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RESEARCH ARTICLE

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Evidence that cervical cancer cells cultured as tumorspheres maintain high CD73 expression and increase their protumor characteristics through TGF-β production

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Abstract

Recently, a link between the biological activity of CD73 and tumorigenicity in solid tumors has been proposed. We previously reported that the generation of adenosine (Ado) by the activity of CD73 in cervical cancer (CC) cells induces transforming growth factor-beta 1 (TGF-β1) production to maintain CD73 expression. In the present study, we analyzed the participation of TGF-β1 in CD73 expression and the development of protumoral characteristics in CaSki CC cells cultured as tumorspheres (CaSki-T) and in monolayers (CaSki-M). Compared with those in CaSki-M cells, CD73 expression and Ado generation ability were

significantly increased in CaSki-T cells. CaSki-T cells exhibited enrichment in the CSC-like phenotype due to increases in the expression levels of stem cell markers (CD49f, CK17, and P63; OCT4 and SOX2), greater sphere formation efficiency (SFE), and an increase in the percentage of side population (SP) cells. Interestingly, compared with CaSki-M cells, CaSki-T cells produced a greater amount of TGF-β1 and presented a marked protumor phenotype characterized by a significant decrease in the expression of major histocompatibility complex class-I (MHC-I) molecules, an increase in the expression of multidrug resistance protein-I (MRP-I) and vimentin, and an increase in the protein expression levels of Snail-1 and Twist, which was strongly reversed with TGF-β1 inhibition. These results suggest that the presence of TGF-β1–CD73–Ado feedback loop can promote protumoral characteristics in the CC tumor microenvironment.

KEYWORDS

cancer stem-like (CSC-like) cells, CD73, cervical cancer, protumor phenotype, TGF-β 1, tumorspheres

1 | INTRODUCTION

Cervical cancer (CC) is the fourth most common type of cancer in women and represents a major public health problem worldwide. In 2020, approximately 600,000 new cases and 340,000 deaths were reported, more than 80% of which occurred in developing countries.[1] Persistent infection by high-risk human papillomavirus (HR-HPV) is one of the main risk factors for the development of low-grade squamous intraepithelial lesions (LSILs), which can progress to high-grade lesions (HSILs) and eventually to CC.[2] The immune response against HPV antigens can eliminate most infections and precursor lesions; however, some women exposed to HR-HPV will develop cancer, suggesting that other risk factors may be involved.[3] Recently, intrinsic factors related to the generation of adenosine (Ado) and the signaling of this nucleoside in tumor cells have been proposed to play important roles in tumor growth, immunosuppression, evasion of the immune response, metastasis, and chemoresistance, among other mechanisms.[4–7] Ado is produced at sites of metabolic stress associated with hypoxia, ischemia, trauma, or inflammation and even in the tumor microenvironment (TME) through the adenosinergic pathway. In the TME, high concentrations of adenosine triphosphate/adenosine diphosphate (ATP/ADP) greater than 50 μM are hydrolyzed to adenosine monophosphate (AMP) by the ectoenzyme CD39 (ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1; EC 3.6.1.5) and subsequently to Ado by the activity of 5′-ectonucleotidase (CD73, EC 3.1.3.5).[8–10] Most of the extracellular signaling activities of Ado are mediated by receptors on the cell membrane that are coupled to G proteins; these receptors are divided into four subtypes (A1R, A2AR, A2BR, and A3R).[11–13]

Purinergic signaling has been associated with the development and maintenance of cancer stem-like (CSC-like) cells, which have the ability to initiate tumors due to their property of self-renewal and unlimited proliferation and play critical roles in tumor metastasis, relapse, and resistance to therapy.[14] Recent evidence indicates that transforming growth factor-beta 1 (TGF-β1) is one of the most prominent extrinsic factors of the microenvironment in solid tumors and initiates a transcriptional program in carcinoma cells that participates in cellular plasticity toward the epithelial-mesenchymal transition (EMT).[15] After EMT activation, cells generally eliminate the expression of epithelial markers, such as E-cadherin, and instead express mesenchymal markers, including vimentin, fibronectin, and certain master transcription factors that induce EMT, such as Zeb1, Twist, Snail, and Slug, which regulate the expression of genes associated with most mesenchymal states of carcinoma cells to facilitate their invasiveness and motility.[16–18] In addition, TGF-β1 present in the TME is a driver of CD73 expression, and the activity of this nucleotidase has been suggested to positively regulate the expression of genes associated with stem cells[19,20] and cellular plasticity in solid tumors.[6]

Several studies have reported that in nonadherent and serum-free conditions, the in vitro tumorsphere formation assay is a functional method to produce tumor cultures enriched in

Significance statement

This study provided the first evidence that high CD73 expression associated with high TGF-β1 production by cervical cancer cells cultured as tumorspheres strongly contributes to the induction of protumoral characteristics related to tumor progressions, such as migration and invasiveness, immune evasion, immunosuppression, and chemoresistance.

subpopulations of CSC-like cells and a microenvironment where cell behavior can be studied under different experimental conditions.[21–25] In addition, a positive correlation has been reported among CD73 expression in tumor cells, the ability to form tumorspheres in vitro, and tumor progression,[26] suggesting a link between increased CD73 expression and tumorigenesis. Our research group has reported that HR-HPV-positive CC tumor cells highly express CD73 in the cell membrane and have a high capacity to generate Ado[27] and that Ado signaling in these tumor cells promotes TGF-β1 production to maintain CD73 expression.[28] Therefore, in this study, we analyzed the participation of TGF-β1 in the expression of CD73 and protumoral markers in CaSki cells cultured as tumorspheres (CaSki-T) and in monolayers (CaSki-M). We found that CSC-like characteristics, including a stem cell phenotype, sphere formation efficiency (SFE), and side population (SP) cells, increased in CaSki-T cells. Likewise, compared with CaSki-M cells, in CaSki-T cells, we observed a significant increase in CD73 expression and the capacity to hydrolyze AMP and generate Ado. Interestingly, CaSki-T cells produced a greater amount of TGF-β and presented a marked protumor phenotype, which was reversed when these cells were cultured in the presence of neutralizing anti-TGF-β, suggesting that the TME is important for maintaining CD73 expression and potentially the production of immunosuppressive factors such as TGF-β1 to favor the protumor phenotype in CC cells. This study provides the first evidence that the TGF-β1–CD73–Ado feedback loop strongly contributes to the induction of protumoral characteristics in CC and may have clinical importance as a therapeutic target.

