

# Annexin A1 peptide and endothelial cell-conditioned medium modulate cervical tumorigenesis

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## Keywords

ANXA1; carcinogenesis; cervical cancer; inflammation; peptide treatment

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Cervical cancer is one of the leading causes of cancer death in women worldwide, and its tumorigenesis can be influenced by the microenvironment. The anti-inflammatory protein annexin A1 (ANXA1) has been reported to be associated with cancer progression and metastasis, suggesting that it plays a role in regulating tumour cell proliferation. Here, we examined the effect of the N-terminal peptide Ac2-26 of ANXA1 on the HaCaT cell line (normal) and HeLa cell line (cervical cancer) co-cultured with endothelium cell-conditioned medium (HMC). Treatment with Ac2-26 decreased proliferation and increased motility of cervical cancer cells, but did not affect cellular morphology or viability. Combined HMC stimulus and Ac2-26 treatment resulted in an increase in apoptotic HeLa cells, upregulated expression of *MMP2*, and downregulated expression of *COX2*, *EP3* and *EP4*. In conclusion, Ac2-26 treatment may modulate cellular and molecular mechanisms underlying cervical carcinogenesis.

Cervical cancer is one of the main gynaecological cancers worldwide, and has been associated with the human papillomavirus (HPV) and/or infection with bacteria such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [1,2]. Cervical neoplasm is associated with persistent HPV in more than 95% of cases, particularly in people with impaired immune system functions [2,3]. In overview, cervical cancer is the seventh most frequent type of tumour and fourth most common in women, disregarding non-melanoma skin cancer [2,4]. Diagnosis may be ascertained using the Papanicolaou test, which assists in the identification of persistent inflammation caused

by the HPV, and in cases of positive results, treatment may be initiated [5].

Besides infections, the cancer microenvironment also influences the tumour development process because it is very different from that of corresponding healthy tissue [6]. The microenvironment has been characterised by its unsettled extracellular matrix composition, increased microvessel density, and abundance of inflammatory cells and fibroblasts with an activated phenotype [6–8]. There is evidence that the cancer progression and development does not rely only on genetic characteristics, but also on the interaction between the cancer and stromal cells, including

## Abbreviations

Ac2-26, peptide of ANXA1; ANXA1, annexin A1 protein; cAMP, cyclic adenosine monophosphate; COX2, cyclooxygenase 2; EP3, prostaglandin E receptor 3; EP4, prostaglandin E receptor 4; HMC, conditioned medium of HUVEC cells; HMCS, non-treated conditioned medium of HUVEC cells; HMCT, treated conditioned medium of HUVEC cells; HPV, human papillomavirus; HUVEC, human umbilical vein endothelial cell; IL, interleukin; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; PGE2, prostaglandin E2; TIMP1, TIMP metalloproteinase inhibitor 1.

endothelial cells, immune cells, fibroblasts, adipocytes and inflammatory cells [6]. The different tumour phases that are associated with inflammation are considered an imminent threat, directly connected to the development process [9]. In the inflammatory scenario, Annexin A1 (ANXA1) has anti-inflammatory effects and therefore plays a key role in the modulation of the inflammatory response.

Annexin A1, which is also expressed by the tumour cells, acts as a modulator of the inflammatory process and has been linked to tumourigenesis [10]. Studies involving ANXA1 and cancer are controversial, but nevertheless indicate that this protein may be a target for new therapeutic interventions and used as a potential biomarker [11]. Its functions are specific to each type of cancer and there is evidence to indicate that its regulation and subcellular localisation are linked to the development, invasion, metastasis, progression and treatment resistance of tumours [12–15].

Structurally, the protein has a C-terminal core, representing 80% of its composition; this portion is common to all members of the annexin superfamily, and has four repeated homologous sequences and the 'type 2' domain for calcium linkage [11]. The variable N-terminal core is unique in length and sequence to each member of the family, and includes potential phosphorylation, glycosylation and peptidase action sites [16,17].

The biological activity of ANXA1 can be reproduced by the first 26 amino acids of the N-terminal core [18] or by some smaller peptides [19]. Since this was discovered, it has become common practice to use these molecules in experimental models of acute [20–23], chronic [24] and systemic inflammation [25], and also in *in vitro* studies using different cancer cell lines [26–29]. Recently, the use of the peptide was evaluated in skin allograft [30] and in inflammatory ocular disease [21,31].

There is evidence of a relationship between ANXA1 expression and cervical tumourigenesis. To ascertain the upregulation of the phosphorylated protein according to disease progression, samples from dysplasia and cervical cancer stages I, II, and III have been used [32]. Other work showed that ANXA1 was downregulated in all stages of the disease [33], and another study, analysing healthy, stage I, II and III, and invasive cancer samples, demonstrated that the protein expression levels corresponded to the disease progression [34].

ANXA1's contributions to tumourigenesis are still not well known, and considering its role in inflammation, it is an important area of research. The available data also point to controversies in the expression of this protein in cervical carcinogenesis, indicating a possible research field.

Considering the important role of ANXA1 in the inflammatory response and in tumours, we analysed the activity of the synthetic peptide of the ANXA1 protein in a cervical carcinoma cell line, along with the conditioned medium of endothelial cells, to help elucidate the processes that occur in the tumour microenvironment and expand understanding of ANXA1 as a therapeutic alternative. The rationale for this co-treatment is that paracrine factors in the conditioned medium of human umbilical vein endothelial cells (HUVECs) simulate the cancer microenvironment, which influences the tumour development process, and is very different from that of corresponding healthy tissue.

