

The impact of CDK9 on radiosensitivity, DNA damage repair and cell cycling of HNSCC cancer cells

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Abstract. Cyclin-dependent kinase 9 (CDK9), mainly involved in regulation of transcription, has recently been shown to impact on cell cycling and DNA repair. Despite the fact that CDK9 has been proposed as potential cancer target, it remains largely elusive whether CDK9 targeting alters tumor cell radiosensitivity. Five human head and neck squamous cell carcinoma (HNSCC) cell lines (SAS, FaDu, HSC4, Cal33, UTSCC5) as well as SAS cells stably transfected with CDK9-EGFP-N1 plasmid or empty vector controls were used. Upon either CDK9 small interfering RNA knockdown or treatment with a pan-CDK inhibitor (ZK304709), colony formation, DNA double strand breaks (DSBs), apoptosis, cell cycling, and expression and phosphorylation of major cell cycle and DNA damage repair proteins were examined. While CDK9 overexpression mediated radioprotection, CDK9 depletion clearly enhanced the radiosensitivity of HNSCC cells without an induction of apoptosis. While the cell cycle and cell cycle proteins were significantly modulated by CDK9 depletion, no further alterations in these parameters were observed after combined CDK9 knockdown with irradiation. ZK304709 showed concentration-dependent cytotoxicity but failed to radiosensitize HNSCC cells. Our findings suggest a potential role of CDK9 in the radiation response of HNSCC cells. Additional studies are warranted to clarify the usefulness to target CDK9 in the clinic.

Introduction

The fate of a cell upon exposure to ionizing radiation depends in great part on their capacity to repair the damage and

maintain genomic integrity (1). For this purpose, the repair of radiation-induced DNA double strand breaks (DSBs), being the most devastating DNA lesions, and cell cycling tightly interrelate in a well-defined manner (1-3).

Although the general coordination and interrelation between DNA damage repair and cell cycling has intensively been documented (4), the detailed interplay of the molecular players is still not well understood. Cyclin-dependent kinases (CDKs), for example, are a family of over 11 members whose activity is controlled by post-translational modifications occurring in CDK-specific manner in the different phases of the cell cycle (5). Owing to their crucial function in cell cycling and DNA damage repair and frequent aberrations of their activities in cancer encouraged an intensive screening for small-molecule CDK pharmacological inhibitors that block CDK activity (6-8). Despite the intensive research regarding CDKs as target for cancer therapy, studies evaluating their role in cancer cell response to irradiation are rare.

CDK9, unlike most other CDKs functioning in cell cycling, is particularly involved in gene transcription (9,10). It is activated by forming a heterodimeric complex with either cyclin T or cyclin K family members and inhibited by both 7SK and HEXIM1 proteins (11). In mammalian cells, two CDK9 isoforms of different molecular weight have been identified. The functional difference between these isoforms remains to be clarified (10). CDK9 together with its cyclin partner (cyclin T or cyclin K) forms a complex known as positive transcription elongation factor b (pTEFb) (12,13). This complex promotes transcription elongation by phosphorylating the CTD of the large subunit of RNA polymerase II (Rpb1-CTD) on S2 (10) or on S5 (14). In turn, pTEFb modulates other cellular functions such as co-transcriptional histone modification, mRNA processing and mRNA export (15,16). A recent study showed that CDK9 together with cyclin K, but not T is involved in maintaining genome integrity after replication stress (17). The connection between CDK9 and cell division has recently been reported in mammalian cells suggesting a direct role for the CDK9/cyclin K complex in checkpoint pathway regulation (17,18). Moreover, coprecipitation studies showed that CDK9/cyclin K complex interacts with ATR, ATRIP and other DNA repair and checkpoint proteins (17).

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Based on its function in cell cycle regulation and DNA damage repair, we hypothesized targeting of CDK9 to potentially modify the cancer cell response to radiotherapy. Therefore, we evaluated the significance of CDK9 inhibition using siRNA technology and a pharmacological inhibitor (ZK304709) (19) for the radioresponse in a panel of human head and neck squamous cell carcinoma (HNSCC) cell lines.

Materials and methods

Antibodies and reagents. Antibodies against GFP (Abcam, Cambridge, UK); BrdU (BD, Heidelberg, Germany); ATM, phospho-ATM (S1981), CDK9, CHK2, phospho-CHK2 (T86), cyclin E, DNA-PK, NBS1, phospho-Rb (S795), phospho-Rpb1 (S2/5) and Rpb1 (Cell Signaling, Frankfurt, Germany); cyclin A, PCNA, RAD50 and Rb (Santa Cruz, Heidelberg, Germany); phospho-histone γ H2AX (S139) (Millipore, Darmstadt, Germany); p53 binding protein 1 (53BP1) (Novus Biologicals, Littleton, CO, USA); cyclin D1 (Zymed Laboratories Inc.); β -actin and anti-mouse IgG FITC (Sigma-Aldrich, Taufkirchen, Germany); HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Amersham, Freiburg, Germany); Alexa 594 anti-mouse and Alexa 488 anti-rabbit (Invitrogen, Cyclin T, Germany) were purchased as indicated. ECL SuperSignal[®] West Dura Extended Substrate was from Pierce (Bonn, Germany); complete protease inhibitor cocktail from Roche (Mannheim, Germany); propidium iodide (PI) from Serva (Heidelberg, Germany); the phosphatase inhibitors Na_3VO_4 and NaF from Sigma-Aldrich, Vectashield/DAPI mounting medium was from Alexis (Grünberg, Germany) and oligofectamine from Invitrogen.

