous ones which have linked high Src activity with decreased susceptibility to anoikis in colon cancer cells [Windham et al., 2002], as well as in rat IECs exposed to TGF-α [Rosen et al., 2001] or transformed with v-Src [Loza Coll et al., 2002]. Conversely, the observation herein that Fak–Src interactions are likewise sustained by an illicit EGFR activity not only provides a basis as to how cell adhesion/Fak-mediated survival signaling may be overridden under anoikis conditions, but also explains the previous observation that only a simultaneous inhibition of Fak and Src can abrogate anoikis resistance in colon cancer cells [Golubovskaya et al., 2003]. Indeed, such sustenance of Fak–Src interactions allows for bypassing the requirement of Fak for survival (this study) and for Src to continue performing its functions as proxy for the cell adhesion/Fak-mediated engagement of the MEK/Erk and/or PI3-K/Akt-1 pathways (this study). Additionally, the sustenance of Src activation and Fak–Src interactions under anoikis conditions may prevent the recruitment/activation of Caspase-8 (the initiator caspase for anoikis) about unbound

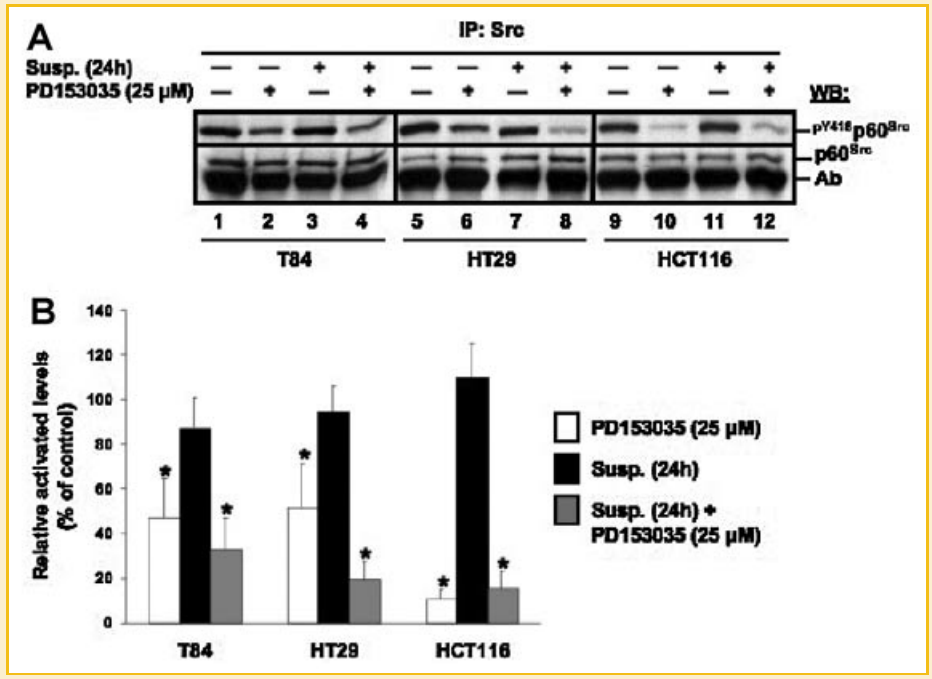

Fig. 9. EGFR activity-mediated activation of Src in intestinal epithelial cancer cells. A: Representative Western blot analyses of Src activation from T84 (lanes 1–4), HT29 (lanes 5–8), and HCT116 (lanes 9–12) cells maintained 24 h in serum-free medium (controls; lanes 1, 5, 9) with the addition of 25 μM PD153035 (lanes 2, 4, 6, 8, 10, 12), and/or kept in suspension (Susp.; lanes 3–4, 7–8, 11–12). Cells were lysed and Src was immunoprecipitated (IP) with specific antibodies. Total proteins from immunoprecipitates (50 μg/well) were separated by SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed (WB) with specific antibodies for the detection of the activated phosphorylated form of Src ($^{pY418}p60^{Src}$). Membranes were thereafter reprobed with the antibodies (Ab) used for IP in order to detect the Src ($p60^{Src}$) protein. B: Same as in (A), except that immunoreactive bands from PD153035 (open columns), suspension (Susp., filled columns), and PD153035 + suspension (gray columns) treatments were semi-quantified and the relative activated levels of Src were thereafter established with the ratios $^{pY418}p60^{Src}/p60^{Src}$, which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.005 \leq P \leq 0.05$) differences with control cultures are indicated by asterisk (*).

integrins [Frisch, 2008]. In any event, these data altogether further support the concept of both EGFR and Src as constituting potential dual, key therapeutic targets for all colon cancer cells [Johnson and Gallick, 2007; Chen, 2008].

