Dickkopf-1 expression is repressed by oncogenic human papillomaviruses (HPVs) and regulates the Cisplatin sensitivity of HPV-positive cancer cells in a JNK-dependent manner

Kristin Frensemeier, Angela Holzer, Karin Hoppe-Seyler, Felix Hoppe-Seyler

Abstract

Oncogenic human papillomavirus (HPV) types control the phenotype of cervical cancer cells through the sustained expression of the viral E6/E7 oncogenes. Here, we show that they strongly restrain expression of the putative tumor suppressor protein Dkk1 (Dickkopf-1) in HPV-positive cervical cancer cells through the restriction of p53 expression by the continuously expressed endogenous E6 oncoprotein. Moreover, our study reveals that compromised Dkk1 expression is linked to increased resistance of HPV-positive cervical cancer cells toward the proapoptotic activity of Cisplatin. Although Dkk1 can act as a Wnt antagonist, the antiapoptotic effect resulting from Dkk1 repression is not linked to an activation of this pathway. Rather, transcriptome and functional analyses uncover that Dkk1 repression leads to a strongly diminished stimulation of c-Jun N-terminal kinase (JNK) signaling which is required for efficient apoptosis induction by Cisplatin in cervical cancer cells. Further, we observed that Dkk1-depleted cervical cancer cells induce senescence under Cisplatin treatment instead of apoptosis, suggesting that Dkk1 levels can strongly influence the phenotypic response of these cells toward Cisplatin. Collectively, these results provide new insights into the virus/host cell crosstalk in cervical cancer cells by identifying Dkk1 as a cellular target which is maintained under strong negative control by the continuous expression of the HPV oncogenes. Moreover, they identify Dkk1 as a critical determinant for the sensitivity of cervical cancer cells toward Cisplatin, showing that Dkk1 repression leads to increased Cisplatin resistance by impairing proapoptotic JNK signaling.

Keywords: cervical cancer, Cisplatin, c-Jun N-terminal kinase, Dickkopf-1, human papillomavirus

Abbreviations: CFA, colony formation assay; CM, conditioned medium; CRISPR, clustered regularly interspaced short palindromic repeats; Dkk1, Dickkopf 1; GSEA, gene set enrichment analysis; HPV, human papillomavirus; JNK, c-Jun N-terminal kinase; log2FC, log2-transformed fold change; NES, normalized enrichment score; PARP, poly(ADP-ribose) polymerase; pRb, retinoblastoma protein; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; SA-β-gal, senescence-associated β-galactosidase; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP end labeling.

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Cancer of the cervix uteri, which was described to induce Dkk1 expression in glioma and together with the E6 leads to proteolytic degradation of the tumor suppressor retinoblastoma protein (pRb), consequently restraining cell cycle control by binding and inactivating the tumor suppressor retinoblastoma protein (pRb). The HPV E6 and E7 oncoproteins are the key drivers of the malignant growth of HPV-positive cancers. While E7 accounts for the deregulation of cell cycle control by binding and inactivating the tumor suppressor retinoblastoma protein (pRb), E6 leads to proteolytic degradation of p53. Consequently, p53-dependent apoptotic responses, such as the proapoptotic p53/PUMA/Bax axis, are impaired, which might also result in increased resistance to cancer therapies.

Increasing evidence indicates an important role for the Dickkopf-1 (Dkk1) protein for human carcinogenesis; however, whether Dkk1 acts as a tumor suppressor or oncogenic factor seems to be highly dependent on the cancer type. Dkk1 is a secreted protein and was originally identified as a head inducer during embryonic development in Xenopus, which was mechanistically linked to its Wnt-inhibitory activity. Active, canonical Wnt signaling results in β-catenin stabilization and its translocation to the nucleus, where it activates the transcription of Wnt responsive genes, including those of Dkk1. As a negative feedback regulator, Dkk1 binds to the Wnt specific LRP5/6 co-receptor and thereby prohibits Wnt activation.

In cervical cancer cells, it was reported that DKK1 is epigenetically silenced and that it acts as a proapoptotic tumor suppressor which—upon ectopic overexpression—can repress the tumorigenicity of HeLa cells. Interestingly, the chemotherapeutic agent Cisplatin, which belongs to the standard care for advanced and recurrent cervical cancers, was described to induce Dkk1 expression in glioma and head and neck cancer cells, and in the latter, Dkk1 repression was reported to contribute to Cisplatin resistance. While the authors of these two studies hypothesized that the proapoptotic activity of Dkk1 may be due to its Wnt-suppressive role, evidence is emerging that Dkk1’s ability to induce apoptosis might be also mediated via β-catenin-independent mechanisms, which potentially includes the involvement of noncanonical Wnt pathways.

