Effects of Metformin on the virus/host cell crosstalk in human papillomavirus-positive cancer cells

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Abstract
Oncogenic types of human papillomaviruses (HPVs) are major human carcinogens. The viral E6/E7 oncogenes maintain the malignant growth of HPV-positive cancer cells. Targeted E6/E7 inhibition results in efficient induction of cellular senescence, which could be exploited for therapeutic purposes. Here we show that viral E6/E7 expression is strongly downregulated by Metformin in HPV-positive cervical cancer and head and neck cancer cells, both at the transcript and protein level. Metformin-induced E6/E7 repression is glucose and PI3K-dependent but—other than E6/E7 repression under hypoxia—AKT-independent. Proteome analyses reveal that Metformin-induced HPV oncogene repression is linked to the downregulation of cellular factors associated with E6/E7 expression in HPV-positive cancer biopsies. Notably, despite efficient E6/E7 repression, Metformin induces only a reversible proliferative stop in HPV-positive cancer cells and enables them to evade senescence. Metformin also efficiently blocks senescence induction in HPV-positive cancer cells in response to targeted E6/E7 inhibition by RNA interference. Moreover, Metformin treatment enables HPV-positive cancer cells to escape from chemotherapy-induced senescence. These findings uncover profound effects of Metformin on the virus/host cell interactions and the phenotype of HPV-positive cancer cells with implications for therapy-induced senescence, for attempts to repurpose Metformin as an anticancer agent and for the development of E6/E7-inhibitory therapeutic strategies.

KEYWORDS
cervical cancer, chemotherapy, human papillomavirus, Metformin, tumor virus
INTRODUCTION

Oncogenic types of human papillomaviruses (HPVs), such as HPV16 or HPV18, are closely linked to the development of prevalent anogenital and head and neck squamous cell carcinomas (HNSSCs), accounting for approximately 5% of the total cancer incidence in humans. Best characterized is the causative role of HPVs for the development of cervical cancer, which each year is diagnosed in approximately 570,000 females and results in over 300,000 cancer deaths. Cervical cancer cells are virtually always HPV-positive and their proliferation depends on the continuous expression of the multifunctional viral E6/E7 oncoproteins. Inhibition of E6/E7 expression in HPV-positive cancer cells leads to rapid and efficient induction of cellular senescence, classically considered to be an irreversible growth arrest. Collectively, these findings indicate that HPV-positive cancer cells are “oncogene addicted” and must maintain viral E6/E7 oncogene expression in order to avoid induction of senescence.

The biguanide Metformin is a widely used drug for the first-line therapy of Type 2 diabetes (DT2) where it primarily acts through reducing hepatic gluconeogenesis. In recent years, Metformin has also raised much interest in the field of oncology. A number of clinical studies indicate beneficial preventive and/or therapeutic effects of Metformin at least on some cancer entities in DT2 patients, and possibly also in nondiabetic patients. However, other reports challenge these findings and question the advantageous effects of Metformin for cancer treatment. The reasons for these discrepancies are still largely elusive and might be explained, for example, by differences in the study setup or by tumor type-specific responses. Conflicting data has also been published for cervical cancer. Metformin was reported to reduce the risk of cervical cancer development in DT2 patients and to decrease cervical cancer specific and overall mortality in cervical cancer patients whereas no effect of Metformin on the course of the disease was described in another study.

Both indirect and direct effects have been proposed to contribute to the antitumorigenic potential of Metformin. As indirect effects, the reduction of serum glucose and insulin concentrations could negatively affect tumor cell growth. Additionally, immunological and antiinflammatory effects may play a role. On the other hand, there are a large number of studies indicating direct antitumorigenic potential, showing that Metformin can act antiproliferative in a broad spectrum of tumor cells in vitro. These also include cervical cancer cells for which different potential mechanisms for mediating Metformin-induced growth suppression were reported, such as repressing Wnt signaling via DVL3 inhibition, decreasing AMPK-GltnAcetylation, or modulating the expression of HMGA2, DEC1 or FOXM1. Interestingly, however, none of these studies addressed the question whether Metformin affects the HPV oncogenes or the virus/host cell crosstalk in HPV-positive cancer cells.