CDK inhibitor. ZK304709 is a pharmacological pan-CDK inhibitor kindly provided by Bayer Pharma AG, Germany (19).

Cell culture. Human SAS, FaDu, HSC4, Cal33, UTSCC5 HNSCC cell lines (a kind gift from R. Grenman, Turku University Central Hospital, Finland, and M. Baumann, Dresden University of Technology, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM, PAA, Cölbe, Germany) containing 10% fetal bovine serum (FBS, PAA) and 1% non-essential amino acids (NEAA, PAA) at 37°C in a humidified atmosphere containing 7% CO_2 . In all experiments, asynchronously and exponentially growing cells were used.

Generation of CDK9-EGFP transfectants. CDK9 PCR fragment (hCDK9-NheI forward: 5'-ctaGCTAGCgcccATG GCAAAGCAGTACGACTCGG-3'; hCDK9-BamHI reverse: 5'-cgGGATCCcgGAAGACGCGCTCAAACCTCCG-3') was amplified using placental DNA (UKD, Dresden, Germany), followed by ligation of CDK9 in frame into NheI/BamHI restriction sites of pEGFP-N1 expression vector (CDK9-EGFP-N1). Stable transfection and selection was performed as described (20).

Colony formation assay. The colony formation assay was applied for measurement of clonogenic cell survival as published (20). In brief, single cells were grown on polystyrene 6-well plates (BD) 24 h prior to irradiation with 0–8 Gy.

After seven days (SAS), 11 days (FaDu), eight days (HSC4), 13 days (Cal33) or 14 days (UTSCC5), cells were fixed with 80% ethanol, stained with Coomassie blue and cell colonies (>50 cells) were counted. Images of representative colonies were scanned using Perfection 4490 Photo scanner (Epson, Meersburg, Germany). Each point on the survival curves represents the mean surviving fraction from three independent experiments.

Radiation exposure. Cells were irradiated at room temperature using single doses of 200 kV X-rays (2, 4, 6 or 8 Gy; Yxlon Y.TU 320; Yxlon; 0.5 mm copper filter; ~1.3 Gy/min, 20 mA) filtered with 0.5 mm Cu. The absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany).

Small interfering RNA-mediated knockdown of CDK9. Human CDK9 small interfering ribonucleic acid (siRNA) (#1 sequence: 5'-GGAGAAUUUACUGUGUUtt-3'; #2 sequence: 5'-GGU GCUGAUGGAAAACGAGtt-3') were obtained from Applied Biosystems (Darmstadt, Germany). Non-specific control siRNA (sequence: 5'-GCAGCUAUAUGAAUGUUGtt-3') was from MWG (Martinsried, Germany). Cells were prepared for siRNA transfection (20 nmol/l) with oligofectamine and were subjected 24 h after transfection to colony formation assay and western blotting as previously published (20).

Immunofluorescence staining. For detection of DSBs, the phosphorylated H2AX-S139 (γ H2AX)/p53 binding protein 1 (p53BP1) foci assay was performed as published (20). γ H2AX/p53BP1-positive nuclear foci of 50 cells were counted microscopically with an Axioscope 2 plus fluorescence microscope (Zeiss) and defined as DSBs. Fluorescence images were obtained using an LSM 510 Meta equipped with Zeiss LSM 510 Software (Zeiss).

DAPI staining. For apoptosis analysis, cells were trypsinized and fixed with 80% ethanol for ≥ 24 h as published (21). Typical apoptotic nuclear shape was analyzed using Vectashield/DAPI mounting medium. Apoptotic nuclei of ≥ 100 cells from 3 independent experiments were counted microscopically using an Axioscope 2 microscope (Zeiss).

Total protein extracts and western blotting. Total protein extracts were isolated as previously described (20). Therefore, cells were lysed using modified RIPA buffer [50 mM Tris-HCl (Carl Roth, Karlsruhe, Germany), pH 7.4], 1% Nonidet-P40 (Sigma-Aldrich, Taufkirchen, Germany), 0.25% sodium deoxycholate (AppliChem, Darmstadt, Germany), 150 mM NaCl (VWR International, Darmstadt, Germany), 1 mM ethylenediaminetetraacetic acid (Merck), complete protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM NaVO_4 (AppliChem), 2 mM NaF (AppliChem)]. Samples were stored at -80°C. Total protein amount was measured using the bicinchoninic acid assay (Pierce, Bonn, Germany). After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, protein detection was performed as published (20).

