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Effect of Sex Steroid Hormones on Tongue Cancer Cells *In Vitro*

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Short Running title: Sex Steroid Hormones in Tongue Cancer

Abstract. Background: Tongue cancer is more common in men than in women. Yet the effects of sex steroid hormones on the behaviour of oral tongue squamous cell carcinoma (OTSCC) are not well known. Matrix metalloproteinase 8 (MMP8) is expressed in OTSCC and can degrade estrogen receptors (ERs). Materials and Methods: Western blot was used to examine the levels of ERβ in OTSCC cell lines (HSC-3 and SCC-25). We evaluated the effects of estradiol and dihydrotestosterone (DHT) on HSC-3 and SCC-25 cell migration, invasion and viability. The effect of estradiol on the invasion of MMP8-overexpressing ($MMP8^+$) and empty vector HSC-3 cells was examined using 3D spheroid invasion assay. Results: Both HSC-3 and SCC-25 cells expressed ERβ. In scratch assay, estradiol, but not DHT, reduced the migration and invasion of HSC-3 and SCC-25 cells. $MMP8^+$ HSC-3 cells showed weaker invasion than empty vector cells, in line with previous reports. However, *MMP8* transfection did not alter the effect of estradiol on HSC-3 cell invasion in spheroid assay. Conclusion: Estradiol inhibited the migration and invasion of OTSCC cells, whereas DHT had no effect. Our data suggest that MMP8 does not modulate the effect of estradiol in OTSCC cells. However, the sex difference in OTSCC incidence might partly be due to protective actions of estradiol in epithelial cell carcinogenesis.

Oral (including lip) cancer accounts for approximately 2% of all cancer cases worldwide (1, 2). Of all oral cancer cases, 90% are squamous cell carcinomas (SCCs), and these are most often situated on the tongue (3, 4). The main risk factors for oral cancer are smoking and alcohol consumption (5), but leukoplakia, erythroplakia and lichen or lichenoid mucosal changes may also increase the risk (6). Early diagnosis and healthy lifestyle choices improve the prognosis (7). Previous studies have shown that the incidence of head and neck cancer, including oral tongue squamous cell carcinoma (OTSCC), is higher in men than in women (8-10).

Estrogens – female sex steroid hormones – are cholesterol-derived C18 steroids and comprise three major members: Estrone, estradiol and estriol. Estradiol is produced in the theca and granulosa cells in the ovaries, whereas estrone and estriol are produced from estradiol in the liver. After synthesis, 17β-estradiol is secreted into the bloodstream, where it binds to sex hormone-binding globulin and albumin. Free estrogens diffuse into target tissues to exert their specific genomic or non-genomic effects (11). Testosterone is an androgen-based male sex steroid hormone. Testosterone is synthesized from cholesterol in Leydig testicular cells and in smaller amounts in, for example, the skin, adipose tissue and adrenal glands (12).

Oral mucosa and salivary glands are sensitive to estrogens (13). The genes encoding estrogen receptors (ERs) α and β are located in different chromosomes, and the two receptor subtypes vary in their structure, particularly the ligand-binding domains are dissimilar (11). Estrogens act *via* nuclear ERs, cytoplasmic/membrane-localized ERs or membrane-associated ERs (14). The expression of the ER subtypes in the oral tissues is partly controversial. ERα is not present in the buccal or gingival epithelium of the mouth or in salivary gland tissue, whereas ERβ is widely detected (13, 15). This suggests that estrogens may act *via* ERβ in oral tissues and explain the

effect of hormonal changes on the oral mucosa and on saliva secretion and composition (13). Korpi *et al.* proposed that matrix metalloproteinase 8 (MMP8) has a protective, probably estrogen-related role in the growth of mobile tongue SCCs (16). Their data showed that expression of MMP8 was positively associated with improved survival of patients, and the tendency was particularly prominent in females. They detected ERβ in OTSCC cells and found that it was cleaved by MMP8 *in vitro*. However, no evidence emerged about whether this cleavage caused functional differences in OTSCC cell response to estrogens.

We aimed to investigate the effects of estrogen and dihydrotestosterone (DHT) on various functions of OTSCC cells *in vitro*. We also examined whether the previously observed proteolytic action of MMP8 on ERβ affected estrogen function in OTSCC cells.

Materials and Methods

Cell lines. A highly aggressive, metastatic OTSCC cell line, HSC-3 (Japan Health Science Research Resources Bank, Osaka, Japan), and a less aggressive, primary OTSCC cell line, SCC-25 (ATCC, Manassas, VA, USA), were used in this study. The creation of MMP8-overexpressing ($MMP8^+$) HSC-3 and SCC-25 cell lines and the corresponding empty vector cell lines has been described by Åström *et al.* (17).

