Universidade do Minho
Escola de Ciências da Saúde

André João Almeida Marques Soares de Albergaria

Transcription Factors and Epigenetics in Breast Cancer: New Findings in *CDH3*/P-Cadherin Gene Regulation

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Tese de Doutoramento
Ciências da Saúde – Ciências da Saúde

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

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(André João Almeida Marques Soares de Albergaria)

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Summary / Resumo

Summary

Breast cancer is the leading cause of cancer amongst women in the westernized world. It is a heterogeneous disease ranging from premalignant hyperproliferation to invasive and metastatic carcinomas. Disease progression is poorly understood but is likely due to the accumulation of genetic alterations leading to widespread changes in gene expression, ultimately affecting cell biology and often increasing growth capacity and survival advantage. Consistent with this, recent studies have shown that different breast tumour subclasses display distinct gene expression profiles. In addition to genetic alterations, there is increasing evidence for gross epigenetic alterations in tumour cells, both at the levels of DNA methylation and histone marks.

Epigenetic alterations target and modulate several important genes in breast cancer, contributing, not only for its initiation, but also for its development and response to therapies. One of the key genes in breast biology and cancer, which expression and molecular function is strongly regulated by epigenetic modifications, is the ligand-activated transcription factor oestrogen-receptor α (ERα), the primary mediator of the ovarian-produced steroid hormone oestrogen action in breast mammary cells. Breast cancer development and progression is, in fact, closely associated with the presence or absence of ERα, being an important prognostic and predictor indicator in this disease. Consequently, the leading drugs used for endocrine therapy of breast cancer, namely anti-oestrogens such as tamoxifen or fulvestrant, block ERα activity. Despite the efficacy of these target agents during short/medium time regimens, the use of endocrine therapy is limited by the onset of drug resistance, normally mediated by epigenetic alterations, in which most patients, who initially respond favourable to endocrine therapy, eventually recur.

In the present thesis we present an immunohistochemical approach involving two ERα-signalling pathways-related transcription factors, FOXA1 and GATA-3, in order to assess whether their expression could be useful as prognostic markers in breast cancer patients. We demonstrated that patients harbouring FOXA1-positive tumours show a better disease-free survival in a 5 years follow-up time and that FOXA1 expression associates with good prognosis clinicopathological features. More importantly, and for the first time, we established that this forkhead-box transcription factor has a power for recurrence risk

stratification among the poor prognostic ERα-negative breast cancer patients, demonstrating the clinical importance of this biomarker in breast cancer prognosis. We still demonstrated that GATA-3 does not constitute a strong predictor for breast cancer disease-free survival nor a good prognostic marker, but was shown to be a robust luminal differentiation marker. Taken together, the expression assessment of FOXA1 and GATA-3 can provide important clinical information, not only regarding the favourable prognostic outcome, but can also constitute an important tool to define and assess the luminal A subtype in breast cancer and, eventually, response to endocrine therapy.

ERα-negative breast cancers are resistant to endocrine therapies and have a worse prognosis than ERα-positive breast cancers. DNA methylation and chromatin remodelling are two epigenetic mechanisms that have been linked with a generation of an aggressive ERα-negative phenotype in breast cancer. Endocrine therapies have been reported to interfere with the expression of ERα-repressed genes, ultimately leading to the ERα-negative phenotype of breast cancer, but most importantly, with the induction of breast cancer cell aggressiveness and invasiveness in specific cell contexts. We have been focussed on studying *CDH3*/P-cadherin gene expression, as an ERα-repressed gene in breast cancer; enlightening a mechanism for its regulation in a breast cancer cell model. The mechanism by which ERα-signalling inhibition led to P-cadherin overexpression was completely unknown until now. Herein, we described a chromatin remodelling (H3K4me2) at *CDH3* gene regulatory region which, being induced by ICI 182,780 treatment, can modulate *CDH3* promoter activity and the expression of a pro-invasive protein in breast cancer, as P-cadherin. Moreover, this study allowed the identification of a new transcription factor, C/EBPβ, that is able to regulate *CDH3* activity in breast cancer cell lines and which expression associates with P-cadherin in breast cancer patient samples.

Overall, the work summarized in this thesis discusses molecular characteristics that may influence the risk for ERα-negative breast cancer patients to recur, also suggesting two new markers which, in addition to ERα assessment, might be useful for predict breast cancer outcome in patients non-responsive to endocrine therapies. Moreover, we demonstrated a chromatin remodelling event provoked by the pure anti-oestrogen ICI 182,780, which is able to increase *CDH3* promoter activity and therefore, the expression of the pro-invasive protein

P-cadherin in breast cancer cells, contributing to the elucidation of how breast cancer cells may acquire aggressive properties after loss of oestrogen signalling

Resumo

O cancro da mama constitui a neoplasia mais frequente entre as mulheres dos países ocidentalizados. A elevada heterogeneidade da doença permite variações fenotípicas que vão desde a hiper-proliferação pré-maligna ao carcinoma invasivo e metastático. Apesar das vias de progressão da doença ainda não se encontrarem totalmente caracterizadas, a causa mais comum e provável será a acumulação de alterações genéticas, as quais, produzem alterações na expressão de vários genes e afectam a biologia da célula, conferindo capacidades de crescimento e de sobrevivência. De facto, tem-se verificado, em estudos recentes, que diferentes sub-grupos de cancro da mama exibem perfis distintos de expressão genética. Associadas a estas alterações genéticas, as modificações epigenéticas ocorridas nas células tumorais, essencialmente ao nível do padrão de metilação do ADN e da acetilação/metilação da cromatina, têm sido alvo de crescente interesse na compreensão do processo da carcinogénese.