Cell cycle analysis. Cells were incubated with 10 mM bromodeoxyuridine (BrdU; Serva) for 10 min, trypsinized

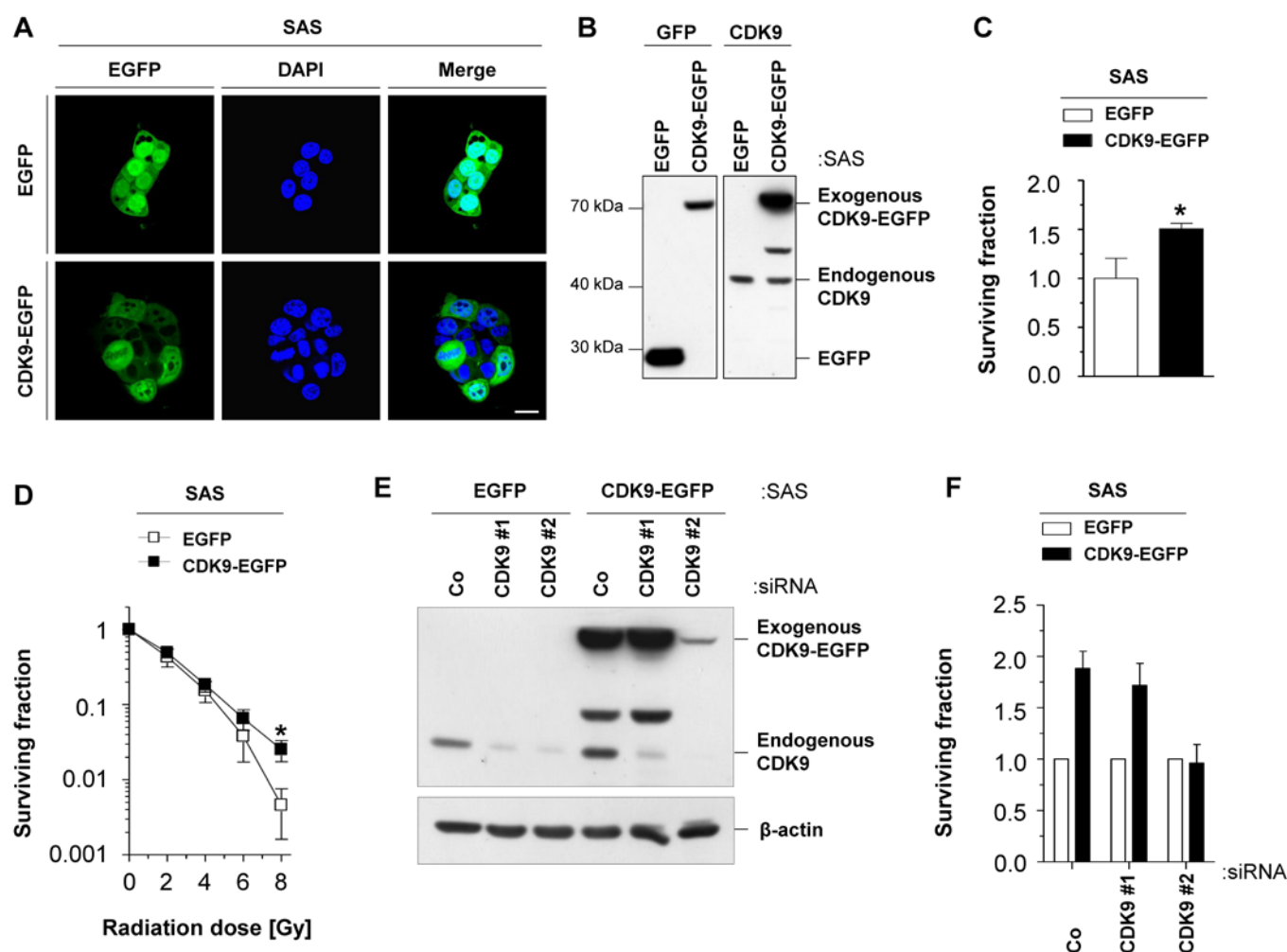


Figure 1. Exogenous expression of CDK9 induces higher basal cell survival and radioresistance in SAS cells. (A) Fluorescence images of EGFP- and CDK9-EGFP- (green) expressing SAS cells. Cell nuclei were stained with DAPI (blue). Bar, 20 μ m. (B) Western blotting of EGFP and CDK9 in EGFP and CDK9-EGFP transfectants. β -actin served as loading control. SAS-CDK9-EGFP and SAS-EGFP transfectants were plated for colony formation and irradiated after 24 h with X-rays (0-8 Gy, single dose). After 7 days, formed colonies were counted microscopically. (C) Plating efficiency and (D) clonogenic radiation survival were calculated. Results represent mean \pm SD (n=3; Student's t-test; *P<0.05). (E) Upon siRNA transfection, whole cell lysates were harvested and western blotting was performed. β -actin served as loading control. (F) Clonogenic radiation survival of SAS-CDK9-EGFP and SAS-EGFP cells upon siRNA transfection. Results show mean \pm SD (n=3; Student's t-test; *P<0.05).

and fixed in 80% ice cold ethanol. Then, cells were prepared for cell cycle analysis as published (22). Detection of BrdU was accomplished with anti-BrdU and anti-mouse IgG FITC antibodies and total DNA staining with propidium iodide (PI) solution. Acquisition of data was performed with a CyFlow (Partec, Münster, Germany). The distribution of cells in the different phases of the cell cycle was analyzed from the DNA dot-blots and histograms using FloMax software.