The cells were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 medium (DMEM/F-12; Life Technologies, Gibco, Paisley, UK) supplemented with 10% foetal bovine serum (FBS; Life Technologies), 50 µg/ml ascorbic acid, 0.4 µg/ml hydrocortisone, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml fungizone amphotericin B (all from Sigma-Aldrich, St. Louis, MO, USA) in a humified incubator at 37°C with 5% CO_2. EDTA-trypsin solution was used to detach the cells (Sigma-

Aldrich).

Estradiol (Sigma-Aldrich) was first dissolved in 70% ethanol and used at concentrations of 20 nM, 100 nM and 500 nM in the experiments, with 100 nM representing the concentration in women. DHT (Sigma-Aldrich) was used at concentrations of 2, 10 and 50 nM, with 10 nM representing the physiological concentration in men.

Western blot. Subconfluent cells were washed twice with PBS and lysed into elution buffer Tris-HCl, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35 (Sigma-Aldrich) with complete protease inhibitor tablets (Sigma-Aldrich). After a total of 2 h on ice and brief sonication, the lysates were clarified by centrifugation at 14000×g for 15 min at 4°C. Protein concentrations were determined by Nanodrop and 30 µg of protein extract was separated under reducing conditions on 12% sodium dodecyl sulphate–polyacrylamide gel. The proteins were transferred into Immobilon P polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA) and blocked with Odyssey® Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA). The primary antibodies rabbit anti-ERβ (1:1,000; Abcam, Cambridge, UK) and mouse anti-β-actin (1:2,000; Abcam) were allowed to bind overnight at 4°C. The membrane was washed thrice for 5 min with TBS-Tween®20 (0.05%), and IRDye® 680RD goat anti-rabbit IgG or IRDye® 800CW goat anti-mouse IgG secondary antibody (1:10,000; LI-COR Biosciences) was allowed to bind for 50 min at room temperature. After removing the secondary antibody and washing the membrane thrice for 5 min with TBS-Tween®20 (0.05%), an Odyssey scanner (LI-COR Biosciences) was used to image the membrane. The band intensities were measured by Fiji (18) and the values obtained for ERβ and β-actin were used to calculate the relative protein levels.

Scratch assay. The 96-well ImageLock plates (Essen BioScience, Ann Arbor, MI, USA) were coated with human tumour-derived protein preparation Myogel (19) mixed with collagen. The mixture was prepared in serum-free DMEM (Life Technologies) with 0.3 mg/ml Myogel and 0.3 mg/ml rat collagen (Corning Inc, Corning, NY, USA). Of the mixture, 50 µl was pipetted into the wells, and the plate was placed in a cell culture incubator (37°C, 5% CO_2, 95% humidity) for 24 h. The cells were then detached and counted with a Scepter automatic cell counter (Millipore Corporation, Billerica, MA, USA). Altogether 25,000 cells in 100 µl complete DMEM/F-12 medium (Life Technologies) were added to each well and left in the cell culture incubator (37°C, 5% CO_2, 95% humidity) for 24 h. WoundMaker (Essen BioScience) was used to achieve homogeneous scratch wounds on the cell layers. After scratching, the medium was removed (migration plate) and 100 µl of serum-free DMEM with or without estradiol (20, 100, or 500 nM) or DHT (2, 10 or 50 nM) was added. In the case of the invasion assay, the medium was removed and 50 µl of Myogel/collagen mixture with/without agents at the concentrations above was added to the wells. The invasion plate was then incubated for 30 min. After the gel solidified, 50 µl serum-free DMEM was added. The wells were monitored using IncuCyte Live-Cell Imaging System (Essen Sartorius, Essen Bioscience), and images were taken at 1-hour intervals. The experiments were performed thrice as described above. To determine the statistical significance, we performed one-way analysis of variance (ANOVA) followed by Bonferroni correction with statistical significance set at $p<0.05$.

Spheroid invasion assay. The 3D-spheroid assay system was used to study cancer cell invasion (20). Spheroid invasion was performed with $MMP8^+$ and empty vector

HSC-3 cells. SCC-25 cells were not examined here because these cells did not form proper, round-shaped spheroids. The spheroids were established in a U-shaped 96-well Clear Ultra Low Attachment Microplate (Corning). One thousand cells in 50 µl of full media were dispensed into each well. The plate was placed in an incubator (37°C, 5% CO_2, 95% humidity), and after 4 days, the tumor spheroids were visible by eye. Then Myogel/fibrinogen mixture was prepared as described in Naakka *et al.* (20) and added to the wells (50 µl). The plate was incubated at 37°C, allowing the gel to solidify for 30 min. After this, 100 µl of complete DMEM with the desired concentration (0, 20, 100, or 500 nM) of estradiol was added to the wells. The spheroids were then imaged daily for 4 days using an inverted light microscope (4× objective). Three spheroids were examined for each condition, and the experiment was repeated four times. Analysis of spheroid invasion area was performed using Ilastik (freeware) and Fiji/ImageJ 1.51 software (18), as described in Naakka *et al.* (20).

