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Integrin-EGFR interaction regulates anoikis resistance in colon cancer cells

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Abstract
Anoikis resistance is an essential property of cancer cells that allow the extra-cellular matrix-detached cells to survive in a suspended state in body fluid in order to metastasize and invade to distant organs. It is known that integrins play an important role in anoikis resistance, but detailed mechanisms are not well understood. Here we report that highly metastatic colon cancer cells showed a higher degree of anoikis resistance than the normal intestinal epithelial cells. These anoikis-resistant cancer cells express high-levels of integrin-α2, β1, and activated EGFR in the anchorage-independent state than the anchorage-dependent state. In contrast, normal intestinal epithelial cells failed to elevate these proteins. Interestingly, a higher co-association of EGFR with integrin-α2β1/-α5β1 was observed on the surface of anoikis-resistant cells. Thus, in the absence of extra-cellular matrix, integrins in association with EGFR activates downstream effectors ERK and AKT and suppress Caspase-3 activation to induce anoikis resistance as was confirmed from the gene-ablation and pharmacological inhibitor studies. Interestingly, these anoikis-resistant cancer cells express high-level of cancer stem cell signatures (CD24, CD44, CD133, EpCAM) and pluripotent stem cell markers (OCT-4, SOX-2, Nanog) as well as drug-resistant pumps (ABCG2, MDR1, MRP1). Altogether, our findings unravel the interplay between integrin-α2β1/-α5β1 and EGFR in anoikis resistance and suggest that the resistant cells are cancer initiating or cancer stem cells, which may serve as a promising target to combat metastasis of cancer.

Keywords Integrin · EGFR · Anoikis resistance · Poly-HEMA · HCT116

Introduction

Attachment of cells to extracellular matrices (ECM) is important not only for physical scaffold but also for their proliferation, differentiation, and survival. Breakdown of such contacts results in *anoikis* or detachment-induced cell death. Anoikis is essential for the maintenance of normal cell and tissue homeostasis. However, resistance to anoikis allows the cancer cells to migrate from their primary site of the organ to a newly distant site leading to metastasis. Several proteins associated with apoptosis are also known to be involved in anoikis but with a difference in their functions in cancer cells and normal cells. BCL-2 over-expression protects cancer cells from anoikis [1]. Contrastingly, normal cells undergoing anoikis show up-regulated BIM and BAD in detached condition [2]. Also, other signaling molecules implicated in anoikis regulation include MAPK/ERK, PI3K/AKT, FAK [3, 4] etc.

Most of the cell-ECM interactions depend on the transmembrane heterodimeric class of receptors, integrins. Integrins have been reported to play an important role in anoikis suppression in different transformed cells [5, 6]. Integrin-α5 subunit is known to suppress anoikis partially in serum-starved HT29 carcinoma cells [7]. However, the role of various integrins in survival or death greatly depends on different cell types and context [6, 8]. Integrin-α4 and -α8β1 have been implicated in promoting anoikis [9, 10]. Despite these discrete reports, the mechanisms by which integrin regulates

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anchorage-independent survival by resisting anoikis are still under the Cinderella of investigations.

The molecular cross-talk between various signaling pathways makes it more complicated to understand the mechanisms of carcinogenesis. Several tyrosine kinase receptors have been reported to be activated by cross-talk with integrins including epidermal growth factor receptor (EGFR), insulin growth factor receptor (IGFR), platelet-derived growth factor receptor (PDGFR), etc. [11, 12]. Deregulated RTK activation confers resistance to anoikis by maintaining/enhancing integrin-mediated cell survival [13–16]. It is generally known that growth factor receptors function properly on ECM-attached cells as integrins create an environment so that the growth factor receptors can activate its downstream signaling in a ligand-dependent manner [17]. Growth factor receptors activation may occur via (i) integrin-ECM ligation; (ii) integrin-mediated phosphorylation of receptor in the absence of soluble growth factor or via (iii) direct physical interaction between the receptor and integrin. Though it is known that integrin-β1-induced EGFR activation is required for adhesion-dependent survival and integrin-α5β3 interacts with PDGFR-β and VEGFR-2 which lead to synergistic signaling effect [11, 18], the detailed role of integrin subunits involved in the regulation of EGFR in the anchorage-independent condition is yet to be explored.

Anoikis resistance is a step towards the metastasis of cancer cells, and this subpopulation of cancer cells are cancer initiating or cancer stem cells (CSC) [19]. $CD44^{+}$/$CD24^{-}$enriched stem like population has been suggested to be associated with anoikis resistance in an anchorage-independent manner [20]. Harrison et al. also reported CSC enrichment by a selection of anoikis-resistant cells or cells expressing the membrane phenotype ESA^{+}/$CD44^{hi}$/$CD24^{low}$ [21]. Various studies showed that anoikis resistance had been associated with increased expression of OCT4, Nanog, and SOX2, important for the maintenance of stemness [22, 23]. Study of CSC linked to anoikis resistance may produce a clear picture of how cancer cells in circulation survive and spread to other organs contributing tomalignancy. In this study, we have demonstrated that anoikis resistant colon cancer cells showed stem cell-like properties and expressed an elevated level of integrin-α2β1/-α5β1 on the cell surface in their detached condition, where integrins interact with EGFR to activate ERK-/AKT-mediated survival pathway and inhibit Caspase-3 activation.

Materials and methods

Cell culture and transfection

Colon cancer cell lines HT29 and COLO205 and normal intestinal epithelial cell line IEC-6 were obtained from NCCS (Pune, India) in 2019 and were authenticated by short-tandem repeat analysis. Colon cancer cell line HCT116 was a gift from Dr. Susanta Roychoudhury (IICB, India) and was authenticated by STR profiling (Cell Sure-Human, Lifecode Technologies). All cell lines were passaged for fewer than 6 months after resuscitation. Cancer cells were routinely maintained in complete DMEM/RPMI 1640 at 37 °C in a humidified incubator containing 5% CO_2. IEC-6 cells were cultured in DMEM medium containing 5% FBS and 10 µg/ml insulin. PcDNA3 vector containing dominant-negative (Dn)-Akt and control pcDNA3.0 vector were separately introduced into HCT116 cells using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's protocol. For endogenous silencing of specific genes, cells were transfected with 300 pmol of control, integrin-α2, integrin-α5, integrin-β1, EGFR, and ERK1/2 siRNAs (Santa Cruz, CA) separately using lipofectamine-2000 for 12 h. Following the transfection cells were provided detachment condition for 24 h and 48 h and harvested for different experiments. For inhibitor study, detached HCT116 cells were treated with 10 µM U0126 and 30 µM LY29004 for overnight and assessed for percent cell death.

Poly-HEMA coating and culture of cells in detached condition

Poly-(2-hydroxyethyl methacrylate) (Poly-HEMA; Sigma, St. Louis, Missouri), has been used to prevent adhesion of cells to tissue culture plates; thus, mimicking the anchorage-independent state of cells. Poly-HEMA powder dissolved in 95% ethanol was used at 20 mg/ml concentration [24]. HCT116, HT29, COLO205, and IEC-6 cells were seeded in FBS containing DMEM/RPMI medium in Poly-HEMA coated tissue culture plates for time-dependent cell viability assay in detached condition using Trypan blue-exclusion method. For cell aggregate formation study, HCT116 and NKE cells were seeded at 2.5×10^{4} cells per well of a Poly-HEMA coated six-well culture plates. Following 24 h and 48 h of detachment cell morphology was observed using phase contrast microscope at a magnification of 10× (Leitz microscope fitted with epifluorescenceilluminator, Germany).

