



OPEN Nuclear and cytoplasmic remodeling of ectocervical cells by high activity of gelsolin

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Our study focuses on gelsolin (GSN), a pivotal and extensively researched protein that interacts with p53 and actin, regulating cytoskeletal remodeling and motility within cellular stress responses (CSR). This research investigates the cytoskeletal changes linked to exceeding intracellular GSN level in cytopathological contexts, specifically examining morphological alterations in ex vivo ectocervical cells. Our analyses reveal significant variations in nuclear and cell size, alongside specific morphological changes linked to high intracellular GSN concentrations. Fluorescence emission from Pap-stained cells was demonstrated as a cost-effective method to assess overall cytoplasmic and nuclear activity. This technique provides valuable insights into cellular stress responses and allows for the differentiation between inflammation- and HPV-related halos and nuclear irregularities. We can hypothesise that perinuclear GSN may be involved in perinuclear halo formation and nuclear remodeling, which are mediated by Ca^{2+} levels and can be altered by HPV infection. We propose the YAP/GSN ratio as a critical factor for epithelial differentiation and stress adaptation, offering a diagnostic marker for malignancy prediction.

Keywords Actin, GSN, Ex vivo epithelial cells, Cytopathology, Papanicolaou fluorescence

Cellular stress responses (CSRs) typically begin with an elevation in intracellular calcium ion (Ca^{2+}) concentration, initiating a cascade of biochemical processes^{1,2}. Cytosolic and nucleoplasmic Ca^{2+} play pivotal roles in regulating a wide range of cellular and nuclear functions^{3–6}. Particularly, Ca^{2+} -sensitive cytoskeletal regulators and actin-binding proteins (ABPs) are dynamically rearranged by specific chemical conditions within the cytoplasm and nucleoplasm. This rearrangement influences the actin network, which is central to regulating morphological transformations and pathological processes in stressed cells^{7–9}.

The cytoskeletal system serves as a fundamental determinant of cellular shape, motility, division, contraction, and intracellular transport¹⁰. It also plays a crucial role in activating apoptotic signaling pathways under Ca^{2+} -sensitive conditions¹⁰. Despite this, the mechanisms linking cytoskeletal dynamics to apoptosis or differentiation in stressed cells remain underexplored. Investigating the intracellular pathways connecting ABP function to cytoskeletal remodeling during apoptosis is essential. In inflammatory conditions, cytoskeletal reorganization is intricately linked to interleukins, cytokines, and elevated intracellular Ca^{2+} levels, which collectively modulate apoptosis factors and induce pathological outcomes¹¹.

Altered ABP function is implicated in chronic inflammation and metaplastic transformations⁸, as nuclear actin-ABP complexes remodel chromatin and histone proteins^{9,12,13}. Understanding nucleoplasmic ABP activity in this context is therefore vital. Notably, cytoskeletal stress responses involve Ca^{2+} -dependent mechanisms^{1,12,13} influencing nuclear and mitochondrial remodeling^{4–6} underscoring their importance in apoptosis regulation.

Recent efforts to identify novel biomarkers in cytopathology have focused on ABPs as potential links between apoptotic processes and morphological transformations¹⁴. Among these, gelsolin (GSN) has emerged as a particularly important ABP due to its multifunctionality^{15,16}. GSN is known to bind p53, forming complexes that regulate cytoskeletal remodeling and motility during CSR. GSN-p53 complexes are localized near the nuclear envelope¹⁵, where GSN contributes to tumorigenesis and invasion^{17–27}. Despite extensive research on GSN's pathological roles, its physiological functions remain less understood.

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Overexpression of GSN has been linked to delayed wound healing²⁸, controlling apoptosis in mosquito midgut cells²⁹, suppression of apoptosis in ovarian cancer cells, and dysregulation of chemosensitivity³⁰. Additionally, GSN overexpression is associated with tumorigenesis, metastasis, and epithelial-mesenchymal transition in bladder cancer, mediated through the inactivation of the Hippo signaling pathway³¹. In cervical cancer, high GSN expression correlates with increased migration and proliferation of cancer cells³², likely due to interactions with the Rho-ROCK-YAP signaling pathways.

Our previous studies have demonstrated that GSN modulates cell division and motility in HeLa cells³³. As we previously hypothesized in our hypothetical model on actin, p53, JMY, and GSN-related cytoplasmic CSR events³⁴, in the case of resting cells, cytoplasmic Ca^{2+} concentration is relatively low, GSN is inactive and remains near the membrane in a complex with PIP2¹⁶, or an actin monomer bond can mobilize it³⁵. Under moderate stress, GSN activation by micromolar Ca^{2+} concentrations induces actin filament capping and rearrangement. Furthermore, p53 competes with actin for GSN binding, leading to filament uncapping. There is a significant negative correlation between the nuclear import of p53 and the total filamentous actin content in the stressed cells, and thus p53 shows perinuclear colocalization with monomeric actin³⁶ and GSN¹⁵. Notably, high intracellular GSN levels slow cell division in epithelial HeLa cells³⁴, which is in a good agreement with the effect of Human Papillomavirus E7 protein (HPV16 E7), that alters the dynamics of the actin cytoskeleton mainly by binding to and inhibiting GSN, leading to an increased cell division³⁷.

In this study, we investigate the fluorescence emission of Pap (Papanicolaou) stained ectocervical cells according to our modified¹⁴ and classical staining methods^{38,39} to obtain more details of cytoplasmic and nuclear activity. Cytoplasmic activity is mainly based on the rearrangement of the cytoskeleton and the enzymatic machinery⁴⁰. Nuclear activity, on the other hand, is mainly related to the transport processes and/or accumulation of proteins or polysaccharides, gene regulation and the restructuring of the nucleoplasm and chromatin^{41,42}. Fluorescence emission of Pap stains^{33,43–46} was previously used to detect bacterial infections and morphologic changes in cytologic specimens. In some histologic studies, hematoxylin-eosin emission^{47,48} has been described as the main component of Pap staining. However, the fluorescence spectroscopic properties of the Pap components have not yet been investigated. Siddiqui et al. found that eosin fluorescence helps to distinguish renal structures and cells in healthy and diseased states, as protein denaturation, necrosis and fibrosis can be easily identified with more detail than classical histopathologic methods⁴⁹.

We used the clinically relevant Bethesda system to find and explain the morphological signs of the cells. The inflammatory process is a physiological response due to a remarkable content of cytokines and inflammatory cells in the sample. Morphologic changes associated with increased cytokine levels indicate inflammatory atypia. The nuclei may be enlarged and show a distinct, fine-grained chromatin; the cytoplasm is often vacuolated, and sometimes clear, small perinuclear halos with blurred borders can be observed^{50,51}. Severe bacterial vaginosis is associated with a high number of bacterial cells, mainly *Gardnerella vaginalis*, which cause severe atypical morphologic signs in the cells at the microscopic level⁵². Low-grade squamous intraepithelial lesions (LSIL) are characterized by lesions of intermediate or superficial cells caused by low- or high-risk HPV. They are usually transient infections with a low risk of malignant progression. In LSIL, nuclear atypia is not uncommon, with nuclear enlargement three times that of normal intermediate nuclei. In addition, hyperchromasia, anisonucleosis, coarse-grained or densely opaque chromatin with divergent nuclear membranes, binucleation or multinucleation may occur. Koilocytosis or dense orangeophilia must be accompanied by nuclear abnormalities. The nuclear contours show great variability from smooth to irregularly spotted with the absence or presence of suspicious nucleoli. Cytoplasmic cavities (koilocytes) are common, defining a broad, sharp, clear perinuclear halo and a highly stained cytoplasm⁵³.

In this study, we focus on ex vivo ectocervical squamous epithelial cells, aiming to bridge the gap between in vitro models and clinically relevant observations. Using viable human epithelial cells collected during routine cervical screening, we investigate morphological and nuclear changes driven by increased cytoplasmic GSN. Additionally, we employ a modified Papanicolaou (Pap) staining protocol combined with fluorescence emission analysis to gain insights into cytoplasmic and nuclear activity. This approach provides a cost-effective means of differentiating changes on Pap-stained cells and can also visualise stress responses or HPV-induced morphological changes.

Results

All data collected in this study were categorized according to the Bethesda System (TBS) criteria, which enabled clear identification of cellular morphological details^{52,54,55}. Cytology samples were obtained from 31 women aged 18–71 years (average age: 48.2 ± 21.5 years; median age: 52 years). The samples were divided into two groups based on menopausal status: premenopausal (14 women) and postmenopausal (17 women). In postmenopausal cases, the decline in estrogen levels may impair GSN function and thus cytoskeletal plasticity⁵⁶. Therefore, we have to perform the analysis within the two groups separately. Cytological analyses using Pap-stained, liquid-based preparations revealed seven mild inflammatory samples and two with severe bacterial vaginosis. Macroscopic observations and cytology reports from Kazincbarcika Hospital (Dept. of Pathology) indicated that seven specimens showed atrophy, one exhibited cervical ectropium, and two showed hyperemia. One specimen presented a combination of atrophy, ectropium, and mild inflammation, while another showed the morphological signs of low-grade squamous intraepithelial lesions (LSIL). Control samples were randomly selected from both pre- and postmenopausal groups and divided into two parts: one subjected to electroporation then incubation and the other incubated without electroporation. To minimize potential cellular alterations, recovery following electroporation was 24 h^{57–59}.

Low-voltage electroporation introducing GSN into viable ex vivo cells

Viable ectocervical squamous cells were subjected to low-voltage electroporation with square-wave impulses to elevate intracellular GSN levels. Alexa568-labeled GSN was successfully introduced into the cells using our electroporation system^{14,34} (Fig. 1A). Following electroporation, GSN primarily localized in the perinuclear region, with an uneven distribution near the cytoplasmic membrane. Additionally, phalloidin-Alexa488-labeled actin filaments displayed distinct, irregular clear regions throughout the cytoplasm (Fig. 1A–D).

GSN affects the dimensions of cells and nuclear irregularities

Ex vivo human ectocervical squamous cells displayed notable morphological changes in response to increased intracellular levels of gelsolin (GSN). These changes were systematically analyzed through brightfield microscopy following a 24-hour incubation period after electroporation. Using our modified Pap (Papanicolaou) staining protocol¹⁴, we quantified the variations in both cell and nuclear sizes under different treatment conditions.

Effects on cell size in premenopausal samples

We studied the changes by 24 h of incubation after the electroporation, which means we were looking for the simultaneous effect of the electroporation and incubation. In premenopausal samples, electroporation with GSN significantly increased the average cell size, rising from $57.23 \pm 16.1 \mu\text{m}$ to $60.14 \pm 16.8 \mu\text{m}$, reflecting a $\sim 5\%$ increase (Fig. 2A,B and K). Conversely, electroporation without GSN led to a slight reduction in cell size, from $57.23 \pm 16.1 \mu\text{m}$ to $53.68 \pm 14.1 \mu\text{m}$ (Fig. 2A,C and K). Similarly, incubation without electroporation caused a slight shrinkage, resulting in an average cell size of $55.49 \pm 10.7 \mu\text{m}$ (Fig. 2A,D and K). Notably, cells were treated with electroporation and GSN exhibited a significant increase in cell size ($\sim 12\%$), $60.14 \pm 16.8 \mu\text{m}$, compared to untreated cells, $53.68 \pm 14.1 \mu\text{m}$, highlighting GSN's substantial role in cytoplasmic expansion. The cytoplasm of cells became rough after the electroporation (Fig. 2C).

