INTERACTION BETWEEN ESTROGEN AND INTERFERON-\(\gamma\) SIGNALING PATHWAYS IN THE REGULATION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II EXPRESSION IN BREAST CANCER CELLS

By

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Activation of the antigen presentation mechanisms by cancer cells is one of the main pathways used by the immune system for tumor detection and suppression. Induction of the expression of molecules of the Major Histocompatibility complex class II (MHC-II) by the interferon-γ (IFNγ) is important for the efficient presentation of tumor antigens. Nevertheless, it has been observed that expression of these molecules is suppressed in tissular contexts where the concentration of estradiol (E2) is high.

In this work we attempted to explain if the down-regulation exerted by estradiol on the expression of the MHC-II molecules in breast cancer cells was mediated by a silencing effect of the estrogen receptor-α (ERα) through a possible estrogen receptor binding site (ERBS) in the locus of promoter IV (pIV) of the master regulator of MHC-II expression, the class II transactivator (CIITA). The breast cancer cell line MDA-MB-231 (ERα-/ERβ-) and its stable transfectants MC2 (ERα+) and VC5 (empty vector) were used as model cell lines. Expression of the MCH-II molecules is controlled by CIITA, and stimulation with IFNγ activates the transcription of the pIV of CIITA. Stimulation of these cell lines with IFNγ induced expression of the MCH-II molecules and addition of E2 repressed such expression only in the MC2 cell line, as observed by flow cytometry analysis. Six other breast cancer cell lines were tested, with only the MCF7 cell line showing a significant inhibition. Then we analyzed if the inhibition of the MHC-II expression was due to a down-regulation of CIITA. Protein analysis performed by western blot and mRNA quantification by RT-qPCR both revealed down-regulation of CIITA in the cells exposed to IFNγ+E2 compared to those treated only with IFNγ. However, reporter gene analysis did not demonstrate any influence of our candidate ERBS in the inhibition of the activation of CIITA-pIV. ChIP-seq analysis of the VC5 and MC2 cell lines for ERα also failed to demonstrate any binding of the receptor anywhere in the vicinity of the CIITA locus. However gene ontology and disease ontology analysis
of the sequencing data showed a higher activation of tumorigenic cellular pathways in the cells treated with IFN\(_γ\) + E2 than in the cells treated only with E2. These results suggest that activation of the inflammatory pathways by IFN\(_γ\) could exert a detrimental effect on the cancer development.

**Key words:** breast cancer, antigenic presentation, CIITA, MHC-II, estrogen, interferon gamma, estrogen receptor binding site.
INTERACTION ENTRE LES VOIES D'ACTIVATION DE L'ESTROGÈNE ET DE L'INTERFÉRON $\gamma$ DANS LA RÉGULATION DE L’EXPRESSION DU COMPLEXE MAJEUR D’HISTOCOMPATIBILITÉ DE CLASSE II DANS DES CELLULES DE CANCER DU SEIN

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Sherbrooke, Québec, Canada, Janvier 2017
L’activation des mécanismes de présentation antigénique par les cellules cancéreuses est l’une des voies principales employées par le système immunitaire pour la détection et la suppression des tumeurs. L’induction de l’expression de molécules du complexe majeur d’histocompatibilité de classe II (CMH-II) par l’interféron-γ (IFNγ) est importante pour la présentation efficace des antigènes tumoraux. Cependant, il a été observé que l’expression de ces molécules est supprimée dans certains tissus dans lesquels la concentration d’estradiol (E2) est élevée.

Dans ce travail, nous avons tenté de déterminer si l’inhibition exercée par l’estrogène (E2) sur l’expression des molécules du CMH-II dans des cellules de cancer du sein est médiée par un effet de silençage du récepteur de l’estrogène-α (ERα) à travers d’un possible site de liaison de récepteur d’estrogène (ERBS) dans le locus du promoteur IV du régulateur clé de l’expression du CMH-II, CIITA. La lignée cancéreuse mammaire cellulaire de cancer de sein MDA-MB-231 (ERα-/ERβ-) et ses transfectants stables MC2 (ERα+) et VC5 (vecteur vide) ont été utilisés comme des lignées cellulaires modèles. L’expression des molécules du CMH-II est contrôlée par CIITA, et la stimulation avec l’IFNγ active la transcription du pIV de CIITA. La stimulation de ces lignées cellulaires avec l’IFNγ induit l’expression des molécules du CMH-II et l’addition d’E2 réprime de cette expression seulement dans la lignée cellulaire MC2, telle qu’elle est observée par analyse de cytométrie de flux. Six autres lignées de cancer de sein ont été testées et seulement la lignée cellulaire MCF7 montrait une inhibition significative. Ensuite, nous avons analysé si l’inhibition de l’expression du CMH-II était due à une régulation de CIITA. L’analyse des protéines effectuée par Western blot et la quantification de l’ARNm par RT-qPCR quantitative ont révélé une inhibition de CIITA dans les cellules exposées à l’IFNγ + E2 par rapport à celles traitées seulement avec l’IFNγ. Cependant, des analyses avec un gène rapporteur n’ont pas démontré une
influence quelconque de notre site de liaison de récepteur d'estrogène candidat dans l'inhibition de l'activation de CIITA-pIV. Des analyses de ChIP-seq dans les lignées cellulaires MC2 et VC5 pour l'ERα n'ont également pas démontré la présence d'une liaison du récepteur dans le voisinage du locus de CIITA. Cependant, des analyses sur l'ontologie des gènes et des maladies sur les données de séquençage ont montré une activation accrue des voies cellulaires cancéreuses dans les cellules traitées avec IFNγ + E2 comparé avec les cellules traitées uniquement avec E2. Ces résultats suggèrent que l'activation des voies inflammatoires par l'IFNγ pourrait exercer un effet plus négatif qu'anticipé sur le développement du cancer.

**Mots clés:** cancer du sein, présentation antigénique, CIITA, CMH-II, estrogène, interféron gamma, site de liaison du récepteur de l'estrogène.
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-Are you ready to rock Jorge?
-Shields-up. Weapons online!
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>BC</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>BCC</td>
<td>Breast Cancer Cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BLS</td>
<td>Bare Lymphocyte Syndrome</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast Cancer gene 1 and 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine MonoPhosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB Binding Protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin ImmunoPrecipitation</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>Chromatin ImmunoPrecipitation followed by sequencing</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II TransActivator</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Responsive Element Binding</td>
</tr>
<tr>
<td>COSMIC</td>
<td>Catalogue Of Somatic Mutations In Cancer</td>
</tr>
<tr>
<td>CTA</td>
<td>Cancer Testis Antigens</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal Carcinoma In-Situ</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>E1</td>
<td>estrone</td>
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<td>estradiol</td>
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<tr>
<td>E3</td>
<td>estriol</td>
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E-box  Enhancer box
EGF  Epidermal Growth Factor
ER  Estrogen Receptor
ERBS  Estrogen Receptor Binding Site
ERE  Estrogen Response Element
ERα  Estrogen Receptor alpha
ERβ  Estrogen Receptor beta
ERR  Estrogen Related Receptors
FIGF  c-Fos Induced Growth Factor
GAS  (interferon) Gamma Activated Site
GEP  Gene Expression Profile
GM-CSF  Granulocyte-Macrophage Colony-Stimulating Factor
GTP  Guanosine TriPhosphate
HAT  Histone Acetyl Transferase
HER-2  Human Epidermal growth factor Receptor 2
HLA-DR  Human Leukocyte Antigen DR
HLA-DM  Human Leukocyte Antigen DM
HSP90  Heat Shock Protein 90
IBC  Inflammatory Breast Carcinoma
IDC  Invasive Ductal Carcinoma
IFNγ  InterFeroN gamma
IFNGR  InterFeroN gamma Receptor
IGF-I  Insulin-like Growth Factor I
IHC  ImmunoHistoChemistry
IL-2  InterLeukin 2
IL-6  InterLeukin 6
IL-11  InterLeukin 11
ILC  Invasive Lobular Carcinoma
IRF-E  Interferon Regulatory Factor-Element
JAK:  JAnus Kinase
KD  Knock-Down
LBD  Ligand Binding Domain
LCIS  Lobular Carcinoma In-Situ
li  Invariant chain
MAPK  Mitogen-Activated Protein Kinase
MEME  Multiple Em for Motif Elicitation
MHC-I  Major Histocompatibility Complex class I
MHC-II  Major Histocompatibility Complex class II
mRNA  messenger RNA
NFkB  Nuclear Factor kappa-light-chain-enhancer of activated B cells
NFY  Nuclear transcription Factor Y
NK  Natural Killer cell
NR  Nuclear Receptor
qPCR  quantitative Polymerase Chain Reaction
pDC  plasmacytoid Dendritic Cell
PDK1  Phosphoinositide-Dependent protein kinase 1
PKA  Protein Kinase A
PKB  Protein Kinase B
PKC  Protein Kinase C
PR  Progesterone Receptor
RNA  Ribonucleic Acid
RT-qPCR  Reverse Transcription quantitative-PCR
SDS-PAGE  Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis
SR  Steroid Receptor
<table>
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<tr>
<td>STAT1</td>
<td>Signal Transducer and Activator of Transcription 1</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor Associated Antigens</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic Epithelial Cells</td>
</tr>
<tr>
<td>TFIIB</td>
<td>Transcription Factor II B</td>
</tr>
<tr>
<td>TFIID</td>
<td>Transcription Factor II D</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumor-specific Antigens</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription Start Site</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal LymphoPoiëtin</td>
</tr>
<tr>
<td>USF1</td>
<td>UpStream transcription Factor 1</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
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<tr>
<td>WT1</td>
<td>Willms Tumor 1</td>
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CHAPTER I
INTRODUCTION

1.1 Historical context

The history of humanity is often defined as the succession of striking events that mark the collective consciousness. Unfortunately, often these events have been great wars; from the Mongol invasions of the XIII century to the world wars of the XX century. These acts were marked by the great, and relative sudden, loss of lives. And yet, when we speak about history we often neglect to consider that illness has been the major cause of death through history, and thus, one of the great factors in the moulding of human civilization. Among all the illness a human can suffer from, infectious diseases are the ones that have had the deepest impact through history. The Black plague pandemic of the XIV century in Europe reduced the population almost by a quarter and the consequent socio-cultural changes dictated the future to come. However, among all these conditions there is one that is inherent to us and has plagued humanity since the very beginning, the cancer. The first recorded depiction of this illness dates back to ancient Egypt, circa 1300 B.C., and clearly describes the characteristics of a breast cancer (BC). Unfortunately for the majority of human history the cause of this condition remained a mystery, and the implementation of archaic treatments was often useless. However with the arrival of the scientific method and the subsequent development of modern medicine during the XIX, XX and XXI centuries, new ways of treating this condition were found. With innovations in surgery methods, such as the general anaesthesia in 1846 by William Morton, and the mastering of mastectomy procedure in the 1880s by Halsted, medicine started an arms race against cancer. Later in the XX century, new tools in the form of chemical compounds were found to preferentially attack the tumor cells. During the post WWII period an old weapon of war, nitrogen mustard, or mustard gas as it was known, was the first drug to be approved in North
America for the treatment of cancer (Gilman, 1963) The compound acts by alkylating the cancer cell’s DNA, thus damaging it and inducing cell death (Goodman et al., 1946). This discovery marked the birth of chemotherapy as a form of treatment for cancer. With the help of these and many other developments humanity was finally able to control to some extent the development of cancer, transforming what was previously a death sentence into a fighting hope for those inflicted by the condition. However as any other arms races we have witnessed in human history, they are never unilateral. Very quickly it was realized that the heterogeneity of cancers was extensive and that they behave quite differently from patient to patient, even in the presence of the same type of cancer. It was then realized that the complexity of the cancer pathology was far greater than we could have ever imagined. Quickly enough it was observed that therapeutic treatments were inducing physiological changes in the tumor that were rendering the treatments less effective. Parallel to these observations, it was realized that tumours were composed of a highly heterogeneous population of cells with different metabolic patterns and cytological characteristics. Elimination of one specific sub-population by a particular treatment was allowing the further development of a different sub-population resistant to the treatment and with different characteristics. This scenario greatly resembles a Darwinian competition within the cancer population where the fittest cells are the ones able to resist the treatments and thus, the ones to survive (Tabassum and Polyak, 2015).

With the progressive reduction of tobacco consumption among the western population breast cancer has taken the place of lung cancer as the leading cause of death among women (Canadian Cancer Society, 2015). According to a report presented by the Public Health Agency of Canada on cancer statistics in 2015, around 42% of Canadian women will develop a form of cancer during their lives, and of these, about 26% will be cases of breast cancer. In total, it is estimated that about 5000 people died from this condition in 2015 in Canada. It is also important to mention that the relative survival
ratio for breast cancer has improved only about 4% from 1992 to 2008 (Canadian Cancer Society, 2015).

Even after 70 years since the beginning of chemotherapy and after thousands of scientific studies, statistics like these remind us that a definite cure for breast cancer, and cancer in general, is still to come and that the continuing investigation of the cancer pathology is as important as it was a century ago.

1.2 Breast cancer pathology and development

Breast cancer pathology is defined as an abnormal cellular growth originating within the breast. Given the highly diverse structural composition of any human organ, breast cancer can arise from any cellular type present in it, from stromal cells (myofibroblastoma) to neurons (neuroendocrine carcinoma), however the vast majority of breast cancers arise from the epithelial cells of the mammary glands, either from the milk ducts or the cuboidal cells (milk secreting cells) (International Agency for Research on Cancer, 2008).

An overview of the structure of the breast is shown in Fig. 1. The classification of breast cancer is a complex procedure that takes into account several characteristics of the tumor such as morphology, origin, extension, and histological and genetic markers. Because of this complexity the classification has undergone several changes over the years. Nevertheless the most common form of classification is based on immunohistocytological observations with the more precise classifications, such as the molecular and genetic markers, being used to clarify the portrait of each specific case. After years of compiled cases, it is possible nowadays to associate each BC profile with a usual outcome (Dai et al., 2015).
According to the histopathology classification of the tumor, breast cancer cases are classified in four main classes (International Agency for Research on Cancer, 2008):

- **Carcinomas in situ contain two categories depending on the origin of the tumor:**

  **Ductal carcinoma in situ (DCIS):** is the most common type of non-invasive breast cancer. The denomination *in-situ* means that tumor is still limited to the interior of the lining of the milk ducts, and that it has not yet spread through the walls of the ducts. Breaching of the ducts allows invasion of the surrounding tissues and more importantly, infiltration into the lymph nodes. Infiltration of the lymphatic system is one of the more usual ways for spreading of the cancer cells to other parts of the body (metastasis).

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**Figure 1. Morphology of the human breast.** 1) Lobules are an assembly of alveolar structures containing specialised cuboidal epithelial cells that secrete milk. 2) The lactiferous duct are connections that form a branched system for the collection of the milk, the ducts join together and drain the milk into an opening in the nipple. 3) Lymph nodes are part of the lymphatic
system and participate to the defense of the breast by circulating the lymphatic fluid containing immune cells. (Picture from www.wisegeek.org)

Detection is usually difficult, as it does not present any of the usual symptoms, such as inflammation or noticeable masses. Most of the time this kind of tumor is hormone receptor positive. Even if it is not life threatening the presence of this kind of tumor is associated with reoccurrence and development of invasive breast cancer.

Lobular carcinoma in situ (LCIS): Similar to the DCIS, this type of cancer occurs when the abnormal cells develop in the milk-making glands (lobules) and remain in-situ. The danger of the LCIS is usually very low and is not life threatening, but it is an indicator that a woman is at increased risk for developing invasive breast cancer in either breast in the future. Given the in-situ characteristic of this tumor, its removal is usually easy and does not require chemotherapy.

- Infiltrating breast carcinomas

Invasive ductal carcinoma (IDC): is the most common type of invasive breast cancer accounting for around 80% of invasive breast cancers. Opposite to the in-situ type, this form of cancer is characterised by infiltrating cells (invasive) that have broken through the wall of the duct and invaded the surrounding breast tissue. The probability of invasion to the lymph nodes is high in this form of cancer.

IDC can be broken down into different subtypes, based on the appearance and behaviour of the cancer cells. These include invasive mucinous carcinoma, tubular
carcinoma, medullary carcinoma and invasive micropapillary carcinoma. In some cases, when the observed morphology of the cancer cells does not present any feature characteristic of a particular type of cancer, the IDC is classified as invasive not otherwise specified or non-specific type.

**Invasive lobular carcinoma (ILC):** Makes up about 10% of invasive breast cancer cases. ILC originates from the milk-making glands (lobules) of the breast and the subsequent invasion is similar to the one in IDC. Particular to this type of cancer is a type of growth in which the cells grow in single lines that lead to a hardening of the breast.

