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E7HPV16 Oncogene and 17beta-Estradiol Stress, Promotes Oncogenic microRNA Expression Patterns, Cell Proliferation and Cervical Intraepithelial Neoplasia 1

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ABSTRACT

Cervical cancer (CC) is the second cause of death by a neoplasia in woman in Mexico. Among the factors that contribute to its development are prolonged infection by a high-risk HPV type and the use of estrogens. It is well known that diagnosis at early stages is extremely important since, in most cases, progression towards carcinogenesis could be prevented, hence the importance of finding candidates that serve as early biomarkers. Several studies have shown that the expression level of the tumor suppressor miR-218 is diminished in CC while oncomiR miR-21 is overexpressed. On the other hand, it has been reported that the Potassium calcium-activated channel subfamily M alpha 1 (*Kcnma1*) oncogene, a known target gene of miR-218, is overexpressed in CC. However, there are few studies on the expression of this oncogene in Cervical Intraepithelial Neoplasia 1 (CIN 1). In this study, the analysis of the K14E7HPV16 carcinogenesis model in young mice (1.5-month-old), showed that a single-dose of 17β-estradiol (E_2) increased both the cell proliferation and the *Bcl-2* oncogene expression, as well as promoted the development of CIN 1. Interestingly, the hormonal stress and the E7 expression, favor the physiological response of the organism in transgenic young mice by decreasing the expression levels of the tumor suppressor miR-218 and increasing the expression of the *Kcnma1* and *Bcl-2* mRNA oncogenes in both, cervical tissue and serum. This work demonstrates the significance of a single E_2 stimulation and the expression of the HPV E7 oncoprotein in the early stage of cervical carcinogenesis. In addition, we provide strong evidence about *Kcnma1* oncogene as a target gene of miR-218 and that both could be used as early circulating biomarkers of CC.

1 | Introduction

Cervical Cancer (CC) occupies the third place among cancers with the highest incidence in women worldwide with 609,669 new cases and more than 296,318 deaths in 2020; 90% of these deaths occur in underdeveloped countries [1]. In Mexico, CC is the second most common type of cancer in women with 10,348 cases documented in 2022 alone and, in turn, represents the second cause of death due to neoplasia with an estimated 4,335 deaths in that same year [1]. It is well known that the main risk factor for CC is a persistent infection by the high-risk human papillomavirus (HR-HPV). However, although HPV is a necessary factor for the development of CC, it is not sufficient to give rise to malignant progression [2, 3]. HPV infection must act in conjunction with other risk factors such as: smoking, an early initiation of sexual life, multiple sexual partners, multiparity, immunosuppression and malnutrition, and one of the most relevant, the chronic use of estrogens [4]. It was described that chronic exposure to estrogens for more than 5 years in HR-HPV infected women favors the development of CC (Brake et al. 2005).

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Summary

This work represents an important study on the early stages of cervical carcinogenesis related to the effect of the E7 oncoprotein of the Human Papillomavirus and the hormonal environment. It is proposed that the validation of this murine model will be useful both for early diagnosis and for establishing possible treatments for early lesions of the cervix.

More than 99% of the CC cases are associated with HR-HPV infection (Mittal et al., 2017), being types 16 and 18 the most frequently detected [5, 6].

E7 oncoprotein plays a key role in cervical carcinogenesis since their continuous expression is necessary for the initiation and maintenance of the malignant phenotype [3, 6–8]. Previous studies have demonstrated that the K14E7HPV16 (K14E7) is a useful mouse model in the study of cervical carcinogenesis with 7-month-old mice developing cervical cancer after 6 months of chronic 17β-estradiol (E_2) stimulus [9–11]. In addition, we have demonstrated that a single E_2 dose within intraperitoneal cavity of 3-month-old K14E7 transgenic mice was sufficient to develop cervical epithelial severe hyperplasia, mild to severe dysplasia and cervical intraepithelial neoplasia (CIN 1) [12].

MicroRNAs, also known as miRNAs, are small noncoding molecules which carry out the negative regulation of the expression of various genes. These molecules are being recognized as possible biomarkers for diagnosis and prognosis of multiple diseases including CC [13, 14]. miR-218, a tumor suppressor miRNA, has been found to be downregulated in several types of cancers, including head and neck, gastric, and breast cancers [15, 16]. Various studies in CC have shown that the expression level of this tumor suppressor miRNA is differentially decreased not only between healthy tissues and tumor tissues but also varies with the degree of cervical lesion and type of cancer ([17, 18] Lung et al., 2015 [16]). On the other hand, increase of oncomiR expression levels leads to cell proliferation and inhibition of apoptosis, inducing cancer invasion and metastasis; as example, the oncomiR miR-21 is upregulated throughout cervical carcinogenesis [17, 18]. In addition, the role of *Bcl-2* gene as oncogene in cervical cancer (REF). These changes in the expression levels of miR-218, miR-21 and *Bcl-2* make them excellent candidates as biomarkers of CC, especially in early stages of carcinogenesis [17, 18].

2 | Materials and Methods

2.1 | K14E7HPV16 (K14E7) Transgenic Mice

Maintenance and genetic construction of K14E7HPV16 (K14E7) transgenic mice have been previously reported ([13, 14] Arvizu-Henandez et al., 2024). K14E7 transgenic mice were maintained in the FVB/n (FvB) inbred strain as transgenic heterozygotes. K14E7 were initially identified according to their phenotype. Additional in situ PCR detection of transgene construction was performed to confirm the genotype (Figure S1). All animals, transgenic and non-transgenic, were housed according to the American Physiological Society's Guiding Principles in the Care and Use of Animals and to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) guidelines. All mice procedures were approved by the Research Unit for Laboratory Animal Care Committee (UPEAL-CINVESTAV-IPN, Mexico; NOM-062-ZOO-1999).

2.2 | Induction of Hormonal Stress (I)

One and a half month-old virgin female transgenic (K14E7) and non-transgenic (FvB) mice were inoculated with a single dose of 833 ng of 17β-estradiol (E_2) (Riley et al., 2010) (Sigma-Aldrich, USA). 18 h after induction, the estrous cycle stage was determined, confirming its transition from Diestrus to Estrus (Figure S2). All induced (I) mice were euthanized in Estrus using a carbon dioxide euthanasia chamber followed by cervical dislocation according to institutional and international regulations (AAALAC; UPEAL-CINVESTAV-IPN, Mexico; NOM-062-ZOO-1999).