Effects on nuclear size in premenopausal samples

The nuclear size of premenopausal samples showed distinct responses to treatment conditions. Electroporation with GSN resulted in a modest reduction in nuclear size from $7.07 \pm 2.4 \mu\text{m}$ to $6.72 \pm 2.4 \mu\text{m}$ (Fig. 2A,B and L). However, electroporation without GSN slightly increased the nuclear size from $7.07 \pm 2.4 \mu\text{m}$ to $7.29 \pm 1.9 \mu\text{m}$ (Fig. 2A,C and L). In contrast, incubation alone led to a significant reduction in nuclear size, shrinking it to

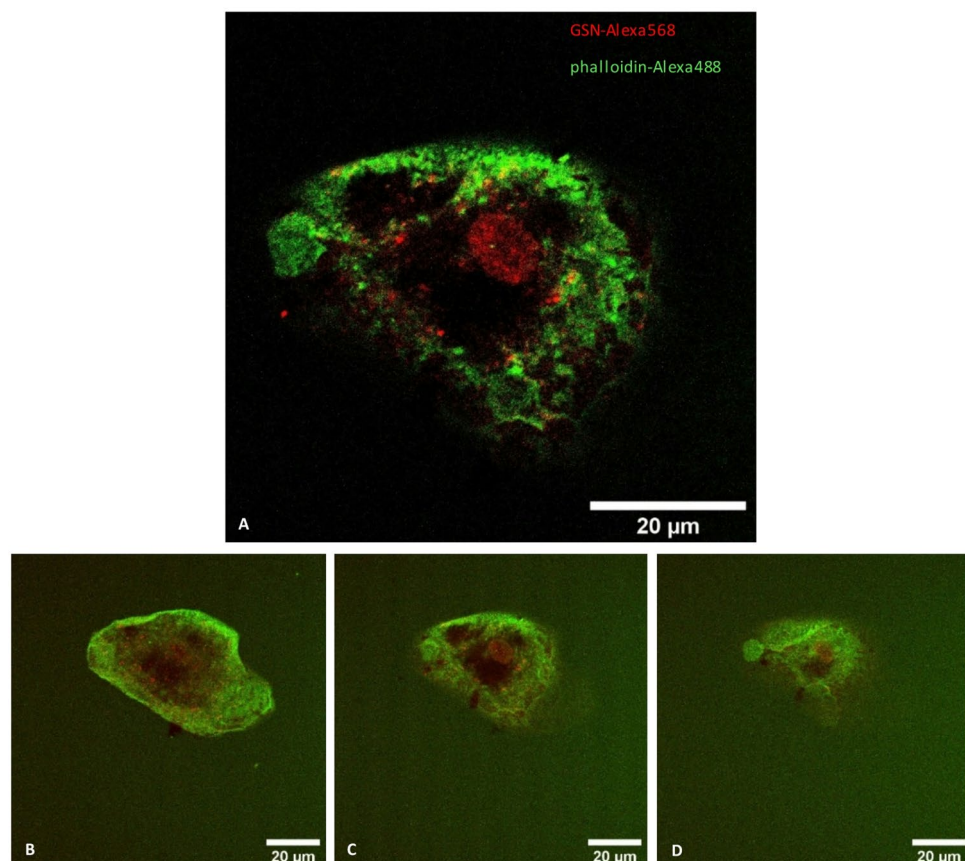
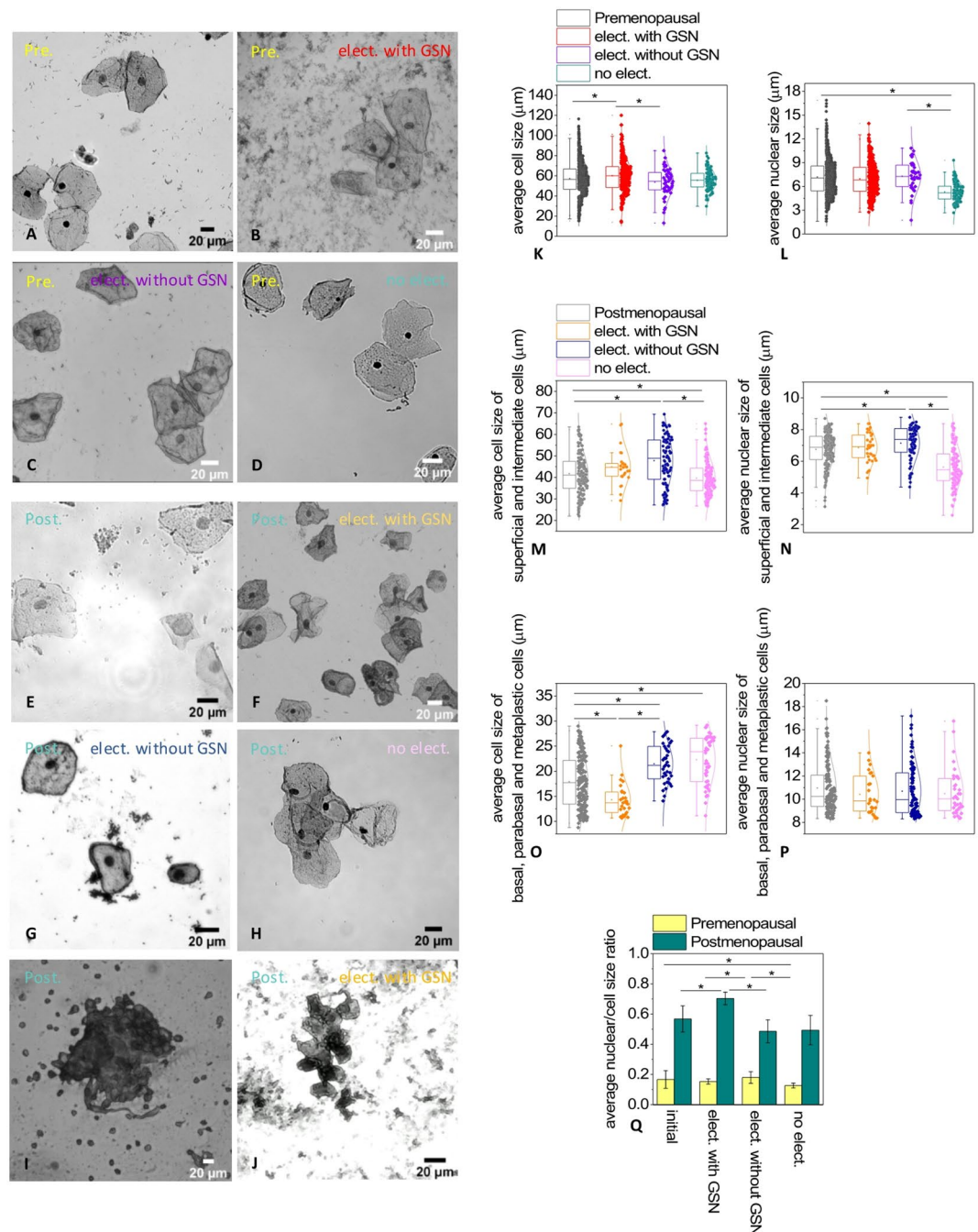


Fig. 1. Confocal fluorescence imaging of ectocervical cells. (A) Cells were electroporated with Alexa568-labeled GSN (red) and subsequently fixed; actin filaments were visualized with phalloidin-Alexa488 (green). (B–D) Z-stack images illustrate clear cytoplasmic volumes in farthest (B), middle (C), and nearest (D) planes.



$5.3 \pm 1.3 \mu\text{m}$ (Fig. 2A,D and L). Interestingly, the simultaneous effects of electroporation and incubation appeared to counteract this reduction, maintaining nuclear size, $7.29 \pm 1.9 \mu\text{m}$, closer to initial values $7.07 \pm 2.4 \mu\text{m}$.

Effects on cell and nuclear size in postmenopausal samples

In postmenopausal samples, the response to GSN was stratified based on cell differentiation. Superficial and intermediate cells demonstrated a minor increase in average cell size after electroporation with GSN, from $41.73 \pm 9.5 \mu\text{m}$ to $44.87 \pm 8.1 \mu\text{m}$ (Fig. 2E,F and M), with both values 25% and 27% lower than those of premenopausal samples, respectively. However, electroporation without GSN caused a more substantial increase in cell size to $47.82 \pm 11.2 \mu\text{m}$ (Fig. 2E, G and M), which is 11% less than in the case of premenopausal samples. Conversely, incubation alone led to a significant decrease in cell size, reducing it to $39.66 \pm 7.9 \mu\text{m}$ (Fig. 2E,H and M), which is 29% lower than in the case of premenopausal samples. The simultaneous effect of electroporation and incubation led to a significantly increased average cell size (by 21%) compared to the impact of incubation only; it was $47.82 \pm 11.2 \mu\text{m}$ or $39.66 \pm 7.9 \mu\text{m}$ (Fig. 2G,H and M) after the electroporation and incubation or after the incubation alone. Nuclear size in these cells exhibited slight variability; electroporation with GSN increased nuclear size marginally from $6.75 \pm 1.1 \mu\text{m}$ to $6.86 \pm 0.9 \mu\text{m}$ (Fig. 2E,F and N), with both values show minor difference than those of premenopausal samples. Electroporation without GSN caused a more pronounced increase to $7.15 \pm 1.1 \mu\text{m}$ (Fig. 2E,G and N). While incubation alone decreased nuclear size

◀ **Fig. 2.** Microscopy imaging and analysis of Pap-Stained ectocervical squamous cells. (A) Intermediate and superficial cells from premenopausal samples show slight size differences prior to and (B) after their electroporation with GSN. (C) Cells after the electroporation without GSN and a subsequent incubation caused a rougher cytoplasm than (D) only after the incubation. (E) Microscopy image shows intermediate and superficial cells from postmenopausal samples prior to and (F) after their electroporation with GSN. (G) Cells after the electroporation without GSN and a subsequent incubation show minor differences from (H) cells after the incubation only. (I) Basal cells from postmenopausal samples (J) show small vacuoles after the electroporation with GSN. (K–P) Box normal diagrams represent data with $\pm$ SD (error bars), average value (points), and median values (lines). Asterisk means statistically significant differences between groups (ANOVA, Bonferroni). Significances were defined as p values < 0.05 . (K) Average cell size of superficial and intermediate cells from premenopausal samples in the initial case (grey points, median = 56.47 μm , $n = 933$), in the case after their electroporation with GSN (red points, median = 59.99 μm , $n = 335$), in the case after electroporation without GSN (purple points, median = 54.61 μm , $n = 50$), in the case after incubation without electroporation (cyan points, median = 55.7 μm , $n = 94$). (L) Average nuclear size of superficial and intermediate cells from premenopausal samples in the initial case (grey points, median = 6.98 μm , $n = 921$), in the case after their electroporation with GSN (red points, median = 6.68 μm , $n = 354$), in the case after electroporation without GSN (purple points, median = 7.26 μm , $n = 38$), in the case after incubation without electroporation (cyan points, median = 5.2 μm , $n = 113$). (M) Average cell size of superficial and intermediate cells from postmenopausal samples in the initial case (light grey points, median = 41.07 μm , $n = 165$), in the case after their electroporation with GSN (orange points, median = 44.7 μm , $n = 26$), in the case after electroporation without GSN (dark blue points, median = 48.89 μm , $n = 96$), in the case after incubation without electroporation (pink points, median = 38.6 μm , $n = 144$). (N) Average nuclear size of superficial and intermediate cells from postmenopausal samples in the initial case (light grey points, median = 6.88 μm , $n = 190$), in the case after their electroporation with GSN (orange points, median = 6.92 μm , $n = 36$), in the case after electroporation without GSN (dark blue points, median = 7.38 μm , $n = 70$), in the case after incubation without electroporation (pink points, median = 5.46 μm , $n = 118$). (O) Average cell size of basal, parabasal, metaplastic cells from postmenopausal samples in the initial case (light grey points, median = 17.72 μm , $n = 176$), in the case after their electroporation with GSN (orange points, median = 13.7 μm , $n = 27$), in the case after electroporation without GSN (dark blue points, median = 21.16 μm , $n = 38$), in the case after incubation without electroporation (pink points, median = 23.9 μm , $n = 48$). (P) Average nuclear size of basal, parabasal, metaplastic cells from postmenopausal samples in the initial case (light grey points, median = 10.24 μm , $n = 148$), in the case after their electroporation with GSN (orange points, median = 9.85 μm , $n = 25$), in the case after electroporation without GSN (dark blue points, median = 9.96 μm , $n = 91$), in the case after incubation without electroporation (pink points, median = 10.03 μm , $n = 39$). (Q) Columns show the average nuclear/cell size ratio (yellow columns, premenopausal)(cyan columns, postmenopausal); the analysis was based on the data from panel K–P, representing average data with $\pm$ SD (error bars), and asterisk means statistically significant differences between groups (ANOVA, Bonferroni). Significances were defined as p values < 0.05 .

