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THE ROLE OF ESTROGEN IN MOUSE GASTRIC STEM CELL HOMEOSTASIS

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

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Declaration of Original Work

I, Aysha Mohamed Yusuf Alkaabi the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*The Role of Estrogen in Mouse Gastric Stem Cell Homeostasis*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Asma Al Menhali, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

To maintain cellular homeostasis, the epithelial lining of the stomach wall continuously fluctuates between cellular proliferation, differentiation, and apoptosis. A key player in this process is the gastric stem cell (GSC). GSCs are located in the isthmus region of the corpus gastric gland and have the potential to proliferate and differentiate. Although several pathways have been identified to regulate stem cell role in several body tissues, little is known about controlling GSC homeostasis. This project aims to study the role of estrogen (E2) in GSC homeostasis using the well-established mouse gastric epithelial progenitor (mGEP) cell line. Our data showed that both estrogen receptor (ER) subunits alpha and beta are expressed in the mGEP cells at mRNA and protein levels. Incubation of mGEP cells with the commonly used selective estrogen receptor modulator (SERM) -tamoxifen- (4-OHT) decreased the cellular viability in a time and concentration dependent manners. Cell viability was not significantly changed in the E2 treated cells. By using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), ER target genes such as insulin-like growth factor 1 receptor (Igf1r), cyclin D1 (Ccnd1), low density lipoprotein receptor (Ldlr), interleukin-6 (Il-6) and vascular endothelial growth factor A (Vegfa) generally showed a concentration dependent decrease of expression when treated with 4-OHT confirming that 4-OHT works as an antagonist to ERα. This well-controlled *in vitro* system helps to understand the impact of E2 signaling on GSC homeostasis. Especially that the effect of 4-OHT on the stomach of breast cancer patients is not fully studied.

Keywords: Estrogen (E2), tamoxifen (4-OHT), mGEP, gastric stem cells (GSCs), stomach homeostasis.

Title and Abstract (in Arabic)

دور الإستروجين في توازن الخلية الجذعية المعدية للفئران

الملخص

للحفاظ على التوازن الخلوي، بطانة جدار المعدة تتقلب بشكل مستمر بين انقسام وتمايز الخلايا، وموت الخلايا المبرمج. اللاعب الرئيسي في هذه العملية هي الخلية الجذعية المعدية (GSC). تقع الخلايا الجذعية المعدية (GSCs) في منطقة البرزخ في الغدد المعدية الموجودة في جسم المعدة ولها القدرة على التكاثر والتمايز. على الرغم من أن تم التعرف على عدة مسارات لتنظيم دور الخلايا الجذعية في العديد من أنسجة الجسم، القليل فقط يعرف عن السيطرة على توازن الخلايا الجذعية في المعدة. يهدف هذا المشروع إلى دراسة دور هرمون الإستروجين (E2) في توازن الخلايا الجذعية المعدية باستخدام أحد أنواع الخلايا الجذعية المعدية من معدة الفئران (mouse gastric epithelial progenitor – mGEP). نتائجنا تشير أن كل من مستقبلات هرمون الإستروجين (ERs) ألفا وبيتا موجودة في خلايا mGEP في مستويات الحمض النووي الريبوزي المرسل (mRNA) والبروتين. معالجة خلايا mGEP مع مغير هرمون الإستروجين الانتقائي (SERM) المسمى بالتاموكسفين (Tamoxifen or 4-OHT) أدى إلى انخفاض في بقاء الخلايا بطريقة تعتمد على وقت المعالجة وتركيز الدواء. في الجانب الآخر، لم يلاحظ أي تغيير بشكل كبير في بقاء الخلايا المعالجة بالإستروجين (E2). كذلك، بالإستخدام النوعي لعكس النسخ تفاعل البلمرة المتسلسل (RT-PCR)، اشارات الجينات المستهدفة لمستقبلات الإستروجين (ERs) مثل مستقبل عامل النمو الذي يشبه الأنسولين 1 (Igf1r)، السيكلين D1 (Ccnd1)، ومستقبل البروتين الدهني منخفض الكثافة (Ldlr)، أنترلوكين 6 (Il-6) و عامل نمو بطانة الأوعية الدموية ألف (Vegfa) عموما انخفاضاً يعتمد ويرتبط بارتفاع تركيز التاموكسفين (4-OHT) مؤكدا أن التاموكسفين (4-OHT) يعمل بطريقة مضادة لمستقبل الإستروجين ألفا (ERα). تساعد هذه الدراسة على فهم تأثير هرمون الإستروجين (E2) على توازن الخلايا الجذعية المعدية (GSCs) خاصة أن تأثير التاموكسفين (4-OHT)على معدة مرضى سرطان الثدي لم تدرس بشكل كامل.

مفاهيم البحث الرئيسية: هرمون الإستروجين، التاموكسفين، الخلية الجذعية المعدية، توازن المعدة

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In the name of Allah, the Most Gracious and the Most Merciful

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Last but not least, I would like to take this opportunity to thank all my friends and colleagues who have given their support and help especially my friends in Bahrain.

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Dedication

To my unique parents for their love and continuous support, my grandmother's soul (Haya Alkaabi) who I miss every day, and to my wonderful family.

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List of Abbreviations

AF	Activation Function
Ccnd1	Cyclin D1
CgA	Chromogranin A
DBD	DNA-Binding Domain
E2	Estrogen or 17β-Estradiol
EC	Enterochromaffin cells
ECL	Enterochromaffin-like cells
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
ERE	Estrogen Response Element
FBS	Fetal Bovine Serum
GF	Growth Factor
GEPs	Gastric Epithelial Progenitors
GI	Gastrointestinal
GSC	Gastric Stem Cell
HDC	Histidine Decarboxylase
HKα	H^+, K^+-ATPase, α
HKβ	H^+, K^+-ATPase, β
IF	Intrinsic Factor
Igf1r	Insulin-like growth factor-1 receptor

Il-6	Interleukin-6
LBD	Ligand-Binding Domain
Ldlr	Low-density lipoprotein receptor
Mal	Mal T-cell differentiation protein
mGEP	Mouse Gastric Epithelial Progenitor
Muc5AC	Mucin 5, subtypes A and C
Muc6	Mucin 6
4-OHT	4-hydroxytamoxifen
SERMs	Selective Estrogen Receptor Modulators
SPEM	Spasmolytic Polypeptide Expressing Metaplasia
SST	Somatostatin
TF	Transcription Factor
Vegfa	Vascular Endothelial Growth Factor A

Chapter 1 : Introduction

1.1 The Stomach

1.1.1 Anatomy and Compartment

The stomach is a secretory organ located in upper part of the gastrointestinal (GI) tract. The adult mouse stomach is divided into three regions from the proximal to distal end: the forestomach; the corpus, and the antrum (Figure 1.1 A). The forestomach is composed of squamous epithelium resembling esophagus[1], while the corpus and antrum make the glandular part of the stomach[2]–[4]. The corpus is the largest part of the stomach that is mainly responsible of gastric acid secretion. The antrum -on the other hand- does not contain acid secreting cells; instead, it is responsible of producing gastrin hormone[5].

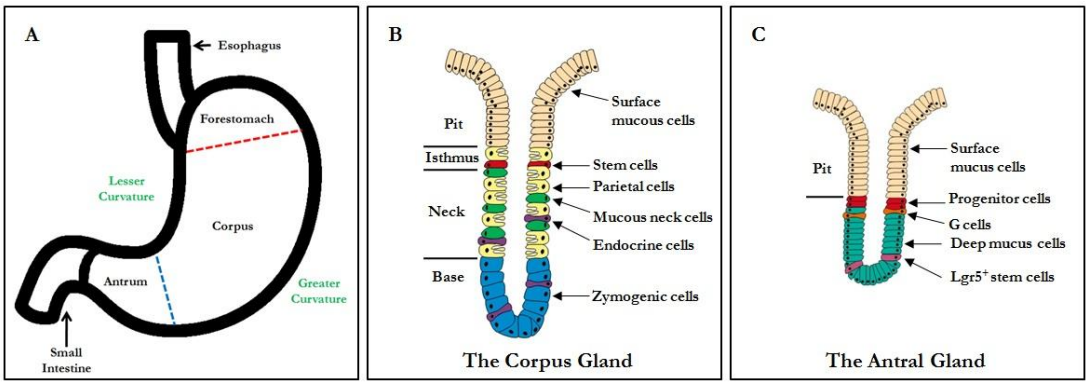

Figure 1.1: Diagram of mouse stomach anatomy. The adult mouse stomach is divided into three regions: forestomach, corpus and antrum (A). The esophagus connects to the forestomach, while the small intestine connects to the antrum. Diagram of a gastric gland from corpus (B), and antrum (C).

1.1.2 Gastric Gland Structure

The structural and physiological building block of stomach glandular part is the gastric gland. The gastric glands represent the basic structural elements of the

gastric epithelium of the corpus and the antrum (Figure 1.1 B and C). The corpus gastric gland is a simple columnar epithelial structure that is composed of four parts: pit, isthmus, neck and base of the gland[3]. The pits occupy the apical region of the gastric glands and contain surface mucous cells. Surface mucus cells secrete mucus to protect the stomach from pathogens, strong acid and digestive enzymes. The corpus glands have short pits while the antral glands have longer pits[2]. The isthmus contains stem cells, which are undifferentiated and highly proliferative cells that give rise to all types of differentiated cells. Below the isthmus is the neck and it contains mucous-producing neck cells which secret mucus and are zymogenic precursor cells. The base region is located at the bottom of the gland and contains slowly dividing cells and digestive enzyme producing cells called zymogenic cells[6], [7]. The hydrochloric acid (HCl) secreting parietal cells are spread along the corpus gland. The acid produced by parietal cells is helpful for food digestion and to make the gastric environment unfavorable for pathogens. Endocrine cells are located at the base of the gastric gland. Enterochromaffin-like cells (ECL) is one type of endocrine cells and responsible for histamine secretion and can stimulate parietal cell acid secretion[8].

In the antrum, the gastric gland is composed of surface mucus cells, deep mucus cells, and G cells that are gastrin producing endocrine cells. Gastrin stimulates the release of acid and histamine from the corpus region[9]. The antral stem cells express the intestinal stem cell marker Lgr5 and are located toward the base of the gland[10].

Several endocrine cells in addition to ECL and G cells are found in the gastric mucosa including Enterochromaffin cells (EC) which secrets serotonin as

neurotransmitter, X (or A-like) cells which secret ghrelin and D cells which secretes somatostatin[11].

Karam and Leblond have identified the life span of the cell lineages in the adult mouse stomach by tracking the cells using ^{3}H-thymidine[7], [12], [13]. Major cell markers and life span are represented in (Table 1.1).

Table 1.1: Cell markers and life span of differentiated mouse gastric epithelial cells

Cell type	Marker	Life span (Days)	Reference
Surface mucus cells	Mucin 5, subtypes A and C (Muc5AC)	3	[7]
Mucus neck cells	Mucin 6 (Muc6) and Trefoil Factor 2 (TFF2)	7-14	[12]
Parietal cells	H^+, K^+-ATPase, α (ATP4A or HKα) and β (ATP4B or HKβ) subunits	54	[13]
Zymogenic cells	Intrinsic Factor (IF)	194	[12]
Endocrine cells	Chromogranin A (CgA)	45-60	[14]
EC	Serotonin	Nd	
ECL cells	Histidine decarboxylase (HDC)	60	[15]
G cells	Gastrin	60	[16]
D cells	Somatostatin (SST)	Nd	
X (or A-like) cells	Ghrelin (GHRL)	Nd	

1.2 The Gastric Epithelial Stem Cells

1.2.1 Salient Features of the Stem Cells

Adult stem cells have two typical features: (1) longevity/the capability of self-renewal and (2) multipotency, or the potential to differentiate into various cell types[17]. The decision of whether or not a stem cell should divide or differentiate is fundamental.

In a series of classical electron microscopic autoradiographic experiments, all gastric cells are believed to be derived from stem cells in the isthmus region of the

corpus gland[18]–[20]. In an interesting report in 1940s, Leblond and colleagues showed that few cells in the isthmus continuously regenerate cells that migrate bi-directionally and differentiate into mature gastric cells[18]. Additionally, in 1960, Corpron identified small, undifferentiated cells with stem cell features including: (1) high nucleus-to-cytoplasm ratio, (2) lack of granules and (3) open chromatin in the isthmus region of rat gland[19]. Karam and Leblond, identified that stem cells are located in the isthmal region of mouse corpus gland[3]. In 2003, Karam *et al.*, identified similar cells in the isthmus of human gastric gland[20].

1.2.2 Gastric Stem Cell Markers

Karam and Leblond were the first to describe the histology of gastric stem cells (GSCs) in 1993[13]. Although that GSCs play an important role in gastric homeostasis and carcinogenesis; the identification and characterization of these cells was a dilemma for decades by a lack of molecular markers. However, in 2007, Barker and Clevers[21] were able to identify the first specific marker of intestinal stem cells, the Wnt target gene Lgr5 by using genetic tracking tools, and fluorescence reporters in mouse modles[1]. Recently, two main molecular markers of normal GSC were identified using molecular approaches: Sox2 which is a marker for the forgut including esophagus, forestomach and the glandular part of the stomach (corpus and antrum)[22], [23], and Lgr5 which is a marker for antral stem cells in addition to intestinal stem cells[23], [24]. The antral $Sox2^{+}$ cells are located above the $Lgr5^{+}$ cells in the base of the gland[25] (Figure 1.1 C). Additionally, Troy is known as a marker for zymogenic cells in the base of corpus gland, in which these cells showed plasticity features and are capable of replenishing entire gastric units by acting as a quiescent or reserve stem cells[26]. Moreover, CD133 (also known as

prominin-1) and CD44 are cell surface markers for normal and cancer GSCs in mouse models and humans cell lines[27]. The hyaluronic receptor CD44 has been identified as a potential GSC marker of the corpus[28]. In addition, Khurana *et al.* found that CD44 labeled a population of small, undifferentiated cells in the isthmus region of the normal gastric gland where the GSCs are usually found[29].

Recently, Vange *et al.* suggested a candidate marker for stem cells in the isthmus region of the corpus gland that is Aspm [Asp (abnormal spindle) homologue, microcephaly-associated (Drosophila)][30]. They developed a method for serial section-navigated laser microdissection to isolate cells from the proliferative isthmus zone of rat gastric mucosa to be used for microarray gene expression analysis. They found that Aspm is expressed mainly in the isthmus region of rat, mouse or human gastric mucosa by immunohistochemical (IHC) staining or *in situ* hybridization[30], [31].

1.2.3 Epithelial Cell Homeostasis: Proliferation and Differentiation

The balance between cell growth, differentiation, and apoptosis is important to maintain homeostasis in any tissue. As a result, any imbalance can give rise to tumor[32]. The continuous regeneration of the stomach occurs due to the proliferation and differentiation of the stem cell in the isthmus region of the corpus gland. Several growth factors found to be expressed in the stomach such as Insulin Growth Factor Binding Protein 2 (Igfbp2)[33], Sonic Hedgehog (Shh)[34], and Heparin-Binding Epidermal-Like Growth Factor (HB-EGF)[35], which are important for the differentiation and proliferation of gastric epithelial cells.

Some critical pathways are known to regulate the self renewal and differentiation of GSCs such as Wnt, Notch and bone morphogenetic protein (BMP)

signaling pathways[36]. Over expression of Wnt signaling cause gastric epithelial cell dedifferentiation[37]. Notch signaling plays a role in replication, cell fate and survival in many tissues. Notch signaling is essential for GSC homeostasis and the inhibition of enteroendocrine cell differentiation[38]. In the intestine, stem cell activity is regulated by cross talk between Notch and Wnt signaling pathways[39]. BMP pathway regulates the proliferation of stomach progenitors and required for parietal cell differentiation[40].

The GSCs have unique features comparing to other stem cells in the GI tract. First, GSCs are located high in the gastric gland comparing to intestinal stem cells which are located in the base of the crypts[41]. Thus, GSCs are more exposed to external stimuli. Second, mature gastric cells can migrate bi-directionally in the gastric gland[41]. Third, there is big variation in the life span of mature gastric cells[41] as shown in (Table 1.1). Finally, corpus stem cells respond to a series of signaling pathway while the intestinal stem cells rely mainly on Wnt signaling to maintain homeostasis. The corpus stem cells does not rely on Wnt pathway however, the antrum stem cells can be considered as a hybrid between corpus and intestinal stem cells as antral stem cells are labeled with Lgr5 (intestinal marker) and are regulated by Wnt signaling pathway[41].

Generally, the multipotent stem cells in the isthmus give rise to three progenitor cell types: pre-pit, pre-neck and pre-parital cells[6]. First, the pre-pit cells migrate upward the pit wall to become differentiated pit cells[7], [42]. Second, the pre-neck cells give rise to neck cells that migrate down through the gland neck and transform into prezymogenic cells, which becomes zymogenic cells later on[43]. Third, the pre-parietal cells yield parietal cells that migrate from the isthmus bi-directionally into either the gland pit or base[44] (Figure 1.2).

It is known that the pre-pit and pre-neck cells have the ability of self-renewal in addition to their differentiation into pit and neck cells, respectively, however, the pre-parietal cells lacks the ability of self-renewal[6].

