

Hpv E6/E7 Silencing Attenuates Cancer Stemness And Enhances Ferroptosis-Associated Responses In Cervical Cancer Cells

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Abstract

Background: Persistent expression of high-risk human papillomavirus (HPV) E6 and E7 oncoproteins is a central feature of HPV-associated cervical carcinogenesis. Cancer stemness and altered redox metabolism are important determinants of malignant persistence, while ferroptosis provides an iron- and lipid-peroxidation-dependent form of regulated cell death. The present study investigated the relationship between HPV E6/E7 expression, stemness-associated characteristics and ferroptosis-related responses in cervical cancer cell models.

Methods: C33A, HeLa, SiHa and CaSki cervical cancer cell models were characterized for E6/E7 expression. A dual E6/E7-specific siRNA was evaluated over 5–40 nM. The selected condition was examined using primary and secondary sphere formation, SOX2/OCT4/NANOG expression, intracellular ROS, lipid ROS, GSH/GSSG, intracellular Fe²⁺ and GPX4/SLC7A11/ACSL4 expression. Ferroptosis induction and rescue were assessed, followed by combined E6/E7 silencing and ferroptosis-modulator treatment.

Results: HPV-positive models showed substantially greater E6/E7 expression than C33A. At 20 nM E6/E7-siRNA, E6 and E7 expression decreased to 0.34 ± 0.03 and 0.37 ± 0.03 while viability remained $89.6 \pm 2.7\%$. Primary sphere formation decreased from $24.6 \pm 2.1\%$ to $11.8 \pm 1.4\%$, and SOX2, OCT4 and NANOG decreased to 0.51 ± 0.05 , 0.56 ± 0.06 and 0.48 ± 0.05 . E6/E7 silencing increased lipid ROS to 2.14 $\pm$ 0.15-fold and decreased GSH/GSSG to 7.1 ± 0.5 , GPX4 to 0.54 ± 0.05 and SLC7A11 to 0.61 ± 0.05 , with ACSL4 increasing to 1.63 ± 0.11 . Combined E6/E7-siRNA and ferroptosis-modulator treatment reduced viability to $39.6 \pm 3.1\%$ and increased lipid ROS to 3.18 $\pm$ 0.21-fold.

Conclusion: The findings support an association between E6/E7 suppression, attenuation of stemness-associated characteristics and increased ferroptosis-associated oxidative stress. The combined treatment produced a greater biological response than either intervention alone in the reported dataset. Formal synergy should not be inferred without a dedicated combination-index analysis.

Keywords: human papillomavirus; E6; E7; cervical cancer; siRNA; cancer stemness; ferroptosis;

GPX4; SLC7A11; ACSL4; RSL3.

1. Introduction

Cervical cancer remains an important malignancy despite the availability of effective preventive and screening strategies. Persistent infection with high-risk HPV is the principal etiological factor, and HPV16 and HPV18 are among the genotypes most strongly associated with cervical carcinogenesis (Bosch et al., 2002; zur Hausen, 2002; Arbyn et al., 2020). Viral persistence alone is not sufficient to explain malignant progression; the phenotype reflects sustained viral oncogene activity together with changes in host-cell proliferation, genomic stability, metabolism, immune interactions and cell-death regulation.

The viral E6 and E7 oncoproteins are particularly important because they interfere with central tumour-suppressive pathways. E6 promotes functional loss of p53-associated responses, whereas E7 disrupts retinoblastoma-family control of cell-cycle progression (Munger et al., 2001; Moody and Laimins, 2010). These activities provide a biological rationale for targeting E6/E7 directly rather than treating HPV-associated malignant cells solely through downstream nonspecific cytotoxic mechanisms.

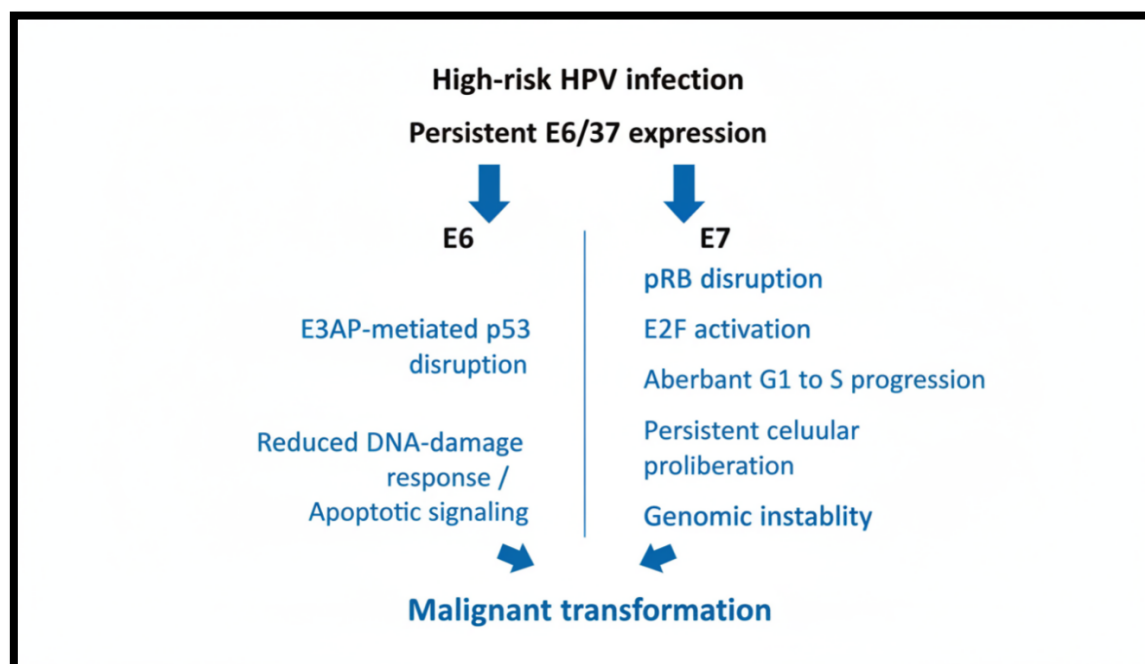


Figure 1. Molecular contribution of high-risk HPV E6 and E7 oncoproteins to cervical carcinogenesis.

RNA interference provides a sequence-directed strategy for reducing expression of disease-associated transcripts. However, siRNA is a macromolecular therapeutic cargo and its effectiveness depends on protection from degradation, cellular uptake, endosomal processing and access to the intracellular RNA-induced silencing machinery (Judge and MacLachlan, 2008; Kanasty et al., 2013). These delivery barriers become particularly relevant when siRNA is combined with a second therapeutic component.

Cancer stemness describes a set of functional and molecular characteristics that include self-renewal-associated capacity, phenotypic plasticity and persistence under selective pressure. Sphere formation is commonly used as a functional surrogate of self-renewal, whereas SOX2, OCT4 and NANOG provide complementary molecular readouts (Al-Hajj et al., 2003; Batlle and Clevers, 2017; Masui et al., 2007; Chambers et al., 2003). The work considers E6/E7-associated stemness as a potential

component of the cellular adaptation that may influence ferroptosis sensitivity.

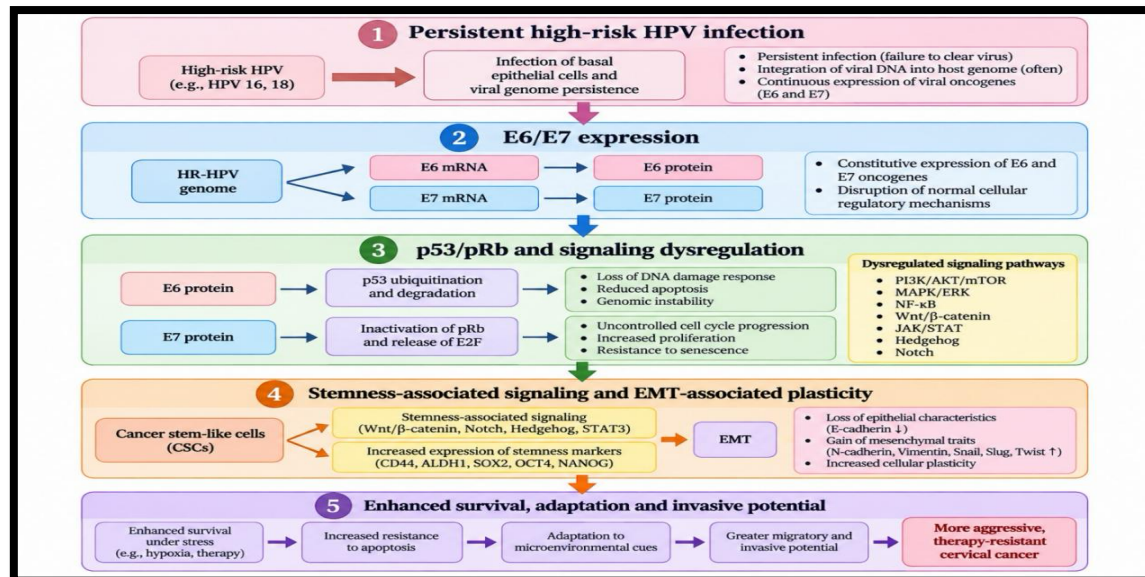


Figure 2. Relationship between HPV E6/E7-mediated molecular dysregulation and cancer-stemness-associated phenotypes.

Ferroptosis is characterized by iron-dependent lipid peroxidation and is mechanistically distinct from apoptosis and necrosis (Dixon et al., 2012; Conrad and Pratt, 2019; Jiang et al., 2021). The SLC7A11–cystine–GSH–GPX4 axis is a central antioxidant defense system that limits phospholipid peroxidation, while ACSL4 influences lipid composition and ferroptosis sensitivity (Doll et al., 2017; Zheng and Conrad, 2020). Consequently, measurements of lipid ROS, GSH/GSSG, GPX4, SLC7A11 and ACSL4 provide a multi-level framework for examining ferroptosis-associated cellular adaptation.