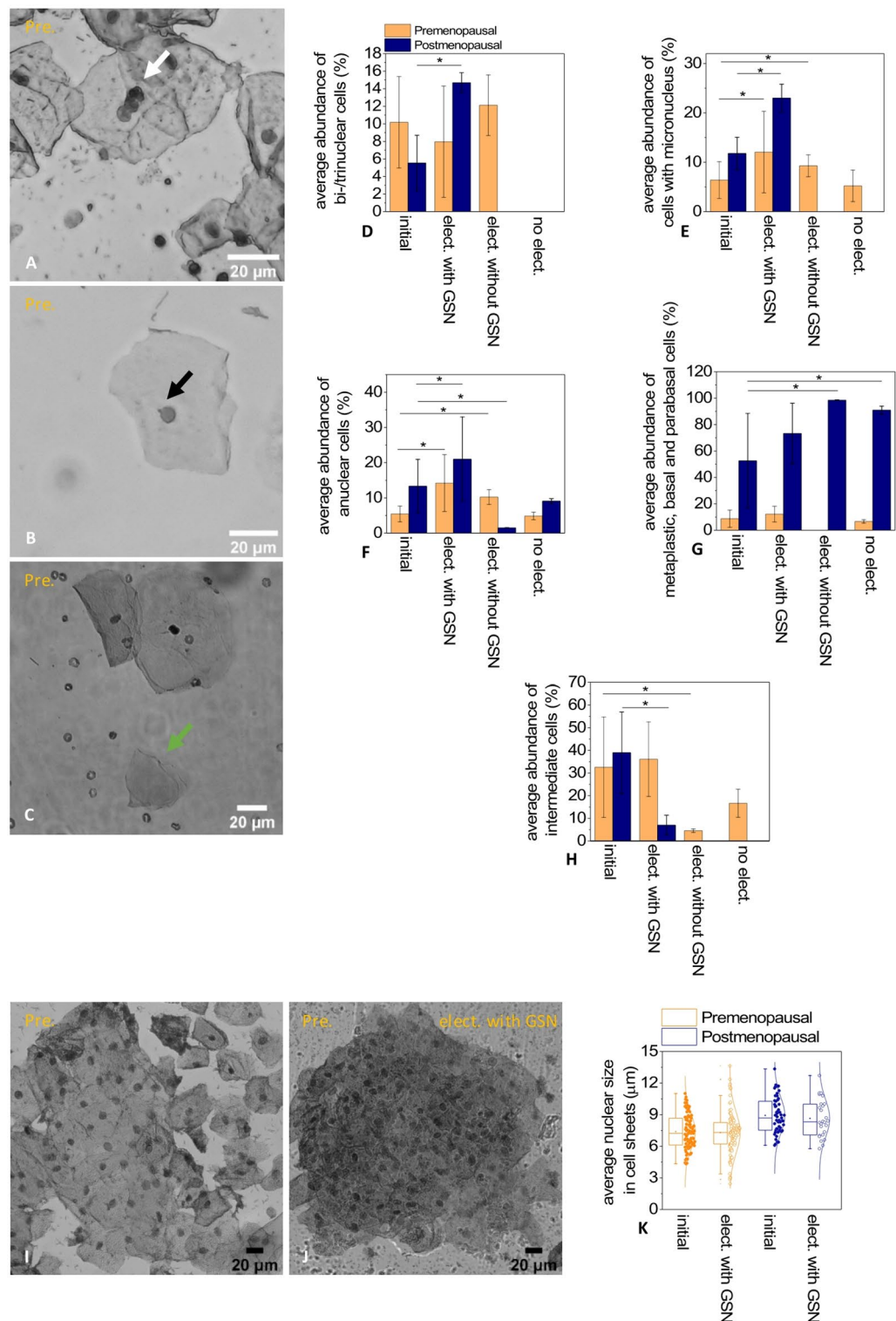
significantly to $5.63 \pm 1.3 \mu\text{m}$ (Fig. 2E,H and N). The effect of electroporation and then the incubation were initialized a significantly increased (27%) nuclear size compared to the impact of incubation only, $7.15 \pm 1.1 \mu\text{m}$ and $5.63 \pm 1.3 \mu\text{m}$ (Fig. 2G,H and N). The nuclear size of the differentiated cells after incubation or electroporation and subsequent incubation showed the same values in pre- and postmenopausal samples. In contrast, basal, parabasal, and metaplastic cells from postmenopausal samples showed unique reactions. Electroporation with GSN caused a notable reduction in cell size, shrinking it from $17.9 \pm 5.2 \mu\text{m}$ to $14.29 \pm 3.2 \mu\text{m}$, with $\sim 2\%$ of cells forming small cytoplasmic vacuoles (Fig. 2I,J and O). Electroporation without GSN, however, significantly increased cell size to $21.47 \pm 3.8 \mu\text{m}$ (Fig. 2O). Similarly, incubation alone also led to an increase in cell size, averaging $22.28 \pm 4.8 \mu\text{m}$ (Fig. 2O). This highlights a contrasting effect of GSN, where its presence during electroporation counteracts the enlargement typically induced by incubation alone. In addition to the enormous changes in cell size, the nuclear size was almost identical in all cases: $10.96 \pm 2.2 \mu\text{m}$ after the electroporation with GSN, $10.42 \pm 1.7 \mu\text{m}$ after the electroporation without GSN, $10.68 \pm 2.2 \mu\text{m}$ after the incubation only; compared to the initial $10.48 \pm 2 \mu\text{m}$ (Fig. 2O).

Nuclear-to-cytoplasmic ratio (N/C)

The nuclear-to-cytoplasmic size ratio (N/C) was also analyzed as an indicator of cellular morphology. In premenopausal samples, incubation without electroporation significantly decreased the N/C ratio from 0.167 ± 0.06 (SEM ± 0.008) to 0.126 ± 0.02 (SEM ± 0.012). However, the combined effects of electroporation and incubation increased the N/C size ratio to 0.18 ± 0.04 , (SEM ± 0.015) partially reversing the effects of incubation alone. Electroporation with GSN produced a lower N/C size ratio (0.153 ± 0.02 ; SEM ± 0.01) compared to electroporation without GSN (0.18 ± 0.04 ; Fig. 2Q). In postmenopausal samples, electroporation with GSN significantly increased the N/C size ratio from 0.568 ± 0.09 (SEM ± 0.085) to 0.703 ± 0.04 (SEM ± 0.149). Electroporation without GSN or incubation alone did not yield significant differences in the N/C size ratio, which remained at 0.486 ± 0.08 (SEM ± 0.08) and 0.493 ± 0.09 (SEM ± 0.11), respectively (Fig. 2Q).

Interpretation of Morphological Irregularities

Further examination revealed nuclear irregularities, including tri-/binucleosis, micronuclei, and anuclear cells (Fig. 3A–C). Electroporation with GSN significantly increased the prevalence of bi-/trinuclear cells in postmenopausal samples, from $5.5 \pm 3.2\%$ to $14.7 \pm 1.1\%$. Interestingly, premenopausal samples exhibited a slight



decrease in bi-/trinuclear cell abundance, from $10.2 \pm 5.2\%$ to $8 \pm 6.3\%$ (Fig. 3D). The presence of micronuclei also increased markedly in both groups following electroporation with GSN, rising from $6.4 \pm 3.7\%$ to $12 \pm 8.3\%$ in premenopausal samples and from $11.8 \pm 3.3\%$ to $23 \pm 3\%$ in postmenopausal samples (Fig. 3E). Besides that, in the case of premenopausal samples the electroporation without GSN also increased the occurrence of micronucleus to $9.3 \pm 2.2\%$ (Fig. 3E). Additionally, the frequency of anuclear cells increased significantly, from $5.4 \pm 2.2\%$, reaching $14.2 \pm 8.1\%$ in premenopausal samples and $21 \pm 12\%$ in postmenopausal samples (Fig. 3F). In both pre- and postmenopausal samples, the electroporation without GSN influenced their abundance; in the premenopausal case, it increased it to $10.2 \pm 2\%$, and in the postmenopausal case, it reduced it to $1.5 \pm 0.02\%$ (Fig. 3F).

The impact of GSN on cellular differentiation was examined by evaluating the abundance of less differentiated cells, specifically metaplastic, basal, and parabasal cells, as a proportion of the total cell population.

◀ **Fig. 3.** Nuclear morphology differences of Pap stained ectocervical squamous cells after the electroporation with GSN. Appearance of (A) trinucleosis (white arrow), (B) micronucleus (black arrow), (C) anuclear cells (green arrow) in samples from premenopausal cases. (D–F) Columns show the average abundance of nuclear irregularities: (D) bi-/trinuclear cells, (E) micronucleus cells, and (F) anuclear cells. (G–H) Show the average abundance of less differentiated cells. Orange columns indicate cells from premenopausal, and dark blue from postmenopausal cases; (G) metaplastic, basal, and parabasal cells; (H) intermediate cells. The analysis was based on the data from microscopy images, representing average data with $\pm$ SD (error bars) and asterisk means statistically significant differences between groups (ANOVA, Bonferroni). Significances were defined as p values < 0.05 . (I) Microscopy image of cells from premenopausal samples formed cell sheets before (J) and after the electroporation with GSN. (K) Box normal diagrams represent nuclear size data of cell sheets from premenopausal cases before (orange filled points, median = 7.22 μ m, $n = 95$) and after the electroporation with GSN (orange opened points, median = 7.3 μ m, $n = 105$), cell sheets from postmenopausal samples before (blue filled points, median = 8.69 μ m, $n = 61$) and after the electroporation with GSN (blue opened points, median = 8.32 μ m, $n = 26$); with $\pm$ SD (error bars), average value (points), median values (lines). There is no statistically significant difference between groups (ANOVA, Bonferroni). Significances were defined as p values < 0.05 .

In postmenopausal samples, electroporation without GSN significantly increased the abundance of these less differentiated cells, rising from $52.56 \pm 35.8\%$ to $98.5 \pm 0.2\%$. The electroporation with GSN did not caused significant differences. Similarly, long-term incubation without electroporation also led to an increase in less differentiated cells, with their proportion reaching $90.9 \pm 3\%$ (Fig. 3G). These findings indicate that the low level of cytoplasmic GSN, postmenopausal cellular differentiation is markedly reduced, leading to an enrichment of less differentiated cells.

In contrast, premenopausal samples exhibited a different pattern. Electroporation without GSN caused a significant reduction in the number of intermediate cells, decreasing from 32.57 ± 22.2 to $4.55 \pm 0.8\%$. Interestingly, electroporation with GSN in postmenopausal samples also led to a notable decrease in the abundance of intermediate cells, reducing their proportion from $39 \pm 18\%$ to $7 \pm 4.4\%$ (Fig. 3H). This suggests that GSN may promote cellular differentiation or counteract the effects of electroporation-induced stress that otherwise inhibits differentiation.