2.3 | Experimental Groups

Each experimental group comprised six-treated animals (FvB or K14E7). Additional groups of untreated-FvB and untreated-K14E7 mice in both stages, Diestrus or Estrus, were included as controls (six animals/group). Three animals from each experimental group were used for RNA purification, the other three animals were used for tissue procurement, paraffin-embedding and subsequent immunohistochemical and in situ PCR analyses. The animals in each group exhibited similar phenotypic and histological characteristics (Figure S3).

2.4 | Estrous Cycle Determination

The estrous cycle was determined in thirty-three 1.5-month-old female mice using exfoliative cytology and the Papanicolaou stain [19]. Cytological preparations were analyzed by bright field microscopy (Figure S4).

2.5 | Serum Samples

Immediately after the mice were killed, 100–500 μl of blood were collected by cardiac puncture from all experimental groups. Blood was allowed to coagulate, centrifugated at 4000 rpm for 10 min and serum was recovered. Samples were stored in TRIzol reagent (Ambion, Life Technologies, USA) at −20°C for later use.

2.6 | Tissue Procurement and Histopathological Analysis

The entire reproductive tract was paraffin-embedded, microtome sectioned and evaluated by an expert pathologist as previously described ([13, 14] Arvizu-Henandez et al., 2024). For each specimen, 5 μm thick histological sections were obtained to prepare distinct slides for light microscopy. The specimens stained with hematoxylin-eosin were evaluated by an expert pathologist. We studied whether the cervix of 1.5-month-old

virgin female transgenic (K14E7) and non-transgenic (FvB) mice single E_2-treated presented histological alterations in comparison with nontreated controls. Tissues were analyzed to determine if there was the presence of hyperplasia, hyperkeratinization, epidermoid metaplasia, inflammatory infiltrate and/or enlarged nuclei in basal and suprabasal epithelial cervical layers [20].

2.7 | Immunohistochemistry and In Situ PCR

Protein detection was performed using the Mouse/Rabbit PolyDetector HRP/DAB Detection System according to the manufacturer's instructions (Bio SB, USA) [13]. Tissue sections were analyzed using primary antibody against PCNA (Santa Cruz Biotechnology, USA). *In situ* PCR was performed as previously described [14]. Negative controls for in situ PCR included reactions without one of the primers and HPVE7-negative tissues. Photomicrographs were obtained using a DFC290 HD digital camera (Leica Microsystems, USA). The images were digitally processed to equalize the bright and contrast of images using the PhotoImpact software (Ulead PhotoImpact SE ver. 3.02; Ulead Systems, U.S.A.). Digital analysis of all captured images was performed as previously described ([13, 14] Arvizu-Henandez et al., 2024).

2.8 | Total RNA Isolation, Synthesis of cDNA and mRNA Quantification by Real-Time Quantitative PCR (RTqPCR)

Total RNA was isolated from serum or cervical tissue obtained from one and a half month-old, estrogen treated- and untreated-transgenic and FvB mice using TRIzol reagent according to the manufacturer's instructions (Ambion, Life Technologies, USA). Three micrograms of total RNA were reverse-transcribed in a 20 µl reaction consisted of 4 µl of 5X first strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM $MgCl_2$], 0.5 mM dNTPs (final concentration), 5 mM dithiothreitol, 15 U RNase Inhibitor (Thermo Scientific, USA), 2.5 µg oligo$(dT)_{12\text{-}18}$ and 200 U SuperScript II Reverse Transcriptase, following the manufacturer's specifications (Thermo Scientific, USA). The relative quantification of Growth Regulating Estrogen Receptor Binding 1 (*Greb1* [21]), Potassium Calcium-Activated Channel Subfamily M Alpha 1 (*Kcnma1* [22]) and B-cell lymphoma 2 (*Bcl-2*) mRNAs by Real-Time PCR was performed using a 7300 Real Time PCR System (Applied Biosystems, USA) as previously described ([13, 14] Arvizu-Henandez et al., 2024). Oligonucleotide primers were designed to be intron spanning and were purchased from Oligo T4 (Mexico) (Table 1). Sequences were obtained from the GenBank database. The amplification of each template was performed in triplicate in one PCR run. *Greb1* expression was determined as an E2-responsive gene. *Gapdh* mRNA level was determined as a housekeeping endogenous control.

2.9 | Quantification of miR-218 and miR-21 in Serum and Tissue by Real-Time Quantitative PCR (RTqPCR)

To detect the levels of mature miR-218 and miR-21 in murine serum and cervical tissue, 1–10 ng of total RNA was reverse-transcribed to cDNA with specific reverse transcription primers using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Stem-loop real-time PCR was used to establish miRNA levels by the TaqMan® MicroRNA assays [miR-218 (ID 000521); miR-21 (ID 000397); Applied Biosystems]. Real-time quantitative-polymerase chain reactions (RTqPCR) were performed using a StepOne Real Time PCR System (Applied Biosystems) following the manufacturer's specifications. The amplification of miR-218 or miR-21 was performed in triplicate in one PCR run. Relative expression levels were normalized to the expression of endogenous control snoRNA202 (ID 001232; Applied Biosystems). The data obtained in RTqPCR assays was analyzed using the equation described by Livak and Schmittgen, $2^{-\Delta\Delta CT}$ [23].

2.10 | Statistical Analysis

The Mann–Whitney U test was used to determine differences of miR-218, miR-21, *Greb1*, *Bcl-2* and *Kcnma1* expression levels within the experimental groups. The statistical analysis of the immunohistochemical assays was carried out from the relative quantification expressed as a percentage of positive signal of PCNA protein and subsequent ANOVA-post hoc Bonferroni analysis. Data are presented as mean ± standard deviation (S.D.) using GraphPad Prism Version 6.01 statistical software (GraphPad Software Inc. USA). Differences of $p \leq 0.05$ were considered statistically significant.

TABLE 1 | Oligonucleotide primers used in RTqPCR assays.