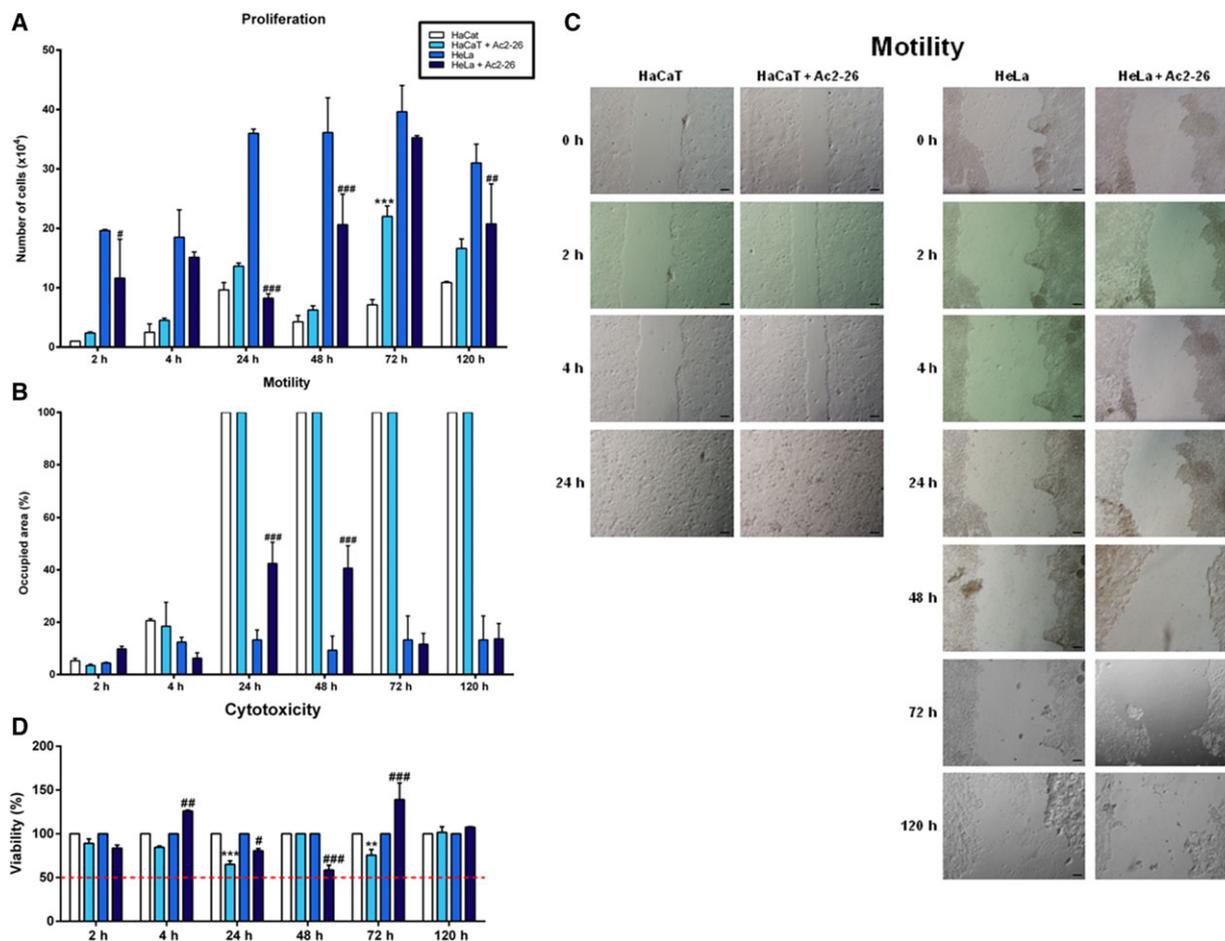
## Results

### Ac2-26 peptide response

Proliferation, motility and cytotoxicity of the human immortalised keratinocyte (HaCaT) cell line and the HeLa cell line (human cervical adenocarcinoma cells infected with HPV18) in response to Ac2-26 peptide treatment were studied. The HaCaT cell line showed an increase in proliferation after 72 h (Fig. 1A), and motility after 24 h, closing the experimental wound, and for this reason the cells detached from the well plate, after 24 h (Fig. 1B and C). In the HeLa cell line, proliferation was decreased after 2, 24, 48 and 120 h (Fig. 1A), while motility was increased after 24 and 48 h (Fig. 1B). Cytotoxicity was not observed in either cell line at any of the experimental times (Fig. 1D). Late apoptosis was decreased in both cell lines after the treatment (Fig. 2A). Gene expression showed an upregulation of all six genes analysed in the HaCaT cell line, and of prostaglandin E receptor 4 (*EP4*), matrix metalloproteinase 2 (*MMP2*) and matrix metalloproteinase 9 (*MMP9*) in the HeLa cell line (Fig. 2B).

### Conditioned medium of endothelial cells (HMC) and Ac2-26 peptide response

In the HaCaT cell line, secreted factors from endothelial cells (HUVECs) without Ac2-26 peptide treatment (HMCS) increased proliferation after 24 h (Fig. 3A). With the combination of secreted factors of endothelial cells and Ac2-26 treatment (HMCT), it was possible to observe an increase of the proliferation at 48 and 120 h, but a decrease at 72 h (Fig. 3B). Motility decreased after 24 h in the HaCaT cells (Fig. 3C,D) after induction with the conditioned medium without (HMCS) and with (HMCT) Ac2-26 peptide treatment.



**Fig. 1.** Response to Ac2-26 peptide treatment of HaCaT and HeLa cell lines in proliferation, motility and cytotoxicity assays. The cells were cultured in complete minimum essential medium (MEM) and treated with Ac2-26 ( $10 \mu\text{g}\cdot\text{mL}^{-1}$ ). (A) Proliferation; (B) motility; (C) images illustrating motility; (D) cytotoxicity.  $P < 0.05$  was considered significant; one symbol,  $P < 0.05$ ; two symbols,  $P < 0.01$ ; three symbols,  $P < 0.001$ : \* vs HaCaT, # vs HeLa; ANOVA followed by Bonferroni's test. Assays were performed with three independent experiments. Error bars indicate SD. Scale bars:  $500 \mu\text{m}$ .