Despite these variations in cell type proportions and other morphological irregularities observed in ectocervical cells, the presence of GSN during electroporation did not cause significant changes in nuclear size or shape when cells were organized in sheets. These cells maintained consistent nuclear dimensions and morphology across treatments. However, electroporation with GSN did induce hyperchromatic nuclei, suggesting increased chromatin condensation or nuclear activity, even in the absence of structural changes (Fig. 3I–K).

GSN affects inflammation and the ROCK pathway

In samples from premenopausal cases with severe vaginosis, cells displayed atypical morphology (Fig. 4A). These cells reacted to electroporation by forming a strict border of hyperchromatic nuclei, indicating heightened nuclear activity or chromatin condensation (Fig. 4B). However, when electroporation was performed with GSN, the samples showed a significant increase in anuclear cells (Fig. 4C,D red arrows). Approximately 10% of the cells also exhibited irregularly asymmetric nuclei (Fig. 4D, cyan arrow), with or without cytoplasmic clearance (Fig. 4D, magenta arrow). These findings highlight the extensive remodeling effects of GSN on nuclear and cytoplasmic structures in inflamed cells.

To identify the primary intracellular signaling pathway linked to GSN's effects on severe inflammation and cytoskeletal remodeling, we investigated the role of the Rho-ROCK pathway. Previous studies have suggested that this pathway can counteract the effects of excessive intracellular GSN by modulating cytoskeletal dynamics⁶⁰. Based on these insights, we introduced fasudil, a potent ROCK pathway inhibitor, to examine the simultaneous effects of GSN and ROCK inhibition in premenopausal samples (Fig. 4E,G).

Cells electroporated with both GSN and fasudil showed distinct morphological changes. The 30% of cells displayed cracked cytoplasm near the nuclei, suggesting a breakdown of cytoplasmic structure induced by the combined treatment (Fig. 4F, pink arrows). In less differentiated cells, the nuclei themselves became cracked or pitted, further indicating significant nuclear remodeling under these conditions (Fig. 4H, pink arrows). These observations underscore the complex interplay between GSN and the Rho-ROCK pathway in regulating cytoskeletal integrity and nuclear morphology during severe inflammatory responses.

Fluorescence emission of the Papanicolaou (Pap) stain indicates nuclear and cytoplasmic activity

Classical Pap-stained ectocervical cell smears from patients with mild inflammation (Fig. 5A–D) and low-grade squamous intraepithelial lesions (LSIL) (Fig. 5E,F) exhibited fluorescence emission under blue light excitation using a FITC filter (excitation at 467–498 nm, emission at 513–556 nm)⁴⁹. Typically, cells display a blue-red color distribution in bright-field light microscopy, which aids in distinguishing differentiated cells (red) from less differentiated cells (blue) (Fig. 5A,C,E). The fluorescence emission provided more detailed images, with green to yellow hues corresponding to the 520–570 nm wavelength range (Fig. 5B,D,F)⁶¹. In these images, differentiated cells emitted higher-intensity green light (Fig. 5B, red arrows), while less differentiated cells exhibited lower fluorescence intensity (Fig. 5B, blue arrows). In LSIL samples, keratin accumulation (Fig. 5F, yellow arrow) or other long-lasting cytoplasmic (Fig. 5B, yellow arrow) components produced a yellowish

cells from severe vaginosis

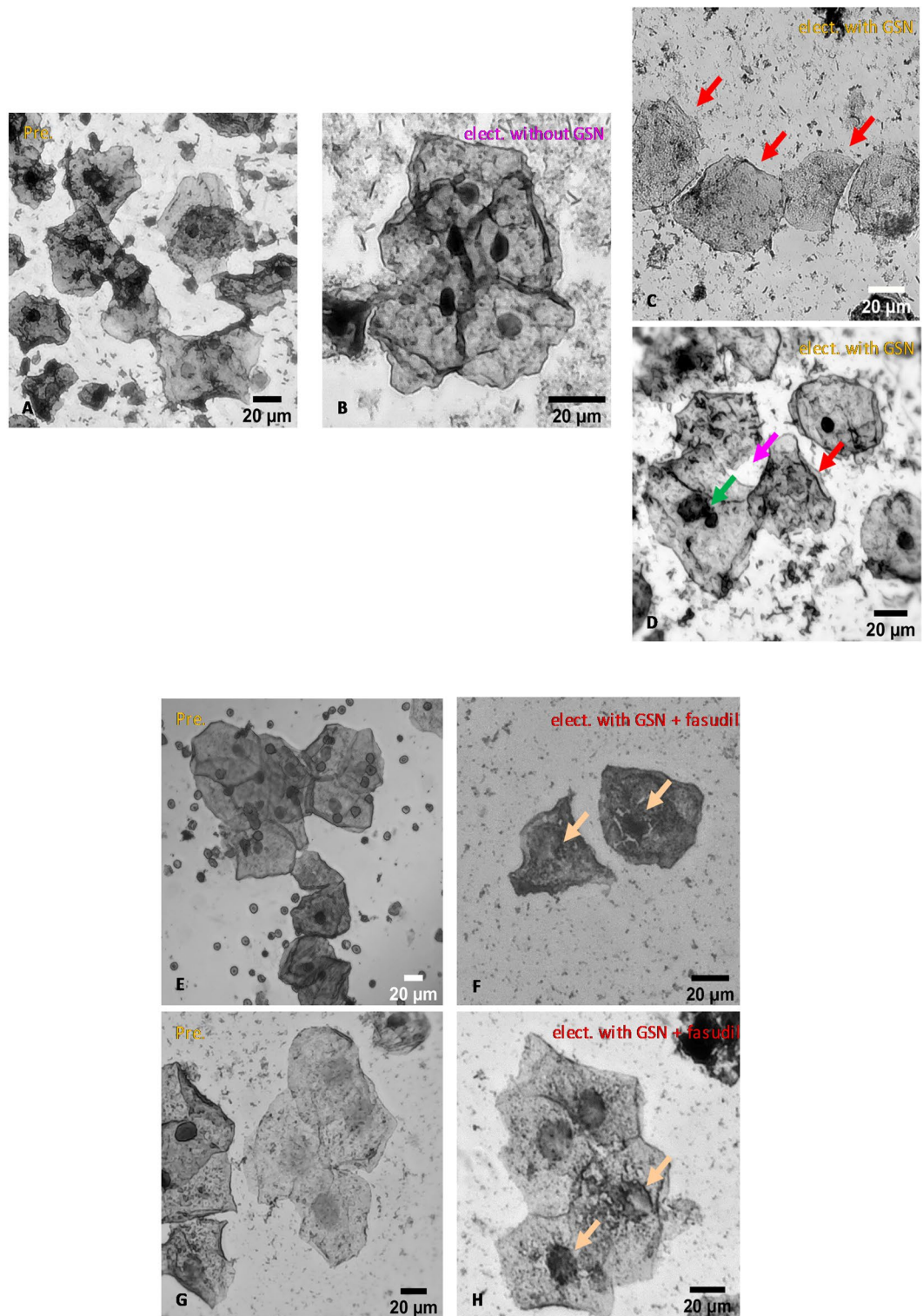


Fig. 4. Uncommon morphological responses of Pap-stained ectocervical squamous cells. Microscopy images of ectocervical squamous cells revealed various uncommon morphological responses under specific conditions, particularly in samples from premenopausal cases with severe vaginosis. (A) Atypical morphological signs observed in untreated cells from these samples. (B) After electroporation without GSN, the cells developed hyperchromatic nuclei with a distinct and well-defined border, indicative of increased chromatin condensation. (C) Significant number of anuclear cells appeared after the electroporation with GSN (red arrows), often accompanied by (D) cytoplasmic clearance (magenta arrow) and asymmetric irregular nuclei (cyan arrow). (E, G) Fasudil is a ROCK pathway inhibitor, in combination with GSN, was administered in cells due to electroporation, and then cells from premenopausal cases developed (F, H) cytoplasmic and nuclear cracks, while less differentiated cells displayed pitted nuclei (pink arrows).

fluorescence emission, consistent with prior observations of keratin's response with specific wavelengths⁶². Hyperchromatic or highly active nuclei displayed low fluorescence emission, whereas nuclei with low activity emitted a greenish fluorescence (Fig. 5A–F). Highly active or irregular hyperchromatic nuclei have low emission, and with low nuclear activity, greenish emission was detected (Fig. 5A–F). Furthermore, some less differentiated cells exhibited vacuolization, which corresponded with high-intensity green fluorescence in the cytoplasm (Fig. 5C,D, green arrows). Inflamed samples frequently showed reactive cells with reduced fluorescence emission and blurred borders in low-staining regions (Fig. 5C,D, yellow arrows). Conversely, in LSIL samples, cells exhibited strict and well-defined halos that demonstrated low fluorescence emission (Fig. 5E,F, white arrows).

To explore fluorescence further, we examined unstained cells incubated in a phenol-red Leibovitz-15 (Lb15) medium. These cells exhibited slight green fluorescence emission, suggesting the medium's phenol-red content interacted with cellular components (Fig. 5G,H). Using an unconventional Pap staining protocol that incorporated liquid-based sampling of ex vivo cells incubated in Lb15, we observed unique fluorescence characteristics. This protocol involved minor adjustments in washing and differentiation steps, leading to brownish-red cell appearances under bright-field microscopy (Fig. 5I,K,M,O,Q and S). Fluorescence imaging of these cells revealed yellowish-orange patterns near the membrane (Figs. 5J,L,N, orange arrows)(in $45 \pm 8\%$ of isolated superficial cells), a feature not observed with classical Pap staining (Fig. 5A,C,E). Some cells also showed yellow fluorescence from their nuclei (Fig. 5N, green arrows)(in $42 \pm 5\%$ of intermediate cells in sheets) and elevated green fluorescence due to the accumulation of cytoplasmic components in vacuoles (in $98 \pm 1\%$ of navicular cells) or perinuclear regions (in $22 \pm 9\%$ of isolated superficial cells)(Fig. 5N, yellow arrows). The cytoplasm of less differentiated, metaplastic (Fig. 5O,P), basal (Fig. 5Q,R), and parabasal cells (Fig. 5S,T) emitted tiny green light (intensities are 20–30% less than in the superficial cells), while their nuclei did not emit fluorescent light (Fig. 5O–T, purple arrows). Additionally, erythrocytes in these samples emitted green light as well (Fig. 5R,T, red arrows).

These observations suggest that the fluorescence emission of Pap-stained ectocervical cells offers detailed insights into nuclear and cytoplasmic activity, enabling the differentiation of cellular states and highlighting specific cytoplasmic and nuclear alterations in response to inflammatory or pathological conditions.