Cell proliferation and viability assays. To investigate cell proliferation and viability, 1,000 HSC-3 and SCC-25 cells were exposed to 20, 100 and 500 nM estradiol in 96-well plate. Plates were imaged by IncuCyte Zoom (Essen Sartorius, Essen Bioscience) and proliferation was detected based on IncuCyte Proliferation Assays for Live-Cell Analysis. After 3 days, the plate was removed from the incubator to room temperature for 15 min before starting the viability assay. Viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) (21). One hundred microlitres of CellTiter-Glo was dispensed into each well. The plate was put on a plate shaker (Heidolph, Schwabach, Germany) at 450 rpm for 5 min and in a plate spinner (Thermo Scientific, Waltham, MA, USA) at 1,000 rpm for an additional 5 min. Finally, the plate was placed in the BMG PHERAstar FS (BMG Labtech, Offenburg,

Germany) plate reader to measure cell viability.

Results

Expression of ERβ in OTSCC cells in vitro. We first confirmed the expression of ERβ in both HSC-3 and SCC-25 cell lines. $MMP8^+$ HSC-3 and SCC-25 cells showed a slight decrease in the level of ERβ compared with the empty vector cell line (Figure 1).

In empty vector SCC-25 cells, a lower narrow band was observed, but it was not present in $MMP8^+$ cells. This might be due to the ability of MMP8 to degrade ERβ. Empty vector cells possibly have a proteolytically processed form of ERβ that is further processed by MMP8, when present. The lower molecular weight form might also be post-translationally differently modified, which might expose it to MMP8 degradation (Figure 1).

Estradiol inhibits the migration and invasion of OTSCC cells whereas DHT has no effect. In HSC-3 cells, estradiol reduced the migration and invasion in IncuCyte scratch wound-healing assay, regardless of the concentration used. However, due to high variation, the difference reached statistical significance ($p<0.05$) only at timepoint 16 h in the invasion assay. In SCC-25 cells, a similar trend was observed; estradiol reduced migration and invasion in dose-dependent manner (Figure 2). DHT did not have an effect on migration or invasion in either of the OTSCC cell lines. Since the results between individual experiments varied markedly, no statistically significant differences were detected (Figure 3).

Overexpression of MMP8 in HSC-3 cells did not change the effects of estradiol

on cells. We used a spheroid invasion model to investigate whether MMP8 modulated the effect of estradiol on HSC-3 cells. In this assay, estradiol had no effect on $MMP8^+$ or empty vector HSC-3 cells (Figure 4A). Invasion by $MMP8^+$ cells was significantly reduced compared with empty vector HSC-3 cells in the spheroid assay (Figure 4B) but MMP8 expression did not change the response of the cells to estradiol.

Estradiol did not have a significant effect on proliferation or viability of OTSCC cells. As estradiol reduced OTSCC cell migration and invasion, we investigated whether it was due to the reduction of cell proliferation or viability. However, estradiol had no effect on the proliferation and viability of OTSCC cells (Figure 5).

Discussion

Incidence of head and neck cancer, including OTSCC, is higher in men than in women and in older populations in general (8-10). However, the incidence of OTSCC is increasing in young women due to unknown aetiology (22). Sex steroid hormones, especially estradiol, play a role in carcinogenesis through binding to estrogen receptors. Ishida *et al.* showed that ER antagonist (tamoxifen), but not agonist (estradiol), induced cell death of cultured oral SCC (23). We investigated the effects of estradiol and DHT on the behaviour of OTSCC cells. As MMP8, which is an enzyme expressed in oral cancer, degrades ERs (16), we aimed to determine whether it has a role in modulating the effects of estradiol on OTSCC cells. Based on our findings, estradiol, but not DHT, reduced the migration and invasion of OTSCC cells, but MMP8 did not modulate this effect.

A total of 354,864 new oral cancer cases (including of the lip) were diagnosed worldwide in 2018, 108,444 of these were diagnosed among women (2). There has

been a slight rise in oral cancer rates among women, whilst incidence among males has been decreasing (24). Moreover, according to an earlier study from the USA, the incidence of tongue cancer increased from 1973 to 2012 among young (<50 years) people, especially women, although most did not smoke or use alcohol compared with previous generations (22). In addition to hormonal differences, there are many other factors that might protect women from OTSCC and other cancer types (8-10). For example, men usually consume less fruit and vegetables than women (25). A meta-analysis by Pavia *et al* examined the association of fruit and vegetable consumption with oral cancer and showed that daily fruit intake reduced the risk of oral cancer by 49% and high vegetable consumption by 50% (26).