In the present study, we aimed to investigate whether there is a crosstalk of the HPV oncoproteins with Dkk1 and to gain insights into the potential role of Dkk1 for the efficiency of Cisplatin-based therapy in cervical cancer cells. We show that Dkk1 expression levels are highly restrained by the endogenous expression of the HPV oncoproteins via a p53-dependent mechanism, indicating that HPV E6 is a key driver in this regulation. In addition, our results reveal that Dkk1 plays a major role for mediating the proapoptotic response of cervical cancer cells towards Cisplatin and, accordingly, Dkk1 repression significantly increases Cisplatin resistance. Further, we provide evidence that this latter effect is mechanistically not linked to an interference with canonical Wnt signaling, but to the induction of c-Jun N-terminal kinase (JNK) signaling. Overall, our findings indicate that the sustained expression of HPV E6/E7 oncoproteins in cervical cancer cells contributes to their apoptosis resistance by continuously downregulating expression of Dkk1. Moreover, Dkk1 is a major determinant for the sensitivity of cervical cancer cells towards Cisplatin by modulating JNK signaling.

### 2 | MATERIALS AND METHODS

#### 2.1 | Plasmids and siRNAs

All plasmids and siRNA sequences are described in detail in the Appendix S1. For RNA interference (RNAi) analyses, siRNAs were transfected at a final concentration of 10 nM using DharmaFECT 1 (Thermo Fisher Scientific, Waltham, Massachusetts), according to the manufacturer’s instructions. For combined E6/E7 and p53 silencing, 10 nM sip53 were transfected on the first day, followed by transfection of 15 nM siE6 or siE6/E7 (HPV18 or HPV16 for HeLa and SiHa, respectively) along with 5 nM sip53 on the second day. Plasmids were transfected using calcium phosphate coprecipitation.
2.2 | Cell culture and treatment conditions

HPV18-positive HeLa and HeLa* (RRID:CVCL 0030), as well as HPV16-positive SiHa (RRID:CVCL 0032) and CaSki (RRID:CVCL 1100) cervical cancer cells were obtained from the German Cancer Research Center (DKFZ) tumor bank (Heidelberg, Germany). All these cell lines have been authenticated within the last 3 years by SNP profiling (Multiplexion GmbH, Heidelberg, Germany) and all experiments were performed with mycoplasma-free cells. Cells were cultivated in Gibco DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (both Sigma-Aldrich, St. Louis, Missouri), 2 mM glutamine and 1 g/L glucose at 37 °C, 5% CO₂ and 21% O₂. Cisplatin (Sigma-Aldrich) was dissolved in 0.9% NaCl/H₂O and JNK-IN-8 (MedChemExpress, New Jersey) was added in DMSO.

Dkk1 knockout (KO) cells were generated from HeLa cells by the CRISPR/Cas9 method following standard procedures. After transfection of the LentiCRISPRv1 plasmid containing Dkk1-specific gRNA, cells were cultured under puromycin (1 μg/mL) selection and after 4 days they were split by serial dilutions in 96-well plates in standard medium to obtain single cell clones. Dkk1 conditioned medium (Dkk1 CM) was generated by transfecting HeLa* cells with 3 μg of the Dkk1 expression vector pCS2-hDkk1-Flag and cell supernatants were harvested after 48 hours, centrifuged for 5 minutes at 1000g to remove cell debris, supplemented in a ratio of 5:1 with fresh DMEM containing 4.5 g/L glucose in addition to standard additives and transferred to HeLa cells for subsequent experiments. Control (Ctrl) CM was derived by transfection of pCS2. For the combined treatment with Cisplatin, parental HeLa and Dkk1 KO HeLa cells were pretreated for 48 hours with Ctrl CM or Dkk1 CM and subsequently treated with Cisplatin.

2.3 | TOPflash/FOPflash luciferase reporter assays

For measuring canonical Wnt signaling activities, cells were transfected in 60 mm dishes with 2 μg TOP- or FOPflash plasmids in addition to 0.5 μg pCMV-β-galactosidase serving as an internal standard. When indicated in the text, 0.5 μg of pcDNA3 or pcDNA3-Wnt3a were co-transfected. Twenty-four hours after transfection, cells were treated with Ctrl CM, Dkk1 CM or LiCl. After 16 hours cells were lysed and luciferase, as well as β-galactosidase activities were measured as previously described.

2.4 | Protein and RNA analyses

RNA extraction, qRT-PCR, protein extraction and immunoblot analyses were performed as previously described and qRT-PCR primer and antibodies are listed in the Appendix S1. For protein analyses in cell supernatants, the cell culture medium was collected from transfected cells, centrifuged for 5 minutes at 1000g to remove cell debris and boiled for 5 minutes at 95 °C in SDS sample buffer. The volume loaded to the gel was determined relative to the respective protein concentrations in the lysates of cells from which the medium was taken. Immunoblots were visualized using the enhanced luminescence (ECL) reagent WesternBright Sirius (Advansta, San Jose, California) in the Fusion SL Detection System (Vilber Lourmat, Germany). Gene expression determined by qRT-PCR was normalized to the internal standard 18S rRNA and the comparative ΔΔCt method was applied for relative quantification of transcript expression.