Hypothesized in vitro model for fluorescence emission after modified pap staining

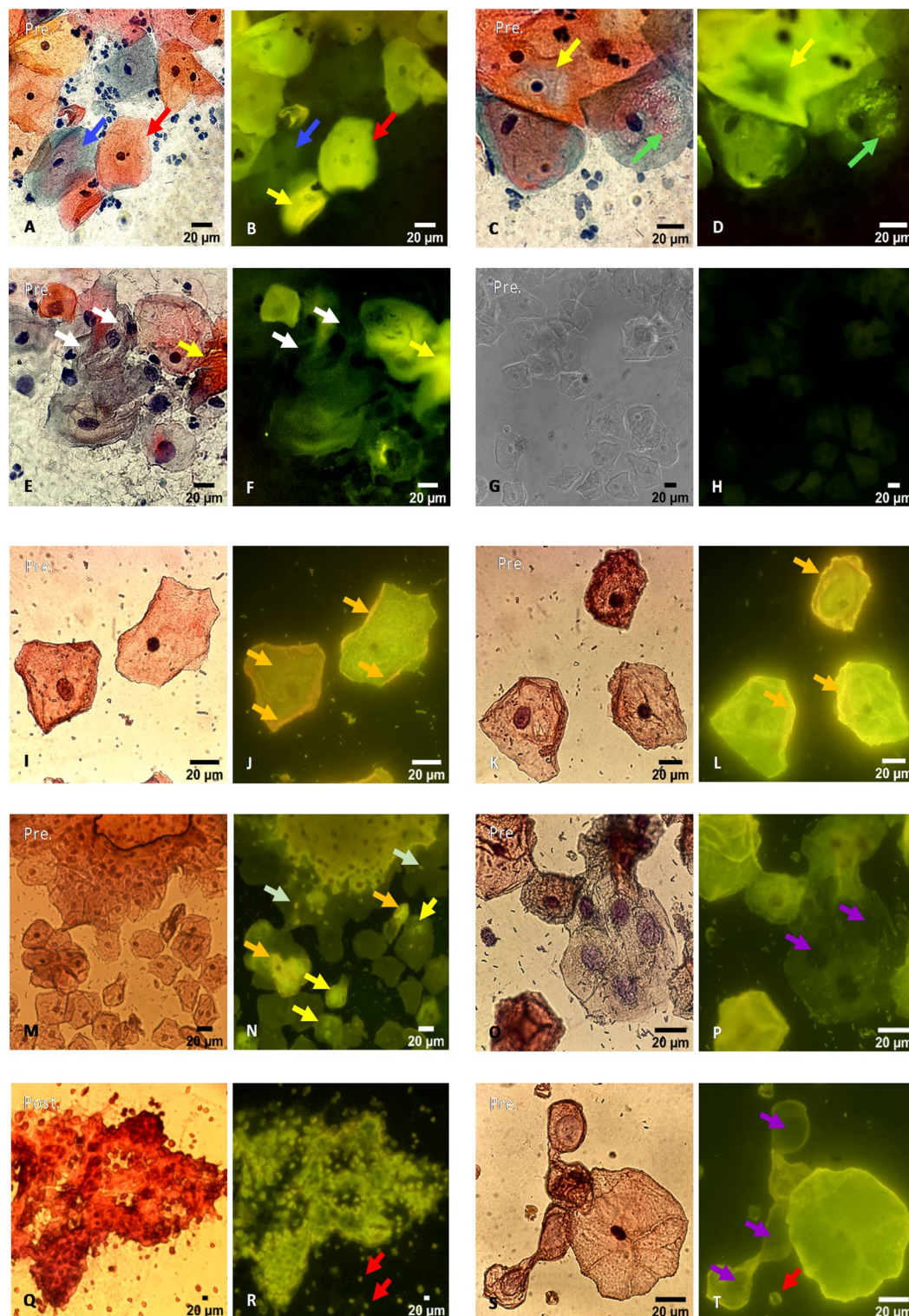
To explain the observed fluorescence emission of cells following the modified Pap staining protocol, we propose an in vitro model based on the chemical composition and interactions of the dyes used in the staining process. In this model we propose the chemical interactions between these dyes and cellular structures, explaining the distinct chromatic and fluorescent properties observed in Pap-stained cells. Specifically, the fluorescence observed in modified Pap-stained cells can be attributed to the unique dye-cell interactions, their affinity for nucleic acids, proteins, and cytoplasmic components, and the adjustment of staining conditions in the modified protocol. Routine cytological Pap staining relies on four dyes applied in three distinct components, each contributing to the specific color and fluorescence properties observed in the stained cells^{39,63}. The first staining component contains hematoxylin (He), which specifically binds to nucleic acids (DNA and RNA) in the cell nuclei. This interaction results in blue to black chromatic patterns that highlight nuclear structures and chromatin organization. Hematoxylin ensures that the nuclear details, including chromatin patterns^{38,63}, are well-defined and prominently visible under both bright-field appears with blue to black colors⁶¹ and fluorescence microscopy. Therefore, the chromatin patterns of the nucleus should be well-defined. The second component, consists of Orange G (OG6) dissolved in 95% ethanol and phosphotungstic acid. This dye binds to acidic proteins and glycogen, producing yellow staining in glycogen-rich areas and orange staining in keratinized regions. The interaction between Orange G and these cellular components is essential for distinguishing cells with keratin or glycogen accumulation, often associated with differentiated or pathological states^{39,62}. The third component, includes Eosin Y and Light Green SF (EA) dissolved in 95% ethanol with phosphotungstic acid and lithium carbonate. Eosin Y stains differentiated superficial cells, nucleoli, cilia, and red blood cells, producing red and pink colors, while Light Green SF imparts a blue-green hue to the cytoplasm of active cells such as endocervical, parabasal, and intermediate squamous cells. The presence of 95% ethanol in the solution prevents overstaining, ensuring optimal color differentiation. Additionally, phosphotungstic acid adjusts the pH of the staining solutions, enhancing the specificity and stability of the color distribution across different cellular components³⁹.

Novel observations in fluorescence emission: The role of modified Pap Staining and Phenol-Red interactions

The emergence of orange fluorescence emission in the modified Pap staining protocol, as shown in Fig. 5, represents a novel finding compared to classical Pap staining. This orange emission was observed when cells were excited with blue light (467–498 nm) using the FITC filter. To understand the origin of this emission, we explored the interactions between phenol-red in the Leibovitz-15 (Lb15) medium and the Pap stain components, as well as their excitation and emission spectra under physiological conditions.

Interaction of phenol-red and pap components

Under physiological conditions (buffer containing 2 mM TRIS, 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM ATP, pH 7.4), phenol-red from the Lb15 medium was found to interact with Pap stain components, altering their excitation and emission spectra (Fig. 6A–C). Among the 1 V/V % of Pap components tested, EA emerged as the leading emitter, with its fluorescence intensity approximately 300 times higher than that of Lb15, He, or OG6 (Fig. 6D). This makes EA a key contributor to the observed fluorescence emission patterns.



Fluorescence characteristics of Lb15 and Pap stain components

All Pap stain components and Lb15 were excitable at 455 nm blue light (Fig. 6A). The emission spectra revealed distinct characteristics for each component.

Lb15 Exhibited emission maxima at 520 nm and a Ca^{2+} -dependent peak at 550 nm (Fig. 6B, red and orange lines, respectively).

He Displayed an uninterpretable emission spectrum without a defined peak above 530 nm. In the presence of Ca^{2+} , He emission was reduced across the 500–600 nm range (Fig. 6B, purple lines).

OG6 When excited at 455 nm, OG6 exhibited a Ca^{2+} -dependent single peak at 540 nm (Fig. 6B, green lines). However, when excited at 520 nm (the emission maxima of Lb15), OG6 produced both green (538 nm) and orange (579 nm) emission peaks in a Ca^{2+} -dependent manner (Fig. 6B, blue lines). Notably, excitation of He at 520 nm did not result in any relevant fluorescence emission (data not shown).

◀ **Fig. 5.** Bright-field and fluorescent microscopy images of ectocervical cells, highlighting the differences between classical and modified Pap staining protocols. Fluorescence emission was excited using blue light FITC filter (excitation at 467–498 nm, emission at 513–556 nm), allowing for the visualization of nuclear and cytoplasmic activity in cells from various pathological conditions. (A–F) Classical Pap stained cells from (A–D) patients with mild inflammation and from (E, F) a patient with LSIL. (B, D, F) Fluorescent micrographs by blue light excitation. Blue arrows indicate less differentiated, and red arrows differentiated cells. Yellow arrows point to the dark areas modified by inflammatory conditions, and white arrows to the LSIL-relevant irregular halos. Green arrows show cytoplasmic accumulation in small vacuoles. (G) Bright-field transmission microscopy images of transparent ectocervical cells and (H) fluorescent images of them were incubated in an Lb15 medium. (I–T) Ectocervical cells were visualized using our modified Pap staining protocol, obtained by liquid-based sampling of ex vivo cells incubated in Lb15, which can accumulate phenol-red, with modified washing and differentiation steps during the preparation. The modification resulted in (I, K, M, O, Q, S) brownish-red appearances in bright field microscopy. (J, L, N, P, R, T) Their fluorescence emission offering more detailed images revealed a range of green to yellow colors, fitting into the 520–570 nm wavelength range. (J, L, N) Fluorescent images show a yellowish-orange pattern near the plasma membrane (orange arrows). A few cells showed yellow emission from the nuclei (green arrows), while others exhibited elevated green fluorescence from cytoplasmic vacuoles or perinuclear volumes (yellow arrows). The cytoplasm of less differentiated cells, (O, P) including metaplastic, (Q, R) basal, and parabasal cells emitted green light. However, their nuclei did not have emission (purple arrows). (R, T) Additionally, erythrocytes in these samples emitted green light, further demonstrating the versatility of this staining method (red arrows).

When 10 V/V % Lb15 was combined with 1 V/V % OG6 and He, simultaneous emission measurements at 467 nm (shortest wavelength of FITC filter transmission) revealed enhanced emission patterns (Fig. 6C). OG6 exhibited two prominent emission peaks at 538 nm and 579 nm, corresponding to green and yellowish-orange light, respectively⁶¹. We observed that the emission maxima of Lb increased by 20% due to adding He and then decreased by 32% due to OG6 addition (Fig. 6C, red line compared to blue lines). These peaks intensified in a Ca^{2+} -dependent manner. Lb15's emission maximum shifted from 518 nm to 522 nm with He addition and further to 526 nm with OG6 addition (Fig. 6C, grey arrows). Interestingly, the presence of Ca^{2+} reduced the emission intensity of Lb15 by 43% when He was added and by an additional 31% with OG6 addition (Fig. 6C, orange line compared to green and yellow lines). Similarly, He emitted local maxima at 558 nm and 535 nm when excited at 465 nm, but both peaks decreased by 10% in the presence of OG6 (Fig. 6C, purple and magenta lines).

EA

was identified as the primary fluorescent component of Pap stain. When excited by a FITC filter (excitation at 467–498 nm, emission at 513–556 nm)⁴⁹, 1 V/V % EA produced an emission peak at 539 nm (Fig. 6D). In the absence of Lb15, 0.1 V/V % EA showed reduced emission, with a 7% decrease due to He addition and an additional 6% decrease with OG6 addition with maxima at 539 nm (Fig. 6E). The integral of peaks between 560 and 600 nm (yellowish-orange emission) decreased by 7% with He and by 8% more with OG6 (Fig. 6E).