As alterações epigenéticas controlam uma série de genes importantes no cancro da mama, contribuindo não só para a sua iniciação, mas também para o seu desenvolvimento e resposta terapêutica. Um dos genes centrais na biologia da glândula mamária e na sua carcinogénese é o receptor de estrogénio (REα), um factor de transcrição que constitui a principal ferramenta que permite a acção da hormona esteróide – estrogénio, sobre as células mamárias. O desenvolvimento e progressão do cancro da mama estão por isso fortemente associados à presença ou ausência de expressão do REα, constituindo este, um importante factor de prognóstico e preditivo da resposta terapêutica endócrina nesta neoplasia. Consequentemente, as principais drogas usadas para terapia endócrina de cancro da mama, nomeadamente as anti-estrogénicas, como o Tamoxifeno e o Fulvestrant, visam bloquear a actividade do REα. Não obstante a eficácia da utilização destes fármacos, o uso da terapia endócrina tem vindo a demonstrar algumas limitações, uma vez que a maioria dos doentes que inicialmente respondem a esta terapia, vêem tardiamente a desenvolver resistência endócrina, eventualmente com recidiva, provocada normalmente por alterações epigenéticas não específicas.

Num contexto de genes associados à via de sinalização mediada pelo REα, estudou-se por imunohistoquímica a expressão de dois factores de transcrição, FOXA1 e GATA-3, no sentido

de avaliar a sua importância como factores de prognóstico em pacientes com cancro da mama. Demonstrou-se que pacientes com tumores positivos para FOXA1 apresentam uma melhor tempo-livre de doença num período de estudo de 5 anos e que a expressão de FOXA1 está associada a perfis clínico-patológicos de bom prognóstico. Demonstrou-se ainda que a expressão de FOXA1, num sub-grupo de tumores REα-negativos, permite a estratificação do risco de recorrência durante o mesmo período de estudo, realçando assim a importância clínica deste factor de transcrição no prognóstico do cancro da mama. Relativamente à expressão da proteína GATA-3, observou-se que este factor de transcrição não constitui nem um factor de prognóstico robusto, nem um indicador de melhor tempo-livre de doença, revelando, no entanto, ser um importante e consistente marcador de diferenciação luminal. Assim, a avaliação da expressão de FOXA1 e GATA-3 poderá ser útil, não só sob o ponto de vista da prática clínico-patológica, no sentido de fornecer uma importante informação relativamente ao prognóstico da doente, mas também como uma ferramenta essencial para definir o subtipo luminal A em cancro da mama.

Os tumores mamários REα-negativos são resistentes a terapias endócrinas e apresentam pior prognóstico relativamente a tumores REα-positivos. A metilação do ADN e a remodelação da cromatina são dois mecanismos epigenéticos que têm sido relacionados com a aquisição de um fenótipo mais agressivo de cancro da mama. Tem sido descrito que as terapias endócrinas podem modelar a expressão de genes reprimidos pelo REα, conduzindo o tumor à aquisição progressiva de um fenótipo REα-negativo, com consequente indução de invasão celular em ensaios *in vitro*. O gene *CDH3*/P-caderina é, no modelo de cancro da mama, um gene reprimido pelo REα, embora o mecanismo pelo qual a inibição da sinalização mediada pelo REα, e resultante sobre-expressão de P-caderina, fosse ainda desconhecido. Demonstrou-se assim que o tratamento de linhas celulares de cancro da mama REα-positivas com o antagonista de REα, ICI 182,780, leva a uma remodelação da cromatina ao nível do promotor do gene *CDH3*, através de uma proeminente marca activadora H3K4me2, a qual se sugere ser causal da indução da expressão da proteína pró-invasiva P-caderina. Este estudo permitiu ainda identificar um novo factor de transcrição com capacidade de regular a actividade do promotor *CDH3* em linhas celulares, e cuja expressão se associa com a P-caderina em tumores de pacientes com cancro da mama.

Em conclusão, o trabalho constante desta tese explora características moleculares que podem influenciar o risco de recidiva de doentes com cancro da mama hormono-negativos e onde a avaliação de dois novos marcadores, conjuntamente com a determinação da expressão de REα, pode ser importante na avaliação do prognóstico de doentes com neoplasia mamária não-responsiva às terapias endócrinas. Demonstrou-se ainda que determinadas alterações da cromatina, induzidas pelo ICI 182,780, são responsáveis pela activação do promotor *CDH3* e consequente expressão de P-caderina em células de cancro da mama. Este estudo permite assim elucidar um mecanismo pelo qual células de cancro da mama adquirem propriedades agressivas após perda de sinalização mediada pelo REα.