Statistical analysis. Data were expressed as means \pm SD of at least three independent experiments. P-values are based on the Student's t-test (Microsoft® Excel 2003). Results were considered statistically significant when a P-value of <0.05 was reached.

Results

CDK9 overexpression enhances the clonogenic survival of HNSCC cells. To better understand the role of CDK9 in clonogenic survival of unirradiated and irradiated cells, we

first stably transfected SAS cells with CDK9-EGFP construct and characterized its cytoplasmic and nuclear localization and expression in whole cell lysates (Fig. 1A and B). We further found that CDK9 overexpression significantly (P<0.05) increased plating efficiency (Fig. 1C) and clonogenic radiation survival (Fig. 1D). To prove CDK9 dependence of these results, we depleted CDK9 expression using siRNA, which resulted in a plating efficiency similar to controls (Fig. 1E and F).

CDK9 knockdown radiosensitizes HNSCC cell lines. To investigate the potential of CDK9 as cancer target in more detail, we next assessed the effects of CDK9 knockdown on plating efficiency and radiation survival in a panel of five HNSCC cell lines. The efficient CDK9 knockdown (Fig. 2A) modified the plating efficiencies rather moderately, except in SAS cells, which showed significantly (P<0.05) reduced survival (Fig. 2B). In combination with irradiation, HSC4, Cal33 and UTSCC5 cells were only slightly altered in the radiosensitivity upon CDK9 depletion, while SAS and FaDu cells were significantly (P<0.05) sensitized to X-ray irradiation (Fig. 2C).