- **Inflammatory breast carcinoma (IBC):** This uncommon type of invasive breast cancer accounts for about 1-3% of all breast cancers. It tends to be more common in younger women, and women of African ancestry. In its early stages, IBC is often mistaken for infection; this is due to a particular inflammatory reaction induced by the tumor cells present in lymph vessels. IBC begins in the milk ducts of the breast and spreads to the lymph vessels. This type of cancer has a higher chance of spreading and it can be more aggressive and harder to treat than invasive ductal or lobular cancer. Treatment usually involves a combination of approaches including chemotherapy, surgery, radiation, and hormone and HER-2 targeted therapy, if appropriate.

- **Pager's disease:** This type of condition is characterised by a cellular growing in and around the nipple. It is an unusual type of breast cancer, accounting for less than 5% of all cases and is more common in women over the age of 50. However
this condition occurs sometimes in combination with another form of breast cancer such as DCIS or IDC.

1.3 Molecular characterization of breast cancer

The classification presented above gives a general description about the localisation of the tumor, its cytological origin and more importantly, about its containment status. However, this tells little about their specific metabolic or genetic features. With the development of simpler and cheaper molecular approaches over the last couple of decades it has become usual to observe the gene expression profile (GEP) of the cells. The GEP allows for further characterisation of the tumor with respect to certain mutations, such as mutations in the breast cancer 1 and 2 genes (BRACA1/2), which will be discussed later. Along with the GEP some specific proteins serve as molecular markers that help with the characterisation of the tumor.

Even though several researchers and organizations have presented different arguments over the years about what genes describe better the cancer phenotype, it was the work of Sorlie and colleagues in the early 2000s (Perou et al., 2000; Sorlie et al., 2001) that established the standard for the molecular subtyping of breast tumors. These works identified five different subtypes, each one associated with a particular set of immunohistochemistry (IHC) markers (Fig. 2A), and grades describing the aggressivity of the cells based on their differentiation status, with well differentiated cells being less aggressive. Sorlies’s classification is based on the status for three hormone receptors identified as IHC markers, these cover: the progesterone receptor (PR), the human epidermal growth factor receptor 2 (HER-2) and the estrogen receptor (ER), with the status of these receptors being crucial in the prognosis of the patients (Fig. 2B) (Dai et al., 2015). Kl67 is a nuclear protein associated with cellular proliferation...
that is normally expressed at low levels in normal breast tissue (Fig. 2A). However the use of KI67 as a molecular marker for breast cancer prognosis has been greatly debated and though it was used by Sorlie and colleagues in their works (Sorlie et al., 2001), the absence of a standard agreement for its quantification has led to its disuse in pathology tests (Inwald et al., 2013). The importance of these factors resides in their ability to induce growth-promoting signals; the progesterone is a steroid sex hormone that participates in the control of the menstrual cycle, pregnancy and embryogenesis and HER-2 belongs to a family of receptor tyrosine kinases that can interact with different signaling molecules, but in most cases induces cellular proliferation (Roy et al., 2009). Estradiol (E2) plays a central role in the breast cancer pathology as it possesses the ability, via the ER, to induce expression of genes encoding growth factors, their receptors and different signaling molecules that can activate cell proliferation and survival stimuli (Shou et al., 2004). Such is the role played by ER that it is called the defining and driving transcriptional factor of breast cancer, since its target genes can direct the cellular growth and the endocrine response. Crosstalk between these three receptors can lead to unusual activation of pathways that exacerbates the growth and division of the cancer cells. For example, activation of the mitogen activated protein kinase (MAPK) by HER-2 induces phosphorylation of ER, thus simulating the binding of E2 and inducing transcription of ER controlled genes (Ali and Coombes, 2002).

A very important fourth marker, this one a genetic mutation, is also used in combination with the previous three to establish the prognosis of the patients; mutations in the Breast cancer 1 and 2 genes (BRCA1/2). Germ line mutations of BRCA1 are present in approximately 90% of familial breast and ovarian cancers and in approximately 50% of familial breast cancers alone. BRCA1 is a nuclear protein of 220 kDa and 1,863 amino acids, it is highly expressed in rapidly proliferating mammary epithelial cells during pregnancy and is down-regulated during lactation (Marquis et al., 1995). Several pleiotropic biological functions have been associated with BRCA1, most of them related to transcriptional regulation, chromatin remodeling, DNA damage repair, cell cycle
regulation, and checkpoint control (Scully and Livingston, 2000). The tumor suppressor role presented by these genes is given by their participation in the DNA repair mechanisms and, therefore, allowing them to play a role in ensuring the stability of the cell's genetic integrity. Dysfunctional mutations in either one of these genes results in DNA damage that may not be repaired properly and as a result, the cells are more likely to develop additional genetic alterations that can lead to cancer.

<table>
<thead>
<tr>
<th>Intrinsic subtype</th>
<th>IHC status</th>
<th>Grade</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A⁺</td>
<td>[ER⁺</td>
<td>PR⁺] HER2-Ki67⁻</td>
<td>1</td>
</tr>
<tr>
<td>Luminal B⁺</td>
<td>[ER⁺</td>
<td>PR⁺] HER2-Ki67⁺</td>
<td>2</td>
</tr>
<tr>
<td>HER2 over-expression⁺</td>
<td>[ER-PR⁺] HER2⁺</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Basal⁺</td>
<td>[ER-PR⁻] HER2⁻, basal marker⁺</td>
<td>3</td>
<td>Poor</td>
</tr>
<tr>
<td>Normal-like</td>
<td>[ER⁺</td>
<td>PR⁺] HER2-Ki67⁻</td>
<td>1</td>
</tr>
</tbody>
</table>

**Figure 2. Breast cancer classification by intrinsic molecular subtype.**

A) Breast cancer subtype dependent on the immunohistochemistry status of the receptors: estrogen (ER), progesterone (PR) and the human epidermal growth factor 2 (HER-2) showing the usual grades and outcomes associated with each subtype. (Perou et al., 2000) B) Usual prognosis in function of the molecular subtypes and the status of BRCA1 (Dai et al., 2015).
Specific inherited mutations in \textit{BRCA1}/\textit{2} increase the risk of female breast and ovarian cancers and they have been associated with increased risks of several additional types of cancer. The effects of mutations in \textit{BRCA1}/\textit{2} are seen even when a person’s second copy of the gene is normal (Campeau \textit{et al.}, 2008). Combination of the status of these four markers is usually the main argument for decision making regarding the treatments to be implemented in the patients, and have been associated with different prognosis (Fig 2B) (Spitale \textit{et al.}, 2009).

The high level of heterogeneity evidenced by Sorlie’s classification reveals a classical characteristic that underlays the very nature of the BC pathology, a high mutational rate that is especially problematic for the treatment of the illness (Osz \textit{et al.}, 2012).

1.4 Tumorigenesis.

The precise molecular mechanisms behind the triggering of tumorigenesis have been intensively studied during the last decades with the discovery of proto-oncogenes by J.M. Bishop and H.E. Varmus during the 80s, establishing a conceptual landmark in the study of cancer (Bishop, 1982; Varmus, 1985). This discovery revealed that cancer is generated by our own genes if mutations in their sequence alter their function. Nevertheless not one modification can start development of cancer by itself, this is greatly due to the redundancy and safety mechanisms of the cells, where failure of one specific pathway is usually compensated by activation of a parallel path or overexpression of a similar one (Gerlinger \textit{et al.}, 2014). Because of this, cancer has been characterised as an aging condition as it takes time for a healthy cell to accumulate the necessary mutations to undermine the safety features and become a tumor. How many of these genetic mutations are necessary to trigger the transformation is still debated, however it has been agreed that transformation from
normal acting cells to cancer proliferation is driven by a relative limited number of genes known as cancer drivers (Kim, 2015).

More precisely, these cancer drivers are defined as genes that are mutated in more than one type of cancer. In a statistical sense this means that every cancer harbors at least one of these mutations, empirically, a tumor tends to contain between two to eight cancer drivers (Vogelstein et al., 2013). Two types of drivers are known today, oncogenes and tumor suppressors. Oncogenic modifications are often missense mutations that change the amino acid residues and alter the protein function. These modifications are more disturbing for the cellular homeostasis when the result is a gain of function. A classic example is the mutation of the KRAS gene, where the mutation of glycine 12 (G12D or G12V) results in an constitutively active k-ras protein that remains in a GTP-bound state (Okudela et al., 2010). In a general manner, gain of function is a hallmark of oncogenic mutations. On the other hand, characteristic tumor suppressor mutations usually result in the disabling of the protein, either as consequence of a missense or nonsense modifications. The most common examples are the mutations of the previously discussed BRCA1 gene. Mutations in this gene are found all over the coding region with no specific mutation being yet identified as more relevant than others among the 389 mutations documented (Vogelstein et al., 2013). In a general manner nonsense mutations in the coding region are typical in a tumor suppressor gene.

A closer look at the function of two of cancer driver genes, will serve to illustrate the reason why this type of genes is primordial in the triggering of the cancer phenotype. The prevalence of mutations in TP53 and PIK3CA in breast cancer is about 33%, this characteristic was observed by a statistical analysis of the mutations presented in the cancer genome database Catalogue of somatic mutations in Cancer (COSMIC) and The Cancer Genome Atlas (TCGA) (Kandoth et al., 2013). The TP53 gene is mutated in 42% of all cancers and its product, p53, plays a central role in the DNA damage response by inducing cell cycle inhibitors that arrest the cellular division, thus giving the
cell time to repair the genomic damage. In the absence of function of p53, these damages are carried through to the next generation of cells, thereby increasing the risk of cellular dysfunction and the proliferation of more damages. The second highly common cancer driver is PIK3CA with mutations in this gene being present in about 18% of all cancers. The product of PIK3CA is the p110 catalytic subunit of the phosphatidylinositol-3-kinase (PI3k). This unit phosphorylates phosphatidylinositol-(4,5)-biphosphate to phosphatidylinositol-(4,5)-triphosphate (PIP3) (Shaw and Cantley, 2006). Mutations in PI3k often result in a gain of function that increases the lipid kinase activity. As described before, gain of function is an oncogenic hallmark and in this case the overproduction of PIP3 by PI3k activates multiple kinases such as the phosphatidylinositol dependant kinase (PDK1) and the protein kinase B (PKB), both of which have been shown to inhibit pro-apoptotic molecules thus conferring abnormal survival capabilities to the cell (Engelman, 2009).

In general, all four molecular subtypes of breast cancer described in Figure 2A (excluding the basal subtype) harbor mutations in Tp53 or PIK3CA, however their prevalence is different and they are remarkably mutually exclusive, but the reason why these mutations are never present together is not yet understood.

1.5 Tumor heterogeneity

One very important factor to consider in the discussion of cancer development is the high level of heterogeneity present within the cell population of the tumor. As important as the activity of the cancer drivers is for cancer development, it is not the only factor to consider. Tumors are rarely composed of monoclonal populations and different genetic expressions from different subpopulations result in different cellular pathways being activated. The effect of cross-talk among these different pathways is a factor impossible to neglect when considering the development and treatment of cancer.
While cancer was studied for many decades as a clonal disease (Nowell, 1976), early studies in murine mammary tumours revealed that certain subpopulations from different sections of the same tumour differ in growth rate, immunogenicity, drug response and ability to metastasize, thereby demonstrating functional and phenotypic heterogeneity (Heppner, 1984). However, even when we are far from understanding the complexity of the interactions that act among these subpopulations, there is growing evidence that cancer cells behave as communities and present several characteristics of ecological behaviour. Due to this, increasing attention is now being directed towards the cooperative behaviour of sub-clones that can influence disease progression, and cancer is now studied as an evolving ecosystem (Kanaseki et al., 2003; Merlo et al., 2006). As expected, these interactions add a greater layer of complexity to therapeutic treatments where, often, tumors presenting a high level of heterogeneity lead to a poor prognosis (Tabassum and Polyak, 2015).

Nevertheless not all of these behaviours act in favour of the survival of the tumor. As in any ecological community there are several kinds of interactions that have been observed in tumors. These interactions can be classified as negative or positive depending on the proliferative capabilities that they give to the tumor (Tabassum and Polyak, 2015). Negative interactions are mainly competitive ones that arise from the limitations in nutrients and oxygen, this type of competition usually results in the elimination of one subpopulation and the expansion of one more fit (Pepper et al., 2009). On the other hand, positive interactions can present different characteristics and can be classified as commensalistic, where one population profits from the other without damaging it or synergistic, where two subpopulations cooperate to produce new characteristics that are not necessarily indispensable for the community, or mutualistic when the subpopulations cooperate to benefit all the parties (Pienta et al., 2008). These positive interactions are of great interest for the treatment of cancer as they can heavily affect the outcome of the patient (Michelson, 1987). For example, targeted therapies that eliminate a tumor subpopulation that exerted a negative interaction over other
subpopulations will favour a later tumor regrowth of the remaining cells, not only because the treatment fails to eliminate the totality of the tumor but also because the remaining subpopulations are no longer under the pressure of a negative interaction (Pepper et al., 2009) (Fig. 3A.). In a similar way, the elimination of a subpopulation that exerts a positive interaction over the tumor community greatly affects their development as these subpopulations often help tumor expansion via the secretion of growth or angiogenic factors such as the c-fos induced growth factor (FIGF) and interleukin 11 (IL-11), respectively (Marusyk et al., 2014). Elimination of a positive interacting subpopulation will result in a rapid decrease of the size of the tumor and a prolonged regression but inevitably will also end up allowing for tumor regrowth because of the same failure to eliminate all the cancer cells.

![Figure 3. Targeted elimination of cancer subpopulations. A) Elimination of a selfish subpopulation exerting a negative interaction over the other subpopulations. In absence of the negative interaction a faster tumor regrowth should be expected. B) Elimination of positive interacting subpopulations that help tumor growth can delay tumor regrowth (Tabassum and Polyak, 2015).](image_url)
The problem presented by the existence of different subpopulations within the tumor constitutes an important challenge for the current treatments, as many of them are based on the precise targeting of cellular characteristics that are only common to some subpopulations. In the breast cancer pathology for example, the treatment with tamoxifen, an anti-estrogenic compound, of ER-positive cases will face two problems: first, the existence of ER-negative subclones among the tumor, these subpopulations will present resistance from the beginning of the treatment and will proliferate better as the competing cells are eliminated. Once the ER-positive cells are eliminated, the ‘second generation’ tumor will stop responding to the treatment thus aggravating the condition. Another possible outcome is the induction of tamoxifen resistance via mutation of the ER-positive cells, induced by the selective pressure of the treatment (Barkhem et al., 2004). Such a scenario would result in the co-existence of two different subpopulations resistant to tamoxifen, a situation presenting a worst prognosis.

As presented before, the status of ER in breast cancer cells is one of the main factors in BC decision treatment and prognosis prediction, however its importance goes beyond the cancer pathology as E2 is a central factor in the regulation of several developmental mechanisms and tissular functions in mammalian organisms.

1.6 Estrogens and estrogen receptors

1.6.1 Normal function

One of the best models for E2-driven transformations is the breast, an organ that by its nature has evolved to be highly sensitive to hormonal variations. During puberty, tissue development is directed by estrogen along with other sexual hormones, the same is
true during pregnancy, when the same hormones initiate further development and activation of functions. Because of these characteristics, estrogen is known as the main sexual hormone and besides its function in the breast, it drives the development of the female reproductive organs and secondary sex characteristics (Barkhem et al., 2004).

The estrogen family of steroidal hormones is composed of three molecules, estrone (E1), estradiol (E2) and estriol (E3). Estradiol is the most common form and its ubiquitous expression throughout the body is most significant during the fertile years. Its activity is replaced by E1 after the onset of menopause and by E3 during pregnancy. Also, E2 has been identified as the hormone possessing the highest affinity for its receptors, making it the most relevant form of the hormone for not pregnant and pre-menopausal women. (Nilsson et al., 2001) For the rest of this work, every mention of estrogen should be understood as referring to the estradiol (E2) form.

E2 is one of the main sexual hormones and as such acts as a messenger; its activity is mainly mediated via a family of dedicated hormone receptors, the estrogen receptors (ERs). Around the 1970s, Jensen and Jacobsen (1967) observed that the biological effects of estrogen had to be mediated by a receptor protein, based on the specific binding of E2 in the uterus. Continued experimentation for the next decade concluded in two groups reporting the cloning of the estrogen receptor (ER) in 1986 (Green et al., 1986). Until 1995, it was assumed that there was only one ER and that it was responsible for mediating all of the physiological and pharmacological effects of natural and synthetic estrogens and anti-estrogens. However, in 1995, a second ER, estrogen receptor beta (ERβ), was cloned from a rat prostate cDNA library (Kuiper et al., 1996). The first ER is now called ERα (Chu and Fuller, 1997).
Estrogen is produced primarily in the ovaries in females and testes in males, and is central to the regulation of growth, development, and physiology of the reproductive system in humans (Swedenborg et al., 2009). The biological function of estrogen is mediated by ERα and ERβ. The signaling and activation or inhibition of estrogen target genes depends on a tightly controlled balance between the expression of ERα and ERβ in the target organs. ERs belong to the superfamily of steroid hormone nuclear receptors (NRs) (Choi and Jeung, 2003; Osz et al., 2012). This family also includes the estrogen-related receptors (ERR), progesterone receptors (PR), androgen receptors (AR), glucocorticoid and mineralocorticoid receptors. Steroid receptors (SRs), such as the ER, are ligand-dependent transcription factors, and they often control cell cycle activities (Weigel and Moore, 2007). ERs in particular have been shown to regulate certain cell proliferation pathways (Lee et al., 2012).