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PAPER IV – Goto T, Takano M, **Albergaria A**, Briese J, Pomeranz KM, Cloke B, Feroze-Zaidi F, Maywald N, Sajin M, Dina RE, Ishihara O, Takeda S, Lam EW-F, Bamberger AM, Ghaem-Maghami S, Brosens JJ. Mechanism and functional consequences of loss of FOXO1 expression in endometrioid endometrial cancer cells. ***Oncogene*** 27: 9-19, 2008.

PAPER V – Ferreira AM, Westers H, **Albergaria A**, Seruca R, Hofstra RMW. Estrogen, MSI and Lynch syndrome-associated tumors. ***BBA - Reviews on Cancer*** 1796: 194, 2009.

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Abbreviations List

5-Aza-dC – 5-aza-2'-deoxycytidine

AF – activating function

AIB1 – amplified in breast cancer-1

AKT – protein kinase B (PKB)

ALB – albumin gene

AML – acute myeloid leukemia

AP - activating protein

APC – adenomatous polyposis coli gene

AR – androgen receptor

BASE – breast cancer and salivary gland expression

BRCA – breast cancer associated gene

C/EBP – CCAAT/enhancer-binding protein

CDH – cadherin gene

CDK – cyclin-dependent kinase

cDNA – complementary deoxyribonucleic acid

CK – cytokeratin

CpG – adjacent cytosine and guanine dinucleotides

CRE – cyclic AMP response element

DBD – DNA binding domain

DCIS – ductal carcinoma in situ

DNA – deoxyribonucleic acid

DNMT –DNA methyltransferase

E2 – 17β-estradiol

E2F1 - E2F transcription factor 1

E-cadherin – epithelial cadherin

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMT – epithelial to mesenchymal transition

ER – oestrogen receptor

ERE – estrogen responsive element

ERK – extracellular signal-regulated kinase

FOX – forkhead-box protein

GRIP – glucocorticoid receptor interacting protein

GST – glutathione s-transferase

H – histone

HAT – histone acetyl-transferase

HDAC – histone deacetylase

HER – human epidermal receptor

HMT – histone methyltransferase

HNF – hepatic nuclear factor

HOX – homeobox gene

HRE – hormone responsive element

HRT – hormonal replacement therapy

HSP – heat shock protein

IDC-NOS – invasive ductal carcinoma – not otherwise specified

ICI – ICI 182,780 (Fulvestrant, Faslodex)

IGF – insulin-like growth factor

IGFR – insulin-like growth factor receptor

JMD – juxtamembrane domain

K - lysine

LAP – liver-enriched activating protein

LDB – ligand binding domain

LEF/TCF – lymphoid enhancer factor/T-cell factor

LIP – liver-enriched inhibitory protein

MAPK – mitogen-activated protein kinase

MGMT – methyl guanine methyltransferase

MISS – membrane-initiated steroid signaling

MMP – matrix metalloproteinase

mTOR – mammalian target of rapamycin

N-cadherin – neural cadherin

NCoA – nuclear receptor coactivator

NCoR – nuclear receptor corepressor

NFAT – nuclear factor of activator T cell

NFkB - nuclear factor kappa beta

NISS - nuclear-initiated steroid signaling

p120ctn – p120-catenin

P-cadherin – placental cadherin

PcG – polycomb group

PgR – progesterone receptor

PI3K – phosphatidylinositol-3-kinase

PLAC1 – trophosblast-specific gene (placenta-specific gene 1)

PTEN – protein tyrosine phosphatase and tensin homolog

RAR – retinoic acid receptor

RB – retinoblastoma

RNA – ribonucleic acid

RTKi – receptor tyrosine kinase inhibitor

SAHA – suberoylanilide hydroxamic acid

SERM – selective oestrogen receptor modulator

SHBG – sex hormone binding globulins

SHR – steroid hormone receptor

SMRT - silencing mediator for retinoid and thyroid hormone receptors

SOCS - suppressor of cytokine signaling

SRC - steroid receptor coactivator

STAT – signal transducers and activator of transcription

TDLU – terminal ductal-lobular unit

TFF – trefoil factor

TGF – transforming growth factor

TIF – transcriptional mediator/intermediary factor

TIMP - tissue inhibitor of metalloproteinase

TNF – tumor necrosis factor

TSA – trichostatin A

TSS – transcription start site

uPA – urokinase-type plasminogen activator

VEGF – vascular endothelial growth factor

WHO – world health organization

WIF - wnt inhibitory factor

XBP1 – X-box binding protein

ZO – zonula occludens

α-SMA – alpha smooth muscle actin

Objectives and Thesis Layout

Objectives and Thesis Layout

The role of transcriptions factors, as key entities for the most fundamental cell functions, has becoming studied as important tools to be used in clinical pathology. Modulation of transcription factor activity, through genetic or epigenetic processes, constitutes the tip of the spear of the cellular gene expression and cellular biology. In breast cancer, where those genetic and epigenetic mechanisms are someway disrupted, transcription factor expression naturally represents important markers of tumour behaviour with pathological value. Moreover, they drive crucial human oncogenic pathways, being used as targets for therapeutic interference in cancer development, progression and in treatment-response prediction.