Moreover, both conditions showed cytotoxicity to these cells only at 48 h (Fig. 3E,F).

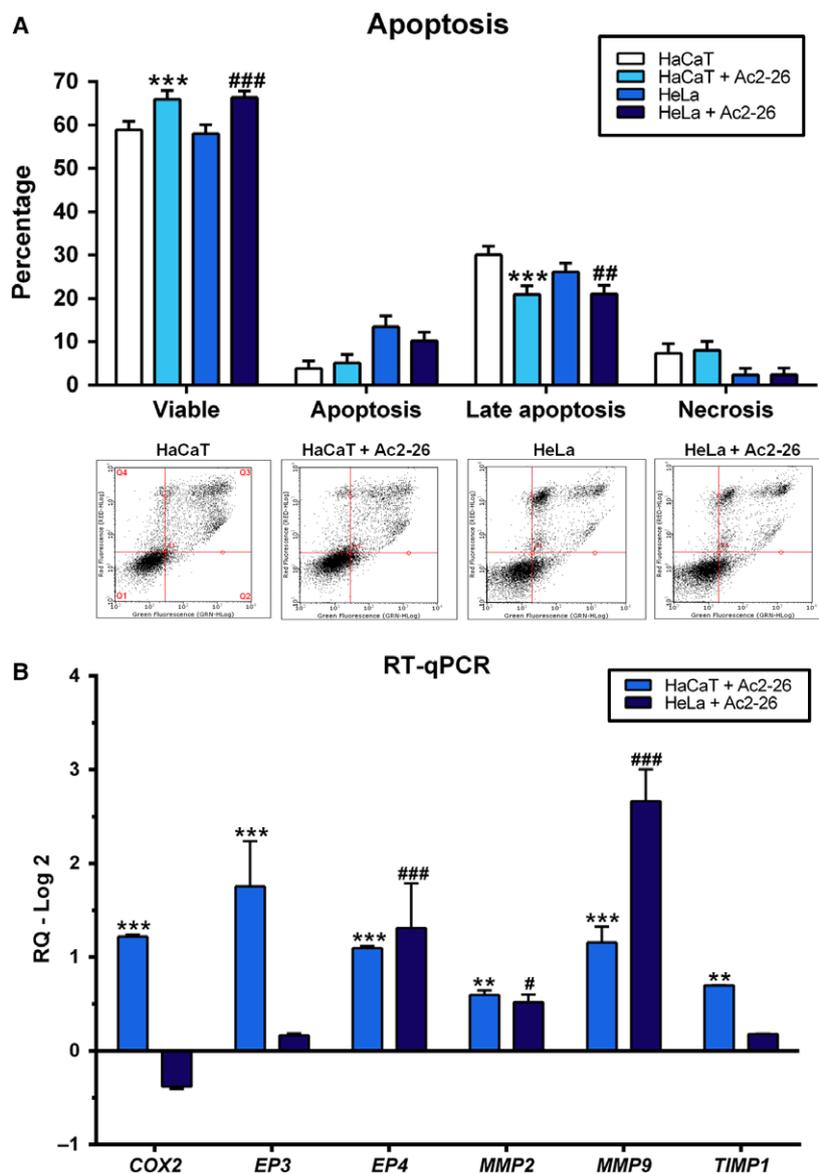
In the HeLa cell line, the secreted factors of endothelial cells without treatment (HMCS) led to a decrease in proliferation after 24 h (Fig. 4A), while with induction with HMC and the peptide treatment there was a decrease in proliferation at 72 h, but an increase at 24 and 48 h (Fig. 4B). In HeLa cells, motility had increased at 4 h after the induction with the conditioned medium without and with the treatment (HMCS and HMCT), but at 24 h there was a decrease after HMCT induction that only became statistically significant in 120 h (Fig. 4C,D). As with the HaCaT cell line, HeLa cells showed cytotoxicity only at 48 h (Fig. 4E,F).

Late apoptotic cells were increased in the HaCaT cell line (Fig. 5A,B) after induction with the conditioned medium without and with the treatment

(HMCS and HMCT), but decreased in the HeLa cells (Fig. 5C) after induction with the conditioned medium without the treatment (HMCS). In the HeLa cells after the induction with the conditioned medium with the treatment (HMCT), it was possible to observe an increase in apoptotic cells, but still showing more viable cells (Fig. 5C,D).

The secreted factors of endothelial cells without the peptide treatment (HMCS) upregulated prostaglandin E receptor 3 (*EP3*), *EP4* and *MMP2* gene expression in the HaCaT cell line, while downregulating cyclooxygenase 2 (*COX2*), *EP3* and *EP4* and upregulating *MMP2* gene expression in the HeLa cell line (Fig. 6A).

The peptide treatment upregulated *COX2*, *EP3*, *EP4* and *MMP9* gene expression in the HaCaT cell line and also upregulated *EP4* and *MMP9* gene expression in



**Fig. 2.** Response to Ac2-26 peptide treatment by HaCaT and HeLa cell lines in an apoptosis assay and gene expression. The cells were cultured in complete MEM and treated with Ac2-26 ( $10 \mu\text{g}\cdot\text{mL}^{-1}$ ). (A) Densitometry and DotBlot apoptosis;  $\gamma$  = cell percentage in 10 000 events. (B) Gene expression.  $P < 0.05$  was considered significant; one symbol,  $P < 0.05$ ; two symbols,  $P < 0.01$ ; three symbols,  $P < 0.001$ : \* vs HaCaT; # vs HeLa; ANOVA followed by Bonferroni's test. Assays were performed with three independent experiments. Error bars indicate SD.