2 | METHODS

2.1 | Tumor cell culture

CC CaSki (HPV-16+) cells obtained from the American Type Culture Collection (ATCC) were cultured under adherent conditions as a monolayer/2D using RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Gibco), 100 U/μl penicillin, and 100 U/μl streptomycin (Gibco) and maintained under sterile conditions at 37°C with 5% CO_2 in a saturated humidity environment. For culture in tumorspheres, CaSki cells were seeded (1×10^4 cells per well) in ultralow-adhesion six-well plates (Corning Costar) using 2 ml of reduced-serum medium (Opti-MEM; Gibco) containing 0.5% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco), 1% nonessential amino acids, GlutaMAX (Gibco), and 20 ng/ml rhEGF and rhFGFb (R&D Systems) and maintained under sterile conditions at 37°C with 5% CO_2 in a saturated humidity environment. On Day 7 of culture, the tumorspheres were mechanically disaggregated and reseeded under the same conditions. After two consecutive reseedings (Day 21 of culture), the tumorspheres were characterized, and the tumor cells within each sphere were analyzed to determine their phenotypic and functional characteristics; the same analyses were conducted for the tumor cells cultured in monolayers.

2.2 | SFE

Cells from monolayer tumor cell cultures or tumorspheres were seeded (1×10^4) in ultralow adhesion plates in Opti-MEM medium (2 ml/well). After 14 days, the number of spheres with a diameter greater than 200 μm was determined using the following formula: % SFE = (total number of spheres/10,000) × 100.

2.3 | Characterization of tumorspheres

After 21 days of culture, the tumorspheres reached an approximate diameter of 200–300 μm. Some were fixed for 24 h in a 4% paraformaldehyde solution and embedded in paraffin, and then a microtome was used to slice 5-μm sections that were subsequently processed for staining with hematoxylin-eosin (H-E). To analyze the structure, the tumorspheres were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 2 h. Subsequently, they were incubated in 1% osmium tetroxide (OsO_4) in PBS and embedded in EPON resin, after which they were sliced into ultrathin sections and stained with 4% uranyl acetate and 0.4% lead citrate. The sections were evaluated in a JEOL 1010 transmission electron microscope operated at 80 kV. Digital images were obtained with a Hamamatsu camera.

2.4 | SP assay

Tumor cells from monolayers or tumorsphere cultures (1×10^6) were incubated for 1 h in the presence or absence of 10 μM Hoechst 33342 (Sigma-Aldrich). In some cases, to block Hoechst efflux, cells were stained in the presence of 50 μM verapamil (Sigma-Aldrich), as previously described.[29] The cells were then washed twice with PBS, and the percentage of cells that excluded the dye was analyzed using a FACSAria IIu flow cytometer (BD Biosciences). The data obtained were analyzed using FlowJo 10 software.

2.5 | Flow cytometry

Tumor cells from monolayer cultures or tumorspheres were analyzed by flow cytometry to determine the expression of core markers, that is, CD49f, CK17, P63, OCT4, and SOX2, using the following antibodies: anti-CD49f-APC (R&D Systems); anti-CK17-AF488 (Bioss); anti-P63-AF647 (Bioss); anti-OCT4 (rabbit anti-human; Abcam); and anti-SOX2 (mouse anti-human, Abcam). For the analysis of CD73, major histocompatibility complex class-I (MHC-I), MRP-1, E-cadherin, vimentin, Snail, and Twist, the following antibodies were used: anti-CD73-PE (mouse anti-human, BD, Pharmingen); W6/32 (mouse anti-human, which recognizes the conformational epitope of the α chains of the human leucocyte antigen (HLA)-A, HLA-B and HLA-C molecules associated with β2-microglobulin), which was generously donated by Dr. Gerd Moldenhauer of the German Cancer

Research Center, Heidelberg, Germany; anti-MRP1 antibody Abcam; anti-E-cadherin-PerCP-Cy 5.5 (BD, Pharmingen); anti-vimentin-Alexa Fluor 488 (BD, Pharmingen; anti-Snail Mouse monoclonal Abcam (Cambridge); and anti-Twist Mouse monoclonal Abcam (Cambridge). The secondary antibodies used were goat anti-rabbit PE (R&D Systems) and goat antimouse Fuorescein IsoTioCyanate (Sigma-Aldrich). For intracellular staining, tumor cells were fixed for 10 min with a 2% paraformaldehyde solution (Sigma-Aldrich) and subsequently permeated with 0.2% saponin (Sigma-Aldrich) supplemented with 2% FBS. For each determination, 3×10^4 events were obtained in a FACSAria IIu cytometer (BD Biosciences), and the data were analyzed using FlowJo 10.

2.6 | mRNA expression

To analyze the mRNA expression of CD73 in CC cells, RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was obtained from 500 ng of RNA using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). CD73 expression was determined by end-point reverse transcription-polymerase chain reaction (RT-PCR). The G6PDH gene was used as an internal control. RT-PCR was performed in a volume of 25 µl in accordance with the instructions of the manufacturer of Master Mix PCR (Promega), and the reactions were run in TC1000-G equipment (DLAB NT). The amplified RT-PCR products were electrophoresed in a 2% agarose gel (Invitrogen). The gel was stained with GelRed (Biotium), and a UV transilluminator (UVP Biodo-H System) was used to visualize the amplified products. The following primers were used: CD73 sense, 5′GCACTATCTGGTTCACCGTGT'3 and CD73 antisense, 5′CCTTCCACACCATTATCAAATTC′3; G6PDH sense, 5′ GCTACGCTCGGATCTTGTTC′3; and G6PDH antisense 5′ CCCAGTGCTTTTCGCTCT′3.

2.7 | Quantification of TGF-β1 and inhibition of TGF-β1 activity

A Quantikine ELISA kit for human TGF-β1 (R&D Systems, Inc.) was used to quantify the TGF-β1 content in the supernatants of CC cell cultures. To neutralize the biological activity of TGF-β1 produced in the cell cultures, anti-TGF-β1, anti-TGF-β2, and anti-TGF-β3 neutralizing antibodies (anti-TGF-β, R&D) were added following the manufacturer's protocol.