In the present study, we show that Metformin leads to a strong inhibition of viral E6/E7 oncogene expression. At the same time, Metformin subverts the senescence response of HPV-positive cancer cells and protects them against the pro senescent effects of E6/E7 inhibition. Moreover, Metformin efficiently protects HPV-positive cancer cells against chemotherapy-induced senescence. These results uncover complex effects of Metformin on the virus/host cell crosstalk in HPV-positive cancer cells, with potential implications for their therapeutic susceptibility.

MATERIALS AND METHODS

Cell culture and treatment conditions

HPV18-positive HeLa (RRID:CVCL_0030), HPV16-positive SiHa (RRID:CVCL_0032), CaSk (RRID:CVCL_1100) and MRI-H-186 (also called CERV-186, RRID:CVCL_5720) cervical cancer cells were obtained from the tumor bank of the German Cancer Research Center (DKFZ), Heidelberg. UDSCC2 (RRID:CVCL_E325) HNSCC cells were a kind gift of Prof. T. Hoffmann, University of Ulm, Germany. All these cells were cultivated at 37 °C in DMEM (Gibco, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Life Technologies), 2 mM l-glutamine, 5.5 mM glucose (if not specified otherwise), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) at 5% CO2. SCC152 (also called UPCI-SCC-152, RRID:CVCL_C058) HNSCC cells were a kind gift of Prof. S. M. Gollin, University of Pittsburgh, PA, and were cultured in MEM containing nonessential amino acids. Cells were cultured at 21% O2 (“normoxia”) or 1% O2 (“hypoxia”); using a hypoxic chamber InvivO2 400 physiological oxygen workstation, Ruskind Technology Ltd, UK. All cell lines have been authenticated using SNP profiling within the last 3 years (Multiplexion GmbH, Heidelberg, Germany). All experiments were performed with mycoplasma-free cells.
Metformin (Enzo Life Sciences, Lausen, Switzerland) and Phenformin (Sigma-Aldrich) were solved in DMEM. PI3K and AKT inhibitors were employed at the following end concentrations: 10 μM AKTi VIII (Sigma Aldrich), 10 μM MK-2206 (Adipogen, Lielstat, Switzerland), 10 μM Ipatasertib (MedChemExpress, Monmouth Junction, NJ), 10 μM GSK690693 (MedChemExpress), 5 μM Auresertib (BioVision, Milpitas, CA), 20 μM LY294002 (Cayman Chemical, Ann Arbor, MI), 5 μM PX-886 (LKT Laboratories, St Paul, MN) and 5 μM GDC-0941 (Selleckchem, Houston, TX).

RNAi experiments were performed utilizing siRNA pools of three different siRNAs each (si16E6/E7; si18E6/E7, si18E6), which efficiently and specifically block HPV16 or HPV18 E6 or E6/E7 oncogene expression, respectively, as described in detail elsewhere.22-24

2.2 | RNA and protein analyses

Protein extractions and immunoblot analyses were performed as previously described,22 with the exception that cells were lysed in CSK-1 buffer (10 nM Pipes pH 6.8, 300 mM NaCl, 1 mM EDTA, 300 mM Sucrose, 1 mM MgCl2, 0.5% TritonX-100), supplemented with protease inhibitor Pefabloc (Merck, Whitehouse Station, NJ), protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 30 minutes on ice. PARP (Poly [ADP-ribose] polymerase) cleavage was detected using the mouse monoclonal antibody “Cleaved PARP” (#9548; Cell Signaling, Danvers, MA). All other antibodies employed in this study are listed in detail elsewhere.23 Enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent, GE Healthcare, UK) was employed for immunoblot visualization using the Fusion SL Detection System (Vilber Lourmat, Germany).