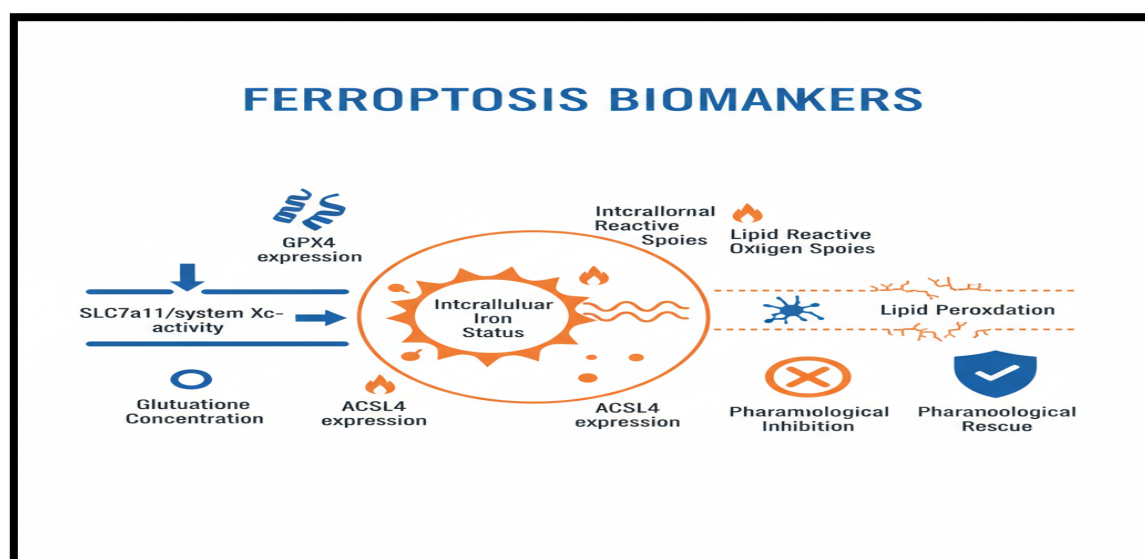


Figure 3. Ferroptosis-associated biomarkers involving iron homeostasis, lipid peroxidation and antioxidant defense.

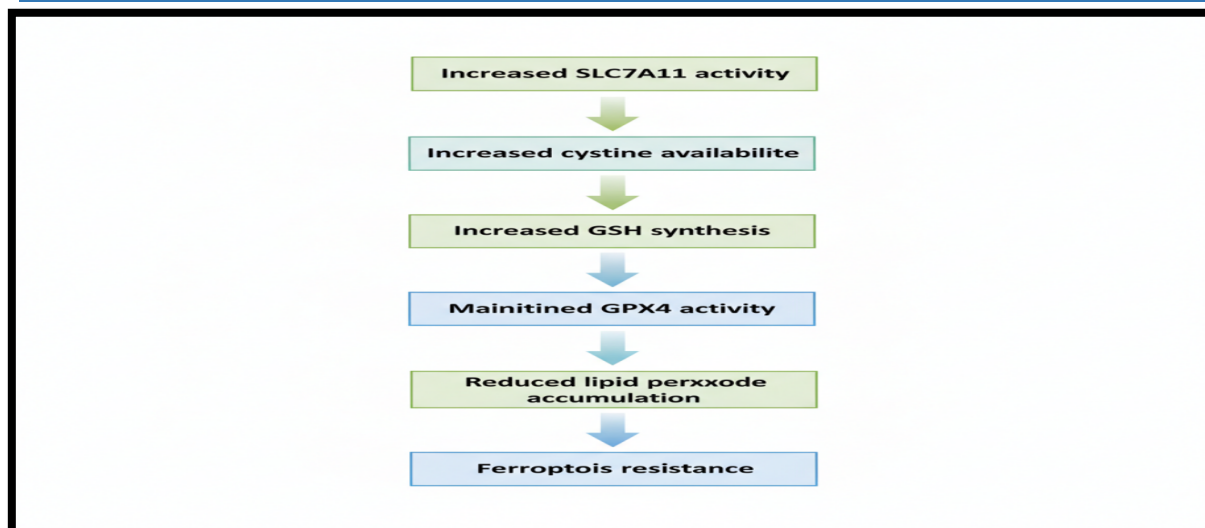


Figure 4. Role of SLC7A11, glutathione and GPX4 in ferroptosis resistance.

The central question addressed here was whether reduction of HPV E6/E7 expression is accompanied by a measurable change in stemness-associated characteristics and ferroptosis-related parameters. A second question was whether E6/E7 suppression modifies the response to a ferroptosis-modulating intervention. The study was therefore organized from baseline molecular characterization to siRNA optimization, functional stemness evaluation, ferroptosis-associated profiling, pharmacological rescue and combined treatment.

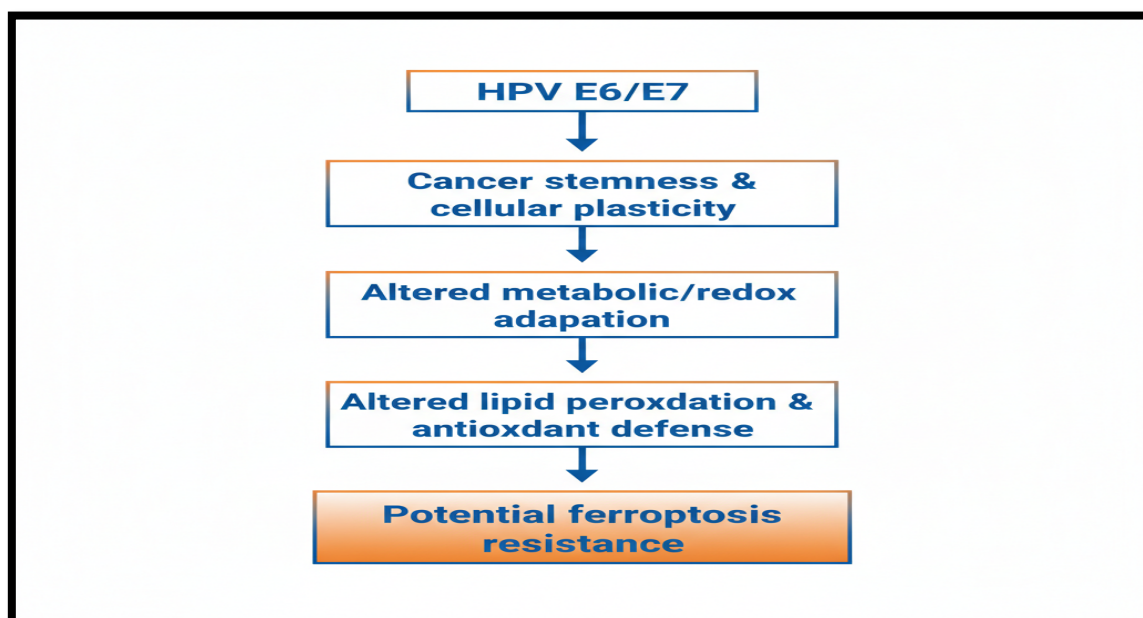


Figure 5. Proposed relationship between HPV E6/E7 expression, cancer stemness and ferroptosis resistance.

1.1 E6/E7 signalling as a determinant of malignant phenotype

The biological activity of E6 and E7 is not limited to the direct disruption of tumour-suppressive proteins. Their persistent expression can reshape transcriptional programmes, cell-cycle progression, DNA-damage responses, metabolic behaviour and interactions with the cellular stress response. This broader view is relevant to the present study because changes in stress adaptation may influence how a cancer cell responds to lipid oxidative damage. The experimental design therefore treated E6/E7 as upstream molecular regulators rather than isolated viral markers.

The use of multiple HPV-associated cell models is important because cervical cancer is heterogeneous. HeLa, SiHa and CaSki differ in genotype-associated biology, cellular background and basal molecular phenotype. The inclusion of C33A provides an HPV-negative comparator, but it does not make the four cell lines biologically identical. Consequently, the study interprets the baseline comparison as a characterization step and places greater emphasis on within-model treatment comparisons.

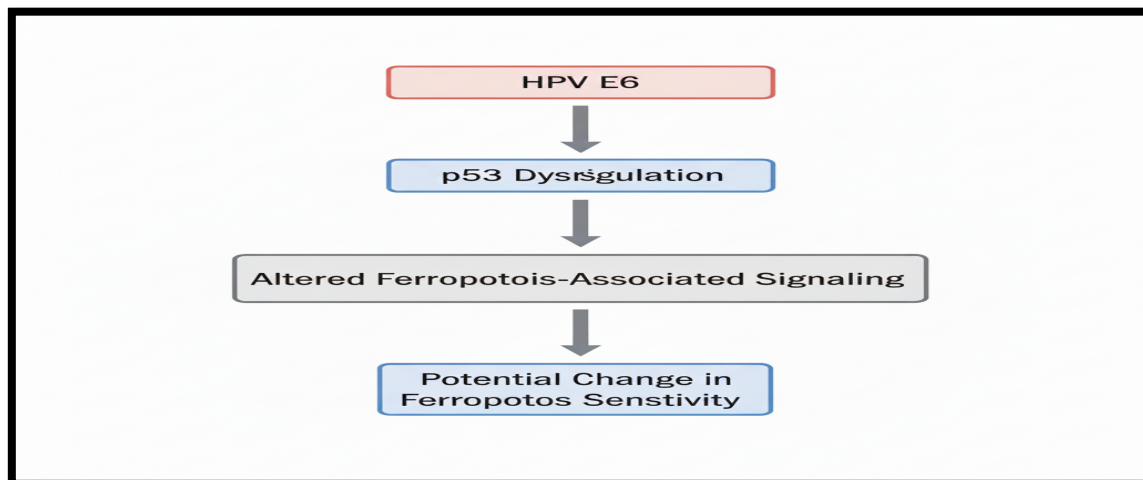


Figure 6. Proposed mechanistic link between HPV E6-mediated p53 dysregulation and ferroptosis sensitivity.

1.2 Cancer stemness and phenotypic plasticity

Cancer stemness is best considered a dynamic phenotype rather than a fixed cellular identity. Stem-like characteristics can be influenced by signalling pathways, metabolic state, microenvironmental stress and treatment pressure. Sphere formation therefore provides a functional assay of self-renewal-associated behaviour, while SOX2, OCT4 and NANOG provide molecular context. Agreement between functional and molecular endpoints increases confidence that an intervention has affected the phenotype being investigated.

In the present framework, E6/E7-associated stemness is not assumed to be synonymous with ferroptosis resistance. Instead, the study tests whether suppression of E6/E7 changes both domains in a coordinated direction. This distinction is important because an observed association can arise from parallel downstream effects rather than a direct causal relationship.