2.8 | Enzymatic activity of CD73

To analyze the hydrolytic activity of CD73 in tumor cells cultured in monolayers or tumorspheres, 1×10^6 cells were cultured in the presence of 5 mM AMP in 100 µl of Opti-MEM medium (Gibco) supplemented with 1% dialyzed FBS. The supernatant was collected after 4 h. The presence of Ado was detected by thin layer chromatography (TLC) and ultraperformance liquid chromatography (UPLC) (UPLC Acquity, Waters). To analyze the samples by TLC, 1 µl of each supernatant was spotted on plates covered with fluorescent silica gel (Sigma-Aldrich Co.). The samples were eluted for 1 h using a mobile phase composed of isobutanol:isoamyl alcohol:ethanol:ammonia:water (9:6:18:9:15), as previously reported,[27] and 5 mM AMP, Ado, and inosine (Ino) (Sigma-Aldrich) were used as standard controls. The compounds were visualized using a UV transilluminator (UVP Biodo-H System). A UPLC system (UPLC Acquity, Waters) was used to quantify the amount of Ado generated in cell cultures in the presence of AMP. Quantitative analysis of samples using standard quantities of synthetic Ado was carried out with Empower 3 software (Waters), as previously reported.[27] Briefly, the mobile phase consisted of 0.5% acetonitrile, 5% methanol, and 94.5% sodium acetate buffer 0.25 M, pH 6.3. Supernatant samples were centrifuged at 13,000 rpm, filtered on Amicon membranes with a cutoff of 3000 Da, and subsequently diluted 1:200 with the mobile phase mixture. The run conditions were as follows: flow rate of 1.0 ml/min, UV detection at 254–260 nm, 2.0 min retention time, room temperature, and a LiChrospher 5-µm RP-18e 100 A (size 125 mm Å~ 4 mm, 5 µm particle size) reversed-phase column. Ado was quantified by comparing the retention time of the sample with that of the synthetic Ado used as a standard.

2.9 | Statistical analysis

The numerical data are presented as the average value ± SEM of three independent experiments. Comparisons were evaluated with multivariate statistical analysis using GraphPad Prism version 7 (GraphPad Prism software). Differences were considered significant when $p < .05$.

3 | RESULTS

3.1 | Culturing CC cells as tumorspheres enriched the population with CSC-like characteristics

The tumorsphere formation assay uses a functional approach to analyze the self-renewal capacity of cells in the presence of subpopulations of CSC-like cells.[25] CaSki cells derived from an epidermoid carcinoma of the cervix were maintained under standard culture conditions, either as a monolayer/2D (CaSki-M) or in ultralow adhesion plates to form tumorspheres/3D (CaSki-T) (Figure 1A). After two consecutive reseedings (21 days of culture), the SFE of the tumor cells was analyzed. Interestingly, compared with that of CaSki-M cells, the SFE of CaSki-T cells increased significantly (Figure 1B). The CaSki-T cells formed through this culture system were compact and generally showed a size greater than 200 µm (Supporting Information: Figure 1A,B). Transmission electron microscopy revealed a large number of intercellular focal adhesions (asterisks in Supporting Information: Figure 1C,D). Compared with CaSki-M cells, CaSki-T

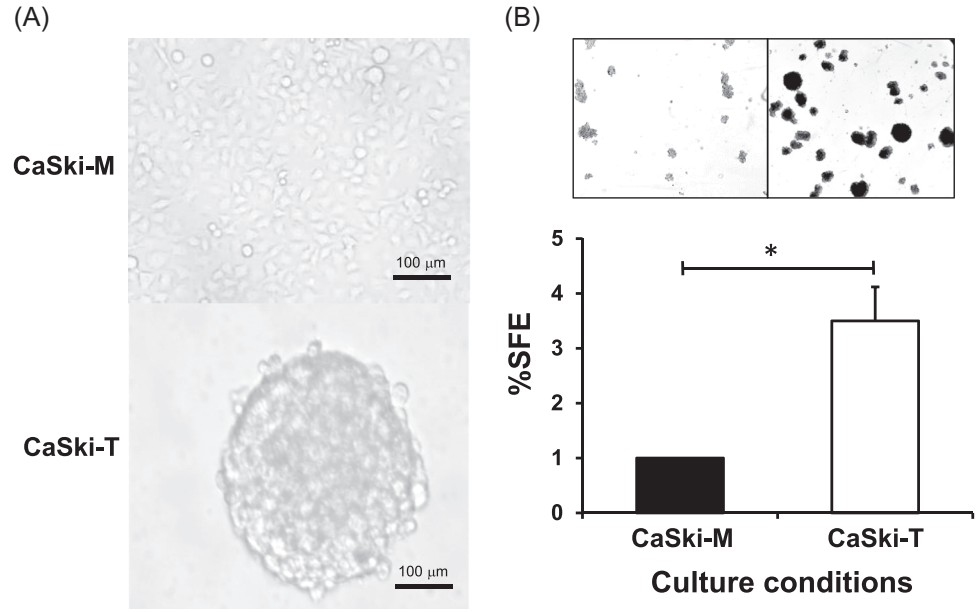

FIGURE 1 CaSki cells in monolayer cultures and as tumorspheres and their sphere formation efficiency. (A) Microphotographs (×40 magnification) of CaSki cells in a monolayer culture (CaSki-M) and as tumorspheres (CaSki-T). (B) Sphere formation efficiency (SFE) of the cells maintained in each culture condition and photographs of the cells after 21 days of culture in ultralow adhesion plates. Representative data from three independent experiments ±SEM. *Significant difference at $p < .01$.

cells exhibited greater expression of the stem cell markers CD49f, CK17 and P63, OCT4 and SOX2 (Figure 2A). In addition, a higher percentage of SP cells was present in CaSki-T cells than in CaSki-M cells, 0.46% and 0.18%, respectively. Verapamil treatment strongly reduced Hoechst efflux, reducing the percentage of SP cells among CaSki-T and CaSki-M cells to 0.093% and 0.070%, respectively (Figure 2B). These results suggest that CaSki cells cultured as tumorspheres increase the CSC-like characteristics of CaSki cells and, therefore, their self-renewal capacity.