Flow cytometry

For studying anoikis assay, adherent and detached cells were labeled with 7AAD and Annexin-V-FITC (BD Pharmingen, San Jose, CA) according to manufacturer's protocol and analyzed in a flow cytometer (FACS Verse, BD). Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. Total 10,000 events were acquired for analysis using CellQuest software (BD Biosciences, San Jose, CA). Expression of human cancer

stem cell markers CD24, CD44, CD133, and EpCAM, were analyzed in the adhered and detached condition of HCT116 cells by flow cytometry studies using CD24-PE, CD44-APC, CD133-FITC, and EpCAM-AF546 tagged antibodies (BD Biosciences). Stem cell differentiation markers, OCT-4-PerCP-Cy5.5, SOX-2-AF647 and Nanog-PE and drug-resistance pumps, MRP1-FITC, ABCG2-PE and MDR1-PE (BD Biosciences), were quantified flow-cytometrically by measuring mean fluorescence intensities.

Immunofluorescence

Adherent cells on coverslips and detached cells attached to poly-L-lysine coated coverslips were fixed with 3.7% *p*-formaldehyde. Cells were blocked with 3% BSA in PBS and incubated with anti- integrin-α2, integrin-α5, integrin-β1 and EGFR antibodies (1:100 in 1%BSA) for overnight at 4 °C, followed by staining with AF-488, -546 and -594 secondary antibodies (1:100 in 1%BSA) (Invitrogen, CA) for 2 h at 4 °C. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) for 15 min. Images were acquired in a confocal microscope (Leica TCS SP8 CLSM) using 63 × magnification. Image analysis was performed with the ImageJ 1.48q Fiji analysis software. For fluorescence intensity comparisons, images were acquired at identical settings and mean intensity concerning a defined area (Region of interest or ROI) was analysed. Surface expression of Integrin subunits (α2, α5, and β1) and EGFR was quantitated from confocal mid-sections of cells with the number of cells analysed separately indicated in the figure legend.

Co-immunoprecipitation and immunoblotting

For whole cell lysates, cells were homogenized in buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, and 1 mM DTT) supplemented with protease and phosphatase cocktail inhibitor. A total of 30–50 µg protein was resolved using SDS-PAGE and transferred to PVDF membrane and probed with specific antibodies. Primary antibodies used were anti-BIM (pAb), BCL-2 (pAb), p-p38MAPK (pAb), p-ERK (mAb), integrin-α5 (pAb), p-EGFR (Tyr^{1173}) (pAb), ERK (pAb), AKT (pAb),activated Caspase-3(pAb) at a dilution of 1:1000; anti-integrin-α2 at 1:500 and integrin-β1 at 1:250 (Santa Cruz, CA); anti-p-Akt (Ser^{473}) at 1:1000 (pAb; Cell Signalling, Danvers, MA), EGFR at 1:1000 (pAb,R & D Biosystem, MN, USA). Secondary antibodies conjugated to HRP were used at 1:10,000 (Sigma). After that, the immunoblots were visualized by chemiluminescence. Equal protein loading and cell fractionated samples were confirmed with anti-β-actin (mAb; Santa Cruz) and anti-α-tubulin antibodies (pAb; Thermo Fischer Scientific, MS) respectively. For co-immunoprecipitation between EGFR and integrin-α2/-α5/-β1, 300 µg protein from cytosolic or membrane fractions was immuno-precipitated using anti-EGFR antibody and protein G plus/protein A-agarose beads (Calbiochem, CA). The immunopurified protein was immunoblotted with anti-integrin-α2, -α5 and -β1 antibodies. Cellular fractionation was performed following the protocol of subcellular fractionation of cultured human cells by bio-protocol (http://www.bio-protocol.org/e754). Cytosolic and membrane fractions of adhered and detached HCT116 cells were collected, studied for Western Blot and co-immunoprecipitation.

Statistical analyses

Values are shown as standard error of the mean (SEM) except otherwise indicated. Comparison of multiple experimental groups was performed by 2-way ANOVA followed by a post hoc Bonferroni modification of multiple comparison t-tests. Data were analyzed, and when appropriate, significances of the differences between mean values were determined by Student's t test. Results were considered significant at $p \leq 0.05$.

Results

Colon cancer cells show anoikis resistance in contrast to normal intestinal epithelial cells

Anoikis resistance is acquired by cancer cells to evade apoptosis in anchorage-independent condition. We utilized an artificial anchorage-independent culture condition using poly-HEMA (PH)-coated plates to mimic the suspended state of cells. Here, we have compared this phenomenon in a battery of epithelial colon cancer cell lines (HCT116, HT29, and COLO205 cells) to that of normal intestinal epithelial cells (IEC-6) to assess their susceptibility to anoikis in detached condition.We observed that HCT116 and HT29 cells were more resistant to anoikis over IEC-6 cells (Fig. 1a) whereas COLO205 showed resistance at the early time point and slowly became sensitive at late time point. From the anoikis assay by Annexin-V-positivity, it was observed that colon cancer cells showed no significant cell death (~ 2–5%) in contrast to their normal counterpart (IEC-6) (~ 19%; Fig. 1b). To find out the reason for such contrasting degree of apoptosis in detached-condition, we checked the expression level of few pro-/anti-apoptotic proteins those play important roles in cell survival. Our results