The complex structure of ERs, containing several functional sites, is evidence of the multiple activities they can execute. Similar to other NRs, ERs are comprised of five domains with distinct functions (Fig. 4A) (Skafar and Zhao, 2008). First, the N-terminus of the A/B domains contains an activation function 1 (AF1) module, which contributes to the transcriptional activity of ERs and is an essential domain for interaction with co-regulators. This domain is prone to post-transcriptional modifications that stimulate AF1 activity (Kong et al., 2003). A central region named C domain encodes for a DNA-binding domain (DBD) that is essential for the binding of ERs to DNA (Geserick et al., 2005). The D domain is a hinge region that includes sequences for nuclear localization signaling and facilitates post-translational modification of ERs. Usually these modifications contribute to the activation of ER signaling in the cells. Finally, at the C-terminal region we find the E/F domain, which contains a ligand-binding domain (LBD) that serves also as an interaction site with co-regulators, in addition to the ligand-dependent activation function 2 (AF2) module. Both modules have the ability to regulate the activity of ERs (Edwards, 2000). Certain differences between the F domain of ERα
and ERβ may be responsible for the selectively transcriptional control of ERs in specific target genes (Skafar and Zhao, 2008).

Binding of different ligands to the LBD (either steroid hormones or synthetic compounds) induces a specific conformation of the receptor thus promoting precise co-regulatory interactions and associations of the function domains AF1 and AF2 (Bramlett et al., 2001; Nilsson and Gustafsson, 2011). In the classical pathway, binding of estrogen to the receptor induces its phosphorylation and the formation of homodimers and heterodimers, that are then translocated into the nucleus (Fig. 4B) (McDevitt et al., 2008). ERs modulate the transcription of target genes by binding to estrogen response elements (EREs) in the DNA sequence (Park et al., 2011). This binding promotes DNA bending and looping, allowing for the interaction with co-regulators and the general transcriptional machinery. Some of these co-regulators are co-activators, co-repressors, co-integrators, and histone modifiers such as acetyltransferases and deacetylases (Edwards, 2000). In the absence of estrogen, ERs remain in an inactivated state thanks to the association with the heat shock protein 90 (Hsp90). This protein has been associated with protein stabilization, binding affinity of receptors to ligands, and signaling cascades (Sanchez, 2012).

1.6.2 Non genomic activities of ERs

Even if the activity of ERs is most often mediated via the binding to the EREs and the consequent chromatin modifications, it is also possible for them to modulate the cellular activity without directly binding to DNA. Characteristic of the non-genomic activities is the fast response times of these pathways, with cellular mechanism responding in seconds to minutes of E2 stimulation compared to hours for the canonical DNA-dependent pathway (Marino et al., 2006). The mechanisms by which the non-genomic
activities are exerted are highly heterogeneous. For instance, inhibition of the expression of the cytokine IL-6 by E2 is mediated by an interaction between ERα and the \( c-rel \) subunit of the NFκB complex, where the ERα prevents NFκB from binding to the IL-6 promoter (Galien and Garcia, 1997). Similar is the activation of ERα by E2 that induces subsequent interaction and activation of the IGF-1 receptor resulting in activation of the MAPK signaling. The majority of the non-genomic activities are mediated by membrane bound ERs (Fig 4B). In these cases other membrane bound receptors such as g-protein coupled receptor 30 (GPR30) or the metabotropic glutamate receptors (mGLUR) interact with the ER via cytoplasmic kinases to transduce signals to targets in the cytosol or in the nucleus (Levin, 2009).

1.6.3 Non-E2-dependent activation of ERs

In the same manner as the ERs can regulate certain cellular activities independently of their DNA binding properties, some activities can be induced in absence of E2 (Bunone \textit{et al.}, 1996). Frequently, this alternative activation of the ERs is exerted by signaling pathways that phosphorylate it, thus imitating the modifications induced by the binding of E2. Some of the factors that can induce such modifications include regulators of the general cellular phosphorylation state, such as protein kinase A (PKA) (Aronica and Katzenellenbogen, 1993) or protein kinase C (PKC) (Ignar-Trowbridge \textit{et al.}, 1996) and certain extracellular signals such as peptide growth factors, cytokines, or neurotransmitters (Chalbos \textit{et al.}, 1994). Among these factors, some are known to completely mimic the effects of E2, for example the epidermal growth factor (EGF) in the mouse reproductive tract (Curtis \textit{et al.}, 1996). Other growth factors which activate ERα signaling include insulin (Newton \textit{et al.}, 1994), insulin-like growth factor I (IGF-I) (Aronica and Katzenellenbogen, 1993), and transforming growth factor (TGF)-\( \beta \) (Ignar-Trowbridge \textit{et al.}, 1996). As mentioned before, a very important example of this is the activation of ERα by HER-2 via phosphorylation, thus leading to ER-activation and
induction of E2-controlled genes in absence of the hormone (Nilsson et al., 2001). This phenomenon is often observed in breast cancer patients that have developed resistance to anti-estrogen therapy.

1.7 Breast cancer resistance to anti-estrogen therapy

The genetic deregulations described above are also very important in the context of tumor survival, as the resistance developed to several types of treatments is dependent on the activation of non-targeted cancer driver mechanisms that allow for cellular proliferation even with active inhibition of the 'original' driver.

Given the importance of E2 for breast cancer development, the targeted treatment of ER has been one of the most usual therapies for this condition. As ER-positive breast cancer represents around 70% of all cases of breast cancer, the ER inhibitor Tamoxifen has been widely used (Jordan, 2006). Tamoxifen acts as an agonist of ER by competing with E2 to bind the receptor. Once bound, Tamoxifen does not inhibit but greatly modifies the activity of ER (Wang et al., 2004). However, about 30% of all cases treated with Tamoxifen show resistance and end up relapsing at a rate of 1-5% per year (Goss et al., 2008).

Different molecular approaches have been used to identify the mechanisms behind this resistance with several different pathways showing some kind of activity responsible for the phenomena. Overexpression of the MYC oncogene, activation of the insulin/Igf receptor signaling and mutation of the PI3k are some of the observed cancer drivers that are activated in the resistance phenotype (Engelman and Janne, 2008)
Figure 4. Schematic representation of ERα structural domains, activation and signaling. A) Functional domains of the ERα. The A/B domain at the N-terminus contains the function domain AF-1 (transcription activation function 1) that interacts with other TF. The C/D domain has a DNA binding function that contains a two-zinc finger structure. The E/F domain at the C-terminus possess the ligand binding pocket and the second function domain AF-2, that interacts with co-activators. B) Activity of the ER can be executed by three different pathways. 1) The classical pathway is mediated by binding of nuclear ER to an ERE, recruitment of co-regulators and subsequent induction of target genes. 2) The secondary pathway is mediated by cytoplasmic or membrane bound ER. Interaction with other membrane bound receptors, such as the mGLUR and cytoplasmic kinases can control transcription factor and regulate gene
transcription. 3) The third pathway is mediated by non-ER receptors such as the GPR30/GPER. Activation of these receptors induce signaling through cytoplasmic kinases and finally regulation of gene transcription (Nilsson and Gustafsson, 2011).

However these back-up cancer drivers are not only activated in breast cancer, but have been also observed in many other cancer types under treatment, further showing the importance of these mechanisms to the proliferative phenotype. Among the cancer drivers in the breast the most usual cause responsible for anti-estrogen resistance is a mutation in the estrogen receptor alpha (ESR1) gene. Certain mutations generate amino acid substitutions that mimic the active conformation of ER in the absence of ligand, thus rendering the cell non-dependent of E2 (Robinson et al., 2013; Toy et al., 2013). Interestingly enough, these mutations are usually not present in the primary tumor and are evidence for Darwinian survival under the selective pressure of anti-estrogen therapy (Kim, 2015).

1.8 Immune response to cancer

All tumors have a common characteristic regarding their relationship towards the immune response, the presence of a complex assembly of immune cells and the consequent cellular interactions established between the two cellular classes (Coussens et al., 2013). Tumor cells become immunogenic early during the tumorigenic process and are able to be eliminated by the immune system. Tumor suppression is mainly the result of cytotoxic activity by activated T cells directed to tumor cells expressing tumor-specific antigens. Tumor cells possess a high mutational rate that generates changes in the proteins, these changes can be identified by the T cells via the antigenic presentation pathway (Dunn et al., 2004). Tumor escape is most often the result of interference of the chronic inflammation mechanism that disrupts the cytotoxic
T cell activity. Such scenario is usually observed in tumors evolving from chronically inflamed tissues, where tissue repair pathways are overexpressed (Coussens et al., 2013). An example of the importance of the T cell immune response for the control of cancer was evidenced when mice lacking a functional immune system were shown to be severely more susceptible to induced cancers. In humans, the effect of interleukin-2 (IL-2), secreted by T-cells, showed an increased ability of the lymphoid compartment to suppress tumors (Schreiber et al., 2011).

1.8.1 Antigenic activation of the immune response

Observations from these and other experiments revealed that the T cell compartment is central to the immune response thanks to its ability to recognize peptide epitopes that are displayed by major histocompatibility complex molecules (MHCs) on the surface of the tumor cells. In theory, three classes of antigens may mediate such cancer rejection. These classes are broad classifications of potential antigens found within tumors. The first class is known as tumor specific antigens (TSAs), these are antigens that are not encoded by the normal genome and may represent either oncogenic viral proteins or abnormal proteins that arise as a consequence of somatic mutations; these are known as neo-antigens (Hanahan and Weinberg, 2000). For the large majority of human tumors without a viral cause, such neo-epitopes are only produced by tumor-specific DNA modifications that result in the formation of new protein sequences. In the case of virus-associated tumors, such as cervical cancer and a subset of head and neck cancers, epitopes derived from viral open reading frames also contribute to the pool of neo-antigens (Schumacher and Schreiber, 2015). During cancer initiation and progression, tumor cells acquire protein-altering mutations that are either responsible for transformation, as the cancer driver mutations mentioned before, or are a by-product of the genomic instability that accompanies cellular transformation, these later ones are known as passenger mutations (Hanahan and Weinberg, 2011). The
alterations that actually result in expression generate mutant proteins with a loss of self-
identity and can be perceived as foreign proteins by the immune system (Heemskerk et al., 2013). Another factor that induces the development of neo-antigens is as a by-
product of anticancer treatments, like chemotherapy or radiation therapy, or by
targeting epigenetic control mechanisms or drugs intervening with DNA repair pathways.

The second class of antigens is known as tumor associated antigens (TAAs). These
TAAs include proteins encoded in the normal genome and may be either normal
differentiation antigens or other normal proteins that are aberrantly expressed.
Overexpressed normal proteins that possess growth/survival promoting functions, such
as Wilms tumor 1 (WT1) (Ohminami et al., 2000) or the breast cancer inducing HER-2
(Kawashima et al., 1999), are examples of TAAs that directly participate in the
oncogenic process. Some TAAs are composed of proteins carrying posttranslational
modifications such as phosphorylation (Doyle et al., 2006). Because TAAs are normal
proteins, their antigenicity depends on abnormal expression levels or abnormal context
to circumvent naturally occurring mechanisms of immunological tolerance (Pardoll,
2003). Along these lines, TAAs usually have lower T cell receptor (TCR) affinity
compared with TSAs or foreign antigens (Stone et al., 2015). Because of this, TSAs
are less likely to be susceptible to mechanisms of immunological tolerance and
therefore may represent more visible targets for immune-mediated tumor control
(Heemskerk et al., 2013).

The final category are the cancer-germline/cancer-testis antigens (CTAs) (Simpson et
al., 2005).This category represents the antigens that are normally expressed in testis,
fetal ovaries, and trophoblasts, but can also be expressed in cancer cells (Simpson et
al., 2005).Because they are encoded in the normal genome but display highly restricted
tissue expression, CTAs have received considerable attention as attractive targets for
immunotherapy (Scanlan et al., 2002). Given the fact that the reservoir of T cells recognizing the non-mutated self-antigens is controlled by the maturation process in the thymus, the neo-antigens constitute an important fraction of the cancer antigens that can elicit the immune response (Gilboa, 1999).

1.8.2 T cell mediated tumor suppression

Shortly after development, tumors become organized and acquire tissue-like characteristics with local and systemic connections with immune cell populations of myeloid and lymphoid origin, each one interacting with the tumor in different patterns that may drive further tumor development to different outcomes (Palucka and Coussens, 2016).

It is important to understand that the interaction between the tumor and the immune system is a dynamic process, guided by the pressure exerted by the immune response, answered by the tumor resistance, and modulated by a damage control mechanism that protects the surrounding tissues (Coussens et al., 2013). When the immune response to cancer is high and the immune-suppression processes are low, the tumor development is kept under control. However, a strong anti-tumor response will induce certain physiological mechanisms designed to inhibit the effector T cells in order to prevent tissue damage and maintain tissue homeostasis (Coussens et al., 2013). In the absence of a precise set of stimuli, the immune response has evolved to be transitory. It is designed to maintain the integrity of the organism and to establish coexistence with the environment by protecting it from external threats. Given that the threats are usually transitory, the suppression often prevails. Multiple pathways of suppression are at play in tumor microenvironments, including cells such as Th2-polarized macrophages, immature and suppressive monocytes, regulatory B cells, and regulatory T cells, as well as certain molecules that are key in the activation of checkpoints that control T cell
differentiation (for example, CTLA-4 and IDO) and effector function (such as PD-1). It has been shown that pharmacological blockade of these inhibitory pathways can tip the balance toward anti-cancer effector T cells (Coussens et al., 2013). The latter ones can be primed or boosted by antigen-presenting cells (DCs) and/or by co-stimulatory signals (for example CD137 ligands). Tumor antigens in general can be presented to T cells in exogenous vaccines, as well as endogenously via DCs that captured dying neoplastic cells. When T cells specific to defined antigens kill neoplastic cells, such a process can enable generation of responses to other antigens, so called epitope spreading. A critical factor in the balance between immunogenicity and suppression is inflammation; this is due to the fact that the type of inflammation can direct the continued immune response to the tumor. A Th1 oriented response is more effective to destroy the tumor, while a Th2/Th17 oriented may result in tumor promotion. (Palucka and Coussens, 2016).

To better understand how the type of immune response can drive the direction of the tumor response, it is important to have a closer look at the interactions and functions of the different compartments of the immune system with the tumor.

The myeloid cells present different functions related to the maintenance of homeostasis of the organism; these functions involve the capture and degradation of antigenic molecules by macrophages or for presentation by dendritic cells (DCs); tissue repair induction by the macrophages, and some effector functions by the mast cells, monocytes and granulocytes, such as release of different cytokines (Tae Chul Moon 2014). However, in some cases tumor cells can alter the steady-state activity of the myeloid immune cells present in the vicinity of the tumor, known as tumor micro-environment (TME) (DeNardo et al., 2011). The cells present in the TME include tissue-resident and blood derived cells and their activity can be regulated by the tumor cells by secreting factors such as interleukin-6 (IL-6) or granulocyte-macrophage colony-
stimulating factor (GM-CSF). These secretions have the ability to increase recruitment and proliferation of immature myeloid cells in proportions that are atypical under physiological conditions (Gabrilovich et al., 2012). The myeloid cells possess an important functional plasticity in response to environmental signals, and can drive the immune response to different outcomes, for example antigen degradation or antigen presentation, when macrophages acquire DC capabilities (Banchereau et al., 2000). Alternatively, they can drive the immune response towards tissue repair rather than inflammation when macrophages are polarized toward type 2 states, and protective or non-protective T-cell immunity when programmed by cancer-derived factors (Balkwill et al., 2005).

Among the myeloid compartment, the dendritic cells play a central role in the immune response, as they are able to trigger both the humoral and cell mediated branches of the immune system by presenting the antigens to B and T lymphocytes (Lanzavecchia and Sallusto, 2001). In the cancer pathology the tumor antigens are presented to T cells either at the tumor sites or at the lymph nodes. The dendritic cells can display these tumor antigens via the classical pathways by major histocompatibility molecules of class I (MHC-I) or class II (MHC-II). Lipid antigens can also be presented via non-classical pathways by CD1 molecules that allow selection of rare antigen-specific T cells (CD4+ or CD8) and NK-T cells (Lanzavecchia and Sallusto, 2001). If the antigenic presentation by dendritic cells induces a better activation of naïve CD8+ T cells, these will differentiate into T cytotoxic lymphocytes (CTL) and direct the immune response towards a Th1 polarized immune response. In contrast, preferential activation of naïve CD4+ T cells will produce helper cells with different cytokine expression patterns and FoxP3+ regulatory cells. These cells have a dampening effect on the cytotoxic activity to prevent autoimmune responses, however this type of response is also less anti-carcinogenic (Zhu and Paul, 2008). In a similar way, non-maturation of the dendritic cells by exposure to interleukin-10 for example, will induce the antigenic presentation for T cell suppression (Pulendran, 2015).
The advantage of a Th1-polarized CD4+ response is its increased cytotoxic effect. This type of response is characterised by secretion of interleukin-2 (IL-2), tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). These cytokines in combination with CD8+ T cells promote macrophage cytotoxic activity (Stout and Bottomly, 1989) and can induce upregulation of antigen processing and expression of MHC molecules in dendritic cells (Lanzavecchia and Sallusto, 2001). On the other hand the Th2-polarised response is characterised by secretion of interleukins-4,-5,-6,-10 and -13. This pattern of secretion can induce T-cell anergy and loss of cytotoxicity, increase the humoral immunity and regulate the tumor-promoting activities of macrophages (DeNardo et al., 2011).