The general aim of the work reported in this thesis is to investigate and discuss how molecular (genetic or epigenetic) determinants may affect breast cancer risk and patient outcome, with a very special emphasis in poor prognosis ERα-negative tumours, as well as, in ERα-positive cell lines where ERα-signalling was abrogated by anti-oestrogens treatment. In doing so, this thesis is organized in individual chapters, each aiming to fulfil a specific goal in the quest to accomplish the general objective of this dissertation, as presented below.

Chapter 1 presents a general introduction that reviews the most current knowledge on breast cancer, aiming to introduce the reader for the research topics presented throughout the following chapters. It briefly summarizes the classification, epidemiology, and treatment of breast carcinomas, while devoting particular attention to the main genetic and epigenetic mechanisms which regulate gene expression in breast cancer. Moreover, a robust explanatory section concerning the role of P-cadherin in mammary tumours is stressed, since the regulation of this gene constitutes a major object of our research.

Chapter 2 focus on the relevance of the expression of FOXA1 and GATA-3 in breast cancer and their special prognostic significance in hormone receptor-negative tumours. Herein, we explored the usefulness of assessing the expression of these two transcription factors to stratify subsets of patients that can have better outcome, among the ERα-negative/poor prognosis breast cancer group.

Chapter 3 encloses a study which major aim was to explore the molecular mechanism linking the ERα-signalling pathway and P-cadherin-regulated expression in breast cancer cells, as previously shown in the past by our group. The mechanism by which ERα-signalling inhibition led to P-cadherin overexpression was completely unknown until now. This chapter shows how chromatin remodelling of *CDH3* gene promoter by anti-oestrogens can modulate the expression of a pro-invasive protein in breast cancer, as P-cadherin. This study still permitted to identify, for the first time, a new transcription factor – C/EBPβ that is able to regulate P-cadherin expression.

Chapter 4 attempts to bring together the findings from Chapters 2 and 3, and thoroughly discuss them in the milieu of other relevant published data. Brief suggestions for future directions to complement our research are presented, together with a few general concluding remarks.

Finally, an appendix section was included, enclosing additional studies performed during the PhD time course.

1. General Introduction

1. GENERAL INTRODUCTION

1.1 – BREAST CANCER EPIDEMIOLOGY AND RISK FACTORS

At the newborn twenty-first century, cancer remains the major public health problem worldwide, accounting approximately for one in four deaths in developed countries (1-2). In 2008, in Europe, excluding non-melanoma skin cancers, there were an estimated 3.2 million incident cases of cancer diagnosed, with approximately 1.7 million cancer deaths (3-4). Breast cancer is by far the most common form of cancer diagnosed in women in both developing and developed countries of the world and it is the cause of death in approximately 20-30% of all females who die from cancer in these countries. In fact, one in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast (5-6). Today, breast cancer affects nearly 430.000 female, being ranked the leading cause of cancer deaths (129.000 in 2008) in European women (3-4) (Figure 1). According with the data from the Portuguese League Against Cancer (www.ligacontracancro.pt), breast cancer is also the first cause of death by cancer in Portuguese women. Although the estimates age-standardised incident rates (103.5/100.000) are above the European standard (94.3/100.000), the Portuguese breast cancer mortality rate (21/100.000) is slightly lower than the European ratio (26/100.000) (3).

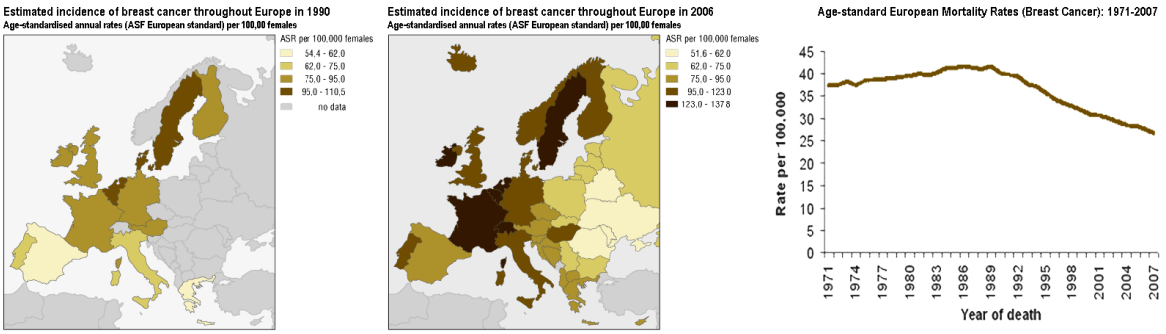

Figure 1. Estimated incidence of breast cancer throughout Europe in 1990 and in 2006. Without exceptions, breast cancer incidence has increased in all European countries in the time interval studied (16 years). However, breast cancer mortality rates in Europe have fallen dramatically since 1989. [Adapted from Cancer Research UK; Globocan 2002; Black *et al.*, 1997 (7); Ferlay J *et al.*, 2007 (3)].