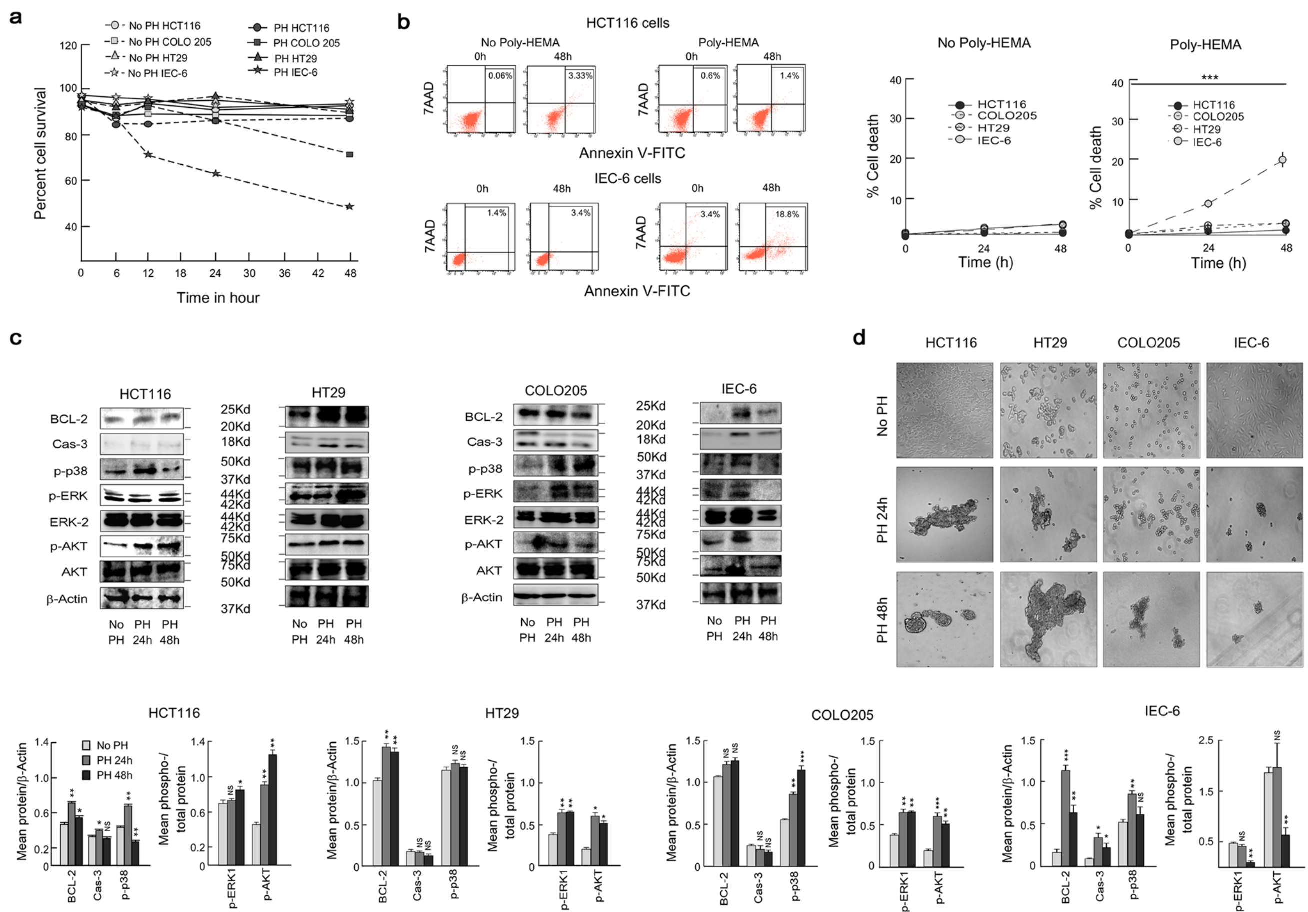


Fig. 1 Colon cancer cells show anoikis resistance in contrast to normal intestinal epithelial cells. **a** The time-dependent survival advantage of cancer (HCT116, COLO 205 and HT 29) and normal (IEC-6) cells was performed in adherent (no PH) and detached (PH) conditions. **b** HCT116 and IEC-6 cells were flow-cytometrically analysed for Annexin-V-FITC/7AAD-positivity, regarded as dead cells, in adherent (no poly-HEMA) and detached (poly-HEMA) conditions (*left*). Flow-cytometric data was represented as percent anoikis (cell death) graphically (*right*). **c** Cell lysates from HCT116, HT 29, COLO 205 and IEC-6 cells in adherent and detached condition were subjected to Western blot analysis for protein expression of BCL-2, activated Caspase-3 (Cas-3), p-p38MAPK, p-ERK, ERK, p-AKT, AKT. 24 h polyHEMA: PH24 h; 48 h polyHEMA: PH48 h (*upper*). β-Actin was used as a loading control. Mean intensities of the proteins normalized to β-Actin and that of phospho-proteins to the total proteins were represented graphically (*lower*). **d** Phase-contrast microscopic images of HCT116, HT 29, COLO 205 and IEC-6 cells in adherent and detached condition (24 h and 48 h); Magnification 10×. Values are mean ± SEM of three independent experiments in each case or representative of a typical experiment. *p < 0.05, **p < 0.01, ***p < 0.001

indicated down-regulation of anti-apoptotic protein BCL-2 and an up-regulation of pro-apoptotic active caspase-3 and p-p38MAPK in detached IEC-6 cells (Fig. 1c) in comparison to that of colon cancer cells.

In contrast, pro-survival proteins like ERK and AKT were found to be activated in anoikis-resistant cancer cells but not in anoikis-susceptible normal intestinal cells (Fig. 1c). Next, to study the morphological changes in a detached state of all cells, they were seeded in poly-HEMA-coated plates, and changes during detachment were observed under a phase contrast microscope. It was observed that in detached condition HCT116, HT29 and COLO205 cells formed large, multicellular aggregates whereas, IEC-6 cells did not form such aggregates rather produced small clusters with the sparse association (Fig. 1d). These results indicate that normal intestinal epithelial cells when detached underwent anoikis by altering the pro-/anti-apoptotic proteins in contrast to cancer cells, which resist it by up-regulating the survival proteins.

Anoikis-resistant cells exhibit higher expression of integrins and EGFR on the cell surface

Since the attachment of cells to the sub-cellular matrix is mediated by the heterodimeric cell surface receptor integrins; we thought to explore their role in anchorage-independent survival. In order to understand their role in anoikis resistance, we intended to compare the expression profile of integrin-α2, -α5 and -β1 in detached cancer cells to that of the adherent as well as to normal epithelial cells. It was observed that in the detached condition, the level of integrin-α2 and -β1 were increased significantly in detached cancer cells, whereas the level of integrin-α5 showed different expression profiles in the three colon cancer cells. Interestingly, normal epithelial cells showed a decreasing trend for all the integrins (Fig. 2a). In addition, EGFR activation was also up-regulated in anoikis-resistant cancer cells but found to be down-regulated in anoikis-susceptible normal cells (Fig. 2a). Our confocal microscopy data in HCT116 cells further supports the notion that detached cancer cells expressed a higher level of integrin-α2, -β1 and EGFR as compared to the normal detached cells (Fig. 2b, *left*). It is noteworthy that the expression levels of all these receptors were found to be significantly higher in anoikis-resistant cells than the anoikis-sensitive cells (Fig. 2b, *right*). Together these data indicated that all these integrins and EGFR might be required for anchorage-independent survival of colon cancer cells.The confocal microscopic images of HCT116 cells (Fig. 2c) and the immunoblot data of different colon cancer cells (Fig. 2d) revealed that integrin-α2, -α5, -β1 and EGFR were expressed at a high level on the surface of anoikis-resistant cells.

