Targeting of the EGFR/β1 integrin connecting proteins PINCH1 and Nck2 radiosensitizes three-dimensional SCC cell cultures

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Abstract. Epidermal growth factor receptor (EGFR) signaling plays an important role in tumor cell resistance to therapy. In addition to ligand binding, mutual and cooperative interactions of EGFR with integrin cell adhesion receptors critically influence proper downstream signaling through a number of bridging adapter proteins. In the present study, we analyzed the role of two of these adapter proteins, called PINCH1 and Nck2, for cellular radioresistance in combination with EGFR-targeting using the monoclonal antibody cetuximab. siRNA-mediated knockdown of PINCH1 or Nck2 resulted in enhanced radiosensitivity of 3D grown human squamous cell carcinoma cell lines FaDu (head and neck) and A431 (epidermis) comparable with effects seen after cetuximab treatment. Combination of knockdown and cetuximab did not result in additive nor synergistic effects regarding clonogenic radiation survival. Modifications in MAPK, Akt and FAK phosphorylation occurred upon cetuximab treatment as well as PINCH1 or Nck2 depletion. We further found this tumor cell radiosensitization to be due to attenuated repair of DNA double strand breaks and altered Rad50 and Nbs1 expression but without changes in other DNA repair proteins such as ATM, DNA-PK and Mre11. Our data suggest that the adaptor proteins PINCH1 and Nck2 critically contribute to cellular radioresistance and proper EGFR signaling in 3D lrECM grown human squamous cell carcinoma cells. Further investigations are warranted to identify the intracellular signaling network controlled by EGFR, PINCH1 and Nck2.

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Introduction

Epidermal growth factor receptor (EGFR) signaling is known to be deregulated in many human tumors (1,2). Causative are EGFR gene amplifications and mutations resulting in receptor overexpression and constitutively active EGFR tyrosine kinase activation. Due to its substantial role in progression and pathogenesis of different carcinomas, huge efforts have been undertaken to develop specific EGFR targeting approaches. Monoclonal antibodies such as cetuximab or tyrosine kinase inhibitors are clinically administered as monotherapy or in multimodal concepts in combination with chemo- and/or radiotherapy (3). Despite promising preclinical data, clinical trials revealed EGFR targeting less effective in prolonging overall survival as expected. Currently, cetuximab is standard of care together with radiotherapy for head and neck squamous cell carcinomas (HNSCC) (4,5). To further optimize the efficacy of anti-EGFR treatment, it is essential to fully understand EGFR-related intracellular signaling.

Receptor tyrosine kinase signaling pathways are structurally and functionally linked with integrin-associated signaling to optimally regulate survival, proliferation, differentiation, adhesion and migration (6-9). Specific adapter molecules connect EGFR and integrins such as Nck2, particularly interesting new cysteine-histidine rich 1 (PINCH1) and integrin-linked kinase (ILK) (10-14). Data suggest that PINCH1 binds to Nck2 via its LIM4 domain and with its LIM1 domain to ILK (13,15). The exact EGFR-integrin interaction and transactivation mechanisms remain to be unraveled. However, ligand-dependent EGFR stimulation and integrin-mediated cell-extracellular matrix (ECM) adhesion seem inevitable for proper channeling of biochemical cues and control of cellular sensitivity to cytotoxic agents (16-19). Intriguingly, EGFR and integrin signaling have been shown to critically contribute to the cellular radiation response and repair processes involved in DNA double strand breaks (DSB), being the most severe in mammalian cells (16,20-24). Furthermore, both EGFR and integrin pathways participate in the repair of radiation-induced DNA lesions involving the key DNA damage recognition and repair proteins ATM and DNA-PK (23,25).

To address the role of the adapter proteins PINCH1 and Nck2 for EGFR signaling, cell survival and cellular radiosensitivity, we investigated in human squamous cell carcinoma (SCC) cells of the hypopharynx (FaDu) and the skin (A431) in a more physiological 3D laminin-rich (lr) ECM-based cell culture model (24,26). We found reduced clonogenic radiation survival of 3D grown SCC cells to the same extent as for PINCH1 or Nck2 knockdown, a finding correlative with impaired DSB repair.

Materials and methods

Antibodies and reagents. Antibodies against PINCH1 (BD Biosciences, Heidelberg, Germany), EGFR, EGFR Y1068, EGFR Y1173, MAPK, MAPK T202/Y204, Akt, Akt S473, Akt T308, FAK, FAK Y397, Src, Src Y416, ATM, DNA-PK, Mre11, Rad50 and Nbs1 (Cell Signaling Technology, Frankfurt, Germany), ATM S1981 (Rockland Immunochemicals Inc., Pottstown, PA, USA), p53 binding protein 1 (53BP1; Novus Biologicals, Cambridge, UK), phospho-Histone H2AX S139 (Millipore, Darmstadt, Germany), β-actin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse (Amersham, Freiburg, Germany) antibodies were purchased as indicated. Coomassie was from Merck (Darmstadt, Germany), complete protease inhibitor cocktail was from Roche Diagnostics (Mannheim, Germany), BCA assay and SuperSignal West Dura Extended Duration Substrate were from Thermo Fisher Scientific (Karlsruhe, Germany), nitrocellulose membranes were from Schleicher & Schuell, and oligofectamine from Invitrogen (Karlsruhe, Germany).