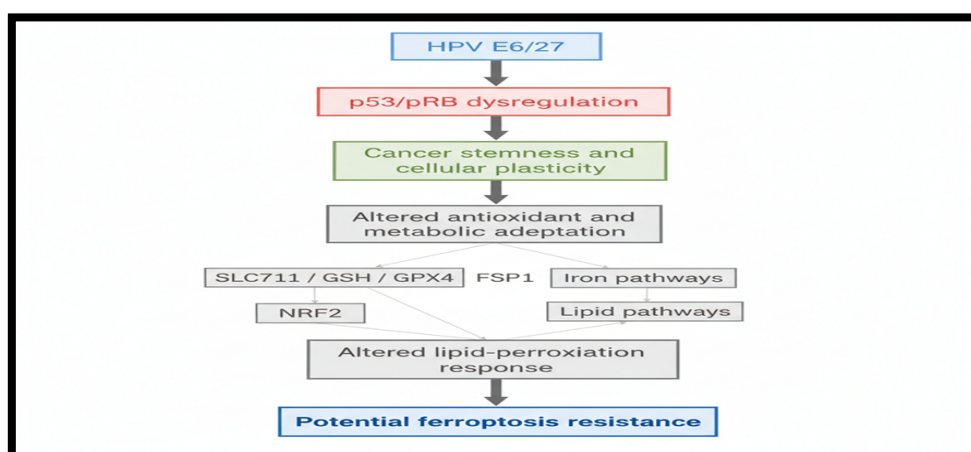


Figure 7. Proposed interplay between HPV E6/E7, cancer stemness, redox adaptation and ferroptosis

1.3 Ferroptosis biology relevant to the study

Ferroptosis is driven by the imbalance between lipid peroxide formation and cellular antioxidant capacity. System Xc⁻ contributes cystine availability for glutathione synthesis, GPX4 reduces phospholipid hydroperoxides, and ACSL4 contributes to the lipid substrate environment that can favour ferroptotic damage. Iron availability further contributes to the oxidative chemistry underlying lipid peroxidation. Because several of these processes can change independently, the study uses a panel rather than a single ferroptosis marker.

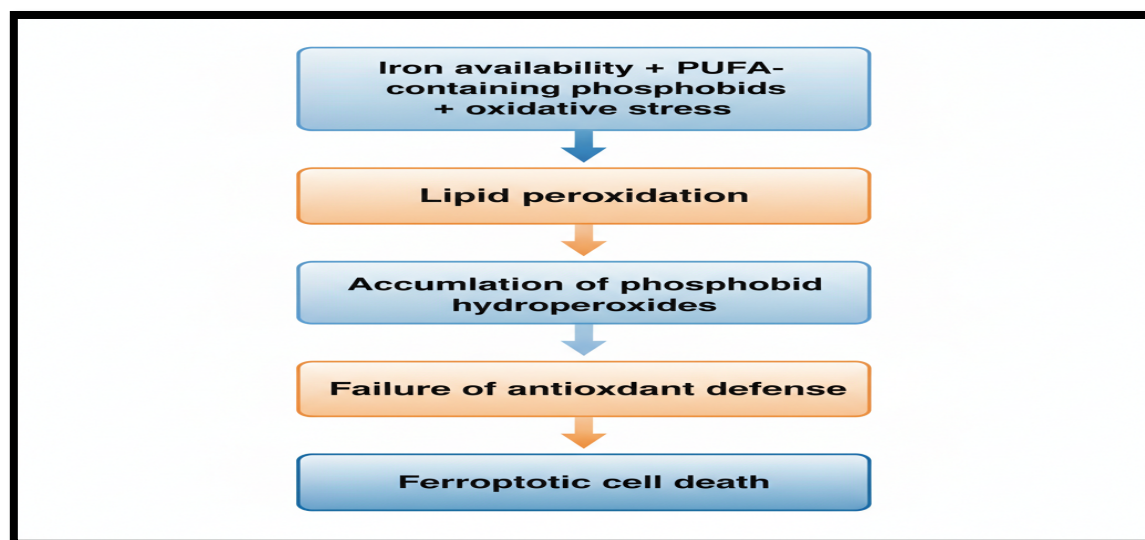


Figure 8. Molecular sequence leading to ferroptotic cell death.

The study also recognizes the role of antioxidant adaptation in cancer. Increased glutathione availability, altered SLC7A11 activity and maintenance of GPX4 can allow malignant cells to tolerate oxidative stress. Conversely, loss of antioxidant capacity together with increased lipid ROS can move cells toward ferroptotic vulnerability. The observed combination of increased lipid ROS and reduced GSH/GSSG, GPX4 and SLC7A11 after E6/E7 silencing therefore provides a coherent biochemical pattern.

1.4 Rationale for combined E6/E7 silencing and ferroptosis modulation

A combined intervention can be rationalized when the two components act at different but biologically connected levels. E6/E7-siRNA targets the oncogenic signal, whereas RSL3 acts on the ferroptosis-protective GPX4 axis. The intention is not to assume that one pathway is completely dependent on the other, but to test whether simultaneous perturbation produces a greater measurable response than either intervention alone.

The rescue experiment is an important part of this rationale. If the reduction in viability and increase in lipid ROS were due solely to nonspecific toxicity, a ferroptosis inhibitor would not be expected to reverse the phenotype in a coherent manner.

2. Materials and Methods

2.1 Study design and experimental logic

The experimental sequence comprised four linked stages. First, E6/E7 expression and ferroptosis-associated parameters were characterized in C33A, HeLa, SiHa and CaSki cells. Second, E6/E7-siRNA concentration was optimized using target suppression and cell viability as selection criteria. Third, the selected condition was evaluated for effects on sphere formation and stemness-associated markers and for changes in oxidative and ferroptosis-related endpoints. Fourth, E6/E7 silencing was combined with ferroptosis modulation and the resulting phenotype was evaluated using viability, lipid

ROS and redox parameters.

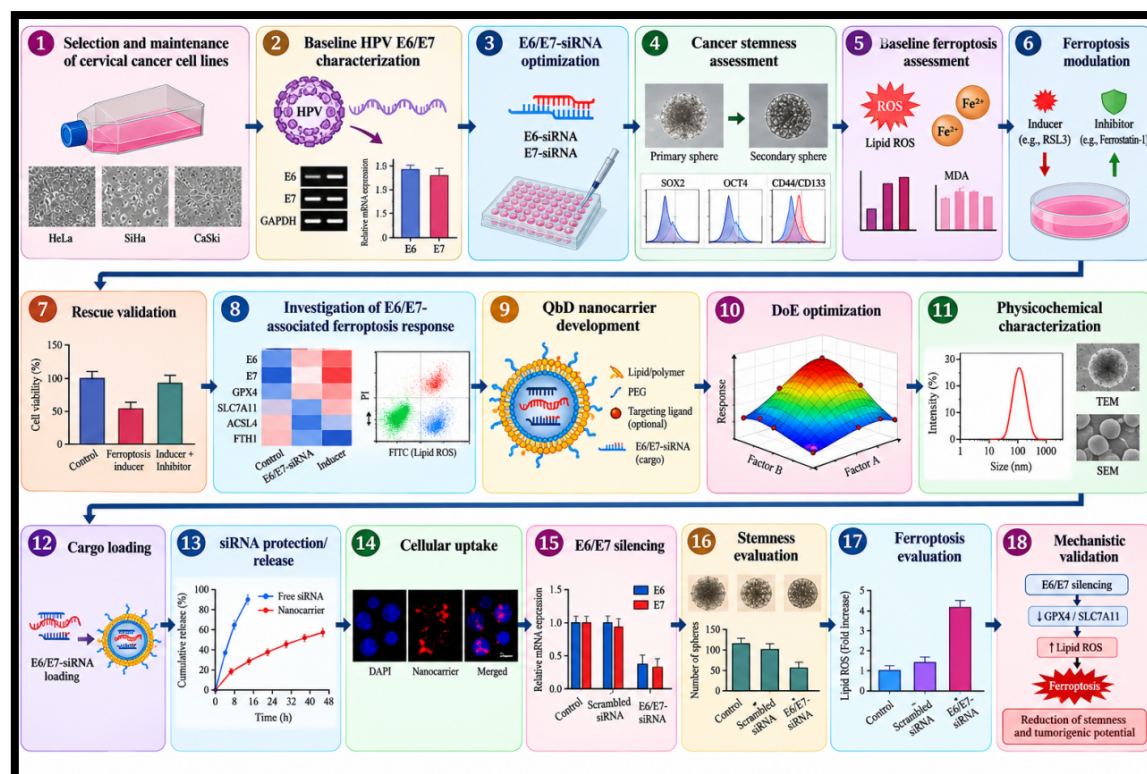


Figure 9. Overall experimental workflow used in the study.

2.2 Cell models and controls

C33A, HeLa, SiHa and CaSki cervical cancer cell models were included. C33A served as the HPV-negative reference model, while HeLa, SiHa and CaSki represented HPV-associated cervical cancer models. The experimental design incorporated untreated controls and scrambled/non-targeting siRNA controls to distinguish target-specific effects from nonspecific effects associated with transfection.

2.3 E6/E7-specific siRNA intervention

The study used a dual E6/E7-specific siRNA strategy. The results record the E6 target sequence as 5'-GCUAACGUAUCGAUCUGAUU-3' and the E7 target sequence as 5'CCAUGAUCGUACGAACUAUU-3', together with a non-targeting scrambled-siRNA control. E6 and E7 expression were evaluated after transfection and the working concentration was selected on the basis of target suppression with acceptable cellular viability.

2.4 Stemness-associated measurements

Primary and secondary sphere formation were used as functional measures of self-renewal-associated behaviour. Sphere-forming efficiency and mean sphere diameter were recorded. SOX2, OCT4 and NANOG were assessed as stemness-associated molecular markers. Western blot analysis was used as a complementary protein-level assessment where reported.

2.5 Ferroptosis-associated measurements

Intracellular ROS, lipid ROS, GSH/GSSG, intracellular Fe^{2+} , GPX4, SLC7A11 and ACSL4 were included. The results specify DCFH-DA for intracellular oxidative stress assessment and C11-BODIPY 581/591 for lipid peroxidation-associated fluorescence. GSH/GSSG and iron were measured using colorimetric assays, while protein endpoints were evaluated by Western blot/densitometric analysis. The integrated panel was intended to distinguish general oxidative stress from changes more directly related to ferroptosis-associated redox control.

2.6 Ferroptosis induction and rescue

RSL3 was identified as the ferroptosis-modulating agent (RSL3), with Ferrostatin-1 used as a rescue/inhibitor condition. The rescue experiment compared a ferroptosis-inducing condition with and without pharmacological rescue using viability, lipid ROS and GPX4 as principal readouts. This approach provides pharmacological support for ferroptosis involvement when the response is partially reversed by the inhibitor.

2.7 Statistical framework

Quantitative results are presented as mean $\pm$ variability as reported. The study methodology describes replicate-based measurement and analysis using analysis of variance with an appropriate post-hoc comparison for multi-group experiments. The reported numerical values and does not introduce additional p-values that are not explicitly present in the source dataset.

2.7 QbD considerations for co-delivery systems

QbD is particularly useful for multi-component nanocarriers because several formulation variables can influence more than one response simultaneously. Increasing polymer content can change particle formation and cargo retention, stabilizer concentration can influence interfacial properties, and surface-modifying components can affect both colloidal behaviour and biological interaction. A factor-by-factor approach may miss these interactions, whereas DoE provides a structured experimental space in which the contribution of selected variables can be quantified.

The present formulation strategy defined the QTPP before optimization. Particle size, PDI, surface charge, siRNA association and RSL3 loading were treated as critical physical or performance attributes. Biological performance was also considered part of the target profile because a formulation that meets particle-size specifications but fails to suppress E6/E7 would not meet the functional purpose of the delivery system.