Integrin-α2/-α5 and -β1 associate with EGFR in anoikis-resistant cells

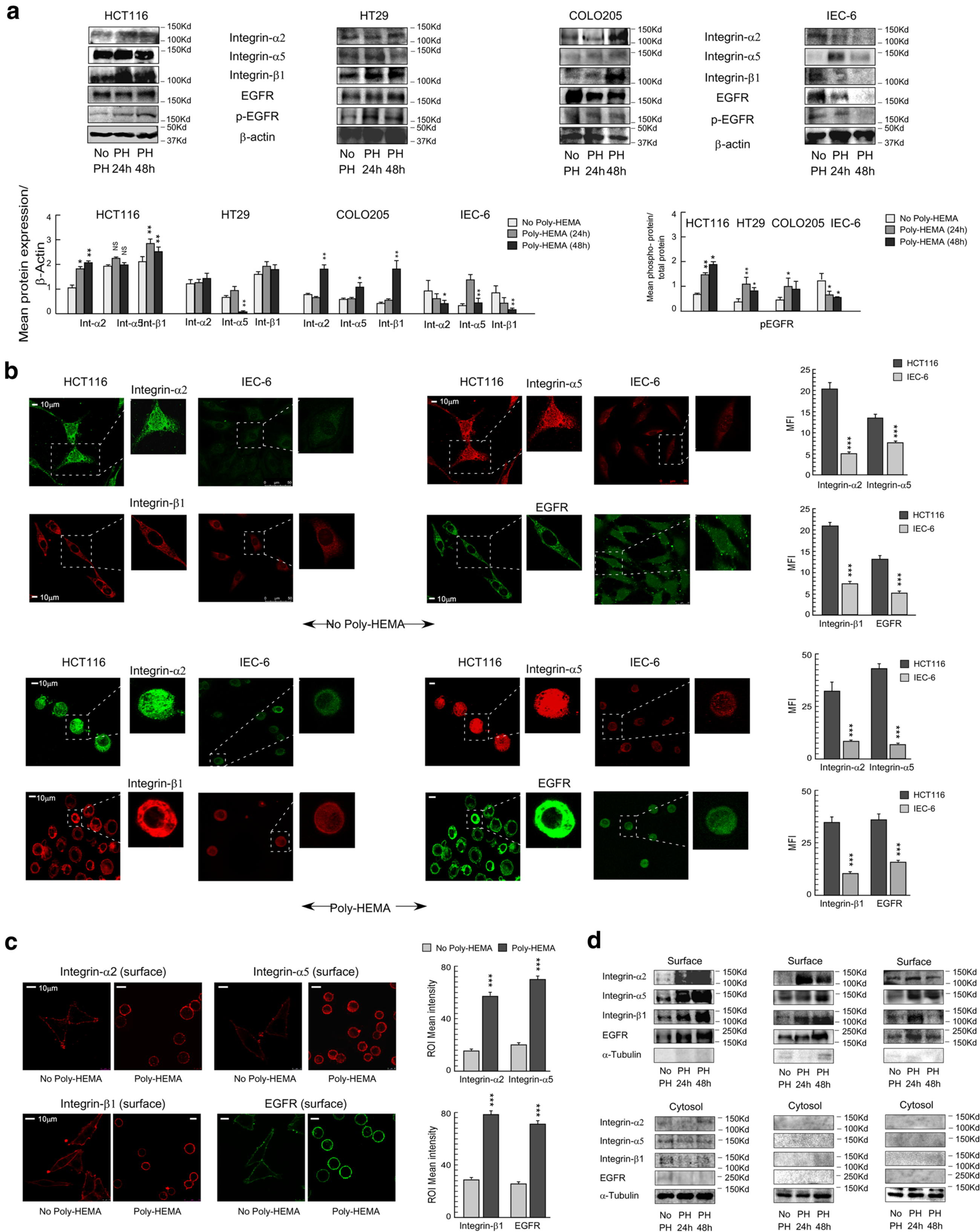
Epidermal growth factor receptors have been reported to be activated by integrin involving crosstalk mechanism (11). This information tempted us to look for any interaction between integrin and EGFR in anoikis-resistant cells. Our confocal microscopic data in HCT116, HT29 and COLO205 showed that integrin-α2/β1 co-localization was more pronounced on the surface of detached cells than in adherent cells (Fig. 3a) but, no co-localization was observed between integrin-α5 and integrin-β1 in HT29 and COLO205 cells (Fig. 3b). Interestingly, there was a significant co-localization between integrin-α2/-β1 and EGFR on the surface of anoikis-resistant HCT116 and HT29 cells (Fig. 3c, e) whereas, detached COLO205 cells exhibited co-localization between integrin-α5/-β1 and EGFR on their surface (Fig. 3d, e).To confirm our observations, we performed co-immunoprecipitation experiments to understand that whether there exists any interaction between individual integrin and EGFR during detachment. We observed an interaction between integrin-α5 and EGFR in the adherent condition in HCT116, which subsides during the detachment (Fig. 3f, *left*). On the contrary, integrin-α2 and integrin-β1 co-associated with EGFR on the course of detachment (Fig. 3f, *left*). A similar association was observed between EGFR and integrin-α2/-β1 and also with integrin-α5/-β1 in HT29 cells (Fig. 3f, *middle*). In contrast, integrin-α5/-β1 co-associated with EGFR in detached COLO205 cells (Fig. 3f, *right*).This entire phenomenon occurs on the cell surface, which dictates the survival signaling during the detachment.

Genetic ablation of integrin-α2/β1 inhibits EGFR-mediated anoikis resistance

By understanding the interaction between the two classes of receptors, we intended to study the functional importance of such interaction in anoikis resistance in HCT116 cells as our model. To accomplish our aim, we performed the anoikis susceptibility following genetic ablation of each of the receptors individually in a detached state. It was observed that siRNA-mediated ablation of integrin-α2, -α5 or -β1 re-insisted significant cell death in anoikis-resistant cells within 48 h of cell detachment (Fig. 4a). Perturbation of EGFR alone also showed a significant death in these cells (Fig. 4a).When integrin-α2 and -β1 were perturbed separately the phosphorylation and activation of EGFR were completely abolished without affecting total EGFR level in detached condition (Fig. 4b, *left and right*). Interestingly, integrin-α5 knock-down did not affect the activation of EGFR (Fig. 4b, *middle*) but, integrin-α5 associated with EGFR in the detached condition of HT29 and COLO205 cells (Fig. 3f, *middle and right*). Also, the genetic ablation of integrin-α5 reversed anoikis resistance (Fig. 4a) indicating that this receptor may be partially involved in anoikis resistance or other survival pathway depending on the cell type. All these phenomena suggest that integrin-α2/-β1 interacted with EGFR to activate it that supports anoikis resistance.

Integrin-EGFR interaction regulates anoikis resistance through the activation of ERK and AKT

Most of the down-stream effector molecules, essential for cell survival, regulated by integrin, are commonly shared by the receptor tyrosine kinase. RAS/MAPK-pathway and


a
HCT116
HT29
COLO205
IEC-6
Integrin-α2
Integrin-α5
Integrin-β1
EGFR
p-EGFR
β-actin
150Kd
100Kd
50Kd
37Kd
No PH PH
PH 24h 48h
Mean protein expression/ β-Actin
Int-α2
Int-α5
Int-β1
NS
No Poly-HEMA
Poly-HEMA (24h)
Poly-HEMA (48h)
Mean phospho- protein/ total protein
pEGFR
b
HCT116
IEC-6
Integrin-α2
Integrin-α5
Integrin-β1
EGFR
10μm
MFI
No Poly-HEMA
Poly-HEMA
c
Integrin-α2 (surface)
Integrin-α5 (surface)
Integrin-β1 (surface)
EGFR (surface)
No Poly-HEMA
Poly-HEMA
ROI Mean intensity
d
Surface
Cytosol
α-Tubulin
250Kd

◂ **Fig. 2** Anoikis-resistant cells exhibit higher expression of integrins and EGFR on the cell surface **a** Protein expression profiles of integrin-α2, -α5, -β1, EGFR and p-EGFR (Tyr1173) from HCT116, HT 29, COLO 205 and IEC-6 cells in adherent (no PH) and detached (PH) conditions (24 h and 48 h) (*upper*). β-actin was used as a loading control. Mean protein expressions normalized to β-Actin and that of phospho-proteins to the total proteins were represented graphically (*lower*). **b** Confocal microscopic images show the total cellular integrin-α2, -α5, -β1, and EGFR in HCT116 and IEC-6 cells in adherent (no poly-HEMA) and detached condition (poly-HEMA) (*left*). AF-488-integrin-α2; AF-488-EGFR; AF-546-integrin-α5; AF-546-integrin-β1, scale bar 10 µm; magnification 63×. Mean fluorescence intensity (MFI) for integrin-α2, -α5, -β1, and EGFR in detached cells were analysed using ImageJ software (n = 15) and represented graphically (*right*). **c** Surface expression of integrin-α2, -α5, -β1 and EGFR in HCT116 cells in adherent (no poly-HEMA) and detached (poly-HEMA) condition was analysed by confocal microscopy (*left*). ROI-Mean fluorescence intensity of integrins and EGFR were calculated concerning a constant area (5–6 region of interest (ROI)/per cell; n = 80) and represented graphically (*right*). Scale bar 10 µm; magnification 63×. **d** Western Blot analysis of integrin-α2, -α5, -β1 and EGFR from the surface (*upper*) and cytosolic fractions (*lower*) of HCT116, HT 29 and COLO 205 cells (*left* to *right* respectively) in adherent (no PH) and detached (PH) conditions (24 h and 48 h). α-Tubulin was used as a loading control. Values are mean ± SEM of three independent experiments in each case or representative of typical experiment *p < 0.05, **p < 0.01, ***p < 0.001