3D cell culture. A431 and FaDu cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, MA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Cölbe, Germany) containing GlutaMAX-I supplemented with 10% fetal calf serum and 1% non-essential amino acids (PAA Laboratories) at 37˚C in a humidified atmosphere containing 7% CO_2. For 3D cell culture, plates were coated with 1% agarose (Sigma) to prevent cell attachment to the bottom of the well. Laminin-rich extracellular matrix (lrECM; Cultrex 3D Culture Matrix; Trevigen, Gaithersburg, MD, USA; BD Matrigel™ Basement Membrane Matrix; BD Biosciences) was added to the cell culture medium to obtain a final concentration of 0.5 mg/ml (26).

Radiation exposure. Irradiation was delivered at room temperature using 2 to 6 Gy single doses of 200 kV X-rays (Yxlon Y.TU 320; Yxlon International, Hamburg, Gemany; dose rate ~1.3 Gy/min at 20 mA) filtered with 0.5 mm Cu. The absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany). The dose-rate was ~1.3 Gy/min at 20 mA, and applied doses ranged from 0 to 6 Gy.

Colony formation assay. Clonogenic survival under three-dimensional (3D) growth conditions was determined in a 3D colony formation assay as published (26). Briefly, single cells were mixed with lrECM (Trevigen) to obtain a final concentration of 0.5 mg/ml and placed in agarose-coated 96-well plates. After 24 h, cetuximab was added to the medium to a final concentration of 5 μg/ml. After 24 h cells received 0 to 6-Gy irradiation. Cetuximab remained in the cell culture medium for the entire growth period. Cells were cultured for 9 days (A431) or 11 days (FaDu). Cell clusters with a minimum of 50 cells were counted microscopically. Plating efficiencies: numbers of colonies formed/numbers of cells plated and surviving fractions (SF): numbers of colonies formed/numbers of cells plated (irradiated) x plating efficiency (unirradiated)) were calculated. Each point on survival curves represents the mean surviving fraction from at least three independent experiments.

siRNA transfection. PINCH1 siRNA (sequence: 5'-GGACC UAUAUGAAUGGUUUtt-3'), Nck2 siRNA (sequence, 5'-GG GAAGAACAAACACUUCAtt-3') and a non-specific control (Co)siRNA (sequence, 5'-GCAGCUAUAUGAAUGUUGUtt-3') were obtained from Ambion (Frankfurt, Germany). siRNA transfection was performed as previously published (24). Twenty-four hours after delivery of 20 nM siRNA using oligofectamine, cells were plated in 3D lrECM. Colony formation assays and western blotting were carried out. Efficient PINCH1 knockdown was confirmed by western blotting, while Nck2 depletion was analyzed on mRNA level by use of RT-PCR.

Total protein extracts and western blotting. Cells cultured in 3D lrECM (Trevigen) were lysed with modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet-P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, complete protease inhibitor cocktail, 1 mM $NaVO_4$, 2 mM NaF]. Total protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Probing and detection of specific proteins with indicated antibodies were performed as previously described (26).

Reverse transcription-PCR. For validating Nck2 knockdown total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). cDNA was prepared with SuperScript™ III reverse transcriptase kit according to the instructions of the manufacturer (Invitrogen). RT-PCR was performed for Nck2 and G3PDH (Nck2-fw, 5'-TGCTGGAC GACTCCAAGAC-3' and Nck2-rev, 5'-AGCCCTTCTTCA GGCTGTTC-3'; G3PDH-fw, 5'-ACCACAGTCCATGCCA TCAC-3' and G3PDH-rev, 5'-TCCACCACCCTGTTGCT GTA-3'; Eurofins MWG Operon, Ebersberg, Germany) using 2 μl of cDNA and HotStar Taq polymerase (Qiagen, Venlo, The Netherlands) according to standard PCR protocols. Results of RT-PCR were analyzed using 1.5% agarose gels (Sigma) with 0.1% ethidium bromide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

Immunofluorescence staining. For detection of residual DNA double-strand breaks (rDSB), the phosphorylated histone H2AX S139 (γH2AX)/p53 binding protein 1 (53BP1) foci assay was performed as published (26). Cells were grown in 0.5 mg/ml lrECM (BD Matrigel™) under 3D conditions for 24 h, irradiated with 0 or 6 Gy and isolated 24 h post irradiation. γH2AX/p53BP1-positive nuclear foci of 50 cells were counted microscopically with an Axioscope 2 plus fluores-