Name	Sequence (5′→ 3′)	Amplicon (bp)
Gapdh F	CATTGGCAATGAGCGGTTC	88
Gapdh R	GGTAGTTTCGTGGATGCCACA	
Greb1 F	ATGGCAGCGTGGATTTTTCTC	88
Greb1 R	GGGCTGTTTGAGTCAGACATTT	
Kcnma1 F	CGACAAACAGAATGCAACAAGG	190
Kcnma1 R	ACTCATGGGCTTGATTTGAATGT	
Bcl-2 F	ACTTCGCAGAGATGTCCAGTCA	177
Bcl-2 R	TGGCAAAGCGTCCCCTC	

3 | Results

3.1 | One-Dose Hormonal Induction (I) Increased *Greb1* Relative Expression in 1.5-month-old Mice

The relative expression of *Greb1* mRNA was measured as a control for hormonal stimulation. As expected, an increase in the relative expression of *Greb1*, an E_2 inducible gene, was observed in one-dose E_2-induced-FvB and K14E7 mice (FvB I and K14E7 I), compared to control groups in diestrus (FvB D and K14E7 D) or Estrous (FvB E and K14E7 E; Figure 1). Notably, a significant increase in the relative expression of *Greb1* was observed in the group of K14E7 I mice as compared to both FvB and K14E7 mice in the Diestrus phase (FvB D and K14E7 D). This result confirms the effect of hormonal treatment.

3.2 | A Single Dose of E_2 Altered the Architecture of the Cervical Epithelium and Increased the Development of Metaplasia in Early Stages of Cervical Carcinogenesis

The histopathology of the exocervix and the Squamous Columnar Junction (SCJ) of the cervical tissue of young mice was analyzed. The exocervical tissue of both the control group (FvB D) and FvB mice in Estrus (FvB E), showed a healthy cervical epithelium according to the stage of the estrous cycle phase in which they are found (Figure 2). Conversely, in the group of one-dose E_2-induced-FvB mice (FvB I), mild hyperplasia can be observed in which the normal structure and organization of the epithelium is lost. In K14E7 experimental groups, the presence of inflammatory infiltrate was observed. Additionally, in the group of K14E7 mice in Estrus (K14E7 E) a mild hyperplasia is observed while in the group of single-dose K14E7 Induced mice (K14E7 I) a severe hyperplasia and a possible Cervical Intraepithelial Neoplasia Type 1 (CIN 1) was observed (Figure 2). In the experimental groups without exogenous hormonal stimulus, the SCJ displayed a well-differentiated stratified squamous epithelium-columnar epithelium junction (SCJ; Figure 2). On the other hand, in the FvB I and K14E7 I mice groups, this area was more difficult to differentiate due to the metaplasia present in the columnar epithelium that begins to stratify at the endocervix level, in comparison with the control group (FvB D mice) in which the normal architecture of the columnar epithelium was preserved. The endocervix of FvB D experimental groups showed normal glandular characteristics, unlike the FvB E mice, where secretions can be observed inside the glands, which could be an indicator of high glandular activity. In the group of FvB mice with hormonal stress (FvB I), glandular hyperplasia is observed due to the formation of several layers of cells in the glandular epithelium whose normal architecture is usually in monolayer. In the case of K14E7 mice, a hypertrophic and hyperplastic epithelium is observed in all groups (K14E7 D, K14E7 E, K14E7 I). Although no significant differences were found in these groups, it can be observed that in the group of 17β-estradiol-induced-K14E7 mice (K14E7 I), there is an increase in the glandular hyperplasia compared with the other groups (Figure 2). These findings indicate that a single dose of E_2 is sufficient to induce alterations in cervical epithelial architecture.

3.3 | Single E_2 Treatment Increased the Cell Proliferation Levels in Exocervix of FvB and K14E7 Mice

Cell proliferation levels were determined based on the expression levels of the proliferation biomarker, PCNA protein (Figure 3). As expected, the exocervix of control group mice (FvB D) showed cells in proliferation restricted to the basal layer of the epithelium while FvB E mice, cell proliferation was not only found in the basal cells but also in suprabasal layer (homogeneously). On the other hand, in the group of hormonally induced mice (FvB I), nuclei with a positive signal can be

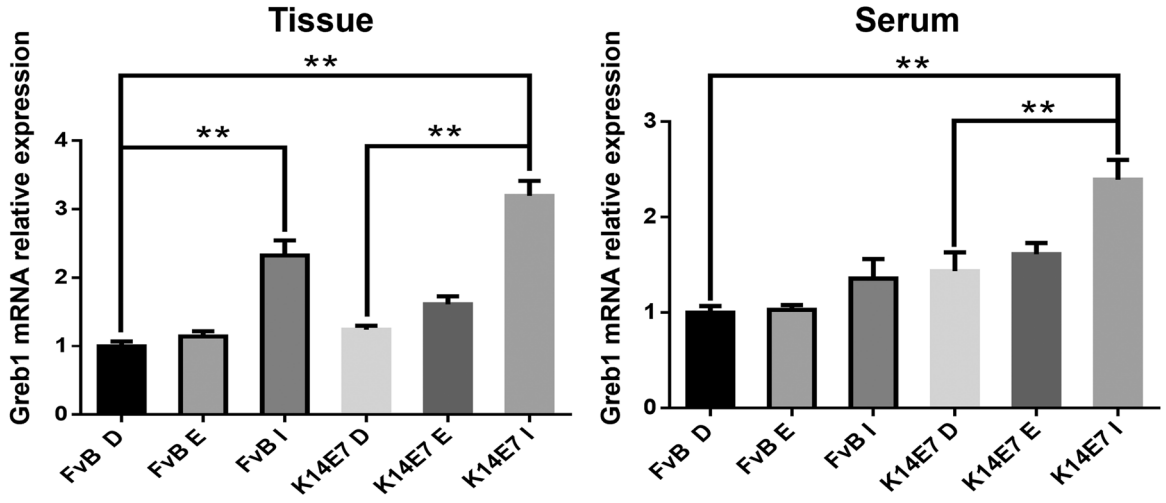

FIGURE 1 | Expression levels of *Greb1*, an estrogen-responsive gene, after single induction with E_2. *Greb1* expression was measured in both murine cervical tissue and serum and normalized using *GAPDH* expression as internal control, as described in Materials and Methods. Bars represent the mean. Error bars represent standard deviation (SD). ** Statistically significant ($p < 0.05$). FvB D: FvB mice in diestrus phase; FvB E: FvB mice in estrus phase; FvB I: E_2-induced FvB mice; K14E7 D: K14E7 mice in diestrus phase; K14E7 E: K14E7 mice in estrus phase; K14E7 I: E_2-induced K14E7 mice. Induction with E2 was as indicated in Materials and Methods. Groups comprising at least three animals of 1.5-month-old each were included.