In Portugal, one in each ten Portuguese women develops this disease during its life, with about 4.500 new cases/year and with 1.500 deaths/year, making breast cancer an important health problem for the Portuguese population. Unfortunately, while favourable trends in the reduction of breast cancer mortality has been established for most of the countries in European Union, Portugal, Spain and Greece represent a notable exception, with an increased risk of dying from breast cancer of 11%, 15% and 7%, respectively (8).

Breast cancer presents a multifactor etiology, leading to a variety of genetic changes that ultimately result in variable biological behaviours among different patients (9). As it happens for most of the human malignancies, where the interaction between environmental factors and genetic profiles dictates the susceptibility for the initiation of the disease, remarkable epidemiologic, clinical and genetic studies have been made in order to define social and biological traits, which can improve the identification of breast cancer risk groups among women (10-11) . There are marked differences in the incidence of breast cancer in different places, the predominant impression being that the disease is more common among Caucasians living in the colder climates and more highly industrialized countries of the Western hemisphere (6). The observed differences in breast cancer incidence rates among countries may reflect demographic variations in modifiable risk factors (9, 12). In fact, the Western diet is associated with both earlier age at menarche and post-menopausal obesity, but other factors such as the sedentary lifestyle and alcohol consumption, also contributes to increased breast cancer risk (11-13). In other hand, childbearing and breastfeeding reduces risk, with greater protection for early first birth and a larger number of births (13). The post-menopausal obesity, the late menopause (after the age of 55 years) and the early menarche (before than 12 of age) are important risk factors, since they increase the breast exposure to elevated levels of oestrogen. Indeed, hormonal influence due to the lifetime exposure of the mammary gland epithelium to endogenous sex hormones has been described as the most well-established risk factor in breast cancer, having a major role in a variety of other female cancers, namely vaginal and endometrial carcinomas, since oestrogens have effects on cell proliferation and DNA damage, as well as in the promotion of cancer growth (6, 9, 14-15). Clinical and experimental data have indicated that exposure to oestrogens is one of the leading causes of sporadic female breast cancer and, in 2002, oestrogen was declared to be a known human carcinogen by the

National Toxicology Program of the National Cancer Institute in USA (6). Several studies have explored the effect of endogenous serum concentrations of hormones and breast cancer risk. Post-menopausal women, who present high serum levels of sex hormone binding globulins (SHBGs) and elevated levels of serum estradiol, show an increased risk for breast cancer development. Similarly, it has been shown that this risk is also increased in women that present high blood and tissue levels of oestrogen and progesterone (12). Moreover, other hormones, such as insulin-like growth factor-1 (IGF-1), have been suggested to potentiate the risk of breast cancer (16). However, nowadays, women face an exogenous source of oestrogens, which are provided by the widespread usage of oral contraceptives and hormonal replacement therapy (HRT), this last being associated with increased risk of breast cancer, especially when comparing its use during short and long periods of time (13, 17).

Other well-established risk factors for breast cancer is the existence of family inherited germline mutations in breast cancer susceptibility genes, like *BRCA1* and *BRCA2*, the most common implicated high-penetrance genes in hereditary breast cancer (6, 18). Approximately 5%-10% of all breast cancer cases result from the presence of mutations on these inherited susceptibility genes within the family history, being the existence of one or more first-degree relatives an established criterion for increased risk of developing the disease (19). Male breast cancer is rare and represents approximately 1% of all cancers in men, causing around 0,1% of male deaths per year. The risk of breast cancer in males carrying *BRCA2* mutations, though small, is probably greater than in men carrying *BRCA1* mutations and it may account for 4-14% of all cases (6, 20). A history of prior breast biopsies, especially if revealing benign proliferative disease, also increases the risk of invasive carcinoma (21).

1.2 – Pathology and Histological Classification in Breast Cancer

The adult female mammary gland consists of a branching tree-like network of ducts, lined by a double layer of epithelial and myoepithelial cells, surrounded by fibroblasts embedded in an extracellular matrix or stroma, mainly composed by a dense fibrous connective tissue, admixed with adipose tissue, and harbouring vascularity. Besides the

structural support that stroma provides to the mammary gland, it is thought that it can play an important role in the dynamic induction of the breast gland structure morphogenesis and differentiation (22-23) (Figure 2).