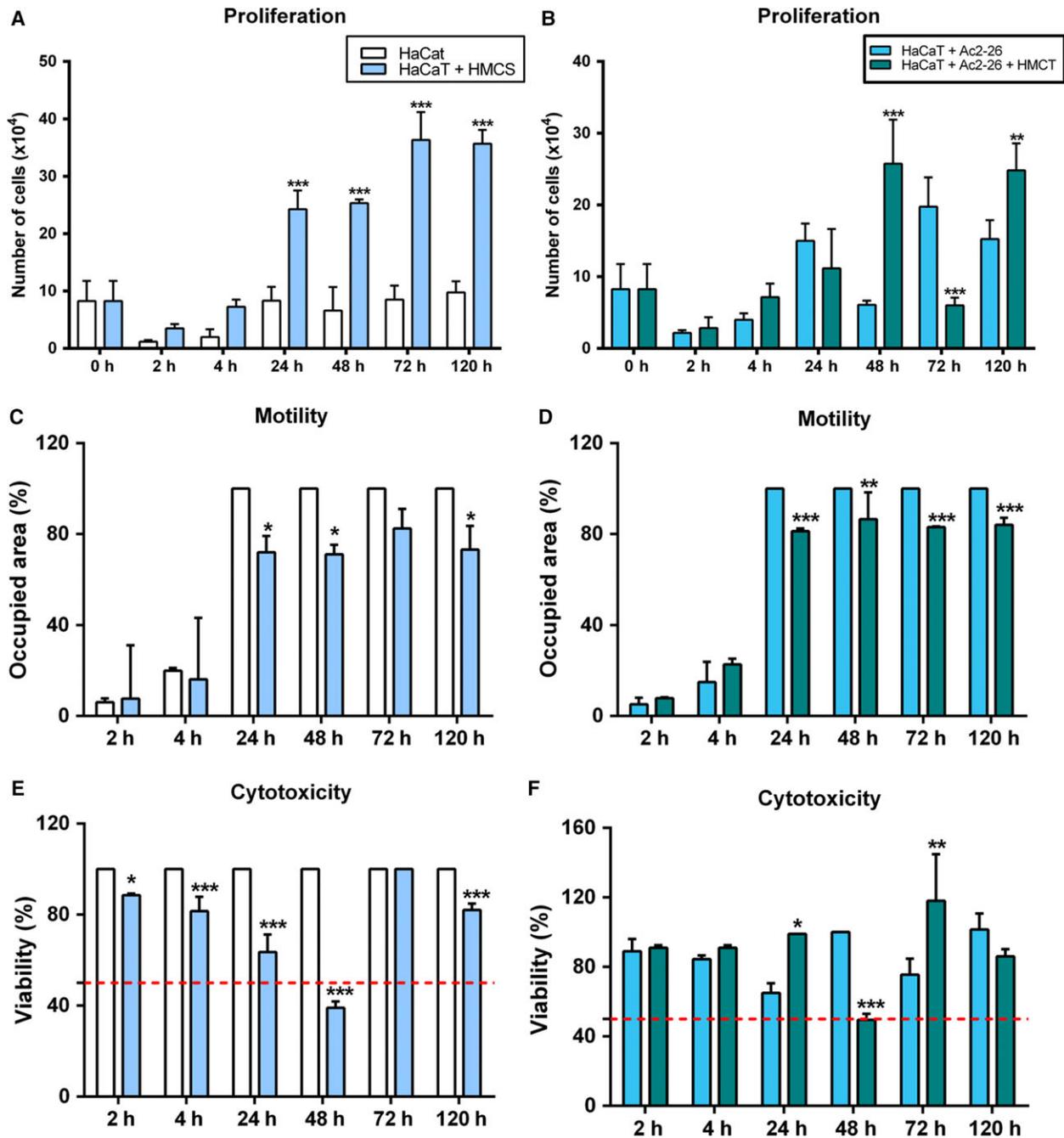
the HeLa cell line. The combination of the secreted factors from endothelial cells with Ac2-26 treatment upregulated *MMP2* gene expression in HaCaT cells, and downregulated *COX2*, *EP3* and *EP4* gene expression, but upregulated *MMP2* gene expression in HeLa cells (Fig. 6B).

## Discussion

Cervical cancer is related to HPV infection, and microbial infection is associated with 20–25% of the cancer cases worldwide [35]. It has been observed that the start of the carcinogenesis is more associated with persistent inflammation, as a result of infections or autoimmunity [36]. Besides the infections, the tumour

microenvironment also plays a role in the tumourigenesis, due to its complex structure, in which different factors may influence and modify the cancer [37–39], and our experiments demonstrate this heterogeneity.