2.8 Biomimetic surface design

The membrane-mimetic component was based on phosphatidylcholine, cholesterol and DSPE-PEG. Phospholipids can contribute to an interface that resembles aspects of biological membranes, while cholesterol can influence membrane packing and stability. DSPE-PEG provides a hydrophilic polymeric component that can modify surface behaviour. The present study does not claim that the formulation reproduces a complete native cell membrane; the term biomimetic is used to describe the membrane-mimetic surface architecture.

The surface layer is relevant to co-delivery because the two therapeutic cargos have different physicochemical characteristics. E6/E7-siRNA is a polyanionic nucleic acid, whereas RSL3 is a small-molecule ferroptosis inducer. A formulation that separates core loading from nucleic-acid association can therefore provide complementary microenvironments for the two components.

2.9 Critical Quality Attributes and biological CQAs

The formulation CQAs included particle size, PDI, morphology, siRNA association, RSL3 loading, release, stability and cellular uptake. Functional CQAs included E6/E7 silencing and biological activity against stemness and ferroptosis-associated responses. This distinction between physical CQAs and biological performance attributes is important because the latter ultimately determine whether a formulation is useful for the intended mechanism.

For siRNA-containing systems, association efficiency alone is insufficient. The nucleic acid must remain intact and biologically active. Similarly, high RSL3 loading does not necessarily indicate rapid or appropriate intracellular availability.

2.10 Co-delivery rationale

Co-delivery is intended to synchronize intracellular exposure to two therapeutic components. A

common carrier may reduce differences in distribution and cellular uptake between free components, while the formulation can be designed to protect siRNA during extracellular handling. However, co-delivery also introduces additional formulation complexity. The amount of each cargo, its location within the carrier, its release kinetics and its biological activity must all be considered.

2.11 Process reproducibility and independent confirmation

A QbD optimization is strongest when the predicted optimum is independently prepared and tested. Independent confirmation helps determine whether the model describes reproducible formulation behaviour rather than a single favourable run. For publication, the optimization record should therefore be accompanied by the actual confirmation-batch data, preparation date, formulation code and analytical replicate structure where available.

3. Results

3.1 Baseline E6/E7 expression

Table 1. Baseline relative expression of HPV E6 and E7 in the investigated cervical cancer cell models.

Cell line	HPV status	E6	E7
C33A	HPV–	0.08 ± 0.02	0.07 ± 0.02
HeLa	HPV18+	1.42 ± 0.11	1.31 ± 0.10
SiHa	HPV16+	1.68 ± 0.14	1.56 ± 0.12
CaSki	HPV16+	1.52 ± 0.13	1.44 ± 0.11

The HPV-positive models showed markedly higher E6 and E7 expression than C33A. SiHa exhibited the highest reported relative E6 and E7 values, followed by CaSki and HeLa. This baseline distribution established a molecular context for examining the biological effects of E6/E7 silencing.

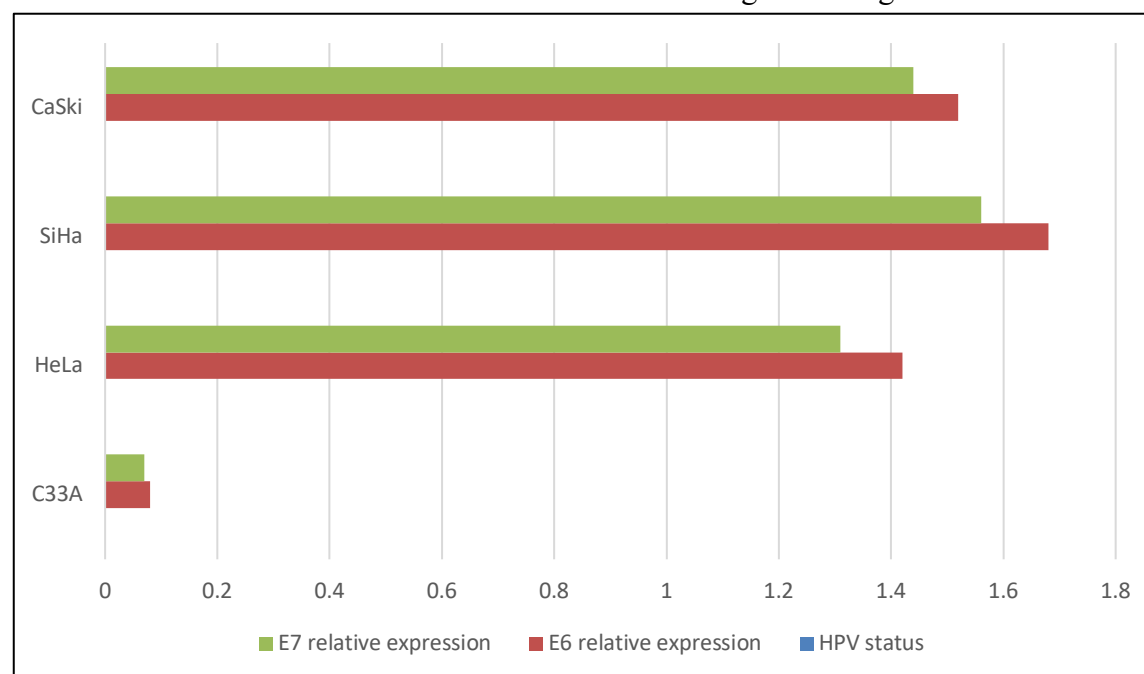


Figure 10. Baseline relative expression of HPV E6 and E7 in cervical cancer cell lines.

3.2 Optimization of E6/E7-siRNA concentration

Table 2. Effect of E6/E7-siRNA concentration on E6/E7 expression and cell viability.

siRNA (nM)	E6 expression	E7 expression	Cell viability (%)
5	0.78 ± 0.05	0.81 ± 0.06	96.2 ± 2.1

10	0.61 ± 0.04	0.65 ± 0.05	93.8 ± 2.4
20	0.34 ± 0.03	0.37 ± 0.03	89.6 ± 2.7
40	0.29 ± 0.03	0.31 ± 0.04	81.5 ± 3.1

Target expression decreased progressively with increasing siRNA concentration. At 20 nM, E6 and E7 expression were reduced to 0.34 ± 0.03 and 0.37 ± 0.03 , respectively, while viability remained $89.6 \pm 2.7\%$. Increasing the concentration to 40 nM produced only a modest additional reduction in target expression but reduced viability to $81.5 \pm 3.1\%$. The investigation selected 20 nM as a practical working condition.

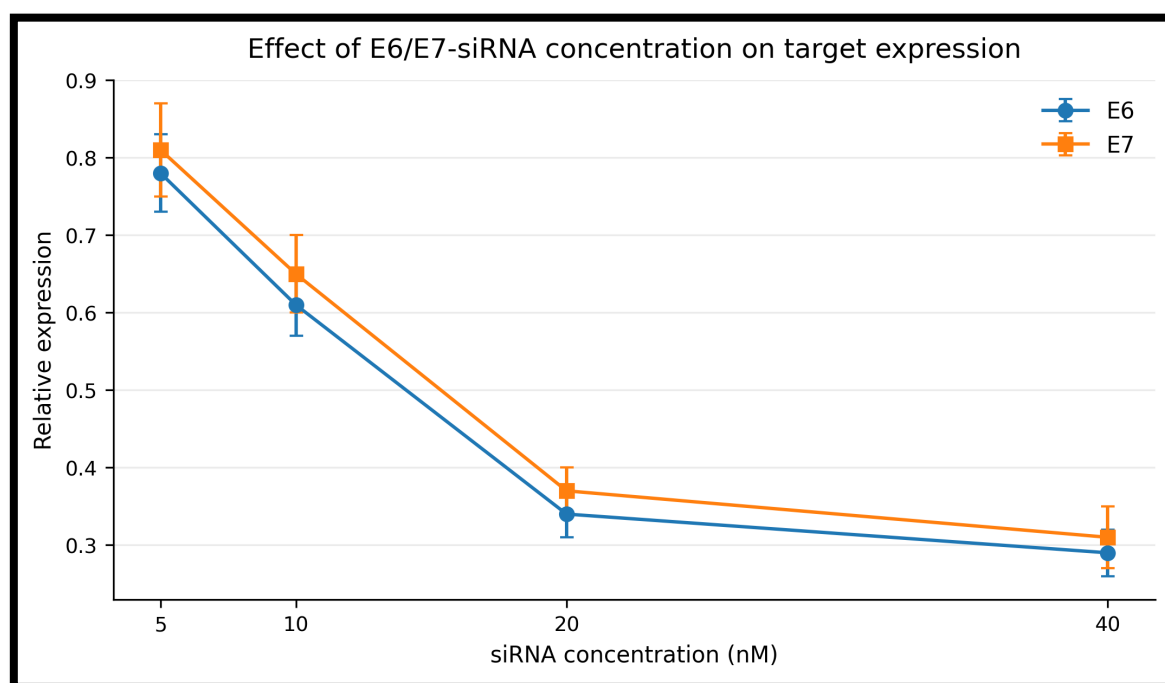


Figure 11. Effect of E6/E7-siRNA concentration on E6 and E7 expression.

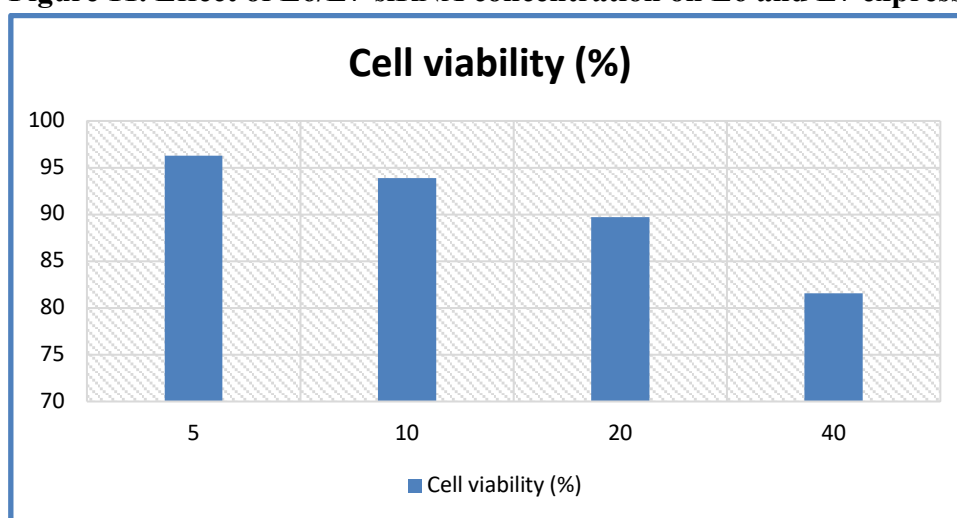


Figure 13. Effect of E6/E7-siRNA concentration on cervical cancer cell viability.