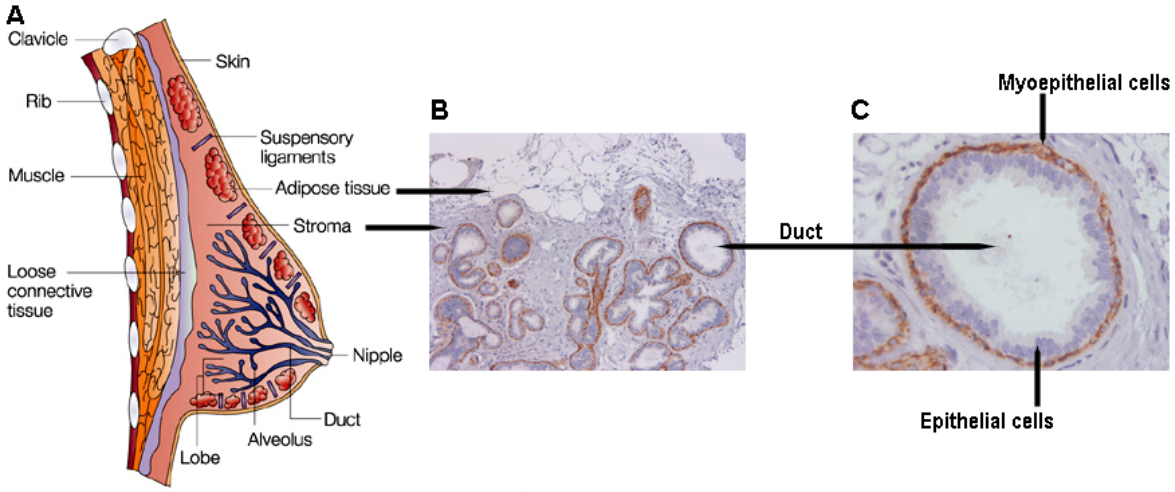

Figure 2. (A) Schematic cross-section of a mature female breast showing the main anatomical structures. Macroscopic diagram of breast structures. [Adapted from Ali S *et al.*, 2002 (24)]; **(B)** and **(C)** Low magnification (100x) of normal breast gland tissue, where a complex net of mammary ducts are surrounded by connective tissue and a microscopic high magnification (400x) of segmental breast duct, showing a clear separation of epithelial and myoepithelial cells, which are immunostained for a myoepithelial marker (P-cadherin).

In a premature development state, lobules exist as alveolar buds, turning into secretory mature structures (acini or alveoli), after menarche, as a response to the ovary release of female hormones, oestrogen and progesterone. These secretory units open into the intralobular terminal duct, which, histologically, presents essentially two layers: an inner layer constituted by a continuous surface of a luminal secretory epithelial cells, and an outer discontinuous layer of prominent basal or myoepithelial cells with large and clear cytoplasm and with contractile properties (25). Due to the contained myofilaments, these cells assist in milk ejection during lactation and provide structural support to the lobules (21). A committed stem cell in the terminal duct is postulated to give rise to both luminal and myoepithelial cells (26). While myoepithelial cells are characterized by expressing P-cadherin, α-smooth muscle actin (α-SMA) and a distinct subset of basal epithelial cytokeratins, luminal epithelial cells can be distinguished by the expression of nuclear receptors for steroid hormones oestrogen and progesterone (ER and PgR), as well as, a

subset of epithelial cytokeratins, such as CK8, CK18, and CK19 (27). More than 90% of the steroid-induced epithelial cell proliferation observed in the non-pregnant gland occurs in the luminal cell type (27).

Frequently, it is at the level of the terminal ductal-lobular unit (TDLU), which, not only constitutes the functional structure of the breast for milk production, but also is highly responsive to hormonal stimulus occurred during development and maturation processes occurring during pregnancy and lactation (22), that many of the known epithelial benign and malignant lesions are observed (28).

The current histological classification of the World Health Organization (WHO) of breast tumours is highly extensive and includes malignant and benign neoplasias of epithelial origin, besides the neoplasias with myoepithelial, mesenchymal and fibroepithelial origin (12). Nearly 95% of breast diseases seem to have a common origin in luminal epithelial cells from the TDLU (12, 29), and can be divided in three major lesions: benign lesions, *in situ* and invasive carcinomas. Although the biologic progression of breast cancer is not yet completely established, essentially in respect to ductal carcinomas, a high number of clinico-pathological and molecular studies seem to point towards a progression from intraductal lesions with atypia or *in situ* carcinomas (12).

A wide variety of benign alterations in ducts and lobules are observed in the breast (26). Benign lesions are usually defined as well-differentiated, with similar morphology of that of its origin and with well demarcated areas of expansion slow growth. According to the subsequent risk of developing breast cancer, these lesions have been divided into non-proliferative breast changes, proliferative breast diseases and atypical hyperplasia (21, 26, 29).

In situ carcinomas represent malignant lesions that were originally classified as ductal or lobular, based on the resemblance of the involved spaces to normal ducts or lobules. However, it is now recognized that varied patterns of growths *in situ* are not related to the site or cell of origin, but rather reflect differences in tumour cell biology, such as whether the tumour cells express the cell adhesion protein E-cadherin or not. By current convention, “lobular” refers to carcinomas of a specific type, and “ductal” is used more generally for adenocarcinomas that have no other designation (21). Independently of the

special designation, both lesions share considerable epithelia proliferation rates associated with malignant cellular features, such as neoplastic proliferation, but without invasion of the natural physical barrier formed by the basement membrane of the duct or lobule. In ductal carcinomas *in situ* (DCIS), myoepithelial cells are preserved, although they may be diminished in number (21). With the advent of mammographic screening, the diagnosis of DCIS rapidly increased from fewer than 5% of all carcinomas to 30% of carcinomas in well-screened populations. In fact, among cancers detected mammographically, almost half are DCIS (30) (Figure 3).