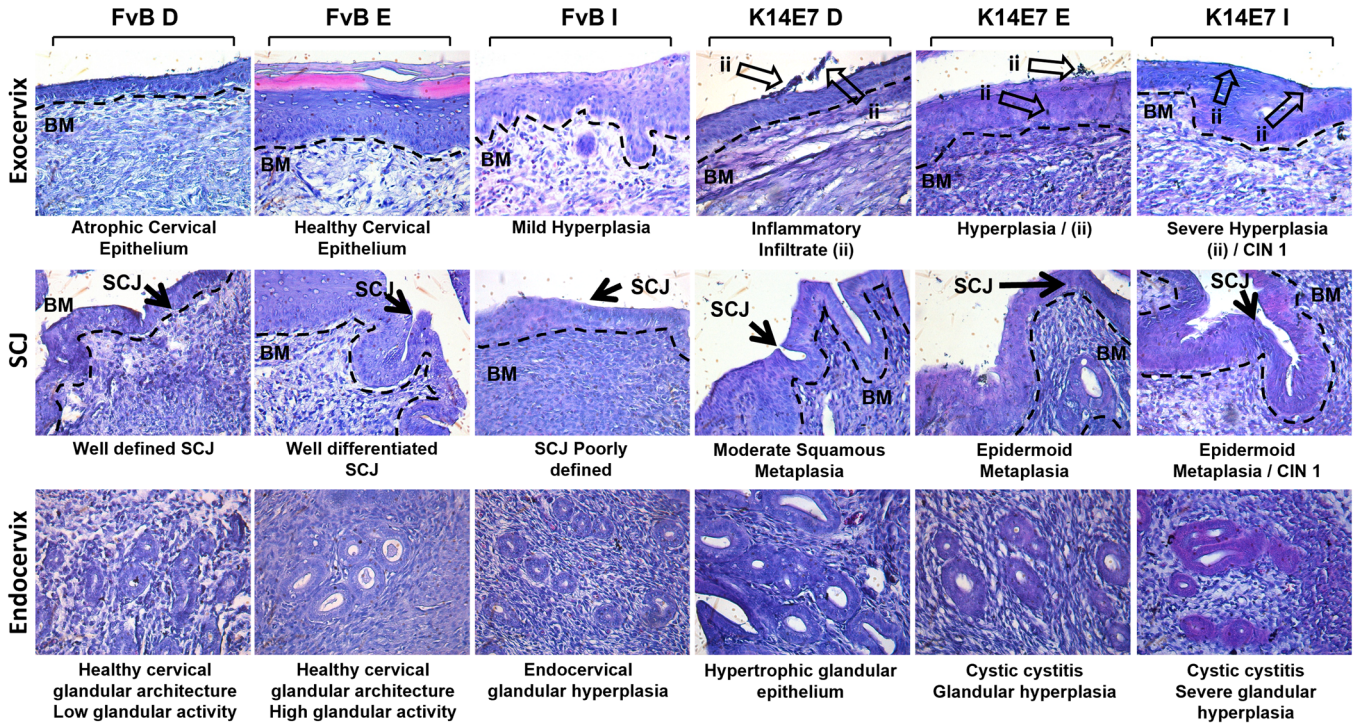

FIGURE 2 | Histopathological analysis of cervical tissue obtained from 1.5-month-old FvB mice (controls) and K14E7 transgenic mice in Diestrus (D), Estrus (E) and E_2-induced mice (I). Black arrows: Squamo-Columnar Junction (SCJ). BM: Basal membrane; ii: inflammatory infiltrate (empty arrows). Amplification: 40×. Groups comprising at least 3 animals each were included.

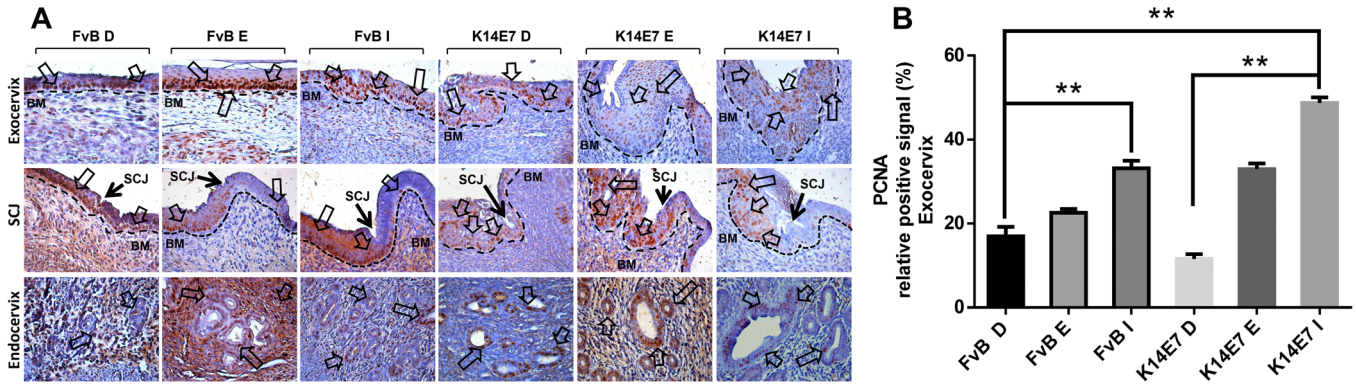

FIGURE 3 | (A) Cell proliferation levels as determined by PCNA protein detection in the cervix of FvB mice (control) and K14E7 transgenic mice in Diestrus (D), Estrus (E) and Estrus Induced with exogenous E_2 (I) at 1.5 months of age. Empty arrows: positive signal for PCNA. Black arrows: Squamo-Columnar Junction (SCJ). BM: Basal membrane. Amplification: 40×. Groups comprising at least three animals each were included. (B) Relative amount of immunohistochemical PCNA positive signal. These results are representative of at least three independent experiments. Statistically significant: ** ($p \leq 0.05$).

observed from the basal layer to the intermediate layer, including the suprabasal layer; it is noteworthy that cell proliferation was distributed randomly. In both, the K17E7 D and K14E7 E experimental groups, cells with a positive proliferation signal were distributed in a random way in almost all layers of the epithelium (Figure 3A); additionally, in the group of K14E7 I mice, an increase in the number of proliferating cells in all layers of the epithelium was observed (Figure 3).