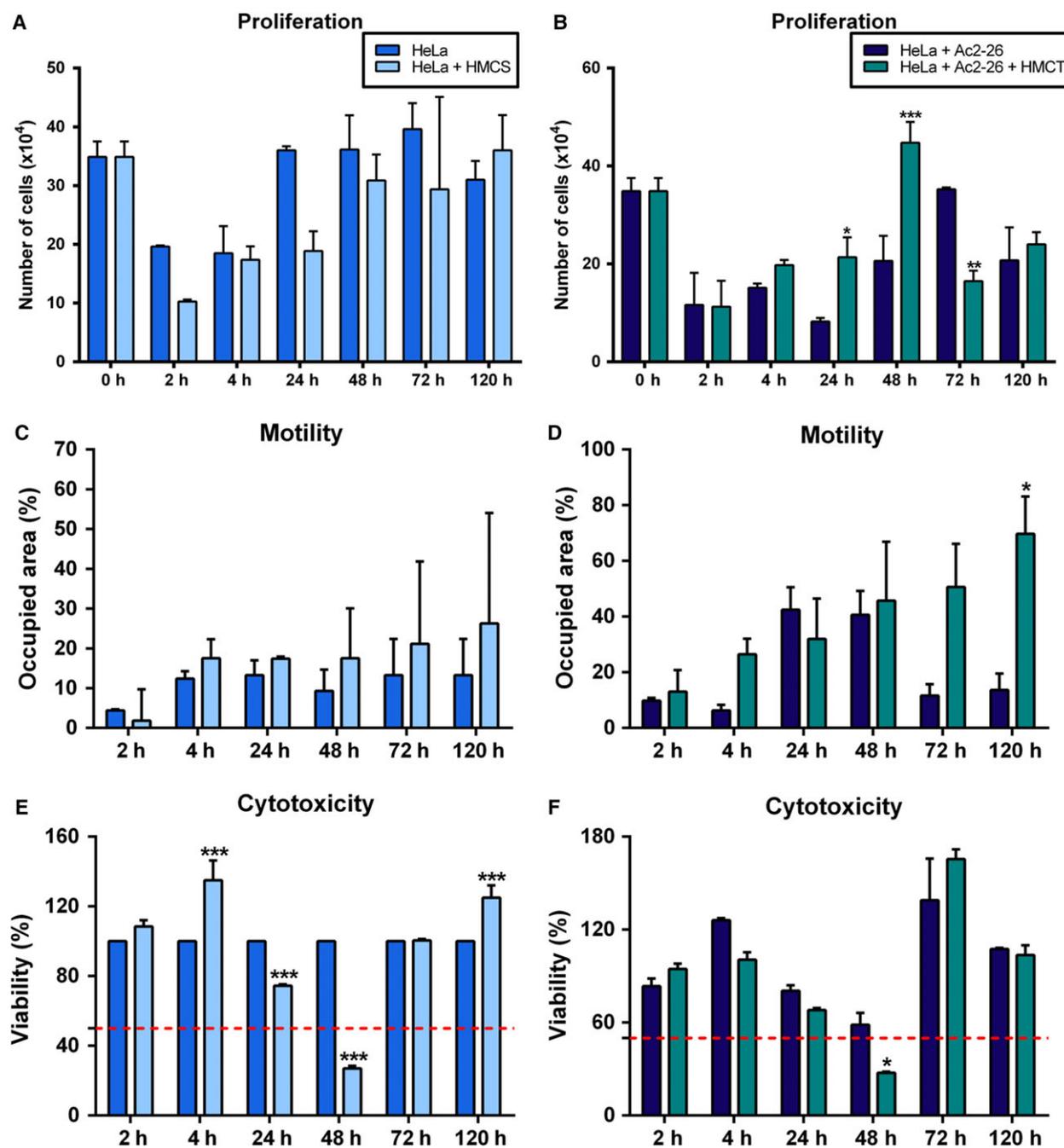
Our results demonstrate that cervical cancer cells exhibit a decrease in growth when treated with the Ac2-26 peptide, and are stimulated by the conditioned medium of endothelial cells. This is important for our understanding of the role of this protein in cancer. These conditions also result in an increase in motility after 120 h, and the maintenance of viability, the stimulation becoming cytotoxic only at 48 h. Regarding apoptosis and gene expression, an increase in apoptotic cells, the downregulation of *COX2*, *EP3* and *EP4*, and the upregulation of *MMP2* were observed.



**Fig. 3.** Response of the HaCaT cell line to conditioned medium induction and Ac2-26 peptide treatment. The cells were cultured in complete MEM and stimulated with conditioned HUVEC cell medium (HMC) (at a ratio of 1 : 1) that was untreated (HMCS; A,C,E) or treated (HMCT; B,D,F) with Ac2-26 (at 10  $\mu\text{g}\cdot\text{mL}^{-1}$ ). (A,B) HaCaT proliferation; (C,D) HaCaT motility; (E,F) HaCaT cytotoxicity.  $P < 0.05$  was considered significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ : (A,C,E) vs HaCaT; (B,D,F) vs HaCaT + Ac2-26; ANOVA followed by Bonferroni's test. Assays were performed with three independent experiments. Error bars indicate SD.

The pro- and anti-inflammatory effects of ANXA1, a protein known to be an endogenous glucocorticoid, depend on the type of ligand, and have been described previously [12]. ANXA1 protein was demonstrated to initiate the p38 mitogen-activated protein kinase