2.5 | Apoptosis assays

For terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) assays, cells were grown and treated on coverslips and subjected to the “In Situ Cell Death Detection Kit” (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Images were acquired on a Cell Observer Microscope (Zeiss, Jena, Germany) and quantification of TUNEL-positive cells relative to DAPI stainings (Roche) from at least five images per condition was performed using an ImageJ macro (Karin Kreta, Light microscopy core facility, DKFZ Heidelberg). Caspase 3/7 activity was assessed for cells treated with Cisplatin in 96-well plates in the presence of Incucyte Caspase-3/7 Green Reagent (Sartorius, Göttingen, DE) according to manufacturer’s instructions and analyzed by Incucyted Life Cell Imaging using IncuCyte S3 2019B software (Sartorius). Caspase 3/7 counts of four images per well at a ×10 magnification were normalized to the cell confluence and measurements were performed in technical triplicates and in three independent repetitions.

2.6 | Colony formation and senescence assays

For colony formations assays (CFAs) and senescence assays (SAs), cells were split after Cisplatin treatment and cultivated for the indicated time periods in standard medium. CFAs were fixed and stained with a crystal violet/formaldehyde solution. SA-β-gal staining was performed as described previously and images were acquired using a ×10 magnification on the EVCOScl Core Cell Imaging System (Thermo Fisher Scientific).

2.7 | Gene expression analyses

Affymetrix technology-based microarray analyses were conducted by the Microarray Unit of the Genomics and Proteomics core facility (GPCF) (DKFZ Heidelberg). Gene expression from untreated and Cisplatin-treated parental HeLa and Dkk1 KO #3 cells (three biological replicates each) were analyzed on Clariom S Assay GeneChips (Thermo Fisher Scientific) according to the manufacturer’s instructions. Gene set enrichment analysis (GSEA v. 4.0.3) of the PID_AP1_Pathway was performed for the comparison of log(2)-transformed fold changes (log2FC) of gene expression in Cisplatin-treated Dkk1 KO to parental HeLa cells. Further details on the analyses of differential gene expression in volcano plots and the
2.8 | Statistical analyses

All experiments were performed at least thrice with consistent results and data for qRT-PCR, TUNEL quantifications and luciferase assays is presented as mean with error bars depicted as standard deviations (SD). Statistical significances were determined by one-way ANOVA using SigmaPlot version 14.0 (Systat Software Inc., San Jose, California) and are indicated for P-values <.05 (*), <.01 (**) and <.001 (**). For transcriptome analyses on Affymetrix GeneChips, basic statistical analyses were performed by the GPCF Microarray Unit, DKFZ.

3 | RESULTS

3.1 | HPV oncogene expression restrains Dkk1 levels in cervical cancer cells

To investigate whether Dkk1 may be a downstream target of the HPV E6/E7 oncogenes, Dkk1 expression levels were analyzed after siRNA-mediated silencing of E6 or E6/E7 in HPV18-positive HeLa and HPV16-positive SiHa and CaSki cervical cancer cells (Figure 1A, B). In all these cells, Dkk1 protein expression was strongly increased upon E6 or E6/E7 silencing, indicating that the sustained HPV oncogene expression efficiently restricts endogenous Dkk1 protein levels. E6 inhibition alone was sufficient to increase Dkk1 concentrations in

FIGURE 1  Dkk1 expression is restrained by HPV E6. (A) HeLa and SiHa cells were transfected with control siRNA (siCtrl) or siRNAs silencing HPV18 or HPV16 E6 and E6/E7 (siE6; siE6/E7), respectively, either alone or concomitantly with siRNA silencing p53 (sip53) expression. Dkk1, p53 and HPV18 (HeLa) or HPV16 (SiHa) E6/E7 protein levels were analyzed by immunobots. β-actin: loading control. (B) Transfection of HPV16 E6 and E6/E7-specific siRNAs and corresponding immunoblot analyses in CaSki cells. (C) DKK1 mRNA expression was determined by qRT-PCR and is indicated by mean log2FC including standard deviations, relative to siCtrl-transfected cells (log2FC = 0). Statistical significance is indicated by asterisks (* P < .05; ** P < .01; *** P < .001; n.s., not significant). (D) Secreted Dkk1 in the cell supernatant of different HPV-positive cancer cells after E6 or E6/E7 silencing compared to the protein levels in the corresponding lysates. HPV E7 is not secreted and served as an internal control that supernatants do not contain intracellular content. (E) Immunoblot analyses of Dkk1 and HPV18 or HPV16 E6 and E7 protein levels in HeLa and SiHa cells, respectively, transfected with two different siRNAs silencing Dkk1 expression, applied either alone (siDkk1 #1; siDkk1 #2) or as pool (siDkk1 #1 + #2). Vinculin: loading control.
parallel with p53 levels (Figure 1A,B), which is interesting as DKK1 was previously reported to be transcriptionally activated by p53. In order to explore the potential regulatory role for p53 in Dkk1 induction upon HPV oncogene repression we combined E6 or E6/E7 silencing with siRNA-mediated repression of p53. Concomitant silencing of E6 or E6/E7 and p53 efficiently blocked the increase in Dkk1 levels (Figure 1A), indicating that E6 profoundly restricts Dkk1 expression via interference with p53-mediated DKK1 transactivation. Accordingly, E6-mediated repression of DKK1 is also observed at the transcript level, where DKK1 mRNA concentrations are significantly increased after E6 or E6/E7 silencing, which again was abolished when p53 was repressed in parallel (Figure 1C). Further, intracellular Dkk1 induction after knockdown of the HPV oncogenes was accompanied by a strong increase of Dkk1 levels in the supernatant of all tested cervical cancer cells (Figure 1D), indicating that also the amount of secreted Dkk1 underlies regulation by HPV E6. Collectively, these findings define Dkk1 as a novel indirect downstream target for the HPV oncogenes, which is repressed via E6-mediated interference with p53. On the other hand, silencing of Dkk1 did not affect HPV E6/E7 protein expression in HeLa or SiHa cells (Figure 1E), indicating that endogenous Dkk1 does not act as an upstream regulator of the HPV oncogenes.