PI3K/AKT-pathways are the common downstream target of these receptors [25, 26]. In addition, we observed that ERK and AKT were over-activated (Fig. 1c), and EGFR was associated with integrin-α2/β1 (Fig. 3f) in anoikis-resistant cells. Logistically, we assumed that integrin-α2/β1-EGFR interaction is activating ERK and AKT during anoikis resistance. To test this, next, we tried to find out the down-stream effector molecules that contribute to the anoikis resistance. As hypothesized, we found that genetic ablation of integrin-α2/-β1 and EGFR blocked ERK (Fig. 5a, b) and AKT (Fig. 5c) activation in detached condition. Ablation of ERK (by siRNA) or AKT (by ΔN-Akt) by genetic manipulation significantly reversed anoikis resistance (Fig. 5d). Consistence with the study, it was observed that the pharmacological inhibitors of MAPK/ERK (U0126) and PI3K/AKT (LY294002) significantly reversed the anoikis resistance in these cells (Fig. 5e). Furthermore, we also showed that perturbation of integrin-α2/-β1 and EGFR significantly reduced BCL-2 activation and enhanced Caspase-3 cleavage (Fig. 5f). Altogether, our data suggest that integrin-α2/-β1/EGFR-mediated ERK/AKT activation promotes anoikis resistance that involves BCL-2 up-regulation and caspase-3 down-regulation.

Anoikis resistant population of colon cancer cells exhibits the property of cancer stem cells

It is well established that a subpopulation of tumor cells, known as cancer stem cells (CSCs), exhibits a profound role in metastasis [27]. During metastasis, this CSCs need to survive during detachment from the primary site and need to travel through the peripheral circulation. Studies reported that CSC populations in colon cancer could be demarcated with specific surface markers such as CD44, CD24, CD133, EpCAM, etc. [28]. We hypothesized that these anoikis-resistant cells exhibit cancer stem cell-like properties. To ascertain their properties, we checked the cell surface signatures of these cells. Our flow-cytometric data indicated a significant increase in $CD24^{-/+}/CD44^{+}$ cell population in the detached condition in comparison to the adherent condition (33% vs. 3%; Fig. 6a). Interestingly, these detached cells also show a higher percentage of CD133/EpCAM-positivity (20% vs. 4%; Fig. 6b). These anoikis-resistant cells express the stemness or pluripotency markers like OCT-4, SOX-2, and Nanog (Fig. 6c). It was also observed that these cells expressed high-levels of drug-resistant proteins like ABCG2, MDR1, and MRP1(Fig. 6d). Altogether, our study implied that these anoikis-resistant cells are cancer initiating or cancer stem cells since they express the stemness related markers.

Discussion

Anoikis is essential for maintaining tissue homeostasis in multicellular organisms, perturbation of which leads to abnormal cell proliferation, ultimately contributing to the development of cancer. Many possible mechanisms responsible for anoikis resistance have been suggested of which integrin/EGFR-mediated pathways are yet to be explored in detail. Study of receptor function becomes significant in the context of different cellular systems since they play an indispensable role in both towards the anoikis sensitivity or resistance. Here, we report the resistance to anoikis in colon cancer cells during their detachment from the basement as well as the variation in the signalling amongst the anoikis-resistant cells in adherent and detached conditions in comparison to their normal counterparts. We observed that highly metastatic colon cancer cells are able to survive after the detachment from the substratum than the normal cells by the process called anoikis resistance. It is known that anoikis and apoptosis are morphologically similar [29] and utilize much similar signalling machinery [30–32]. We observed a similar