Several studies have demonstrated the protective role of $MMP8^+$ in various types of cancer (27), including oral cancer (16). Korpi *et al.* proposed that some of the actions of $MMP8^+$ in OTSCC might be related to its ability to cleave ERβ. In our *in vitro* models, the cells with MMP8 overexpression did not behave differently from the empty control cells during estradiol treatment (16). However, in western blot, we observed a slight difference in the expression pattern of ERβ in $MMP8^+$ OTSCC cells compared with controls, which might have been due to proteolytic actions of MMP8. Regardless of this, the actions of MMP8 on ERs may not alter the effects of estradiol on tongue carcinoma cell functions such as motility. However, it is possible that the action of MMP8 on ERs plays some modulatory role in the early steps of cancer progression, for example, during malignant transformation. In our empty vector and $MMP8^+$ HSC-3 cell lines, the transduction procedure itself might have caused some changes in the cell response to estradiol because even the control cell line (transduced with empty vector) was not affected by estradiol in all experiments and the responses of both control and $MMP8^+$ cells to estradiol varied markedly between individual experiments.

Although our viral transduced HSC-3 cells did not respond to estradiol, we found that in parental HSC-3 and SCC-25 cell lines, estradiol, but not DHT, reduced cell migration and invasion. Hence, estradiol may act protectively in OTSCC and partly explain why women less frequently have oral cancer. In line with this reasoning, Lindblad *et al.* showed that a male cohort exposed to estrogen had a reduced risk of gastric cancer (28).

In conclusion, our study reveals that estradiol may have some protective role in tongue cancer by reducing cancer cell migration and invasion. In addition to lifestyle choices, this might partly explain why females in general less frequently have tongue cancer. This protective effect is not affected by the presence of MMP8, at least not in aggressive tongue carcinoma cells. As the incidence of OTSCC in young women seems to be on the rise for unknown reasons, it is important to pay attention to all factors that might affect the levels of female sex steroid hormones in oral tongue mucosa.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Johanna Peltonen, Katja Tuomainen and Tobias Sallinen carried out the experiments and interpreted the results. Islaam Faress and Pirjo Åström designed the study, carried out the experiments and interpreted the results. Ilida Suleymanova performed the analysis of the experiments. Ahmed Al-Samadi and Tuula Salo designed the study and interpreted the results. All Authors critically reviewed the article.

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Figure legends

Figure 1. Expression of ERβ was confirmed by western blot in both HSC-3 and SCC-25 cell lines. SCC-25 cells showed a small lower molecular weight band, which disappeared in $MMP8^+$ cells (A). $MMP8^+$ HSC-3 (B) and $MMP8^+$ SCC-25 (C) cells showed slightly reduced levels of ERβ compared with the empty vector cell line. The densitometer value for ERβ in SCC-25 cells was measured from the upper band only. Data are means ± standard deviation of two blots.