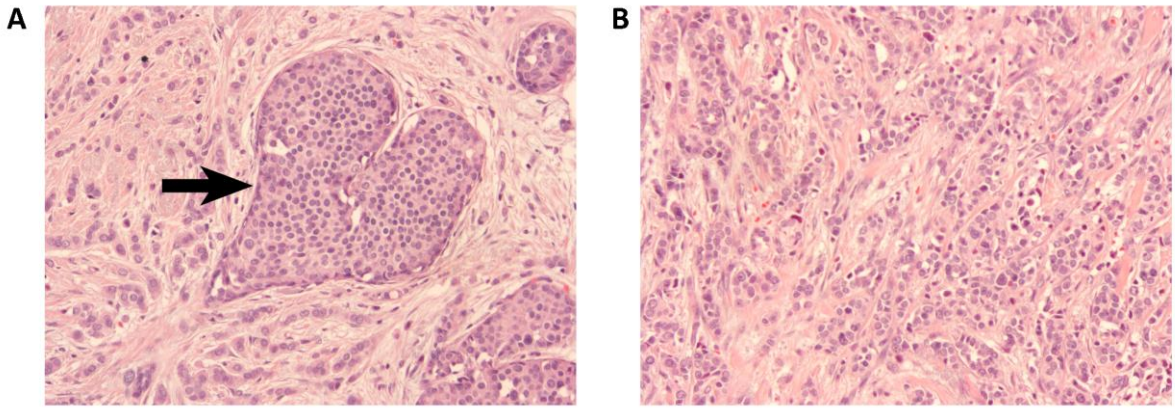

Figure 3. Haematoxylin-eosin staining of breast carcinomas cross-sections. **(A)** Ductal carcinoma *in situ* (DCIS), arrow (200x); **(B)** Invasive ductal carcinoma (IDC-NOS) (200x).

In contrast with DCIS, invasive breast carcinomas constitute a group of malignant epithelial tumours characterized by invasion of adjacent tissues and a marked tendency to metastasize to distant sites. The most frequent histological type of the invasive ductal carcinoma (ductal carcinoma NOS – Not Otherwise Specified) represents around 70 to 80% of all invasive breast cancers. It is a heterogeneous group of tumours that do not exhibit distinct morphological characteristics to be classified in a more specific way, as invasive lobular carcinomas, tubular carcinomas and mucinous carcinomas. The invasive lobular carcinoma, which constitutes the second more frequent histological type, represents 5 to 15% of breast invasive carcinomas and is associated with lobular carcinoma *in situ* in 90% of the cases. The other types of breast carcinomas, presenting specific morphological characteristics in at least 90% of the tumour mass, are considered special histological types. Breast carcinomas of special type include invasive tubular, mucinous, cribriform and

micropapilar carcinomas, metaplastic carcinoma and medullary carcinoma, among others (12, 21).

1.3 - Prognostic Factors and Therapeutic Strategies in Breast Cancer

The outcome for women with breast cancer varies widely. Many women have a normal life expectancy, whereas others have only a 10% chance of being alive in 5 years after diagnosis. With the exception of women presenting distant metastasis or with inflammatory carcinoma, which are both poor prognosis features, patient prognosis is determined by the pathologic examination of the primary carcinoma and of the axillary lymph nodes. Prognostic information is, therefore, important in counselling patients about the likely outcome of their disease and choosing appropriate treatment (21). The pathological examination includes cytological and histological assessment of some classical anatomo-pathologic parameters, which give valuable prognostic and predictive information. The major prognostic factors are the histological type (invasive *versus in situ* disease), axillary lymph nodes *status* and tumour size (10). In the absence of distant metastasis, which represents the major threat to breast cancer patient outcome, the axillary lymph node status is the most important prognostic factor for invasive carcinoma. With no nodal involvement, the 10-year disease-free survival rate is close to 70-80%, falling to 35 to 40% with one to three positive nodes, and 10-15% when more than 10 nodes are positive. Also, the size of the metastasis within the nodes are of proven prognostic importance (21). Tumour size is another powerful independent prognostic marker, with larger size associated with a worse outcome. The prognostic and predictive importance of tumour size is of greatest relevance in node-negative breast cancer patients, since this is the subgroup that should be treated if other prognostic markers suggest clinical aggressiveness (12). Women with node-negative carcinomas, with <1 cm in size, have a 10-year survival rate of 90%, whereas survival drops to 77% for cancers >2 cm (21). The histological grade, histological subtype, lymph vascular invasion, proliferative rate, DNA content and the response to neoadjuvant therapy (an alternative approach in which the patient is systemically treated before surgery), are also considered as minor prognostic and predictive factors of breast cancer (21).

Traditional therapeutic strategies for breast cancer include surgery, chemotherapy, and radiotherapy. The primary goal of breast cancer surgery is to remove the cancer lesion itself, as well as the regional axillary lymph nodes, in order to assess the extent of disease spreading and, therefore, helping in the further decision of therapy regimens. Actually, new options such as sentinel lymph node biopsy, where one to three key lymph nodes are removed and tested before any others are excised, are reducing the need for full axillary lymph node resection, particularly in women with early-stage disease (9). Systemic adjuvant therapies, like chemotherapy and radiotherapy, given to patients that complete their surgery, is designed to eradicate clinically undetectable microscopic deposits of cancer cells that may have spread from the primary tumour, usually result in decreased recurrences and improved patient survival (9-10).