RNA extractions and quantitative real-time PCRs were performed as described.22 For E6/E7 mRNA measurements, primers were employed which recognize all three transcript classes coding for HPV16 or HPV18 E6 or E6/E7.22 Relative quantification was performed by the comparative Ct (2^(-ΔΔCt)) method.25

2.3 | Proteome analyses by mass spectrometry and GSEA analyses

For proteome analyses, SiHa cells were treated in triplicates for 24 hours with 7.5 mM Metformin. Technical details for protein sample preparations and mass spectrometry-based quantitative proteome analyses are provided in detail elsewhere.24 The proteomics data are deposited to the ProteomeXchange Consortium via the PRIDE27 partner repository with the dataset identifier PXD011095. Gene set enrichment analysis (GSEA) was performed using GSEA v. 4.0.3 and MSigDB v. 7.1. Symbols and average log2FC values of three replicates of all detected proteins (5930) were loaded into GSEA to perform preranked enrichment analysis with the following parameters: number of permutations, 1000; enrichment statistic, weighted; max size: exclude larger sets. 500; min size: exclude smaller sets, 15; normalization mode, meandiv; seed for permutation, timestamp. This resulted in 14 735 gene sets of the MSigDB being used in the analysis. Significantly enriched gene sets were screened manually for further investigation.

To investigate possible modulations of p53 targets, genes that are regulated by p53 with high confidence28 were used to create a gmx file for subsequent GSEA. This gene set “P53 targets high confidence” was loaded into the GSEA software and its enrichment was examined for the proteome data of Metformin-treated SiHa cells (Table S1). For investigating the enrichment of this gene set after siRNA-mediated E6/E7 repression in cervical cancer cells, genes that were significantly differentially expressed in a corresponding transcriptome analysis of HeLa cells29 were analyzed by GSEA.

2.4 | Proliferation, senescence and colony formation assays

Real-time analyses of cellular proliferation rates were performed using the IncuCyte S3 live-cell imaging system (Essen BioScience, Hertfordshire, UK) as described.30 HeLa and SiHa cells were labeled with nuclear restricted mKATE2 fluorescent protein after the protocol provided by the supplier (Essen BioScience), treated with different concentrations of Metformin, as specified in the text, and labeled nuclei were counted over time. Four images per well were acquired every 4 hours at a magnification of 10x and analyzed using the IncuCyte S3 2019B software. Experiments were performed at least thrice in triplicates with consistent results.

For senescence detection, cells were stained for SA-β-Gal activity as described and visualized by the EVOSx Core Cell Imaging System (Thermo Fisher Scientific, Dreieich, Germany) with 20-fold magnification. For senescence assays in Figure 5, cells were transfected with E6/E7-inhibitory siRNAs or control siRNA (siContr-1), cultivated in the absence or presence of Metformin, and stained for SA-β-Gal activities 5 days after transfection. All senescence assays were performed independently at least thrice with consistent results.

Colony formation assays (CFAs) were performed as previously described.23 Briefly, cells were cultured for 24 hours under Metformin or under hypoxia and subsequently treated with 10 μM Etoposide or, depending on the cell line, with 0.1 μM or 0.2 μM Doxorubicin (see experimental schemes in Figure 6B and Figure S6B). The cells were grown for further 48 hours under Metformin or hypoxia, splitted and cultured for 7 to 12 days under normoxia in drug-free medium. Colonies were fixed and stained with formaldehyde-cresyl violet. Control cells were treated accordingly, but consistently kept under normoxia without Metformin. CFAs were performed independently at least thrice with consistent results.

2.5 | Statistical analyses

SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA) was used for statistical tests. For comparison of relative mRNA levels upon Metformin treatment, a one-sample t-test was performed with the test mean set to zero. Shapiro-Wilk was used for normality statistic and the alpha value was set to 0.05. Values of $P \leq 0.05$ (*) and $P \leq 0.01$ (**) were considered statistically significant.
RESULTS

3.1 Metformin represses HPV E6/E7 oncogene expression

In order to investigate the possible effects of Metformin on viral E6/E7 expression, HPV-positive cervical cancer cells were treated with increasing doses of Metformin. In all tested cell lines, Metformin efficiently downregulates HPV E6/E7 oncoprotein levels in a dose- (Figure 1A) and time-dependent (Figure 1B) manner. The downregulation of E6/E7 expression by Metformin is further observed upon treatment with another biguanide, Phenformin, which may also have the potential to be repurposed for cancer treatment\(^{31}\) (Figure S1). The downregulation of E6/E7 by Metformin is not limited to cervical cancer cells, but is also detected in HPV16-positive HNSCC cells in a dose- (Figure S2A) and time-dependent (Figure S2B) fashion, and is also observed at the transcript level (Figure S2C). Release kinetics reveal that E6/E7 repression by Metformin is reversible in that a change to cell culture medium devoid of Metformin restores E6/E7 expression in a time-dependent manner, both in HPV-positive cervical cancer cells (Figure 1D) and HPV-positive HNSCC cells (Figure S2D).