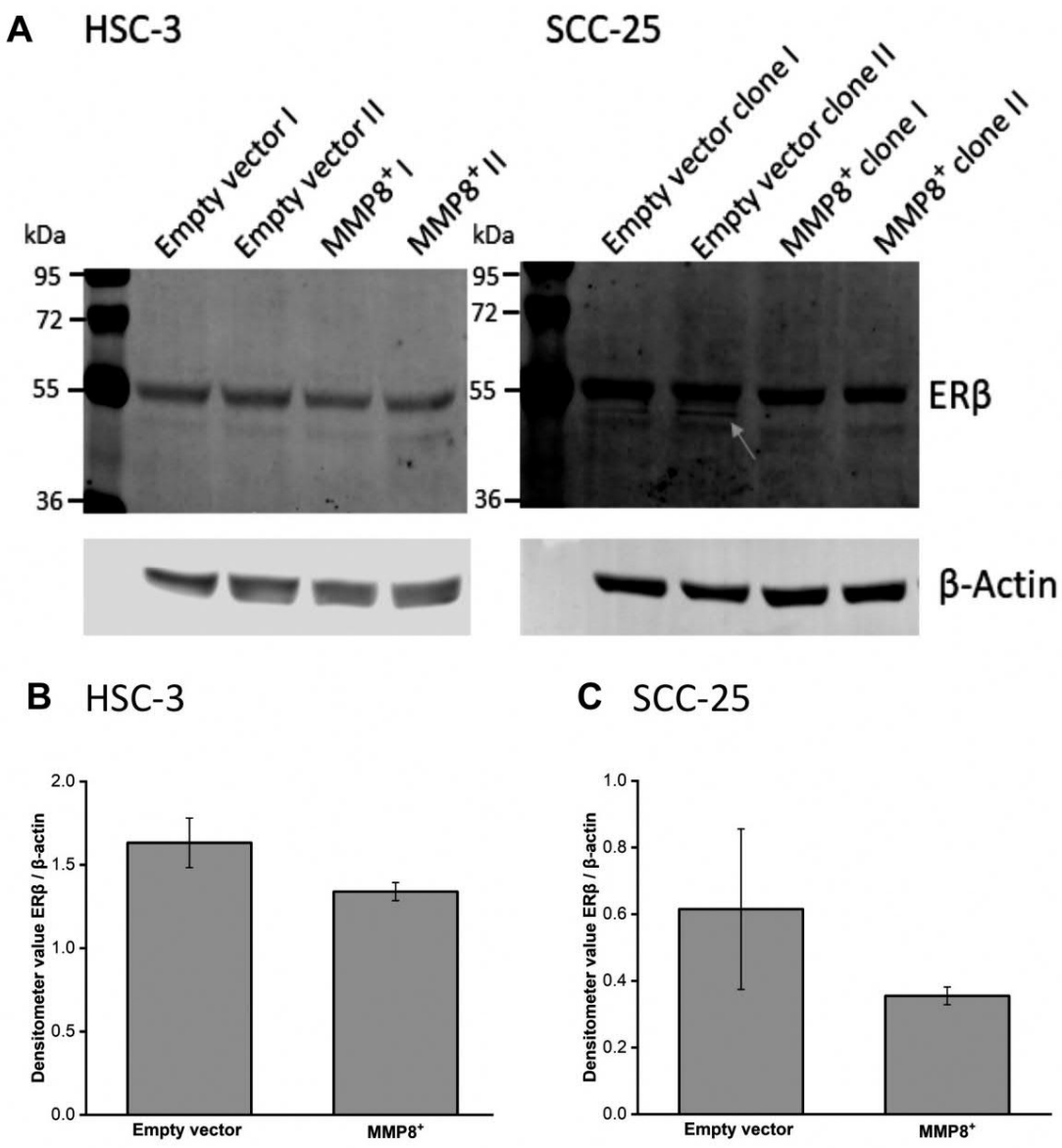
Figure 2. Effect of estradiol (EST) on migration (A) and invasion (B) of HSC-3 and SCC-25 cells in IncuCyte scratch wound-healing assay. Estradiol reduced the migration and invasion of HSC-3 and SCC-25 cells. Data are means ± standard deviation of wound closing percentages.

Figure 3. Effect of dihydrotestosterone (DHT) on migration (A) and invasion (B) of HSC-3 and SCC-25 cells in IncuCyte scratch wound-healing assay. DHT did not significantly affect migration and invasion of HSC-3 cells (left) or SCC-25 cells (right). Data are means ± standard deviation of wound closing percentages.

Figure 4. A: Effect of estradiol on invasion of $MMP8^+$ and empty vector HSC-3 cells in the spheroid assay. Invasion by $MMP8^+$ cells was significantly lower than that of empty vector HSC-3 cells. B: The area under the curve (AUC) was calculated from the areas of the spheroid curves shown in part A. Estradiol had no significant impact on either type of transfected cell in both assays. Data are means ± standard deviation of spheroids area and the corresponding AUC.

Figure 5. Effect of estradiol on proliferation and viability of HSC-3 and SCC-25 cells in IncuCyte proliferation assay (A) and luminescent cell viability assay (B). Estradiol had no effect on the proliferation nor on viability of HSC-3 or SCC-25 cells. Data are means ± standard deviation of cell confluence normalized to day 0 (A) and luminescent value normalized to control (B).

Figure 1


A
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36
ERβ
β-Actin
B
HSC-3
C
SCC-25
Densitometer value ERβ / β-actin
Empty vector
MMP8+