3.3 Primary and secondary sphere formation

Table 3. Effect of E6/E7 silencing on primary sphere formation.

Treatment	Sphere-forming efficiency (%)	Mean sphere diameter (μ m)
Control	24.6 ± 2.1	168 ± 12

Scrambled siRNA	23.9 ± 1.9	164 ± 11
E6/E7-siRNA	11.8 ± 1.4	103 ± 9

Table 4. Effect of E6/E7 silencing on secondary sphere-forming efficiency.

Treatment	Secondary sphere-forming efficiency (%)
Control	18.7 ± 1.8
Scrambled siRNA	18.1 ± 1.6
E6/E7-siRNA	7.9 ± 1.1

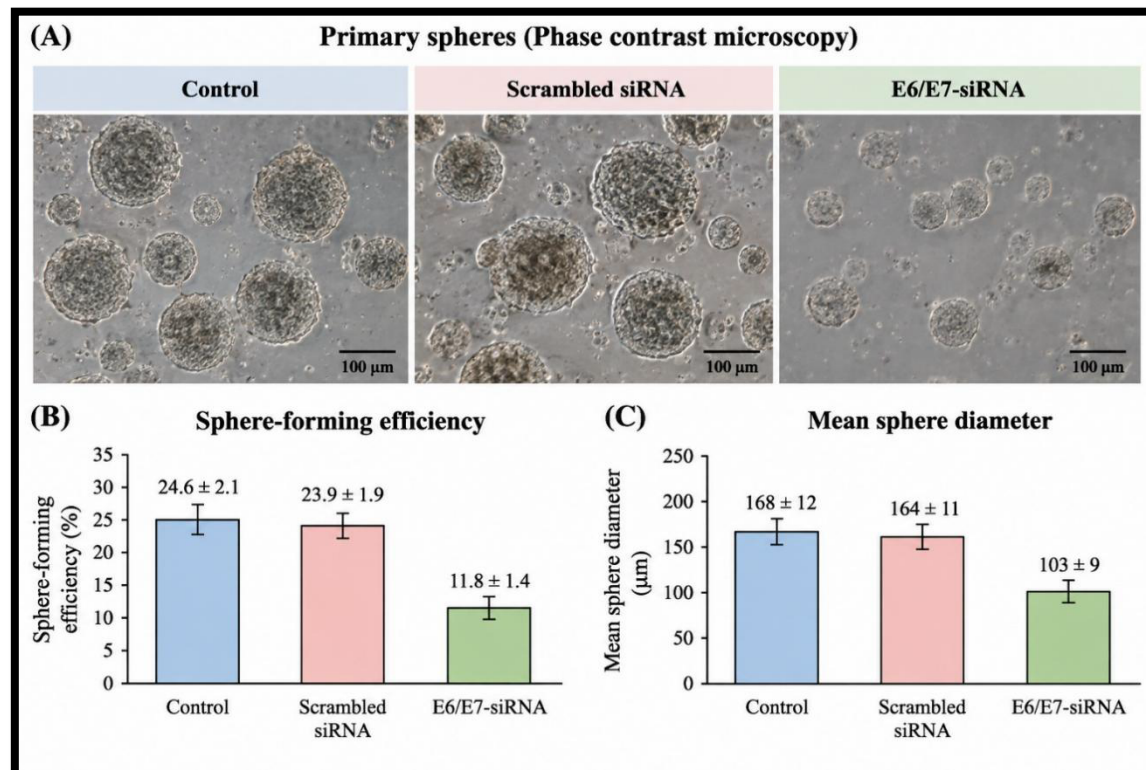
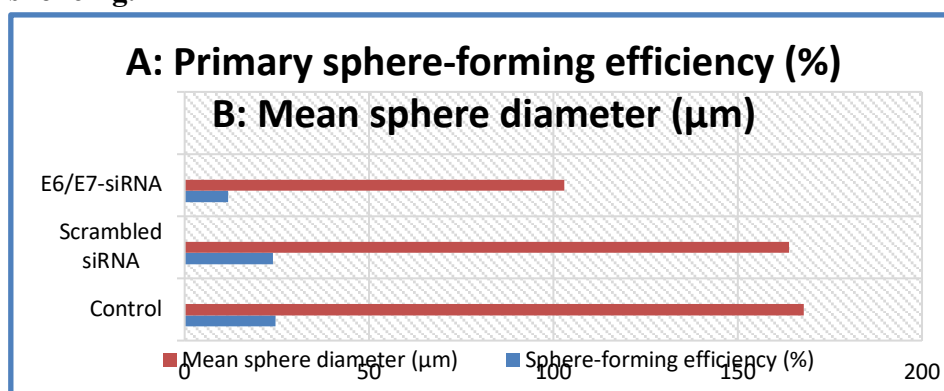


Figure 14. Representative microscopic images of primary sphere formation following E6/E7 silencing.



Primary sphere-forming efficiency decreased from $24.6 \pm 2.1\%$ in control cells to $11.8 \pm 1.4\%$ after E6/E7 silencing. Mean sphere diameter also decreased from $168 \pm 12 \mu\text{m}$ to $103 \pm 9 \mu\text{m}$. Secondary sphere formation showed a similar direction, decreasing from $18.7 \pm 1.8\%$ in controls to $7.9 \pm 1.1\%$ after E6/E7 silencing. The preservation of the direction across primary and secondary assays supports a reduction in self-renewal-associated capacity rather than an isolated change in one culture passage.

3.4 Stemness-associated markers

Table 5. Relative expression of SOX2, OCT4 and NANOG after E6/E7 silencing.

Treatment	SOX2	OCT4	NANOG
Control	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.09
Scrambled siRNA	0.96 ± 0.07	0.97 ± 0.08	0.95 ± 0.07
E6/E7-siRNA	0.51 ± 0.05	0.56 ± 0.06	0.48 ± 0.05

E6/E7 silencing reduced SOX2, OCT4 and NANOG expression to 0.51 ± 0.05 , 0.56 ± 0.06 and 0.48 ± 0.05 , respectively. Scrambled-siRNA treatment remained close to control values. The molecular pattern therefore paralleled the reduction in sphere formation and provides a second level of evidence for attenuation of stemness-associated characteristics.

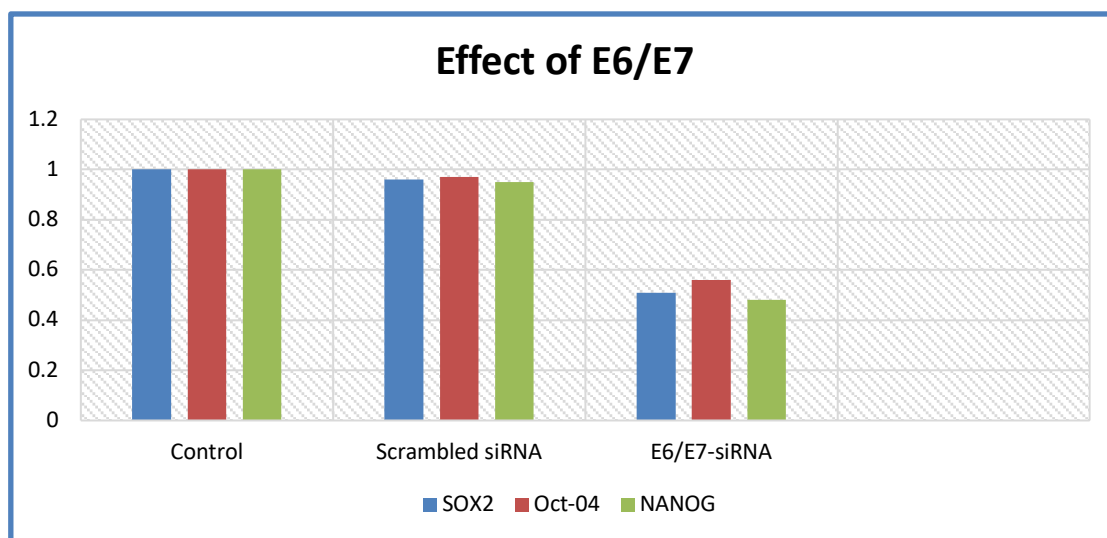


Figure 16. Effect of E6/E7 silencing on SOX2, OCT4 and NANOG expression.

3.5 Baseline ferroptosis-associated phenotype

Table 6. Baseline intracellular oxidative, lipid-peroxidation and redox parameters.

Parameter	C33A	HeLa	SiHa	CaSki
ROS (fold change)	1.00 ± 0.08	0.84 ± 0.06	0.78 ± 0.05	0.82 ± 0.06
Lipid ROS (fold change)	1.00 ± 0.07	0.71 ± 0.05	0.65 ± 0.05	0.68 ± 0.05
GSH (μmol/mg protein)	8.4 ± 0.5	10.1 ± 0.6	10.8 ± 0.7	10.4 ± 0.6
GSSG (μmol/mg protein)	1.2 ± 0.1	0.91 ± 0.08	0.84 ± 0.07	0.88 ± 0.07
GSH/GSSG	7.0 ± 0.5	11.1 ± 0.7	12.9 ± 0.8	11.8 ± 0.7
Fe ²⁺ (relative units)	1.00 ± 0.08	0.83 ± 0.06	0.79 ± 0.05	0.82 ± 0.06

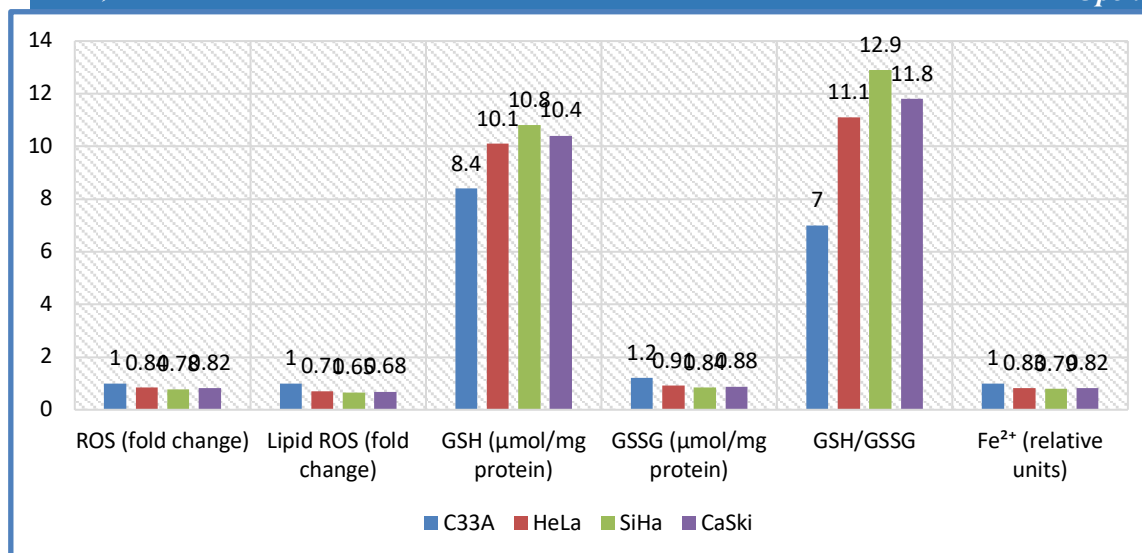


Figure 17. Baseline Ferroptosis-Associated Parameters in C33A, HeLa, SiHa and CaSki Cell Lines

Source: Researcher's Data Analysis, 2021

Table 7. Baseline GSH concentration and change relative to C33A.