We recently found that hypoxia leads to a strong downregulation of viral E6/E7 oncoprotein expression through a mechanism that can be counteracted by unphysiologically high glucose supply (25 mM)
and which depends on the hypoxia-induced activation of AKT kinase.\textsuperscript{26} To assess whether this pathway is functionally involved in the downregulation of HPV oncoprotein expression by Metformin, we tested the effects of varying glucose concentrations and of AKT inhibition. As observed under hypoxia,\textsuperscript{26} the Metformin-induced downregulation of E6 and E7 levels is efficiently counteracted by high

**FIGURE 2** Effects of glucose, AKT inhibitors and PI3K inhibitors on Metformin-induced E6/E7 repression. (A) Immunoblot analyses of E6/E7 levels in HeLa and SiHa cells treated for 24 hours with the indicated doses of Metformin under varying glucose concentrations. Vinculin, representative loading control. (B) Immunoblot analyses comparing E6/E7, phospho-AKT (P-AKT\textsuperscript{S473}) and total AKT expression levels in Metformin- and hypoxia-treated cervical cancer cells. HeLa and SiHa cells are cultivated for 24 hours under normoxia in the absence of Metformin (left panels), under 2.5 mM or 5 mM Metformin, respectively (central panels), or under hypoxic conditions (1% O\textsubscript{2}) (right panels). Additionally, high glucose (25 mM) or 10 \(\mu\)M of the AKT inhibitor AKTi VIII are added when indicated. Vinculin, representative loading control. (C) Analyses of the effects of a spectrum of AKT inhibitors (AKTi VIII, MK-2206, Ipatastatib, GSK-690693, Afuresertib) and PI3K inhibitors (LY294002, PX-886 and GDC-0941) on Metformin (central panel)- and hypoxia (right panel)-induced E6/E7 repression and on P-AKT\textsuperscript{S473}, P-AKT\textsuperscript{T308} and total AKT levels. HeLa cells are cultivated for 24 hours under normoxia in the absence of Metformin (left panel), under 2.5 mM Metformin (central panel) or under hypoxic conditions (1% O\textsubscript{2}) (right panel). Additionally, high glucose (25 mM), AKT inhibitors or PI3K inhibitors are added when indicated (for individual concentrations of the inhibitors please refer to Section 2). Vinculin, β-Actin, representative loading controls.
FIGURE 3  Gene set enrichment analysis of quantitative proteome analyses in Metformin-treated HPV16-positive SiHa cervical cancer cells. (A) Enrichment plot of the gene set representing the "cervical cancer proliferation cluster" in SiHa cells treated for 24 hours with 7.5 mM Metformin. Average log2FC (fold change) values of three replicates of the proteome analyses are used as input for preranked gene set enrichment analysis (GSEA) with 1000 permutations. (B) Gene symbols and average log2FC values of proteins which are present in the ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER gene set and are measured in the proteome analysis for the present study. Color scale indicates log2FC, blue: log2FC = −1.0, red: log2FC = 1.0. (C) Volcano plot of log2FC values vs −log10(p-value) of all proteins detected in the proteome analysis (blue) and of the 102 proteins of the ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER (red). Further marked are strongly downregulated proteins, which were also identified in other studies to be linked to cervical cancer. FDR, false discovery rate; FWER, family-wise error rate; NES, normalized enrichment score [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 4  Proliferation and senescence response of HPV-positive cancer cells under Metformin treatment. (A) Cell counts of viable HeLa and SiHa cells treated with increasing doses of Metformin (Met) for the indicated time periods. Cell numbers are determined by live cell imaging employing the IncuCyte S3 live-cell imaging system. (B) Cell counts of HeLa and SiHa cells over 5.5 days which are treated with 2.5 mM Metformin for 2 days (red triangles) or for 3 days (red diamonds) and subsequently released from treatment by changing to Metformin-free medium. Dashed vertical lines mark the time points for medium exchange after 2 days or 3 days. The respective control cells are continuously grown in the absence of Metformin, also with medium change after 2 days (black triangles) or after 3 days (black diamonds). (C) Senescence assays. Upper panels: SiHa cells are transfected with control siRNA (siContr-1) or E6/E7-inhibitory siRNAs (si16E6/E7) and analyzed for SA-β-Gal staining (blue color) 7 days after transfection. Lower panels: Sa-β-Gal assays of SiHa cells treated for 72 hours with 2.5 mM Metformin and analyzed for SA-β-Gal activities 7 days after initiation of treatment (see experimental scheme at the lower right). (D) Immunoblot analyses of HPV16/18 E6, E7 and p53 levels in HeLa and SiHa cells upon inhibiting E6/E7 and E6 expression by RNAi (each left panel) or by treatment with 2.5 mM or 5 mM Metformin, respectively, for 24 hours (each right panel). siContr-1, control siRNA; β-Actin, representative loading control. (E) Immunoblot analyses of HPV16/18 E6, E7, PARP and cleaved (cl.) PARP expression in HeLa and SiHa cells treated for up to 72 hours with the indicated Metformin doses. β-Actin, representative loading control [Color figure can be viewed at wileyonlinelibrary.com]
glucose levels (Figure 2A). Metformin affects phospho-AKT levels in a cell type-dependent manner, leading to an increase in HeLa (Figure 2B, left panel) and to a decrease in SiHa cells (Figure 2B, right panel). Notably, whereas the AKT inhibitor AKTi VIII can efficiently counteract hypoxia-linked E6/E7 repression, this effect is not observed for Metformin-induced E6/E7 repression (Figure 2B), indicating that the latter effect is AKT-independent.