These different outcomes of the immune response have led to a change in the classical view, according to which the immune cells simply facilitate tumor rejection, and has been replaced by a more complex view, where the lymphocytes can present both tumor-promoting and tumor-inhibiting properties (Coussens et al., 2013). The best example of this phenomenon is the link between the type of inflammation and the associated type of Th immune response, and how each of these inflammation profiles shows opposing effects on tumors. Th2-associated chronic inflammation promotes neoplastic cell survival, angiogenesis, tissue remodeling, and metastasis, while Th1-associated acute inflammation, triggers neoplastic cell destruction. As the immune response pressures the tumor cells, several mechanisms are implemented by the cancer cells to escape elimination. First, the cells become antigen-edited as the more immunogenic cells are killed off by the immune system. Then the cells that escape elimination become less immunogenic. This progression is exerted by two mechanisms. The main factor is the down-regulation of MHC molecules while a great proportion of them also activate intrinsic gene expression programs that are directed to suppress T cells and stimulate myeloid cells. These two characteristics being typical of a Th2 oriented response. Cytokines related to this kind of response include transforming growth factor β (TGFβ); IL-4, -13, -8, and -10; thymic stromal
lymphopoietin (TSLP); and indoleamine 2,3-dioxygenase (IDO) (Coussens et al., 2013). This allows for the recruitment of FoxP3+CD4+ regulatory T cells, Th2 CD4+, Th2-polarized macrophages and monocytes, and B regulatory cells (Balkwill et al., 2005). Induction of a Th2-oriented response activates myeloid cells, thus increasing synthesis of angiogenic (e.g., VEGF), growth and/or survival (e.g., EGF, TNFα) factors that in turn induce the proliferation of epithelial cells, as well as enzymes controlling the remodelling of tissue (e.g., metallo-, cysteine, and serine proteases). These activities have a high pro-tumorigenic ability in that they help the TME by favouring neoplastic cell survival and sustained proliferation (Balkwill et al., 2005). At the same time activation of macrophages and monocytes by Th2 also increases expression of molecules, e.g. inducible nitric oxide synthase or Arginase 1, that directly and indirectly suppress CD8+ T cell proliferation and cytokines such as IL-10 that inhibit DC maturation and antigen cross-presentation to T cells (Ruffell et al., 2014). In conclusion, the Th2-oriented immune response favours a particular type of inflammatory response that induces a TME that is tumor promoting and immune supressing whereas Th1-oriented response inducing the acute inflammatory response increases cytotoxic activities and leads to a more efficient tumor suppression (Wang et al., 2009; Wu et al., 2009).

1.9 Antigenic presentation and regulation

Antigenic presentation by the DCs is at the heart of the initiation of the immune response, however DCs are not the only cell type capable of executing this task and antigenic presentation by other types is also highly relevant for the tumor suppression. In the presence of a Th1 oriented response, the acute inflammation mechanisms are triggered, IFNγ secretion is induced and several pathways with different and important anti-tumoral effects are activated such as the increased tumoral susceptibility of NK cells and the growth arrest mediated by IRF-1 (Li et al., 2012).
Figure 5. Antigenic processing and presentation by MHC-I and MHC-II molecules. A) MHC-I antigenic processing. Endogenous proteins are captured in the cytoplasm by the proteolytic complex of the proteasome,
the resulting peptides are translocated into the endoplasmic reticulum by antigen transport proteins (TAP), where they bind to the antigen presenting groove of the MHC-II molecules, the MHC-II+Ii+antigen complex is then fused with the cellular membrane. (Murat and Tellam, 2015) B) MHC-II antigenic processing. The MHC-II molecules are assembled in the endoplasmic reticulum and joined by the Ii peptide that prevents binding to endogenous peptides in the reticulum. The MHC-II+Ii complex separates from the reticulum in a vesicle. Exogenous antigens are captured by the cell in an endosome where they are digested by proteolytic enzymes. Then the endosome fuses with the vesicle and feeds the peptides to the MHC-II molecules that exchanges the remaining CLIP peptide for the exogenous antigen. Finally, the MHC-II+Ag complex is fused with the cellular membrane (Neefjes et al., 2011).

However the most relevant activity of IFN\(\gamma\) is the induction of expression of MHC-II molecules by the epithelial cells, as in the absence of IFN\(\gamma\), the expression of these molecules is limited to the DC, macrophages and B cells (ten Broeke et al., 2011). This secondary antigenic presentation mechanism greatly increases the number of cells that are able to present neo-antigens and other tumor antigens to the immune system.

The precise mechanism behind the antigenic loading process and the final surface expression of MHC-II molecules starts with the synthesis and assembly of the transmembrane \(\alpha\)-and \(\beta\)-chains in the endoplasmic reticulum to form the heterodimeric molecule of MHC class-II (MHC-II). Once the MHC-II molecule is assembled it associates with the invariant chain (Ii) that binds the heterodimer by its antigen-binding groove (Bertolino and Rabourdin-Combe, 1996). The resulting complex (MHC-II + Ii) is transported through the Golgi apparatus to an endosomal compartment. The vacuole carrying the MHC-II+Ii complex is known as the MHC class II compartment (MIIC)
(Denzin et al., 2005). Once in the cytoplasm, the li is digested by resident proteases, leaving a residual peptide linked to the binding site of the MHC molecule, this peptide is known as the class II-associated li peptide (CLIP). Then the MHC-II+CLIP complex is joined by a chaperone protein that facilitates the replacement of CLIP for an antigenic peptide, this chaperone also belongs to the MHC family and is known as HLA-DM. In the classical pathway the antigenic peptide (Ag) is derived from an exogenous macro-antigen (protein) that is internalised by phagocytosis and digested in the endosome. The resulting endosome containing the Ag is fused with the MIIC where they bind MHC-II molecules at antigen-binding groove. The MHC-II+Ag complexes are then transported to the plasma membrane, where they present their peptide cargo to the TCR present on the CD4+ T cells (Fig. 5) (Denzin et al., 2005; Anders et al., 2011).

As mentioned before, MHC class I molecules usually present cytosolic peptides of intracellular origin, whereas MHC class II molecules acquire peptides that are generated by proteolytic degradation in endosomal compartments. The source for these peptides is usually exogenous material that is endocytosed from the extracellular environment, but very importantly it can be also endogenous components, such as plasma membrane proteins, components of the endocytic pathway and cytosolic proteins that access the endosomes by autophagy (Villadangos and Schnorrer, 2007). This alternate cross-presentation for the MHC-II molecules is most important in the cancer context as it increases the frequency of presentation of tumoral antigens present in the cytosol.

Under normal conditions the expression of MHC-I molecules is constitutive in most tissues and contributes to the maintenance of homeostasis by allowing a continual probing of the cells by the immune system (Kurts et al., 2010). Meanwhile the expression of MHC-II molecules is restricted to antigenic presenting cells (APCs) such as the dendritic cells, B cells and thymic antigen presenting cells (Neefjes et al., 2011). However, MHC class II expression can be induced in the majority of tissues by certain stimuli, mainly the cytokine IFNγ (Ting and Trowsdale, 2002). Cell types that can be
induced to express MHC-II molecules include the mesenchymal stromal cells (Romieu-Mourez et al., 2007), fibroblasts and endothelial cells (Geppert and Lipsky, 1985). It is important to note that non-MHC-II expressing cells can express it in the absence of co-stimulatory molecules in order to maintain peripheral tolerance (Kreisel et al., 2010). As we can see, the antigenic presentation function mediated by the MHC-II molecules is crucial to the activity of dendritic cells, whose main role is precisely the presentation of antigens to the lymphocytes and yet their expression is not constitutive and is only induced under certain conditions. This may be explained by the fact that MHC-II molecules have been identified as one of the main markers in autoimmune conditions, thus it is easy to see why their expression should be kept in check (Fernando et al., 2008).

The control of the MHC-II expression is executed mainly at the transcriptional level, via a conserved regulatory module situated 150–300 bp upstream of the transcription-initiation site in all MHC class II genes (Reith and Mach, 2001; Ting and Trowsdale, 2002). This regulatory module is known as the SXY box and consists of four sections: the S, X, X2 and Y boxes. These boxes are usually present in a highly conserved order, orientation and spacing. This SXY module is found among several vertebrate species expressing MHC-II molecules, in the promoters of the genes encoding the α- and β-chains. The SXY modules are also usually found in the promoters of the genes encoding invariant chain (Ii) and the non-classical MHC class II molecules, such as HLA-DM and HLA-DO (Reith et al., 2005).

Experiments performed in B cell lines that presented regulatory defects in MHC class II expression contributed to the discovery of four key trans-acting factors that regulate the transcription of MHC class II genes by interacting with the SXY module. Failure of this mechanism results in a condition known as bare lymphocyte syndrome (BLS), a type of hereditary immunodeficiency, where no MHC-II genes are expressed (Reith and Mach, 2001). The four factors discovered in the BLS are CIITA, regulatory-factor-X5 (RFX5), RFX-associated protein (RFXAP) and RFX-associated ankyrin-containing
protein (RFXANK) (Steimle et al., 1993; Steimle et al., 1995; Durand et al., 1997; Masternak et al., 1998; Nagarajan et al., 1999). Mutations in any one of these regulatory factors will result in a BLS phenotype. These factors are dedicated to the transcription of MHC class II genes and related genes and they are essential for its expression as only residual MHC class II expression is retained in BLS suffering mice that lack RFX5 or CIITA (Chang et al., 1996; Clausen et al., 1998).

These factors regulate the activation of MHC-II expression via the assembly of a multi-proteic complex known as the enhanceosome. First, three of the factors, RFX5, RFXAP and RFXANK form the heterotrimeric RFX complex that binds the X box of the SXY module (Reith et al., 1988; Steimle et al., 1995). RFX nucleates the assembly of a higher-order nucleoprotein complex through cooperative binding interactions with the X2-box specific cyclic-AMP-responsive-element-binding protein (CREB), the Y-box-specific nuclear transcription factor Y (NFY) and an unknown factor that binds the S box (Moreno et al., 1999; Muhlethaler-Mottet et al., 2004). Finally, CIITA is recruited by multiple synergistic protein–protein interactions (Masternak et al., 2000). Once the enhanceosome is fully assembled, CIITA induces the activation of the transcription of MHC class II genes (Ting and Trowsdale, 2002).

In this sense, CIITA functions as a non-DNA-binding co-activator coordinating multiple events that are required for the activation of transcription. The activation of transcription by CIITA happens in different manners: first, it can recruit general transcription-initiation components such as the transcription factor II D and B (TFIID and IIIFB) (Fontes et al., 1999); it can also induce the phosphorylation of RNA polymerase II (Spilianakis et al., 2003); third, it interacts with the positive transcription elongation factor b (P-TEFb) (Kanazawa et al., 2000); and can recruit co-activators that alter chromatin accessibility by inducing histone acetylation or methylation (Zika and Ting, 2005). One final important option is by recruiting the chromatin-remodelling factor Brahma related gene 1 (BRG1) (Pattenden et al., 2002).
Figure 6. MHC-II enhanceosome structure. Regulation of the MHC-II expression is mediated by CIITA via enhanceosome. Binding of the regulatory factors RFX, CREB and NFY to the X, X2 and Y boxes respectively of the SXY module along with an unidentified factor that binds the S box form the enhanceosome. This complex serves as a binding platform for the recruitment of CIITA and triggering of the transcriptional machinery (Reith et al., 2005).

These properties are the reason why CIITA merits the name of master regulator of MHC-II expression. Given that the regulation of MHC-II complex depends so much on CIITA then it becomes evident that also the regulation of CIITA needs to be precisely controlled.
1.10 CIITA regulation

The control of CIITA expression is performed mainly at the level of transcription, however it can also be modulated by modifications that alter the stability of the mRNA and the protein (Schnappauf et al., 2003; De Lerma Barbaro et al., 2005). Transcription of CIITA is controlled via three different promoters, which are known as promoters I, III and IV (pI, pIII and pIV) from their position in the gene (Fig. 7) (Muhlethaler-Mottet et al., 1997). Promoters pI, pIII and pIV possess each one a different first exon that is spliced to the shared downstream common exons 2 to 19. This alternative splicing produces three types of mRNA differing only at their 5′ ends. All three mRNAs have a translation-initiation codon in the common second exon, however, pI and pIII contain a separate initiation codon in their first exons. The presence of these alternative initiation codons leads to specific N-terminal extensions of 101 and 24 amino acids, respectively, producing three CIITA isoforms that differ at their N-terminus.

The differential activation of these three promoters is dependent in tissue specificity, cytokine induction and developmental state. Under normal conditions pI is active in myeloid cells such as DC and IFNγ-activated macrophages. While pIII is mainly active in lymphoid cells, it is also active in plasmacytoid dendritic cells (pDC), a subclass of DC (Landmann et al., 2001). The pIV is constitutively active in thymic epithelial cells (TEC) but is more important for inducing CIITA in non-hematopoietic cells (Fig. 7) (Muhlethaler-Mottet et al., 1997; Muhlethaler-Mottet et al., 1998). Nevertheless this normal activation pattern can be modified sometimes, for example, all three promoters were reported to be inducible by IFNγ, and both pI and pIII were found to be active in DCs. Also, pIII can be active in certain tumours of non-haematopoietic origin (Piskurich et al., 1999; Goodwin et al., 2001). It is important to remark that this ‘division of labour’ between the promoters is highly specific and is much more precise in vivo than was anticipated from earlier in vitro studies (Reith et al., 2005).
Figure 7. Differential activation of CIITA promoters. The three active promoters of CIITA (pI, pIII and pIV) are induced under different conditions and in different cell types. For myeloid cells such as DC and IFNγ activated macrophages the activation is directed by pI. In lymphoid cells and plasmacytoid dendritic cells (pDC) the activation is exerted by pIII, a subset of DC. pIV is constitutively active in thymic epithelial cells (TEC) but is more important for inducing CIITA in non-hematopoietic cells. In certain cases IFNγ induction of pIIV is blocked in tumors due to epigenetic modifications (Reith et al., 2005).

1.11 Induction of CIITA-pIV by IFNγ

In order to put the antigen presentation mechanism in context with the breast cancer pathology, it is important to remember that the immune response triggered by the DCs will induce the activation of Th1 cells. As it was mentioned before, these cells will in turn activate the inflammatory response via the secretion of different cytokines including the IFNγ. This cytokine will activate pIV of CIITA and induce the expression of MHC-II
molecules in order to confer a transient "APC" ability to the epithelial cells that will help
with the pathological identification of the tumor (Banchereau et al., 2000).

With this in mind, it is time to take a closer look at the role of pIV of CIITA. A conserved
300 bp proximal region of the promoter is necessary for its activation by IFNγ and
contains three regulatory elements: an IFNγ-activated-site (GAS), an enhancer box (E-
box) and an IFN-regulatory factor element (IRF-E). (Muhlethaler-Mottet et al., 1998;
Piskurich et al., 1999). The GAS and E box are bound cooperatively by signal
transducer and activator of transcription 1 (STAT1) and upstream transcription factor 1
(USF1). The IRF-E is co-occupied by IRF1 (Muhlethaler-Mottet et al., 1998; Dong et
al., 1999) and IRF2 (Xi et al., 1999; Xi et al., 2001). Activation of the interferon gamma
receptor (IFNR) by its ligand induces phosphorylation of STAT1 by the receptor-
associated protein tyrosine kinases Janus kinase 1 and 2 (JAK1 and JAK2). Phosphorylation of STAT1 induces its dimerization and subsequent translocation to the
nucleus where it acts as transcription factor by binding targeted promoters, including
pIV. The expression of IRF-1 is also induced by IFNγ and generates a delayed induction
of the CIITA expression relative to the rapid induction of expression of genes that are
controlled only by STAT1 (Steimle et al., 1994; Morris et al., 2002).

At the chromatin level, activation of pIV is mediated by the remodelling of the local
structure. This is in part executed by BRG1, which is the ATPase subunit of some
ucleosome remodelling complexes (Pattenden et al., 2002) and STAT1 which
increases chromatin accessibility by inducing histone acetylation (Morris et al., 2002).