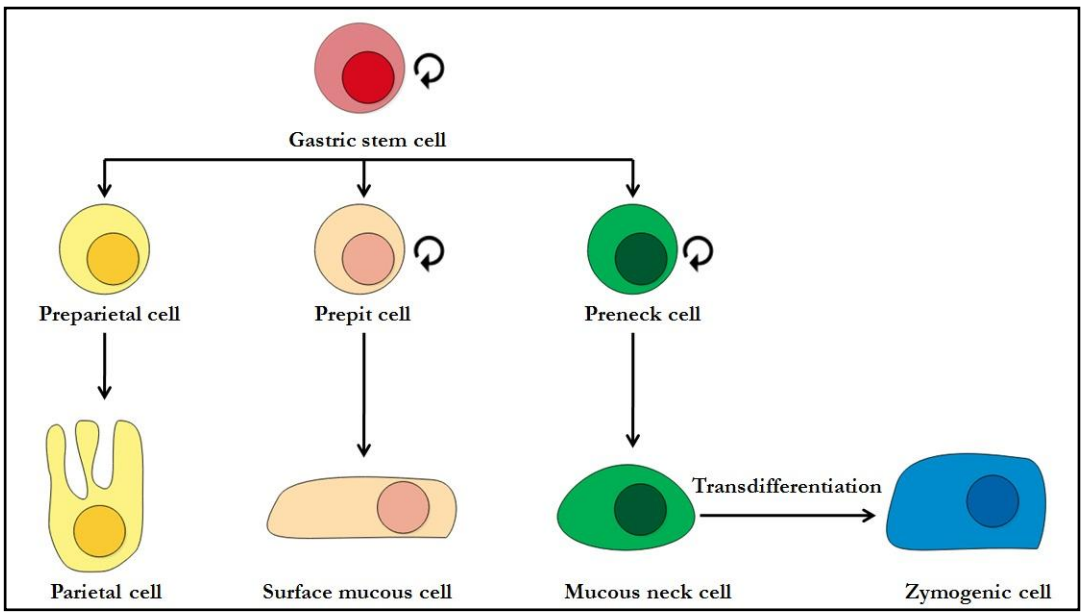

Figure 1.2: Self renewal and differentiation of gastric stem cells (GSCs). The GSC give rise to three progenitor cell types: pre-parital, pre-pit, and pre-neck cells. The pre-parietal cells yield parietal cells (acid-secreting cells). The pre-pit cells differentiate into surface mucus cells. The pre-neck cells give rise to neck cells which becomes zymogenic cells later on. GSCs, pre-pit and pre-neck cells have the ability of self-renewal (circled arrow), while pre-parietal cells lack this ability.

It is hard to study the progenitor cells of the mouse gastric epithelium because of many reasons; (i) they are found among heterogeneous population of differentiated cells[3], (ii) they have small size and found in relatively small number, (iii) it is difficult to study the progenitor cells as primary culture due to their limited *ex vivo* life span and they cannot be subcultured easily[45]. In the next section, unique genetically modified mouse GSCs will be discussed with highlighting its potentials in studying GSCs and its role in mucosal homeostasis.

1.2.4 Mouse Gastric Epithelial Progenitor (mGEP) as a Model

The mGEP cell line has been established from the stomach of an 18-month-old FVB/N transgenic mouse by expressing an oncogene, the simian virus (SV40) large T antigen under the control of H,K-ATPase β-subunit gene, *Atbp4* regulatory element in the parietal cell linage[45]. This resulted in pre-parietal cells amplification because of the block in their differentiation into acid secreting parietal cells[46], additionally, the differentiation of preneck cells into zymogenic cells has been blocked at the stage of neck cells[45] (Figure 1.3).

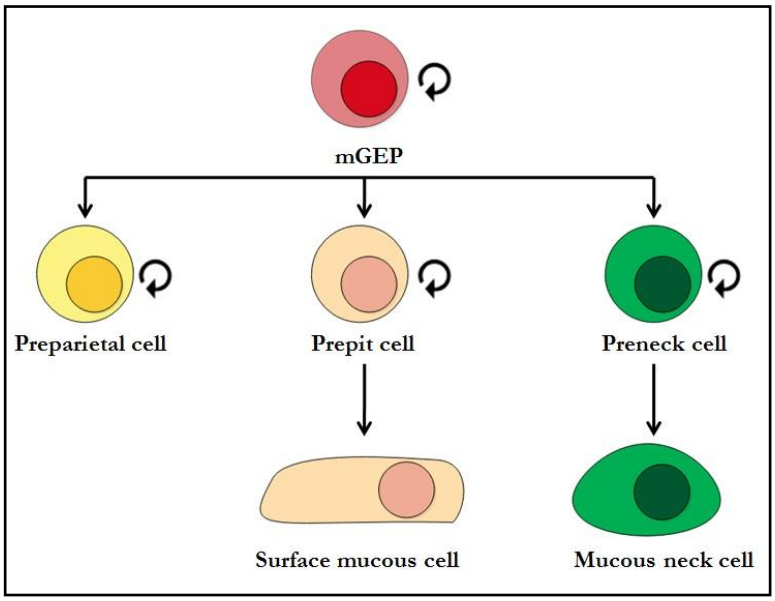

Figure 1.3: Self renewal and differentiation of mouse gastric epithelial progenitor (mGEP). mGEP cell give rise to three progenitor cell types: pre-parital, pre-pit, and pre-neck cells. The pre-pit cells differentiate into surface mucus cells and the pre-neck cells give rise to neck cells. These pre-parietal cell lacks the ability to differentiation into parietal cell and the mucus neck cell no longer transdifferentiate into zymogenic cell as the normal GSCs.

Many evidence indicated that this mGEP cell line showed features resembling gastric epithelial progenitors (GEPs) in the intact mouse stomach[6] such as the large nucleus-to-cytoplasm ratio, the numerous free ribosomes, and the few small membrane-bond organelles: rough endoplasmic reticulum, mitochondria, and

Golgi apparatus. Moreover, the epithelial nature of the cells had revealed a typical polyhedral appearance with narrow intercellular spaces and the nuclei were characterized by noted nucleoli and much diffuse chromatin. Furthermore, none of the differentiated gastric cell markers such as H,K-ATPase, chromogranin A, intrinsic factor, and GSII and UEA-I lectins was detectable in these cells[45].

Farook *et al.* documented that the doubling time of cultured mGEP cells is around 40 h in a medium containing fetal bovine serum (FBS)[45]. mGEP cells were treated with different hormones to investigate there effect of cell growth. Cells were cultured in the presence of 0.5, 1, 2 nM of hydrocortisone, oestrogen or retinoic acid. The results showed that hydrocortisone and oestrogen stimulated cell proliferation while retinoic acid had an inhibitory effect on the cell population[45]. Therefore, the mGEP cell line is a useful model to study the mechanisms involved in the control of proliferation of GSCs. Although that the effect of estrogen in mGEP proliferation was studied[45], still no study is done to show the effect of estrogen in mGEP cell differentiation.

1.3 Estrogen

1.3.1 Estrogen Hormone (Ligand)

Estrogen is one of the sex hormones that were discovered by Allen and Doisy in 1923 among other ovarian “estrus-stimulating” hormones[47]. Estrogen is a small, carbon-rich steroid molecule produced in the ovaries and other tissues[48] by the process of testosterone aromatization[49]. Estrogen is produced in extra-ovarian tissues including testis[50], brain[50] and adipose tissue[51]. Recently, it has been shown that estrogen is produced in gastric parietal cells of rat stomach by the

expression of synthase enzyme (aromatase) in these cells[52].

Estrogen regulates reproductive functions and have a wide range of biological effects on the cardiovascular, immune, musculoskeletal and central nervous system in both females and males[53]. Estrogen plays a preventive role against mood changes, hot flushes and Alzheimer's disease[54]. On the other hand, many researchers strongly supported that estrogen has a definitive role in the development and progression of breast cancer[55]–[57]. The active form of estrogen in the body is 17β-estradiol (E2) (Figure 1.4), and although the two metabolites of E2, estrone and estriol are considered as high affinity ligands, they are much weaker agonists on estrogen receptors (ERs) comparing to E2[58].

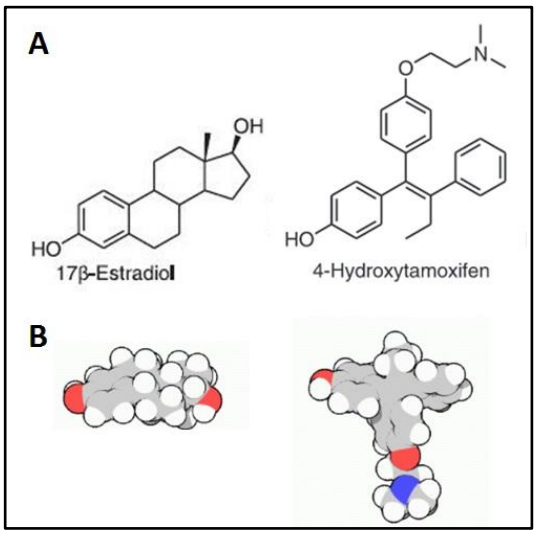

Figure 1.4: 17β-Estradiol (E2) and 4-hydroxytamoxifen (4-OHT). Molecular structures[58] (A) and the 3D structures[48] (B) of the endogenous estrogen E2 and 4-OHT. 4-OHT mimics the shape of E2, however, it has an extra chain that is important to its antagonist action[48]. Atoms color: white for hydrogen, gray for carbon, red for oxygen, and blue for nitrogen.

1.3.2 Estrogen Receptors (ERs)

Estrogens exert their physiological effects by binding to the ERs, which are members of the steroid/thyroid nuclear receptor superfamily[59]. The breaking finding was made in the late 1950s by Elwood Jensen who discovered the estrogen binding protein that is known today as ER alpha (ERα)[60]. Jensen discovered the presence of ER in human mammary cancer cells, and then many researchers have also discovered the existence of ER in gastric cancer cells, suggesting that estrogen plays a role in regulating these cells[61]. Nearly, after three decades later of Jensen discovery, the first ERα knock out mouse was created in 1993[62]. The surprising thing was the ability of these knock down mice to survive without this receptor which was thought to be sole mediator of estrogen signaling. The characterization of the ERα knockout mice showed that another ER is present which is known as ER beta (ERβ)[63], and this discovery raised another question of whether ERα knockout mice can survive because ERβ can substitute the function of ERα. Therefore, ERβ knockout were made in addition to the double ERαβ knockouts[64]. The results showed that life is possible without either or both ERs, however, the reproductive functions are severely defected[65]. The expression of ERβ have been detected in many tissues like cardiovascular system, central nervous system, urogenital tract, immune system, lung, kidney and GI tract[66]. Recently, *Tachibana et al.* documented that ER α and β are expressed in mouse cornea[67].

Mouse ERs are the products of different genes located at different chromosomes. Mouse ERα gene is located on chromosome 10 position (A1)[68] while mouse ERβ gene is located on chromosome 12 position (D1-D3)[69] as shown in Figure 1.5.

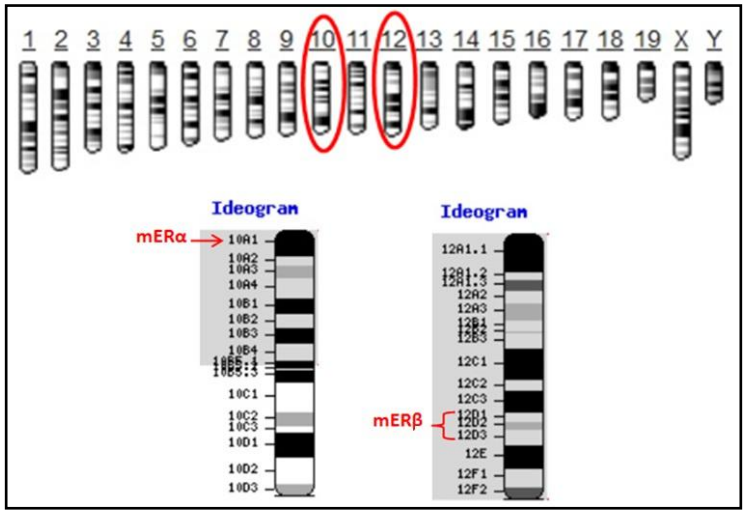

Figure 1.5: The cytogenetic location of mouse ERα and ERβ

ERα and ERβ belong to group of nuclear receptors called the steroid/thyroid hormone superfamily, members of which share a common structural organization[59]. These receptors are consisting of three independent but interacting functional domains: the A/B domain or the NH_2-terminal domain, the C or the DNA-binding domain (DBD), and the D/E/F or ligand-binding domain (LBD) which occurs in the COOH-terminus (Figure 1.6). The two ERs consist of two main conserved domains, the DBD and the LBD. "ERα and ERβ share homologous regions in the DNA and ligand binding domains (~96% and ~58% amino acid homology respectively)"[70]. The NH_2-terminal domain is not conserved in both ERs. The C domain/DBD contains two zinc figure structure that is important for receptor dimerization[71] and contains the nuclear localization signal[72]. The D domain contains the hinge region and may play a role in transcriptional repression[72]. Additionally, there is two activation functions (AF) to facilitate and activate transcription process; AF-1 located in the NH_2 terminus of the receptor and the AF-2 that is located in the COOH-terminal LBD. AF-1 is constitutively active,

however, AF-2 activation is ligand dependent[73]. Interestingly, both ERs have similar affinities to E2 and bind to the same DNA response elements[58].

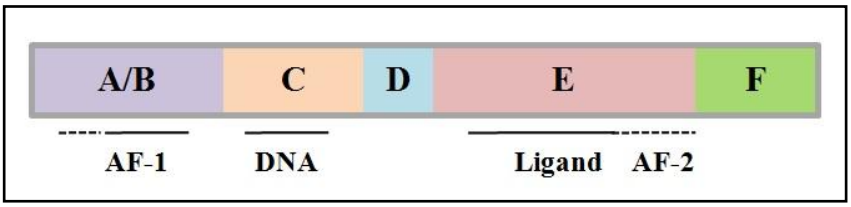

Figure 1.6: Schematic representation of the structure of estrogen receptor (ER) as an example of nuclear receptors. The A/B domain at the NH_2 terminus contains the AF-1 site where the transcription factors bind. The C domain composed of two-zinc finger structure that binds to DNA, and the D/F domain at the COOH terminus contains the ligand binding site in addition to the AF-2 domain that directly binds to coactivator peptides[73].

Although that it is well known that ERs are nuclear receptors, it has been firmly noticed that small population of ERs have been localized in the plasma membrane as well as in discrete cytoplasmic organelles such as mitochondria and the endoplasmic reticulum[74]. The plasma membrane ERα and ERβ exist within caveolar rafts, and these ERs do not contain any *trans*-membrane domain[75]. Therefore, the ability of ERs to associate with the plasma membrane could be because of their association with membrane proteins and/or by post-translational modification of lipids to ERα[76]. "Approximatly 5-10% of total cellular ER is found at the plasma membrane in many cells"[74]. This percentage includes both ERα and ERβ, but their localization varies depending on cell type. For example, in vascular endothelial cells, both ERs exist as homodimers and heterodimers at the membrane of these cells[77]. In contrast, in other cells such as breast cancer cells, mainly ERα is found at the plasma membrane with few ERβ present[78].

1.3.3 ERs Signaling Pathways

ERs regulate growth, development, homeostasis and differentiation in different cell types through the regulation of large number of target genes[59]. Estrogens diffuse through the plasma membrane of target cells to activate different signaling pathways and trigger transcription of target genes. Two different types of signaling can be mediated by E2, known as genomic and non-genomic pathways[49], [75]. In the genomic pathway, E2 binds to ER and induces activation of gene transcription with or without the presence of estrogen response element (ERE) in the promoter region of the target gene. In contrast, the non-genomic pathway depends on the nuclear ERs and the membrane associated ERs[75], [76] which results in cytoplasmic alternation and regulation of gene expression[76], [79].

The two main types of signaling pathways (genomic and non-genomic) are further divided into four different ER signaling pathways as shown in Figure 1.7. The first three pathways are genomic while the last one is non-genomic. Pathway 1: The classical mechanism of ER (ERE-dependent genomic action): involves the translocation of ligand-bound ERs to the nucleus, thus induce conformational changes and cause receptor dimerization, and binding of the receptor complex to ERE in the promoter region of target genes[75], [80]. The ERE is a palindromic sequence in the promoter region of the target gene and it was shown that it function in an orientation and distance-independent manner[81]. Pathway 2: ERE-independent genomic action: takes place when the promoter region of target gene lacks ERE, and involves an association between ligand-bound ERs and DNA through DNA-binding transcription factors (TFs)[82]. This mechanism is called “transcriptional cross-talk”[83]. Examples of such TFs are stimulating protein-1 (Sp-1) which induce the

transcription of low-density lipoprotein (LDL) receptor[84], cyclin D1[85],endothelial nitric oxide sinthase (eNOS)[86], c-*fos*[87], and retinoic acid receptor-1 α genes[88]. Another example is the interaction between ERα and nuclear factor-κB (NF-κB) which regulates the interleukin-6 (IL-6) promoter[89]. Several genes are activated by the interaction of ERs with Fos and Jun proteins at activator protein-1 (AP-1) binding sites. Ovalbumin[90], insulin-like growth factor (IGF)-1 receptor[91], cyclin D1[92], collagenase[93] are examples of genes activated via AP-1. Pathway 3: Ligand-independent genomic action: in the absent of estrogens, growth factors (GF) such as insulin-like growth factor (IGF) and epidermal growth factor (EGF) activate protein-kinase cascades, leading to phosphorylation (P) and activation of nuclear ERs at EREs[75], [80]. Pathway 4: Non-genomic actions: these actions are too rapid that they cannot depend on the activation of RNA and protein synthesis[75], while the genomic actions of steroid hormones take longer time and occur usually after at least 2 hours after E2 stimulation[94]. The non-genomic actions are believed to be mediated by membrane associated ERs. Usually the membrane associated ER exist as monomers but form dimmers in response to estrogenic compounds[74]. The non-genomic actions are mostly associated with activation of different protein-kinase cascades, moreover, they can indirectly influence gene expression by activating signal transduction pathways that act on specific TFs[75]. The non-genomic actions include the immobilization of intracellular calcium[95] and activation of different signaling pathways such as mitogen-activated protein kinase (MAPK) signaling pathway[96] and phosphoinositol (PI) 3-kinase signaling pathway[97] in different cell types including breast cancer cells.