To gain more insights into this regulation, we tested five AKT inhibitors with different modes of action (AKTi VIII and MK-2206 are allosteric inhibitors; Ipatasertib, GSK-690693 and Afuresertib are ATP-competitive inhibitors) as well as three different inhibitors of PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) (LY294002, PX-886 and GDC-0941), a kinase which acts as upstream activator of AKT.32 The AKT and PI3K inhibitors do not appreciably affect E6/E7 expression per se (Figure 2C, left panel). Remarkably, however, whereas all AKT inhibitors efficiently counteract hypoxia-linked E6/E7 repression (Figure 2C, right panel), they do not interfere with Metformin-induced E6/E7 repression (Figure 2C, central panel). In contrast, all PI3K inhibitors efficiently counteract both hypoxia- and Metformin-mediated E6/E7 repression (Figure 2C). Thus, active PI3K is a key determinant for Metformin-induced HPV oncogene expression whereas AKT seems not to be involved in this process—in contrast to its critical role for hypoxia-induced E6/E7 repression.

### 3.2 Modulation of the proteome of HPV-positive cancer cells by Metformin

To assess the effects of Metformin on the proteome composition of HPV-positive cancer cells, the relative changes in protein abundances were assessed in a mass spectrometry (MS)-based proteomics analysis26 in Metformin-treated HPV16-positive SiHa cells (Table S1). Interestingly, employing preranked GSEA33 and the molecular signatures database (MSigDB) 7.124 the gene set ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER35 was found to be significantly negatively enriched in Metformin-treated SiHa cells, compared to untreated control cells (Figure 3A). Expression of this gene set is highly correlated with E6/E7 mRNA levels and viral DNA load in invasive cervical cancer biopsies and is linked to an unfavorable clinical prognosis.35 Out of 140 factors contained in this gene set, 102 were detected in our proteome analysis (Figure 3B) with the majority being downregulated (Figure 3C), reflecting a strong negative enrichment. Several of the most strongly downregulated factors from this set, such as ASF1B,26 CDC20,27UBE2C28 and ATAD229 (Figure 3B,C), are reported to exert oncogenic activities in cervical cancer cells and/or to be linked to cervical cancer initiation, progression or invasion.