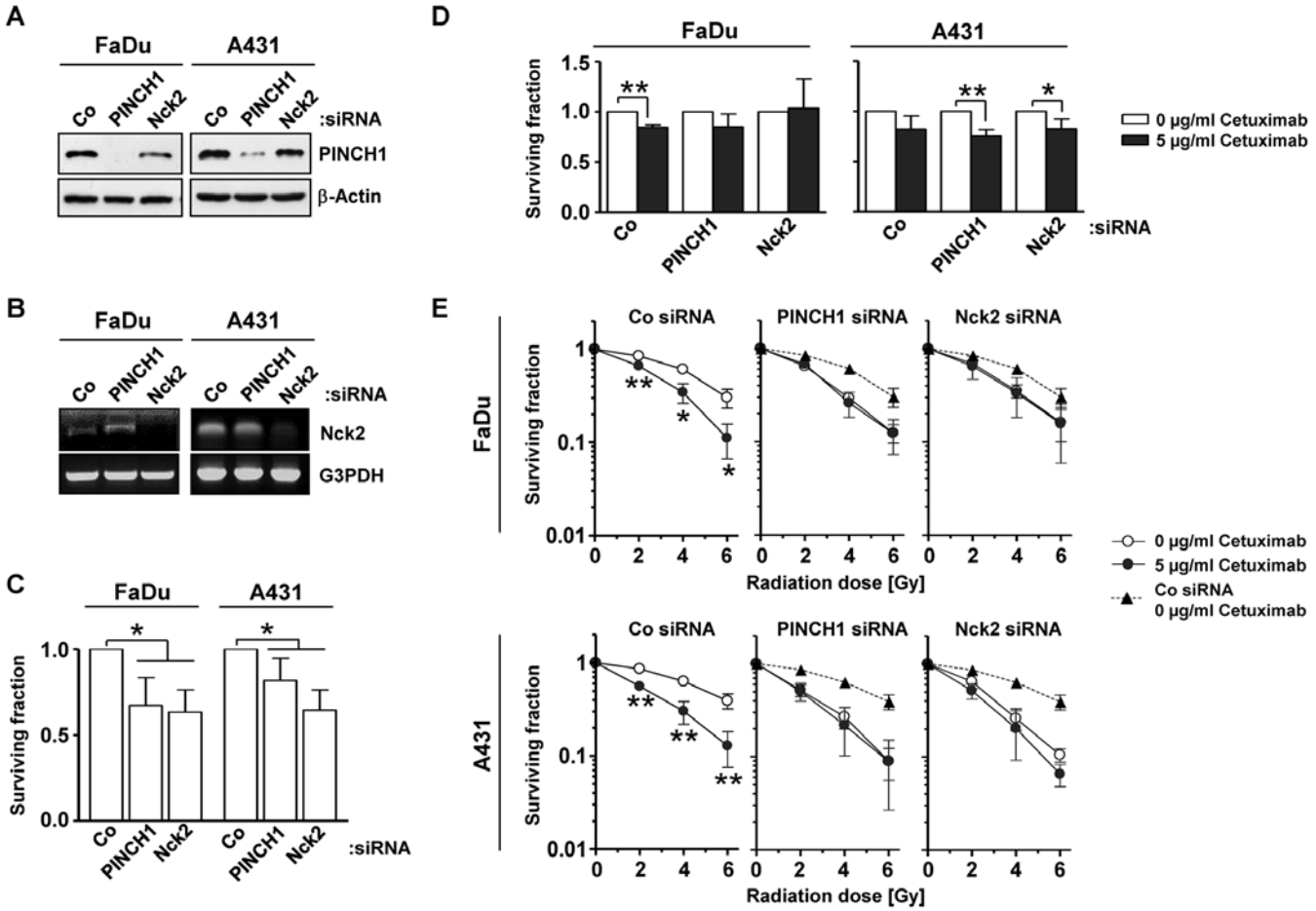

Figure 1. Cetuximab treatment and PINCH1 or Nck2 knockdown result in similar enhancement of radiosensitivity in 3D lrECM grown FaDu and A431 SCC cells. (A) PINCH1 western blot analysis of PINCH1- and Nck2-siRNA treated cells. β-actin served as loading control. (B) Nck2 reverse transcription-PCR products of PINCH1- and Nck2-siRNA treated cells. G3PDH served as control. (C) Basal clonogenic survival of 3D FaDu and A431 cell cultures under PINCH1 or Nck2 knockdown. (D) Clonogenic cell survival of PINCH1 or Nck2 knockdown FaDu and A431 cell cultures exposed to cetuximab. (E) Clonogenic radiation survival of 3D-grown PINCH1 and Nck2 knockdown cell cultures additionally treated with cetuximab 24 h prior to irradiation. Results show mean ± SD (n=3; t-test; *P<0.05; **P<0.01). Co siRNA, non-specific siRNA control.

cence microscope (Carl Zeiss AG, Jena, Germany) and were defined as DSB.

Stimulation with EGF. Cells cultured in 3D lrECM (Trevigen) were serum starved for 24 h followed by a 1 h-treatment with 5 µg/ml cetuximab and 15-min stimulation with 10 nM EGF before cells were harvested and whole cell lysates were used for western blotting.

Data analysis. Data were expressed as means ± SD of at least three independent experiments. To test statistical significance, Student's t-test was performed using Microsoft® Excel 2003. Results were considered statistically significant at P-values of <0.05.