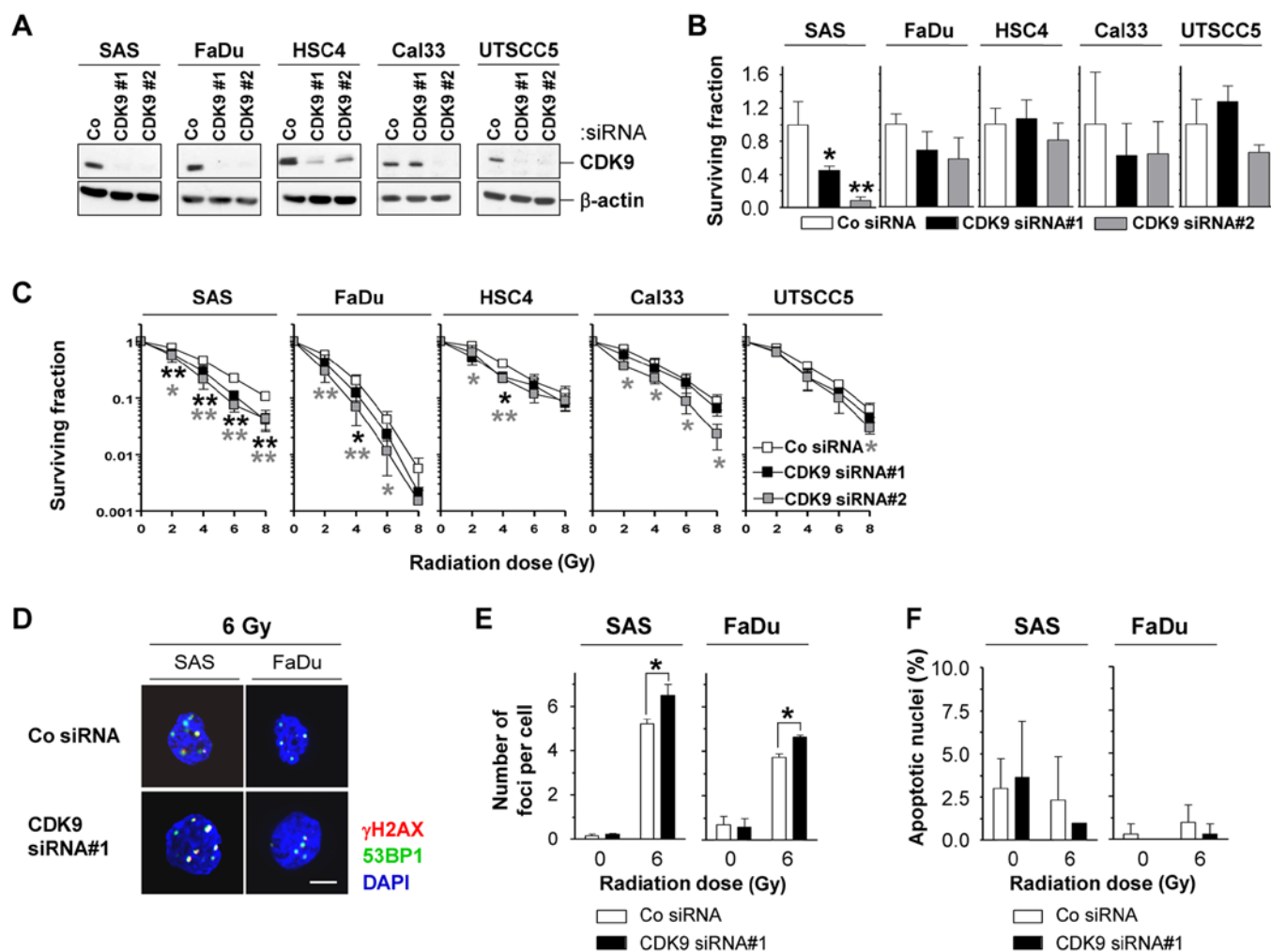


Figure 2. CDK9 knockdown modulates the radiosensitivity of HNSCC cells. (A) Cells were transfected (CDK9 siRNA#1, CDK9 siRNA#2; Co siRNA) and efficient CDK9 depletion was confirmed by western blotting. β -actin served as loading control. (B) Plating efficiencies after CDK9 knockdown. Results show mean \pm SD (n=3; Student's t-test; * P <0.05; ** P <0.01). (C) Clonogenic radiation survival of indicated HNSCC cell lines upon CDK9 knockdown and X-ray irradiation (0–8 Gy, single dose). Results show mean \pm SD (n=3; Student's t-test; * P <0.05; ** P <0.01). (D) Representative images show γ H2AX/p53BP1 double staining for residual DSBs. DAPI was used for nuclear staining. Bar, 10 μ m. (E) Number of γ H2AX/p53BP1-positive foci per cell at 24 h after irradiation (50 nuclei were counted). Results show mean \pm SD (n=3; t-test; * P <0.05). (F) CDK9 siRNA#1-transfected cells (non-specific siRNA used as control) were irradiated with X-rays (0 or 6 Gy, single dose) and the percentage of apoptotic nuclei per 100 cells was determined. Results show mean \pm SD (n=3).

In line with the CDK9-dependent radiosensitization occurred a significantly (P <0.05) increased number of γ H2AX/53BP1-positive foci per cell in 6-Gy irradiated CDK9 knockdown SAS and FaDu cell cultures relative to controls (Fig. 2D and E). Enhanced rates of apoptosis were not found after CDK9 knockdown alone or in combination with irradiation (Fig. 2F).

CDK9 depletion fails to modulate DNA repair proteins. In SAS and FaDu cells showing the strongest radiosensitization by CDK9 silencing, we analyzed the expression and phosphorylation patterns of various proteins involved in DNA repair 15 min after 6 Gy X-rays (Fig. 3A). Surprisingly, we found no significant alterations in expression and phosphorylation of DNA repair proteins upon CDK9 knockdown in SAS and FaDu cells (Fig. 3B).

CDK9 knockdown modulates cell cycling in SAS and FaDu cells. Next, we sought to investigate whether CDK9 play a

role in cell cycling and found that CDK9 knockdown in SAS and FaDu cells, respectively, results in a significant (P <0.05) increase in G1 phase cells and a significant (P <0.05) decrease in the S phase cell population (Fig. 4A). No changes were observed in the G2/M phase cell population (Fig. 4A). In combination with irradiation, CDK9-depleted SAS and FaDu cells showed no significant alterations in their cell cycle distribution (Fig. 4B).

Silencing of CDK9 impacts on cell cycle protein expression.

Next we analyzed the expression and phosphorylation of a panel of cell cycle regulatory proteins and of Rpb1 upon CDK9 knockdown (Fig. 5A). Interestingly, in line with CDK9 depletion, we found a rapid decline in the level of cyclin D1, an induction of cyclin E, and a slight reduction in Rb S795 phosphorylation in both SAS and FaDu cell lines as compared to controls (Fig. 5B). Moreover, levels of RB, Rpb1-CTD, or phospho-Rpb1-CTD(S2/5) remained stable.