Cell line	GSH (μmol/mg protein)	Change vs C33A
C33A	8.4 ± 0.5	Reference
HeLa	10.1 ± 0.6	+20.2%
SiHa	10.8 ± 0.7	+28.6%
CaSki	10.4 ± 0.6	+23.8%

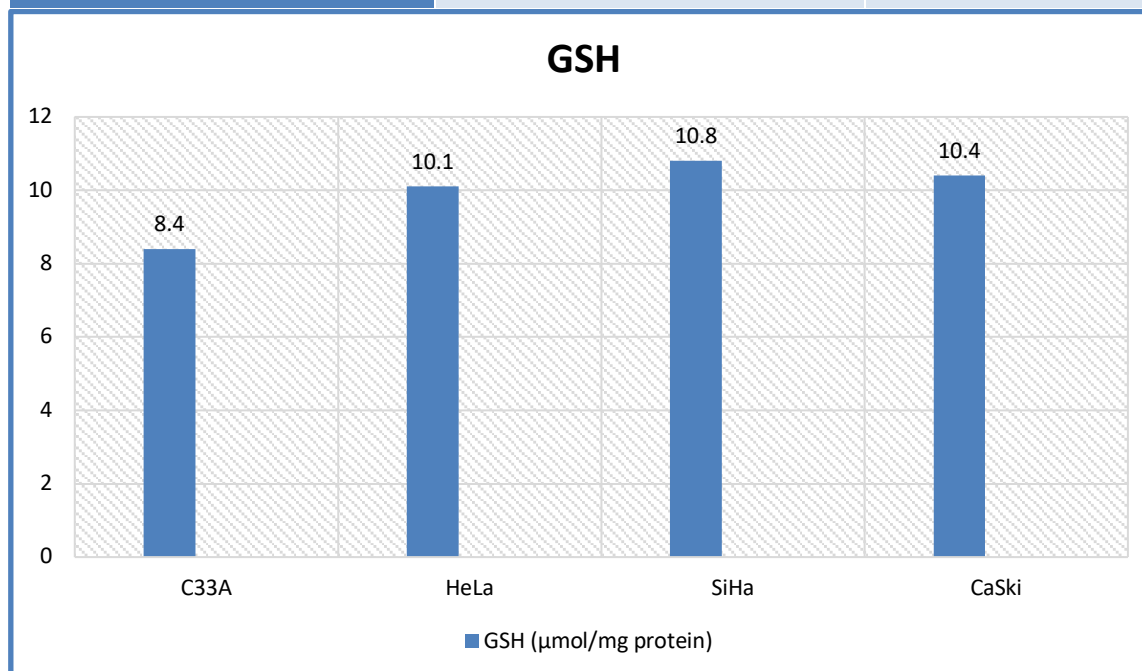


Figure 18. Baseline GSH concentration in cervical cancer cell lines.

Source: Researcher's Data Analysis, 2021

Table 8. Baseline intracellular Fe²⁺ and change relative to C33A.

Cell line	Fe ²⁺ relative units	Change vs C33A
C33A	1.00 ± 0.08	Reference
HeLa	0.83 ± 0.06	−17.0%
SiHa	0.79 ± 0.05	−21.0%
CaSki	0.82 ± 0.06	−18.0%

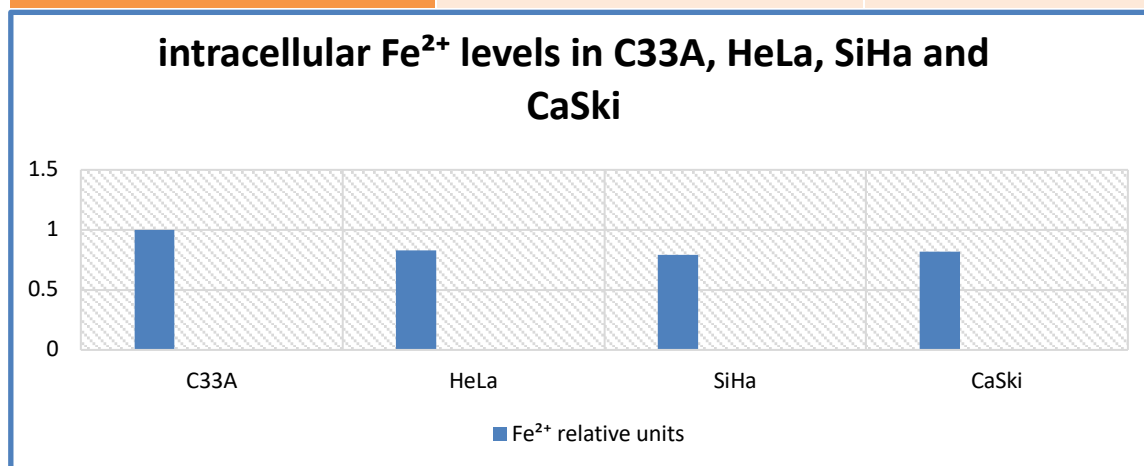


Figure 19. Baseline intracellular Fe²⁺ levels in C33A, HeLa, SiHa and CaSki cervical cancer cell lines

Source: Researcher's Data Analysis, 2021

Table 9. Baseline GPX4, SLC7A11 and ACSL4 expression.

Cell line	GPX4	SLC7A11	ACSL4
C33A	0.72 ± 0.06	0.68 ± 0.05	1.00 ± 0.08
HeLa	1.18 ± 0.09	1.12 ± 0.08	0.78 ± 0.06
SiHa	1.31 ± 0.10	1.25 ± 0.09	0.69 ± 0.05
CaSki	1.24 ± 0.09	1.18 ± 0.08	0.73 ± 0.06

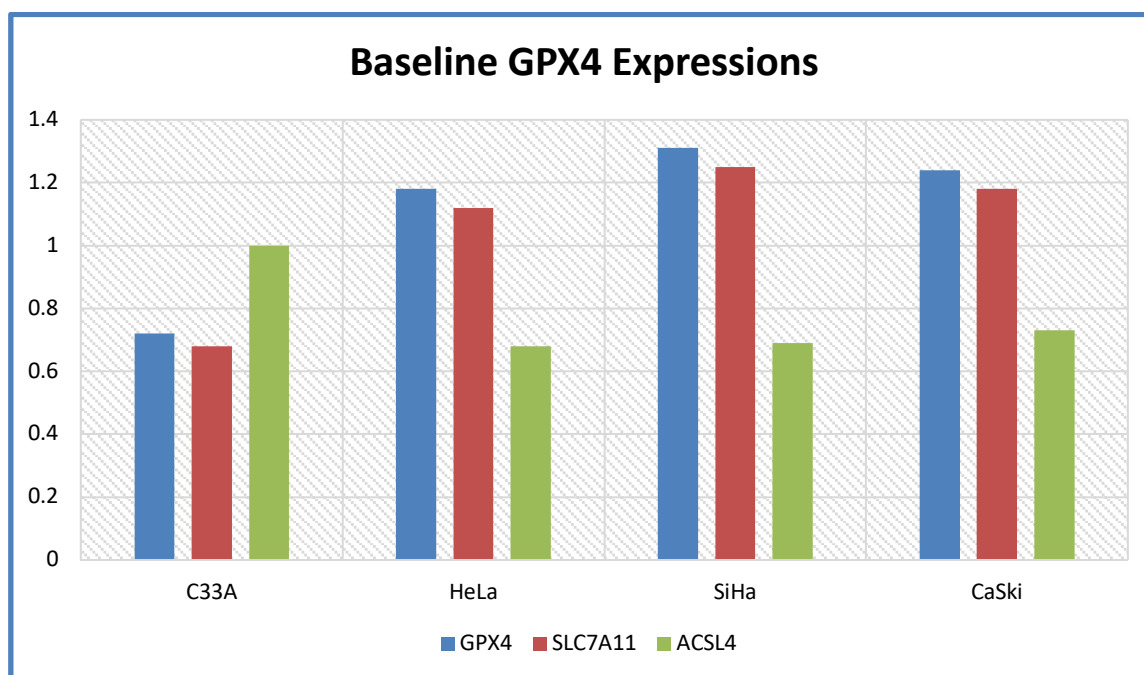


Figure 20. Baseline GPX4 expression in C33A, HeLa, SiHa and CaSki cervical cancer cell lines.

Source: Researcher's Data Analysis, 2021

The baseline dataset showed distinct redox and ferroptosis-associated profiles across the four cell models. Relative to C33A, HPV-positive models showed differences in ROS, lipid ROS, GSH/GSSG and intracellular Fe^{2+} . Protein-level differences were also observed for GPX4, SLC7A11 and ACSL4. These findings emphasize that ferroptosis-associated phenotypes are cell-line dependent and should be interpreted in relation to matched controls.

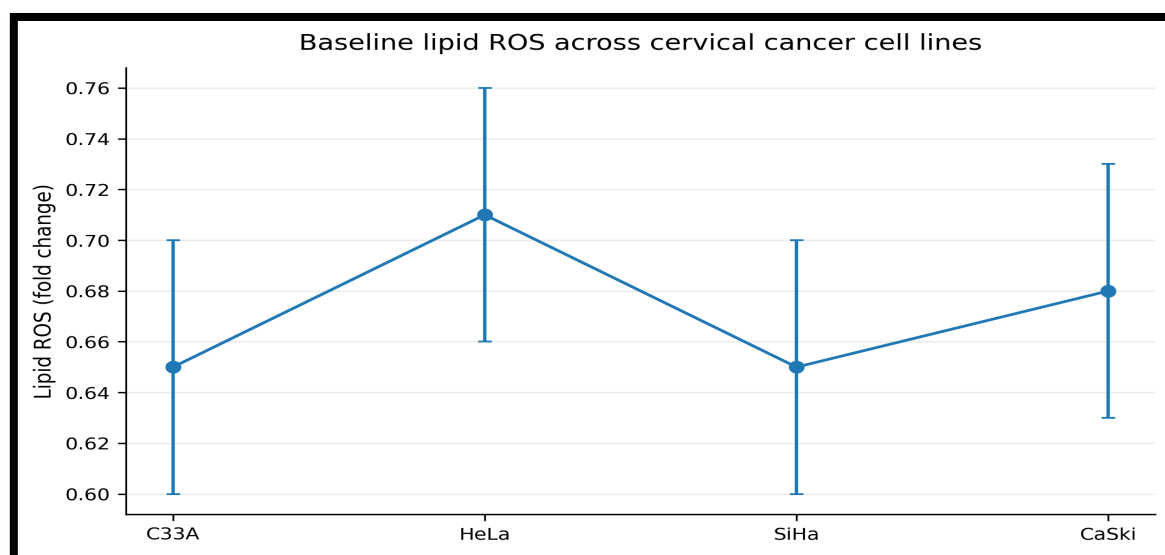


Figure 21. Baseline lipid ROS across cervical cancer cell lines.