Results

Depletion of the adapter proteins PINCH1 and Nck2 enhances the radiosensitivity of 3D grown SCC cells. We commenced the present study by measuring the effect of PINCH1 or Nck2 knockdown in SCC cells without and in combination with the monoclonal anti-EGFR antibody cetuximab. While the efficient PINCH1 (Fig. 1A) and Nck2 (Fig. 1B) knockdown alone caused significantly (P<0.05) reduced clonogenic survival in both cell lines (Fig. 1C), its combination with cetuximab resulted in only minor cell line-specific alterations of clonogenicity relative to controls (Fig. 1D). Intriguingly, depletion of PINCH1 or Nck2 enhanced the radiosensitivity of FaDu and A431 cells compared to siRNA controls (Fig. 1E) and, notably, to the same extent as observed for the combination of cetuximab plus X-ray irradiation (Fig. 1E). These data indicate PINCH1 and Nck2 to play an important role in the cellular response to radiation and to serve as critical determinants of EGFR associated downstream signaling.

Cetuximab differentially impacts on EGFR downstream signaling upon PINCH1 or Nck2 knockdown. To optimally assess the inhibitory efficacy of cetuximab on EGFR and its downstream signaling, we serum-starved our 3D cell cultures. Upon EGF stimulation, EGFR tyrosine (Y)1068 and Y1173 phosphorylation were induced while cetuximab effectively prevented this induction (Fig. 2A). In spite of the effective EGFR blocking, increased MAPK threonine (T)202/Y204 and Akt S473/T308 phosphorylation was detected upon EGF application in cetuximab-treated FaDu cultures comparable to untreated controls (Fig. 2A). In contrast, FAK and Src, which