In FvB D and FvB E experimental groups, the Squamous Columnar Junction (SCJ) showed no major differences in cell proliferation levels (between squamous and columnar epithelium); however, FvB I mice showed proliferating cells in the basal and suprabasal layers of the squamous epithelium but also in the columnar epithelium (Figure 3A). In the case of the K14E7 groups, an increase in proliferation was observed in the columnar epithelium as compared with FvB D mice. Likewise, it can be observed that in the squamous epithelium of the SCJ (metaplasia) proliferating cells are randomly distributed in almost all layers of the epithelium in the three K14E7 experimental groups. Once again, the highest levels of proliferation were shown by the K14E7 experimental groups compared to the FvB mice (Figure 3), particularly after a single dose of E_2. In the endocervix, an increase in proliferation levels in the glandular epithelium was observed in all experimental groups with respect to the control group (FvB D; Figure 3). As described for

exocervix and Squamous Columnar Junction, it can be observed that in the three groups of K14E7 mice there is a tendency to increase proliferation in the glandular epithelium as compared to the FvB mice. Notably, in the K14E7 I mice, several layers of proliferating cells can be observed associated to the glandular epithelium. Our results demonstrate that single E_2 treatment increased the cell proliferation levels in cervical tissue of FvB and K14E7 mice.

3.4 | The Expression of HPV E7 Under Hormonal Stress Modifies the Expression Profile of the Tumor Suppressor miR-218 and *Kcnma1* Oncogene in Early Stages of Cervical Carcinogenesis

miR-218 expression levels were determined using the FvB D mice as controls (Figure 4A). A decrease was observed in FvB E mice; however, FvB I mice showed an increase after hormonal induction (Figure 4A). In contrast, and expected, the relative expression levels of miR-218 were significantly decreased in all K14E7 experimental groups (K14E7 D, K14E7 E, and K14E7 I). Similar results were observed in serum samples (Figure 4A). On the other hand, the relative expression of *Kcnma1* oncogene was increased in FvB E and K4E7 D experimental groups (Figure 4B). In contrast, a significant decrease was observed in the group of FvB I mice (Figure 4B). Surprisingly, a significant decrease in the expression of this oncogene was observed in both K14E7 E and K14E7 I mice. Serum samples showed similar results (Figure 4B). The results obtained using the K14E7 murine model support previous findings that propose miR-218 as a suppressor of the *Kcnma1* oncogene.

3.5 | Hormonal Stress and the E7 Oncogene Increase the Expression of the miR-21 Oncomir and the *Bcl-2* Oncogene

miR-21 oncomiR expression levels were determined in both tissue and serum of the six experimental groups. Interestingly, the hormonal environment increased miR-21 expression in FvB E, FvB I, K14E7 D, K14E7 E, and K14E7 I experimental groups (Figure 5A). It is worth noting that the hormonal induced

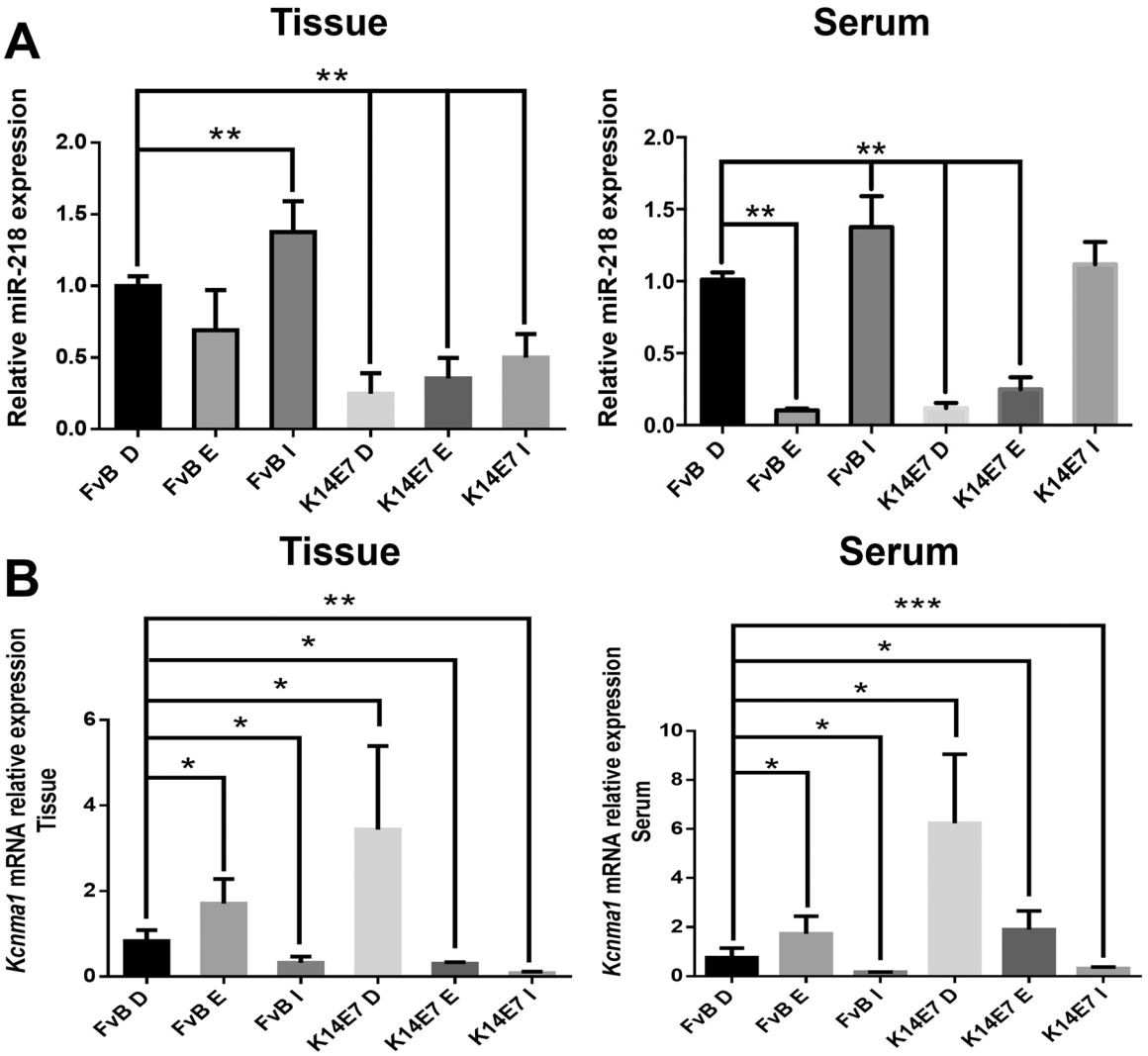

FIGURE 4 | Determination of expression levels of the tumor suppressor miR-218 (A) and the oncomiR miR-21 (B) in tissue and serum obtained from murine experimental groups. Bars represent the mean. Error bars represent standard deviation (SD). * Statistically significant ($p \leq 0.01$). ** Statistically significant ($p \leq 0.05$). Groups comprising at least three animals each were included.