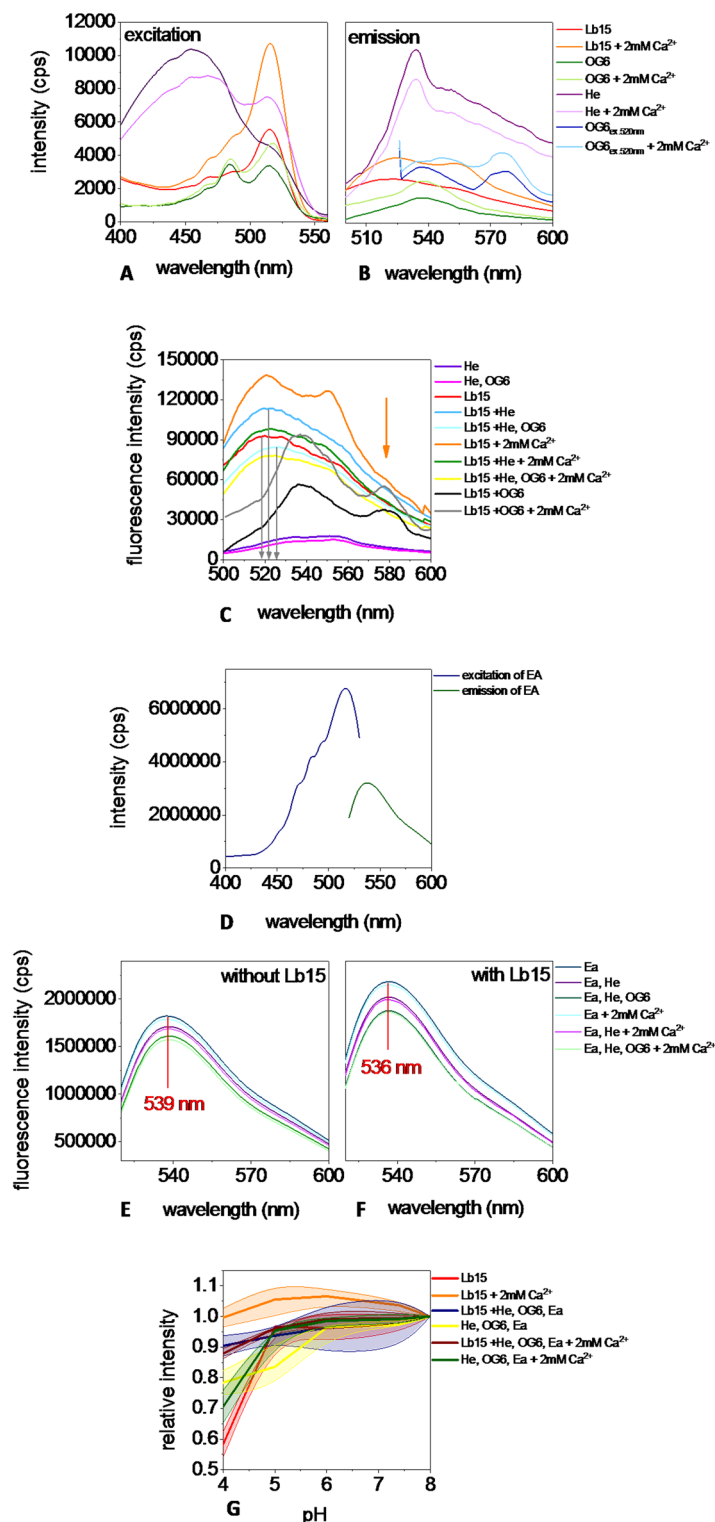
In the presence of 2 mM Ca^{2+} , all EA emission peaks decreased by 2–4%. However, when Lb15 was present, EA emission increased by 20%, with a blue shift of its maximum to 536 nm. Subsequent addition of He and OG6 reduced this enhanced intensity by 9% and 7%, respectively (Fig. 6F). The integral of peaks between 560 and 600 nm decreased by 13% due to He addition and by an additional 8% with OG6. Beside that, in the presence of 2 mM Ca^{2+} , all emission peaks of EA were decreased by 2% (Fig. 6E,F).

pH sensitivity of Pap stain components

The fluorescence intensity of Lb15 alone and Pap stain components in the presence of Ca^{2+} was sensitive to acidic conditions only at pH levels below 5.0, where intensities decreased by 40% and 30%, respectively (Fig. 6G). Below pH 6.0, the intensity of Pap stain components without Ca^{2+} decreased by 20%, but in most other cases, emission of Lb15 and Pap components remained stable between pH 4.0 and 8.0. Interestingly, the presence of Ca^{2+} increased Lb15 emission by 10%.

GSN increases cytoplasmic activity and generates intra-cytoskeletal strength

The fluorescence emission of superficial cells from premenopausal samples was analyzed using the modified Pap staining protocol, with excitation by blue light. When incubated overnight without electroporation, these cells exhibited a moderate and homogeneous green fluorescence emission (Fig. 7A,B). Notably, the Ca^{2+} -responsive yellow or orange emission patterns observed in earlier analyses (Fig. 5I–N, orange arrows) were absent under these conditions. When cells underwent electroporation with buffer followed by overnight incubation, their cytoplasm appeared smoother under bright-field microscopy, yet the fluorescence emission pattern remained consistent with that observed prior to treatment (Figs. 5I–T and 7C and D). This suggests that electroporation with buffer alone does not significantly alter the fluorescence or cytoplasmic activity. In both premenopausal and postmenopausal samples, electroporation with GSN followed by overnight incubation produced remarkable morphological and fluorescence changes (Fig. 7E–J). These cells developed strictly confined dark cytoplasmic regions (intensities are 30–80% lower than in other parts of the cytoplasm) (Fig. 7E–J, red arrows) and various yellowish-orange fluorescence emissions (Fig. 7E–J, orange arrows). Electroporation with fasudil elicited a distinct response in cells. Many granules with defined green fluorescence emissions appeared in the cytoplasm, as shown in Figs. 7K,L (blue arrows). In premenopausal samples subjected to electroporation with both GSN and fasudil, the cells exhibited significant structural changes. Bright-field microscopy revealed black granules



and remarkable cytoplasmic cracks, as described earlier (Fig. 4E–H). Despite these changes, the fluorescence emission was minimal, with tiny green light emitting from the cytoplasm (Fig. 7M,N, green arrows).

Discussion

To provide a comprehensive interpretation of clinically relevant disorders and actin content of squamous epithelial cells, we have created a hypothetical model (Fig. 8) summarizing the pathways that may be responsible for GSN-mediated changes in cytoplasmic and nucleoplasmic activity.

Our findings suggest that excessive GSN localizes near the nucleus following electroporation and may degrade the actin network through filament severing and capping¹⁵. GSN inactivation, mediated by reduced cytoplasmic Ca^{2+} levels and its binding to PIP2, may anchor GSN to membrane domains^{16,35}. In premenopausal samples, estrogen likely enhances PI3K-Akt and PI3K-PIP3 signaling pathways in superficial and intermediate

◀ **Fig. 6.** Fluorescence emission of Lb15 and Pap stain components He, OG6, and EA. Spectra represent the average of three independent measurements, under the buffer conditions of 2 mM TRIS, 100 mM KCl, 1 mM Mg_2Cl_2 , 0.2 mM ATP, pH7.4. (A) Excitation (emission side was at 560 nm) and (B) emission (excitation side was at 455 nm, excludes blue lines) spectra in identical 1 V/V % of Lb15, and OG6 or He in the absence (red, green, purple lines, respectively), or presence of 2 mM Ca^{2+} (orange, light green, magenta lines, respectively). OG6 was also excited with 520 nm light (blue lines). (C) The simultaneous emission spectra of 10 V/V % of Lb15 in the presence of various combinations of 1 V/V % OG6, He and 2 mM Ca^{2+} were excited at 465 nm (red, blue, light blue, orange, green, and yellow lines). The Ca^{2+} -dependent emission of Lb15 and OG6 (black and gray lines) show a defined peak at 580 nm (orange arrow). The emission maxima of Lb15 was at 518 nm and shifted to 522 nm by adding He, then to 526 nm by OG6 (grey arrows). The excitation of He at 465 nm was emitted with a maximum at 558 nm and another local maximum at 535 nm (purple line), and both peaks were decreased in the presence of OG6 (magenta line). (D) The emission of 1 V/V % EA excited at 465 nm (green line) was 300 times higher than the intensity of other components on panels A and B. The EA is a multicomponent dye that can be excited (blue line) with a wide range of 450–530 nm (the emission side was at 550 nm). (E) In the absence of Lb15, the emission of 0.1 V/V % EA was excited at 465 nm (blue line), modified by He addition (purple line), and then by OG6 addition (green line) with maxima at 539 nm. (F) In the absence of Lb15, EA emission had a maximum peak at 539 nm. Light blue, magenta, and light green lines indicate their spectra in the presence of 2 mM Ca^{2+} . (G) The fluorescence intensity of Lb15 alone (red line) or Pap stain components (He, OG6, EA) in the presence of Ca^{2+} (green line) was decreased under pH5.0. Pap stain components in the absence of Ca^{2+} were decreased slightly under pH6.0 (yellow line). Lb15 in the presence of 2 mM Ca^{2+} (orange line) and Lb15 with Pap stain components (dark blue line) in the absence or presence of Ca^{2+} (wine line) did not react to the change of pH between 8.0 to 4.0.

cells, increases cell diameters. In this scenario, PIP3 inhibits physiological level of GSN. However, the Ca^{2+} influx induced by electrical stress activates the excess GSN, leading to increased cell size and adhesion^{56,64}. Nuclear size decreased in resting cells during incubation, but electrical stress due to electroporation increased it. Nuclear size increases with increasing nuclear-cytoplasmic dynamics and nuclear activity such as transport, chromatin remodeling and stress responses, and a large nuclear size is an appropriate sign of less differentiated cells. In some cases, altered nuclear morphology could affect chromatin organization and physiology of diseased cells^{65,66}. Electroporation with GSN counteracted the electrical stress, reducing nuclear size. Additionally, GSN downregulates YAP, a protein that promotes cell proliferation^{31,67}. Electrical stress can activate the Rho signaling pathway, inducing YAP nuclear import⁶⁸ and supports intranuclear transport, slows cell differentiation, and causes increased nuclear size⁶⁵. In addition, the increase in cell diameter may be related to the flattening of the cells due to their increased adhesion to the surface, so that the flattening of the cell nuclei also increases their diameter. Resting cells in postmenopausal samples showed a decrease in both cell and nuclear sizes during incubation, likely due to the absence of estrogen-enhanced PI3K-Akt signaling. The moderate Ca^{2+} flux triggered by electrical stress activated GSN, leading to increased cell size and adhesion^{56,64}. However, when GSN levels exceeded free Ca^{2+} , GSN binding to PIP2 inhibited subsequent Ca^{2+} flux, suppressing IP3 signaling and reducing cell size^{69,70}. Less differentiated cells in postmenopausal samples, obtained primarily from atrophic tissue with low estrogen levels, exhibited greater size variability in response to treatments compared to superficial and intermediate cells. Their nuclear size, however, was less affected, likely due to higher YAP protein content^{71,72}. As we have previously observed, GSN cannot affect the nuclear size of in vitro HeLa cells³⁴, but it decreases their cell length within an hour after electroporation with GSN, followed by slow cell division, which can be interpreted by the severing and capping of short filaments. Here we aim to show that excess intracellular GSN increases cell size in premenopausal samples, and differentiated ex vivo cells and decreases cell size in postmenopausal samples, and less differentiated cells. Among other factors, HeLa cells in vitro can be interpreted as less differentiated cells of postmenopausal samples due to the absence of estrogen. However, the effect of the 24-hour incubation could be a stress for the less differentiated cells of postmenopausal samples, increasing their availability for surface binding, so that their cell size increases. For differentiated cells, in vitro incubation may have a calming effect by reducing their nuclear size. To interpret the nuclear irregularities observed in cells from pre- and postmenopausal cases, we propose that estrogen/GSN ratio can be a critical determinant of nuclear remodeling. The heightened severing and capping activity of GSN may disrupt normal mitotic regulation, as the insufficient formation of YAP-tubulin complexes⁷³ makes cells more susceptible to bi- or trinucleosis. Regardless of estrogen levels, stressed cells with increased YAP expression can experience compromised nuclear envelope integrity and chromatin stability^{74,75}. This, combined with elevated GSN activity, can result in micronuclei formation or anucleosis.

The lower number of intermediate cells in postmenopausal samples after electroporation with GSN is probably influenced by low estrogen levels. In addition, stress activation of the Rho-YAP signaling pathway in these samples promotes a less differentiated looking cellular morphology, while the relative frequency of differentiated cells decreases. Cells in sheets, which possess multiple intercellular junctions, did not respond to GSN electroporation.