1.12 Objectives and hypothesis

So far E2 and MHC-II molecules have been presented as acting at opposite ends of
the spectrum in the cancer pathology. E2 acts as a tumorigenic factor that activates
cellular pathways that induce cell growth and division, while the MHC-II molecules are
central participants in the identification of the tumor cells thanks to their antigenic presentation activity. However, some studies have already suggested the existence of a closer relation between the estrogenic pathways and the antigenic presentation. The basis for this idea relies in several epidemiological observations that have shown certain inflammatory auto-immune conditions, such as rheumatoid diseases with their higher incidence among the female population (Lockshin, 2001). Systemic lupus erythematosus (SLE) has also higher incidence among the female population, with women being around four times more prone than men to develop the condition (Cutolo et al., 2014). Along these lines, pregnancy has also been observed as a factor influencing the development of auto-immune conditions, about 50% of women suffering from rheumatoid arthritis see a remission of their condition during the duration of the gestation, a period when the systemic concentration of E2 is increased (Ostensen et al., 2011). These observations have been related to neuroendocrine influence on the immune system via the effects of the gonadal steroids, mainly by the anti-inflammatory role of androgens, the double function of estrogens (support B cells and inhibit macrophages and T cells), the increased peripheral conversion of androgens to estrogens in inflammatory tissues (androgen drain) and generally disturbances of the gonadal axis (Straub et al., 2013). Nevertheless, the precise mechanisms that intersect the hormonal and the immune pathways remain an ongoing work.

Among these observations, a statistical analysis from a study performed on a group of 112 cases of breast cancer that measured the expression of HLA-DR, and the co-chaperons HLA-DM and Ii, suggested the existence of an inverse correlation between the expression level of ERα and the level of MHC-II expression by the breast cancer cells, as cells expressing the hormonal receptor had a significantly lower expression of one of more of the MHC-II related proteins, thus suggesting a role for hormone regulation on MHC-II expression (Oldford et al., 2006). This observation comes to support previous works that have already attempted to explain the molecular mechanism behind this down-regulation. Nevertheless many of these works have come
to certain contradictory results. Benveniste and colleagues (2004), reported a down-regulation of MHC-II expression independent of CIITA but mediated by a decreased acetylation of the MHC-II locus induced by a lower binding of CBP. However these studies also reported an inability of E2 antagonists such as ICI and competitors such as Tamoxifen to restore the E2 induced down-regulation of MHC-II (Adamski and Benveniste, 2005). Meanwhile Drover and colleagues (Mostafa et al., 2014), reported also an E2 induced down-regulation of MHC-II, but mediated by an inhibition of the CIITA activation via the IFNγ signaling pathway where ICI was able to restore the E2 effect on the MHC-II expression (Mostafa et al., 2014). These discrepancies leave the place for further exploration, as it seems plausible that the definitive mechanism has not been yet discovered.

In recent years it has been possible to observe genome wide modifications and activities for several transcription factors thanks to next generation sequencing techniques. Among these observations, studies for ERα in different cell lines have revealed ERBS in previously unknown positions that regulate target genes that were previously though as non-hormonal dependent. Results from one of these analysis in the MCF7 breast cancer cell line, obtained by a chromatin-immunoprecipitation followed by sequencing (ChIP-seq), revealed the presence of a possible ERBS sequence in the locus of CIITA (Fig. 8) (Brunelle M., personal communication). Having in mind that a definitive mechanism for the MHC-II inhibition induced by E2 seems to still be elusive and considering the genetic regulatory functions of ERα, it was hypothesized that this ERBS could act as CIITA silencer, thus inhibiting the expression of MHC-II molecules.

Further analysis of published results from sequencing experiments (i.e. ChIP-seq, DNAse hypersensitivity) have revealed contradictory results for the ERα binding near the CIITA locus. Benveniste and colleagues (2005) reported but did not show binding
of ERα in the CIITA locus only after dimerization with ERβ. However, most of the observed data for BCC lines did not present any binding in the region. Such discrepancies are hard to explain and could be consequences of differential tissue expression, different cell lines or altered cancer expression, or simply due to technical differences between the different laboratories. Nevertheless, the production of more sequencing information helps in the construction of a more solid and richer database for further experiments. Also, the BCC line MDA-MB-231 remains less studied compared to other model cell lines, and information derived from our experiments will constitute a first entry for ChIP-seq data from this particular model under these conditions, moreover the MC2 transfectants, which are positive for ERα, represent a cleaner model for the observation of the ERα behaviour in the absence of ERβ.

Based on the information gathered and presented, we hypothesize that the expression of MHC-II molecules in breast cancer cells is modulated by estradiol (E2) via binding of estrogen receptor-α (ERα) to an estrogen receptor binding site (ERBS) in the intronic section of CIITA-pIV. In order to test our hypothesis we established if the presence of our candidate sequence for ERBS in the CIITA-pIV locus, as identified by the ChIP-seq observations (Fig. 8), inhibits the activation of CIITA-pIV in the presence of E2 via luciferase assays. Then we defined the position of the ERBS using bioinformatics motif prediction tools to find shorter sequences that could harbor the ERBS. These sequences were then cloned into new reporter-constructs and tested for CIITA inhibition in the presence of E2. Finally, to prove the binding of ERα to the CIITA locus, we performed ChIP-qPCR assays for the hormone receptor at the pIII and pIV. Further analysis was performed on the histone modifications at the CIITA locus to better understand the epigenetic modifications induced by E2. Also, genome wide ChIP-seq analysis of the ERα binding in the presence of E2 and IFNγ gave us better understanding of the behaviour of ERα in the inflammatory context for BC cells.
Figure 8. ChIP-seq in the MCF7 cell line. ChIP-seq data showing enriched regions for different histone modifications and binding of ERα around the CIITA locus. CIITA-pIII and pIV positions are approximate (Brunelle M, personal communication).

The significance of this work mainly resides in the importance of understanding the molecular mechanisms governing the antigenic presentation pathway, mediated by the MHC-II molecules and controlled by CIITA. Analysis of these pathways will help to better understand the immune system regulation and activation, not only in the cancer pathology, but for all contexts of immune response such as autoimmune conditions, a field where the regulation of MHC-II molecules is of vital importance. The results from this work could potentially lead to the development of therapeutic treatments targeting the newly discovered mechanisms.
2.1 Cell culture

The ERα/ERβ-negative breast cancer cell line MDA-MB-231-clone-10A (ATCC HTB-26), and stable transfectants MC2 (MDA-MB-231 clone 10A with ESR1-NM_000125) and VC5 (MDA-MB-231 clone 10A with empty transfection vector) established from it, were produced by Dr. Craig Jordan (MacGregor Schafer et al., 2000) and were a kind gift of Dr. Sheila Drover. Cells were grown in DMEM phenol red free medium (Wisent) supplemented with 10 % heat inactivated and charcoal-treated Cosmic Calf Serum (Hyclone), plus L-glutamine-penicillin-streptomycin mixture (Wisent). MC2 and VC5 were selected with 500 μg/mL G418 (Wisent).

Breast cancer cell lines MCF7 (ATCC HTB-22) and T47D (ATCC HTB-133) were a generous gift of Dr. Nicolas Gévry, cell line BT-474 was also a gift of Dr. Sheila Drover, cell lines ECC-1 (CRL-2923), ZR-75-1 (ATCC CRL-1500) and MDA-MB-361 (ATCC HTB-27) were acquired from the ATCC. MCF7 was cultivated in the same medium as the MDA-MB-231, VC5 and MC2. Cell lines T47D, ECC-1 and ZR75 were all cultivated in RMPI-1640 phenol red free medium (Wisent) supplemented with 10 % heat inactivated and charcoal-treated Cosmic Calf serum (Hyclone), plus L-glutamine-penicillin-streptomycin mixture (Wisent). The cell line BT-474 was cultivated in a modified DMEM phenol red free medium (Wisent) supplemented with 25 mM HEPES (BioShop), 10 % heat inactivated and charcoal-treated Cosmic Calf serum (Hyclone), plus L-glutamine-penicillin-streptomycin mixture (Wisent). MD-MB-361 was cultivated in Leibovitz’s L15 phenol red free medium, supplemented with 20 % heat inactivated and charcoal-treated Cosmic Calf serum (Hyclone), plus L-glutamine-penicillin-
streptomycin mixture (Wisent). This cell line was maintained in atmospheric air conditions at 37°C, all other cell lines were maintained at 5 % CO₂ and 37°C.

2.2 Transfections

MDA-MB-231, MC2 and VC5 cell lines were transiently transfected using FuGeneHD (Promega) at 4 µl/µg DNA. Previous to transfection, the cells were incubated for 30 minutes in Opti-MEM (ThermoFisher) reduced serum medium and left overnight after the transfection before reverting to charcoal-stripped DMEM.

2.3 Stimulations

For cytokine stimulation, the culture medium was replaced with fresh medium containing 100 U/ml IFNγ (Prospec), with or without E2 10⁻⁷ M (Sigma). As control for the E2, the same volume of EtOH (0.1 % v/v) was added.

2.4 Western blotting.

Whole cellular extract was obtained by freeze-thaw and mechanical sheering cycling in lysis buffer (600 mM KCl, 2 0mM Tris-HCl, 20 % glycerol) with complete-mini protease inhibitor cocktail (Sigma). Protein concentration was measured by optic density absorption at 595 nm using Bradford reagent (BioRad).

For the immunoblots of MHC-II and ERα 15 µg of protein were used, whereas 45 µg were used for CIITA. 7.5 % TGX FastCast stain-free acrylamide (BioRad) was used for CIITA and 10 % for all other proteins. Wet transfer to PVDF membrane was done in a
Mini Trans-Blot block according to manufacturer’s protocol (BioRad) at 350 mA constant current for 1h at 4°C. Blots were blocked for 1 h at room temperature with blocking buffer (5 % powdered milk, 50 mM Tris-base, 0.15 M NaCl, 0.1 % Tween-20), primary antibody or antiserum were diluted in blocking buffer and incubated with the blot overnight at 4°C. Revelation was carried out with appropriate HRP conjugated-secondary antibody 1/5000 (Pierce) and SuperSignal West-Femto substrate (ThermoFisher). Total protein in the blot was quantified via the stain-free system (BioRad) for which a trihalo compound present in the acrylamide mixture binds the tryptophan residues of the proteins in the gel when activated by UV radiation. This trihalo compound is covalently bound to the proteins and thus transferred along with them into the blot. A picture of the total transferred proteins (excited by UV light) was captured with a ChemiDoc (BioRad) device and used to normalize the targeted protein signal per lane. CIITA was detected using rabbit antiserum K5D (Camacho-Carvajal et al., 2004) and MHC-II molecules were detected using antibody XD5 against HLA-DR β-chain (Cresswell, 1985). FoxA1 and TLE3 were detected using antibodies ab40868 and ab94972 (Abcam), respectively.

2.5 Flow Cytometry

Adherent cells were removed from the plates using phenol red free 0.05 % trypsin solution (Life technologies), washed in PBS and stained using anti-HLA-DR specific antibody D1.12, a generous gift from Dr. R. Accolla (Carrel et al., 1981), or anti-HLA-DR monoclonal antibody L243 (ATCC HB55) from hybridoma supernatant, a generous gift from Dr. J. Thibodeau. Secondary staining was done using goat anti-mouse IgGF(ab’)2 coupled to Alexa-647 (ab150115 Abcam). Analysis was performed on a FACSCalibur device (Beckton Dickinson).
2.6 ChIP assays

VC5 and MC2 cells were stimulated with IFN\(\gamma\) (100 U/\(\mu\)l), E2 (10\(^{-7}\) M), IFN\(\gamma\) + E2, or EtOH (0.1 %v/v) (for incubation times see Fig. 14A). Then cells were fixed with PBS-formaldehyde 1.1 %, harvested and lysed in SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1). Raw chromatin was sheared by sonication in a Bioruptor 300 (Diagenode). ER\(\alpha\) bound chromatin was precipitated using antibody ER\(\alpha\)-H184 (Santa Cruz) and protein A Dynabeads (Novex). Precipitated chromatin was purified using a QIAquick PCR purification kit (Qiagen). As negative control for specific binding, no-antibody precipitations were performed. A fraction of the sheered chromatin was directly purified and used as normalizing factor (Input) for qPCR analysis.

2.7 Library preparation for Illumina sequencing

Genomic fragments of 250-350 bp from ChIP purified chromatin were selected and purified by differential ratio of DNA/PEG using AMPure beads (Beckman Coulter). Linker sequences were added to the purified fragments using T4 DNA ligase and barcode sequences were included by PCR reaction using Phusion HF (NEB) master mix in a CFX-96 Real Time system (BioRad). Final verification of the size and concentration of the library was performed in a 2100-Bioanalyzer (Agilent). Sequencing of the libraries was performed by the Institut de Recherches Cliniques de Montreal (IRCM) in an Illumina HiSeq 2000 device.

2.8 ChIP-seq analysis

Raw sequencing results were filtered and analysed with tools provided by the Galaxy-GenAP platform (https://genap.ca/). Sequences were sorted by quality score with
FASTQ-quality trimmer. Alignment was executed using the Bowtie algorithm against the human genome (Hg19) and enrichment positions (peaks) were determined by statistical significance evaluated by the MACS2 algorithm. Cistronic relations between the peaks for ERα were further analysed using the Genomic Regions Enrichment of Annotations (GREAT) tool for GenOntology (GO), DiseaseOntology (DO) annotations and physiological pathways. Binding motif analysis was performed using the MEME-ChIP application (Bailey et al., 2006).

2.9 Quantitative PCR (ChIP)

Purified genomic DNA from ChIP essays was quantified by qPCR using Omni Klentaq DNA polymerase (Enzymatics) and SyBr Green PCR buffer. Amplification was performed in a 7500 Real Time PCR system (Applied Bioscience). 5X PCR buffer was composed of 250 mM Tris-HCl, 80 mM (NH₄)₂SO₄, 17.5 mM MgCl₂, 0.125 % Brij 58. Enrichment for specific genomic positions was normalized against Input control and reported as % of Input.

2.10 Quantitative RT-PCR (mRNA)

MDA-MB-231, VC5 and MC2 cells were stimulated with IFNγ (100 U/μl), E2 (10⁻⁷ M), IFNγ + E2, or EtOH (0.1 % v/v) and incubated as for ChIP essays. Cells were lysed in the plate and total RNA was purified with Absolutely RNA microprep (Agilent). Reverse transcription was done using Expand reverse transcriptase (Sigma) according to manufacturer’s protocol. Amplification was performed in a 7500 Real Time PCR system (Applied Bioscience). 5X PCR buffer (250 mM Tris-HCL, 80 mM (NH₄)₂SO₄, 27.5 mM MgCl₂, 0.125 % Brij 58).
2.11 Reporter gene assays

The luciferase reporter genes carrying CIITA promoters III and IV and the intronic region were constructed on a pGL3-Basic backbone (Promega). The pGL3-Control vector carrying both a SV40 promoter and enhancer was used as positive control and the pGL3 Basic vector as negative control. Normalisation for transfection efficiency was performed by co-transfecting the cells with a plasmid coding for secreted alkaline phosphatase (SEAP) driven by a CMV promoter (CMV-SEAP Adgene #24595). The morning after the transfection, the medium was removed and replaced with fresh medium containing the stimulants IFNγ and/or E2 as described in the text. After 48 h of stimulation, 1 ml of medium was recovered for SEAP quantification. Cells were washed and lysed using lysis buffer (25 mM Tris, 2 mM DT, 1 % Triton X-100, 10 % glycerol) and whole cellular extract was harvested for luciferase activity. 20 µl of cellular extract were transferred to a microplate and mixed with 100 µl luciferin buffer (1.07 mM (MgCO₃)₄Mg(OH)₂, 2.67 mM MgSO₄, 0.1 mM EDTA, 270 µM Coenzyme A, 530 µM ATP, 33.3 µM DTT, 470 µM D-luciferin potassium). Luminosity was evaluated in a Lumat LB9507 (EG&G Berthold) device.

For SEAP quantification, a fraction of the culture medium was transferred to a 96 wells plate, mixed with p-nitrophenyl phosphate solution (SigmaFAST pNPP) according to manufacturer’s protocol and incubated for 30 min at room temperature. Optical density was evaluated at 405 nm in a uQuant spectrophotometer (BioTek instruments). Optimal volume of medium is determined a priori by a standard curve. Activity of the reporter gene is normalised against the SEAP density and reported as a unidimensional value.
2.12 Depletion experiments by shRNA

The ERα-shRNA expressing vectors (ERα sh3300, ERα-sh10074, shFoxA1 and shTLE3) were a kind gift from Dr. N.Gévry. Lentiviral supernatant was produced by co-transfecting Hek293T cells with psPAX2 packing protein, pMD2G VSV G envelope and shRNA vector according to protocol from the Trono laboratory (http://tronolab.epfl.ch/). The viral supernatant was harvested after 36h, filtered and used to infect MDA-MB-231, VC5 and MC2 cells.
3.1 Expression of major histocompatibility complex class II is differentially modulated by E2 in BCC lines.