### 3.2 | Dkk1 contributes to Cisplatin-induced apoptosis in cervical cancer cells

In order to investigate the functional significance of Dkk1 for the response of HPV-positive cancer cells towards Cisplatin, HeLa, CaSki and SiHa cells were treated with two different Cisplatin doses when Dkk1 expression was silenced. Immunoblot analyses show that Dkk1 protein expression was induced by Cisplatin, which was accompanied by increased expression of the apoptosis markers cleaved (cl.) (PARP) poly(ADP-ribose) polymerase and cl. Caspase 9 (Figure 2A). Silencing of Dkk1 reduced the Cisplatin-induced increase of both cl. PARP and cl. Caspase 9, indicating that Dkk1 contributes to the proapoptotic

![Figure 2](image_url)

**Figure 2** Dkk1 repression decreases Cisplatin-induced apoptosis in cervical cancer cells. (A) HeLa (left panel), CaSki (central panel) and SiHa cells (right panel) were transfected with control siRNA (siCtrl) or a pool of two Dkk1-targeting siRNAs (siDkk1 #1 + #2) and were subsequently treated with two different doses of Cisplatin (CDDP). Immunoblots depict expression of Dkk1, PARP (cleaved (cl.) and uncleaved forms) and cl. Caspase 9 (Casp.9). β-actin: loading control. (B) TUNEL assays of siCtrl- or siDkk1 #1 + #2-transfected HeLa and SiHa cells under treatment with 15 µM CDDP (scale bar: 50 µm) (left panel). The percentage of TUNEL-positive cells was quantified relative to the number of DAPI stained cells (representing total cell numbers) and is depicted by mean values along with standard deviations (right panel). Asterisks indicate statistical significance (*P < .05; **P < .001; n.s., not significant).
effect of Cisplatin in cervical cancer cells. This was corroborated by employing TUNEL assays for apoptosis detection (Figure 2B). Compared to control siRNA-transfected HeLa cells of which ~16% became apoptotic, only ~2.4% of the cells stained TUNEL-positive upon Dkk1 silencing. A decrease in TUNEL-positive cells upon Dkk1 repression was also detectable in Cisplatin-treated SiHa cells, although apoptosis induction in control siRNA-transfected cells was lower and the difference to siDkk1 #1 + #2-transfected cells was not statistically significant. Overall, the results provide evidence that Dkk1 augments Cisplatin-induced apoptosis in cervical cancer cells.

3.3 | Dkk1 knockout HeLa cells are protected from Cisplatin-induced apoptosis and undergo a switch to cellular senescence

To study this issue in more depth, we generated Dkk1 knockout (KO) HeLa cells using the CRISPR/Cas9 technology. After single cell cloning, we chose four independent stable Dkk1 KO clones for further analyses (HeLa Dkk1 KO #1 to #4). As observed before, treatment of parental HeLa cells with 10 μM Cisplatin lead to enhanced Dkk1 expression and induction of apoptosis as indicated by the increase in cl. PARP and cl. Caspase 9 levels (Figure 3A). In contrast, under the same experimental conditions, induction of these apoptosis markers was not detectable in Dkk1 KO cells, indicating protection from Cisplatin-induced apoptosis. In line with this observation, all four Dkk1 KO clones showed lower Caspase 3/7 activity under Cisplatin treatment compared to parental HeLa cells in live cell imaging analyses (Figure 3B). Moreover, TUNEL assays revealed that only 1% to 2% of the Dkk1 KO cells underwent apoptosis, while in parental HeLa cells ~12% of the cells became apoptotic (Figure 3C). In accordance with the results obtained after transient Dkk1 repression, these findings support the notion that Dkk1 is a critical regulator of Cisplatin-induced apoptosis in cervical cancer cells.