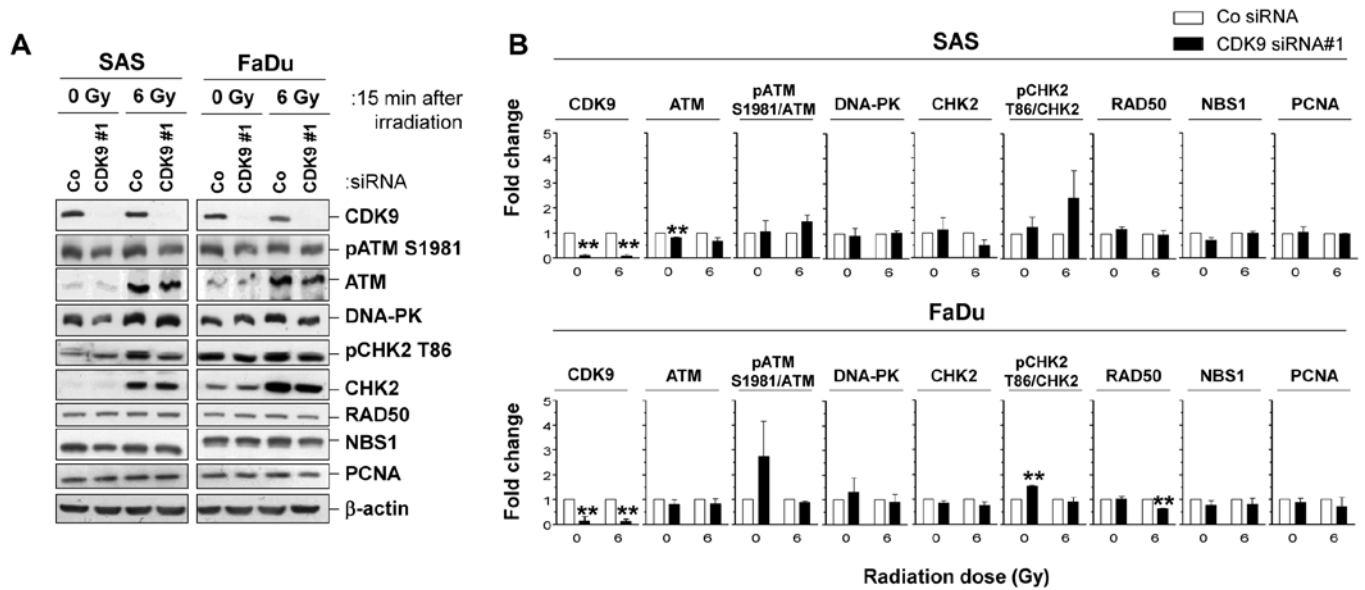


Figure 3. CDK9 depletion does not alter DNA damage repair protein expression. (A) Whole cell lysates of CDK9-depleted cell cultures (non-specific siRNA used as control) were harvested 15 min after irradiation (0 or 6 Gy, X-rays, single dose) and analyzed by western blotting using indicated antibodies. β -actin served as loading control. (B) Densitometric analysis of the western blots. Data were normalized to β -actin. Results show mean \pm SD (n=2; Student's t-test; **P<0.01).

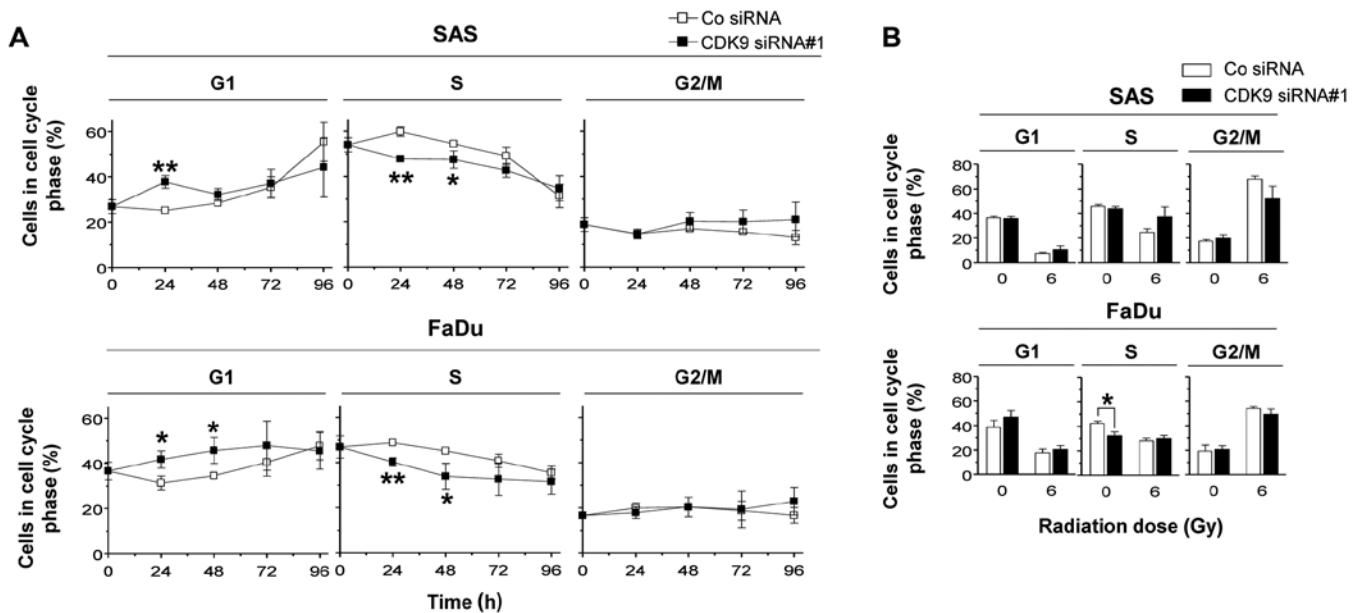


Figure 4. Depletion of CDK9 changes in cell cycle distribution of SAS and FaDu cells. (A) Cell cycle analysis of CDK9-depleted SAS and FaDu cell cultures as compared to controls. At indicated time-points, cells were pulse-labeled with BrdU and fixed. Cell nuclei were extracted, immunostained with anti-BrdU/FITC and counterstained with PI. Results show mean \pm SD (n=3; Student's t-test; *P<0.05; **P<0.01). (B) Cell cycle analysis of unirradiated and 6 Gy-irradiated CDK9-depleted SAS and FaDu cells. After 14 h, cells were pulse-labeled with BrdU and fixed. Cell nuclei were extracted, immunostained with anti-BrdU/FITC and counterstained with PI. Results show mean \pm SD (n=3; Student's t-test; *P<0.05).