Antigenic presentation of tumorigenic antigens is a vital mechanism for an efficient immune response to breast cancer. This process is mainly executed by the MHC class-I molecules that present peptides of intracellular origin. However, molecules of the MHC class-II family, specialised in the presentation of peptides of extracellular origin, also play an important role, as they present material from surrounding cells and in some cases present cytoplasmic peptides through an alternate pathway (Villadangos, 2001). However this antigenic presentation is sometimes compromised in some breast cancer patients who possess an unusually high concentration of E2 in this gland (Oldford et al., 2006). We therefore decided to analyse multiple BCC lines for IFN$\gamma$-induced expression of HLA-DR (human MHC-II isotype) molecules at the cell surface level in the presence or absence of E2. All of the BCC lines used were chosen for being positive for the expression of ER$\alpha$ according to the literature, however the expression of this factor was not confirmed in the laboratory. The cells were stimulated with IFN$\gamma$ (100 IU/ml), IFN$\gamma$ (100 IU/ml) + E2 (10 nM), or EtOH (0.1 % final volume). The latter was added to the non-stimulated cells as E2 was dissolved in 100% EtOH. The IFN$\gamma$ was dissolved in molecular grade water. The cells were then incubated for 56h before being harvested and analyzed by flow cytometry (FCM). Fig. 9A shows a typical result for the MC2 cell line. A shift in the mean fluorescence intensity (MFI) of the expression in HLA-DR molecules of the cellular population was indicative of the modulation. EtOH treated cells (MFI: 7.1) were considered negative for HLA-DR expression, meanwhile the IFN$\gamma$-
induced sample showed a high expression of HLA-DR (MFI: 207.7) and IFN$_{\gamma}$+E2 (MFI: 59.9) showed a clear inhibition of the HLA-DR expression. Among the different BCC lines tested, we observed that the expression of HLA-DR was only statistically different in the MCF7 and the MC2 (MDA-MB-231 ER$\alpha$+) lines (Fig. 9B). Interestingly enough, both cell lines presented a lower induced expression of HLA-DR compared to the other cell lines. In two cases, the ECC-1 and BT-474, the cells did not respond to the IFN$_{\gamma}$ induction. These results suggest that E2 negatively regulates the expression of MHC-II molecules in some, but not all ER$\alpha$ positive cell lines.

Figure 9. HLA-DR expression in different BCC lines. The presented cell lines were stimulated with EtOH, IFN$_{\gamma}$ or IFN$_{\gamma}$+E2, and incubated
for 56h before harvesting. Staining was done with primary anti-HLA-DR IgG D1-12 (1/200) and secondary anti-rabbit IgG Alexa647 (1/250). Detection of fluorescence is done in the FL4 channel. A) Typical histogram for flow cytometry staining for HLA-DR in the MC2 line. The quantifying measure is the mean value of the population expressed as the Mean-fluorescence-intensity (MFI). B) HLA-DR expression in different BCC lines compared to the MC2 line. (*: p<0.05). Results are presented as average ± standard deviation of two biological duplicates. MC2 is a stable transfectant of MDA-MB-231 containing an ERα wild type sequence while VC5 is a control stable transfectant containing only the vector.

3.2 E2-induced down-regulation of HLA-DR expression is dependent on ERα and mediated by down-regulation of CIITA

Given that the transactivator of class II (CIITA) is known as the master regulator of MHC-II expression and its modulation directly affects the expression of MHC-II molecules (Reith and Mach, 2001), we decided to test if the down-regulation of the MHC-II molecules was caused by a lower expression of CIITA at the protein level. MDA-MB-231 (ERα-/ERβ-) and its stable transfectants MC2 (w.t. ERα+) and VC5 (vector), were stimulated with EtOH, IFNγ or IFNγ+E2 as described previously. From our preliminary studies we knew that the CIITA protein expression reaches a peak at 24h of IFNγ stimulation (data not shown). Thus the cells were harvested at this time point and whole protein was extracted for western blotting against CIITA (Fig. 10A bottom) using antiserum K5D (Camacho-Carvajal et al., 2004) and ERα using antiserum H-184 (Santa Cruz Biotech) (Fig. 10A top). As expected, only the stable transfectant MC2 cells express ERα (Fig. 10A top, lanes 1-3). The expression of ERα seems to be increased by the introduction of IFNγ (Fig. 10A top, lane 2), however the presence of
IFNγ+E2 induces a strong decrease of ERα (Fig. 10A top lane3). This is very likely a consequence of induced degradation of the receptor following its activation by E2 (Barkhem et al., 2004), this effect was also observed by us in samples treated only with E2 (data not shown).

**Figure 10. E2-induced inhibition of CIITA expression.** Cell lines MDA-MB-231, MC2 and VC5 were stimulated with EtOH, IFNγ or IFNγ+E2 as
described above, then incubated for 24h and harvested for protein extraction and SDS PAGE. Whole protein was blotted for ERα using rabbit antiserum H-184; CIITA was blotted using antiserum K5D. A) Western blot against ERα (top) and CIITA (bottom). B) Quantification of the CIITA blot by ImageLab (Biorad), the CIITA expression of the IFNγ sample was normalised to 100% for each cell line; each WB band was normalized against the total amount of protein in the lane. C) RT-qPCR for CIITA expression in cells stimulated with IFNγ, E2 or IFNγ+E2 for 4h. Results are presented as average ± standard deviation of three technical replicates. M: stained molecular weight marker, m: unstained molecular weight marker for stain-free protocol (BioRad).

Analysis of CIITA expression showed no signal for the solvent alone (Fig. 10B, lanes 1, 4 and 7), an increase in the protein abundance with the IFNγ stimulation (Fig. 10B, lanes 2, 5 and 8) for the three cell lines and more importantly it revealed a strong down-regulation in the presence of E2 (Fig. 10B, lane 3) for the MC2 line only. This down-regulation was not observed for the other two cell lines, MDA-MB-231 (Fig 10B, lanes 5-6) and VC5 (Fig 10, lanes 8-9), both negative for ERα. Inhibition of CIITA expression was also observed by qPCR in the MC2 line in the presence of IFNγ+E2 (Fig 10C lanes 1-4), while not observed in the VC5 (Fig. 10C lanes 5-8). Taken together these results suggest that the down-regulation of HLA-DR expression observed previously is mediated by a decrease in the expression of CIITA.

To further study the dependency of CIITA down-regulation on signaling via ERα, we decided to perform a knockdown for the latter. MDA-MB-231, MC2 and VC5 were incubated in the presence of a lentivirus expressing a short-hairpin RNA targeting the ERα. Two different shRNA for ERα (sh3300 and sh10074) were tested and the empty
viral vector pLKO was used as control. After 24h incubation with the lentivirus, the cells were washed and incubated in the presence of IFNγ or IFNγ+E2 for another 24h before harvesting for protein extraction. Replicate wells were incubated for a total of 56h for FCM analysis of HLA-DR surface expression. Only the ERα-sh10074 showed knockdown activity in MC2 cells (Fig. 11A top, compare lanes 1-3 with 7-9). Protein analysis by western blot was only performed for the MC2 samples (ERα+) and the VC5 (ERα-) samples treated with sh10074 (Fig. 11B). The results showed that ERα knockdown restored the expression of CIITA to IFNγ levels (Fig. 11A, lanes 8-9) and that this restoration translated into an increased expression of HLA-DR molecules at the cellular surface, as it was observable in the FCM analysis (Fig. 11C). Taken together, these results suggest that E2-induced inhibition of HLA-DR molecules is dependent on ERα and mediated by down-regulation of CIITA protein expression.

3.3 Down-regulation of CIITA gene expression may be induced by the silencer activity of ERα

ERα is a well-known ligand-dependent transcription factor, whose main activity is the modification of the chromatin landscape to allow the recruitment of the transcriptional machinery (Barkhem et al., 2004). In the absence of activation, chromatin presents a closed environment that prevents proteins, such as polymerases, from accessing the DNA. In order to allow the transcription of E2-induced genes, ERα induces chromatin modification that facilitates access to the DNA strand by the transcription machinery. In this context, ERα works more often as an enhancer of genetic activation; however, it has been observed that ERα can also work as a silencer by inducing modifications in the chromatin that prevent transcription (Jangal et al., 2014).
Figure 11. CIITA expression in ERα depleted cells. The model cell lines MC2 and VC5 were depleted for ERα via shRNAs (pLKO, sh3300 and sh10074). 24h after transduction of shRNA the cells were stimulated with EtOH, IFNγ or IFNγ+E2 as described above, and harvested 24h after for...
protein extraction or 56h later for FCM. A) Western blot for ERα (top) using rabbit antiserum H-184 (Santa Cruz) and CIITA using antiserum K5D (bottom). B) Quantification of the CIITA blot (Fig. 3A bottom) by ImageLab (Biorad). CIITA expression of the IFNγ sample was normalised to 100% for each cell line; each WB band was normalized against the total amount of protein in the lane. C) HLA-DR cell surface expression was analysed by FCM using L243 monoclonal antibody for the ERα-KD cells. Results are presented as average ± standard deviation of two biological duplicates. (*: p<0.05)

Chip-seq analysis performed for ERα in the presence of E2 in MCF7 (Brunelle M, personal communication) revealed not only the presence of a binding site in the first intron of CIITA, approximately 4 kb downstream of the pIV, but also the presence of several histone modifications that are normally associated with an open chromatin state characteristic of an active genetic region (Fig. 8). Given that the presence of ERα seems to induce down-regulation of CIITA gene expression, we wondered if this response might be attributable to a previously unknown silencing activity of ERα for CIITA.

In order to test if the binding of ERα in the intronic region of CIITA had any influence on its transcriptional activity, we decided to perform luciferase assays. To that end we isolated and cloned the region around the candidate sequence for the estrogen receptor-binding site (ERBS). The sequences for promoters III and IV of CIITA (CIITA-pIII and CIITA-pIV, respectively) were also isolated and cloned. The CIITA-pIII should not be, or very weakly, induced by IFNγ and was used as a negative control for the promoter activity, while the CIITA-pIV is strongly induced by it (Dong et al., 1999). Using these sequences, six different constructs of reporter plasmids were elaborated; two constructs carried either one of the two CIITA promoters alone (Fig. 12B left) and the four others contained one of the CIITA promoters along with the candidate ERBS. This ERBS was cloned into the plasmid in the same orientation as the CIITA promoter (fwd)
or in the reverse sense (rev) (Fig. 12B right). If the candidate ERBS acted indeed as silencing sequence, then its activity should be independent of its orientation.

**Figure 12. Luciferase assay for CIITA intronic candidate ERBS.** pGL3 luciferase reporter plasmids were constructed to evaluate the level of promoter activation. A) Schematic representation of the position of the promotors.
ERBS in the CIITA locus, not to scale B) Schematic maps for the pGL3 constructs. Left: constructs carrying either CIITA-pIV or pIII. Right: based on the previous construct, the candidate ERBS was introduced in either forward (fwd) or reverse (rev) orientation, 3’ to the polyA signal. C) Luciferase activity dependent on the construct used. The MC2 line was transfected with the pGL3 constructs, and 24h later the cells were stimulated with EtOH, IFN\(\gamma\) or IFN\(\gamma\)+E2 as described previously for 48h before being processed for protein extraction. Promoter activation was measured by the luciferase activity. None: construct containing no promoter. SV40: simian virus 40 promoter used as constitutive promoter. Luciferase activity was normalised to the SEAP concentration. Results are presented as average ± standard deviation of three biological replicates.

The constructs were then co-transfected into the three model cell lines (MC2, VC5 and MDA-MB-231) along with a normalizing construct that expresses in a constitutive manner secreted alkaline phosphatase (SEAP) (Addgene 24595). The cells were incubated with the transfectant solution for 24h before being washed and stimulated with EtOH, IFN\(\gamma\) or IFN\(\gamma\)+E2 for 48h. After incubation, the culture supernatant was recovered to measure the SEAP concentration. Then the cells were lysed and the whole cellular extract recovered for luciferase activity, measured in a Luma LB 9507 luminometer. The luciferase expression was normalized to the SEAP concentration to compare the promoter activity between the different conditions (Fig. 12C). The SEAP concentration was determined by hydrolysis of p-nitrophenyl phosphate and subsequent measure of absorbance at 405nm. The results showed that none of the CIITA-pIII constructs were induced by the IFN\(\gamma\) stimulation (Fig. 12C, lanes 8, 11 and 14), while the CIITA-pIV constructs were readily activated (Fig. 12C, lanes 17, 20 and 23). However, for the CIITA-pIV constructs E2 was able to inhibit the activity of the promoter in all cases, even in the absence of the candidate ERBS (Fig. 12C, lanes 18,
21 and 24). The positive control used was a construct carrying a highly active SV40 promoter (Fig. 12C, lanes 4-6). However as is the case with the majority of the promoters of viral origin, the SV40 was affected by the IFN\(\gamma\) stimulation (Harms and Splitter, 1995). This effect is somehow expected, as IFN\(\gamma\) is one the most important cytokines involved in the antiviral response, nevertheless the usefulness of the SV40 construct as reference point is still valid. As a whole, the results from the luciferase expression come to corroborate the idea that HLA-DR inhibition is mediated by a down-regulation of the CIITA expression induced by the presence of E2, but would contradict the original hypothesis that the regulation is dependent on the candidate ERBS in the first intron of CIITA.

3.4 Analysis of distal regulatory CIITA elements by ChIP-PCR

In order to identify other possible locations where ER\(\alpha\) could bind near the CIITA locus, we proceeded to study the published information and looked for different regions that showed evidence of ER\(\alpha\) binding, transcription factor binding, IFN\(\gamma\) related factors or histone modifications suggestive of active chromatin states. An important part of the information was taken from the results presented by Bremner and colleagues (Fig. 13B), who focused on the role of BRG1 in the long distance regulation of the CIITA locus (Ni et al., 2008). From these compiled observations, we came up with 14 different sites around the CIITA-pIV position, which we considered as candidate sites for an ER\(\alpha\)-regulatory element (Fig. 13A). To identify the presence of a probable ERBS among these 14 new candidates, we performed ChIP-quantitative PCR (qPCR) analysis. For these experiments only the cell lines MC2 and VC5, as a negative control, were used, as the scale of the experiment would have made the use of the three cell lines a very complex endeavour. The cells were stimulated with EtOH (30min), IFN\(\gamma\) (5h30min), E2 (30min) or IFN\(\gamma\)+E2 (5h+30min) (Fig. 13A). Then the cells were crosslinked with paraformaldehyde, the chromatin extracted and immunoprecipitated
for ERα using the polyclonal serum H-184 (Santa Cruz Biotech) and purified with protein-A Dynabeads (Life technologies). The obtained DNA fragments were amplified and analysed by qPCR. Well described ERBS from SKG3, IGFB4 and TFF1 genes were used as positive controls, and an intragenic region of the TFF1 gene without ERα binding activity (TFF1-b) was used as a negative control (Jangal et al., 2014).

Unfortunately, analysis of the enrichment of the sequences (Fig. 14B) did not suggest the presence of an ERBS among the candidate regions, as their enrichment was significantly lower than the one observed for the positive control sequences. Even if compared with the binding region for the SKG3 gene, a region that presented the lowest enrichment among the positive controls. Also, the difference between the ERα positive and negative cell lines was not significant either. Furthermore, it is important to note that neither of the controls remained constant among the treatments, with the TFF1a and IGFBP4 showing a significant decrease in the enrichment with the combination of IFNγ+E2. Also, the IGFBP4 control showed an important enrichment in all cases, even in the absence of added E2. This might be explained by the action of the remaining E2 left in the serum after the depletion with activated charcoal. The impossibility to completely eliminate the endogenous E2 from the serum presents a significant problem, as it is impossible to have a control totally free of E2. More possible consequences of this problem will be discussed later on.

3.5 Inhibition of the ERα partners TLE3 and FoxA1 does not prevent down-regulation of the HLA-DR expression

To better understand the ramifications of ERα activity in the down-regulation of CIITA, we decided to inhibit the expression of two known partners of ERα, TLE3 and FoxA1, these two being often present in the regulatory sequences of ERα target genes. The
co-repressor TLE3 has been shown to repress the expression of ERα-regulated genes in the absence of E2 by maintaining the chromatin in a low acetylated state that prevents its transcription. The recruitment of TLE3 to the regulatory elements of ERα is dependent on the transcription factor FoxA1, that is also known to work as a pioneer factor for ERα binding and in general is known to play an important role in the E2 signaling pathway in several BCC lines (Jangal et al., 2014).

Figure 13. Primer pair positions for the regions of interest in the CIITA locus. A) Positions of the amplified regions are numbered with

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respect to the beginning of the promoter-IV (pIV). The top red arrowheads show the position of the CIITA promoters, the bottom arrowheads show the position of the regions amplified by the respective primer pairs. B) Example of the published data used to find the regions of interest (Ni et al, 2008). Chip-qPCR showing the enrichment positions for the presented factors in the presence or absence of IFNγ in the CIITA locus. *, ** and ***, values are greater than fivefold above background at the control site (-70 kb).