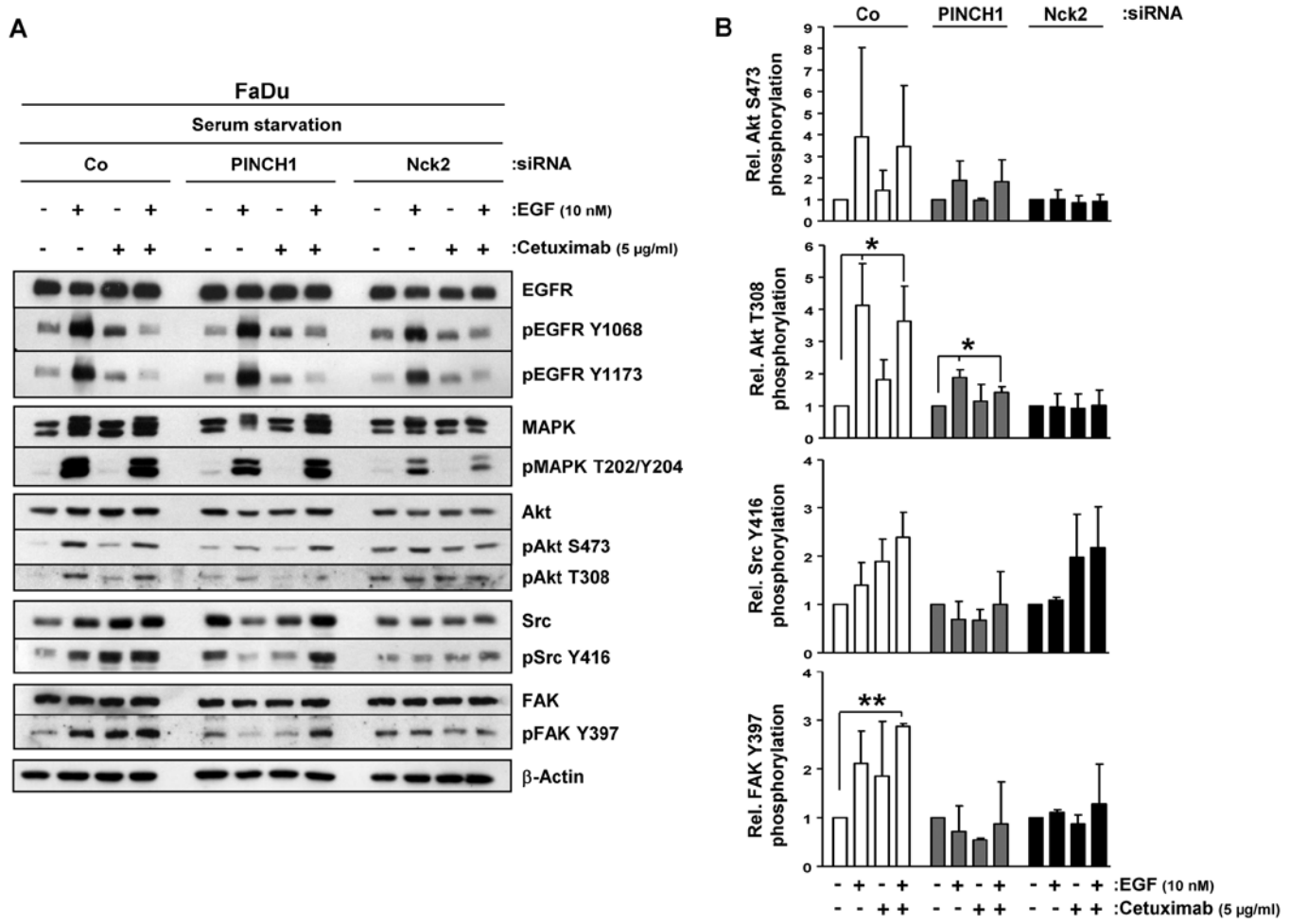

Figure 2. Cetuximab prevents EGF-induced EGFR phosphorylation and fails to block downstream signaling. (A) 3D lrECM grown PINCH1 or Nck2 knockdown cell cultures were serum-starved for 24 h, treated with cetuximab for 1 h and then stimulated with EGF for 15 min. Western blot analysis on whole-cell lysates and detection of EGFR and associated signaling molecules. β-actin served as loading control. (B) Fold-change of Akt S473 and T308, FAK Y397 and Src Y416 phosphorylation upon normalization to total protein expression under EGF stimulation and/or cetuximab treatment relative to untreated control (mean ± SD; n=2; t-test; $^{**}P<0.01$). Co siRNA, non-specific siRNA control.

have been shown to locate downstream of EGFR, demonstrated enhanced phosphorylated upon EGF exposure, which even increased when cetuximab was applied (Fig. 2).

When combined with PINCH1 or Nck2 depletion, the phosphorylation pattern of EGFR remained similar to controls (Fig. 2A). In contrast to PINCH1 depletion, MAPK showed attenuated phosphorylation upon EGF and cetuximab exposure under Nck2 knockdown relative to siRNA controls (Fig. 2). Independent from cetuximab, phospho-Akt S473/T308 was slightly induced by EGF in PINCH1 knockdown cultures and marginally induced under all tested conditions in Nck2 depleted cells (Fig. 2). Similar patterns were observed for Src and FAK phosphorylations. PINCH1 silencing prevented induction of Src and FAK phosphorylation by EGF but enabled strong phosphorylation and in cetuximab-treated, EGF-exposed cells (Fig. 2). Nck2 depletion facilitated stimulation of Src Y416 phosphorylation upon EGF and EGF/cetuximab without affecting FAK phosphorylation. These data suggest a function of PINCH1 and Nck2 in EGFR signaling.

EGFR signaling is modulated in 3D PINCH1 and Nck2 knockdown cultures cell line-dependently. In the next step, we investigated EGFR signaling in 10% serum, 3D lrECM grown cell cultures to find signaling modifications that contribute to the enhanced radiosensitivity seen upon cetuximab treatment and PINCH1 or Nck2 knockdown. Cetuximab and PINCH1 or Nck2 knockdown caused reduced Y1068 and unchanged Y1173 phosphorylation of the EGFR in 3D lrECM FaDu cultures (Fig. 3). While Akt serine (S)473 and Scr Y416 stayed stable, MAPK T202/Y204 and Akt T308 phosphorylation were significantly diminished by cetuximab but not PINCH1 or Nck2 depletion in FaDu cells (Fig. 3). In A431 cells, EGFR, Akt T308 and Src Y416 phosphorylation remained largely unmodified upon cetuximab or knockdowns, while MAPK T202/Y204 and Akt S473 showed reduced phosphorylation due to PINCH1 or Nck2 knockdown or cetuximab, respectively (Fig. 3). The only protein kinase showing similar modifications in both cell lines upon knockdown and cetuximab was FAK at its Y397 autophosphorylation site (Fig. 3). These data demonstrate differential impact of cetuximab and PINCH1 or Nck2 depletion on EGFR signaling in 3D lrECM cell cultures grown in 10% serum. Furthermore, the inconsistencies in signaling modifications in the two tested SCC cell lines