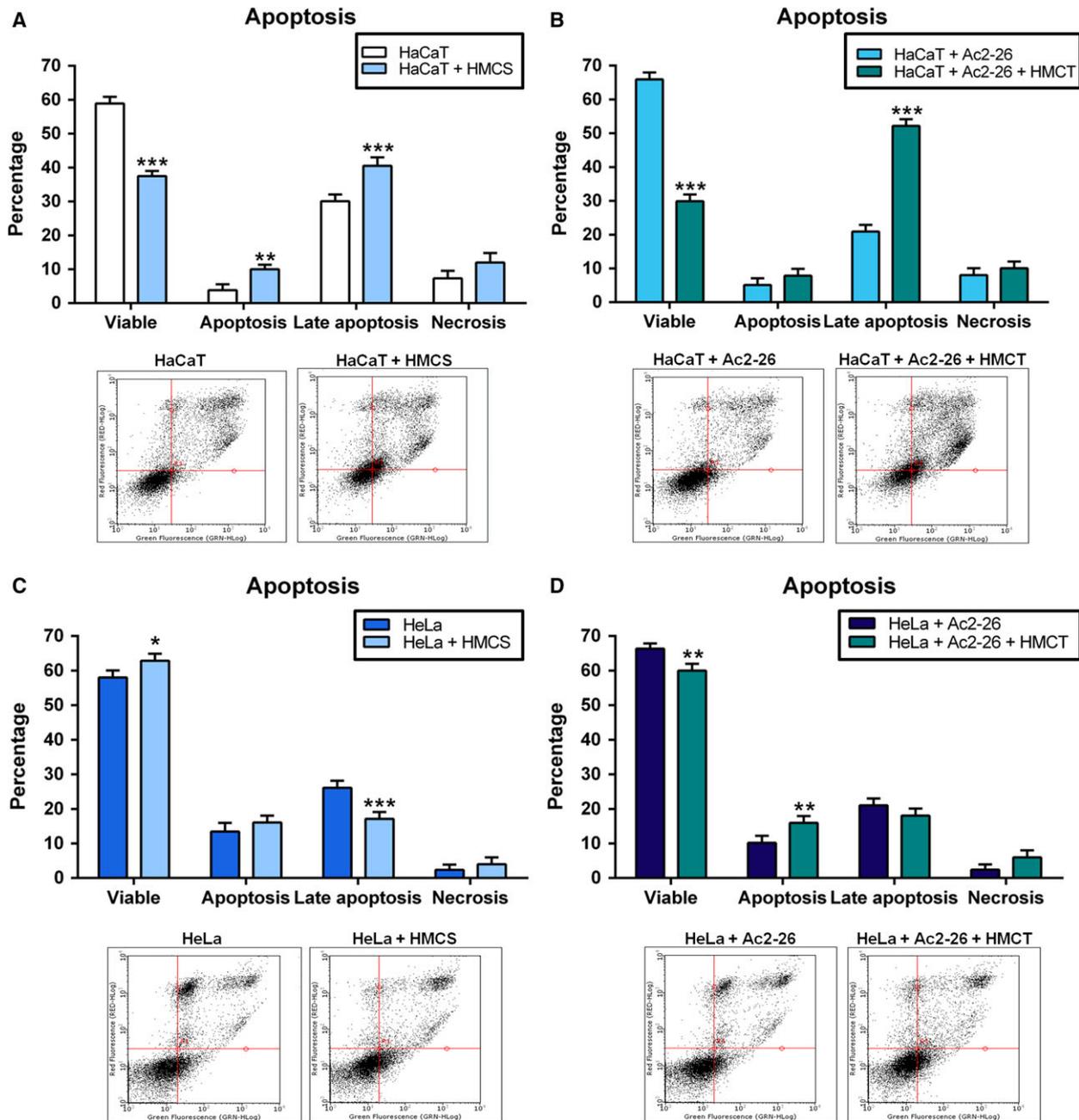
(MAPK) pathway and the Ac2-26 peptide promotes the initiation of the c-Jun N-terminal protein kinase (JNK) pathway [40]. The MAPK family pathway is one of the most important cascades responsible for reading an extracellular stimulus and unleashing a response in the



**Fig. 4.** Response of the HeLa cell line to conditioned medium induction and Ac2-26 peptide treatment. The cells were cultured in complete MEM and stimulated with conditioned HUVEC cell medium (HMC) (at a ratio of 1 : 1), untreated (HMCS; A,C,E) or treated (HMCT; B,D,F) with Ac2-26 (at  $10 \mu\text{g}\cdot\text{mL}^{-1}$ ). (A,B) HeLa proliferation; (C,D) HeLa Motility; (E,F) HeLa cytotoxicity.  $P < 0.05$  was considered significant;  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ : (A,C,E) vs HeLa (B,D,F) vs HeLa + Ac2-26; ANOVA followed by Bonferroni's test. Assays were performed with three independent experiments. Error bars indicate SD.

cells, and one of these pathways is the JNK pathway, a signalling cascade known to be activated by stress [41]. The JNK cascade may act by phosphorylating different molecules, among them p53 [42]. Through this action,

we could suggest that the proliferation decrease after the Ac2-26 treatment and HMC stimulation may be through activation of the MAPK family and targeting of p53, resulting in cell cycle arrest.



**Fig. 5.** Response of HaCaT and HeLa cell lines to conditioned medium induction and Ac2-26 peptide treatment. The cells were cultured in complete MEM and stimulated with conditioned HUVeC cell medium (HMC) (at a ratio of 1 : 1), untreated (HMCS; A–C) or treated (HMCT; B–D) with Ac2-26 (at  $10 \mu\text{g}\cdot\text{mL}^{-1}$ ). (A,B) HaCaT densitometry and DotBlot apoptosis; (C,D) HeLa densitometry and DotBlot apoptosis;  $y =$  cell percentage in 10 000 events.  $P < 0.05$  was considered significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ : (A–C) vs Control of each group; (B–D) vs Control + Ac2-26 of each group; ANOVA followed by Bonferroni's test. Assays were performed with three independent experiments. Error bars indicate SD.

The proliferation decrease found in this study is corroborated by refs [26,27], which also observed this phenomenon in cancer cell lines after treatment with ANXA1. One of the authors indicated that ANXA1 may act as a tumour suppressor, possibly acting on

the extracellular signal-regulated kinases 1 and 2 pathway, also a signalling cascade from the MAPK family [27]. This response may result in overexpression of this pathway, which modifies the expression of the molecules involved in the cell cycle, resulting in arrest in



observe an increase in apoptotic cells after the peptide treatment and stimulation with HMC.

There are studies with ANXA1 showing its pro- and anti-inflammatory responses and also associating its expression with metastasis [50]. During the invasion process, it is suggested that a cell population, called 'leader' cells, invade the adjacent matrix, opening space for following cells [51]. It is necessary that the 'leader' cells secrete factors in order to initiate the process, which will provide a path for the other cells; one of these factors is matrix metalloprotease [52]. Boudh-*raa et al.* [53] have observed that ANXA1 shows expression patterns related to specific tumour types, and they have associated the protein cleavage with melanoma cell aggressiveness. The same group showed that in melanoma cells, the administration of Ac2-26 peptide activates formyl peptide receptors and *MMP2* gene expression [54]. In our current work, the level of *MMP9* gene expression equalled the control, after HMC stimulation and peptide treatment, and the upregulation of *MMP2* was also observed in the healthy HaCaT cells; therefore this pattern could be due to the secreted paracrine factors of the endothelium, possible as an attempt to restore a healthy environment.