Interestingly, similar to parental HeLa cells, the colony formation capacity of the Dkk1 KO cells was also suppressed under Cisplatin treatment (Figure 3D). This suggests that the Dkk1 KO cells, which were protected from apoptosis, induce another antiproliferative mechanism. At least in part, this is due to the induction of cellular senescence, as indicated by the typical morphological changes of senescent HeLa cells (cellular enlargement and flattening, cytoplasmic extensions) as well as by positive staining for the senescence marker senescence-associated beta-galactosidase (SA-β-gal) (Figure 3E). In contrast, only very few of the parental HeLa cells survived and became senescent after Cisplatin treatment. Collectively, these results indicate that Dkk1 depletion protects HeLa cells against Cisplatin-induced apoptosis, and leads to a phenotypic switch toward cellular senescence.

3.4 | Cisplatin resistance of HeLa Dkk1 KO cells is not linked to the activation of canonical Wnt signaling

Since Dkk1 is known to be a potent inhibitor of canonical, β-catenin-dependent Wnt signaling, which was previously associated with enhanced Cisplatin resistance, we explored the role of this pathway for the Dkk1-dependent Cisplatin response in HeLa cells. To this end, we generated Dkk1 conditioned medium (Dkk1 CM) by transfection of a Dkk1 expression vector (pCS2-hDkk1-Flag) into a laboratory HeLa variant, referred to as HeLa*, which—among other differences in gene expression—exhibits undetectable Dkk1 protein levels (Figure 4A,B). Dkk1 was expressed in the cell lysate and supernatant of pCS2-hDkk1-Flag, but not in vector control (pCS2)-transfected HeLa* cells (Figure 4B) which were used to generate control conditioned medium (Ctrl CM). Both conditioned media were transferred to HeLa cells (Figure 4A) and the TOP-/FOPflash luciferase reporter system was employed to measure canonical Wnt signaling activity.

In line with a previous report, HeLa cells did not exhibit basal canonical Wnt activities, as no activation of TOPflash above FOPflash was detectable (Figure 4C). In contrast, when co-transfected with an expression vector for the established Wnt signaling activator Wnt3a, TOPflash activities increased ~14-fold in Ctrl CM-treated HeLa cells. Notably, Dkk1 CM treatment strongly counteracted this effect, showing that Dkk1 is able to repress Wnt3a-activated canonical Wnt signaling in HeLa cells. Next, TOPflash activities were compared in parental HeLa cells and Dkk1 KO clones after Cisplatin treatment, as well as after Wnt3a overexpression (Figure 4D). None of the Dkk1 KO clones exhibited detectable basal Wnt activities, alike parental HeLa cells. Overexpression of Wnt3a resulted in enhanced luciferase activities in parental HeLa and all tested Dkk1 KO HeLa cells (with some clonal variability in the extent of induction), showing that canonical Wnt signaling is, in principle, inducible in these cells. Contrarily, Cisplatin treatment did not affect TOPflash activities, neither in parental HeLa cells nor in Dkk1 KO cells. Moreover, the levels of total β-catenin and nonphosphorylated β-catenin (the active form for mediating Wnt-induced transcription) are not affected under Cisplatin treatment (Figure 4E). In contrast, treatment with the Wnt signaling activator LiCl increases the expression of both forms of β-catenin in parallel to TOPflash activation (Figure S1). Taken together, these findings indicate that the increased resistance of Dkk1 KO cells towards Cisplatin is not linked to an activation of canonical Wnt signaling caused by the depletion of the negative Wnt regulator Dkk1.

3.5 | Cisplatin resistance in Dkk1-depleted cells is associated with decreased JNK/AP-1 signaling

To further elucidate the mechanisms underlying the regulatory role of Dkk1 in Cisplatin-mediated apoptosis, the transcriptomes of Cisplatin-treated parental HeLa cells and one selected Dkk1 KO clone, Dkk1 KO #3, were assessed on Affymetrix GeneChips. The experimental conditions and the accompanying phenotypic responses of the cells are depicted in Figure 5A. Differential gene expression was determined by pairwise comparisons. Volcano plot analyses indicate major significant global transcriptional changes in Cisplatin-treated parental HeLa cells, which were less pronounced in Dkk1 KO cells (Figure 5B). Interestingly, with JUN, FOSL1 and FOSB, several members of the
AP-1 family were among the strongest upregulated factors in parental HeLa cells when treated with Cisplatin and this effect was less noticeable in the Dkk1 KO cells. Gene set enrichment analyses (GSEA) comparing the datasets from Cisplatin-treated Dkk1 KO and parental HeLa cells (4vs2) revealed that genes from the AP-1 transcription factor network (PID_AP1_Pathway) were negatively enriched in Dkk1 KO cells with a normalized enrichment score (NES) of -2.05 (Figure 5C). In total, 26 out of 69 genes from this pathway...
showed significant ($P < .05$) differential expression in this dataset (Figure 5D). Comparison of these components for all four datasets further indicates that the AP1-complex components JUN, ATF3, FOSL1, FOSB, FOS and JUNB were strongly induced upon Cisplatin treatment in parental HeLa cells, but less in Dkk1 KO HeLa cells (Figure 5D).