In the case of prolonged inflammation, actin-binding proteins play a key role in cytoskeletal remodeling, may reduce nuclear activity, play a role in carcinogenesis, or disrupt intercellular barriers^{13,14,76}. In samples with severe inflammation, such as vaginosis, morphological abnormalities have been observed, such as severe cytoplasmic clearing and asymmetric nuclear irregularities. While electroporation without GSN partially mitigated these abnormalities, excessive intracellular GSN amplified their frequency. Interestingly, extracellular GSN, also known as plasma GSN (pGSN), acts as an immunomodulatory protein that binds lipopolysaccharides

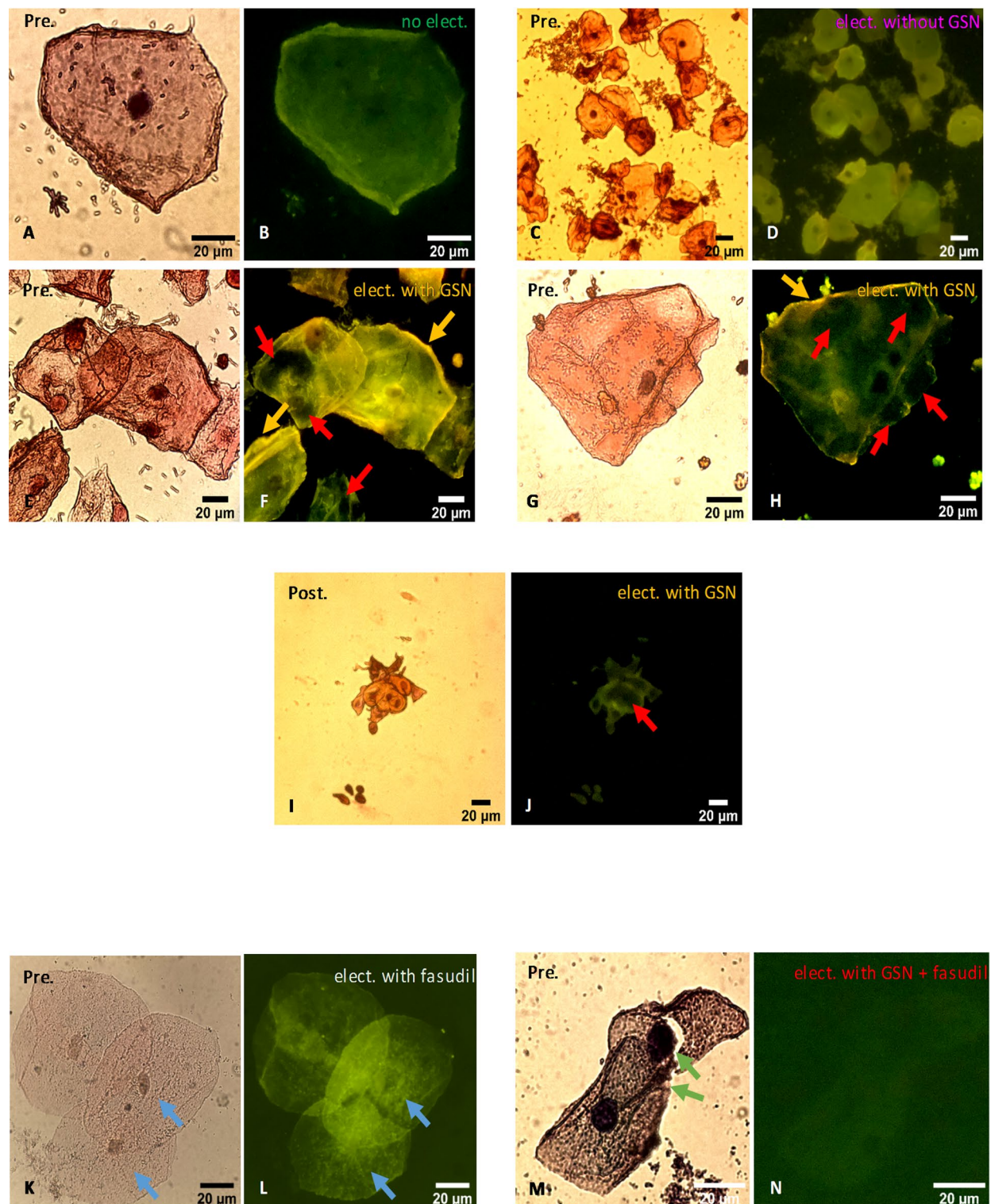


Fig. 7. Bright-field and fluorescent microscopy images of modified Pap-stained ectocervical cells due to GSN. The emission was excited with blue light (FITC filter). (A, B) Premenopausal samples incubated overnight without electroporation, (C, D) while electroporation with buffer followed by overnight incubation. (E–H) Cells from premenopausal cases, after the electroporation with GSN followed by incubation. Clear-bordered dark cytoplasmic regions are indicated with red arrows, and yellowish-orange fluorescent signals are indicated with orange arrows. (I, J) Cells from postmenopausal cases, after the electroporation with GSN followed by incubation. A red arrow indicates the clear, bordered dark cytoplasmic region. (K, L) Cells from the premenopausal sample responded to the electroporation with fasudil. Blue arrows indicate intensive green emitting vacuoles. (M, N) Cells from premenopausal samples reacted significantly due to electroporation with GSN and fasudil. (M, N) Cells from premenopausal samples showed remarkable cracks (green arrows) with minimal green cytoplasmic emission after the electroporation with GSN and fasudil.

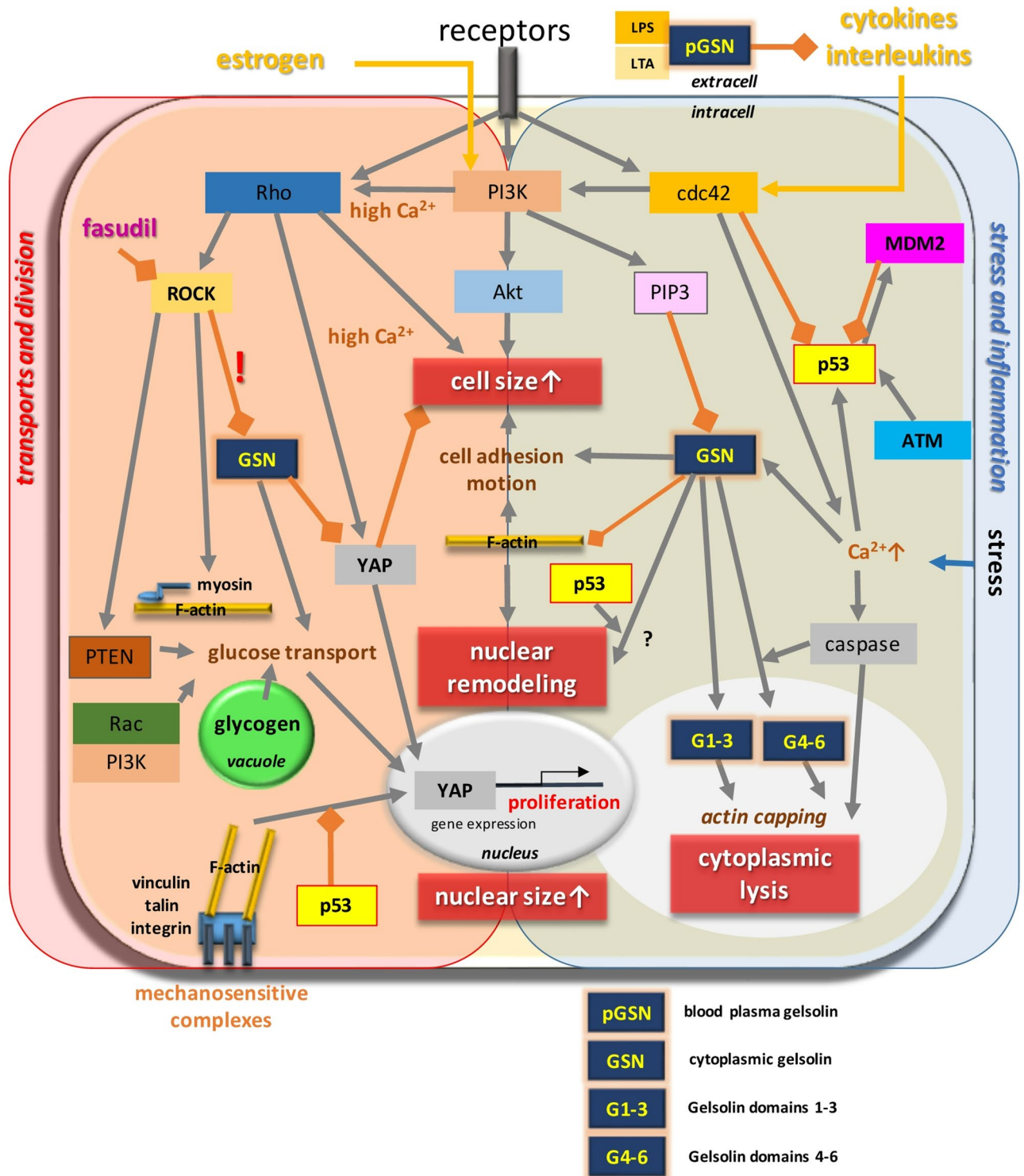


Fig. 8. A hypothetical model to explain GSN-modified cellular processes. Significant morphological changes are highlighted in red boxes. The symbol “?” denotes an unknown process, while “!” indicates potential indirect or direct inhibitory signals.

(LPS) and lipoteichoic acid (LTA)^{77,78}. Besides that, cytokines and interleukin activate the Cdc42 signaling pathway, leading to gradients of caspases and proteases in the cytoplasm, triggered by elevated intracellular Ca^{2+} ^{78,79}. GSN, cleaved by caspase-3 into G1-3 and G4-6 fragments, acts as a functional capping protein^{79,80}, which can inhibit p53⁸¹ or activate YAP⁸².

Under inflammatory conditions, PI3K activation inhibits GSN function through PIP3^{56,83}, while the activation of the ATM-MDM2 pathway increases p53 levels⁸⁴. Moreover, Cdc42 inhibits p53⁸⁵ reducing GSN-p53 complex

formation³⁴, thereby promoting nuclear remodeling. The concurrent high capping activity of GSN, along with proteolysis from caspase cascades, can lead to stringent cytoplasmic clearance.

Treatment with fasudil, which downregulates Rho-ROCK signaling, inhibits Rho-YAP signaling and halts YAP nuclear transport⁸⁶. However, fasudil and GSN together can generate high cytoplasmic activity and reinforce the actin network through the stiffness of shortened actin filaments, independent of MLCP-blocked myosin⁸⁷. The flattening effect may also enhance nuclear import of residual YAP and other proteins⁶⁸, leading to disruptions in lamin-linked nucleoplasmic and perinuclear cytoplasmic actin filaments^{75,88}.