**Figure 14. ChIP-qPCR enrichment for target sequences.** A) Cell culture conditions for ChIP. The MC2 and VC5 model lines were

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stimulated with EtOH, IFNγ, E2 or IFNγ+E2, as shown. After incubation, the cells were fixed with formaldehyde and harvested to perform ChIP for ERα using antiserum H-184. B) After DNA purification, the regions of interest around the CIITA locus as shown in Fig. 13A were amplified by qPCR. SKG3, TFF1a and IGFBP4 are positive controls for known ERα binding sequences, TFF1b is a negative control without ERα binding activity. Results are presented as average ± standard deviation of three biological replicates.

In order to inhibit the activity of these two factors, we decided to use shRNA for both proteins. The MC2 and VC5 cell lines were transduced with lentiviral vectors containing the shRNA sequences and 24h after infection, the cells were stimulated with EtOH, IFNγ, E2 or IFNγ+E2 as indicated above. After 56h of incubation, the cells were harvested for HLA-DR surface expression by FCM. The efficiency of TLE3 and FoxA1 knockdowns was not directly observed by us, but it was tested by Jangal and colleagues (Jangal et al., 2014). Analysis of HLA-DR expression (Fig. 15A) showed a very interesting behaviour. The knock-down of TLE3 resulted in a very important inhibition of IFNγ effect on HLA-DR expression (Fig. 15A, lanes 5 and 14), observable in both cell lines, with the remaining HLA-DR expression being at the same level of the IFNγ+E2 stimulation, which showed a strong inhibition as usual (Fig. 15, lanes 6 and 15). The inhibition of the IFNγ stimulation by itself can be explained if we consider that TLE3 has an inhibitory activity for ERα. Thus in the absence of TLE3 the activity of ERα is exacerbated, consequently being able to reach the same inhibitory levels of added E2 with only the endogenous E2 remaining in the serum. Again the impossibility to have a completely E2-free serum represents a problem as the effect of the TLE3 depletion could have being explained better in an E2-free condition. The depletion for FoxA1 also caused a lesser inhibition of the HLA-DR expression, but contrary to the results observed for the TLE3 KD, failed to prevent the inhibition of the HLA-DR expression.
Taken together, these results do not seem to contribute to the hypothesis that ERα-mediated down-regulation of CIITA is due to its chromatin remodelling activity.

Figure 15. HLA-DR expression in TLE3/FoxA1-KD cells A) The model cell lines MC2 and VC5 were depleted for TLE3 and FoxA1 via shRNAs. 24h after viral transduction the cells were stimulated with EtOH, IFNγ or IFNγ+E2 as described above, and harvested 56h later for FCM of cell surface expression of HLA-DR using L243 monoclonal antibody. Results
are presented as average ± standard deviation of two biological duplicates (*: p< 0.05). B) Western blot analysis of FoxA1 expression in MDA-MB-231, MC2, VC5, and MCF7 cell lines. Cells were stimulated for 48h as described previously and harvested for protein extraction. Whole protein was blotted using antibody ab40868 (abcam). 25 µg total protein were used for all lanes 1 to 9 and 12 µg for lane 10. M: molecular weight marker.

Previous studies showed that the MDA-MB-231 cell line was a non FoxA1 expressing line (Robinson and Carroll, 2012), to clear any doubt whole protein extract from the cell was blotted for FoxA1 using the antibody ab27328 (abcam) (Fig. 15B). The results showed that even if the expression of FoxA1 in the MDA-MB-231 is lower than in MCF7 cells the protein is still expressed. However this lower expression could have an impact in the overall ability of ERα to function, as the lower quantity of FoxA1 could leave some target sequences empty.

3.6 Genome-wide analysis of ERα binding by ChIP-seq

Given that local analysis of the CIITA locus did not lead to the identification of any region that presented any significant evidence for the existence of an ERBS, we decided to extend our analysis to a genome-wide observation of ERα binding activity in the MC2 cell line under our stimulating conditions. To this end we performed a chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis for ERα. Given the characteristics of the ChIP and the cost of the sequencing, only the MC2 cell line was used, as any of the other two cell lines, MDA-MB-231 or VC5, would have given no information about the behaviour of ERα. The cells were stimulated with EtOH, IFNγ, E2, or IFNγ+E2 and then harvested for chromatin extraction as described above (Fig. 14A). The DNA was precipitated using the ERα-specific antiseraum H-184 (Santa
Cruz), purified with protein-A Dynabeads (Life Technologies), sequenced by Illumina system and analysed with the GenAP-Galaxy suite (https://genap.ca, Jacques et al, manuscript in preparation). The enrichment peaks resulting of the statistical analysis by MACS-2 identified the ERα-binding sites genome-wide for our four stimulating conditions (Fig. 16). However the total number of signals found for the E2 condition is significantly lower than is usual for an ERα positive cell line. In comparison, a similar analysis done with the same parameters with the same workflow for the MCF7 cell line, gave 2166 and 3363 signals for a pair of replicate experiments (data not shown). Furthermore, the combination of IFNγ+E2 increased the number of signals genome-wide. Analysis of the peak-calls using the Hypergeometric optimization of motif enrichment (HOMER) tool revealed 180 extra genes presenting ERα motifs with the combination of E2+IFNγ than with E2 alone. Stimulation with IFNγ by itself did not show any significant activation of the ERα binding, with the total number of signals being slightly higher than that of EtOH. Nevertheless this small difference could be the result of the same activity observed in the IFNγ+E2 sample with respect to the E2 only sample, as the EtOH sample is not totally free of E2 as has been mentioned before. This result could suggest that the IFNγ signaling pathway could be somehow interconnected with the E2 pathway in ways still not understood.
Figure 16. **Call peak modulation for different treatments.** Quantity of peaks identified for each treatment after execution of statistical analysis of enrichment by MACS2.

Observation of the ERα binding signals in the UCSC genome browser for two control genes, TFF1 and IGFBP4 (Fig. 17 top), revealed a typical binding signal at the expected positions, however the same observation for the CIITA and the HLA-DR loci did not show any indication of ERα activity in the vicinity (Fig. 17 bottom). Taken together this result would come to support the idea that ERα mediated down-regulation of MHC-II expression is not dependent of its chromatin remodelling activity as it was already suggested.
Figure 17. ChIP-seq for ERα at the CIITA and HLA-DR loci. BedGraph visualized in the UCSC genome browser showing the enrichment peaks for ERα. IGFBP4 and TFF1 (top) show typical signals of ERα enrichment as shown by the arrowheads. In TFF1 the ERBS sequence is found in an enhancer at a distal position at the 5' end of the gene locus. CIITA and HLA-DRA locus (bottom) do not present any signal of enrichment. Black boxes indicate the position of genes.
3.7 Genomic analysis of ERα ChIP-seq results reveals changes in the binding motifs of ERα with the combination of IFNγ+E2

In ChIP-seq experiments a way of confirming the quality of the precipitation for the factor of interest is usually done by executing an analysis of the enriched sequences for a de novo motif finding. This analysis works by looking for conserved sequence patterns among the enriched sequences, and then by comparing its findings to an existing database of known DNA-binding motifs for several DNA-binding factors, such as ERα in the present study. Analysis of our results for the MC2 cell line were done by the MEME-ChIP suite (Bailey et al., 2009) for the de-novo motif finding and compared by the TOMTOM tool against known motifs. The results confirmed the presence of motifs belonging to ERα (ESR1) and ERβ (ESR2) (Fig. 18C and 18D) in both treatments containing E2, and no ERα/β motifs were found for the EtOH or IFNγ treatments (Fig. 18A and 18B). Finding of ERβ motifs is not unexpected as both estrogen receptors have been shown to have similar DNA-binding specificities (Nilsson and Gustafsson, 2011). However, the estrogen receptor motifs were not the only ones identified by the analysis; for the E2 stimulation, motifs for PPARγ, LHX6/89 and Gfi1 (Fig. 18C) were also identified, while for the IFNγ+E2 stimulation, motifs for FoxI1, RunX1, RunX3, NFATC1 and NFATC2 were also identified (Fig. 18D). Taken together, these results help to prove the efficiency of the ChIP experiment and show an interesting activity of ERα in presence of IFNγ, as it would seem that activity of the receptor is increased by the cytokine.

3.8 Genomic analysis of ERα ChIP-seq results show that IFNγ inclusion increases tumorigenic phenotype.

In order to have a better idea of the cellular pathways affected by E2 and IFNγ in the MC2 line, we decided to analyse the binding signals (peaks) resulting from the MACS2
analysis via GREAT (Genomic Regions Enrichment of Annotations Tool) (McLean et al., 2010).

**Figure 18. ERα binding motifs identified by MEME.** The sequences for the ERα peaks established by MACS2 for the MC2 line were fed to the MEME-ChIP suite for motif analysis. The resulting analysis shows the motifs discovered and the DNA binding factors that are associated with it.

This tool allows for the prediction of function of cis-regulatory regions. Previous to this analysis, the ERα peaks had to be remapped from the original Hg38 genome to Hg19, as the current version of GREAT only works with Hg19, because the number of annotations for this genome version is much higher than for Hg38. For this, the BED file was transformed via the UCSC genome browser tool Batch Coordinated Conversion (liftOver). The resulting analysis for the MC2 line was compared to results obtained for the MCF7 line and analysed by GREAT in the same manner. The overall characterisation of the binding, as shown by the distance of the ERα-peak to the
nearest TSS (Fig. 19A), was similar between the MC2 and the MCF7 lines. Analysis of
the distance of the enrichment sites to the nearest TSS (Fig. 19B) was also similar
between the treatments and typical for an ERα binding pattern. The same analysis also
revealed that most Binding sites were associated with two genes at the same time (Fig.
19C), the consequence of this association is difficult to explain as the fact that they are
in proximity to several TSS simultaneously does not directly mean that they exert a
control over the gene. Moreover, the results given by the Mouse Phenotype conditions
(Fig. 20A) and the GO Biological process (Fig. 20B) present a low number of common
processes between the two cell lines. These results are unexpected as the two lines
being BCC of similar origin were expected to behave in a more similar manner.
Nevertheless, it is important to note that accumulated mutations and changes in the
karyotype are a characteristic of tumoral cells and they can severely alter their
metabolical behaviour (Kim, 2015). On the other hand the results obtained for the MC2
cells in the IFNγ+E2 condition showed some significant and intriguing differences to the
cells treated with E2 alone. First of all is the fact that more GO Biological processes
were able to be associated with the peak calling pattern of the IFNγ+E2 treatment than
with E2 alone. More remarkably however, is the fact that the mouse phenotype
conditions associated with the IFNγ+E2 binding pattern presents conditions more
closely related to breast cancer that the ones observed for E2 alone (Fig. 20B). Not
only are these conditions closer to the breast cancer phenotype but the probability of
them of being truly related to the genes associated to the peak pattern is higher as
shown by the higher binomial p-value. These results together show a similar behaviour
in the binding distance of ERα and a somehow less similar group of biological process
and mouse phenotypic conditions between the MC2 and the MCF7 lines, but more
importantly a closer association of the ERα affected genes to the breast cancer
phenotype when the IFNγ is present.
Figure 19. Genome-wide average distances of the ERBS respective to the nearest TSS. The sequences for the ERα peaks established by MACS2 for the MC2 line were fed to GREAT suite for prediction of cis-regulatory regions. A) Absolute distance distribution of the ERBS to the nearest TSS. B) Relative distance distribution of the ERBS to the nearest TSS. C) Number of ERBS peaks associated with 0, 1 or 2 genes.
Figure 20. Biological function and pathological conditions associated to the ERα binding pattern as determined by GREAT. The enrichment peaks for the ERα in the MC2 line were fed to the GREAT
CHAPTER IV
DISCUSSION

Antigenic presentation of tumoral antigens is a vital mechanism for tumor suppression. In this work we attempted to explain the molecular basis behind the down-regulation of the MHC-II molecules expression induced by E2 in IFNγ-stimulated BC cells. We were able to observe that the inhibition is exerted via activation of ERα and is mediated by inhibition of CIITA. Decreased expression of the MHC-II molecules in high E2 contexts have been observed in auto-immune conditions and breast cancer in several clinical studies (Oldford et al., 2006). It has also been observed frequently in breast cancer cases and is considered one of the main mechanisms participating in immune escape (Barkhem et al., 2004). MHC-II down-regulation has been also related to autoimmune conditions with modulations of E2 concentrations during the life cycle of human females (Nilsson and Gustafsson, 2011). These observations led to several works trying to explain the mechanism behind this inhibition, many of them arriving at different conclusions that included or not involvement of CIITA expression (Adamski et al., 2004). Some works on tumor cells have even shown MHC-II inhibition in the absence of E2 and presented the down-regulation as due to epigenetic modifications in the CIITA-pIV (Holling et al., 2006). A previous work by Drover and colleagues attempted to explain the E2-induced inhibition of MHC-II expression via binding of ERα to the CIITA-pIIV locus, but did not analyze binding at the candidate sites and suggested that the inhibition was exerted by interference of the IFNγ pathway at the level of STAT1 (Mostafa et al., 2014). However, analysis of publicly available data from ChIP-sequencing experiments showed ERα binding in an intronic region of the CIITA locus (Fig. 8) (Brunelle M., personal communication). This observation led us to hypothesize that ERα could bind to a previously unknown ERBS with a silencing activity on CIITA expression.
Analysis of the expression of MHC-II molecules in different BCC lines positive for ERα expression demonstrated a cell dependent inhibition by E2. Among seven cell lines analysed here, only the MCF7 and the MC2 lines presented a significant inhibition of MHC-II expression in the presence of E2 (Fig. 9B). It is important to mention that these two cell lines also presented a significantly lower MHC-II expression in the absence of E2 compared to the rest of the tested cell lines (Fig. 9B). This phenomenon could be a consequence of the remaining E2 present in the serum after the charcoal-dextran stripping, as this method probably does not eliminate the E2 completely. As a consequence, ERα activation in the non-E2-added control could result in constant inhibition of the MHC-II expression leading to the observed results. Absence of inhibition in the majority of the cell lines tested is hard to explain but could be a consequence of interference between ERα and ERβ, as it has been shown that activation of ERβ induces degradation of ERα (Nilsson and Gustafsson, 2011). It could also be a consequence of altered ER pathways due to tumoral mutations and aberrant cellular pathways. Interference between the two ERs was the main reason for us to perform these experiments in the MC2 model, as it is only positive for ERα.

The impossibility to have a completely E2-free medium posed a constant problem for the present study, mainly as it prevented us from explaining the lower expression of MHC-II in the MC2 and MCF7 lines. The best option would be to employ a chemically defined medium without serum as culture medium. Culture in this kind of medium is often hard for the cells, as absence of growth factors from the serum makes the cellular proliferation more difficult. Nevertheless, the cell lines could probably be forced to adapt to this type of medium, but elimination of unfit clones and stress induced by lack of growth factors could have unexpected consequences on the experiments. In the eventual impossibility to culture the cells in such a medium, it could be useful to find a way to measure the E2 concentration in the serum as this could allow us to have a better idea of the amount of E2 remaining in the serum after depletion. This could also
help us to produce more constant batches of serum and have a better understanding of the results observed. Evaluation of the E2 concentration by a sandwich-ELISA, or a competitive radioimmunoassay could be possibilities, however these systems remain expensive and laborious options.

Further analysis of the MC2 line revealed that the down-regulation of MHC-II molecules was dependent on the inhibition of CIITA, as observed by western blot (Fig. 10A) and RT-qPCR (Fig. 10C) analysis. These results come to contradict previous results by Benveniste and colleagues, who did not observe decreased expression of CIITA in the presence of added E2, either in mRNA or protein levels, but reported reduced levels of CBP, thus altering the function of the enhanceosome, as CREB is part of it (Adamski et al, 2004). Reduction of CBP decreases acetylation of the histones and maintains the chromatin in a closed conformation (Jangal et al., 2014). Benveniste and colleagues performed the experiments mainly in mouse endothelial brain cells (IBE), a cell system that can therefore not be directly compared to our own work (Adamski et al., 2004). Subsequent analysis presented by the same team showed that CBP inhibition was due to activation of the JNK pathway by ERα, as elimination of this mechanism resulted in restoration of the MHC-II inhibition (Adamski and Benveniste, 2005). However our results coincide partially with the ones presented by van den Elsen and colleagues, who suggested that the down-regulation was dependent on CIITA but due to CpG methylation in the pIV for T lymphoma cells (Holling et al., 2006). Similar results were also reported by Morris and colleagues in the trophoblastic cell lines JEG3 and JAR (Morris et al., 2002). Even if these results were not performed with added E2, they present a recurrent mechanism of MHC-II inhibition in cancer cells. Furthermore, there is no reason to suppose that these experiments were completely devoid of E2, as it was mentioned before, this hormone is normally present in the serum used in cell culture. With the idea that even the remaining E2 left after the striping could be enough to activate the inhibition, it is possible that the observations described by these two teams could be related to E2. The presence of similar result across different cell types
gives strength to the idea that this type of epigenetic modification could be responsible for the inhibition. The status of the CpG methylation in our cell lines was not tested, but could constitute part of the experiments during the continuation of the project.