In accordance with these datasets, validation experiments assessing JUN, JUNB, FOSL1, FOS and ATF3 transcripts upon Cisplatin treatment by qRT-PCR revealed a decreased stimulation of these genes in all four independent Dkk1 KO clones compared to parental HeLa cells (Figure 5E). This regulation was also reflected on the protein level: Cisplatin strongly induced total and Serine (Ser) 63- and Ser73-phosphorylated c-Jun as well as ATF3 and Fra-1 (encoded by FOSL1) concentrations in parental HeLa cells. This effect was strongly reduced in all four Dkk1 KO clones (Figure 5F).

JNK is a key upstream regulator of multiple AP-1 family members, raising the possibility that the Dkk1-dependent effects on Cisplatin-induced apoptosis are mediated through modulating this important signaling hub. Indeed, Cisplatin-linked phosphorylation and therefore activation of JNK p46 and p54 isoforms, which bind to and phosphorylate c-Jun on Ser63 and Ser73 was regulated in parallel with the analyzed AP-1 factors, whereas total JNK levels remained unaffected. These results indicate that Dkk1 can promote Cisplatin-induced JNK phosphorylation. In line, treatment of Dkk1 KO cells with Dkk1 CM augments Cisplatin-induced JNK phosphorylation (Figure S2). We did not observe a stimulation of JNK phosphorylation by treatment with Dkk1 CM in the absence of Cisplatin, suggesting that this effect is indirect. Collectively, these findings show that Dkk1 depletion leads to a decreased activation of the JNK signaling pathway in response to
FIGURE 5  Legend on next page.
Cisplatin treatment, which may be a potential mechanism behind the increase in apoptosis resistance.

### 3.6 Cisplatin-induced apoptosis is impaired upon suppression of JNK activity

In order to test whether JNK activation is indeed a decisive factor for the Dkk1-dependent apoptosis induction under Cisplatin treatment, we blocked JNK activity in HeLa cells, either by transfection of a well-characterized JNK1/2-targeting siRNA or by treatment with JNK-IN-8, a selective pan-inhibitor for JNK1, JNK2 and JNK3. We concentrated on the analysis of the ubiquitously expressed JNK1 and JNK2 isoforms, since JNK3 expression is restricted to heart, brain and testis. As expected, siJNK1/2 downregulated total and phosphorylated JNK levels, while JNK-IN-8 did neither affect JNK expression levels nor the induction of its phosphorylation by Cisplatin (Figure 6A), as it interferes with the catalytic sites of JNKs. Whereas Cisplatin treatment results in the phosphorylation of the JNK substrate c-Jun on Ser63 and Ser73 in control siRNA-transfected or solvent (DMSO)-treated cells, this effect was substantially reduced by both siJNK1/2- or JNK-IN-8-mediated JNK inhibition (Figure 6A), further corroborating that both inhibitory treatment strategies efficiently block JNK signaling.

Intriguingly, JNK inhibition profoundly restrained apoptosis induction by Cisplatin, as indicated by the substantial decrease in cl. PARP and cl. Caspase 9 (Figure 6A). In accordance, TUNEL assays revealed significantly reduced numbers of apoptotic cells after Cisplatin exposure, when JNK activity was blocked by chemical inhibition (Figure 6B) or by siRNA (Figure S3A). To investigate whether the key role of JNK activation for Cisplatin-induced apoptosis is a general phenomenon in cervical cancer cells, we further tested CaSki and SiHa cells, which also exhibited reduced apoptosis under Cisplatin treatment upon transient Dkk1 repression (Figure 2). In accordance with the results obtained in HeLa cells, blocking JNK activity with JNK-IN-8 also impaired Cisplatin-induced c-Jun phosphorylation and strongly diminished the amounts of cl. PARP and cl. Caspase 9 in both CaSki and SiHa cells (Figure 6C). This was accompanied by a significant decrease of apoptotic SiHa cells in TUNEL assays (Figure 6D). Likewise, the induction of cl. PARP and cl. Caspase 9 by Cisplatin was impaired upon siRNA-mediated JNK silencing also in CaSki and SiHa cells (Figure S3B). Overall, these results show that JNK activity plays a key role for Cisplatin-mediated apoptosis induction in cervical cancer cells and downregulation of Dkk1 provides these cells with increased Cisplatin resistance by interfering with JNK activation.

### 4 DISCUSSION

The present work reveals that the expression of the putative tumor suppressor protein Dkk1 is continuously restrained in cervical cancer cells by the sustained expression of the HPV oncogenes. Furthermore, we provide evidence that Dkk1 plays an important role for the proapoptotic response of cervical cancer cells towards Cisplatin treatment.