Figure 2

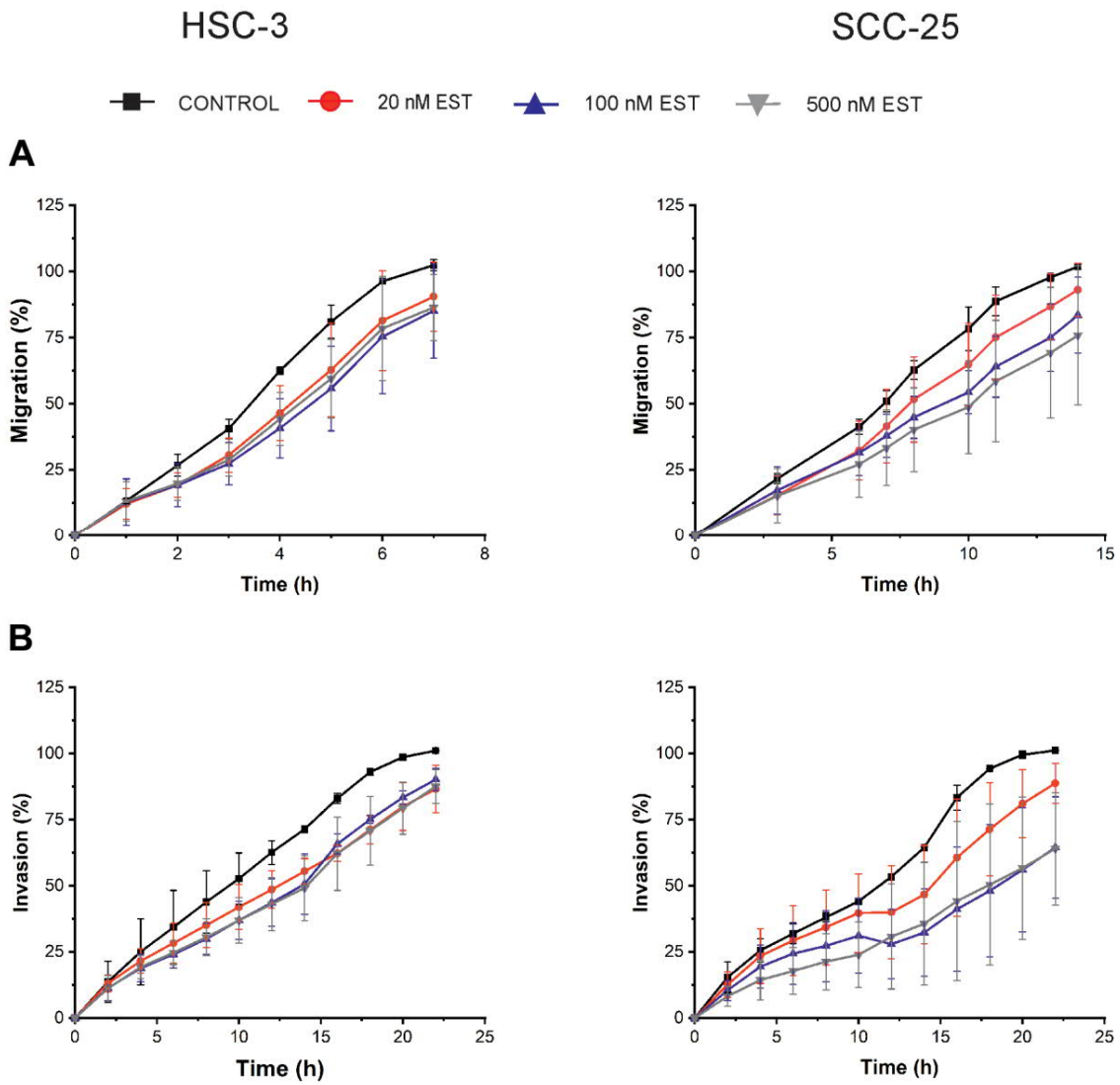

HSC-3
SCC-25
CONTROL
20 nM EST
100 nM EST
500 nM EST
A
Migration (%)
Time (h)
B
Invasion (%)
Time (h)