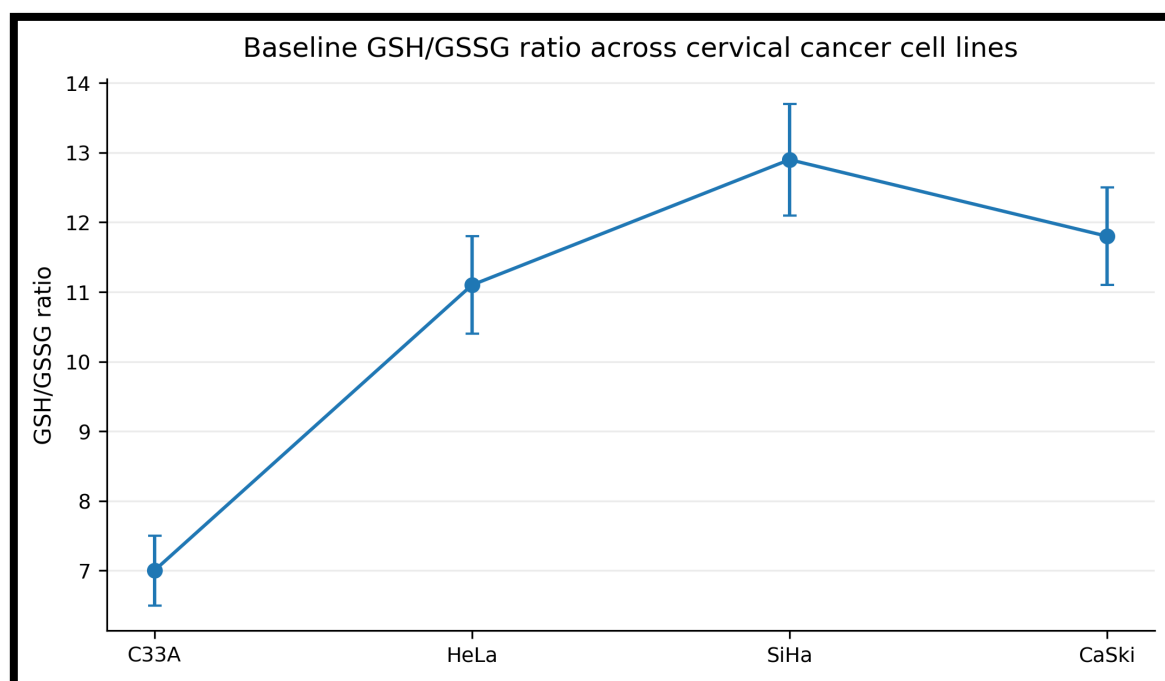


Figure 22. Baseline GSH/GSSG ratio across cervical cancer cell lines.

3.6 Effect of E6/E7 silencing on ferroptosis-associated cellular responses

Table 10. Effect of E6/E7 silencing on viability, ROS, lipid ROS, GSH/GSSG and Fe^{2+} .

Parameter	Control	Scrambled siRNA	E6/E7-siRNA
Cell viability (%)	100.0 ± 3.2	97.8 ± 3.0	69.4 ± 3.8
ROS (fold)	1.00 ± 0.07	1.03 ± 0.08	1.68 ± 0.11

Lipid ROS (fold)	1.00 ± 0.08	1.02 ± 0.07	2.14 ± 0.15
GSH/GSSG	12.8 ± 0.8	12.5 ± 0.7	7.1 ± 0.5
Fe ²⁺ (relative units)	1.00 ± 0.06	1.02 ± 0.07	1.42 ± 0.09

Table 11. Effect of E6/E7 silencing on GPX4 expression.

Treatment	GPX4	Change vs control
Control	1.00 ± 0.07	—
Scrambled siRNA	0.97 ± 0.06	−3.0%
E6/E7-siRNA	0.54 ± 0.05	−46.0%

Table 12. Effect of E6/E7 silencing on SLC7A11 expression.

Treatment	SLC7A11	Change vs control
Control	1.00 ± 0.08	—
Scrambled siRNA	0.96 ± 0.07	−4.0%
E6/E7-siRNA	0.61 ± 0.05	−39.0%

Table 13. Effect of E6/E7 silencing on ACSL4 expression.

Treatment	ACSL4	Change vs control
Control	1.00 ± 0.07	—
Scrambled siRNA	1.02 ± 0.08	+2.0%
E6/E7-siRNA	1.63 ± 0.11	+63.0%

Figure 23. Effect of E6/E7 silencing on lipid ROS.

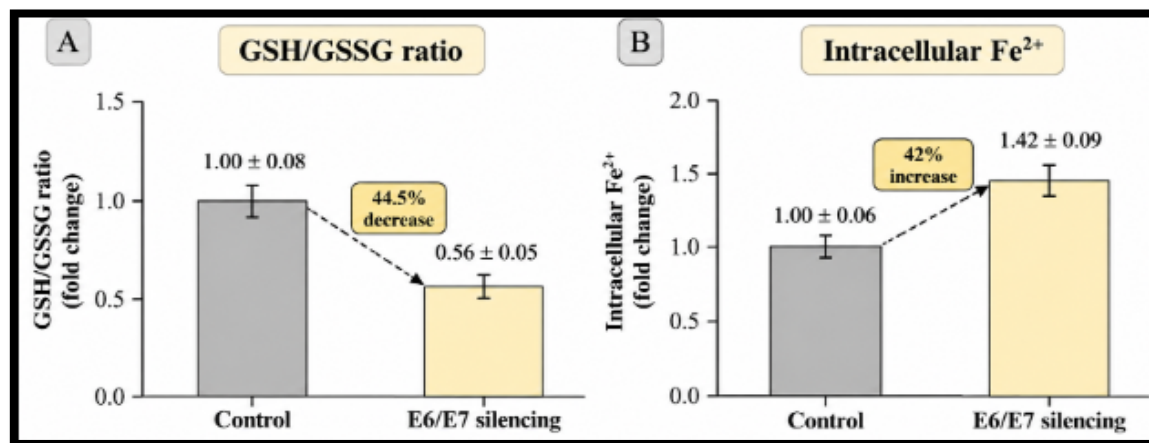


Figure 24. Effect of E6/E7 silencing on GSH/GSSG and intracellular Fe²⁺.

Figure 13. Effect of E6/E7 silencing on viability and ferroptosis-associated cellular parameters. E6/E7 silencing reduced viability from 100.0 ± 3.2% to 69.4 ± 3.8%. Intracellular ROS increased to 1.68 ± 0.11-fold and lipid ROS to 2.14 ± 0.15-fold. GSH/GSSG decreased from 12.8 ± 0.8 to 7.1 ± 0.5, while Fe²⁺ increased to 1.42 ± 0.09 relative units. At the molecular level, GPX4 and SLC7A11 decreased by approximately 46% and 39%, respectively, whereas ACSL4 increased by approximately 63%.

3.7 Integrated relationship between stemness and ferroptosis-associated endpoints

Table 14. Integrated effects of E6/E7 silencing on sphere formation and ferroptosis-associated endpoints.

Treatment	Sphere formation (%)	Lipid ROS	GPX4	SLC7A11
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Control	24.6 ± 2.1	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.08
Scrambled siRNA	23.9 ± 1.9	1.02 ± 0.07	0.97 ± 0.06	0.96 ± 0.07
E6/E7-siRNA	11.8 ± 1.4	2.14 ± 0.15	0.54 ± 0.05	0.61 ± 0.05

The simultaneous reduction in sphere formation and stemness markers with increased lipid ROS and decreased GPX4/SLC7A11 provides an integrated phenotype linking the two major biological domains examined. This relationship should be interpreted as an association supported by convergent endpoints rather than proof that stemness directly causes ferroptosis resistance. The experimental design nevertheless provides a mechanistic framework for testing that relationship.

3.8 Baseline comparison of ferroptosis-related parameters

Table 15. Baseline intracellular ROS, lipid ROS, GSH and ferroptosis-associated protein expression.

Cell line	Intracellular ROS	Lipid ROS	GSH	GPX4	SLC7A11	ACSL4
C33A	1.00 ± 0.15	1.00 ± 0.15	69.0 ± 6.0	1.00 ± 0.15	1.00 ± 0.15	1.00 ± 0.15
HeLa	2.95 ± 0.28	2.35 ± 0.22	25.0 ± 4.5	1.78 ± 0.14	1.75 ± 0.14	1.60 ± 0.14
SiHa	2.20 ± 0.22	1.95 ± 0.20	37.0 ± 5.0	1.50 ± 0.16	1.48 ± 0.15	1.42 ± 0.14
CaSki	1.75 ± 0.18	1.45 ± 0.18	49.0 ± 5.0	1.30 ± 0.12	1.18 ± 0.13	1.08 ± 0.13

3.9 Ferroptosis induction and rescue

Table 16. Validation of ferroptosis induction and pharmacological rescue.

Treatment	Cell viability (%)	Lipid ROS	GPX4
Control	100.0 ± 3.1	1.00 ± 0.07	1.00 ± 0.08
Ferroptosis-inducing treatment	55.2 ± 3.4	2.46 ± 0.16	0.48 ± 0.04
Ferroptosis-inducing treatment + rescue	82.7 ± 3.0	1.39 ± 0.11	0.79 ± 0.06

The ferroptosis-inducing treatment reduced viability to 55.2 ± 3.4%, increased lipid ROS to 2.46 ± 0.16-fold and reduced GPX4 to 0.48 ± 0.04. Addition of the rescue condition increased viability to 82.7 ± 3.0%, reduced lipid ROS to 1.39 ± 0.11-fold and increased GPX4 to 0.79 ± 0.06. Partial reversal of the phenotype provides pharmacological evidence consistent with ferroptosis involvement.