Additionally, further factors, such as TWEAK-receptor TNFRSF12A/Fn14,40 OLFM141 and ID142 which are particularly strongly repressed by Metformin (Figure 3C) but not contained in the ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER set, are linked to cervical cancer progression or unfavorable prognosis. Collectively, these findings indicate that E6/E7 repression by Metformin treatment of SiHa cells results in the downregulation of potentially oncogenic cellular factors, which are linked to E6/E7 expression in vivo and are associated with poor disease outcome in cervical cancer patients.
3.3 Metformin induces reversible growth inhibition of HPV-positive tumor cells and allows evasion from senescence

In line with its well-documented antiproliferative potential, Metformin inhibits the growth of HPV18-positive HeLa and HPV16-positive SiHa cells in a dose-dependent manner (Figure 4A). Notably, however, this growth inhibition is reversible and reculturing the cells in Metformin-free medium allows them to resume proliferation (Figure 4B). The reversibility of the growth arrest indicates that Metformin treatment impairs senescence induction in HPV-positive cancer cells, which is classically defined as an irreversible growth arrest, under conditions where E6/E7 expression is repressed. In support of this notion, whereas E6/E7 repression by RNA interference (RNAi) efficiently causes senescence, as indicated by the positive staining for the senescence marker senescence-associated-β-Galactosidase (SA-β-Gal) and by the typical morphological alterations of senescent cervical cancer cells (cellular enlargement and flattening, long cytoplasmic projections), senescence induction is severely impaired under antiproliferative Metformin treatment (Figure 4C).

Inhibition of E6/E7 expression by siRNA leads to a strong positive enrichment of p53 inducible target genes in HPV-positive cancer cells (Figure S3A), as expected from interfering with E6-mediated...
p53 degradation. Notably, in strong contrast, GSEA of the proteome 
data under Metformin-induced E6/E7 repression (Table S1) reveals 
that there is no significant enrichment for p53-induced factors 
(Figure S3B). These findings raise the possibility that there is a differ-
ential regulation of p53, a critical factor for senescence induction in 
HPV-positive cancer cells, under the two treatment conditions. 
Indeed, immunoblot analyses reveal that—in contrast to the strong 
p53 induction upon siRNA-mediated E6/E7 repression—there is no 
p53 induction under Metformin in both SiHa and HeLa cells and p53 
levels even fall below those of untreated control cells (Figure 4D).

Finally, we also observed that particularly under prolonged treat-
ment with higher Metformin doses, the cell counts of HeLa and SiHa 
cells fall below those at the starting point of the kinetic (Figure 4A). 
As Metformin has also the potential to act proapoptotic under certain 
conditions (see Section 4), we analyzed the expression of the apo-
ptosis marker cleaved PARP (Poly [ADP-ribose] polymerase) in 
Metformin-treated HeLa and SiHa cells. Kinetic analyses show an 
increase of cleaved PARP over time (Figure 4E) which correlates with 
the onset of the decline in cell numbers, indicating that the cytotoxic 
effect observed under prolonged treatment with higher Metformin 
doses is linked to the induction of apoptosis.

3.4 Metformin counteracts the prosenescent 
effects of E6/E7 inhibition

Targeted E6/E7 inhibition is considered to be an attractive strategy 
for the therapy of HPV-positive cancers and intense research efforts 
are ongoing to identify corresponding approaches that could be trans-
lated into the clinic. The antisenescent activity of Metformin in 
HPV-positive cancer cells raised the question whether the drug may 
also interfere with the prosenescent effect of E6/E7 inhibitors. To this 
end, we blocked E6/E7 expression by RNAi, in the presence or 
absence of Metformin. Notably, and in stark contrast to the efficient 
induction of senescence upon E6/E7 silencing in the absence of Met-
formin (Figure 5, upper right panels), HPV-positive cancer cells mas-
sively evade senescence when they are concomitantly treated with 
Metformin (Figure 5, lower right panels). These results show that Met-
formin can efficiently block the prosenescent effects of targeted 
E6/E7 inhibition.