3.2 | CC cells cultured as tumorspheres exhibited increased expression of CD73 and adenosinergic activity

The expression and activity of CD73 in tumor cells are influenced by conditions in the cellular microenvironment.[30] To determine whether CD73 expression was different in CaSki cells cultured in monolayers or as tumorspheres, CD73 expression in CaSki-M and CaSki-T cells was analyzed by flow cytometry. Compared with CaSki-M cells, CaSki-T cells exhibited increased levels of CD73 protein (Figure 3A) and mRNA (Figure 3B). This increase was associated with a greater capacity of CaSki-T cells to generate Ado from AMP hydrolysis (black arrows, Figure 3C upper). The Ado concentrations detected in CaSki-M and CaSki-T supernatants were 81.9 ± 6.5 and 121.23 ± 8.9 nM, respectively (Figure 3C down). However, the addition of APCP (selective inhibitor of CD73) in both cell cultures strongly decreased the ability of tumor cells to hydrolyze AMP. The Ado concentrations detected in the presence of APCP were 19.6 ± 3.9 and 40.24 ± 5.9 nM, respectively (Figure 3C lower).

3.3 | CC cells cultured as tumorspheres produce high amounts of TGF-β1

TGF-β1 is an important factor in maintaining CD73 expression in CC tumor cells.[28] accordingly, TGF-β1 levels were assessed in CaSki-M and CaSki-T cultures. The TGF-β1 content in the culture supernatant of CaSki-T cells was significantly higher than that in the culture supernatant of CaSki-M cells (Figure 4A). To determine the influence of this factor on CD73 expression, a neutralizing anti-TGF-β antibody was added to the cells when culturing. Interestingly, a significant reduction in CD73 was observed in CaSki-M (>25%) and CaSki-T (>15%) cells (Figure 4B), suggesting that TGF-β1 produced by CC cells helps maintain CD73 expression in an autocrine manner.

On the other hand, we have previously reported that CaSki cells downregulated CD73 expression by using a pSIREN vector containing a siRNA targeting CD73 (CaSki-pS-siRNA-CD73), which strongly reduced TGF-β production.[28] Therefore, we analyzed TGF-β1 production by these cells when cultured as tumorspheres. Interestingly, compared with tumorspheres formed by CaSki cells transfected only with pSIREN vector (CaSki-pS-T), CaSki-pS/siRNA-CD73-T cells formed only noncompact cellular aggregates (Supporting Information: Figure 2A) and showed a reduced SFE percentage (Supporting Information: Figure 2B). In addition, we detected lower TGF-β1 production in the culture supernatant of these cells than in

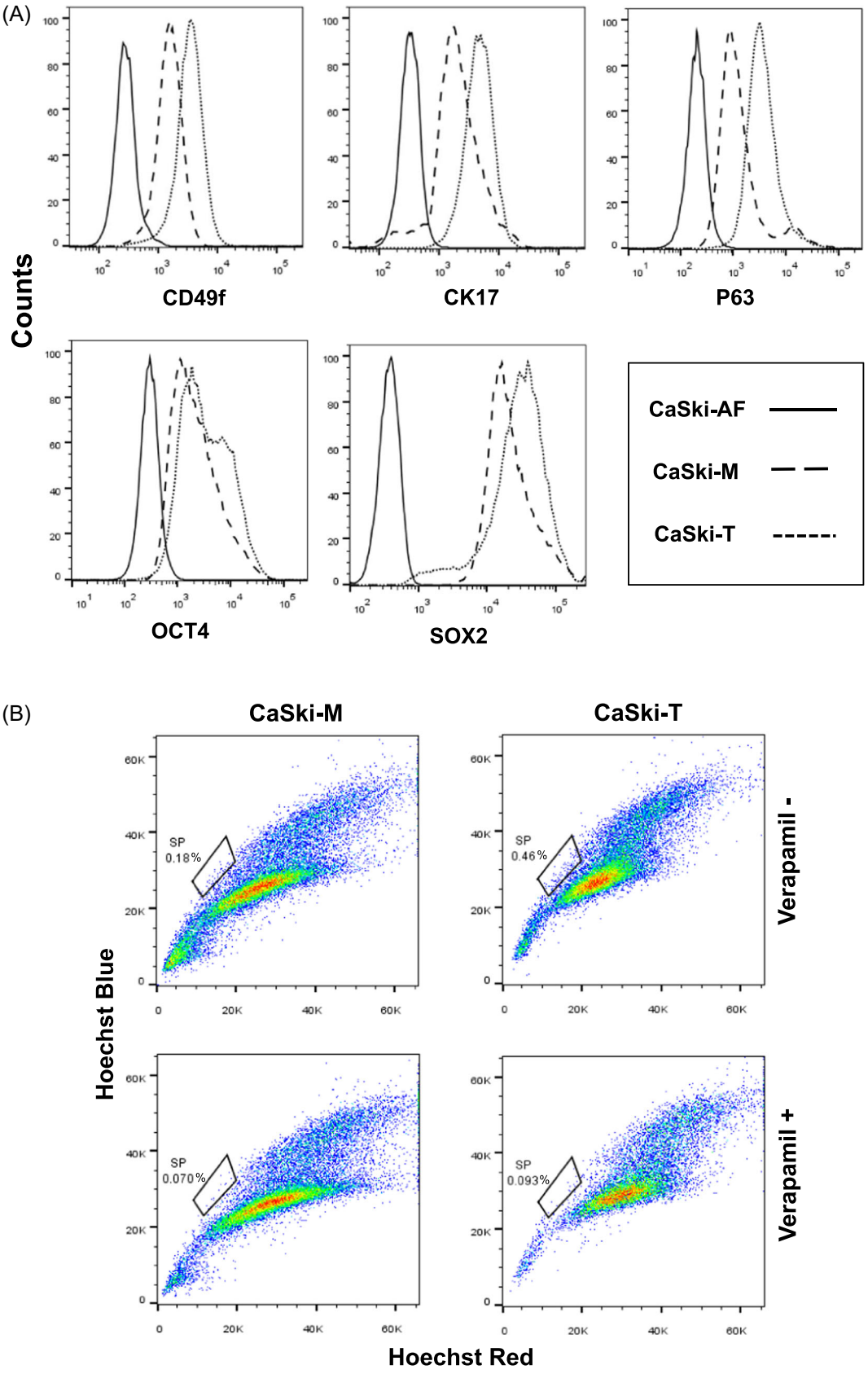

FIGURE 2 (See caption on next page)