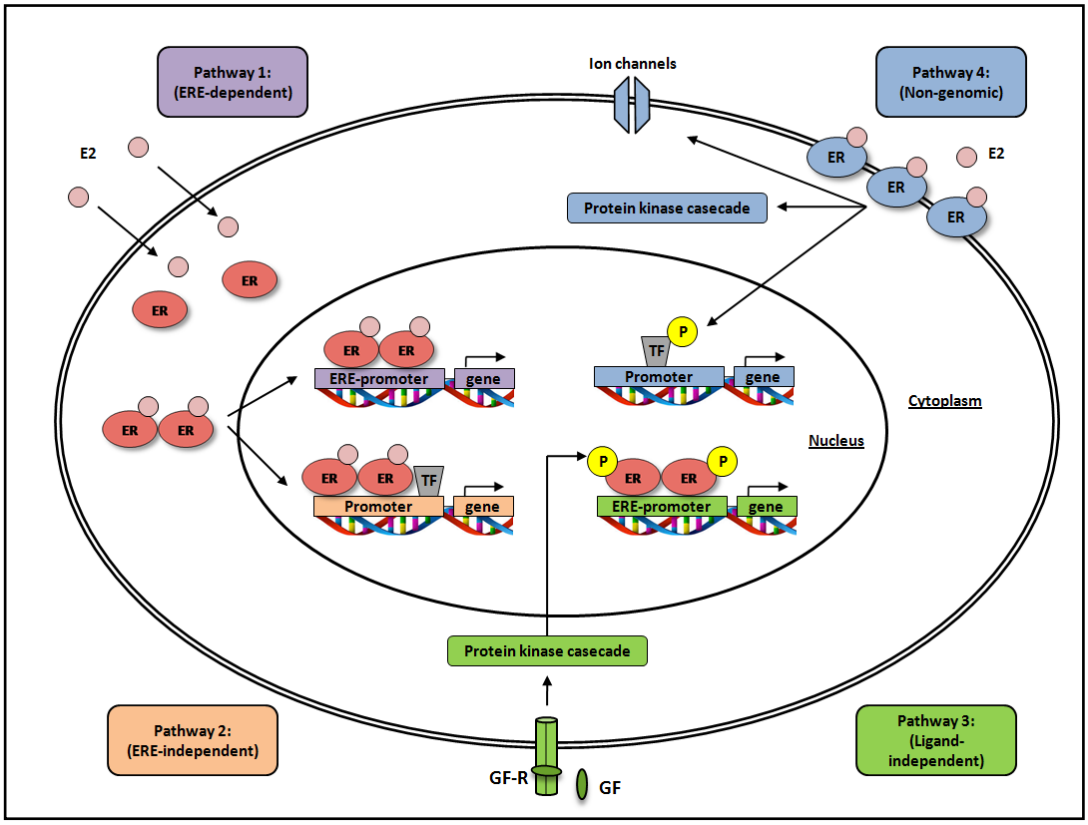

Figure 1.7: Estrogen receptors (ERs) signaling pathways. There are four ERs signaling pathways. Pathway 1: The classical mechanism for ER (ERE-dependent genomic action): the nuclear initiated E2 signaling mediated through ERs leads to transcriptional changes in estrogen target genes with EREs. Pathway 2: (ERE-independent genomic action) similar to pathway 1 except that the promoters of target genes do not contain ERE. Pathway 3: Ligand independent genomic action, GFs activate protein kinase casecade in the absent of E2 leading to phosphorylation and activation of nuclear ER at EREs. Pathway 4: Non-genomic pathway, the membrane initiated E2 signaling leads to different cytoplasmic actions including the regulation of ion channels, activating different protein casecade proteins, and phosphorylation of TFs. (E2: 17β-estradiol, ER: estrogen receptor, ERE :estrogen response element, GF: growth factor, GF-R: GF-receptor, P: phosphorylation, TF: transcription factor).

Generally, the final gene responses activated by ERs depends on number of conditions such as the combination of TF bound to the gene promoter, the cellular localization of ERs, the levels of co-regulator proteins, and the nature of the extracellular stimuli[75]. "All of the steps in transcriptional activation of ER dependent genes, i.e., ligand binding, ER dimerization, DNA binding, and the interaction with cofactors, appear to be influenced by phosphorylation of ER."[73].

1.3.3.1 ERs Target Genes

The study of the relation between the molecular and physiological functions of ERs requires deep understanding of the group of genes regulated in each cell and tissue[49]. Examples of ER target genes include: insulin-like growth factor (Igf)-1 receptor (Igf1r), cyclin D1 (Ccnd1), low-density lipoprotein (Ldl) receptor (Ldlr), interleukin-6 (Il-6), mal T-cell differentiation protein (Mal), and vascular endothelial growth factor A (Vegfa).

Igf1r has many effects on cellular growth and metabolism[91] and can stimulate proliferation of breast cancer[98]. It is known that E2 activates ER, however, other growth factor pathways like Igfs also can activate ER, specifically ERα through nongenomic membrane associated activity[99]. E2 and Igf are quite related in which E2 can activate Igf pathway via ER's genomic and nongenomic functions. Additionally, any blockage of ERα function can inhibit Igf functions and vice versa[72]. Igf functions are contributed to proliferation, angiogenesis, metastasis and resistance to apoptosis[100]. "The IGF system consists of two closely related receptors insulin receptor (IR) and the type I IGF receptor (IGF1R) and three ligands (IGF-I, IGF-II, and insulin)"[72]. Structurally, ER have two activation function domains (AF-1 and 2) as shown in Figure 1.6. ER lacking AF-1 can be activated with E2 but not with growth factors, while ER lacing AF-2 can be activated with growth factors but not E2[101].

Ccnd1 is a well known ER target gene especially in breast cancer cells, as it is important for cell progression through the G1 phase of the cell cycle[102]. Ccnd1 plays a critical role in cell cycle as it accumulates before entering S-phase[103]. Baldin *et al.* demonstrated the localization of Ccnd1 in the nucleus, and they found

that it disappeared from the nucleus when the cell entered S-phase[103]. They found that when anti-Ccnd1 antibodies were used, cells were prevented from entering S-phase. This suggested that Ccnd1 is an important target of proliferative signals in cells cycle[103].

Ldlr mediates the uptake of cholesterol and plays a role in lipoprotein metabolism. The Ldlr is synthesized in the rough endoplasmic reticulum and then transported through Golgi apparatus to the plasma membrane. The mature Ldlr (with O-linked carbohydrate chains) is guided to the "coated pits" on the cell surface which is rich in clathrin and interact with Ldlr protein. After that, the Ldlr can bind LDL particles. Within few minutes of LDL-particle-receptor complex formation, the complex internalized through the process of endocytosis and metabolized through the Ldlr-mediated endocytosis pathway[104]. Moreover, Ldlr is activated by estrogens and leads to lower plasma LDL cholesterol level in postmenopausal women and reduce the risk of cardiovascular diseases[105].

Il-6 is a multifunctional cytokine that plays an important role in host defense mechanisms, immune response and inflammation, , release of acute-phase response proteins, and help in immature osteoclasts activation process[106]–[108]. Il-6 is produced by number of cells such as monocytes/macrophages, fibroblast, epithelial and endothelial cells[109]. Although Il-6 deficient mice do not show any problems in development, absence of Il-6 affects immune response in these mice. Interestingly, ovariectomy in Il-6 deficient mice ablated the osteoporosis that occur in normal mice indicating that Il-6 plays an important role in mediating bone loss when E2 level is low[110]. Il-6 plays a vital role in regulating E2 activity through stimulation of some enzymes like aromatase, steroid sulphatase and 17β-hydroxysteroid

dehydrogenase[111]. Additionally, studies suggested a positive correlation between Il-6 and ERα expression in breast cancers in a way thought to be stem cell mediated[112].

Mal is one of ER target genes[113] that encodes T-lymphocyte maturation protein and plays a role in T-cell differentiation[114]. In addition, it works as tumor suppressor gene that functions in membrane trafficking processes in polarized epithelial cells[115]. Mal is considered as DNA methylation marker in human gastric cancer[116].

Vegf family in mammals consists of five members: Vegfa, Vegfb, Vegfc, Vegfd and placenta growth factor (PGF)[117], [118]. Vegf and its receptor (Vegf-r) are pro-angiogenic factors that are secreted by tumors. Interestingly, endogenous E2 has the ability to regulate the expression of Vegf-r in non-cancer and cancer ovarian cell lines[119]. Vegfa is one of ER target genes and it stimulates vascular endothelial cell growth, tubular formation and migration. Vegfa is a well know key player in tumor angiogenesis[120], for example ERα gene and Vegfa are involved in endometrial cancer[121].

Table 1.2: List of some ERs target genes

ERs Target Genes	**References**
Insulin growth factor 1 receptor (Igf1r)	[91]
Cyclin D1 (Ccnd1)	[102]
Low density lipoprotein receptor (Ldlr)	[104]
Interleukin-6 (Il-6)	[106]–[108], [112]
Mal, T-cell differentiation protein (Mal)	[113]
Vascular endothelial growth factor A (Vegfa)	[119]

1.3.4 Tamoxifen and ERs

Generally antiestrogens are classified into two main groups: (type I) which has mixed estrogenic/antiestrogenic effects like structural derivatives of the triphenylethylene type of drugs including tamoxifen and its metabolites, and (type II) which are pure antiestrogens[98]. Tamoxifen and type I antiestrogens belong to a group of molecules known as selective estrogen receptor modulators (SERMs) as they have agonist/antagonist effects depending on cell type[122], [123]. These SERMs work by forming a receptor complex that is incompletely converted to the activated form[124]. As a result, because of the imperfect changes in the tertiary structure of the receptor, the complex is partially active in initiating gene activation[125]. Tamoxifen mimics the shape of E2 (Figure 1.4) however, it has an extra chain that is important for its antagonist action[48]. The agonist action of antiestrogen can be beneficial in some tissues like bones and cardiovascular system; however, it has bad effect in tissues like breast and uterus as it leads to cancer formation[58].

In the stomach, there are only few studies showing the impact of tamoxifen on the gastric mucosa [126], [127]. Whether this effect is inhibitory or stimulatory on target genes is yet to be studied.

1.3.4.1 Tamoxifen and Breast Cancer

E2 plays an important role in activating genes not only responsible for the growth and differentiation of different tissues but also in responsible for breast and uterine carcinogenesis[128], [129]. There is a strong link between breast cancer and ERα and therefore, there is routine checkup for the expression of this receptor in the

pathological diagnosis of breast cancer. In addition, it is very common to use antiestrogens like tamoxifen in order to treat breast cancer patients[58]. In history, tamoxifen was first clinically developed as a treatment of breast cancer in 1970s[130]. Thereafter, in 1985, the Food and Drug Administration (FDA) approved the use of tamoxifen as an adjuvant therapy with chemotherapy in postmenopausal women with node-positive breast cancer[98]. "Seventy percent of women with ERα-positive breast cancer benefit from tamoxifen"[131]. These women are considered to have ER-rich tumors and therefore, they response to endocrine therapy, while the rest with ER-negative tumors were not responding[132].

Tamoxifen works as an antagonist to E2 in the breast while it has some estrogenic properties in tissues like bone and works to provide signals for bone maintenance[48], [98].

The active metabolite of tamoxifen is known as 4-hydroxytamoxifen (4-OHT) (Figure 1.4)[133]. It has been noticed that the nature of the ligand bounded to the ER affects the interaction between ER and EREs. For example, it was found that both E2-ER and 4-OHT-ER bound a singlet ERE with similar affinity[134]. In contrast, at saturation, 4-OHT-ER binds half the sites comparing to E2-ER binding. Therefore, the nature of the ligand can alter the binding affinity of ER to DNA[98].

1.3.4.2 Tamoxifen Effect on Mouse Stomach

Tamoxifen as a SERM is used clinically in chemotherapeutic, anti-osteoporotic and many other therapeutic strategies[135], [136]. Some researchers suggest that tamoxifen induce gastric cancer[137]. Most gastric cancers occur because of bacterial colonization of the stomach especially by *Helicobacter pylori*,

which cause death (atrophy) of parietal cells, differentiation change (metaplasia) in zymogenic cells and induce proliferation of stem cells[138]. In an interesting report, Huh *et al.* identified that tamoxifen cause parietal cell death and metaplasia[126]. They found that mice treated with 5mg/20g body weight of tamoxifen for three constitutive days undergo parietal cell atrophy, increase of progenitor cell proliferation and dedifferentation of zymogenic cells. The increase of progenitor cell proliferation plus the zymogenic cell dedifferentiation are characteristic features of spasmolytic polypeptide expressing metaplasia (SPEM)[139] as demonstrated in (Figure 1.8). This induced SPEM was completely reversible within two weeks of tamoxifen treatment. It is not clear how tamoxifen induce parietal cell atrophy, but the proton pump inhibitor, omeprazole, partially rescue the effect of tamoxifen which suggest that tamoxifen action needs active acid secretion[126].

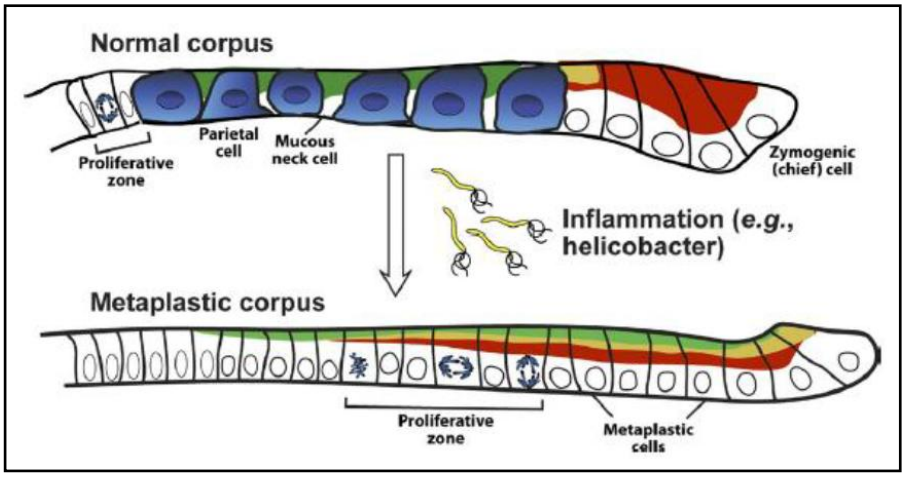

Figure 1.8: Mechanisms of spasmolytic polypeptide expressing metaplasia (SPEM) in the corpus. Features of SPEM includes parietal cells lost, expansion of the proliferative zone, expression of mucus neck cell markers such as spasmolytic polypeptide/TFF2 (shown in green) more toward the base of the gland, and co-expression of zymogenic cell markers such as pepsinogen C (shown in red) with neck cell markers[9].

As it is well known that tamoxifen can function as agonist or antagonist to estrogen depending on cell type, mice treated with the agonist E2 did not develop SPEM and this estradiol could not rescue SPEM induced by tamoxifen. Moreover, sex of the mice did not affect the response to tamoxifen. All together, the SPEM caused by tamoxifen is not ER or sex dependent[126].

1.4 Goals of the Master Thesis

Although the characterization and the functions of the gastric corpus cells have been well studied, the pathways controlling cell proliferation versus differentiation are not fully understood. This project aims to study the role of E2 or 4-OHT on the well-established mouse gastric epithelial progenitor (mGEP) cell line. Our hypotheses are that E2 pathway is required for gastric stem cell homeostasis and 4-OHT acts as an antagonist to ER in mGEP cells. Therefore, the role of E2 or 4-OHT on the mGEP cells has been studied at the cellular proliferation as well as differentiation levels using molecular techniques. In addition, several ER target genes have been studied as well. This study is crucial, especially that the E2 impact on gastric stem cell homeostasis in a well-controlled *in vitro* system is unknown.