These results suggest that CDK9 plays a role in cell cycle regulation.

Pharmacological inhibition of CDK9 mediates cytotoxicity but not radiosensitization. In addition to siRNA-mediated CDK9 inhibition, we finally explored how plating efficiency and radiation survival are affected by the pharmacological CDK9/CDK2/CDK1 inhibitor ZK304709 in SAS and FaDu cells (Fig. 6A). While ZK304709 reduced the plating efficiency

of SAS and FaDu cells concentration-dependently, neither cell line could be radiosensitized when pretreated with 50 nM ZK304709 (Fig. 6B).

Discussion

Optimization of multimodal therapy concepts is essential to improve cancer patient outcome. Therefore, the identification of new and promising molecular targets is necessary. Among

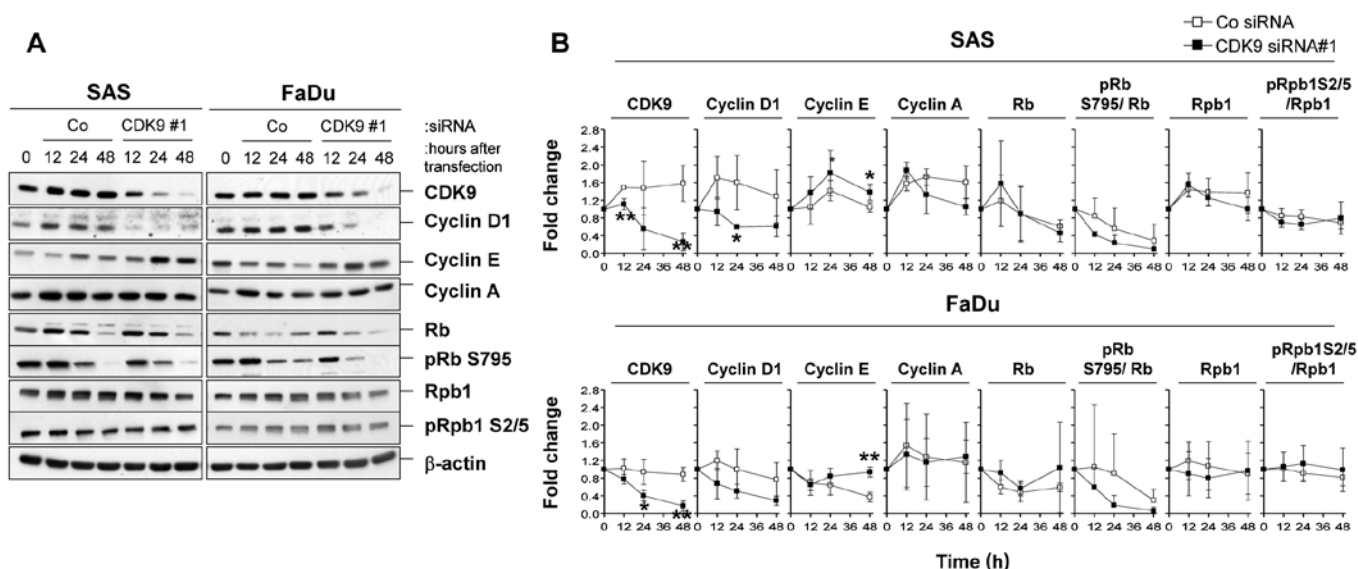


Figure 5. CDK9 contributes to cell cycle protein expression. CDK9 siRNA#1-transfected cell cultures (non-specific siRNA used as control) were irradiated (0 or 6 Gy, X-rays, single dose), lysed in modified RIPA lysis buffer at indicated time-points and analyzed by SDS-PAGE and western blotting. (A) Western blots for proteins regulate the cell cycle. β -actin served as loading control. (B) Densitometric analysis of the western blots. Results were normalized to 0 h values. Results show mean \pm SD (n=3; Student's t-test; *P<0.05; **P<0.01)

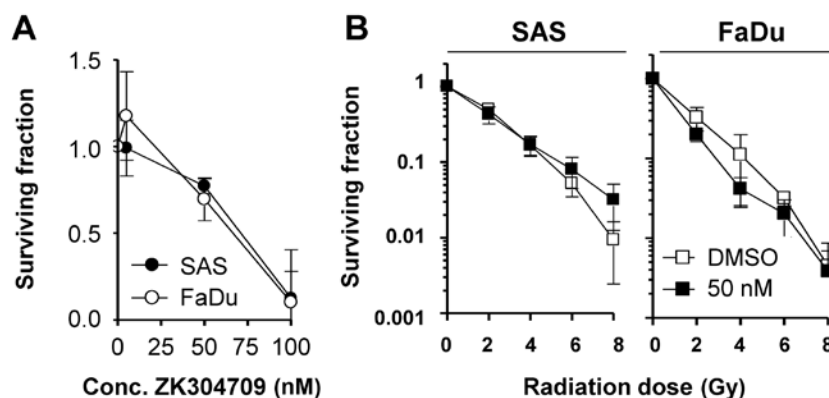


Figure 6. Pharmacological CDK9 inhibition fails to radiosensitize HNSCC cells. (A) Cytotoxic effects of 5, 50 and 100 nM ZK304709 incubated over 24 h relative to DMSO. Results show mean \pm SD (n=3; Student's t-test). (B) After a 24-h pretreatment with 50 nM ZK304709 or DMSO, cells were irradiated with X-rays (0-8 Gy) and clonogenicity was determined. Results show mean \pm SD (n=3; Student's t-test).

the hallmarks of cancer (1), the unlimited proliferation capability of cancer cells is an important characteristic and cell cycle regulators are promising candidates for targeted therapy. In this study, we investigated the role of CDK9 for the cellular radiation response of HNSCC cells.