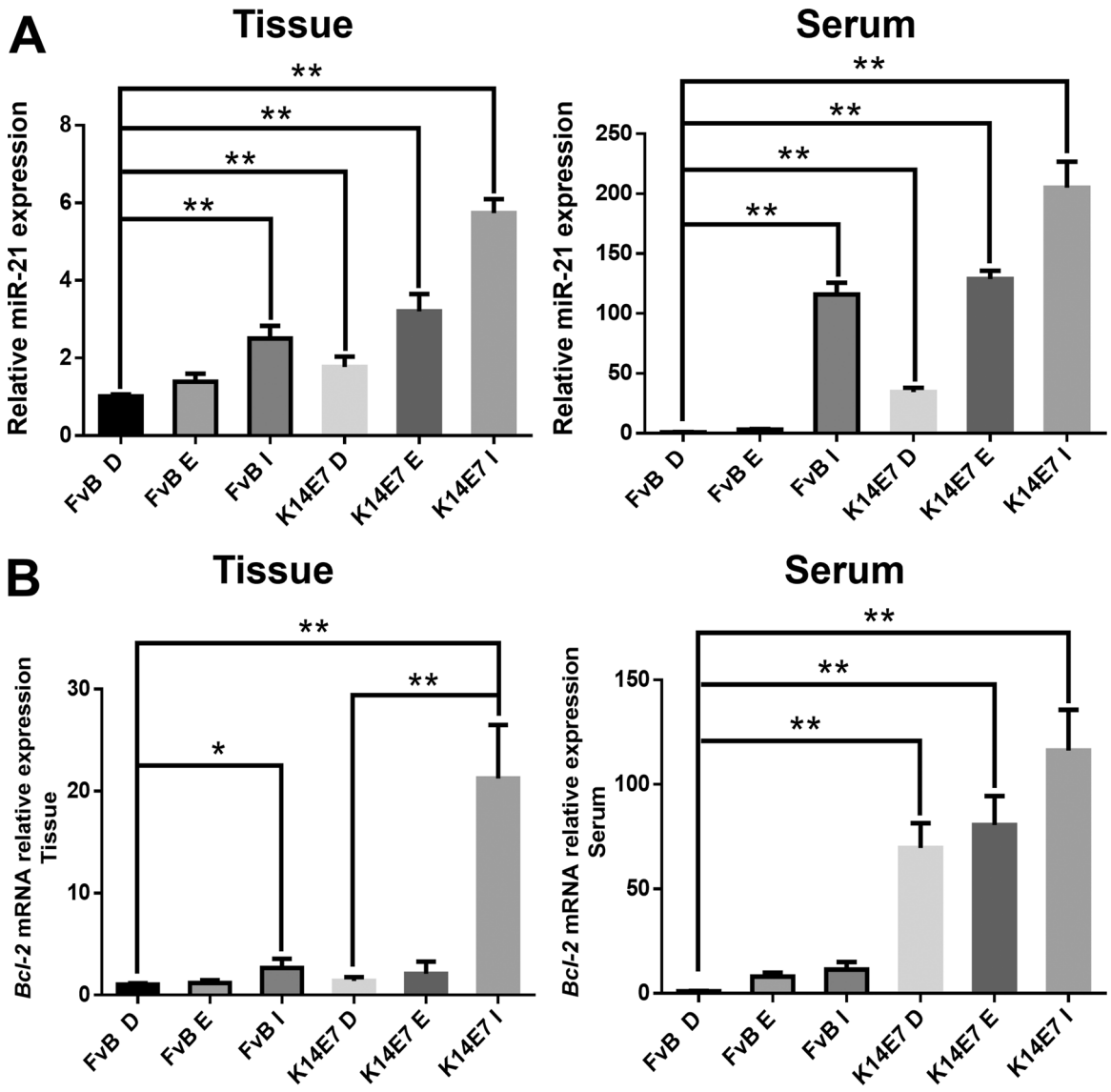

FIGURE 5 | Determination of expression levels of *Kcnma1* (A) and *Bcl-2* (B) oncogenes in cervix of murine experimental groups. Bars represent the mean. Error bars represent standard deviation (SD). Statistically significant ($p \leq 0.01$). ** Statistically significant ($p \leq 0.05$). Groups comprising at least three animals each were included.

groups, FvB I and K14E7 I, showed the highest levels of expression, being the induced transgenic mice group, the one that showed the highest expression (Figure 4B). These results were positively correlated with those obtained in samples from serum. As expected, a significant increase in the relative expression of *Bcl-2* oncogene was observed mainly in the K14E7 I mice both, in tissue and serum (Figure 5B). According to our findings, a single hormonal stimulation and the E7 oncogene increase the expression of the miR-21 oncomiR and the *Bcl-2* oncogene, both molecules proposed as early molecular biomarkers in cervical carcinogenesis.

4 | Discussion

Previous studies have shown that the HPV16 E7 oncoprotein in conjunction with 6-month chronic 17β-estradiol (E_2) treatment led to cervical neoplasia (9, 10). Recently, we observed that 3-month-old estrogen-treated K14E7 mice develop squamous metaplasia and CIN1, similar to human disease (Arvizu-Henandez et al., 2024). However, the effect of the HPVE7 oncoprotein has not been evaluated during the very early stages of carcinogenesis after a single exposure to 17β-estradiol (E_2). In the present work, young K14E7 mice (1.5 months of age, equivalent to 15–20 years in humans) expressing the HPV16 E7 oncoprotein, which simulates HR-HPV infection in young women, were exposed to hormonal stress through a single dose of E_2. These mice presented several characteristics suggesting that could represent an early CC carcinogenesis model.