The PI3-K/Akt-1 pathway has been so far the most characterized for its roles in cell survival [Altomare and Testa, 2005]. In contrast, much more remains to be understood of the cell survival roles of the MEK/Erk pathway [Shaul and Seger, 2007]. The requirement of PI3-K/Akt-1 and/or MEK/Erk for survival is cell, tissue, and differentiation state context-dependent [Altomare and Testa, 2005; Vachon, 2006; Shaul and Seger, 2007]. In IECs, PI3-K/Akt-1 is crucial for cell survival regardless of the state of differentiation, whereas MEK/Erk is required for the survival of differentiated cells, but not undifferentiated ones [this study; Gauthier et al., 2001; Vachon et al., 2002; Dufour et al., 2004; Harnois et al., 2004; Vachon, 2006; Bouchard et al., 2007, 2008]. In addition, we have recently shown that the non-requirement of MEK/Erk in undifferentiated IECs survival is due to the non-to-marginal involvement of this pathway in the process [Bouchard et al., 2008]. Such differentiation state-distinct roles of the PI3-K/Akt-1 and MEK/Erk pathways in IEC survival are related to the proxy functions of Src in the cell adhesion/Fak-mediated engagement of these two pathways [this study; Fig. 11; Bouchard et al., 2007, 2008]. In this respect, our findings herein indicate that T84, HT29, and HCT116 cells do not necessarily behave as either undifferentiated or differentiated IECs, in addition to exhibiting distinct signaling behavior among themselves.

In light of these above considerations, it is noteworthy that our data roundly account for the signaling behavior of T84 cells with regards to their requirement of Src, MEK/Erk, and PI3-K/Akt-1 for their survival, as well as their non-requirement of Fak to this effect (Fig. 11A). Indeed, the inhibition of EGFR, but not Fak, induces apoptosis in adhering T84 cells, in addition to consequent negative impacts on Fak–Src interactions and activation of Src, MEK/Erk, and PI3-K/Akt-1 (this study). This indicates that the autocrine-driven EGFR signaling in these cells overrides altogether cell adhesion/Fak-dependence for survival not only under anoikis conditions, but even while adhering (Fig. 11A). However, our findings do not account completely for the signaling behavior displayed by HT29 and HCT116 cells. First, the requirement of Fak for the survival of adhering HT29 and HCT116 cells, but not while kept in suspension (Fig. 11B), suggests that the autocrine-driven EGFR signaling in these cells can not override cell adhesion/Fak-dependence survival while they are adhering—in contrast to T84 cells (Fig. 11B). Nevertheless, autocrine EGFR signaling remains functional in adhering HT29 and HCT116 cells, as evidenced by the inhibition of EGFR, which induces apoptosis and the down-activation of Src/MEK/Erk, while they are adhering (this study). This in turn suggests that HT29 and HCT116 cells are both cell adhesion/Fak- and EGFR-dependent for survival when adhering, whereas EGFR signaling