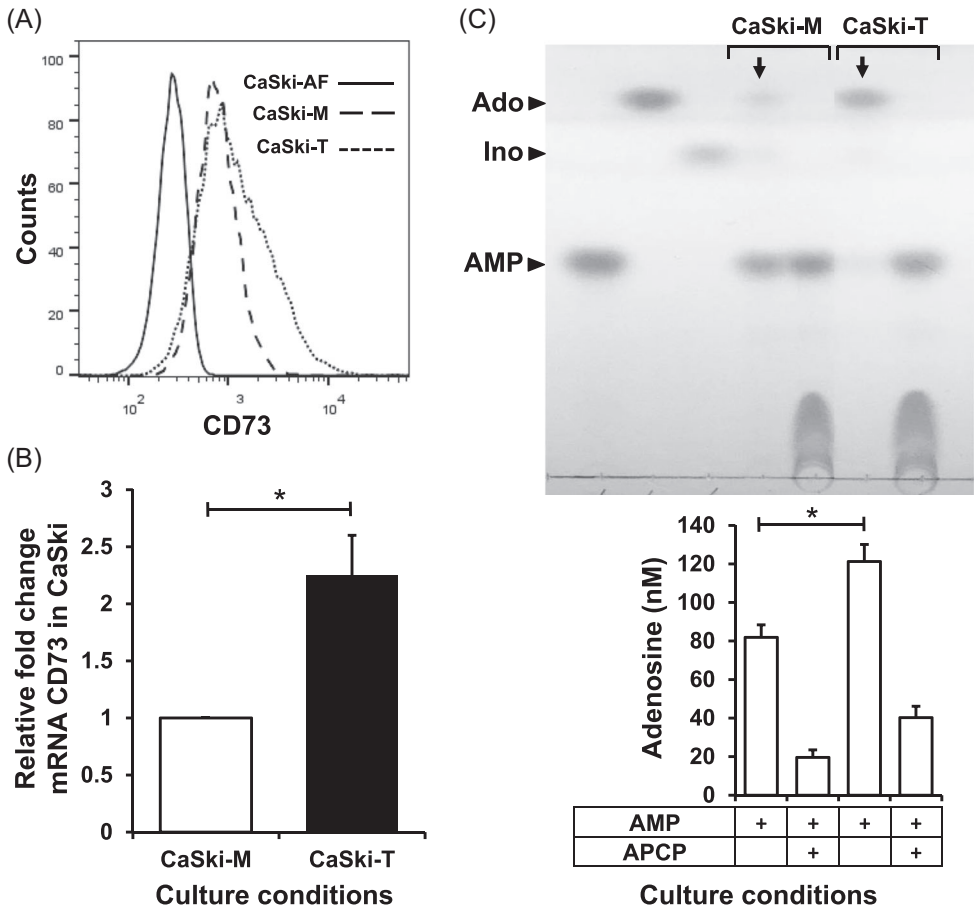

FIGURE 3 Expression and activity of CD73 in CaSki cells cultured in monolayers and as tumorspheres. Expression of CD73 protein (A) and CD73 mRNA (B) in CaSki cells cultured in monolayers (CaSki-M), which was normalized to 1, or as tumorspheres (CaSki-T). (C) The ability of CaSki-M and CaSki-T cells to generate Ado via AMP hydrolysis was analyzed by thin layer chromatography (TLC) (upper) and ultraperformance liquid chromatography (UPLC) using standard concentrations of synthetic Ado (lower). Representative data from 3 independent experiments ±SEM. CaSki-AF, CaSki autofluorescence. *Significant difference at $p < .01$.

the culture supernatant of CaSki-pS-T cells (Supporting Information: Figure 2C).

3.4 | TGF-β favors a protumor phenotype in CC cells cultured as tumorspheres

TGF-β plays an important role in the plasticity of tumor cells, including the regulation of EMT and the induction of protumoral mechanisms such as evasion of the immune response, migration, invasion, and chemoresistance, among others.[31,32] Therefore, to determine whether this factor, produced in greater quantities by CaSki-T cells than CaSki-M cells, was important for maintaining the protumoral phenotype, the expression levels of E-cadherin, vimentin, MHC-I, MRP-1, Snail-1, and Twist were analyzed in CaSki cells maintained in both culture conditions.

Compared with CaSki-M cells, CaSki-T cells exhibited a significant increase in the expression levels of proteins related to the EMT state, such as vimentin, Snail, and Twist, and a reduced expression level of N-cadherin (Figure 5A). In addition, compared with CaSki-M cells, CaSki-T cells exhibited a significant decrease in the expression of MHC-I molecules and increased expression of MRP-1 (Figure 5B). Interestingly, the addition of neutralizing anti-TGF-β antibody to the tumorsphere cultures significantly modified the expression of the

FIGURE 2 Stem markers and side populations among CaSki cells cultured in monolayers and as tumorspheres. (A) The expression of stem cell markers (CD49f, CK17 and P63, OCT4, and SOX2) in CaSki cells cultured in monolayers (CaSki-M) and as tumorspheres (CaSki-T) is shown. CaSki-AF, autofluorescence of CaSki cells. Representative results from three independent trials. (B) CaSki-M or CaSki-T cells were stained with Hoechst 33342 and analyzed by flow cytometry to assess side population (SP) cells, a subpopulation with a high dye exclusion capacity. Plots of total cells and cells stained with Hoechst 33342 are shown; the SP cells are grouped in a quadrangle. Verapamil was used to block Hoechst efflux from SP cells. Representative results from three independent trials are shown.