Figure 3

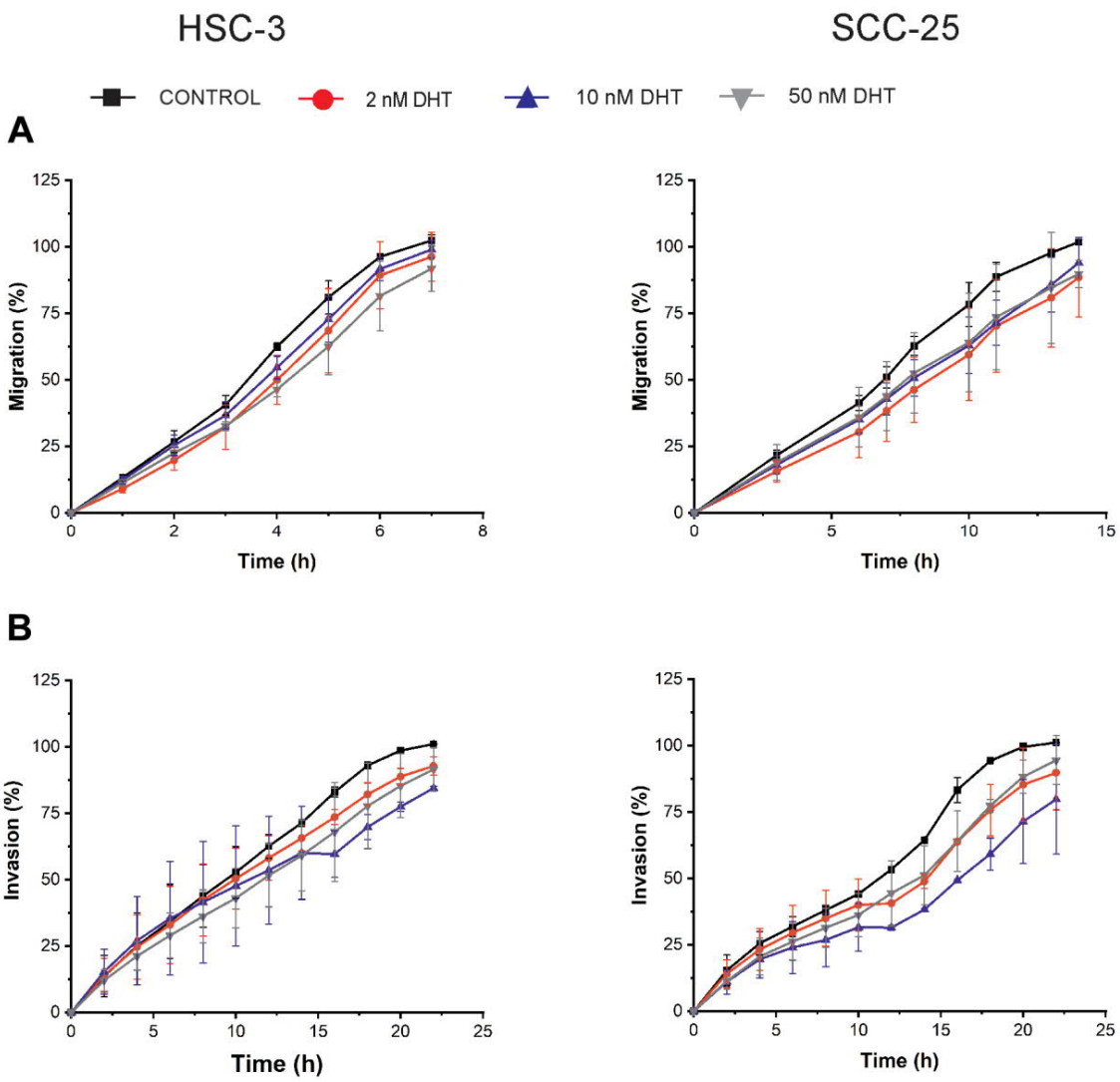

HSC-3
SCC-25
CONTROL
2 nM DHT
10 nM DHT
50 nM DHT
A
Migration (%)
Time (h)
B
Invasion (%)
Time (h)