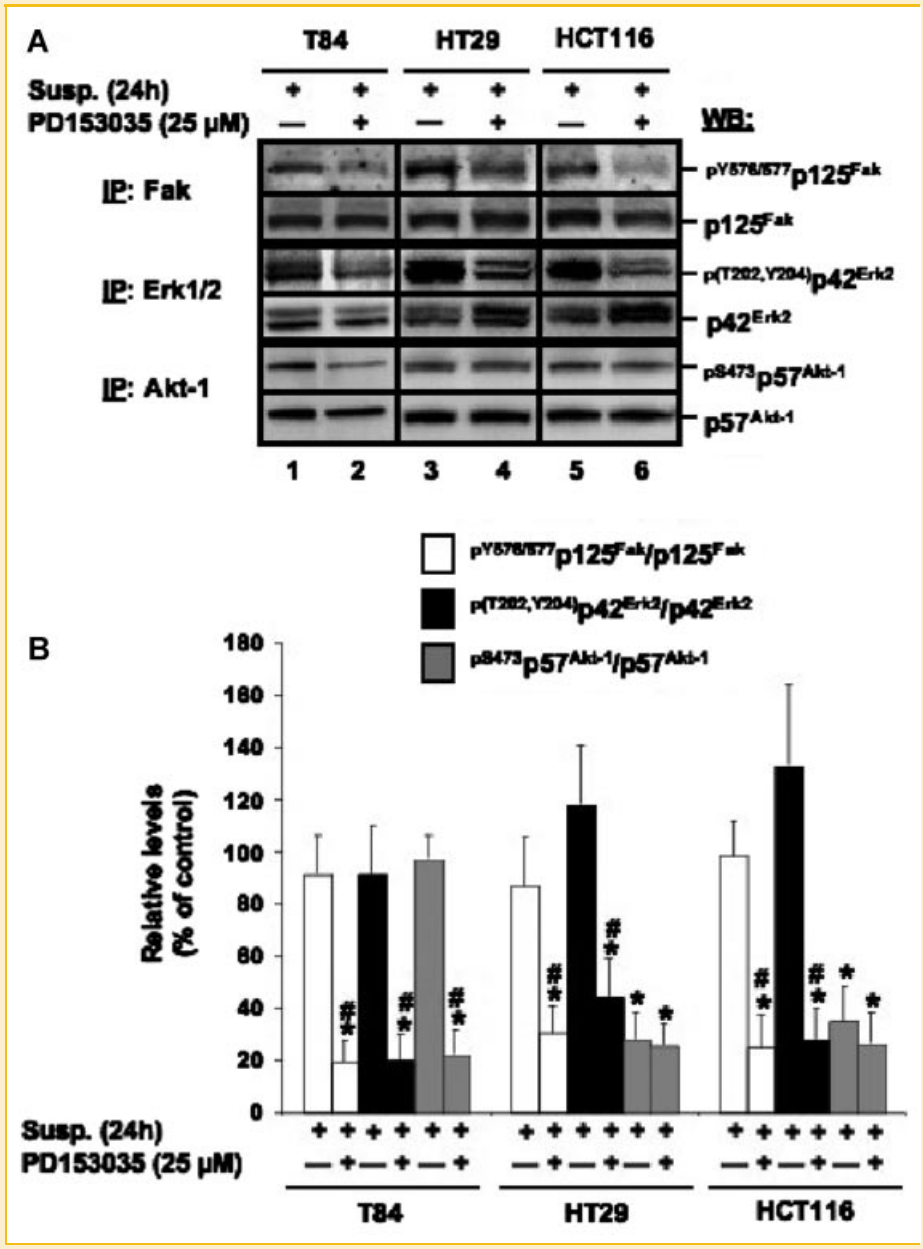

Fig. 10. Roles of EGFR on Fak–Src interactions, MEK/Erk, and PI3-K/Akt-1 activation, in "dedifferentiated" intestinal epithelial cancer cells. A: Representative Western blot analyses of Src-mediated phosphorylation of Fak on residues Y576/577, as well as Erk1/Erk2 and Akt-1 activation, from T84 (lanes 1 and 2), HT29 (lanes 3 and 4), and HCT116 (lanes 5 and 6) cells, which were maintained 24 h in serum-free medium (controls; not shown) with 25 μM PD153035 (lanes 2, 4, 6) and/or kept in suspension (Susp.; lanes 1–6). Cells were lysed and Fak, Erk1/Erk2, and Akt-1 were immunoprecipitated (IP) with specific antibodies. Total proteins from immunoprecipitates (50 μg/well) were separated by SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed (WB) with specific antibodies for the detection of the Src-mediated phosphorylated form of Fak ($^{pY576/577}p125^{Fak}$), or of the activated phosphorylated forms of Erk1/Erk2 ($^{p(T202,Y204)}p42^{Erk2}$) and Akt-1 ($^{pS473}p57^{Akt-1}$). Membranes were thereafter reprobed with the antibodies used for IP in order to detect the Fak ($p125^{Fak}$), Erk1/Erk2 ($p42^{Erk2}$), and Akt-1 ($p57^{Akt-1}$) proteins. B: Same as in (A), except that immunoreactive bands were semi-quantified. The relative Y576/577 phosphorylated levels of Fak, as well as the relative activated levels of Erk-2 and Akt-1, were thereafter established with the ratios $^{pY576/577}p125^{Fak}/p125^{Fak}$ (open columns), $^{p(T202,Y204)}p42^{Erk2}/p42^{Erk2}$ (filled columns) and $^{pS473}p57^{Akt-1}/p57^{Akt-1}$ (gray columns), which were in turn compared to those of control cultures × 100 (expressed as "% of control"). Statistically significant ($0.005 \leq P \leq 0.05$) differences with control cultures are indicated by asterisk (*). Statistically significant ($0.005 \leq P \leq 0.05$) differences between suspension- and suspension + PD153035-treated cultures are indicated by (#).