Pro-carcinogenic functions have been attributed to the *COX2* gene, supporting the strategy of gene suppression; some of the processes related to *COX2* are apoptosis inhibition, proliferation increase, and induction of angiogenesis [55–57]. The available literature shows that this gene may be triggered by different factors, for instance, bacterial lipopolysaccharide, interleukin (IL)-1 $\beta$ , IL-2, tumour necrosis factor  $\alpha$ , epidermal growth factor and transforming growth factor  $\beta$ ; and it may be blocked by anti-inflammatory factors, such as corticosteroids, IL-13, IL-10 and IL-4 [55,58,59]. In this work, it was possible to observe that the peptide treatment and HMC stimulation downregulated *COX2* gene expression in the cervical cancer cells. Prostaglandin E2 (PGE2) is one of the most abundant COX2 products due to the fact that PGE2 exists in all cell types [55]. Besides stimulation of pain and inflammation, PGE2 participates in proliferation, apoptosis, and metastasis mechanisms [60,61]. PGE2 initiates its actions through specific G-protein-coupled membrane receptors, namely EP1, EP2, EP3 and EP4, with each of them initiating a specific cellular pathway [62]. The EP2 and EP4 receptors stimulate adenylate cyclase to increase cyclic adenosine monophosphate (cAMP) production, a glycogen synthase kinase-3 activator involved in the cell cycle [55]. cAMP promotes  $\beta$ -catenin phosphorylation and degradation by the proteasome 26S, stopping the cell cycle [55]. The EP3

receptor has the opposite action to that described above; its interaction with PGE2 leads to the downregulation of adenylate cyclase and cAMP decrease [60].

The culture of HeLa cells with HMC stimulation showed that the treatment did not alter cell motility, but it did result in a decrease in cell proliferation, and in *COX2*, *EP3* and *EP4* gene expression. The remaining production of PGE2 could be acting through the EP4 receptor, culminating in a decrease of cellular growth after treatment, which could be interfering with apoptosis, since a lower number of cells was observed in this cellular process.

Our research studied for the first time the interaction of ANXA1 with the conditioned medium of endothelial cells, an attempt to simulate the tumour microenvironment. It has been possible to observe the likely existence of a complex interaction, and the diverse array of secreted factors may influence cellular and molecular mechanisms in different ways. The diverse types of tumours exhibit different biological roles and signalling pathways when ANXA1 is involved, and this contradiction is explained by the differential expression patterns and biological behaviours of this protein [11]. In addition, calcium binding to ANXA1's C-terminal core, which binds to the phospholipids, may determine the expression specificity and promote its functional diversity [16].

We suggest that along with the tumour cell secreted paracrine factors, ANXA1 diminishes proliferation and does not alter the cellular cytotoxicity. It was possible to observe an increase in apoptotic cells and a downregulation of *COX2*, *EP3* and *EP4*. Altogether, these data provide further insight into the protective effect of ANXA1 and its mimetic peptides in cervical tumorigenesis, and more research needs to be carried out to confirm its use.

## Materials and methods

### Cell culture

Three cell lines were used, HUVECs, HaCaT and HeLa cells. The HUVEC cell line was cultured in MEM/Earle medium (Cultilab, Brazil), pH 7.5, supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA), and 1% L-glutamine (200  $\mu$ M) (Sigma-Aldrich, St Louis, MO, USA). The HaCaT cell line was cultured in MEM/Earle medium (Cultilab), pH 7.5, supplemented with 10% fetal bovine serum (Cultilab), 1% antibiotic/antimycotic (Invitrogen), 1% L-glutamine (200  $\mu$ M) (Sigma-Aldrich), 1% non-essential amino acids (10 mM; Sigma-Aldrich), and 1% sodium pyruvate (100 mM; Sigma-Aldrich). The HeLa

cell line were cultured in MEM/Earle medium (Cultilab), pH 7.5, supplemented with 10% fetal bovine serum (Cultilab), 1% antibiotic/antimycotic (Invitrogen) 1% L-glutamine (200  $\mu\text{M}$ ; Sigma-Aldrich), and 1% non-essential amino acids (10 mM; Sigma-Aldrich). A total of  $10^6$  cells from each cell line were seeded in 75  $\text{cm}^2$  culture flasks and kept at 37 °C in an atmosphere of 5%  $\text{CO}_2$ .

We did all the experiments with another cervix cell line (SiHa – ATCC, Manassas, VA, USA) treated with the peptide, but without the co-treatment with conditioned medium of endothelial cells (HUVECs), and the results were the same with the HeLa; for this reason we did not continue with the co-treatment.

### Cellular co-culture

The co-culture was performed with the conditioned medium of the HUVEC (HMC) cell line, which was used in the co-cultures with the other two cell lines. The HMC was obtained after HUVEC culture when the cells had acquired 80% confluence. The medium (HMC) was collected after 24 h of culture, from the HUVEC cell line medium without treatment (HMCS) and with treatment (HMCT). The treatment was performed with the N-terminal Ac2-26 peptide of ANXA1 (Ac-MVSEFLKQAWFIENEEQEYVQTVK) [63], at a concentration of 10  $\mu\text{g}\cdot\text{mL}^{-1}$  [27]. The co-culture was performed at a 1 : 1 dilution, according to Rodrigues-Lisoni *et al.* [64].