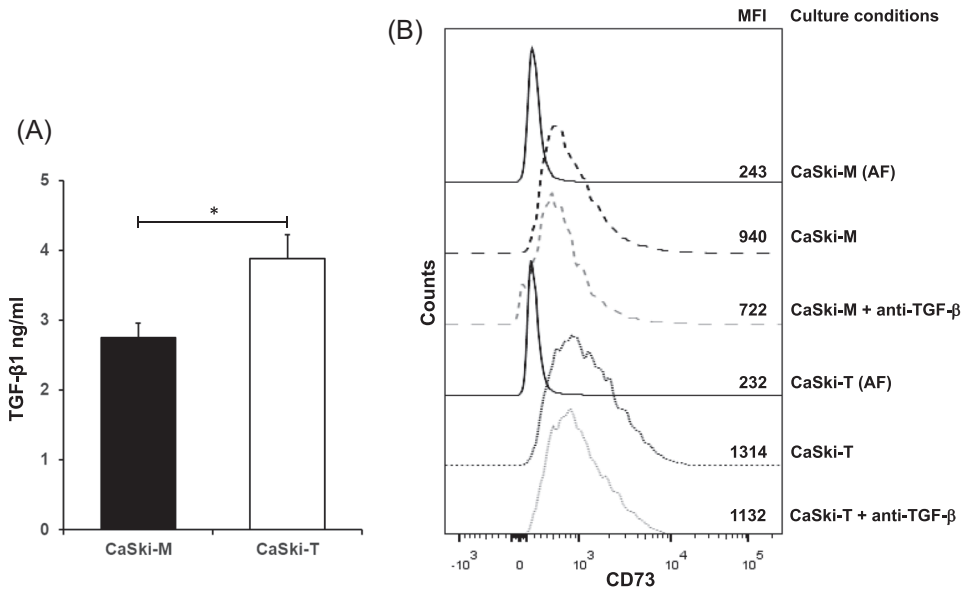

FIGURE 4 Transforming growth factor-beta (T1GF-β1) produced by CaSki cells cultured in monolayers and as tumorspheres and their CD73 expression. (A) The contents of TGF-β1 in the culture supernatants of CaSki cells cultured in monolayers (CaSki-M) or as tumorspheres (CaSki-T). (B) CD73 expression in CaSki-M and CaSki-T cells cultured in the presence or absence of a neutralizing anti-TGF-β antibody. Representative data from three independent experiments ±SEM. *Significant difference at $p < .05$. AF, autofluorescence; MFI, mean fluorescence intensity.

protumoral markers, decreasing vimentin, Snail, Twist, and MRP-1 expression and increasing E-cadherin and MHC-I expression to levels comparable to those observed in CaSki-M cells (Figure 5A,B). Moreover, compared with CaSki-T cells, we also observed that CaSki-pS/siRNA-CD73-T cells strongly modified the levels of proteins related to the EMT state, decreasing vimentin, Snail, and Twist and increasing E-cadherin (Supporting Information: Figure 3). These results suggest that TGF-β1 produced in the microenvironment of tumorspheres is important in the induction of protumoral characteristics in CaSki-T cells.

4 | DISCUSSION

The role of TGF-β1 in the progression and metastasis of various types of cancer, including prostate, breast, colorectal, liver, and CCs, is well known.[33–35] For CC, TGF-β1 has been suggested to play a dual role during disease development, either as an anti-oncogenic factor in precancerous cervical lesions or as a promoter in advanced stages.[36,37] In fact, the presence of TGF-β1 in the tissue and plasma of patients with CC is positively correlated with the expression of HR-HPV oncogenes[38] and with the degree of disease progression.[39–42] However, the molecular mechanisms by which the production of TGF-β1 promotes tumorigenesis in CC have not been completely elucidated. A growing body of evidence supports the protumorigenic role of CD73 and Ado signaling in solid tumors.[6] In this context, we provided evidence that CC cell lines positive for HPV-AR infection, mainly CaSki cells, express significantly higher levels of CD73 on the membrane than cells negative for HPV infection, which is associated with a greater ability to generate Ado and to inhibit the proliferation, activation, and effector function of cytotoxic T lymphocytes (CTLs) through interactions with its A2AR.[27] In addition, we also reported that Ado signaling through A2AR and A2BR in CC tumor cells plays an important role in the induction of TGF-β1 secretion and expression. Likewise, we demonstrate that TGF-β1 produced through this pathway maintains CD73 expression in CC tumor cells.[29] Therefore, in this study, we analyzed the participation of TGF-β1 in CD73 expression and in the development of protumoral characteristics in CC CaSki cells cultured as tumorspheres (CaSki-T) and in monolayers (CaSki-M). CaSki cell tumorspheres are compact structures formed by cells joined together by focal adhesions. The expression of CD73 and the capacity to generate Ado were significantly higher in CaSki-T cells than in CaSki-M cells. As reported in previous studies,[43–45] CaSki-T cells were enriched in CSC-like characteristics due to increases in the expression of stem cell markers (CD49f, CK17 and P63, OCT4 and SOX2), greater SFE, and an increase in the percentage of PS cells. CaSki-T cells also produced higher amounts of TGF-β1 than CaSki-M cells and exhibited a markedly protumoral phenotype characterized by a significant increase in the expression levels of proteins related to the EMT state, such as vimentin, Snail, and Twist, and a reduced expression level of N-cadherin. In addition, CaSki-T cells exhibited a significant decrease in the expression of MHC-I molecules and increased expression of MRP-1. Interestingly, these phenotypes were reversed when a neutralizing anti-TGF-β antibody was added to the tumorsphere cultures, decreasing vimentin, Snail, Twist, and MRP-1 expression and increasing E-cadherin and MHC-I expression to levels comparable to those observed in CaSki-M cells. These results suggest that the three-dimensional microenvironment generated in tumorspheres is important for maintaining the expression of CD73 and