By means of overexpression, siRNA and pharmacological inhibition of CDK9, we found radioprotection by CDK9 overexpression as well as cell line-dependent radiosensitization which could not be recapitulated with the CDK9/CDK2/CDK1 inhibitor ZK304709. Accordingly, we observed that silencing of CDK9 perturbed the repair of irradiation-induced residual DSBs in SAS and FaDu cells. These findings coincide with our own data showing a strong correlation between clonogenic radiation survival and the number of residual DSBs (20,23). Regarding apoptosis, several studies suggested that CDK9 inhibition induces apoptosis in cancer cells (24,25). However, we could not observe any induction in apoptosis following CDK9 depletion in either SAS or FaDu cells.

CDK9 is the catalytic part of pTEFb which stimulates transcription elongation by phosphorylating the CTD of the large subunit (Rpb1-CTD) of RNA polymerase II (RNAPII) (14,16,26). Previous studies reported a possible selective regulatory role of CDK9 on the expression of a restricted subset of genes instead of all genes controlled by RNA polymerase II (9). Therefore, it is possible that CDK9 may specifically regulate one or more DNA damage response proteins. Similar to our study showing no obvious DNA damage repair protein modification upon CDK9 silencing, Yu and Cortez demonstrated that depletion of CDK9 lacked significantly up or down regulation of DNA damage response genes using genome-wide expression analysis (10). Although these results cannot explain how CDK9 modulates the DNA damage repair of DSBs, one might speculate that CDK9 plays a direct role in maintaining genomic integrity via yet to be determined mechanisms (10).

In contrast to DNA repair, our results suggest a function of CDK9 in cell cycling. CDK9 depletion delayed cell cycle

transition based on elevated G1 phase and declined S phase cell populations. Similarly, Cai and colleagues reported that reduction of CDK9 was associated with changes in cell cycle distribution that are consistent with cell cycle delay (24). The S phase retardation after CDK9 silencing seems to be a consequence of the accumulation of cells in the G1 phase. Mammalian cells exhibit variation in their response to irradiation as they move through the cell cycle. Cells in the G2-M phase are the most radiosensitive and S phase cells are the most radioresistant ones while G1 phase cells show moderate radiosensitivity (27). Accordingly, the retardation of S phase population may contribute to the enhanced radiosensitivity of cancer cells upon CDK9 knockdown.

On the molecular level, Rb is one of the known substrates for CDK9 (28). Therefore, it is not surprising that Rb was found to be hypophosphorylated after silencing of CDK9. In addition, CDK9 depletion was associated with a remarkable decrease in cyclin D1 levels in SAS and FaDu. Together with its binding partners CDK4 and CDK6, cyclin D1-dependent kinase activity promotes G1 phase progression by phosphorylating and inactivating Rb (29,30). Because of the central role of Rb in cell cycle progression, especially during the G1 phase, the observed cell cycle changes after CDK9 knockdown seem to be attributed to Rb hypophosphorylation (24). On the contrary, the level of cyclin E, another G1 phase cyclin, was elevated after CDK9 depletion which might indicate an attempt to tune the cell cycle disturbance due to suppression of cyclin D1-dependent kinase activity in the G1 phase (31).

Despite the well-known role of CDK9 in phosphorylating Rpb1-CTD of RNA polymerase II on S2 and S5 residues (8,14,16,26) we unexpectedly observed unaffected phosphorylation of Rpb1-CTD (S2/5) after depletion of CDK9. One possible explanation is that other CDKs such as CDK12 and CDK13 also regulate, like CDK9, transcription elongation by phosphorylating Rpb1-CTD (32,33). This may explain why CDK9 was dispensable for Rpb1-CTD phosphorylation but cannot reveal the CDK9-related changes in cyclin D1 and cyclin E expression.

Finally, the pharmacological multi-target inhibitor ZK304709 was used in combination with radiotherapy to stress the need of CDK9 for clonogenic radiation survival in a more clinically relevant approach (19). Although ZK304709 showed inhibitory efficacy in human tumor xenografts as well as in human pancreatic carcinoma models (19,34), ZK304709 mediated cytotoxicity without enhanced radiosensitivity in the tested HNSCC cells.

These findings suggest a critical role of CDK9 in the cellular response to ionizing radiation. However, further examinations are required to better understand the molecular circuitry of CDK9 involvement level in DNA damage repair and cell cycle regulation and explore the potential of CDK9 targeting for the clinic.

Acknowledgements

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