alone becomes sufficient for survival when these cells are under anoikis conditions. This situation is reminiscent of what has been previously shown in rat IECs [Rosen et al., 2001]. It is thought that components of the EGFR signaling system may become fully available for optimal activation in absence of cell adhesion in IECs, as opposed to when they are adhering. Although efficient EGFR signaling has been reported to require active integrin-mediated cell adhesion in various cell types, there are nonetheless several examples whereby cell adhesion can down-regulate RTK signaling through mechanisms which remain to be fully understood

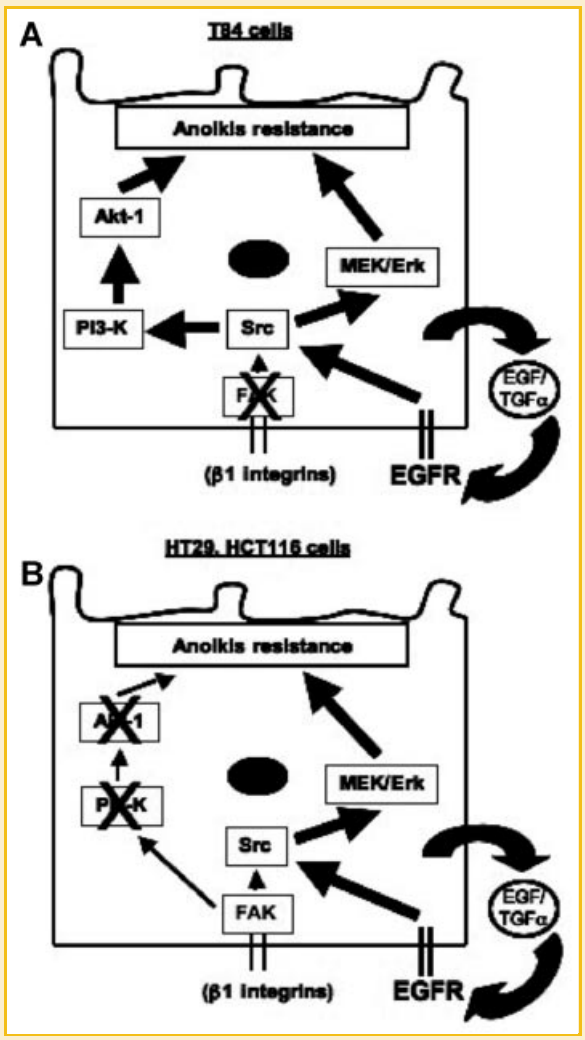

Fig. 11. EGFR-mediated anoikis resistance in "dedifferentiated" intestinal epithelial cells. Schematic drawing of (A) T84 and (B) HT29, HCT116 cells, summarizing the results of the present study. A: In T84 cells, Src acts as a cornerstone for the cell adhesion (β1 integrin)/Fak-mediated engagement of MEK/Erk and PI3-K/Akt-1, as in undifferentiated IECs. However, T84 cells require both pathways for survival, as differentiated IECs do. In this context, an autocrine-driven EGFR activity under anoikis conditions allows for the sustenance of both pathways through Src, while maintaining Fak–Src interactions and thus bypassing altogether the requirement of Fak for survival—even while adhering. B: In HT29 and HCT116 cells, MEK/Erk remains Src-dependent whereas PI3-K/Akt-1 does not (but remains cell adhesion (β1 integrin)/Fak-dependent), as in differentiated IECs. While HT29 cells require Fak, MEK/Erk, and PI3-K/Akt-1 for their survival, like differentiated IECs, HCT116 do not require PI3-K/Akt-1. In both HT29 and HCT116 cells, an autocrine-driven EGFR activity under anoikis conditions nevertheless allows for the sustenance of MEK/Erk through Src while maintaining Fak–Src interactions, thus bypassing the requirement of Fak and PI3-K/Akt-1 for survival—but not when they are adhering. Hence, despite distinctions in signaling behavior not necessarily related to undifferentiated or differentiated IECs, "dedifferentiated" intestinal epithelial cancer cells that are good-to-high expressors of EGF/TGF-α commonly display an autocrine-driven, EGFR-mediated sustained activation of Src under anoikis conditions, consequently conferring anoikis resistance. Thick arrows: pathways stimulated by autocrine-driven, EGFR activity. X, signaling molecule/pathway not required for survival.