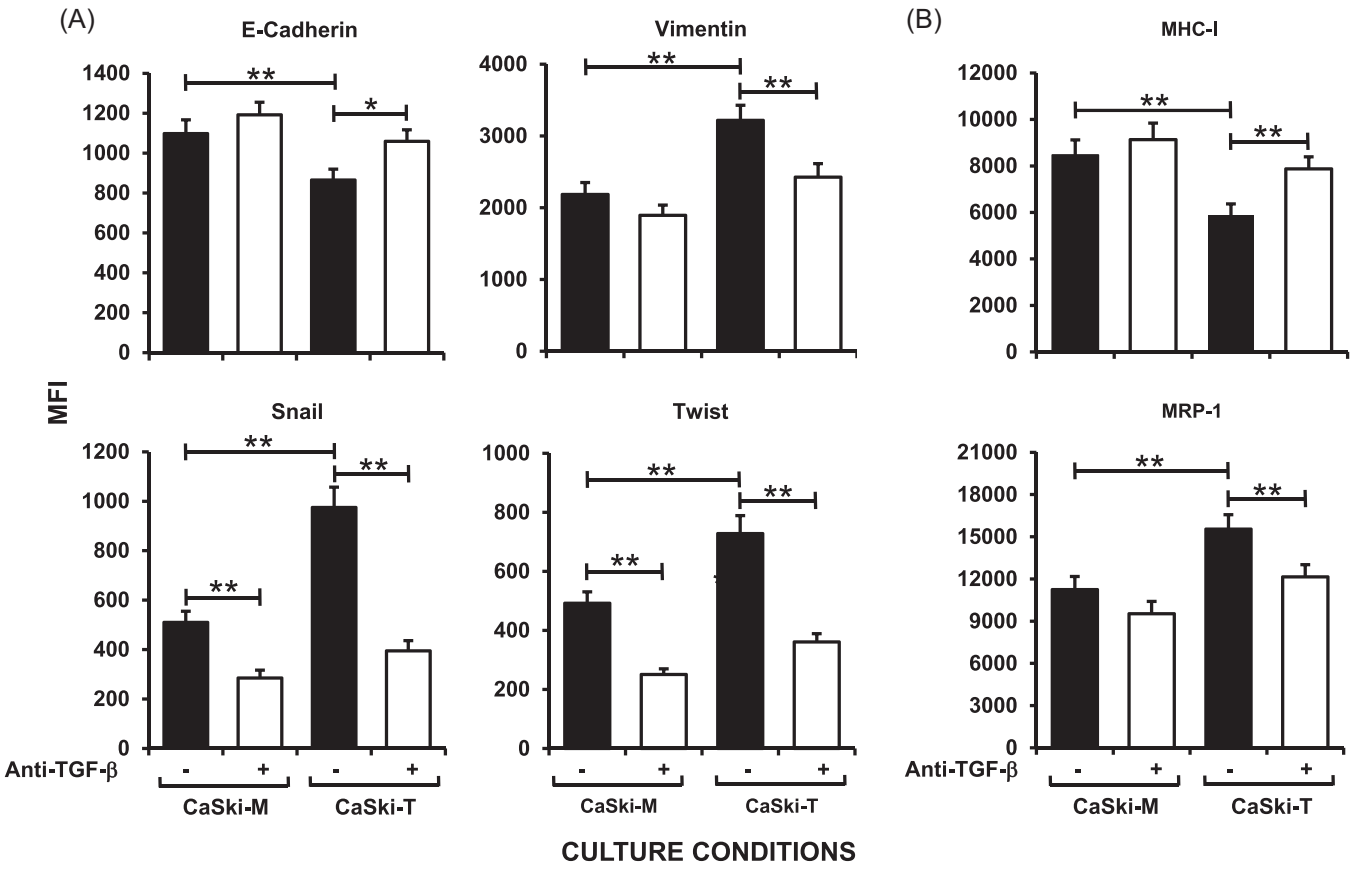

FIGURE 5 Anti-TGF-β reverses the protumor phenotype in CaSki cells cultured as tumorspheres. Cultures of CaSki cells in monolayers (CaSki-M) or as tumorspheres (CaSki-T) were maintained for 14 days in the presence or absence of neutralizing anti-TGF-β antibody⊡ (A) Expression levels of proteins related to the EMT state: E-cadherin, vimentin, Snail, and Twist. (B) Protein expression levels of MHC-I and MRP-1. Representative data from 3 independent experiments ±SEM. *·**Significant differences at $p < .05$ and $p < .01$, respectively. EMT, epithelial-mesenchymal transition; MHC-I, major histocompatibility complex class-I; MRP-1, multidrug resistance protein-1; TGF-b1, transforming growth factor-beta 1.

potentially the production of immunosuppressive factors such as TGF-β1 to favor a protumor phenotype in CaSki cells. In fact, the transcriptional activation of CD73 within the TME is primarily influenced by hypoxic conditions mediated by the activation of hypoxia-inducible factor 1α (HIF-1α)[46] and the presence of immunosuppressive factors, such as TGF-β 1, that can stabilize HIF-1α.[47]

A large number of studies conducted with different types of cancer have shown that the biological activity of CD73 is closely related to stem cell promotion and the expression of genes associated with EMT,[19,20] metastasis,[48,49] evasion of the immune response,[6] and tumor escape.[50,51] However, unlike that in most solid cancer types, CD73 expression in cervical tumors, as well as tumors of the genitourinary system such as ovarian serous cystadenocarcinoma (OV), testicular germ cell tumors (TGCT), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS), bladder urothelial carcinoma (BLCA), kidney chromophobe (KICH), and prostate adenocarcinoma (PRAD), is decreased compared to normal tissues, probably due to the common embryological origin (the intermediate mesoderm) of the reproductive and urinary systems.[4,6] Nevertheless, we have observed that independent of the HPV genotype, cell lines derived from CC tumors can exhibit different CD73 expression levels and Ado generation capabilities.[27] Additionally, differences in cellular phenotypes have been reported, probably due to the microenvironment of origin; for instance, SiHa and HeLa cell lines show a mesenchymal-like phenotype, while CaSki cells, which were obtained from omentum metastases, exhibit an epithelial phenotype.[52] In addition, CD73 function can differ in the same cell type from different tissues and within tumors, and the tumor cells themselves can differ in relation to CD73 expression compared to adjacent stromal cells. The expression and activity of CD73 may also diverge depending on the stage of tumor development and different factors in the microenvironment leading to tumor progression.[4,6]