Fluorescence microscopy of Pap-stained cells offered valuable insights into cellular and nuclear activities, as well as the accumulation of various cytoplasmic components. Regardless of pH conditions, significant green fluorescence emission was observed, attributable to Pap dye components. This fluorescence was inversely proportional to the average cytoplasmic enzyme activity, likely reflecting cytoplasmic accumulation of aging proteins stained with eosin⁴⁹. The cells with high fluorescence emission resulting from the emission of eosin due to the aging of proteins in the less active areas of the cytoplasm. Eosin is an acidic dye that binds electrostatically to the basic amino acid residues of proteins (Hys, Arg and Lys) at acidic and neutral pH⁸⁹. Therefore, the reduction of enzymatic activity during cell aging and differentiation causes proteins to undergo structural reorganization, exposing more binding sites for eosin and thus higher green emission. Differentiated cells subjected to stress accumulated keratin, emitting yellow fluorescence, while less differentiated cells with glycogen-filled vacuoles exhibited bright green emission. Both effects may result from OG6 binding to these components^{39,62}. In inflamed samples, differentiated cells displayed blurred halos with low green fluorescence. due to the eosin-binding gradients from the cytoplasm to the nucleus. The modified Pap staining protocol enhanced visualization of stressed cells by enriching ectocervical squamous cells with phenol-red from the Lb15 medium during incubation, resulting in low green fluorescence. A key distinction between classical and modified Pap staining was the orange fluorescence emission near membranes in differentiated cells and scattered orange emission in less differentiated cells. This phenomenon may indicate complex formation between Pap dye components and phenol red, driven by high Ca^{2+} levels. Spectroscopic data suggest that phenol red interacts with OG6 in a Ca^{2+} -dependent manner, shifting OG6 emission to the yellowish-orange range (580 nm) and inducing red shifts that point to complex formation. OG6 binding to proteins further shifts emission to the orange range (590–600 nm). The overall benefit of the modified Pap staining protocol in combination with fluorescence emission could be that all morphologic changes and differences in nuclear and cytoplasmic activity seen in both HPV positive and negative Pap stained samples can be examined. Fluorescence may have increased sensitivity and improved specificity, it may help the detection of cellular components that are difficult to visualize with conventional light microscopy, and it may be faster and more efficient than some other specialized staining techniques. The inhibition of the ROCK-YAP pathway by fasudil promotes glycogen production, impairs YAP degradation⁹⁰, blocks glucose transport⁹¹, and increases glycogen-filled vesicle numbers.

Cells from LSIL samples demonstrated the same low fluorescence emission in perinuclear strict halos as seen in the strict cytoplasmic volumes of stressed superficial cells with elevated cytoplasmic GSN levels. This observation supports the hypothesis that perinuclear GSN may play an important role in the formation of perinuclear halos and nuclear remodeling, which are mediated by Ca^{2+} levels³⁴ and can be altered by HPV infection^{37,92}. This increased GSN level likely contributes to the formation of highly active perinuclear halos, supported by inflammatory caspase machinery⁹².

Conclusion

This study highlighted the morphological changes induced by excessive cytoplasmic GSN levels in ectocervical epithelial cells from both pre- and postmenopausal cases, with a focus on inflammatory conditions and altered signaling pathways. The fluorescence emission of the components of the Pap stain can indicate the differentiation state of the cells and the different degree of accumulation of cytoplasmic components. The reduction of enzymatic activity and the low plasticity of the cytoskeleton during cell aging and differentiation leads to a structural loss of proteins, which exposes more binding sites for the green emitting eosin. Differentiated cells exposed to stress accumulated keratin and emitted yellow fluorescence, while less differentiated cells with glycogen-filled vacuoles showed bright green emission of OG6. Fluorescence emission from Pap-stained cells was demonstrated as a cost-effective method to assess overall cytoplasmic and nuclear activity. This technique provides valuable insights into cellular stress responses and allows for the differentiation between inflammation- and HPV-related halos and nuclear irregularities. We can hypothesise that perinuclear GSN may be involved in perinuclear halo formation and nuclear remodeling, which are mediated by Ca^{2+} levels and can be altered by HPV infection. Additionally, we predict a sensory role for the ROCK-YAP signaling pathway, which indirectly inhibits GSN expression and activity. GSN was shown to increase cell diameters, decrease nuclear size, and remodel the nucleus through its regulation of nuclear division. The balance of the cytoplasmic YAP/GSN ratio emerged as a critical factor in determining epithelial cell differentiation and stress adaptation. The contribution of the GSN/YAP ratio, which does not obviously change the volume of the nuclei, but may cause a flattening or a rounder shape of the nuclei. This ratio could serve as a potential diagnostic marker for predicting malignant transformations, offering new insights into cellular responses to stress and the progression of disease.

Methods

Protein

Recombinant human GSN was obtained using the pSy5 construct, provided by Prof. Robert C. Robinson's lab (RIIS, Okayama University, Japan; and VISTEC, Rayong, Thailand). The protein was expressed in *E. coli* BL21 Rosetta cells, induced with 1 mM IPTG, and purified using Ni-NTA affinity chromatography under imidazole buffer conditions, as described previously^{34,93}. The purified protein was dialyzed against a 6 mM EGTA-containing

buffer and stored in Ca^{2+} -free buffer A (0.2 mM ATP, 2 mM TRIS, pH 7.4). For fluorescence microscopy, GSN was labeled at target cysteine residues (Cys93, Cys188, Cys201, Cys304, and Cys645) with Alexa568–maleimide (Life Technologies/Thermo Fisher, Waltham, MA, USA), achieving labeling efficiency of 40–50%.

Ex vivo epithelial cells

Ectocervical squamous cells were obtained during routine cervical screenings following protocols approved by the Regional Research Ethical Committee of the Clinical Centre at the University of Pécs (7531-PTE2019). All methods were carried out in accordance with relevant guidelines and regulations. After the examination, the cytology brush was placed in 5 mL Leibovitz-15 (Lb15) medium (Sigma-Aldrich). Samples were incubated on ice for 16 h, allowing cells to sediment. Viable cells were harvested by centrifugation at $2000 \times g$ for 5 min. The pellet was split into two fractions, each resuspended in 5 mL of fresh Lb15 medium: one for low-voltage electroporation with GSN³⁴ and the other for immediate staining.

Pap staining protocols

The classical Pap staining was performed at the Department of Pathology using a routine protocol with a Leica Biosystems kit (Deer Park, IL, USA). Smears on slides were fixed in 96% ethanol for three 10-minute immersions, followed by rehydration in distilled water and 10 min in Harris hematoxylin. A quick differentiation step in HCl (4 s) was followed by tap water equilibration (10 min), isopropanol rinses (2×1 min), and sequential application of OG6 (4 min), isopropanol (2×1 min), and EA50 (3 min). The final steps included washes in isopropanol (2×1 min) and xylene (2.5 min) before slides were mounted with coverslips.

The modified Pap staining used the Pap Red stain kit (Abcam, Cambridge, UK) and began with liquid-based sampling of cells incubated in Lb15 medium. Cells were fixed with 96% ethanol (50 μL) and dried for 10 min at room temperature. After sequential rehydration in 70% ethanol and distilled water (3 times each), slides were treated with hematoxylin (10 min), tap water (2 min), and distilled water (3 times). Further steps included OG6 (4 min), EA (4 min), and extensive rinsing in 96% ethanol. Finally, slides were dehydrated in 96% ethanol (2×2 min), dried, and mounted with coverslips.

Phalloidin labeling

Viable cells were allowed to recover overnight after electroporation before fixation and labeling with Alexa488–phalloidin. Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min. After additional PBS washes, cells were stored in a permeabilization solution (5% BSA, 0.1% TritonX in PBS) for 30 min. For actin labeling, cells were incubated with Alexa488–phalloidin (0.3 μL in 200 μL PBS) for 1 h. After a final PBS wash, samples were mounted with coverslips. Notably, this protocol resulted in the loss of approximately 80% of cells from glass slides.

Patients

Patients provided informed consent for sample donation by signing the Hungarian Acceptance Claim, as approved by the Regional Research Ethical Committee of the Clinical Centre at the University of Pécs (7531-PTE2019). All methods were carried out in accordance with relevant guidelines and regulations. Cytological categories followed The Bethesda System (TBS) guidelines, including definitions for normal, inflammatory, severe bacterial vaginosis, and LSIL cases^{50,51}. Cytology samples were collected from 31 Hungarian women, with an average age of 48.2 ± 21.5 years. Patients were grouped into premenopausal (14 women) and postmenopausal (17 women) categories based on age and hormonal status. Analysis identified 7 mild inflammatory samples, 2 severe bacterial vaginosis cases, and additional specimens with macroscopic or cytological abnormalities: 7 atrophic, 1 with cervical ectropium, 2 hyperemic, and 1 with a combination of atrophy, ectropium, and mild inflammation. A single sample exhibited LSIL morphology (validated by the Department of Pathology, University of Pécs Medical School). Control samples included two premenopausal and two postmenopausal cases. Each control was divided: one part underwent electroporation with a buffer-only solution, while the other was incubated without electroporation. Electroporation required a 24-hour incubation period to recover, as intracellular changes may occur when ex vivo epithelial cells remain in vitro for over two days^{57–59}.

Low-voltage electroporation to increase the intracellular level of GSN

Our low-voltage electroporation system³⁴, was developed using an ArduinoUno® board. This system delivered 5 V, 0.46 mA current in 1 s ON/6 s OFF square-wave pulses, administering 92 μF at 5 V/cm for 10 min to transfect cells with JMY protein. Compared to standard electroporation protocols that deliver 50–270 mC of charge, this setup administered only 39 mC. Cells were suspended in Leibovitz-15 medium supplemented with 250 nM GSN and placed in sterile Falcon tubes. Tubes were modified by adding two holes in their lids, where stainless steel electrodes ($\varnothing$ 0.2 mm) were inserted and fixed with silicone glue. Electrodes, positioned 1 cm apart, were connected to the board's outputs, and output current was measured at the electrodes' ends. Electroporation was conducted in a sterile environment. The efficiency of this method is around 40% (Supp. 1).

Fluorescence spectroscopy

Fluorescence emission and excitation spectra were measured using a Fluorolog-3 spectrofluorometer (Horiba, Jobin Yvon, Palaiseau, France) with 3 nm slits. Measurements were performed at various wavelengths depending on the specific experimental setup.

Microscopy

Microscopy analyses were conducted post-fixation and labeling. Morphology and fluorescence emissions were examined using a Nikon Eclipse Ti2-E STORM microscope with a Nikon C2 detector and Hamamatsu Orca-

Flash 4.0 camera, with excitation provided by 488 nm and 561 nm lasers. Fluorescence emissions from Pap-stained samples were analyzed with an Olympus IX 71 inverted light microscope using 10x and 40x objective lenses and excitation through a FITC filter (excitation at 467–498 nm, emission at 513–556 nm). Images were collected with a coloured (Nikon DL24) camera whose settings were automatically changed to achieve the best exposure and resolution. The background intensity varies from sample to sample and depends on the bacterial activity, the amount of cell debris and other debris in the background of ex vivo sample.

Image analysis

Microscopy images were quantitatively analyzed using ImageJ software (developed by NIH, Bethesda, MA, USA). Cell and nuclear sizes were measured by determining the average diameter across five independent directions per cell and then calculating population averages from 20 to 50 cells per sample. The differences in size represent the two-dimensional differences in diameter and appear to be independent of changes in volume. We were unable to measure the diameter and cell size of epithelial cells that overlapped when collected in sheets in suspension and cells that were partially refolded.

Statistics

Statistical analyses were performed using Origin 2018 (OriginLab Corp., Northampton, MA, USA). Box diagrams illustrated GSN treatment effects on cell populations, with significance tested using ANOVA and Bonferroni correction. Differences were considered statistically significant at $p < 0.05$, as indicated in the figure legends.

Data availability

The manuscript contains all data generated within this study.

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Author contributions

Conceptualization, Krisz. K., B. F. and D. S.; Data curation, K. T. and D. S.; Formal analysis, Krisz. K. and D. S.; Funding acquisition, B. F. and D. S.; Investigation, B. F. and D. S.; Methodology, K. T., Z. S., N. B., O. K.; Project administration, L. C. and D. S.; Resources, B. F., Kálm. K., and D. S.; Software, K. T. and D. S.; Supervision, B. F., Kálm. K. and D. S.; Validation, Krisz. K. and N. B.; Visualization, O. K., K. T. and D. S.; Writing – original draft, Krisz. K., B. F. and D. S.; Writing – review & editing, Krisz. K., Kálm. K. and L. C.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The protocols were approved by the Regional Research Ethical Committee of the Clinical Centre at the University of Pécs (7531-PTE2019).

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-20822-z>.

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