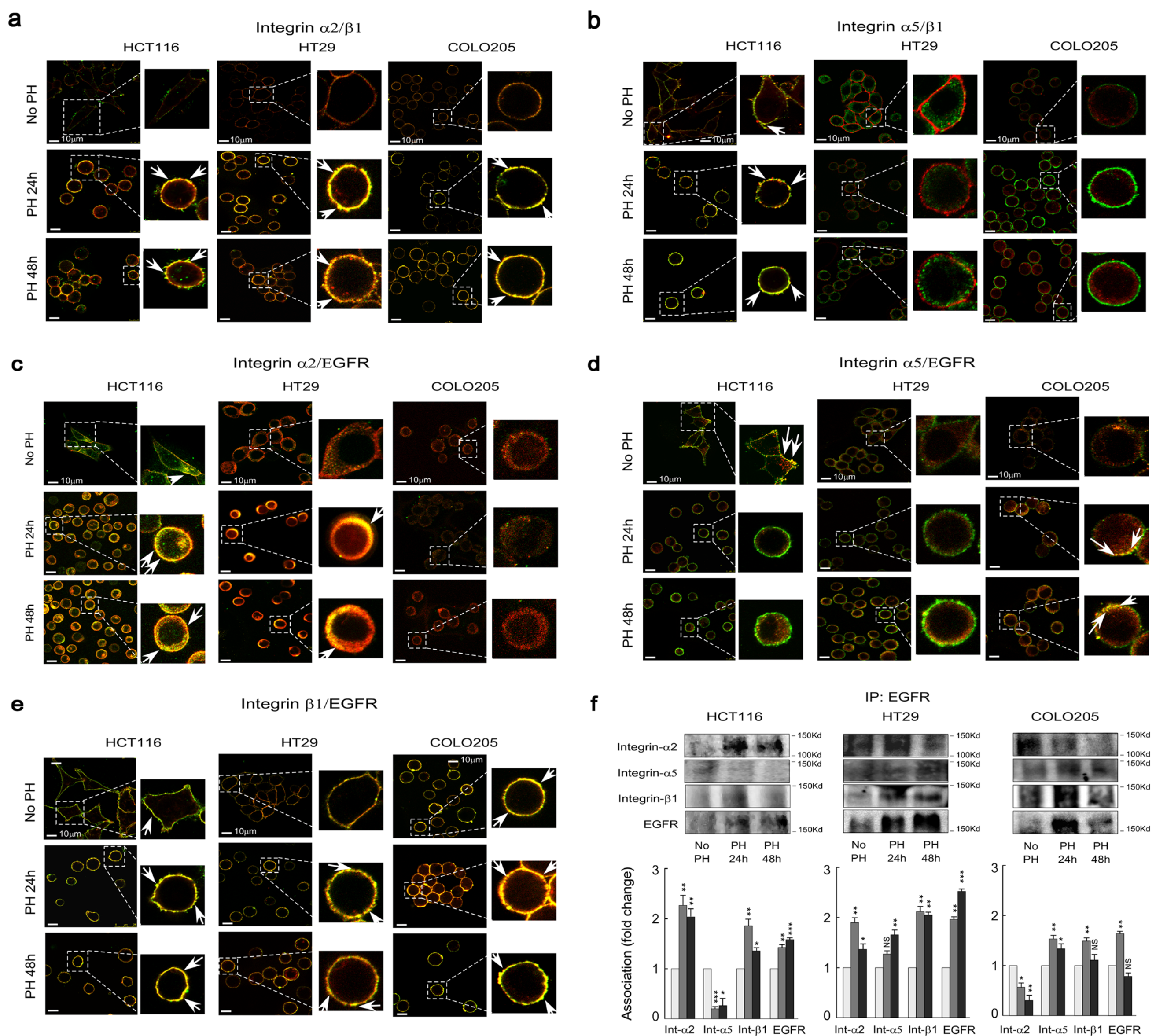


Fig. 3 Integrin-α2/-α5 and -β1 associate with EGFR in anoikis-resistant cells Confocal microscopic images of **a** integrin-α2 and -β1, **b** integrin-α5 and -β1, **c** integrin-α2 and EGFR, **d** integrin-α5 and EGFR, and **e** integrin-β1 and EGFR were represented to show the co-localization of two proteins on the surface of adherent (no PH) and detached (PH) HCT116 cells, HT 29 and COLO 205. The co-localized yellow pixels in the merge images are indicated with white arrowheads. Scale bar 10 μm; magnification 63×. **f** Co-immunoprecipitation studies of integrin-α2, -α5, -β1 with EGFR were performed and detected by Western blotting, from the membrane fraction of adherent (no PH) and detached (PH) conditions of 24 h and 48 h HCT116, HT 29 and COLO 205 cells (*upper*) and represented graphically (*lower*).Values are mean ± SEM of three independent experiments in each case or representative of typical experiment *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

phenomenon that anoikis-susceptible cells (IEC-6) express a higher level of pro-apoptotic protein and lower level of anti-apoptotic and survival proteins in detached condition. On the other hand, anoikis-resistant colon cancer cells showed the opposite trends.

Since the adhesion of cells to ECM is mediated by integrins [33], it is important to study the contribution of integrins in anoikis resistance. There are conflicting reports showing that integrin α2β1 directly promotes anoikis of breast cancer and hepatoma cells [34]; whereas, integrin α5β1 has been shown to suppress anoikis in the ligand-bound state [6]. Moreover, the involvement of different classes of integrins has been showed by Beausejour et al. where anoikis suppression in intestinal epithelial cells depends on the differentiation status of the cells [35]. Here, we report the anoikis-suppressing function of

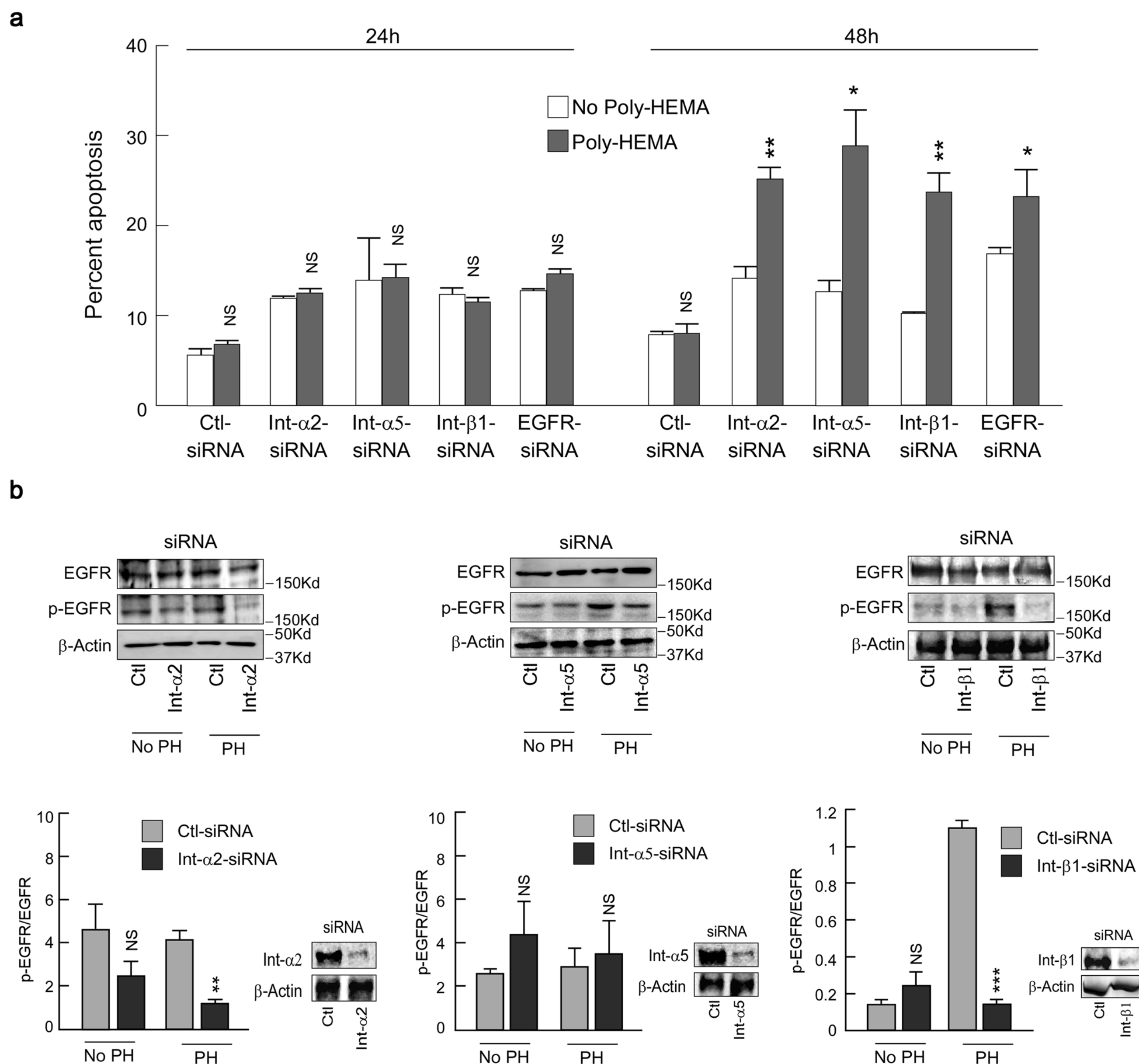
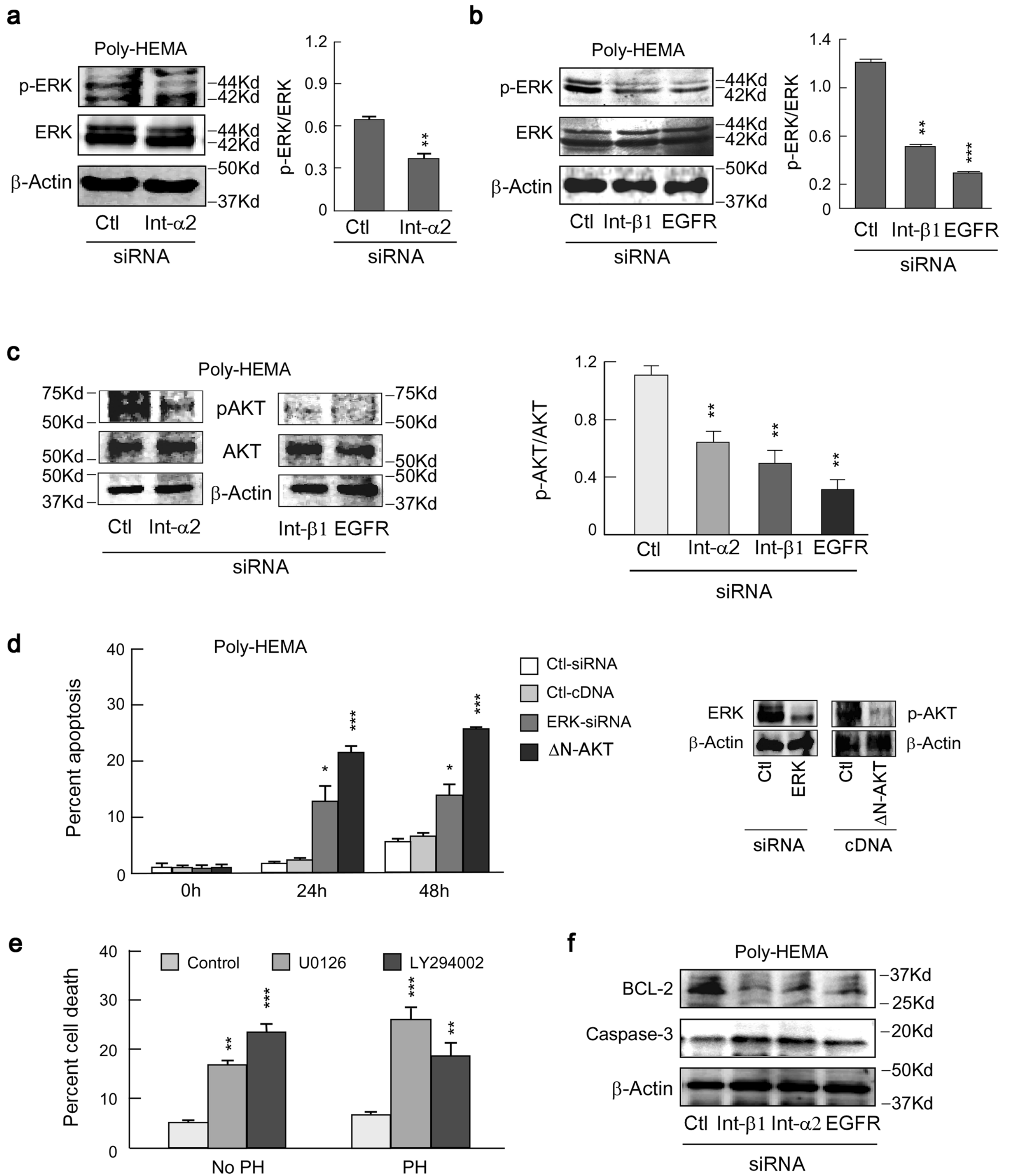