We found that silencing of endogenous viral E6 oncogene expression alone is sufficient to relieve Dkk1 from the negative regulation by the HPV oncogenes, leading to strongly enhanced Dkk1 expression levels in cervical cancer cells. Further, RNAi analyses reveal that the induction of p53 upon E6 silencing is required for the increase of Dkk1 levels, indicating that E6 represses Dkk1 expression through its interference with p53 function. In line with this notion, DKK1 was reported to contain a putative p53 binding site in its promoter and to be a direct transcriptional target gene for p53, as was assessed by ectopic overexpression of wildtype p53. Consistent with this data we observed that the induction of Dkk1 expression upon E6 silencing is detectable not only at the protein, but also at the transcript level. In this context, it is important to note that only a small subset of the over 3500 postulated p53 target genes is significantly affected by silencing the HPV oncogenes in cervical cancer cells. This may, for example, be due to the fact that a significant portion of the postulated p53 target genes have been identified by p53 overexpression studies and genes with low affinity p53 binding sites could not be repressed by p53.

Figure 5 Dkk1 depletion interferes with Cisplatin-induced JNK/AP-1 activation in HeLa cells. (A) Transcriptome analyses of untreated or Cisplatin-treated parental HeLa and Dkk1 KO #3 HeLa cells by Clarion S assays (Affymetrix). Schematic representation of the treatment conditions along with the accompanying cellular phenotypic responses. (B) Volcano plots showing differential gene expression between Cisplatin-treated parental HeLa and Dkk1 KO #3 HeLa cells by Clarion S assays (Affymetrix). Schematic representation of the treatment conditions. Each data point represents the log_{10}FC of a single gene in relation to its log_{10}FC in untreated HeLa cells. Error bars indicate standard deviations. (C) Gene Set Enrichment Analysis (GSEA) for the PID_AP1_Pathway was applied for log_{10}FC of gene expression comparing CDDP-treated Dkk1 KO HeLa to parental HeLa cells (4vs2). NES, normalized enrichment score. (D) Significantly regulated genes from GSEA results are depicted for all pairwise comparisons of datasets in a heatmap. (E) Transcript levels of JUN, JUNB, FOSL1, FOS and ATF3 measured by qRT-PCR after treating parental HeLa and Dkk1 KO HeLa cells (#1 to #4) for 16 hours with Cisplatin. Mean log_{10}FC values represent the relative expression levels of CDDP-treated cells to respective untreated cells. Error bars indicate standard deviations. Statistical significance of differential expression in Dkk1 KO HeLa cells compared to parental cells is depicted by asterisks (*P < .05; **P < .01; ***P < .001; n.s., not significant). (F) Immunoblot analyses of Cisplatin-treated or untreated parental HeLa and Dkk1 KO HeLa cells (#1 to #4) for expression levels of total c-Jun, phosphorylated (p-) c-Jun (Ser63 and Ser73), Fra-1, ATF3 and phosphorylated (p-) (Thr183/Tyr185) and total protein of JNK isoforms p46/p54. Vinculin: loading control.
be unresponsive to the endogenous p53 amounts which are restored after E6 silencing. Our study, however, clearly shows that DKK1 represents a relevant p53 target gene in HPV-positive cervical cancer cells, which is activated by endogenous p53 after its release from the E6-dependent negative regulation. It is important to note that besides a direct effect on DKK1 transcription, p53 has the potential to act at the epigenetic level.

Cisplatin-based chemotherapy is the treatment of choice for advanced or recurrent cervical cancers and intrinsic or acquired Cisplatin resistance represents a major hurdle for efficient treatment. Previous reports on the role of Dkk1 for the response of tumor cells towards Cisplatin yielded controversial results, possibly due to cell type-specific differences, with studies linking DKK1 expression to either increased resistance or increased sensitization towards Cisplatin treatment. Our results reveal that in cervical cancer cells both RNAi-induced Dkk1 repression as well as CRISPR/Cas9-mediated Dkk1 knockout severely compromise Cisplatin-induced apoptosis, indicating that Dkk1 expression is a critical determinant for mediating the proapoptotic effects of Cisplatin in these cells.

Whereas in previous reports the Cisplatin-sensitizing effects of Dkk1 have been discussed to be possibly linked to its Wnt-suppressive abilities, our data does not support this notion, since we did not detect corresponding alterations in the activity of canonical Wnt signaling.

Rather, our results indicate that Dkk1 exerts its proapoptotic effect in cervical cancer cells through activation of JNK/AP-1.
signaling, since transcriptome analyses and validating qRT-PCR and immunoblot analyses disclose that the Cisplatin-induced activation of this pathway is severely impaired upon Dkk1 depletion. This is reflected by the substantially reduced increase in the expression or phosphorylation of several, potentially proapoptotic members of the AP-1 protein family in Cisplatin-treated Dkk1 KO HeLa cells. Moreover, the Cisplatin-induced phosphorylation of both isoforms of the c-Jun upstream kinase JNK (p46 and p54) is strongly diminished in Dkk1 KO HeLa cells. This indicates that Dkk1 plays a major role for JNK activation in cervical cancer cells in response to Cisplatin, although this occurs in an indirect manner, as treatment with Dkk1 CM alone did not directly affect JNK phosphorylation. The existence of a functional link between Dkk1 and JNK is also supported by a study in osteosarcoma cells which reported a positive correlation between Dkk1 expression and increased JNK phosphorylation.