3.5 Metformin interferes with prosenescent 
chemotherapy

Next, we tested whether Metformin may also interfere with pro-
senescent effects of chemotherapy (CT). We have previously shown 
that hypoxia (1% O2) allows HPV-positive cancer cells to evade 
CT-induced senescence, an effect which is linked to the impairment 
of mTORC1 (mechanistic Target of Rapamycin Complex 1) signaling in 
hypoxic cells. Metformin is known to block mTORC1 signaling as 
well. This is also observed under our experimental conditions in 
HPV-positive cervical cancer and HNSCC cells, as indicated by the 
efficient downregulation of phospho-p70S6K, phospho-S6 and 
phospho-4E-BP1 levels under Metformin treatment (Figure S4). We 
thus compared the phenotypic responses of HPV-positive cancer cells 
towards prosenescent CT, either under normoxia (21% O2), under 
hypoxia (1% O2) or under Metformin treatment. Exposure of cervical 
cancer cells to Etoposide efficiently induces senescence in normoxic 
cells (Figure 6A). As expected, this response is severely impaired 
under hypoxia, resulting in the outgrowth of nonsenescent cells 
staining negative for SA-β-Gal activity and lacking the morphological 
alterations typical for senescence. Notably, alike hypoxia, treatment 
with Metformin also allows HPV-positive cells to evade Etoposide-
induced senescence (Figure 6A).

Since senescence is an irreversible growth arrest, evasion from 
senescence should lead to an increased colony formation capacity. 
Consistently, compared to cells treated with Etoposide under normoxia, 
the colony formation capacity of HPV-positive cancer cells which were 
treated with Etoposide under hypoxia and subsequently recultivated 
under normoxia is substantially increased (Figure 6B). Notably, 
compared to Etoposide treatment alone, the colony formation capacity 
of HPV-positive cancer cells is also strongly enhanced after the 
concomitant application of Etoposide and Metformin in normoxic cells 
(Figure 6B). These effects are also observed upon cotreatment with 
Etoposide and Metformin in the absence of glucose (Figure S5A,B). 
Further, corresponding results are obtained for Doxorubicin, also show-
ning that senescence is impaired (Figure S6A) and colony formation 
capacity is substantially increased upon cotreatment of Doxorubicin 
with Metformin (Figure S6B), corroborating that Metformin interferes 
with CT-induced senescence in HPV-positive cancer cells.

4 DISCUSSION

Metformin is currently under intense discussion to be repurposed for 
cancer therapy and a large number of clinical trials examining the anti-
tumorigenic potential of Metformin are ongoing. The results of the 
present study reveal that Metformin exerts profound effects on the 
phenotype of HPV-positive cancer cells, which have not been 
reported before. Firstly, Metformin strongly represses viral E6/E7 
oncogene expression, both at the transcript and protein level. Prote-
ome analyses disclose that this effect is linked to the significant down-
regulation of cellular factors which correlate with E6/E7 expression in 
cervical cancer tissues and which are linked to an unfavorable clinical 
prognosis. Mechanistically, active PI3K signaling was found to be 
required for Metformin-induced E6/E7 repression, however, other 
than under hypoxia, E6/E7 downregulation does not appear to be 
AKT-dependent. Secondly, Metformin induces a reversible prolifera-
tive stop and allows HPV-positive cancer cells to avoid senescence. 
Thirdly, Metformin blocks the senescence response of HPV-positive 
cancer cells towards targeted E6/E7 inhibition by RNAi, indicating 
that it has the potential to interfere with the prosenescent activity of 
prospective therapeutic E6/E7 inhibitors. Fourthly, Metformin also 
can efficiently counteract senescence induction by chemotherapeutic 
agents, enabling HPV-positive cancer cells to evade prosenescent CT.
It is interesting that phenotypic responses of HPV-positive cancer cells towards Metformin exposure or under hypoxia show both similarities and differences. Both treatment regimens induce a strong downregulation of viral E6/E7 expression, however, other than under hypoxia, AKT inhibitors do not counteract Metformin-induced viral oncogene repression. Since Metformin-induced E6/E7 repression was still found to be PI3K-dependent, other downstream targets of PI3K than AKT would appear to be involved in this regulation, an issue that warrants further experimental exploration. Notably, compared to the very efficient senescence response upon siRNA- or HPV E2-mediated E6/E7 downregulation, E6/E7 repression under Metformin treatment is accompanied by impaired senescence, as is also seen under hypoxia. The potential of Metformin to interfere with senescence induction is well documented in the literature and, for example, is an important consideration for clinical investigations testing Metformin as an antiaging drug.