### Pharmacological treatment

The cell lines were cultured in complete medium, as described above, and subsequently, submitted for co-culture with HMC and treated with the N-terminal Ac2-26 peptide of ANXA1 (Ac-MVSEFLKQAWFIENEEQEYVQTVK) [63], at a concentration of 10  $\mu\text{g}\cdot\text{mL}^{-1}$  for six different time points (2, 4, 24, 48, 72 and 120 h) to perform the proliferation, wound healing, and cytotoxicity assays. After the analysis of these experiments one time point was chosen to continue the other assays. The experimental groups are described below:

HaCaT	HeLa
HaCaT + Ac2-26	HeLa + Ac2-26
HaCaT + HMCS	HeLa + HMCS
HaCaT + Ac2-26 + HMCT	HeLa + Ac2-26 + HMCT

This study was performed to analyse the response of Ac2-26 peptide treatment in cancer cell line, and in that way we developed the experiments only with the cells and the peptide. After analysing the results and in an attempt to mimic the tumour microenvironment we added the conditioned medium of endothelial cells (HMCS and HMCT), along with the peptide treatment,

and observed if in these conditions the peptide would exhibit different results.

### Proliferation assay and cellular morphology analysis

To analyse cell proliferation in the HaCaT and HeLa cells, a growth curve was performed. To count the number of cultured cells, they were seeded at a concentration of  $3 \times 10^4$  in 1 mL of complete medium for 24 h. After this period, the medium was replaced with a serum-free medium, with the purpose of maintaining the same cellular phase. After a further 24 h, this medium was replaced again with complete medium, with the addition of specific HMC and Ac2-26 peptide, according to the experimental groups. The cells were analysed and counted at six different time points (2, 4, 24, 48, 72 and 120 h). The cellular morphology was evaluated with inverted microscopy, using an Olympus CKX41 (Olympus, Tokyo, Japan).

### Wound healing assay

The HaCaT and HeLa cells were distributed in 12-well plates; after reaching adherence and confluence, a wound was made in the centre of the well. Subsequently, the cells were subdivided according to the experimental groups and analysed at 0, 2, 4, 24, 48, 72, and 120 h. The cellular motility was monitored with images obtained using a photographic camera coupled to the microscope. The wound areas were determined in six different microscopic fields and quantified using 'IMAGEJ software (National Institute of Health - NIH, Bethesda, MD, USA).

### Cytotoxicity and viability assay (MTS)

The cells were subdivided into the experimental groups and handled according to the manufacturer's protocol for the CellTiter 96<sup>®</sup>AQUeous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), which uses the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], and an electron coupling reagent (phenazine ethosulfate). MTS reagent was added and absorbance at 490 nm was recorded using an ELISA plate reader. The analyses were made at the times of 2, 4, 24, 48, 72, and 120 h, and were evaluated by comparing the cellular viability across the experimental groups, as well as  $\text{IC}_{50}$  calculus (inhibitory concentration to 50% of the cells).

### Flow cytometry

The cells were subdivided into the experimental groups and handled according to the manufacturer's protocol for the FITC Annexin V Apoptosis Detection Kit I (BD

**Table 1.** Primers for the studied genes.

Oligonucleotide	Sequence
COX2 anti-sense	5' AGAAGGCTTCCCAGCTTTTG 3'
COX2 sense	5' ATTCCCTTCCTTCGAAATGC 3'
EP3 anti-sense	5' TCTCCGTGTGTCTTGCAG 3'
EP3 sense	5' AGCTTATGGGGATCATGTGC 3'
EP4 anti-sense	5' CCAAACCTGGCTGATATAACTGG 3'
EP4 sense	5' CGAGATCCAGATGGTCATCTTAC 3'
MMP2 anti-sense	5' CCGTCAAAGGGGTATCCATC 3'
MMP2 sense	5' AAGTCTGGAGCGATGTGACC 3'
MMP9 anti-sense	5' ATTCGACTCTCCACGCATC 3'
MMP9 sense	5' TTGTGCTCTTCCCTGGAGAC 3'
TIMP1 anti-sense	5' TTTTCAGAGCCTTGGAGGAG 3'
TIMP1 sense	5' ACTGTTGGCTGTGAGGAATG 3'

Pharmingen™, São Paulo, SP, Brazil), to investigate viability, apoptosis, late apoptosis and necrosis, and utilised Guava Easy Cyte (Millipore, Barueri, SP, Brazil) equipment to perform the analysis.

### Selection of the genes

The genes were previously selected from potential markers in the tumour microenvironment, evaluated in the studies of [64,65]. The metabolic pathways from these potential markers were evaluated and some related genes were also chosen. The gene selections took into consideration those that were potentially involved in the tumour invasion and inflammatory processes.

The genes selected were: *COX2* (also known as prostaglandin-endoperoxide synthase 2; *PTGS2*), *EP3* (or *PRGER3*), *EP4* (or *PTGER4*), *MMP2*, *MMP9* and TIMP metalloproteinase inhibitor 1 (*TIMP1*). The specific primers for each transcript were designed with PRIMER3 (<http://primer3.ut.ee/>), as shown in Table 1.

### Quantitative real time PCR

The reactions were performed in the 7500 Fast Real-Time PCR system thermocycler (Applied Biosystems, Foster City, CA, USA); all the reactions were carried out in a final volume of 20 µL with 100 ng of cDNA, SYBR® Green PCR Master Mix, and 100 nM of each primer (F and R). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was the best endogenous control gene when tested alongside actin beta (*ACTB*). The analyses were performed from the cycle threshold ( $C_t$ ) of each sample according to [66].

### Statistical analysis

All assays were performed with three independent experiments. PRISM version 6.0 (GraphPad Software Inc., San Diego, CA, USA) was used to perform all statistics. The Kolmogorov–Smirnov normality test was used to analyse

distribution. Variance analysis (ANOVA) was used for between group comparisons, followed by the appropriate parametric or non-parametric *post-hoc* test. Statistical significance was set at a probability value of less than 0.05.

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### Conflict of interest

The authors declare no conflict of interest.

### Author contributions

LTC designed and performed all experiments and wrote the manuscript. JP contributed to the analysis of the flux cytometry. BRC performed the experiments with the quantitative PCR. EHT contributed to writing the manuscript. SMO supported the experimental development and contributed to writing the manuscript. FCRL coordinated the project, and contributed to the experiments and writing of the manuscript.

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