Figure 4

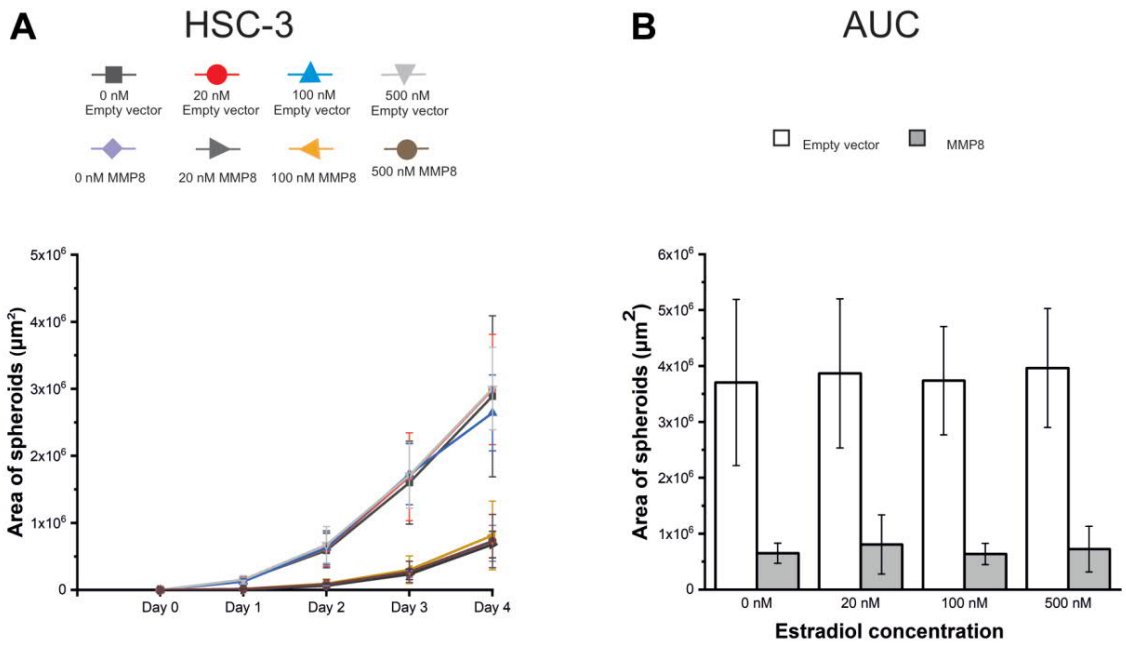
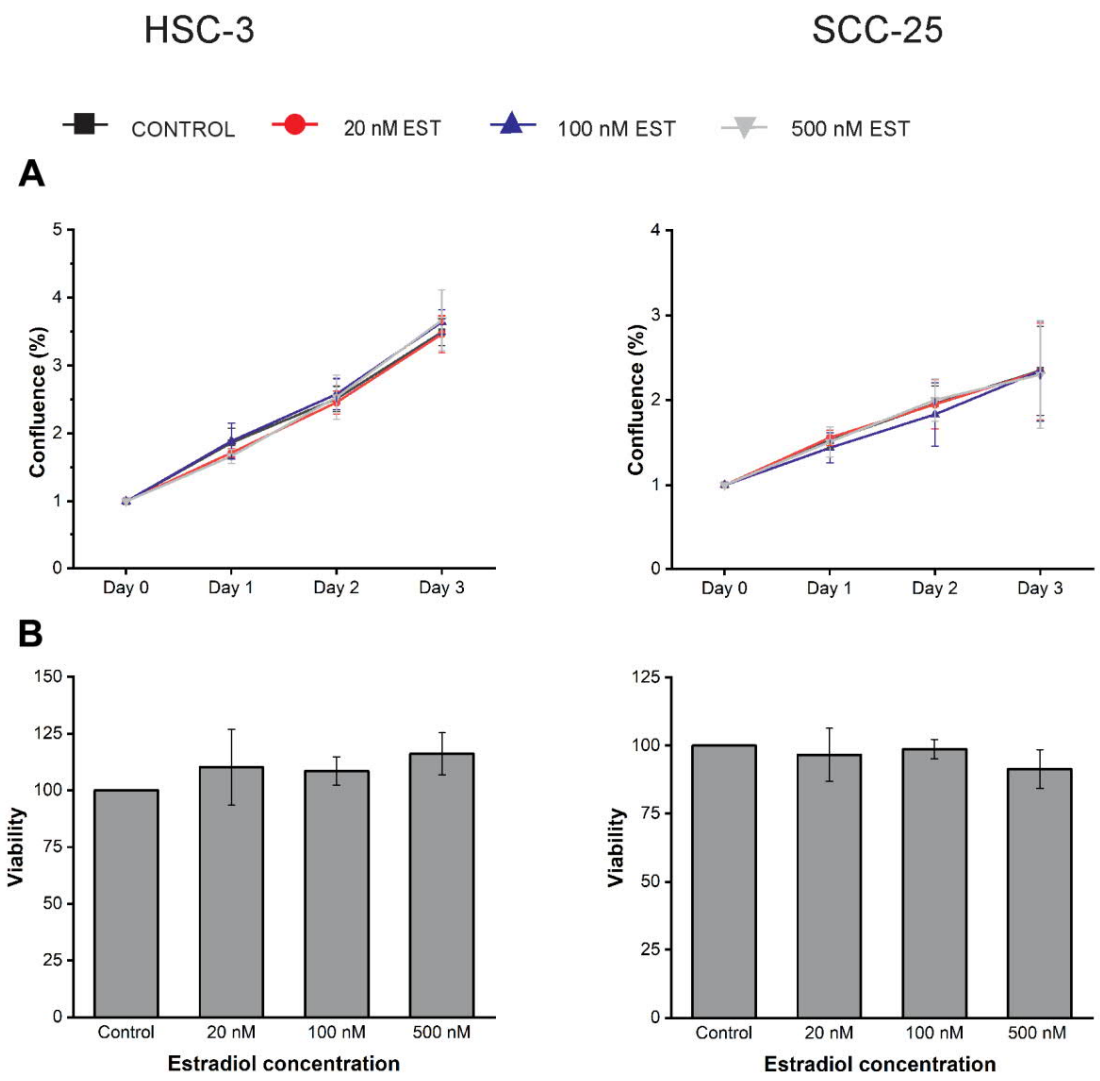

A
HSC-3
0 nM Empty vector
20 nM Empty vector
100 nM Empty vector
500 nM Empty vector
0 nM MMP8
20 nM MMP8
100 nM MMP8
500 nM MMP8
Area of spheroids (µm²)
5x10⁶
4x10⁶
3x10⁶
2x10⁶
1x10⁶
0
Day 0
Day 1
Day 2
Day 3
Day 4
B
AUC
Empty vector
MMP8
Area of spheroids (µm²)
6x10⁶
5x10⁶
4x10⁶
3x10⁶
2x10⁶
1x10⁶
0
0 nM
20 nM
100 nM
500 nM
Estradiol concentration

Figure 5


HSC-3
SCC-25
CONTROL
20 nM EST
100 nM EST
500 nM EST
A
Confluence (%)
Day 0
Day 1
Day 2
Day 3
B
Viability
Control
20 nM
100 nM
500 nM
Estradiol concentration