Chapter 2 : Methods

2.1 Cells and Treatment

mGEPs (passages 21-24) were obtained from Prof. Sherif Karam (UAEU). Cells were cultured in appropriate cell culture medium RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM of L-Glutamin (Life Technologies) and 100 U/ml / 50 µg/ml penicillin/streptomycin (Life Technologies). Cells were maintained in humidified incubator at 37^{o}C in a 5% CO_2 atmosphere.

mGEP cells were treated with different concentrations of estrogen (β-estradiol or E2) (Sigma) or tamoxifen (4-OHT) (Sigma Aldrich). Cells were cultured in 6-well plates at a density of 200,000 cells/well using media supplemented with 10% FBS. Then, cells were starved for 24 h before the treatment. RPMI-1640 medium (Sigma) Supplemented with 0.1% FBS, 2 mM L-Glutamine and 100 U/ml / 50 µg/ml penicillin/streptomycin was used to starve the cells. For dose-course analysis, cells were treated with 0.5, 1, 2.5, 5, 10, 50, and 100 µM of E2 or 4-OHT for 24 and 48 h. Three control cells were used; (i) cells grown in RPMI-1640 medium supplemented with 0.1% FBS plus 0.1% ethanol which is the concentration of ethanol in E2 preparation (abbreviated as CE), (ii) cells grown in RPMI-1640 medium supplemented with 0.1% FBS plus 1% ethanol which is the concentration of ethanol in 4-OHT preparation (abbreviated as CT) and (iii) cells grown in RPMI-1640 medium supplemented with 0.1% FBS (abbreviated as C). mGEP cells treated with different concentrations of E2 or 4-OHT were examined for morphological changes using EVOS XL Core Cell Imaging System (Life Technologies) which is equipped with an indigenous camera and pictures were taken with 40X magnification.

2.2 Cell Viability

mGEPs were seeded in triplicate in 96-well plates at a density of 6,000 cells/well using media supplemented with 10% FBS. After 24 h of culture, cells were starved using media supplemented with 0.1% FBS. After another 24 h, cells were treated for various times (24 or 48 h) with increasing concentrations of E2 or 4-OHT (0.5, 1, 2.5, 5, 10, 50, and 100 µM). Control cells were treated with vehicle (ethanol); control for E2 (0.1% ethanol) and control for 4-OHT (1% ethanol). The effect of drugs on cell viability was determined using Cell Cytotoxicity Kit (Abcam) according to the manufacturer's specifications. The results are representative of an average of 5 and 4 independent experiments for 24 and 48 h, respectively. The data was presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which is assumed to be 100%.

2.3 Immunocytochemistry (ICC)

mGEP were stained and imaged using Olympus microscope IX83. Primary antibodies used for immunostaining were: mouse monoclonal anti-estrogen receptor alpha antibody (1:50, Cat. ab9269, Abcam), mouse monoclonal (1:100, Cat. ab288, Abcam) and rabbit polyclonal (1:100, Cat. ab3576, Abcam) anti-estrogen receptor beta antibodies. Estrogen receptor beta peptide (Cat. ab5018, Abcam) was used for competition and control experiments with the rabbit polyclonal anti-ER beta antibody (Cat. ab3576, Abcam) that reacts with this peptide. The competition experiment was done using a solution with equal weights per unit volume of peptide and corresponding antibody. Secondary antibodies were goat anti-mouse IgG H&L (Cy3 ®) preadsorbed (Cat. ab97035, Abcam) and goat anti-rabbit IgG H&L (Cy3 ®)

preadsorbed (Cat. ab6939, Abacm). Fluoroshield mounting medium with DAPI (Abcam) was used as a nuclear marker to stain the nucleus.

The cells were fixed with 3% formaldehyde in 1 X PBS for 20 min, washed three times with PBS, incubated with 1% Bovine Serum Albumin (BSA) for 45 min, and permeabilized with 0.1% triton X 100 in PBS for 10 min. Then the slides were incubated with the primary antibodies for overnight, washed three times with PBS, incubated with the secondary antibody for 1 h and then washed three times with PBS. Finally, DAPI with media was added to the slides. In the negative control slides, BSA was used in place of the primary antibody.

2.4 RNA Extraction and RT-PCR Analysis

RNA was isolated from the vehicle of treated cells in 6-well plates using TRIzol® (Life Technologies). Equal amounts of RNA (500 ng) RNA was reverse-transcribed using the iScript™ cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer.

RT-PCR was used for the semi-quantitative analysis for the proliferation and differentiation markers and ER target genes. RT-PCR was performed using one tenth (2 µL) of cDNA synthesized by the iScript™ cDNA synthesis kit (Bio-rad). RT-PCR used the Go Taq Flexi DNA Polymerase kit (Promega), according to manufacturer's instruction. The RT-PCR products were separated by 2% agarose gel and visualized by ethidium bromide staining using Gel Doc™ EZ Imager (Bio-rad). The experiment was repeated twice. Primer sequences and product size are shown in (Tables 2.1 and 2.2). RNA signals intensities on RT-PCR were analyzed and quantified using

ImageJ v.1.45 software (http://rsbweb.nih.gov/ij/), after normalization to GAPDH signal intensities.

2.5 Statistical Analysis

The statistical analysis were done using SPSS version 20. Data were reported as group mean ± SD. The data were analyzed via one-way ANOVA followed by LSD's *Post-Hoc* multiple comparison test (to compare all groups). Significance for statistical comparisons was set at $p< 0.05$.

Table 2.1: Mouse oligonucleotides primers for RT-PCR reactions

	Gene	Forward	Reverse	Product size (bp)
Receptors				
1	ERα	5'-GACCAGATGGTCAGTGCCTT-3’	5'-ACTCGAGAAGGTGGACCTGA-3'	205
2	ERβ	5'-CAGTAACAAGGGCATGGAAC-3'	5'-GTACATGTCCCACTTCTGAC-3'	243
Differentiation markers				
3	HKα	5'-TGTACACATGAGAGTCCCCTTG-3'	5'-GAGTCTTCTCGTTTTCCACACC-3'	157
4	HKβ	5'-AAC AGA ATT GTC AAG TTC CTC-3'	5'-AGA CTG AAG GTG CCA TTG-3'	140
5	IF	5'-CTTGGCCCTGACCTGTATGT-3'	5'-TAGGTTGCTCAGGTGTCACG-3'	191
6	Muc5AC	5'-GTGGTTTGACACTGACTTCCC-3'	5'-CTCCTCTCGGTGACAGAGTCT-3'	103
7	Muc6	5'-AGCCCACATTCCCTATCAGC-3'	5'-CACAGTGGAAGATTGCGAGAG-3'	192
8	TFF2	5'-TGCTCTGGTAGAGGGCGAG-3'	5'-CGACGCTAGAGTCAAAGCAG-3'	137
9	HDC	5'-CGTGAATACTACCGAGCTAGAGG-3'	5'-ACTCGTTCAATGTCCCCAAAG-3'	182
10	CgA	5’-AAGAAGAGGAGGAGGAAGAGG-3’	5’-TCCATCCACTGCCTGAGAG-3’	149
11	Sst	5’-TCTGCATCGTCCTGGCTTTGG-3’	5’-TCATTCTCTGTCTGGTTGGGCTC-3’	160
Stem cell markers				
12	CD44	5'-CACCATTGCCTCAACTGTGC-3'	5'-TTGTGGGCTCCTGAGTCTGA-3'	116
13	Sox2	5’-GCGGAGTGGAAACTTTTGTCC-3’	5’-CGGGAAGCGTGTACTTATCCTT-3’	157
Internal control				
14	GAPDH	5'-TCAAGAAGGTGGTGAAGCAGG-3'	5'-TATTATGGGGGTCTGGGATGG-3'	350

Table 2.2: Oligonucleotides primers for ER target genes

	Gene	Forward	Reverse	Product size (bp)
1	Insulin growth factor 1 receptor (Igf1r)	5-GTGGGGGCTCGTGTTTCTC-3	5-GATCACCGTGCAGTTTTCCA-3	127
2	Cyclin D1 (Ccnd1)	5-GCGTACCCTGACACCAATCTC-3	5-CTCCTCTTCGCACTTCTGCTC-3	183
3	Low density lipoprotein receptor (Ldlr)	5-TGACTCAGACGAACAAGGCTG-3	5-ATCTAGGCAATCTCGGTCTCC-3	118
4	Interleukin-6 (Il-6)	5-CCAAGAGGTGAGTGCTTCCC-3	5-CTGTTGTTCAGACTCTCTCCCT-3	118
5	mal, T-cell differentiation protein (Mal)	5-TTTGTGAGTTTGTCTTTGGAGGC-3	5-CCGCCATGAGTACCAATTATGT-3	157
6	vascular endothelial growth factor A (Vegfa)	5-CTGCCGTCCGATTGAGACC-3	5-CCCCTCCTTGTACCACTGTC-3	233

Chapter 3 : Results

3.1 Expression of Mouse ERα and ERβ in mGEP Cell Line

The expression of mouse ERα and ERβ in mGEP cell line was tested at both mRNA and protein levels by using RT-PCR (Figure 3.1) and ICC (Figure 3.2), respectively. The results confirmed the expression of both receptors. Further sequencing for ERα and ERβ amplicons was done and results are shown in (Supplementary Table S.1 and S.2).

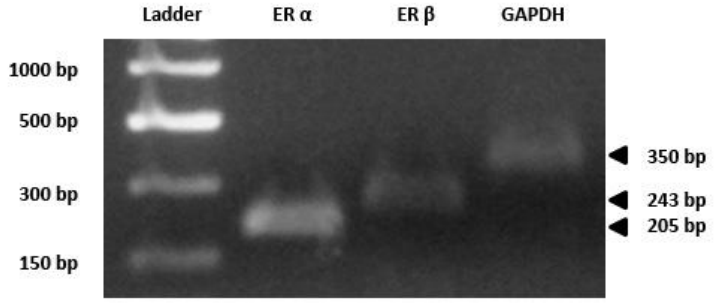

Figure 3.1: Expression of mouse ERα and ERβ in mGEP cells. RT-PCR products for ERα (lane 1) with band size of 205 bp and ERβ (lane 2) with band size of 243 bp. GAPDH band is shown in (lane 3) with band size of 350 bp.

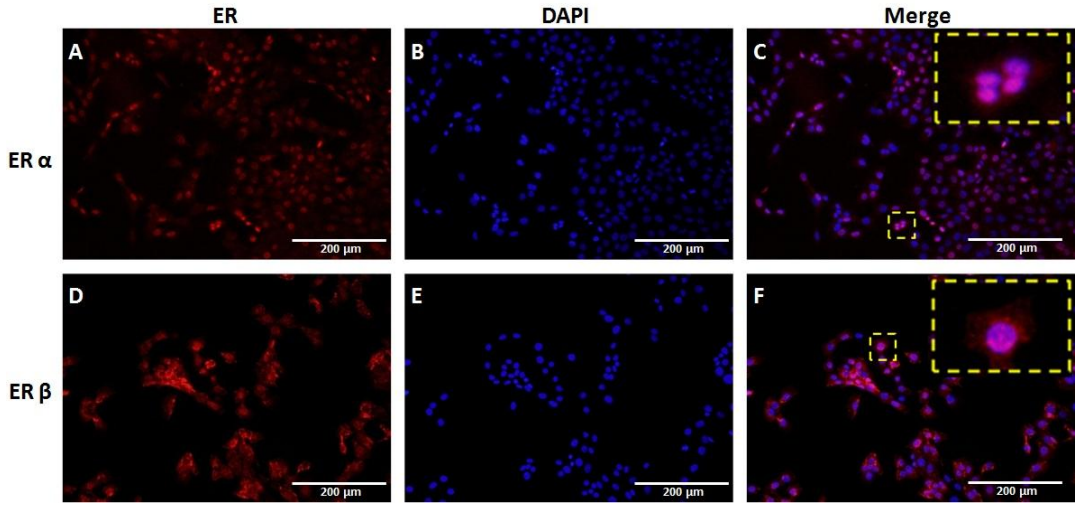

Figure 3.2: Cellular localization of mouse ERα and ERβ in mGEP cells. Both ERs are expressed in the nuclei and cytoplasmic region of mGEP cells. Antibodies used: (A) mouse monoclonal anti-ERα (ab9269), and (B) mouse monoclonal anti-ERβ (ab288). Images taken at 20X magnifications.

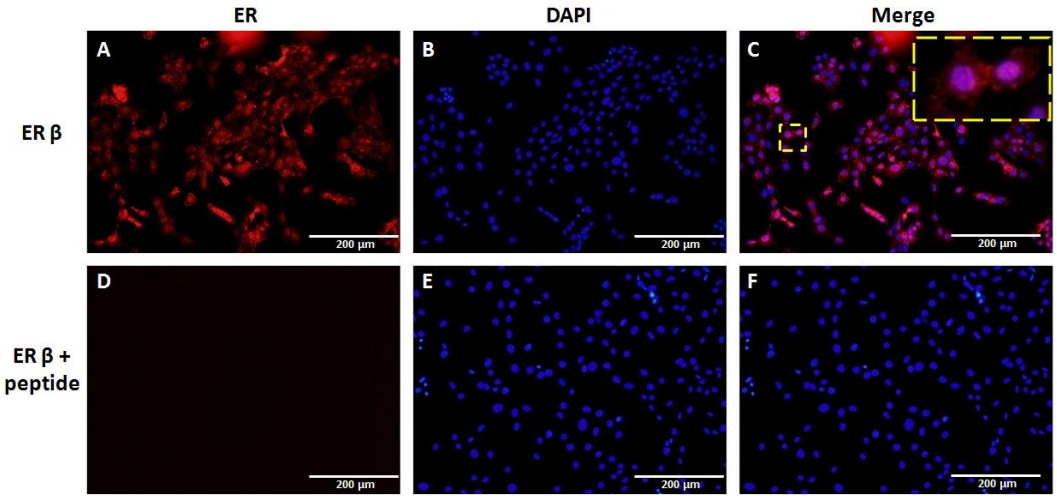

Figure 3.3: Cellular localization and specificity determination of mouse ERβ in mGEP cells using rabbit anti-ERβ. ERβ is expressed in the cytoplasmic and nuclear region of mGEP cells (A-C). No detection of ERβ when rabbit anti-ERβ plus its blocking peptide were used for competition (control) experiment (D-F). Antibodies used: (A) rabbit polyclonal anti-ERβ (ab3576), and (D) rabbit polyclonal anti-ERβ (ab3576) plus its corresponding peptide (ab5018). Images taken at 20X magnifications.

Both mouse ERα and ERβ are expressed in the nuclei and cytoplasmic regions of mGEP cells (Figure 3.2). Expression of mouse ERβ using rabbit anti-ERβ antibody is shown in (Figure 3.3 A-C). ERβ is expressed both in the cytoplasmic and nuclear regions of mGEP cells. ERβ blocking peptide was used for the competition experiment with the rabbit anti-ERβ antibody (Figure 3.3 D-F). ERβ peptides blocked all the ERβ in the cells, therefore, no signal was detected in the competition experiment and therefore, conforming the specificity of the antibody.

3.2 Effect of Estrogen or Tamoxifen in mGEP Cellular Viability

The effect of different concentrations of E2 or 4-OHT 0.5, 1, 2.5, 5, 10, 50 and 100 μM on the viability of mGEP cell line was tested. The results indicated that E2 does not alter the cellular viability after 24 and 48 h treatment comparing to controls. On the other hand, 4-OHT decreased cellular viability in concentration and time dependent manners (Figure 3.4 A-B). Therefore, 4-OHT has an antagonistic effect on mGEP viability comparing to E2.

After 24 h treatment, 4-OHT concentrations of 2.5 and 5 μM caused a significant reduction in cell viability (70.5 and 49.5 %, respectively). Moreover, higher 4-OHT concentrations of 10, 50 and 100 μM caused complete loss in cell viability (0%). Similarly, after 48 h treatment, 4-OHT concentration of 2.5 μM caused a significant reduction in cell viability (9.1 %). In addition, higher 4-OHT concentration of 5, 10, 50 and 100 μM caused loss of cell viability (0%). Higher concentrations, above 5 μM of 4-OHT appear to have a toxic effect on cell growth and viability.