On the other hand, in nonadherent and serum-free conditions, the in vitro tumorsphere formation assay has been considered a functional method to produce tumor cultures enriched in subpopulations of CSC-like cells and a microenvironment where cell behavior can be studied under different experimental conditions.[21–25] However, a recent in silico analysis carried out by Iser et al.[43] related to CD73 expression on cells derived from CSC-enriched tumorspheres using microarray gene expression profiles of 7 GSE datasets from the

Expression Omnibus Database (GEO) reported that protocols for CSC induction or isolation, as well as the maintenance conditions or exposure to treatments, presented significant differences among the studies analyzed. In addition, similar to their results obtained from SiHa cells, they reported that in human primary cervical cells isolated from healthy tissue, the human anaplastic thyroid cancer cell line (THJ-11 T cells),[53] and breast cancer cell lines from human primary tumors,[54] a decrease in the CD73 expression of CSC-enriched tumorspheres compared to monolayer cells was evident. In contrast, the CaSki CC cell line[55] and human prostate cancer stem cell (PCSC)[56] primary lines showed enhanced CD73 expression in spheres compared to monolayers, while in a cell line derived from human primary HPV-negative pharyngeal squamous tumor cells[57] and primary cells from human pancreatic ductal adenocarcinoma (PDAC),[58] no significant differences were observed between spheres and monolayers. However, in accordance with the study by Bajaj et al.,[55] we also found enhanced CD73 expression in CaSki tumorspheres compared to monolayers; however, we detected a significant increase in TGF-β1 production in spheres than in monolayer cell cultures, which was associated with an increased expression level of proteins related to the EMT state in these cells, such as vimentin, Snail, and Twist, and a reduced expression level of N-cadherin. Interestingly, this phenomenon was reversed when neutralizing anti-TGF-β1 was added to cell cultures, and when CaSki cells with downregulated CD73 expression were cultured as tumorspheres, lower TGF-β1 production was detected in the culture supernatant, which was associated with decreased levels of vimentin, Snail, and Twist and an increased level of E-cadherin in these cells, indicating the importance of CD73 expression and increased TGF-β1 production to promote the protumoral characteristics of CaSki cells when cultured as tumorspheres.

Similar results were obtained in tumorspheres formed by ovarian cancer cells, where high expression of CD73 and increased expression of transcription factors related to EMT, such as Snail-1, Snail-2, and Twist-2, were observed when compared with those in epithelial cells of the Fallopian tube.[20] In addition, deregulation of the CD73 gene resulted in the low expression of key EMT genes, such as Snail-1, Twist-2, and Zeb-1, and a decrease in the mesenchymal phenotype of tumor cells, allowing a decrease in N-cadherin and vimentin and an increase in E-cadherin.[20] In our study, the increase in protumoral characteristics observed in CaSki-T cells was largely dependent on the autocrine production of TGF-β1 because inhibition of this factor in cell cultures strongly reversed the manifestation of these characteristics in tumor cells. Indeed, TGF-β signaling is a key event contributing to the invasion and dissemination of tumor cells because of the induction of genes associated with EMT (Snail-1, Snail-22, Zeb-1/2, and Twist) and transcriptional repression of E-cadherin.[59] In CaSki-T cells, a significant increase in the expression of vimentin and the transcription factors Snail-1 and Twist was noted, which was significantly higher in CaSki-T cells than in CaSki-M cells. Snail-1 expression is correlated with the degree of tumor evolution, lymph node metastasis in various types of tumors, and a poor prognosis for patients with metastatic cancer. Recent studies indicate that Snail-1 causes metabolic reprogramming, induces CSC-like traits in tumor cells, and promotes drug resistance, tumor recurrence, and metastasis.[60] Therefore, the increase in protumor characteristics observed in CaSki-T cells may be related to Snail-1 overexpression resulting from the culture conditions. Therefore, in subsequent studies, analyzing whether high CD73 expression and the promotion of protumoral characteristics, such as overexpression of proteins related to EMT associated with high TGF-β1 production, occur in other CC cell lines cultured as tumorspheres will be interesting.

In the context of tumor development and progression, at least two important functions of CD73 have been described: (a) the catalytic activity of this enzyme to generate Ado from AMP hydrolysis and (b) the ability of CD73 to promote adhesion and modulate cell migration. Accordingly, the low CD73 expression found in CC spheres has been hypothesized to be related to tumor progression, leading to tumor migration and invasiveness.[43] Interestingly, our results provide evidence that high CD73 expression in CC spheres, which is related to high TGF-β1 production, also results in the promotion of protumoral characteristics related to tumor progression, such as migration and invasiveness, immune evasion, immunosuppression, and chemoresistance, suggesting that both functions of CD73 can be relevant in CC progression.

In a comprehensive molecular study of CC, the *TGFBR2* gene, which is present exclusively in cervical squamous tumors, was reported to be significantly mutated in more than 70% of the tumors analyzed,[61] illustrating the clinical importance of this route as a therapeutic target. In agreement with the present study, the alterations reported in the TGF-β signaling pathway in CC[61] can contribute significantly to the activity of the adenosinergic pathway and therefore promote tumorigenesis in the CC TME.

5 | CONCLUSION

This study provided evidence that high CD73 expression associated with high TGF-β1 production by CC cells cultured as tumorspheres strongly contributes to the induction of protumoral characteristics related to tumor progression, such as migration and invasiveness, immune evasion, immunosuppression, and chemoresistance.

AUTHOR CONTRIBUTIONS

Rosario García-Rocha and **Monserrat Carrera-Martínez**: Investigation, methodology, writing – original draft. **Katia Alhelí Monroy-Mora**, **María de los Ángeles Ponce-Chavero**, **María Luisa Escobar-Sánchez**, and **Gabriela Molina Castillo**: Investigation, methodology. **Christian Azucena Don-López, Juan José Montesinos-Montesinos, Benny Weiss-Steider**, and **Jorge Hernández-Montes**: Supervision, visualization. **Rommel Chacón-Salinas**, **Luis Vallejo-Castillo**, and **Sonia Mayra Pérez-Tapia**: Resources, validation. **María de Lourdes Mora-García** and **Alberto Monroy-García**: Conceptualization, Funding acquisition, writing – review and editing. All authors were involved in drafting the article or revising it critically for important

content, and all authors approved the final version to be submitted for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data displayed in this publication, including Supporting Information, are available from the corresponding author upon request.

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