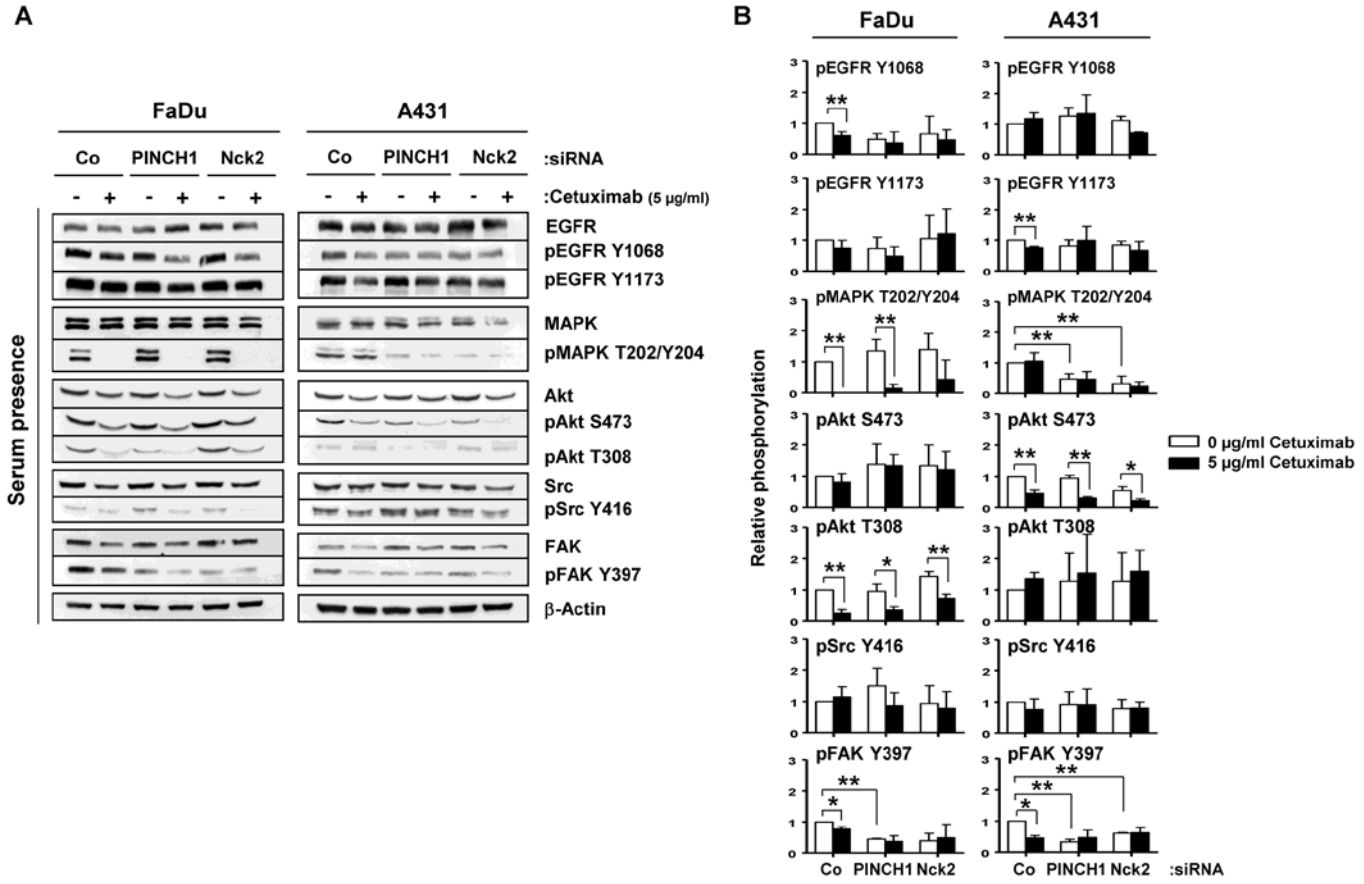

Figure 3. Cetuximab and PINCH1 or Nck2 depletion affects differentially EGFR signaling in 3D lrECM cell cultures. (A) Western blot analysis on whole-cell lysates from serum (10%) grown, 3D PINCH1 or Nck2 knockdown cell cultures treated with cetuximab 1 h prior to lysis and detection of EGFR and associated signaling molecules. β-actin served as loading control. (B) Densitometric analysis of western blot analyses shown in A. Phosphorylation was normalized to total protein expression (mean ± SD; n=3; t-test; $^{*}P< 0.05$; $^{**}P<0.01$). Co siRNA, non-specific siRNA control.

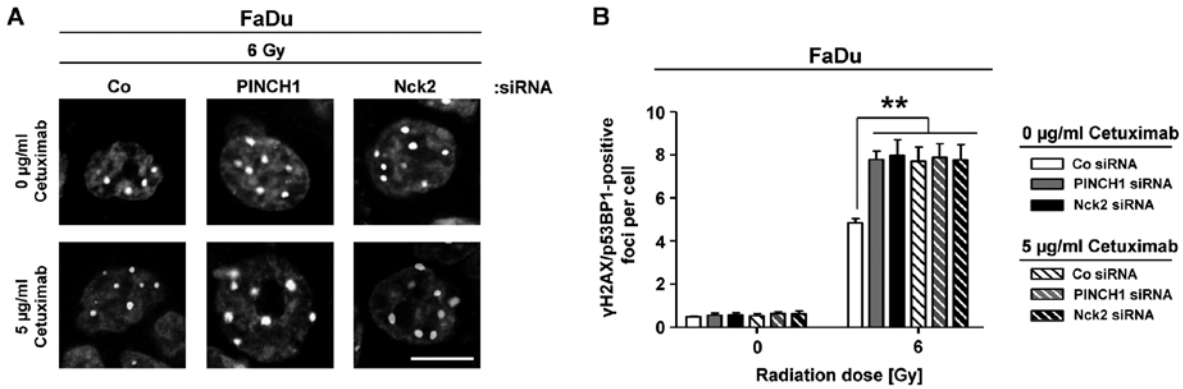

Figure 4. Cetuximab treatment, PINCH1 and Nck2 knockdown result in increased DNA double strand break numbers in X-ray exposed 3D lrECM grown FaDu cell cultures. (A) Representative photographs show γH2AX/p53BP1 double staining for residual DSB. DAPI was used for nuclear staining. Scale bar, 10 μm. (B) Number of γH2AX/p53BP1-positive foci per cell 24 h after 6-Gy irradiation (50 nuclei were counted). Results show mean ± SD (n=3; t-test; $^{**}P<0.01$). Co siRNA, non-specific siRNA control.

cannot explain the similarity in radiosensitization as result from cetuximab treatment or PINCH1 or Nck2 depletion.