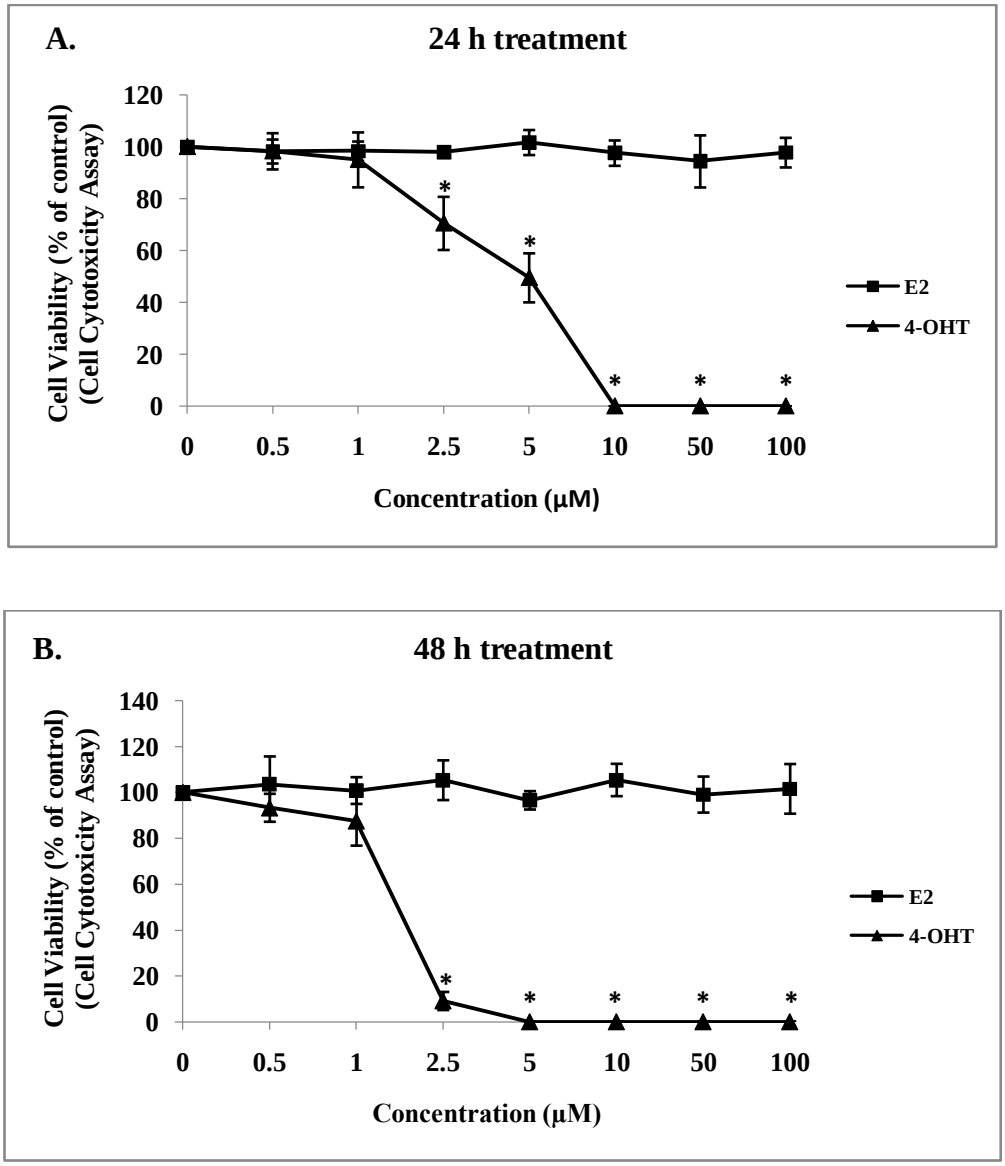

Figure 3.4: Cellular viability of mGEP after (A) 24 h and (B) 48 h treatment with E2 or 4-OHT. E2 does not alter the cell viability after 24 or 48 h treatment. 4-OHT decreased cellular viability in concentration and time dependent manners. Data represent the mean of five and four independent experiments for 24 and 48 h treatment, respectively, carried out in triplicate. Statistical analysis for cell viability data was performed using one-way ANOVA followed by LSD's *Post-Hoc* multiple comparison test (*$p < 0.05$).

3.3 Morphological Changes in mGEP Cells Treated with Estrogen or Tamoxifen

Microscopic observation of mGEP cells treated with E2 for 24 and 48 h showed that there was no change in the shape and number of cells using 0.5, 1, 2.5, 5, and 10 μM of E2 (Figure 3.5 and 3.7). However, the number of mGEP cells treated with 4-OHT decreased when the concentration of the drug increased after 24 and 48 h treatment. Noticeably, cells treated with 4-OHT underwent morphological changes which occurred at concentrations of 10 μM after 24 h, and 5 and 10 μM after 48 h of treatment, respectively, such as rounding and shrinkage, which are characteristic of apoptotic cells (Figure 3.6 and 3.8). Additionally, total cell death occurred at higher 4-OHT concentrations (50 and 100 μM) at both times, 24 and 48 h (data not shown).

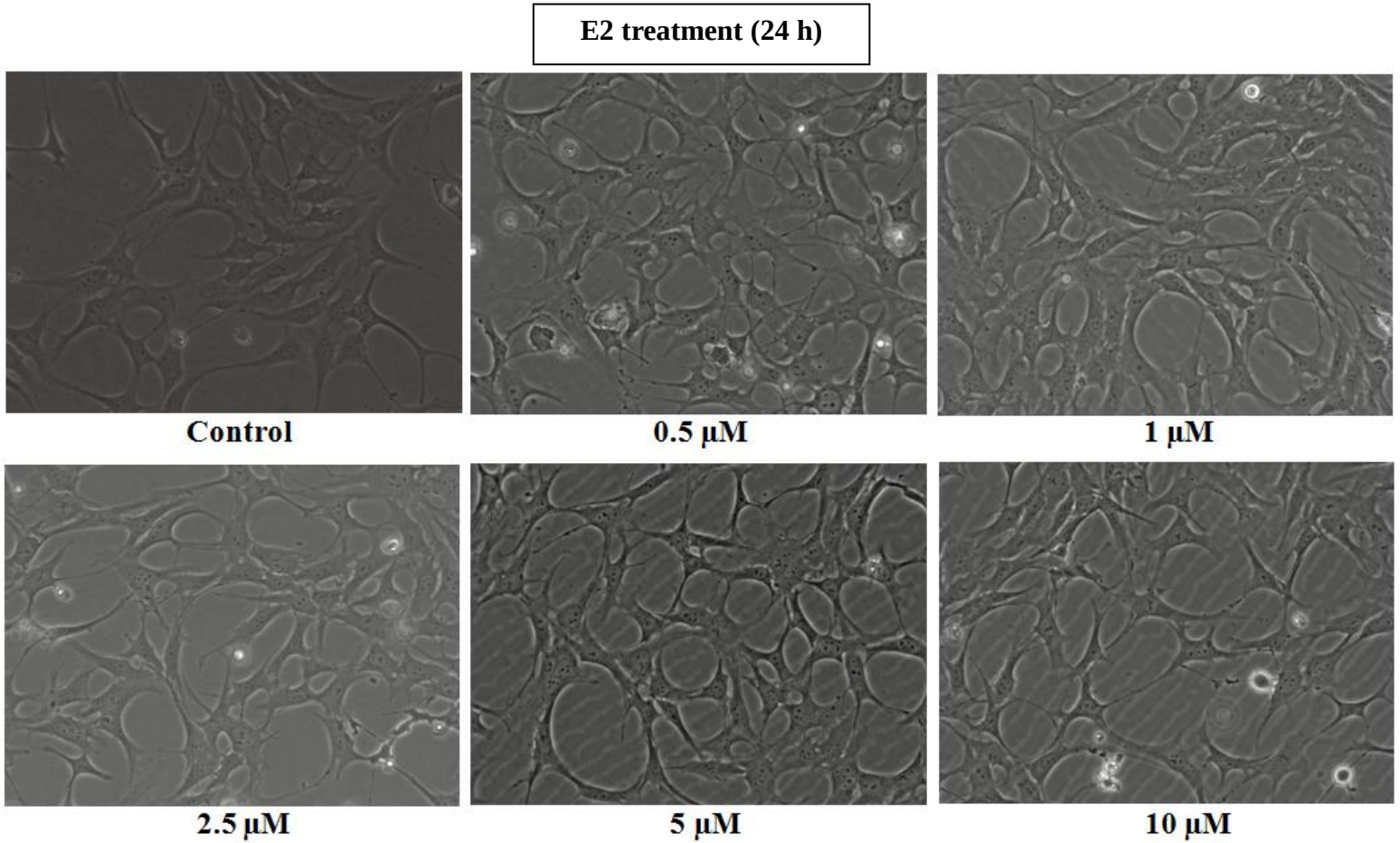

Figure 3.5: Morphology of mGEP cells treated with E2 after 24 h. Photos showed that there was no morphological changes in mGEP cells after E2 treatment for 24 h. Images taken at 40X magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies).

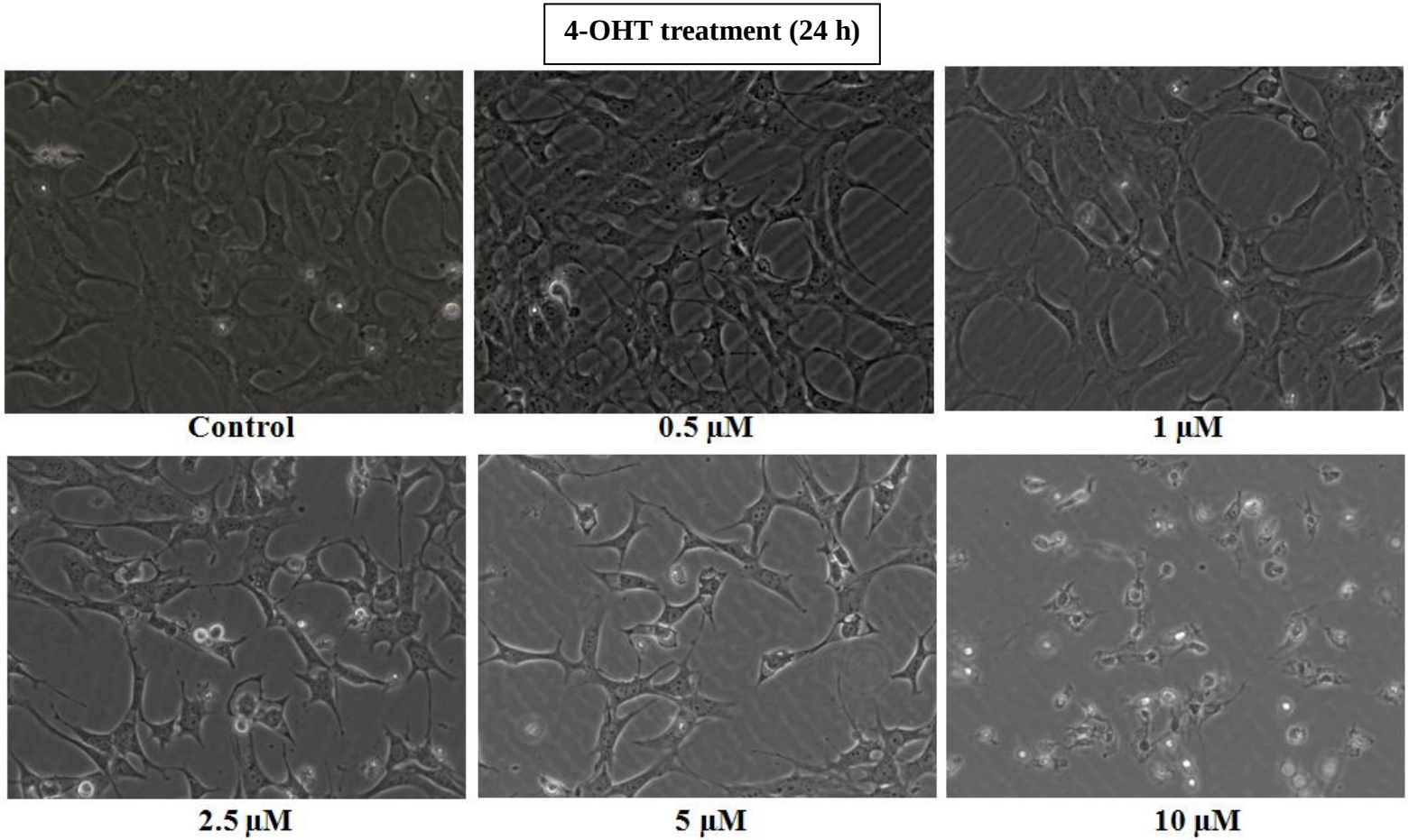

Figure 3.6: Morphological changes in mGEP cells treated with 4-OHT after 24 h. The number of cells decreased as the 4-OHT concentration increased. Additionally, some morphological changes occurred at dose of 10 µM such rounding and shrinkage, which are characteristic of apoptotic cells. Images taken at 40X magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies).

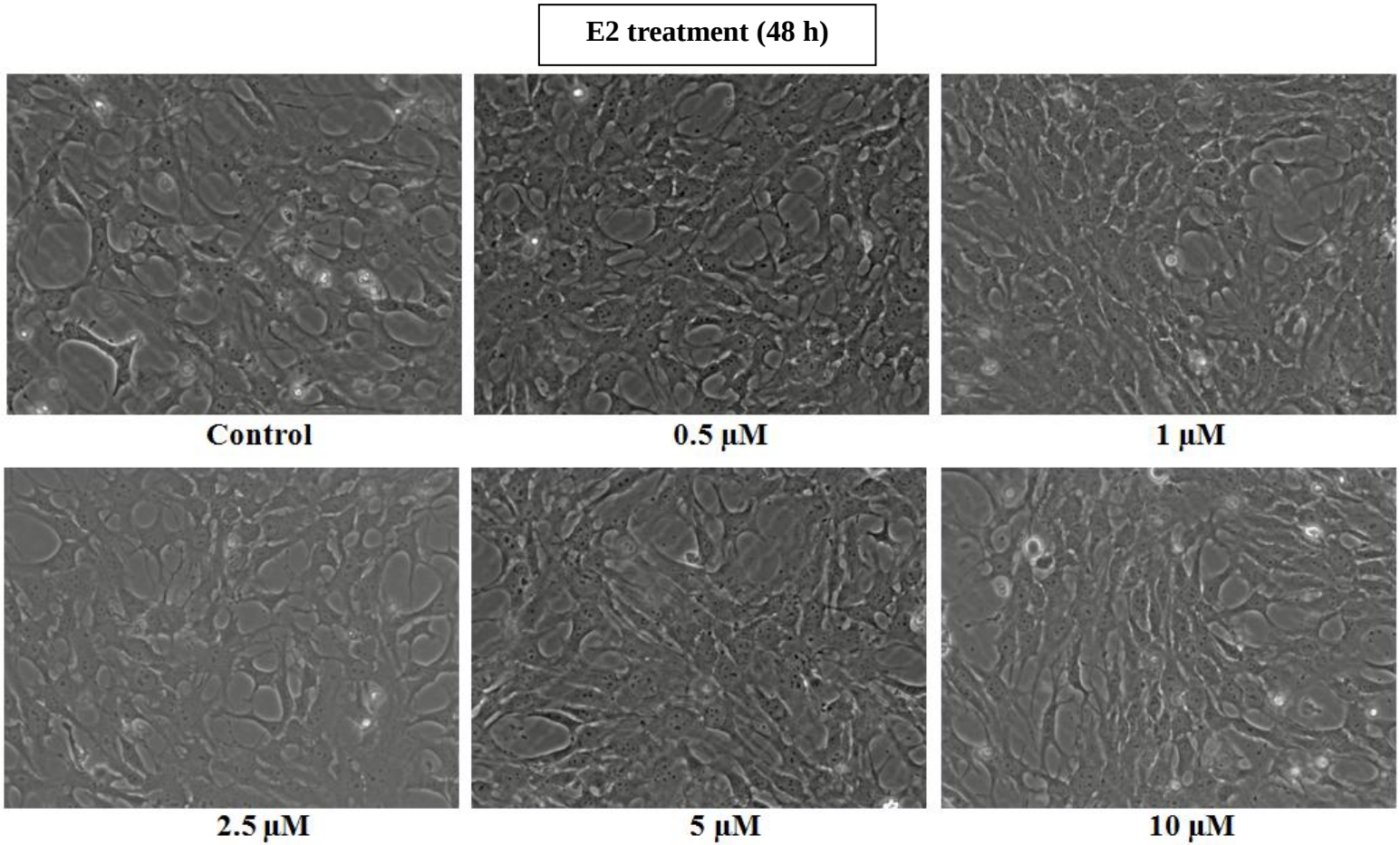

Figure 3.7: Morphology of mGEP cells treated with E2 after 48 h. Photos showed that there was no morphological changes in mGEP cells after E2 treatment for 48 h. Images taken at 40X magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies).

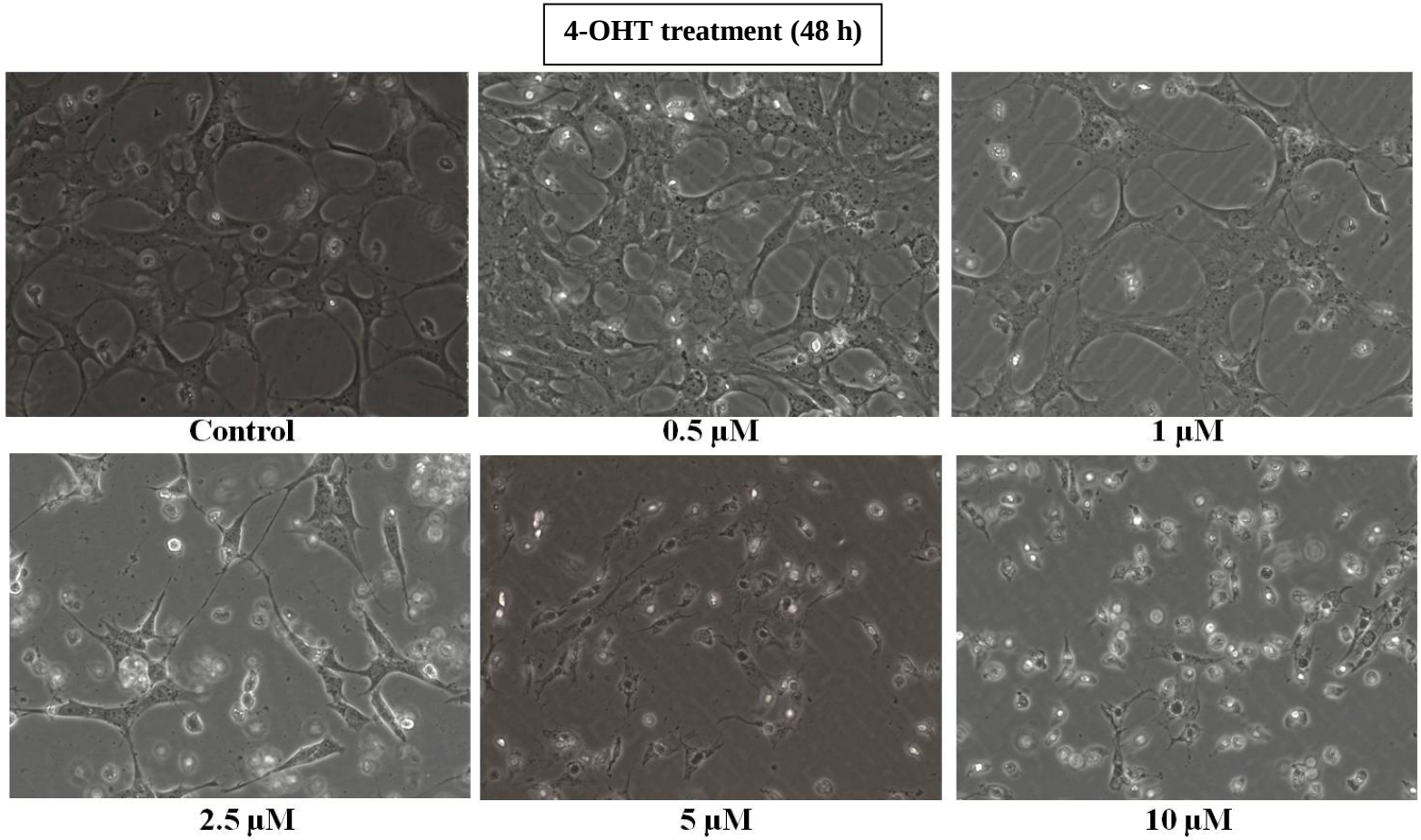

Figure 3.8: Morphological changes in mGEP cells treated with 4-OHT after 48 h. The number of cells decreased as the 4-OHT concentration increased. Additionally, some morphological changes occurred at dose of 5 and 10 µM such rounding and shrinkage, which are characteristic of apoptotic cells. Images taken at 40X magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies).