Unlike the results just mentioned, previous results from Drover and colleagues showed that the inhibition of MHC-II expression was indeed induced by the presence of E2 and dependent on ER$\alpha$. However, it was mediated through inhibition of the IFN$\gamma$ activation pathway at the level of STAT1 via blocking of the JAK1 pathway (Mostafa et al., 2014). These results present certain similarities with the ones from van den Elsen and colleagues, as the JAK1 and JNK pathways both interact with the MAPK mechanism, this one being activated by E2 (Holling T, et al. 2006). The experiments performed by Drover and colleagues were executed in the MC2 cell line that we used for our own experiments, thus further study of the interaction of the JAK1 and JNK pathways in MC2 could be interesting.

The presumed silencer activity of the ERBS in the intronic region downstream of pIV was measured by reporter gene expression, but failed to demonstrate any effect of the candidate region in the activation of CIITA-pIV (Fig. 12C, lanes 16 -24). However, it was previously reported by Benveniste and colleagues (2004) that association of ER$\alpha$ to the MHC-II promoter was dependent on the presence of ER$\beta$, as binding to the promoter region was only possible after dimerization of the two ERs (Adamski and Benveniste, 2005). Even if the binding was observed in a different promoter, it is possible that our ERBS could only be bound under the same conditions. Repetition of the luciferase experiments in either a double positive cell line (ER$\alpha$/ER$\beta$) or an ER$\beta$-complemented MC2, would be a viable experiment in the continuation of the project. It is important to note that Drover and colleagues also found potential ERBS in the CIITA-pIV locus using bioinformatic predictions (Mostafa et al., 2014) but we were unable to see any binding of ER$\alpha$ at the proposed target sequences, neither by ChIP-qPCR (Fig. 14) nor ChIP-seq (Fig. 17).
Nevertheless, inhibition of MHC-II expression by E2 proved to be dependent on ERα, both by the fact that only the MC2 cell line is affected by E2 and by the fact that suppression of ERα via shRNA interference restored expression of the MHC-II molecules to non E2 levels (Fig. 11C, lanes 7–9). However, similar to the results presented by Drover and colleagues (Mostafa et al., 2014), the inhibition of ERα in the MC2 cell line did not restore the MHC-II levels to the VC5 and MDA-MB-231 levels (Fig. 11C, lanes 16–18). Analysis by western blotting revealed an almost complete elimination of ERα expression (Fig. 11A, lanes 7–9), however shRNA interference mechanisms are never completely efficient and the remaining ERα expression could be sufficient to inhibit CIITA expression. It is important to consider that in such a case both the expression of ERα and the E2 concentration would be greatly reduced and if even under these conditions the inhibitory mechanism is still sufficient to maintain a constant inhibition of MHC-II expression, then the mechanism is highly sensitive and strongly active. The same experiment was performed in the MCF7 cell line, with similar results being obtained as suppression of ERα expression led to a partial restoration of MHC-II expression induced by IFNγ. Interestingly enough, two different shRNAs were used for the interference of ERα; ERα-sh3300 worked only in the MCF7 line (results not shown), while ERα-sh10074 worked only in the MC2 line (Fig. 11A, lanes 7-9). This result is most probably a consequence of the difference in the sequence of the endogenous ERα expressed by the MCF7 and the sequence used to clone the ERα in the MDA-231 line to produce the MC2 stable transfectant (MacGregor Schafer et al., 2000). Analysis of the sequences for each shRNA revealed that the sh3300 targeted the 5′ terminus of ERα, while the sh10074 targeted an intragenic sequence in the middle of the ERα coding sequence. The 5′ terminus being an untranslated sequence, it is probably absent from the plasmid thereby preventing the activity of the shRNA, however the reason why the sh10074 is not functional in MCF7 and only is active in
MC2 cannot be explained at the moment as the intragenic sequence should be present in the MCF7 line too.

Following the impossibility to see any effect from our first candidate sequence for ERBS, we analysed the CIITA locus at 14 different positions by ChIP-qPCR for ER\(\alpha\). According to the literature, these positions presented evidence of active chromatin, such as histone acetylation and binding of transcription factors such as ER\(\alpha\), IRF1 and STAT1 (Ni et al., 2008). The results partially confirmed the observations from the luciferase assays, as no enrichment for the original candidate sequence was observed. Likewise no binding of ER\(\alpha\) could be observed in the CIITA locus at any of the other targeted sequences including the promoters. One possible explanation for the absence of binding in the observed regions is a bias in the selected sequences. As explained before, the targeted sequences were chosen for presenting active chromatin characteristics, however we were trying to observe a possible silencing effect of the ER\(\alpha\), and in this context it is most probable that the chromatin should have acquired characteristics of closed conformation, still some of the sequences tested presented ER\(\alpha\) binding according to results from the UCSC genome browser. This particularity could be explained by differences in the cell lines, for example, as we mentioned before Benveniste and colleagues (2005) observed binding or ER\(\alpha\) in the MHC-II promoter in ER\(\alpha\)+/ER\(\beta\)+ cells (Adamski and Benveniste, 2005)

Furthermore, analysis of the suppression of the ER\(\alpha\) partners TLE3 and FoxA1 gave some interesting results. TLE3 is a transcriptional co-repressor for several transcription factors by maintaining a low state of histone acetylation (Verdone et al., 2006; Duque et al., 2009). In the context of ER\(\alpha\), this low acetylation state is maintained in the absence of E2 (Jangal et al., 2014). Suppression of TLE3 by shRNA interference in the MC2 cell line resulted in an almost total inhibition of MHC-II expression induced by
IFN\textsubscript{\gamma}, in both, IFN\textsubscript{\gamma} alone and IFN\textsubscript{\gamma}+E2 samples, with the expression still being above the negative control (Fig. 15A, lanes 4-6). At first it was thought that suppression of TLE3 removed the repressor state kept over the chromatin thus allowing for traces of E2 to activate the inhibitory mechanism of ER\textsubscript{\alpha} on the MHC-II expression. However this explanation cannot account for the results observed in the VC5 line, where the expression of MHC-II was also brought down. Given that VC5 is ER\textsubscript{\alpha} negative, the deletion of TLE3 should have not presented any major effect. However, we do not know if the suppression of TLE3 inhibits the MCH-II expression at the level of CIITA or at the MHC-II level. Analysis of mRNA for CIITA and MHC-II genes under suppression of TLE3 should give us a better idea about the workings of this interference.

Even in the impossibility to establish a link between E2 and the down-regulation of MHC-II mediated by a silencing effect of ER\textsubscript{\alpha} at the CIITA level, as observed via the reporter gene assays (Fig. 12C) and the ChIP-qPCR results (Fig. 14B) where no association of ER\textsubscript{\alpha} was observed, the results from the ChIP-seq revealed some interesting behaviours. First of all, the increased binding of ER\textsubscript{\alpha} with the combination of IFN\textsubscript{\gamma} and E2 compared to the E2 alone, as we were able to observe by the difference between the raw numbers of peaks in the two treatments (Fig. 16). This effect could be a consequence of the activation of different cellular pathways induced by IFN\textsubscript{\gamma} that generate modifications in the chromatin structure needed for the activation of cytokine target genes. In such a case the reordering of the chromatin could open ERBS previously closed in the absence of IFN\textsubscript{\gamma}. It is also possible that activation of MAPK proteins by IFN\textsubscript{\gamma} could induce a simultaneous activation of ER\textsubscript{\alpha} in parallel to the E2-activated ER\textsubscript{\alpha}, such a scenario could account for the increased number of signals in the IFN\textsubscript{\gamma}+E2 treatment, but not for the modification of the binding preferences of ER\textsubscript{\alpha}. It is also possible that activation of TFs by the IFN\textsubscript{\gamma} pathways could induce association of ER\textsubscript{\alpha} with some of these factors. As described above, in some cases ER\textsubscript{\alpha} can bind to non-ERBS sequences that present certain similarity to classical ERBS. It is also possible for ER\textsubscript{\alpha} to tether to other TFs that have already bound their DNA sequences.
(Nilsson and Gustafsson, 2010). These mechanisms could help to explain the differences in the binding preferences and frequencies of ER$\alpha$ in the presence of IFN$\gamma$+E2 compared to E2 alone. However the precise explanation of how or if these mechanisms are in place in this precise case cannot be given with the results at hand.

The modification of the binding preferences of ER$\alpha$ were better observed by the differences between the gene ontology and disease ontology analysis between the two treatments, where inclusion of IFN$\gamma$ showed an increased activity of cancer related mechanisms (Fig. 20). These results come to coincide with previous publications that have found several correlations between the inflammatory pathways and cancer proliferation. As presented in the introduction, different inflammatory profiles affect cancer proliferation differently. Acute inflammation associated with a Th1 response induces a higher level of cytotoxic activity with anti-tumoral effects, while a chronic inflammation response induces tissue repair mechanisms with tumoral proliferating activities (Coussens et al., 2013). In this sense, it was unexpected to observe that IFN$\gamma$, as part of the acute inflammatory response, was instead presenting a stronger cancer related phenotype. RNAseq experiments could be performed to clarify if the genes associated by the gene-ontology analysis are being activated or supressed. It is important to note however, that the tumor suppressor activity of IFN$\gamma$ is most usually linked to its ability to trigger the cellular response of the immune system. Such a scenario would imply that even if IFN$\gamma$ has a global anti-tumoral effect, it could also have a tumoral proliferating effect at the intracellular level. Such a competitor effects in the cancer phenotype exerted by the same cytokine would add, without doubt, more complexity to the understanding and treatment of this condition. Thus understanding of the changes in the genetic expression exerted by the IFN$\gamma$ in combination with E2 is a fundamental topic to study.
Further analysis of the binding motifs obtained with the MEME suite (Fig. 18) also revealed some interesting relations. In the presence of IFNγ and E2, binding motifs for factors RunX1 and RunX3 appeared. The RunX protein family, composed of RunX1, 2 and 3, are transcription factors possessing a Runt DNA-binding domain. Every one of these factors is related to cellular differentiation and development of hematopoiesis, bone, and epithelial formation. Activation of these factors has been observed in several types of cancer, such as translocation and activation of RunX1 in human leukemia, however it is important to note that RunX3 is often considered a tumor suppressor gene and mutations in it are usually present in breast cancer cells (Chimge and Frenkel, 2013). Parallel to this, it is has been observed that IFNγ can induce activation of RunX2 in human mesenchymal stem cells (Duque et al., 2009). These observations combined with our results are intriguing, as they seem to indicate a double effect of IFNγ, even at the intra-cellular level. On one hand activating tumor proliferating pathways via RunX1, while suppressing them via activation of RunX3 on the other.

It is important to mention that the number of peaks observed genome-wide was relatively low compared to the number of peaks usually observed in similar experiments. A consequence of these particularities maybe a lower level of confidence about the observed binding motifs, as this analysis depends on the number of sequences (from the peaks) used to average the frequency with which a particular motif appears. It is possible that new motifs could have been observed, if more sequences had been available. However the reason why the number of peaks was low is not clear at the moment.

A recent work presented by Franco and colleagues (2015) revealed an interesting interaction between TNFα-activated NF-κB activity and ERα genetic control. They showed that the TNFα pathway alters E2-signaling to induce rearrangement of NF-κB and FoxA1 binding sites genome-wide, thus inducing the activation of latent ERBS that activate different patterns of genetic expression and influence cancer development.
(Franco et al., 2015). These altered patterns have been shown to increase the expression of a number of genes associated with proliferation, invasion and metastasis in BCC (Frasor et al., 2009; Ross-Innes et al., 2012). However, these results are more likely mediated by the physical interaction between ER\(\alpha\) and NF-\(\kappa\)B and combined binding at enhancers (Kalaitzidis and Gilmore, 2005; Pradhan et al., 2012; Franco et al., 2015). So far, observations like these have not been established between ER\(\alpha\) and TFs from the IFN\(\gamma\) pathway. Modification of ER\(\alpha\) binding pattern was shown to be dependent on NF-\(\kappa\)B and FoxA1 for the activation of the latent ERBS; for us it would be interesting to observe if the pathways for both TFs are active and functional in the MDA-MB-231 and MC2 lines. Information about the status of these mechanisms would be of great help for the understanding of E2 interference of IFN\(\gamma\) pathway as it is possible that these factors also collaborate for the activation of latent ER\(\alpha\) silencer sequences.

The results from Franco’s and colleagues (2015) are of particular interest to us, as TNF\(\alpha\) seems to alter the genetic expression profile in a similar manner to IFN\(\gamma\). Given that TNF\(\alpha\) and IFN\(\gamma\) are both cytokines characteristic of the acute inflammatory response, the idea of them having redundancy effects in the BCC is not unrealistic. Very interesting is also the dual effect presented by TNF\(\alpha\) in combination with E2 as both act as anti-proliferative and proliferative stimulant of tumor expansion depending on the specific cancer type (Franco et al., 2015). This behaviour also holds some similarity to the double effect of IFN\(\gamma\) mentioned before, as pro- and anti-tumorigenic by the recruitment of different RunX factors in the presence of IFN\(\gamma\) and E2.
CHAPTER V
PERSPECTIVES

The results obtained so far are interesting and seem to reinforce the growing evidence about the relationship between inflammation and the cancer pathology. This relationship, already witnessed in clinical observations, has been the object of several works over the past decade that have tried to decipher the molecular basis behind this relationship. To complete the observations already obtained, corroborate the results and give a more elaborate perspective of the possible mechanisms behind the crosstalk between the E2-activated ERα and the inhibition of the MHC-II expression, additional experiments could be performed.

Repetition of the ChIP-seq analysis in the MC2 cell line to confirm the observations from the first experiment would give us a higher level of confidence about the first results. At the same time, execution of the same ChIP-seq experiment in the MCF7 cell line would allow us to observe if the alterations in the ERα-binding affinity are kept or not between the cell lines, as these two were the only ones to show inhibition of the MCH-II expression in the presence of E2. Within this line of thought it would be interesting if the complementation of the MC2 line with ERβ would generate results similar to the ones observed for the MCF7 (ERα+/ERβ+).

In line with the results reported by Franco and colleagues (2015), it would be interesting to study the behaviour of ERα with TNFα in the MC2 cell line as absence of ERβ should alter the binding preference for certain genes. Given the fact that ERβ has been reported before as having certain anti-tumorigenic effects it would be interesting to see
the alterations in the cistromic profile and if the genome-wide binding would give a GO analysis presenting a less tumorigenic pattern. In a general manner, analysis of the behaviour of ERα in the presence of one or several cytokines of the acute inflammatory response such as IL-1, IL-6, IL-8 and IL-11 would help us to create a more complete and realistic picture of the cancer behaviour. It is evident that the same type of analysis should be performed with cytokines of the chronic inflammatory response, more so as this kind of response has been shown to be cancer proliferative. Among the inflammatory cytokines there are some that would present a particular challenge as they participate in both, the acute and inflammatory response such as the IL-1 and IL-6 (Feghali and Wright, 1997). The cistromic modifications induced by them would be without doubt intriguing. Analysis of RNA-seq and GRO-seq for this conditions would also present a much deeper picture of the cellular activity and would create a more robust profile of the cistromic behaviour.

Even if the results from the present work did not help to improve the understanding of the inhibition exerted by E2 on MHC-II expression, they remain interesting even if somehow partial, and need to be retested before any significant conclusion could be elaborated. Nevertheless they seem to greatly agree with previous works about the alteration of ERα behaviour in the inflammatory context. Continuation of this project in its present form, as a genome-wide analysis of the modifications induced by IFNγ and other cytokines on the behaviour of E2-activated ERα, is interesting as these experiments could not only help to construct a better picture of the cancer and inflammatory pathologies but potentially could participate to the creation of new treatment protocols that would take into account the specific inflammatory environment of not only each patient but also of different cell subpopulations of the same tumor.
# Annex I. Primer Sequences

ChIP-qPCR primer pairs sequences for the CIITA locus

<table>
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<th>Primer Name</th>
<th>Relative Position to CIITA-plIV (kbp)</th>
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**ChIP-qPCR primer pairs sequences for known ERBS**

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### ANNEX II. shRNA SEQUENCES

**Interference RNAs sequences for knock-down experiments**

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### ANNEX III. RT-qPCR PRIMER SEQUENCES

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<tr>
<td>Hs-HLA-DRA-ex4F</td>
<td>CCTGGGCCTGACTGTGG</td>
</tr>
<tr>
<td>Hs-HLA-DRA-ex5R</td>
<td>CTATAGGGCTGGAAAATGCTGAAG</td>
</tr>
<tr>
<td>VS40-Lc-HSHPRT-F2</td>
<td>GACTTTGCTTTCTTGGTCA</td>
</tr>
<tr>
<td>VS40-Lc-HSHPRT-R2</td>
<td>GGTGTATTTTGTCTTTCC</td>
</tr>
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