PINCH1 and Nck2 knockdown hampers DNA double strand break repair. Based on these observations and in line with radiosensitization, a highly significant and similar increase in the number of γH2AX/53BP1-positive foci was observed in 6-Gy-irradiated cetuximab-treated or PINCH1 or Nck2 knockdown FaDu cell cultures relative to corresponding controls (Fig. 4). Approximately 3 additional rDSB were detectable under the different conditions.

To better understand the underlying mechanisms of this elevated rDSB rate, we analyzed expression and phosphorylation of a variety of key proteins of the DNA damage recognition

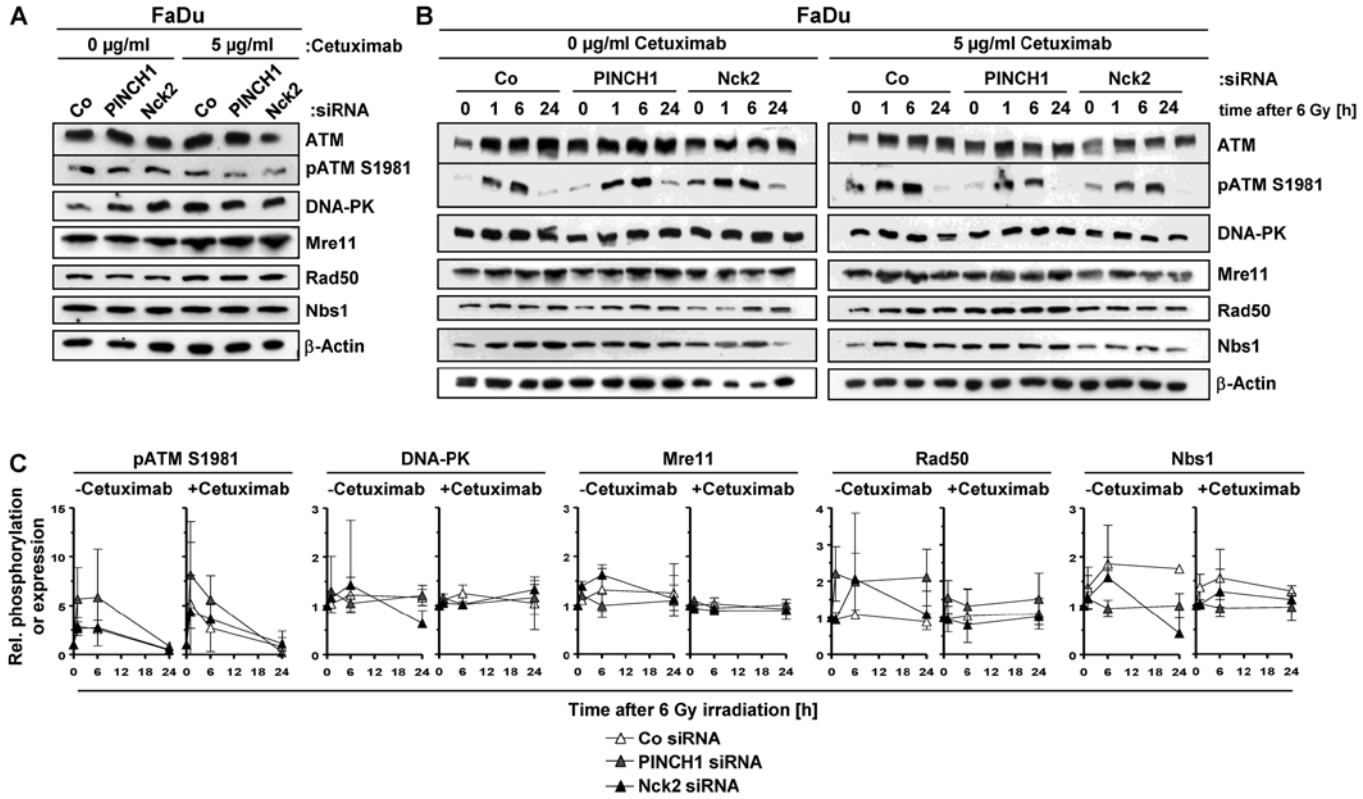

Figure 5. Expression of DNA repair-associated proteins remains largely unchanged upon cetuximab treatment and PINCH1 or Nck2 knockdown in 3D lrECM cell cultures. (A) Western blot analysis on 3D FaDu PINCH1 or Nck2 knockdown cultures treated with cetuximab for 1 h and detection of different DNA repair molecules. β-actin served as loading control. (B) Western blot analysis on whole-cell lysates of 3D grown FaDu PINCH1 or Nck2 knockdown cell cultures treated with cetuximab 1 h prior to 6-Gy irradiation and harvested 0, 1, 6 or 24 h thereafter. Detection of DNA repair proteins. β-actin served as loading control. (C) Densitometric analysis of western blot analyses shown in B. Phosphorylation was normalized to total protein expression (mean ± SD; n=2). Co siRNA, non-specific siRNA control.

and repair machinery in cetuximab-treated and untreated cells as well as upon PINCH1 or Nck2 silencing. Notably, a pattern of alterations in the tested proteins that provides an explanatory basis for the increased number of rDSB and radiosensitization upon PINCH1 or Nck2 knockdown or cetuximab administration was not observed (Fig. 5). Remarkable were the enhanced ATM S1981 phosphorylation in PINCH1 knockdown cells and the differences in Rad50 and Nbs1 expression under PINCH1 and Nck2 depletion between cetuximab absence and presence (Fig. 5B and C). These observations suggest that EGFR blocking using cetuximab or depletion of the adaptor proteins PINCH1 or Nck2 promotes radiosensitization, which emanates from hampered rDSB repair involving yet to be identified mechanisms.