In the exocervix of the three experimental groups expressing the E7 oncoprotein (K14E7 D, K14E7 E, and K14E7 I), inflammatory infiltrate was evident. This is important since chronic inflammation is a risk factor for cancer, as recognized by the National Cancer Institute [24]. Moreover, in K14E7 E mice cervical hyperplasia was observed, probably due to a synergy effect between E7 and E_2. Finally, and being the experimental group of greatest interest, K14E7 I mice showed, in addition to

the inflammatory infiltrate, severe hyperplasia that could indicate a possible CIN 1 according to the classification standards for this type of lesion [11] (Figure 2). The exocervix of control (non-transgenic) mice, exposed to hormonal stress (FvB I), showed histopathological changes in the architecture of the epithelium such as mild hyperplasia, suggesting that a single dose of estrogen was sufficient to promote an increase in epithelial cell proliferation (Figure 2). This was confirmed using the cell proliferation marker PCNA (Figure 3). The murine cervical squamous columnar junction (SCJ) showed that in the presence of the E7 oncoprotein, this zone becomes less differentiated in comparison with FvB mice (Figure 2), since the columnar epithelium begins to stratify, an effect that is increased with the presence of E_2 leading to an epidermoid metaplasia as compared with FvB. In FvB control mice under one-doses 17β-estradiol stress and in all K14E7 experimental groups endocervical glandular hyperplasia was observed as well as the presence of cystic cystitis (Figure 2).

The histopathological changes (increase in cell proliferation and abnormal modification of the epithelium architecture, premalignant lesions) observed in the exocervix, the SCJ and the endocervix coincide with other reports using chronic estrogen treatments ([10, 11, 25] Gómez-Gómez et al., 2003 [9, 14]).

As mentioned earlier, we observed that after one-single dose of E_2, cell proliferation increases homogeneously in different layers of the FvB I exocervix. In comparison with the FvB D control group, in the exocervix of K14E7 experimental groups, we observed both an increase of cell proliferation and a heterogeneous distribution of proliferating cells in different layers of the epithelium (Figure 3). Interestingly, in SCJ, proliferating cells were detected in the columnar epithelium. This coincides with what was previously reported in the literature [7, 11, 26].

In this work, we studied the expression levels of miR-218, a tumor suppressor miRNA and the oncomiR miR-21. Under all three experimental conditions, we observed that in cervical tissue, the presence of E7 was sufficient to significantly reduce the expression levels of the tumor suppressor miR-218 with respect to control groups (Figure 4A). This aligns with different works in which it was demonstrated that the expression of miR-218 is significantly reduced in patients infected with HPV16 [17, 27, 28]. This is the first report suggesting that E7 inhibits the expression of this tumor suppressor miRNA early in cervical carcinogenesis. Thus, Martínez et al. (2008) observed that, in cervical carcinoma cells, the E7 oncoprotein is capable of reducing miR-218 expression levels as observed in the present work. This could be achieved at the epigenetic level [29], since it is known that E7 induces the epigenome reprogramming through DNMT1, increasing promoter DNA methylation patterns ([3] Mittal et al., 2017 [15]).

Interestingly, our results show that the levels of miR-218 tend to rise slightly in FvB I as compared with FvB D mice (Figure 4A). This could be explained as a response of the organism since these are very young mice without E7 oncoprotein effect, which probably try to compensate the increase in cell proliferation and histopathological alterations by raising the levels of miR-218 tumor suppressor as it was observed previously detecting other microRNAs in same mice at early time of chronic hormonal treatment [14]. It is noteworthy to mention that it has been seen that estradiol is capable of stimulating the biogenesis of miRNAs through different mechanisms and signaling pathways, increasing their expression levels [30]. On the other hand, significant differences were observed in the expression of this miRNA in the serum of FvB I mice (increased expression) and K14E7 D and E mice (diminished expression) compared to the FvB D control group. Although the overall behavior of miR-218 expression in serum tends to mirror that observed in tissue, slight variations were present that did not reach statistical significance. This variability in serum could result from differences in extracellular vesicle-mediated transport, miRNA stability in circulation, or systemic homeostatic mechanisms [18, 31]. Despite this, the overall behavior of miR-218 expression tends to be consistent between tissue and serum, as observed in other contexts, such as temperature regulation [32]. This suggests that miR-218 could still serve as a promising noninvasive biomarker for early diagnostic cancer methods [12].

According to the inversely proportional relationship between the expression of miR-218 and KCNMA1 (Figure 4), this work supports that of Oeggerli et al. [33] suggesting that *Kcnma1* oncogene is a target of tumor suppressor miR-218. In our model, it was observed that in the group of control mice in Estrus (FvB E), the hormonal stimulus with E_2 increased the expression of *Kcnma1* (also known as Slo gene) and decreased that of miR-218, probably due to the estrogen response elements in the Slo promoter [34]. Surprisingly, FvB I mice showed the lowest *Kcnma1* expression levels, while the sole presence of E7 (K14E7 D) was sufficient to significantly increase the expression levels of this oncogene. On the other hand, in the K14E7 E and K14E7 I experimental groups, the expression of *Kcnma1* is significantly decreased ($p \leq 0.01$), contrary to what was observed by Ramírez et al. Ramírez et al., [35]. This discrepancy may be attributed to mice age and longer E_2 treatment used in that work (4- and 7-month-old chronically E_2-treated FvB and K14E7 mice), while in this work, we analyzed young mice with a very short time of estrogen exposure. A plausible explanation for the decrease in *Kcnma1* expression levels in the K14E7 mouse groups is the regulation of the expression of this gene by miR-218. These findings favor the hypothesis that *Kcnma1* could be a target gene of miR-218, according to what was reported in previous studies using bioinformatics methods carried out by Venkataraman et al. Venkataraman et al., [36] our work being, to date, the first experimental work that supports this bioinformatic study.