Fig. 4 Genetic ablation of integrin-α2/β1 inhibits EGFR-mediated anoikis resistance. **a** HCT116 cells transfected with control, integrin-α2/-α5/-β1 and EGFR-siRNA were allowed to be in adherent or detachment for 24 h and 48 h followed by a flow-cytometric assessment of cell death. **b** Western blot analysis of p-EGFR (Tyr1173) concerning total EGFR in control, integrin-α2/-α5/-β1-siRNA transfected HCT116 cells in adherent (no PH) and detached (PH) conditions (*upper*) were performed. Mean intensities of p-EGFR normalized to EGFR were determined and represented graphically (*lower*). Transfection efficiency of integrin-α2, -α5 and -β1 siRNAs were confirmed by immunoblots. β-actin was used as loading control. Values are mean ± SEM of three independent experiments in each case or representative of typical experiment $*p<0.05$, $**p<0.01$, $***p<0.001$

integrin-α2β1/-α5β1 in colon cancer cells in basement-detached condition. In adherent condition, integrin-α5β1 helps to transmit the survival signal to HCT116 cells, whereas survival in adherent-condition requires integrin-α2β1 for COLO205 cells. Hence, it can be concluded that integrin heterodimers play an important role in the detached condition of cancer cells depending on the context of the cells and induce the pro-survival signaling; thus, resisting anoikis.

There are a plethora of studies indicating the co-ordination between integrins and growth factor receptors in cancer cells [36, 37], but there are lacunae of information regarding the interaction between integrins and EGFR in anoikis resistance.We observed that the levels of the integrins-α2, -α5, -β1 and EGFR increased significantly



on the cell surface of colon cancer cells. These tempted us to hypothesize that integrin and EGFR may interact or co-ordinate in order to provide survival signaling to the anoikis-resistant cells. Our genetic manipulation and biochemical studies suggest that in adherent condition, where basement matrix is present, integrin-α5β1 interacts with EGFR on the cell surface to transmit the survival signaling, and their association is suppressed during detachment in the absence of the matrix. On the other hand, in detached condition, integrin-α2β1 co-associates

◀**Fig. 5** Integrin-EGFR interaction regulates anoikis resistance through the activation of ERK and AKT Western blot depiction of p-ERKs and ERKs in **a** integrin-α2, **b** integrin-β1- and EGFR siRNA-transfected cells in detached (poly-HEMA) condition. Mean intensities of p-ERK normalized to total ERK were represented graphically (**a**, **b**, *right panels*). **c** The level of total AKT and p-AKT (Ser473) were analysed in integrin-α2, -β1 and EGFR ablated cells in detached (poly-HEMA) condition (*left*). Phospho-AKT was normalized to total AKT and mean intensities were represented graphically (*right*). β-actin was used as a loading control. **d** Control-/ERK1/2-siRNA or control vector/ΔN-AKT-cDNA transfected cells in detached conditions of 24 h and 48 h were analysed flow-cytometrically for percent cell death. *Right panel* represents the transfection efficiencies. **e** HCT116 Cells treated overnight with pharmacological inhibitors of MAPK/ERK (U0126 10 μM), and PI3K/AKT (LY294002 30 μM) were analysed flow cytometrically for percent cell death compared to control set. **f** The levels of BCL-2 and Caspase-3 were analysed in integrin-α2, -β1 and EGFR ablated cells in detached (poly-HEMA) condition by western blotting. β-actin was used as a loading control. Values are mean ± SEM of three independent experiments in each case or representative of typical experiment $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

with EGFR dictate the pro-survival signaling. However, in COLO205 cells, it was observed that anoikis resistance is promoted by the association of integrin-α5β1 with EGFR. This allows the highly-metastatic cells to resist anoikis when they are detached from the primary site, flow through the peripheral circulation and home at a distant site. Alanko *et al.* suggested that integrin signaling is not restricted to cell–ECM adhesions.They identified an endosomal pathway away from the plasma membrane that involved Rab21-mediated endocytosed integrin-induced FAK signaling that suppressed anoikis [38]. Our genetic ablation studies confirmed that integrin-α2β1-mediated EGFR activation is required for anoikis resistance. The direct physical association of integrin-α2β1 and EGFR has also been reported in cell–cell contact sites in serum-depleted condition [39]. Our work specifically proved the association of integrin-α2β1 with EGFR in a detached condition, which may be the reason for EGFR activation in anoikis-resistant cells. Our findings are also supported by that of Demer's et al. who reported the role of EGFR in anoikis resistance in colon cancer cells [40]. COLO205 cells require association of integrin-α5/β1 with EGFR to survive in a detached condition which is in consistent with the findings by Haenssen et al., who observed that integrin-α5 is required for ErbB2 activation in anoikis resistance of human mammary epithelial cells [41]. To some extent, our prediction is also supported by the finding of Moro et al. where the association of EGFR with integrin-αVβ3 and -β1 on the membrane caused EGFR activation at specific residues which also involved the activation of Src and p130Cas [42]. At the down-stream of integrin/EGFR survival signaling, ERK and AKT play an indispensable role. All this information also indicate that both the receptors converge into the RAS-ERK and PI3K-AKT pathways to support the anoikis resistance [43–46]. Our prediction of receptors-mediated ERK/AKT activation also confirms the results of Demers et al, where EGFR-regulated MEK/ERK or PI3K/AKT signaling is involved in intestinal epithelial cancer cells anoikis resistance [40]. In the down-stream pathway, it was observed that ablation of integrin/EGFR alters BCL2-mediated Caspase-3 activation to switch from anoikis resistance to susceptible.