JNK is a key upstream regulator of the AP-1 complex, acting either directly via phosphorylation or indirectly via the multitude of feedback regulation mechanisms, which can lead to increased expression of AP-1 factors. We found that multiple AP-1 components are differentially regulated in Cisplatin treated HeLa cells upon Dkk1 depletion, however, as their activity is determined by the complex variety of different combinations via their homo- or heterodimerization, it remains to be deciphered in future studies which of these specific factors may ultimately affect Cisplatin-mediated apoptosis. In the present work, we therefore focused on the functional analysis of the overarching signaling hub JNK and its significance for the Cisplatin resistance of cervical cancer cells.

JNK activation has been linked to the apoptosis response of cancer cells before, however, both pro- or antiapoptotic activities have been reported, depending on the cellular context. This also includes a possible role of JNK in the context of Cisplatin-induced apoptosis which has been reported to be either increased or decreased by JNK in different tumor models. Our study clearly demonstrates that the efficacy of apoptosis induction by Cisplatin in cervical cancer cells is substantially reduced upon blocking JNK signaling either genetically by RNAi or chemically with the irreversible small molecule JNK inhibitor JNK-IN-8. Consequently, these results provide direct evidence that JNK activity plays a critical role for efficient induction of apoptosis in these cells in response to Cisplatin treatment. This conclusion is further corroborated by a previous study reporting that the long-term generation of Cisplatin-resistant HeLa strains by chronic exposure to the drug was linked to an impairment of JNK/AP1 signaling. Collectively, our findings indicate that the induction of proapoptotic JNK signaling in response to Cisplatin is Dkk1-dependent, thereby providing a mechanistic explanation for the increased Cisplatin resistance of cervical cancer cells when Dkk1 is downregulated.

Another intriguing observation which warrants further investigation is our finding that HeLa Dkk1 KO cells are still growth inhibited under Cisplatin treatment, however, other than in parental HeLa cells, the underlying antiproliferative mechanism in Dkk1-depleted cells is not apoptosis, but the induction of cellular senescence. This observation indicates that the phenotype of cervical cancer cells in response to Cisplatin treatment can be strongly influenced by the expression levels of Dkk1. Whereas the underlying molecular mechanism remains to be elucidated, it is noteworthy that interference with JNK activity has been reported to possess potential to promote cellular senescence. Thus, the much more pronounced activation of JNK by Cisplatin in the presence of Dkk1 may block senescence induction in parental HeLa cells, whereas the Dkk1 depletion in HeLa Dkk1 KO cells may favor a senescent response by disabling a strong JNK activation.

This finding is not only interesting under mechanistic aspects concerning the antiproliferative effects of Cisplatin in cervical cancer cells, but could also be relevant for the clinic. On the one hand, therapy-induced senescence aims to target cancer cells by stopping their proliferation and inducing their clearance by the immune system. On the other hand, emerging evidence shows that senescence can also have nonbeneficial consequences, such as the secretion of protumorigenic factors or enhanced chemotherapy resistance. Consequently, apoptosis has been proposed to be a more beneficial therapeutic outcome than senescence. Although it is conceivable that different phenotypic responses of cancer cells towards treatment may influence the clinical outcome, this hypothesis awaits further exploration. Thus, the observation that Dkk1 depletion can lead to a switch from Cisplatin-induced apoptosis towards senescence may also have implications for current attempts to develop Dkk1 inhibitors for the therapy of tumors in which Dkk1 is believed to exert oncogenic activities, which includes their application in combination schemes with Cisplatin.

In conclusion, the present study provides new insights into the virus/host cell crosstalk in cervical cancer cells, revealing that expression of the putative tumor suppressor Dkk1 is strongly restricted by oncogenic HPVs. Moreover, it identifies Dkk1 as a critical determinant for an efficient cellular response toward Cisplatin treatment, showing that Dkk1 repression interferes with Cisplatin-induced proapoptotic JNK signaling and thereby protects HPV-positive cancer cells from Cisplatin-mediated apoptosis.

**AUTHOR CONTRIBUTIONS**

Kristin Frensemeier: Conceptualization, Data Curation, Interpretation of Data, Methodology. Angela Holzer: Methodology. Karin Hoppe-Seyler: Conceptualization, Data Curation, Interpretation of Data, Writing-Original Draft. Felix Hoppe-Seyler: Conceptualization, Data Curation, Interpretation of Data, Writing-Original Draft. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT
The data and other items supporting the results of the study will be made available upon reasonable request. The Affymetrix data have been deposited to the Gene Expression Omnibus (GEO) database with the dataset identifier GSE200748.

ORCID
Felix Hoppe-Seyler https://orcid.org/0000-0002-1864-300X

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