Discussion

EGFR downstream signaling is influenced by a variety of extra- and intracellular factors. Among the intracellular factors, there exist adapter proteins that structurally bridge EGFR and integrins while their functional role in signal transduction and cancer cell therapy resistance is less clear (6,9,14). Focusing on the focal adhesion proteins PINCH1 and Nck2, we investigated the function of these two proteins in EGFR signaling and cellular radiation response of SCC cells grown under more physiological 3D lrECM conditions. In the present study, we show that: i) single knockdown of PINCH1 or Nck2 resulted in enhanced radiosensitivity of SCC cells comparable with effects seen after cetuximab treatment alone; ii) modifications in MAPK, Akt and FAK phosphorylation occurred upon cetuximab treatment as well as PINCH1 or Nck2 depletion; and iii) tumor cell radiosensitization by cetuximab and PINCH1 or Nck2 silencing is in accordance with attenuated repair of DNA double strand breaks.

A connection between cell adhesion, cell-ECM interactions and cancer therapy resistance has been demonstrated by a large number of reports from our group and others (18,19,23,24,27-29). Focal adhesion proteins seem to be part of fundamental signaling hubs as their depletion or pharmacological inhibition cause reduced cell viability and enhanced therapy resistance in different tumor models (8,18,19,24,26,28-31). Compared to PINCH1 not much is known about the function of the adapter protein Nck2. PINCH1 is critically involved in regulating cell shape, attachment, spreading and motility (15,32,33). In line with previous *in vitro* and *in vivo* data (30,34), knockdown of PINCH1 resulted in enhanced radiosensitivity of the head and neck squamous cell carcinoma cell line FaDu and the epidermoid cancer cell line A431. In addition to Nck2 being involved in IGF-1 signaling (15), regulating cell migration (35,36) and apoptosis (37), we show that Nck2 is also key for EGFR downstream signaling and clonogenic radiation survival.

Despite the cell line dependency of the shown effects, both PINCH1 and Nck2 determine the involvement and

the phosphorylation of a spectrum of EGFR downstream located mediators ranging from MAPK via Akt to FAK. In HT-1080 fibrosarcoma cells, Chen *et al* (38) documented a regulating role of PINCH1 in MAPK phosphorylation resulting in increased levels of the proapoptotic protein Bim to trigger activation of the intrinsic apoptosis pathway. We observed this phenomenon of reduced MAPK phosphorylation in PINCH1$^{-/-}$ embryonic mouse fibroblasts but not in human cancer cells, nor under 3D lrECM cell culture conditions, which points at a great dependence of MAPK phosphorylation on growth conditions and the cancer cell model (30). For cell survival, radiochemosensitivity, adhesion and spreading, PINCH1 serves as interacting platform for Akt and protein phosphatase 1α as well as RSU-1 at its LIM5 domain (34,39,40). In primitive endoderm cells, PINCH1 regulates JNK activation via RSU-1 and Bax activity via integrin signaling (41). Nck2 also links to apoptosis regulation in case of UV-radiation through enhanced caspase-3 and PARP cleavage (37). While modification of FAK upon EGFR inhibition is known (16,42), alterations of FAK phosphorylation by PINCH1 are novel and similarly observable for Nck2. These data strongly indicate that EGFR signaling is more complex than first thought. PINCH1 and Nck2 mediate differential connections with downstream signaling proteins on the basis of a unique, yet to be identified proteome expressed in the tested cell lines.

Notably, this concept is obviously not applicable for the closely associated endpoints clonogenic radiation survival and DNA double strand break repair. Both SCC cell lines are similarly radiosensitized and display a similar number of unrepaired DSB. These data are surprising and indicate that a particular part of the intracellular signaling network is greatly overlapping between PINCH1 and Nck2. Possibly, as both proteins shuttle between focal adhesions and cell nucleus, the DNA damage response is critically controlled by these proteins. While PINCH1 seems to interact with the nuclear transcription factor Wilms tumor 1 protein (43), Nck1 is supposed to be carried into the nucleus by the suppressor of cytokine signaling 7 as this protein possesses a nuclear import and export sequence (44). Upon DNA damage, Nck1 expression impacts on cell cycle blockage and interferes with ATM/ATR signaling. Nck2 also has a nuclear function in acting as repressor of gene transcription of jun/fos promoter elements induced by v-Abl (45).

In summary, our data suggest that the adapter proteins PINCH1 and Nck2 critically participate in the regulation of cellular radiosensitivity and EGFR function and downstream signaling in 3D grown human SCC cells. Future work is warranted to provide detailed information on the molecular circuitry how PINCH1 and Nck2 control EGFR signaling and cellular radiosensitivity.

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