[Danielsen and Maihle, 2002; Mitra and Schlaepfer, 2006; Normanno et al., 2006; Moschos et al., 2007]. Such contrasting, dual influence of cell adhesion on RTK activity appears to be associated with the specific repertoire of integrins expressed by any given cell type. Cancer cells in general show gross, heterogeneous alterations in their expression of integrins [Mitra and Schlaepfer, 2006; Moschos et al., 2007], and colon cancer cells are no exception to this [Lussier et al., 2000; Thomson et al., 2003; Le Tourneau et al., 2007]. Hence, the difference of behavior between HT29, HCT116, and T84 cells with regards to their requirement (or not) of Fak for survival, as well as the demonstrated capacity of EGFR signaling to fully compensate for loss of Fak activation in HT29 and HCT116 cells only under anoikis conditions (this study), may be caused at least in part by distinctions in functional integrin repertoires. In this respect, additional studies are needed to identify the full complement of integrins expressed by these cells. Alternately, RTK ligands such as EGF/TGF-α may be expressed in membrane-bound forms, thus adding further complexity to the activation processes of their receptor complexes [Higashiyama et al., 2008]. To this effect, it remains unclear how exactly RTKs manage to bind their ligands and activate when cells are deprived of adhesion [Danielsen and Maihle, 2002; Grossmann, 2002; Reddig and Juliano, 2005; Normanno et al., 2006]. Therefore, further studies are required to better understand how autocrine EGFR activation is regulated in intestinal epithelial cancer cells, in both cell adhesion and loss of adhesion contexts.

Second, the requirement of PI3-K/Akt-1 for the survival of HT29 cells while adhering, but not when kept in suspension, along with their requirement of EGFR activation/activity and MEK/Erk for survival when adhering (Fig. 11B), suggest that the sustenance of MEK/Erk activation by autocrine EGFR signaling under anoikis conditions becomes sufficient to compensate for a down-activation of PI3-K/Akt-1. In this respect, the forced expression of a constitutive active mutant of MEK1 is sufficient to render rat IECs anchorage-independent for growth [Boucher et al., 2004]. Alternately, but not exclusively, additional survival pathways may be concomitantly stimulated by EGFR signaling in support of MEK/Erk, when these cells are kept in suspension. The basic non-requirement of PI3-K/Akt-1 for the survival of HCT116 cells, along with their requirement of EGFR activity and MEK/Erk for survival regardless of whether they are adhering or not (Fig. 11B), support this scenario. Indeed, EGFR signaling in other cell systems has been shown to stimulate the PKCδ and/or Rac/Pak1 pathways in conjunction with MEK/Erk, in order to enhance the inhibitory phosphorylation of the pro-apoptotic Bcl-2 homologs Bad and Bim, and therefore protect against apoptosis/anoikis [Quadros et al., 2006; Sastry et al., 2006]. Incidentally, the JAK/STAT pathway is also commonly activated by EGFR signaling in cancer cells, including those of the colon, thus adding yet another cell survival-promoting pathway in support of MEK/Erk [Danielsen and Maihle, 2002; Henson and Gibson, 2006; Normanno et al., 2006]. Hence, further studies in the cell survival roles of MEK/Erk in IECs and colon cancer cells, as well as in the identification of pathways additionally stimulated by EGFR in these, should provide a better accounting of the signaling behavior of the three intestinal epithelial cancer cells studied herein—and of colon cancer cells in general. In any event, these data altogether provide a signaling basis to explain the apparent contradictory conclusions of

previous reports concerning to the roles of Src-mediated stimulation of MEK/Erk and/or PI3-K/Akt in colon cancer cells and transformed rat IECs, as well as the requirements of these two pathways for the survival of these cells [Loza Coll et al., 2002, 2005; Windham et al., 2002; Golubovskaya et al., 2003].

In conclusion, the findings herein further underlie the ''dedifferentiated'' state [Zweibaum and Chantret, 1989], as well as the heterogeneous nature, of intestinal epithelial cancer cells. Much remains to be understood as to how EGFR signaling can confer anoikis resistance to cancer cells. Nonetheless, this study has demonstrated that colon cancer cells which are good-to-high EGF/TGF-α expressors commonly display an autocrine-driven, EGFR-mediated sustained activation of Src under anoikis conditions, thus conferring anoikis resistance at least in part through a consequent sustenance of Fak–Src interactions and MEK/Erk activation. In this respect, the present findings altogether provide further mechanistic understanding of anoikis resistance in intestinal epithelial cancer cells. Additionally, we identify an EGFR/Src/MEK/Erk pathway as a crucial determinant of colon cancer cell survival under anoikis conditions, bypassing the need for Fak and/or PI3-K/Akt-1 to this effect. Further studies, in conjunction with the present findings, should provide a greater knowledge not only of the regulation of cell survival and anoikis in intestinal epithelial cancer cells, but of the roles of anoikis dysregulation in intestinal tumorigenesis as well.

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