miR-21 is a well-characterized oncomiR that regulates various pathways involved in tumorigenesis, including cell proliferation, apoptosis, and metastasis. It is well known that this oncomiR is frequently overexpressed in several cancers including cervical cancer [37] in fact, HPV-positive cervical cancer samples show increased miR-21 expression throughout the cervical carcinogenesis, making it a notable biomarker [37]. Here, we demonstrated that a single dose of E_2 increases miR-21 levels in FvB I mice (Figure 4B). As expected, we also found in all K14E7 experimental groups (K14E7 D, K14E7 E, and K14E7 I) a strong upregulation of miR-21 compared to controls. Our results indicate that the HPV16 E7 oncoprotein alone induces miR-21 expression in vivo, with E_2 acting synergistically to further enhance this effect. It is well established that E7 expression and E_2

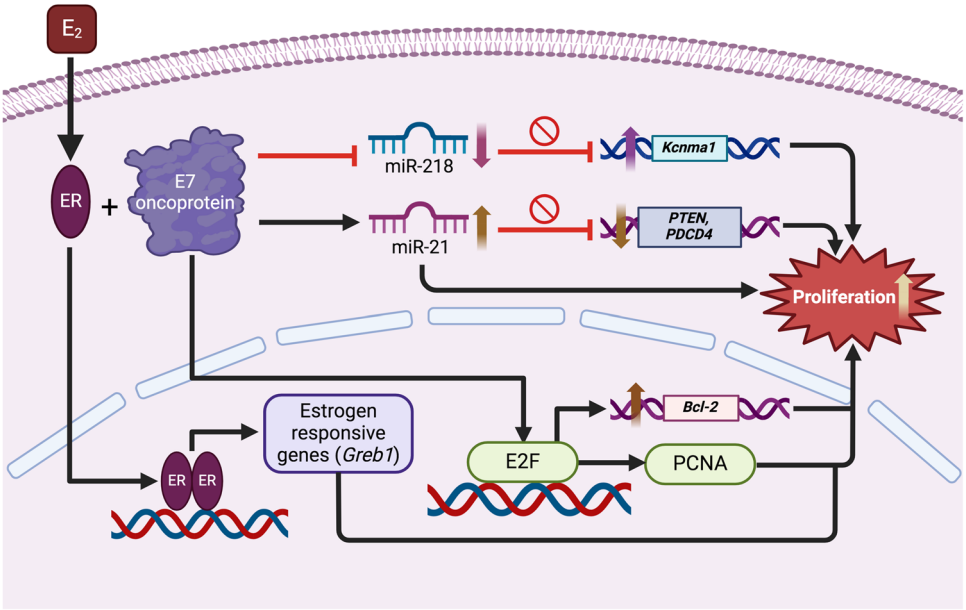

FIGURE 6 | A schematic model of the mechanism underlying the role of 17β-estradiol (E_2) and E7 oncoprotein in early stages of cervical carcinogenesis. We confirmed the effect of E_2 through the determination of the expression of the estrogen-responsive gene *Greb1*. Our results indicate that HPV16 E7 oncoprotein and E_2 decrease the miR-218 expression levels and induce the overexpression of the oncomiR miR-21. In addition, *Kcnma1* and *Bcl-2* oncogenes overexpression increases cell proliferation levels and induces histopathological cervical alterations in the cervix of 1.5-month-old transgenic mice.

activate antiapoptotic pathways, which, in turn, stimulate the AP-1 transcription factor and drive the expression of genes involved in cell proliferation, including miR-21. This synergistic interaction between E7 and hormonal stress likely amplifies miR-21 expression, promoting oncogenic processes.

Some of the primary targets of miR-21 are *PTEN* (phosphatase and tensin homolog), a key negative regulator of the PI3K/AKT pathway, and *PDCD4* (programmed cell death protein 4), a tumor suppressor critical for apoptosis. The downregulation of these targets by miR-21 contributes to increased cell proliferation, resistance to apoptosis, and heightened invasiveness, processes that are central to cervical and other cancers [38].

In our study, the observed upregulation of miR-21 in K14E7 I mice may partly explain the elevated levels of cell proliferation and the inhibition of apoptosis associated with early cervical carcinogenesis.

On the other hand, it has been reported that the overexpression of *Bcl-2* is not only related to elevated cell proliferation levels but also with resistance to apoptosis in E7-transfected cells. Additionally, E_2 inhibits apoptosis partially by the induction of *Bcl-2* transcription [37]. Using a similar early cervical carcinogenesis model system, we have recently detected this *Bcl-2* upregulation in mouse cervical tissue, as well as the generation of CIN 1 lesions in 3-month-old K14E7 mice, suggesting that, in addition of a single estrogenic treatment, chronic activity of the E7 oncoprotein was necessary to induce upregulation of *Bcl-2* in mouse cervical tissue (Arvizu-Hernandez et al., manuscript in preparation). Accordingly, in this work, we found that *Bcl-2* mRNA expression levels were increased in FvB I mice and mainly in K14E7 I mice, confirming the response to 17β-estradiol induction in very young animals (Figure 5B).

In conclusion, a hormonal stimulus with a single dose of 17β-estradiol and the E7 oncoprotein expression was sufficient to increase cell proliferation levels and induce histopathological cervical changes (possible CIN 1) in the cervix of 1.5-month-old transgenic mice. These effects were reinforced by a decrease in miR-218 level and by the overexpression of the oncomiR miR-21. In addition, the single-dose hormonal stimulus and E7 expression can favor the expression of the *Kcnma1* and *Bcl-2* oncogenes, allowing increased cell proliferation, severe hyperplasia, and epidermoid metaplasia. These findings demonstrated that hormonal stress via single E_2-dose and the E7 oncoprotein expression contribute to the development of early cervical lesions, as well as genetic (*Kcnma1, Bcl-2*) and epigenetic (miRNAs) alterations. Circulating miR-218, miR-21, *Kcnma1* and *Bcl-2* expression may be useful early diagnostic biomarkers of Cervical Cancer (Figure 6).

Author Contributions

All authors contributed to the study conception and design. P.G. and R.O.D advised the development of this work. The maintenance and care of murine strains, material preparation, experimental procedures, data collection and analysis were performed by E.A.H. and R.O.D. The first draft of the manuscript was written by E.A.H., R.O.D. and P.G. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethics Statement

All mice procedures were according to the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) regulations and approved by the Committee of the Laboratory Animal Research and Care Unit (UPEAL-CINVESTAV-IPN, Mexico; NOM-062-ZOO-1999).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data included in this study are available upon request by contacting the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.