3.4 Expression of Mouse ERs, Proliferation and Differentiation Markers after Estrogen or Tamoxifen Treatment

Semi-quantitative RT-PCR was performed to measure the expression of ERs, proliferation and differentiation markers after 24 (Figure 3.9) and 48 h (Figure 3.10) treatments with E2 or 4-OHT. All comparisons of gene expression of treated cells were against the control of each treatment. Gene expression was compared to that of the corresponding control (CT or CE) after normalization to GAPDH signal intensities. The corpus region of the stomach was used as positive control for gene expression.

The following results are description for different markers after 24 h treatment (Figure 3.9 and Supplementary Table S.3). After 24 h treatment with E2, ERα expression was highest at concentration of 2.5 μM (2.8 fold-increase) while other E2 concentration showed ≈ 2 fold-increase comparing to control (CE). For the cells treated with 4-OHT, the expression of ERα showed 2 fold-increase at dose 1 μM comparing to control (CT). No change was observed at dose 2.5 and 5 μM while the expression decreased to 0.4 fold at concentration of 10 μM. ERβ gene expression was compared to control (C) for both E2 and 4-OHT treatments since there was no expression for both controls CT and CE. For cells treated with E2, ERβ expression was less than the control while ERβ expression increased with 4-OHT treatment. ERβ expression was 3.3, 2.5, 3.8 and 4 fold-increase with increased 4-OHT concentration (1, 2.5, 5 and 10 μM, respectively).

For differentiation markers, only muc5AC and muc6; markers of surface mucous and mucous neck cells, respectively, were found to be expressed in the treated cells. For cells treated with E2, the expression of muc5AC was below the

level of control (CE) for 1,5,10 μM while it was close to control at 2.5 μM. The expression of muc5AC after 4-OHT treatment showed 2.4, 2.4, 5.8 and 4.1 fold-increase for 1, 2.5, 5 and 10 μM of 4-OHT when compared to control (CT).

Muc6 gene expression was compared to control (C) for both E2 and 4-OHT treatments since there was no expression for both controls CT and CE. For cells treated with E2, there was a reduction in the expression at lower concentrations while there was a 1.7 fold-increase in the expression at concentration of 10 μM. Oppositely, the expression of muc6 showed 3.1 fold-increase at 1 μM of 4-OHT and then it showed a reduction in muc6 expression at higher 4-OHT concentrations. No expression of other differentiation markers (HKα, HKβ, IF, TFF2, HDC, CgA, Sst) was observed in the control and treated cell of different concentrations of E2 or 4-OHT after 24 h treatment (data not shown except for HKα and IF in Figure 3.9).

CD44, a stem cell marker does not show a remarkable change in expression when treated with E2 and 4-OHT. For the corpus stem cell marker Sox2; it showed the highest fold change by 4.4 fold-increase at 1 μM concentration of 4-OHT then it decreased as the concentration of 4-OHT increased. On the other hand, the expression of Sox2 was high at all concentrations of E2 comparing to CE, with highest fold increase at 2.5 μM (3.9 fold) and lowest at 5 μM (1.8 fold). The highest expression of Sox2 for both treatments was at 2.5 μM and 1 μM of E2 and 4-OHT, respectively, indicating that these concentrations influence stem cell potency.

Next, the gene expression results after 48 h treatment (Figure 3.10 and Supplementary Table S.4) showed a 2.1 fold-increase in ERα expression when

treated with 1 μM of 4-OHT (similar to 24 h treatment) while the expression was similar to control for other 4-OHT and all E2 concentrations.

ERβ expression at 1 μM of E2 was highest among E2 concentrations showing 4.7 fold-increase, additionally, ERβ expression at concentration of 2.5 and 5 μM were lower than the expression at 1 μM, however, at 10 μM the expression increased by 2.5 folds comparing to CE. ERβ expression was lowered at 2.5 and 5 μM of 4-OHT comparing to CT, while the expression at 1 and 10 μM was close to CT.

For differentiation markers, only muc5AC and muc6 were found to be expressed in treated cells after 48 h treatment which was consistent with 24 h treatment. For cells treated with E2, muc5AC showed highest expression (3.4 fold-increase) at 1 μM comparing to CE and other higher concentrations of E2. Then the expression of muc5AC decreased in concentration dependent manner at 2.5, 5 and 10 μM comparing to CE. Muc5AC expression showed 1.7 fold-increase at 1 μM of 4-OHT while there was no change in the expression at higher concentrations of 4-OHT comparing to CT.

For cells treated with E2, the level of muc6 expression was almost the same as CE. Muc6 expression was doubled at 5 μM of 4-OHT while no much change in the expression of cells treated with 1, 2.5 and 10 μM comparing to CT. No expression of other differentiation markers (HKα, HKβ, IF, TFF2, HDC, CgA, Sst) was observed in the control and treated cell of different concentrations of E2 or 4-OHT after 48 h treatment (data not shown except for HKα and IF in Figure 3.10).

The expression of CD44 was not changed at 1 μM of E2 comparing to CE and it showed 1.7 fold-increase at 2.5 μM and then it decreased at higher E2

concentrations. CD44 expression decreased as the concentration of 4-OHT increased; indicating that stem cell potency is lowered after 48 h treatment as the concentration of 4-OHT increased.

For the corpus stem cell marker Sox2, the expression at 1 μM of E2 was similar to CE while it decreased at higher E2 concentrations. Sox2 expression showed 2 fold-increase at 1 μM of 4-OHT then it decreased at higher 4-OHT concentrations inducting a reduction in stem cell potency marker.

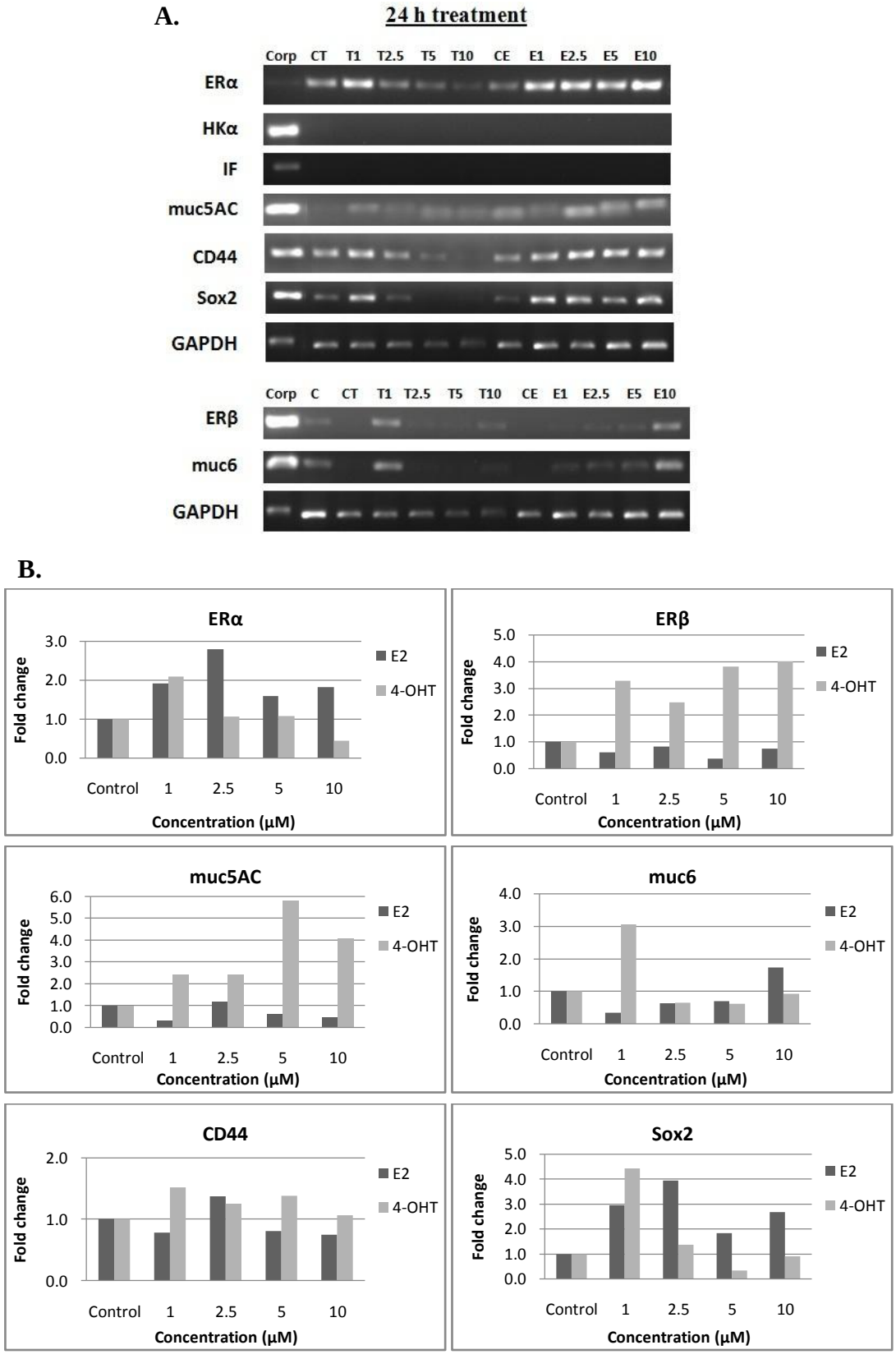

Figure 3.9: Representative RNA expression of ERs, differentiation and proliferation markers after 24 h of E2 or 4-OHT treatment in mGEP cells, when compared to controls. RNA expressions analyzed by RT-PCR (A) were quantified by densitometry after normalization to GAPDH signal intensities (B). (Corp: corpus, C:

control with 0.1% FBS, CT: control for 4-OHT with 0.1% FBS and 1% EtOH, T: 4-OHT treatment followed by the concentration used in µM, CE: control for E2 with 0.1% FBS and 0.1% EtOH, E: E2 treatment followed by the concentration used in µM).

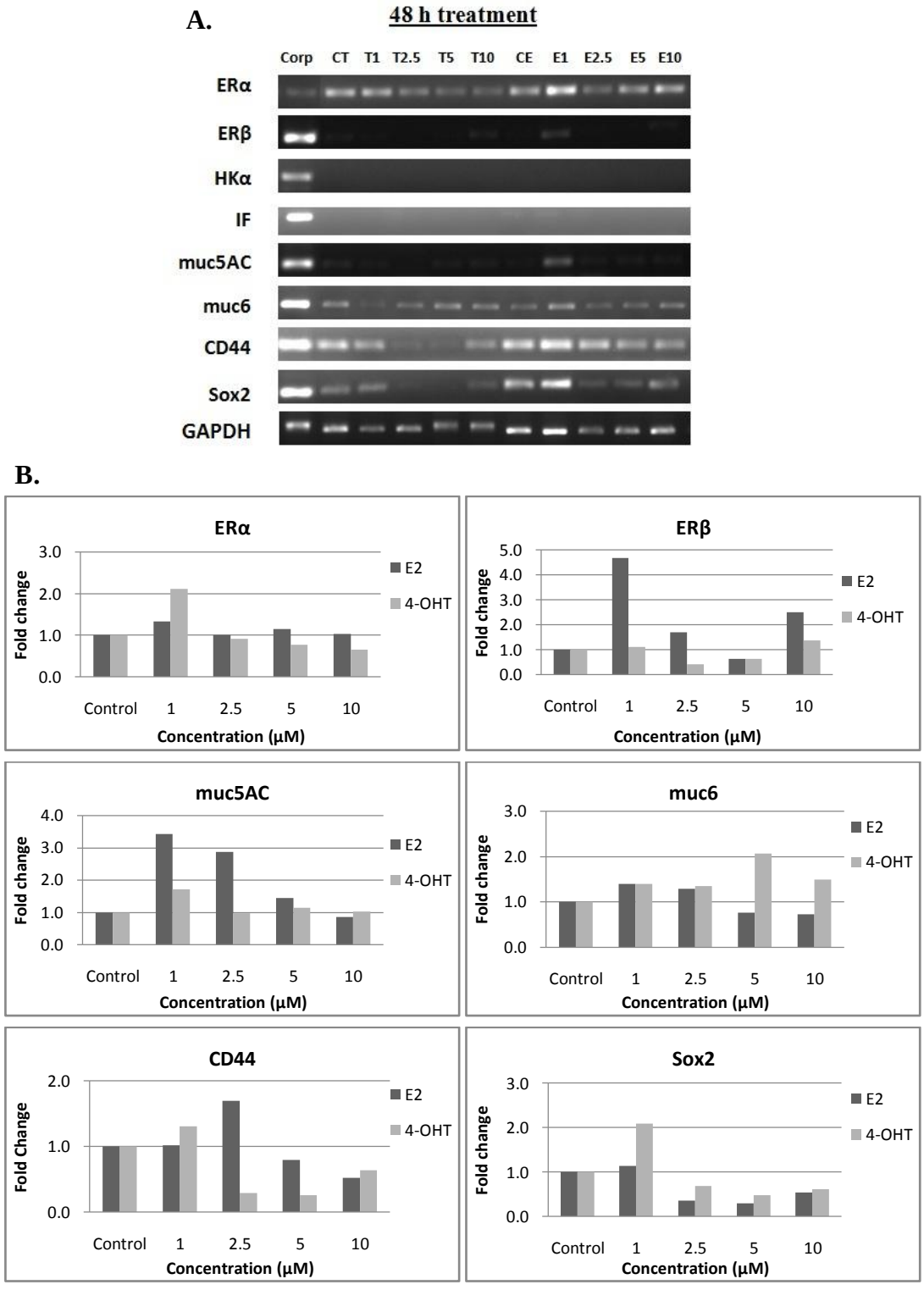

Figure 3.10: Representative RNA expression of ERs, differentiation and proliferation markers after 48 h of E2 or 4-OHT treatment in mGEP cells, when compared to controls. RNA expressions analyzed by RT-PCR (A) were quantified by

densitometry after normalization to GAPDH signal intensities (B). (Corp: corpus, CT: control for 4-OHT with 0.1% FBS and 1% EtOH, T: 4-OHT treatment followed by the concentration used in μM, CE: control for E2 with 0.1% FBS and 0.1% EtOH, E: E2 treatment followed by the concentration used in μM).

3.5 Expression of ER Target Genes after Estrogen or Tamoxifen Treatment

Expression of ER target genes were examined using RT-PCR after 24 (Figure 3.11) and 48 h (Figure 3.12) treatments with E2 or 4-OHT. All comparisons of gene expression of treated cells were against the control of each treatment. Gene expression was compared to that of the corresponding control (CT or CE) after normalization to GAPDH signal intensities. The corpus region of the stomach was used as positive control for gene expression.

The expression of ER target genes after 24 h treatment is shown in (Figure 3.11 and Supplementary Table S.3). The expression of Igf1r at different E2 concentrations was similar to CE. The expression of Igf1r showed 1.8 fold-increase at 1 μM of 4-OHT while there was no change in gene expression at higher 4-OHT concentrations when compared to CT.

For Ccnd1, there was a reduction by 0.5 fold at 1 μM then the expression was close to CE at higher E2 concentrations. The expression of Ldlr showed 1.7 fold-increase at 2.5 μM of E2 while the expression at other concentrations were similar to CE. Il-6 gene expression was compared to control (C) for both E2 and 4-OHT treatments since there was no expression for both controls CT and CE. The expression of Il-6 was low at 1 μM and then it showed a gradual increase until it reached 1.8 fold-increase at 10 μM of E2.