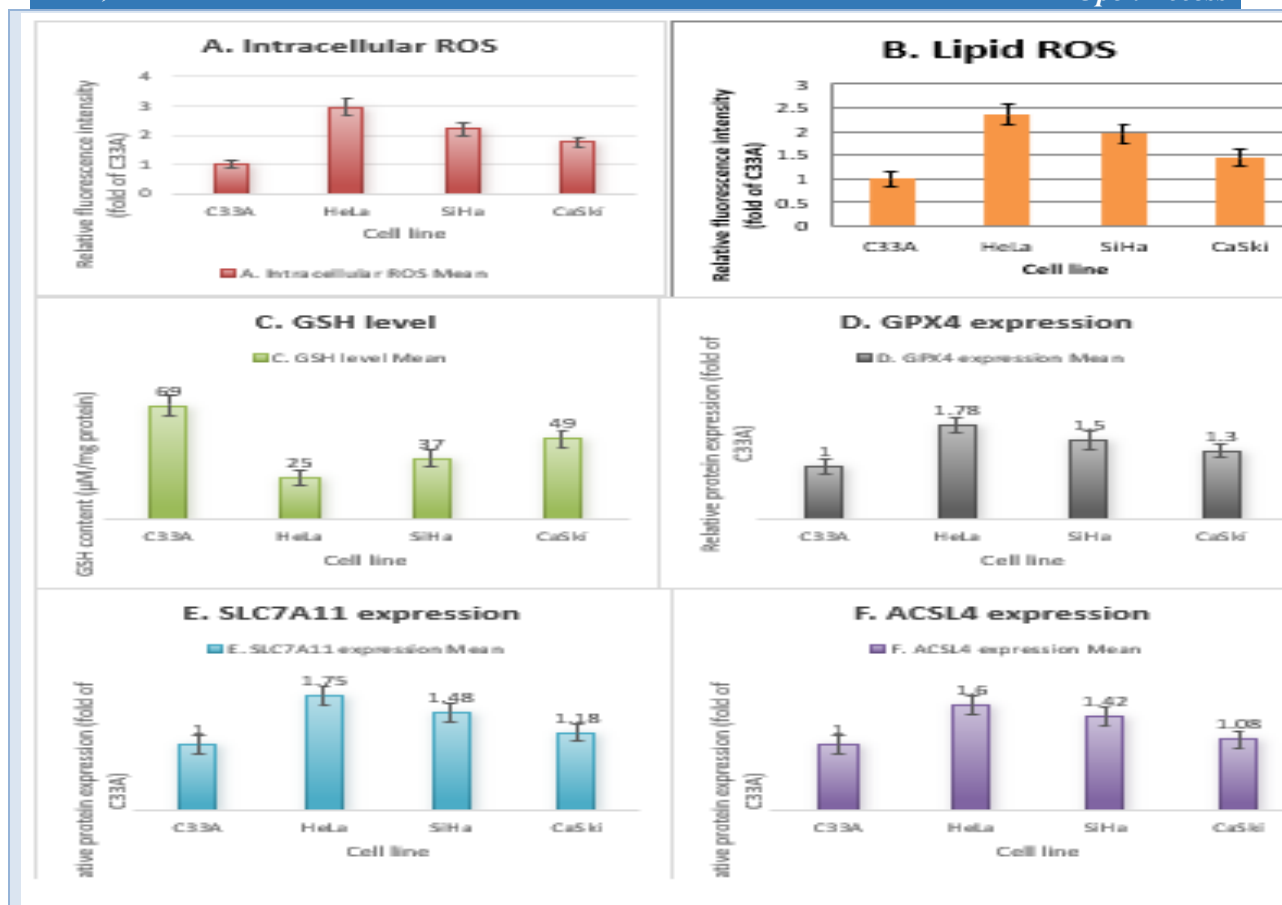


Figure 25. Baseline ferroptosis-associated parameters in C33A, HeLa, SiHa and CaSki cervical cancer cell lines. (A) Intracellular ROS, (B) lipid ROS, (C) GSH level, (D) GPX4 expression, (E) SLC7A11 expression and (F) ACSL4 expression.

Source: Researcher's Data Analysis, 2021

3.10 Combined E6/E7 silencing and ferroptosis modulation

Table 17. Comparative effect of E6/E7 silencing and ferroptosis modulation.

Treatment	Cell viability (%)	Lipid ROS	GSH/GSSG
Control	100.0 ± 3.2	1.00 ± 0.08	12.8 ± 0.8
Scrambled siRNA	97.8 ± 3.0	1.02 ± 0.07	12.5 ± 0.7
E6/E7-siRNA	69.4 ± 3.8	2.14 ± 0.15	7.1 ± 0.5
Ferroptosis modulator	64.8 ± 3.5	2.31 ± 0.17	7.6 ± 0.5
E6/E7-siRNA + ferroptosis modulator	39.6 ± 3.1	3.18 ± 0.21	5.4 ± 0.4

The combined condition produced the strongest reported reduction in viability and the greatest increase in lipid ROS, with a concurrent decrease in GSH/GSSG. Relative to the individual E6/E7-siRNA and ferroptosis-modulator conditions, the combined response was therefore greater across the reported endpoints. Because no formal combination-index analysis is presented, the correct interpretation is an enhanced combined response rather than demonstrated pharmacological synergy.

4. Discussion

The findings provide a coherent sequence from HPV oncogene expression to functional and molecular changes associated with stemness and ferroptosis. The higher E6/E7 expression observed

in HPV-positive cell models is consistent with the central role of these viral proteins in HPV-associated transformation (Munger et al., 2001; Moody and Laimins, 2010). Direct suppression of E6/E7 reduced sphere-forming capacity and stemness-associated markers, indicating that the viral oncogene program is linked to phenotypic characteristics relevant to cellular plasticity.

The sphere-forming findings are particularly informative because both primary and secondary assays showed the same direction of change. However, sphere formation should be interpreted as a functional surrogate and not as an exclusive assay for cancer stem-cell identity. The accompanying reductions in SOX2, OCT4 and NANOG provide molecular support, while future work could extend the phenotype using additional lineage and self-renewal assays.

The ferroptosis-associated dataset adds a second layer to the interpretation. E6/E7 silencing increased lipid ROS and intracellular ROS, reduced the GSH/GSSG ratio, decreased GPX4 and SLC7A11, and increased ACSL4. This combination is directionally compatible with reduced antioxidant protection and increased susceptibility to lipid-peroxidation-driven cell death. GPX4 is a major suppressor of phospholipid peroxidation, whereas ACSL4 can shape the lipid substrate environment relevant to ferroptosis (Doll et al., 2017; Jiang et al., 2021).

The observed change in intracellular Fe^{2+} after E6/E7 silencing is also compatible with altered ferroptosis-associated iron handling, although a single colorimetric endpoint cannot define the complete cellular iron pool. The study therefore appropriately combines iron measurements with lipid ROS, GSH/GSSG and protein markers rather than relying on iron alone.

The pharmacological rescue experiment strengthens the interpretation because the ferroptosis-associated phenotype was partially reversed by the rescue condition. This is important when distinguishing ferroptosis-related effects from general cytotoxicity. Nevertheless, rescue experiments do not establish a complete causal pathway, and complementary genetic or lipidomic validation would further strengthen the mechanistic conclusion.

The combined E6/E7-siRNA and ferroptosis-modulator treatment produced a larger response than either intervention alone. This result is biologically consistent with a two-level intervention in which viral oncogenic signalling is suppressed while a lipid-peroxidation-dependent death pathway is simultaneously engaged. The results deliberately avoid calling this effect synergy, and that distinction is maintained here because formal combination-index calculations were not reported.

From a translational perspective, the results suggest that HPV oncogene suppression and ferroptosis modulation can be investigated as complementary rather than mutually exclusive strategies. However, the work remains an in-vitro cell-model study. Questions regarding selectivity for malignant versus non-malignant cervical cells, delivery specificity, pharmacokinetics, tissue distribution and in-vivo safety remain outside the present experimental scope.

5.1 Methodological interpretation of the integrated phenotype

The study illustrates the value of evaluating a biological hypothesis at several levels. E6/E7 expression provides the initiating molecular variable; sphere formation and SOX2/OCT4/NANOG provide functional and phenotypic measures; ROS, lipid ROS, GSH/GSSG and Fe^{2+} describe the redox environment; and GPX4, SLC7A11 and ACSL4 provide molecular indicators linked to ferroptosis regulation. The convergence of these endpoints is more informative than any single measurement considered in isolation.

The use of scrambled siRNA is also important because transfection itself can influence cellular physiology. The relatively similar values between untreated and scrambled groups in the reported datasets support the interpretation that the larger changes observed after E6/E7-siRNA are associated with target-directed intervention. Nevertheless, a non-targeting control cannot completely exclude

sequence-independent effects, and independent siRNA sequences would provide an additional validation layer.

The study also separates induction from rescue. A ferroptosis-inducing treatment increased lipid ROS and reduced viability, while the rescue condition moved these endpoints toward control values. This pharmacological triangulation is useful because lipid ROS can increase during several forms of cellular stress. The rescue response therefore adds specificity to the interpretation, although it should be complemented by orthogonal genetic and biochemical evidence in future work.

Finally, the combined treatment result should be viewed as a hypothesis-generating observation that motivates the nanocarrier study. The larger response may reflect pharmacological interaction, additive effects, increased intracellular stress or other pathway relationships. Formal interaction analysis is required before a quantitative synergy claim can be made.

5. Strengths and limitations

The principal strength of the study is the use of multiple orthogonal endpoints. E6/E7 expression was evaluated at the target level, stemness was assessed through both sphere formation and marker expression, and ferroptosis was examined using oxidative, lipid-peroxidation, redox, iron and protein-level endpoints. The use of scrambled siRNA and pharmacological rescue conditions further improves interpretability.

Important limitations include the use of established cervical cancer cell models, the absence of in-vivo validation, and the need for more complete mechanistic dissection of the link between E6/E7 and ferroptosis. Combination effects should be quantified using formal pharmacological interaction methods in future studies. Additional controls, including non-malignant cervical epithelial cells and mechanistically distinct ferroptosis inhibitors, could also help define specificity.

6. Conclusion

HPV E6/E7 silencing was associated with a coordinated reduction in stemness-associated characteristics and an increase in ferroptosis-associated oxidative stress in cervical cancer cell models. The selected 20 nM siRNA condition reduced E6/E7 expression while retaining comparatively greater viability than the highest tested concentration. E6/E7 silencing reduced primary and secondary sphere formation and decreased SOX2, OCT4 and NANOG expression. In parallel, lipid ROS and intracellular ROS increased, GSH/GSSG, GPX4 and SLC7A11 decreased, and ACSL4 increased. Ferroptosis rescue partially reversed the induced phenotype, and combined E6/E7 silencing with ferroptosis modulation produced the strongest reported response. These findings establish the biological rationale for the subsequent development of a nanocarrier designed to co-deliver E6/E7-siRNA and RSL3.

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