Anoikis resistance is a hallmark of metastatic cancer cells [47], and metastasis occurs in a sub-population of cells in tumor mass, known as cancer stem cells (CSC) [27]. Cancer stem cells are well known for their property of self-renewal, differentiation, and drug-resistance [48–50]. Several signature markers have been used for the identification of colorectal cancer stem cells such as CD133, CD24, CD29, CD44, CD166 (ALCAM), EpCAM, Lgr5, etc. [28]. We have shown that the anoikis-resistant cells are the cancer stem cell-enriched populations acquiring high-level of cancer stem cell signatures (CD24, CD44, CD133, EpCAM) and pluripotent stem cell markers (OCT-4, SOX-2, Nanog) as well as drug-resistant pumps (ABCG2, MDR1, MRP1) during detachment. These results are supported by the findings of others that anoikis-resistance is associated with high-level expression of OCT4, Nanog and SOX2, the transcription factors required for the maintenance and development of stem cells [22, 23]. Besides, our findings also implied that the anoikis-resistant cells are drug-resistant as they express high-level of drug-resistant pumps ABCG2, MRP1, and MDR1. Together, it can be suggested that cancer initiating or cancer stem cells are anoikis-resistant cells which can migrate through body circulation and thus, contributes to distant metastasis.

Henceforth, our study suggests that anoikis-resistant cancer cells are cancer stem cells population that are pluripotent and drug-resistant. Such anoikis resistance is acquired by the activation of EGFR through its interaction with integrins in absence of a basement matrix. Thus, our findings underline the understanding of the mechanism of disseminated cancer cell survival, which can contribute to the development

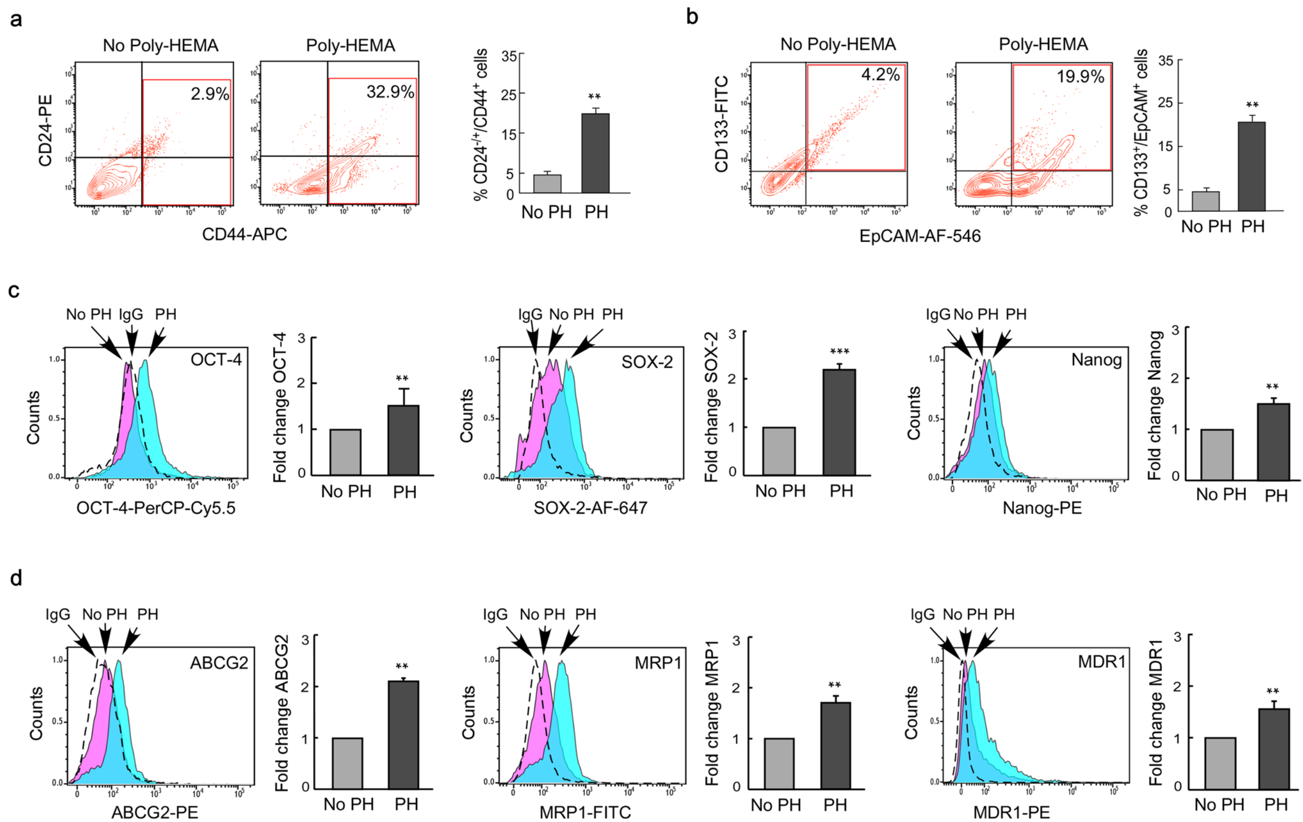


Fig. 6 Anoikis resistant population of colon cancer cells exhibits the property of cancer stem cells. Percentage of cancer stem cell signature molecules **a** CD24$^{-/+}$/CD44^{+} and **b** CD133^{+}/EpCAM^{+}in adhered (no poly-HEMA) and detached (poly-HEMA) HCT116 cells were analyzed flow-cytometrically (*left*) and represented graphically (*right*). CD24-PE; CD44-APC; CD133-FITC; EpCAM-AF-546. **c** Flow-cytometric histogram overlay depicting intensities for pluripotent stem cell markers OCT-4, SOX-2 & Nanog in adhered (no PH) and detached (PH) HCT116 cells. OCT-4-PerCP-Cy5.5; SOX-2-AF-647; Nanog-PE. Mean fluorescence intensities (MFIs) of the proteins were represented graphically in terms of fold change in *right panels* to the histograms. **d** Flow-cytometric histogram overlay depicting intensities for drug-resistant pumps ABCG2, MRP1 & MDR1 in adhered (no PH) and detached (PH) cells. MRP1-FITC; ABCG2-PE; MDR1-PE. Mean fluorescence intensities (MFIs) of the markers were represented graphically in terms of fold-change in *right panels* to the histograms. Values are mean ± SEM of three independent experiments in each case or representative of typical experiment $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

of effective strategies to combat cancer cells survival and metastasis.

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Author contributions DG formulated the project, designed and executed most of the experiments, analyzed related results and wrote the manuscript; TS performed the immunostaining experiments and analyzed the results; SB assisted in cell-culture maintenance; SC analyzed experimental results; SD performed cell viability assays and Western blot experiments; PK analyzed experimental results; AA helped conceptually and gave critical suggestions; TD designed the experiments and edited the manuscript; GS conceptualized the project, designed the experiments, edited the manuscript.

Compliance with ethical standards

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

Research involving human participants and animals This article does not contain any studies with human participants or animals performed by any of the authors.

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