Vegfa showed increased gene expression when treated with E2 comparing to CE. Vegfa expression was increased by 4.5 folds at 2.5 μM of E2, the expression increased by 3 folds at 1 and 10 μM of E2 while the expression was doubled at 5 μM comparing to CE.

The expression of Ccnd1, Ldlr, Il-6 and Vegfa were highest at 1 μM of 4-OHT (1.8, 2.3, 3.7 and 3.8 fold-increase, respectively) then the expression gradually decreased as the concentration of 4-OHT increased. For Il-6, no expression was found at 2.5, 5 and 10 μM of 4-OHT. Additionally, No expression of Mal was detected in the control and treated cell of different concentrations of E2 or 4-OHT after 24 h (Figure 3.11 A).

The expression of ER target genes after 48 h treatment is shown in (Figure 3.12 and Supplementary Table S.4). Igf1r expression was similar to CE for E2 treated cells at all concentrations. Ccnd1 showed highest expression at 10 μM of E2 (1.6 fold-increase) while there was no change in gene expression at other E2 concentrations when compared to CE. No change in Ldlr was observed when treated with E2 when compared to CE.

The expression of Igf1r, Ccnd1 and Ldlr was highest at 1 μM of 4-OHT with 1.8, 1.7 and 2.2 fold-increase, respectively, then the expression gradually decreased as the concentration of 4-OHT increased. No Ldlr expression at 5 and 10 μM of 4-OHT.

Il-6 showed an increase gene expression when treated with E2 comparing to CE. Il-6 expression increased at 1 and 2.5 μM by 1.6 and 4.1 folds then it decreased to 2.3 and 2.5 folds at 5 and 10 μM of E2 comparing to 2.5 μM of E2. No change in

the expression of Il-6 at 1 μM of 4-OHT when compared to CT, then there was no Il-6 expression at higher 4-OHT concentrations.

Vegfa expression was lowered when treated with E2 comparing to CE. There was a decrease in Vegfa expression when treated with 1 μM 4-OHT and no expression was found at 2.5, 5 and 10 μM of 4-OHT.

No expression of Mal was detected in the control and treated cell of different concentrations of E2 or 4-OHT after 48 h of treatment (Figure 3.12 A).

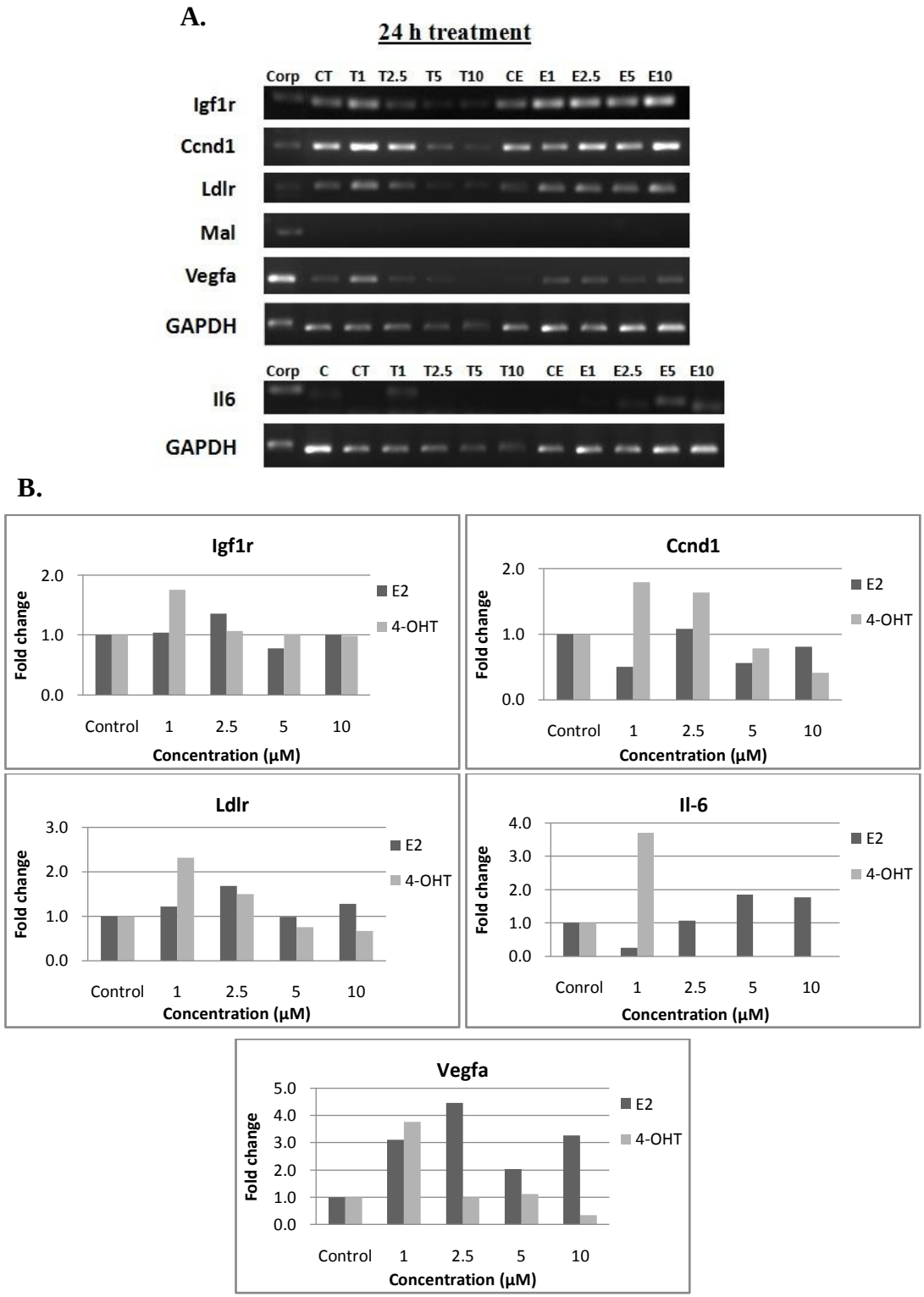

Figure 3.11: Representative RNA expression of ER target genes after 24 h of E2 or 4-OHT treatment in mGEP cells, when compared to controls. RNA expressions analyzed by RT-PCR (A) were quantified by densitometry after normalization to GAPDH signal intensities (B). (Corp: corpus, C: Control with 0.1% FBS, CT: control for 4-OHT with 0.1% FBS and 1% EtOH, T: 4-OHT treatment followed by the concentration used in µM, CE: control for E2 with 0.1% FBS and 0.1% EtOH, E: E2 treatment followed by the concentration used in µM).

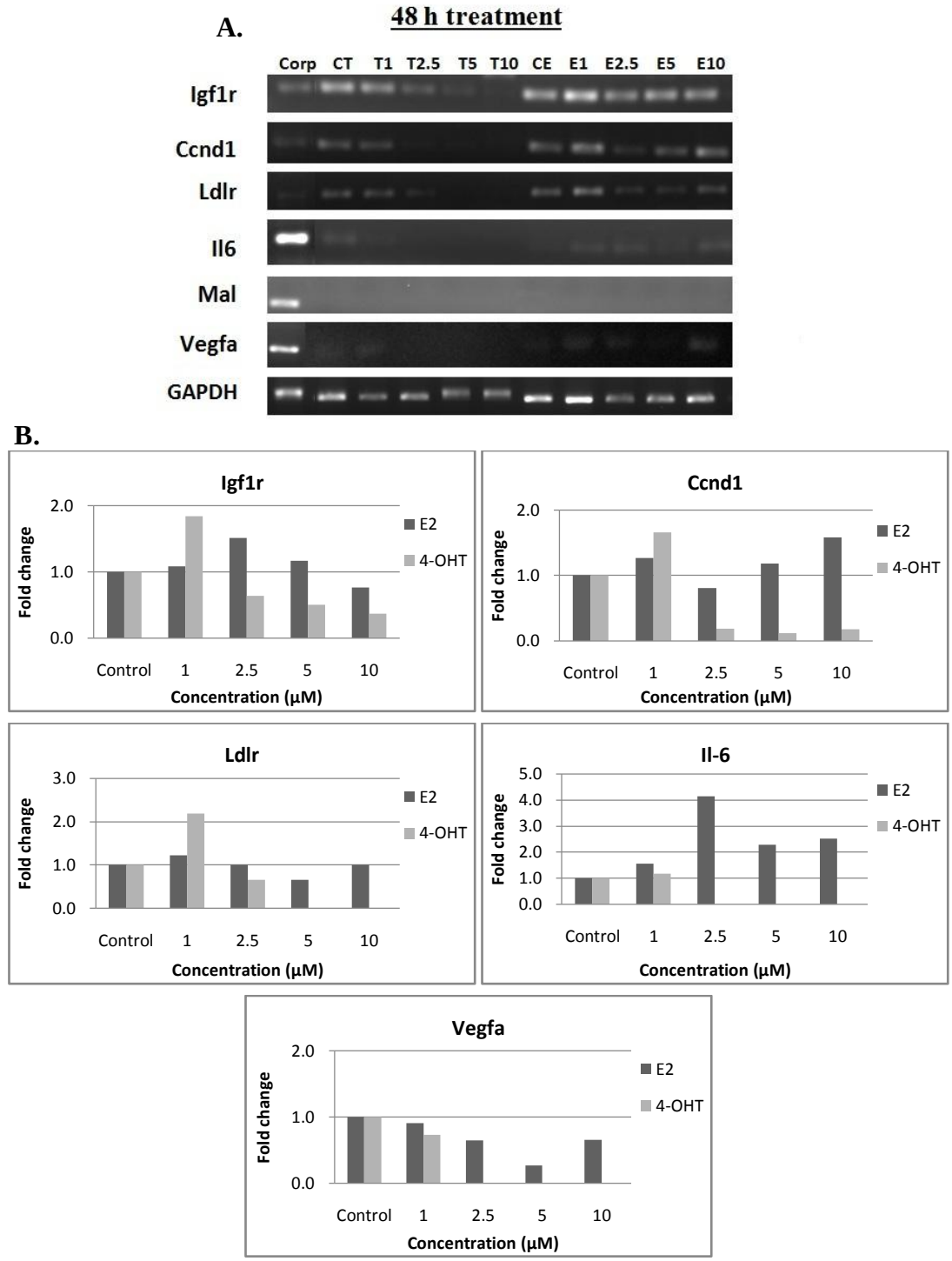

Figure 3.12: Representative RNA expression of ER target genes after 48 h of E2 or 4-OHT treatment in mGEP cells, when compared to controls. RNA expressions analyzed by RT-PCR (A) were quantified by densitometry after normalization to GAPDH signal intensities (B). (Corp: corpus, CT: control for 4-OHT with 0.1% FBS and 1% EtOH, T: 4-OHT treatment followed by the concentration used in µM, CE: control for E2 with 0.1% FBS and 0.1% EtOH, E: E2 treatment followed by the concentration used in µM).

Chapter 4 : Discussion

Estrogens regulate the growth and differentiation of target tissues through the mediation of ERα and ERβ. The oncologic importance of ERs in cancers of classical target tissues like breast and uterine endometrium has been well studied[55], [57], [140]. However, little is known about the effect of E2 and ERs on stomach homeostasis. To do so, this project aims to study the role of ERs in mouse GSCs using cultured mGEP cell line which is a useful tool to study the effect of E2 hormone -*in vitro*- in conditions that are independent of neural, hormonal and blood flow factors. It is important to know that mGEP cells are genetically engineered cells that show similar features to normal GSCs however they are not identical.

In the present study, the expression of ERα and ERβ in mGEP cells were demonstrated at both mRNA and protein levels, (Figures 3.1 and 3.2), respectively. The size and nucleotide sequence of amplicons were identical with expected sizes and sequences as shown in (Supplementary Tables S.1 and S.2). Immunostaining experiments showed that both ERα and ERβ are expressed in the nuclei and cytoplasmic regions of mGEP cells. Confirmation of the specificity of ligand-receptor binding was done using antibody with its blocking peptide (Figure 3.3).

The expression of ERα and ERβ in mGEP cells can be linked to a previous study by Campbell-Thompson *et al.*, in which they found that mRNA of both ER types are detected in rat stomach[70]. Additionally, they determined the distribution of ER proteins in rat stomach in which they found that both ERs are localized in the nuclei of parietal cells and epithelial cells in the progenitor zone[70]. These results

are consistent with our findings that ERs are expressed in mGEP cells which resembled stem cells in the progenitor zone of the stomach glands.

Cell viability study was carried out to examine the effect of E2 or 4-OHT on mGEP cells. E2 concentrations from 0.5 μM to 100 μM did not significantly affect the cell viability in mGEP cells after 24 and 48 h treatment comparing to controls (Figure 3.4 A-B). Cells treated with the SERM (4-OHT) showed decreased cellular viability in concentration and time dependent manners (Figure 3.4 A-B). Thus, 4-OHT had an antagonistic effect on cell viability comparing to E2.

When treated cells were observed under the microscope, there was no change in the shape and number of cells treated with 0.5, 1, 2.5, 5, and 10 μM of E2 for 24 and 48 h (Figures 3.5 and 3.7). On the other hand, the number of mGEP cells treated with 4-OHT decreased in time and concentration dependent manners. Moreover, cells treated with high 4-OHT concentrations showed morphological changes such as rounding and shrinkage, which are characteristics of apoptotic cells (Figures 3.6 and 3.8).

Collectively, the cell viability results and morphological changes of mGEP cells demonstrated that increasing E2 concentrations did not affect the viability and morphology of mGEP cells when compared to controls. However, these results showed that increasing concentrations of 4-OHT suppressed cellular viability and showed some characteristics of apoptotic cells. These results are similar to the study carried by Lippman and Bolan (1975) as they were the first to demonstrate that 4-OHT inhibited the growth of the ER-positive MCF-7 breast cancer cell line, and this effect can be reversed by the addition of E2. Additionally, the action of E2, was not specifically dramatic comparing to controls[141]. Similar results were also

demonstrated by Ruffy *et al.* (2006) where they found a delay in cell proliferation in normal human dermal fibroblasts when treated with 4-OHT *in vitro*[142].

The gene expression analysis of mGEP cells was determined before and after E2 or 4-OHT treatments at two time points (24 and 48 h). Treatment with 4-OHT induced ERα expression at 1 μM concentration both after 24 and 48 h treatment by 2 folds. Then there was a reduction in ERα expression at higher 4-OHT concentrations comparing to 1 μM demonstrating that low 4-OHT can induce ERα while higher concentrations did not. Mostly, all E2 concentrations induce higher expression of ERα with the highest expression at 2.5 μM after 24 h (2.8 fold) while the expression of ERα was the same after 48 h treatment. These results suggest that higher 4-OHT concentrations do not induce ERα expression comparing to E2 especially after 24 h treatment (different expression after 4-OHT or E2 treatments). After 24 h treatment, ERβ induced mainly by 4-OHT comparing to E2 while the induction of gene expression occurred after 48 h treatment with E2 and not 4-OHT. From our results, it has been noticed that ERα and ERβ have different pattern of expression with E2 or 4-OHT treatments. This can be explained that both ERs have different transcriptional activities with certain ligand, promoter and cell type[143]. Therefore, when both ERs co-expressed in cells, ERβ can antagonize ERα dependent transcription[143].

After 24 and 48 h treatments, mGEP cells expressed RNA for muc5AC (surface mucus cells) and muc6 (mucous neck cells) as the only expressed markers for differentiated gastric cells. This expression of muc5AC and muc6 in mGEP correspond to the ability of the cells to differentiate into surface mucous and mucous neck cells as shown in (Figure 1.3). No expression of other differentiation markers (Table 2.1) was found to be expressed in mGEP cells before and after the treatments.

After 24 h treatment, 4-OHT induced the expression of muc5AC while E2 treatment did not induce any. However, after 48 h, E2 induced muc5AC expression more than 4-OHT.

Muc6 expression showed 3 fold-increase at 1 μM 4-OHT after 24 h treatment while it showed 2 fold-increase at 5 μM 4-OHT after 48 h treatment. No remarkable increase in the expression of muc6 for E2 treated cells after 48 h while the expression showed gradual increase after 24 h. Therefore, the expression of muc6 is not showing any pattern when treated with E2 or 4-OHT.

RT-PCR showed that mGEP cells expressed muc5AC and muc6 markers in the control and treated cells. The expression of these markers in control cells can be explained by the ability of stem cells to express mRNA for differentiation markers but not expressed the corresponding proteins, which appear later in the differentiated cells and the expression of such markers represent precursors for differentiated cells[144]–[146].

The expression of Sox2 after 24 h of E2 or 4-OHT treatment resembled to the expression of ERα where highest expression were at 2.5 μM of E2 and 1 μM of 4-OHT. This indicates that these concentrations induced the expression of potency markers of mGEP comparing to other concentrations. Similarly, after 48 h treatment, Sox2 showed highest expression at 1 μM of 4-OHT which was correlated with ERα expression. Then the expression of Sox2 decreased as the concentration of 4-OHT increased indicting that there is a reduction the expression of potency markers in mGEP both after 24 and 48 h.