It is interesting that—in contrast to the strong induction of p53 expression in HPV-positive cancer cells upon inhibition of E6/E7 expression by RNAi—p53 levels are not increased when E6/E7 expression is inhibited by Metformin. This latter observation points at a functional key role of p53 for the differential senescence response under these two experimental conditions, in line with the well-recognized importance of p53 for senescence induction upon E6/E7 repression in HPV-positive cancer cells. Moreover, it is notable that active mTORC1 signaling can play an important role for efficient senescence induction in several cell models, including in cervical cancer cells in response to E6/E7 repression or prosenescent CT, and both hypoxia as well as Metformin (this work) can severely impair mTORC1 signaling in HPV-positive cancer cells. Importantly, our results further indicate that the antisenescent effect exerted by Metformin is dominant over the prosenescent potential of HPV oncogene repression, since Metformin can also efficiently block the senescence response of HPV-positive cancer cells towards targeted E6/E7 inhibition by RNAi.

The results of the present study also have implications for the possible use of Metformin to treat HPV-induced cancers. In principle, the observed effects of Metformin on the phenotype of HPV-positive cancer cells could be therapeutically advantageous or disadvantageous—and whether Metformin eventually acts anti-tumorigenic may be determined by the relative balance of these effects. For example, the repression of the E6/E7 oncogenes by Metformin could support immune evasion of HPV-positive tumor cells by downregulating viral antigen expression, a response, which would also pose a problem for immunotherapeutic approaches targeting E6/E7-derived antigens. However, on the other side, Metformin can enhance the efficiency of immunotherapy by its effects on the tumor microenvironment. Furthermore, prospective therapeutic E6/E7 inhibitors, which theoretically should allow a highly selective attack on HPV-positive cancer cells might face the obstacles that—under Metformin treatment—the cellular senescence response is impaired and, furthermore, the therapeutic targets of the E6/E7 inhibitors may no longer be expressed in the tumor cells. Additionally, whereas the antiproliferative effects of Metformin on HPV-positive cancer cells could be beneficial for interfering with tumor growth, they may also increase their resistance towards chemotherapeutic agents, which preferentially target proliferating cells.

Moreover, the observation that Metformin can counteract the prosenescent activities of chemotherapeutic drugs in HPV-positive cancer cells may pose another problem, in view of the evidence that chemo- and radiotherapy (CT, RT) exert their anticancer effects not only through inducing tumor cell death but also through causing senescence (“therapy-induced senescence, TIS”). The here observed potential of Metformin to interfere with TIS is remarkable in regard of the fact that a large fraction of the clinical trials testing Metformin for its anticancer activity is performed by combining Metformin with CT or RT. However, whereas some researchers consider TIS beneficial for cancer therapy, others point at the possibility that, rarely, tumor cells can escape from TIS and eventually can exhibit a more aggressive growth behavior.

It also should be noted that Metformin not only can block cell growth but also has the potential to induce apoptosis at higher doses, a response that has been reported to be enhanced under low glucose levels. This notion is supported by the proliferation analyses of HPV-positive cancer cells in the present study, indicating that prolonged treatment with higher doses of Metformin induces apoptotic cell death. Furthermore, several chemotherapeutic drugs can act prosenescent at lower concentrations and proapoptotic at higher concentrations. These considerations add another level of complexity to the spectrum of possible phenotypic responses of cancer cells towards Metformin, since both the concentrations of drugs and glucose can be heterogeneously distributed in solid tumors due to alterations in microenvironmental conditions, for example, as result of differences in blood perfusion, alterations in oxygen supply or metabolic heterogeneity of tumor cells.

Collectively, this work provides new insights into the effects of Metformin treatment on the virus/host cell interactions and the phenotype of HPV-positive cancer cells. It also forms a basis for future studies. For example, it will be important to further delineate the factors participating in the PI3K-dependent, but AKT-independent repression of the E6/E7 oncogenes through Metformin and to explore whether this regulatory circuit might be exploitable for therapeutic purposes. In addition, whereas the present investigations delineate the effects of Metformin on viral oncogene expression and the senescence response of HPV-positive cancer cells, it will also be interesting to examine its influence on their apoptosis regulation, particularly in combination with proapoptotic CT and RT.

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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 mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011095.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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