The expressions of ER target genes are mostly related to the expression of ERα especially after 4-OHT treatment. Since the highest ERα expression was at 1 μM of 4-OHT (≈2 fold-increase), the highest expression of Igf1r, Ccnd1, Ldlr, Il-6 and Vegfa was at 1 μM treatment with 4-OHT after 24 and 48 h. Additionally the fold change in Igf1r, Ccnd1, Ldlr after 1 μM treatment with 4-OHT was the same after 24 and 48 h treatment which is linked unchanged ERα expression after 1 μM 4-OHT treatment after 24 and 48 h. In most ER target genes, there was a reduction of expression as the concentration of 4-OHT increased while no such dramatic reduction occurred with E2 treatment. This suggest that 4-OHT and E2 work oppositely in inducing ER target genes indicating that 4-OHT acts as antagonist to E2 in mGEP cells.

Igf1r did not show a remarkable change when treated with E2 after 24 and 48 h, however, there was a gradual reduction in Igf1r expression when treated with 4-OHT for 48 h. This indicates that E2 treatment maintain the proliferation and growth of mGEP cells through Igf1r functions while this proliferation maintenance is lost in case of 4-OHT which is correlated with cell viability results (Figure 3.4). Kahlert *et al.* demonstrated that ERα and not β is required to induce the activation of Igf1r[147], which is consistent with our results.

Ccnd1 expression was higher with E2 treatment than 4-OHT treatment after 48 h in which E2 keep the proliferation status of mGEP cells while there was a reduction in proliferation with 4-OHT treatment where the expression of Ccnd1 was lowered as the concentration of 4-OHT increased. This is consistent with the results by Sabbah *et al.* where they found that E2 induce cell proliferation in target tissues by inducing Ccnd1 through the G1 phase of the cell cycle[148].

The expression of Ldlr did not show a remarkable change when treated with E2 after 24 and 48 h while there was a reduction in Ldlr expression when treated with 4-OHT especially after 48 h. Therefore, E2 can maintain the level of Ldlr in mGEP cells while 4-OHT did not, which strengthen our hypothesis that 4-OHT works as an antagonist to ER in mGEP cells. The high expression of Ldlr is important in cholesterol transport from the serum into the cells, used in cell proliferation and steroid hormone synthesis[149] probably like E2 hormone.

Il-6 expression was highly induced with 1 μM of 4-OHT while there was no expression at higher 4-OHT concentrations. E2 induced the Il-6 expression both after 24 and 48 h. This indicates that E2 is important to induce and maintain Il-6 expression suggesting a role of E2 in defense mechanism and immune response. Additionally, Il-6 only expressed at 1 μM of 4-OHT both after 24 and 48 h treatments which is correlated with high expression of ERα at 1 μM of 4-OHT both after 24 and 48 h. These results are consistent with studies that suggest positive correlation between Il-6 and ERα[112].

Mal was not expressed in the control and treated cells after 24 and 48 h treatment indicating that this gene is not functional in mGEP cells.

Vegfa as a pro-angiogenic factor showed a concentration dependent decrease when treated with 4-OHT for 24 and 48 h (more reduction after 48 h than 24 h). This finding is consistent with previous *in vitro* report in which 4-OHT caused delayed cell proliferation and decreased production of VEGF in normal human dermal cells[142]. For E2 treated cells, Vegfa expression reduced after 48 h comparing to 24 h which is correlated to the reduction in ERα expression after 48 h comparing to 24 h.

Chapter 5 : Conclusion and Future Directions

This thesis aimed to study role of E2 in mouse gastric stem cell homeostasis. This study showed that both ERα and ERβ are expressed in mGEP cells at mRNA and protein levels. The cell viability of mGEP cells treated with 4-OHT decreased in a time and concentration dependent manners while no change in cell viability occurred in the E2 treated cells. This study suggests that 4-OHT works as an antagonist to ERα as it cause a reduction in the receptor expression as well as reduction in ER target genes like Igf1r, Ccnd1, Ldlr, Il-6 and Vegfa in time and concentration dependent manners, as well as the reduction in cell viability comparing to E2. No such reduction in ER target genes was observed in E2 treated cells. Only the differentiation markers of surface mucous cells and mucous neck cells (muc5AC and muc6, respectively) were expressed upon E2 and 4-OHT treatments indicating the ability of mGEP cells to differentiate only into these two cell lineages.

Although this study examined different aspects of the effect of E2 or 4-OHT on mGEP cells, there are still several remaining areas to be studied and questions to be answered. First, further study on the mechanism of cell death in mGEP cells treated with 4-OHT is needed to indicate whether 4-OHT is inhibiting cell proliferation or inducing cell death. For example, caspase 3 or Tunel assay can be used to test the mechanism of cell death. Second, cell cycle studies are interesting to know where the cells are arrested after treatment. Further study of the proliferation quantification (counting cells after treatments) could be done in addition to the viability study to conclude if proliferation decreased or not. Third, a mechanistic study could be done at protein level using western blot analysis for proliferation and differentiation markers in addition to ER target genes. Forth, it was demonstrated

that the effect of 4-OHT on the stomach is related to the pH and acid secretion[126], therefore, studying the effect of 4-OHT after altering the pH of the media could be done. Also it will be useful to use pure antagonist for ER like fulvestrant (Faslodex)[150] or specific antagonist to each ER such as ERα antagonist, methyl-piperidino-pyrazole (MPP) and ERβ antagonist, pyrazolo [1,5-a] pyrimidine (PHTPP)[151] to study the effect by targeting both or either ERs. Additionally, a competing experiment between 4-OHT and E2 can be done to find if cells behaves differently once both treatments used together. Moreover, the idea of this project can be done using primary culture to compare between mGEP cells and primary culture of gastric stem cells in response to E2 or 4-OHT. Finally, cells can pretreated with inhibitors for non-genomic and ligand independent pathways to differentiate between direct and indirect E2 signaling activation/inhibition.

4-OHT is used as a treatment for breast cancer patients as it antagonizes the ERs in the breast and inhibits the transcription of ER target genes that are involved in carcinogenesis. At the same time, it is know that 4-OHT affects the stomach of breast cancer patients as ERs are expressed in the stomach. However, the effect of 4-OHT on the stomach of breast cancer patients is not fully studied. Therefore, this well-controlled *in vitro* system helps to understand the impact of E2 or 4-OHT on GSC homeostasis especially for the patients taken 4-OHT. Further elaboration of this study can have medical applications about the pros and cons of 4-OHT treatment for cancer patients.

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Appendix

Sequencing of Mouse ERα and ERβ

The purified RT-PCR products for mouse ERα and ERβ (Figure 3.1) were sequenced with appropriate amplification primers in an automated DNA analyzer (3500 Genetic Analyzer, Applied Biosystems). Basic Local Alignment Search Tool (BLAST) was used to identify amplification products by comparing the query sequences with the sequences in the database. BLAST website: [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. Nucleotide sequences of amplicons were identical with those of reported for mouse ERα and ERβ.

Supplementary Table S.1: BLAST results for mouse ERα amplicon.

Sequence	>**ER-α** TCAGCATCTATTCTGATATGATCCTTCTAGACCCTTCAGTGAAGCCTCAATGATGGGCTTATTGACCAACCTAGCAGATAGGGAGCTGGTTCATATGATCAACTGGGCAAAGAGAGTGCCAGGCTTTGGGGACTTGAATCTCCATGATCAGGTCACCTTCTCGAGTA			
Match	*Mus musculus* estrogen receptor 1 (alpha)			
	Transcript variant 1, mRNA	Transcript variant 2, mRNA	Transcript variant 3, mRNA	Transcript variant 4, mRNA
Accession number	NM_007956.5	NM_001302531.1	NM_001302532.1	NM_001302533.1
Query coverage	96%			
E-value	8e-73			
Identity	99%			

Supplementary Table S.2: BLAST results for mouse ERβ amplicon.

Sequence	>**ER-β** GCGAGAATAGTCGTCCCGGGTGTAAGAACTGCTGTTGAAATCTGATTGTT CCCGCTTTCTGGTAAATCCTCATCTCGGGGTCTAGTGTGCTCGAAGAGGA AGTAAAGCATGAGGGCTCCCAGAAACTTCCAGTCCAGTGACGGCCAGGT GGAGGCGGATGATACAATGATGGTCAAGTGGGGACATGTACA	
Match	*Mus musculus* estrogen receptor 2 (beta) (Esr2)	
	Transcript variant 1, mRNA	Transcript variant 2, mRNA
Accession number	NM_207707.1	NM_010157.3
Query coverage	94%	
E-value	9e-29	
Identity	82%	

Normalization of RT-PCR Gels

Supplementary Table S.3: Representation of RT-PCR gel of different gene expression in control and E2 or 4-OHT treated cells after 24 h treatment after normalization to GAPDH signal intensities (quantified using ImageJ). (C: control with 0.1% FBS, CT: control for 4-OHT with 0.1% FBS and 1% EtOH, T: 4-OHT treatment followed by the concentration used in μM, CE: control for E2 with 0.1% FBS and 0.1% EtOH, E: E2 treatment followed by the concentration used in μM).

Genes	**Control or treated cells**										
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
ERα	-	6932	11489	4375	2532	1037	3712	11311	12554	10853	13594
GAPDH	-	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
ERα/GAPDH	-	0.960	2.013	1.024	1.034	0.423	0.626	1.199	1.752	0.999	1.145
	-	1.0	2.1	1.1	1.1	0.4	1.0	1.9	2.8	1.6	1.8
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
ERβ	14815	0	17755	10050	8858	9345	0	5457	5559	3938	8374
GAPDH	15635	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
ERβ/GAPDH	0.948	0.000	3.111	2.352	3.618	3.814	0.000	0.578	0.776	0.362	0.705
	1.0	0.0	3.3	2.5	3.8	4.0	0.0	0.6	0.8	0.4	0.7
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
muc5AC	-	17960	34302	25496	35401	24836	43782	22824	62165	48181	39506
GAPDH	-	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
muc5Ac/GAPDH	-	2.487	6.011	5.967	14.457	10.137	7.389	2.419	8.674	4.435	3.328
	-	1.0	2.4	2.4	5.8	4.1	1.0	0.3	1.2	0.6	0.5

Genes	Control or treated cells										
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
muc6	40425	0	45256	7166	3864	5806	0	8253	11706	19582	53372
GAPDH	15635	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
muc6/GAPDH	2.585	0.000	7.931	1.677	1.578	2.370	0.000	0.875	1.633	1.802	4.496
	1.0	0.0	3.1	0.6	0.6	0.9	0.0	0.3	0.6	0.7	1.7
	C	CT	T1	T2.5	T5	T10	CE	E1	E2.5	E5	E10
CD44	**-**	8690	10451	6461	4063	3143	6714	8383	11187	9904	10037
GAPDH	**-**	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
CD44/GAPDH	**-**	1.203	1.832	1.512	1.659	1.283	1.133	0.888	1.561	0.912	0.845
	-	1.0	1.5	1.3	1.4	1.1	1.0	0.8	1.4	0.8	0.7
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Sox2	**-**	3370	11769	2754	384	1042	2922	13707	13956	9785	15633
GAPDH	**-**	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
Sox2/GAPDH	**-**	0.467	2.062	0.645	0.157	0.425	0.493	1.453	1.947	0.901	1.317
	-	1.0	4.4	1.4	0.3	0.9	1.0	2.9	3.9	1.8	2.7
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Igf1r	**-**	7197	9984	4554	2470	2418	6354	10529	10441	9099	12142
GAPDH	**-**	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
Igf1r/GAPDH	**-**	0.997	1.750	1.066	1.009	0.987	1.072	1.116	1.457	0.838	1.023
	-	1.0	1.8	1.1	1.0	1.0	1.0	1.0	1.4	0.8	1.0

Genes	Control or treated cells										
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Ccnd1	-	12029	17076	11639	3218	1688	9102	6936	11938	9399	14702
GAPDH	-	7223	5706	4273	2449	2450	5925	9436	7167	10864	11871
Ccnd1/GAPDH	-	1.665	2.993	2.724	1.314	0.689	1.536	0.735	1.666	0.865	1.238
	-	1.0	1.8	1.6	0.8	0.4	1.0	0.5	1.1	0.6	0.8
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Ldlr	-	7052	12901	6245	1796	1602	4790	9349	9744	8696	12302
GAPDH	-	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
Ldlr/GAPDH	-	0.976	2.261	1.461	0.733	0.654	0.808	0.991	1.360	0.800	1.036
	-	1.0	2.3	1.5	0.8	0.7	1.0	1.2	1.7	1.0	1.3
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Il-6	34668	0	46813	0	0	0	0	5577	17074	44507	46616
GAPDH	15635	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
Il-6/GAPDH	2.217	0	8.204	0.000	0.000	0.000	0.000	0.591	2.382	4.097	3.927
	1.0	0.0	3.7	0.0	0.0	0.0	0.0	0.3	1.1	1.8	1.8
	C	**CT**	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Vegfa	-	3491	10362	2037	1314	394	813	4029	4393	3015	5309
GAPDH	-	7223	5706	4273	2449	2450	5925	9436	7167	10864	11872
Vegfa/GAPDH	-	0.483	1.816	0.477	0.536	0.161	0.137	0.427	0.613	0.278	0.447
	-	1.0	3.8	1.0	1.1	0.3	1.0	3.1	4.5	2.0	3.3

Supplementary Table S.4: Representation of RT-PCR gel of different gene expression in control and E2 or 4-OHT treated cells after 48 h treatment after normalization to GAPDH signal intensities (quantified using ImageJ). (C: control with 0.1% FBS, CT: control for 4-OHT with 0.1% FBS and 1% EtOH, T: 4-OHT treatment followed by the concentration used in μM, CE: control for E2 with 0.1% FBS and 0.1% EtOH, E: E2 treatment followed by the concentration used in μM).

Genes	**Control or treated cells**									
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
ERα	9736	8570	5506	4295	4122	8195	12815	3528	5830	6739
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
ERα/GAPDH	0.783	1.654	0.718	0.602	0.515	0.588	0.782	0.564	0.671	0.606
	1.0	2.1	0.9	0.8	0.7	1.0	1.3	1.0	1.1	1.0
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
ERβ	6850	3196	1718	2491	6072	2255	12367	1717	889	4501
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
ERβ/GAPDH	0.551	0.617	0.224	0.349	0.758	0.162	0.755	0.275	0.102	0.405
	1.0	1.1	0.4	0.6	1.4	1.0	4.7	1.7	0.6	2.5
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
muc5AC	7263	5174	4369	4746	4789	3521	14197	4539	3178	2392
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
muc5AC/GAPDH	0.584	0.999	0.569	0.665	0.598	0.253	0.867	0.726	0.366	0.215
	1.0	1.7	1.0	1.1	1.0	1.0	3.4	2.9	1.4	0.9

Genes	Control or treated cells									
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
muc6	11436	6483	9528	13606	11028	7122	11690	4112	3392	4131
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
muc6/GAPDH	0.920	1.251	1.242	1.906	1.376	0.511	0.714	0.657	0.390	0.371
	1.0	1.4	1.4	2.1	1.5	1.0	1.4	1.3	0.8	0.7
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
CD44	11819	6415	2097	1723	4859	10796	12939	8213	5338	4484
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
CD44/GAPDH	0.950	1.238	0.273	0.241	0.606	0.775	0.790	1.313	0.615	0.403
	1.0	1.3	0.3	0.3	0.6	1.0	1.0	1.7	0.8	0.5
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Sox2	10234	8874	4318	2793	4047	10485	13913	1664	1894	4517
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
Sox2/GAPDH	0.823	1.712	0.563	0.391	0.505	0.752	0.849	0.266	0.218	0.406
	1.0	2.1	0.7	0.5	0.6	1.0	1.1	0.4	0.3	0.5
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Igf1r	12554	9648	4921	3259	2963	11567	14710	7848	8396	7090
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
Igf1r/GAPDH	1.010	1.862	0.641	0.456	0.370	0.830	0.898	1.255	0.967	0.637
	1.0	1.8	0.6	0.5	0.4	1.0	1.1	1.5	1.2	0.8

Genes	Control or treated cells									
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Ccnd1	7481	5185	855	495	842	7694	11482	2788	5656	9743
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
Ccnd1/GAPDH	0.602	1.001	0.111	0.069	0.105	0.552	0.701	0.446	0.651	0.876
	1.0	1.7	0.2	0.1	0.2	1.0	1.3	0.8	1.2	1.6
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Ldlr	7563	6887	3075	0	0	10141	14614	4565	4151	7753
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
Ldlr/GAPDH	0.608	1.329	0.401	0.000	0.000	0.728	0.892	0.730	0.478	0.697
	1.0	2.2	0.7	0.0	0.0	1.0	1.2	1.0	0.7	1.0
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Il-6	12827	6256	0	0	0	7768	14241	14468	11118	15664
GAPDH	12435	5182	7673	7140	8012	13936	16382	6255	8686	11123
Il-6/GAPDH	1.032	1.207	0.000	0.000	0.000	0.557	0.869	2.313	1.280	1.408
	1.0	1.2	0.0	0.0	0.0	1.0	1.6	4.1	2.3	2.5
	CT	**T1**	**T2.5**	**T5**	**T10**	**CE**	**E1**	**E2.5**	**E5**	**E10**
Vegfa	17860	12618	0	0	0	5023	5309	3308	1406	3567
GAPDH	11769	11321	6383	3867	949	10699	12412	10866	11217	11623
Vegfa/GAPDH	1.518	1.115	0.000	0.000	0.000	0.469	0.428	0.304	0.125	0.307
	1.0	0.7	0.0	0.0	0.0	1.0	0.9	0.6	0.3	0.7