Besides these therapeutic strategies, isolated or in association with the referred prognostic factors, there is a number of other factors that, being also predictive of outcome, potentially direct therapies against particular molecular targets, like nuclear hormone receptors, growth factors and their tyrosine-kinase receptors (31).

1.3.1 – Hormonal Receptors: ER and PgR

Breast cancer is usually a hormone-dependent tumour. More than 100 years ago, George Beatson showed that ovariectomy in pre-menopausal women resulted in metastatic breast cancer remission and improved prognosis (32). Discovery of the involvement of the ovarian hormone oestrogen in stimulating breast cancer growth paved the way for the development of therapies that inhibit oestrogen synthesis or block its receptor (33). Oestrogens may affect carcinogenesis by acting either as initiators, causing direct DNA damage by hydroxylated oestrogen metabolites, or as promoters, inducing growth and survival of initially transformed cells (34-35). Through the binding with high affinity to oestrogen receptor (ER), oestrogens can regulate the growth of breast cancer, influencing gene expression (Figure 4A) and cellular phenotypic changes, defining ER expression in breast cancer as critical for tumour progression (36). In other hand, the absence of ER expression within a mammary tumour impairs the usage of an ER-target therapy to treat breast cancer. While *in vivo* studies, using human breast epithelium implanted into athymic

nude mice, have shown that epithelial proliferation is induced by oestrogens in a dose-dependent manner, progesterone, either alone or in combination with oestrogen, had no effect on epithelial proliferation (37). However, in contrast with these findings, it has been demonstrated that patients following an oestrogen-HRT regimen show a lower risk of developing breast cancer when compared with the ones that follow a combined administration of oestrogen plus progestins (38). Current assays use immunohistochemistry to detect nuclear steroid hormone receptors, a finding that is commonly correlated with a low breast cancer histological grade, better patient outcome and is an important predictor of response to hormonal therapy (21, 39). Eighty percent of carcinomas that are ER and progesterone receptor (PgR) positive respond to hormone manipulation, whereas only about 40% of those with either ER or PgR alone are able to respond. ER-positive tumours are also less like to respond to chemotherapy. Conversely, cancers that fail to express one of these markers have less than 10% likelihood to respond to hormonal therapy but are more likely to respond to chemotherapy agents (21).

There are two subtypes of ER: ERα and ERβ. These two receptor subtypes vary in structure, and their encoding genes are on different chromosomes (40). Though, ERβ is highly homologous to ERα in its DNA- and ligand binding domains, being equally activated by 17β-estradiol (E2), and inhibited by anti-oestrogens (24, 40). However, although all studies agree that ERβ is expressed in breast cancer cells, its involvement in breast carcinogenesis is highly controversial. It has been suggested that a change of ERα/ERβ ratio during tumour development is more relevant than the absolute levels of ERα or ERβ, a hypothesis supported by the finding that, in ER-positive breast cancers, the mean ratio ERα/ERβ is higher than in normal tissue (41-42).

Since most breast carcinomas are, at least initially, hormone responsive, systemic endocrine therapy is an established strategy for adjuvant breast cancer treatment (43). Current endocrine therapies of breast cancer are based on three main known mechanisms of action, all of them targeting the ER signaling pathway (39, 44): 1) antagonizing ER function by competitive binding (selective oestrogen receptor modulators – SERMs and pure antiestrogens); 2) downregulating ER (pure antiestrogens); and 3) reducing levels of synthesized estrogen (aromatase inhibitors).

Extensive research on the function and structural conformation of ER, together with the known protective effects of oestrogens in various tissues, have allowed the elucidation of the agonist/antagonist activity of different ligands, ultimately leading with the development of new classes of SERMs, drugs that act like E2 agonists in certain tissues, such as bone, cardiovascular system, brain and lipid metabolism, but antagonizing oestrogen action in others, like breast and ovaries (24, 39, 44-46). Tamoxifen, the first prototypic SERM (9), was originally developed as an oral contraceptive (47), but the potential of its anti-oestrogenic action was recognized and it has now become the first-line endocrine agent for breast cancer treatment. Tamoxifen can reduce the risk of breast cancer in women at high risk for developing the disease and is beneficial in pre- and post-menopausal women whose tumours are ER-positive (24, 44, 48), being responsible by 26% of reduction in the annual recurrence rate and by 14% reduction in the death rate by breast cancer (9). Unfortunately, the treatment with tamoxifen is not effective for more than 5 years, since development of resistance is a very common event (49). The biologic activity of tamoxifen ranges from full oestrogen agonist to partial and full antagonist, depending on the species studied and the target tissue assessed. Therefore, although tamoxifen causes tumour regression in some women with metastatic disease (48), this range of activity may account for some of the undesirable effects of tamoxifen, such as increased endometrial proliferation and a slightly increased risk of endometrial carcinoma (50). A SERM, like Tamoxifen, has the possibility to bind to either ERα or ERβ, but due to differences in the transactivating functions (AF), the SERM-ER complexes can be altered, resulting in increased or decreased oestrogenicity (Figure 4B). It is known that the ligand programs the ER conformation, so that coactivators or corepressors can bind to the external surface of a SERM-ER complex and, therefore, activating or repressing transcriptional activation of oestrogen target genes (51-53). Advances have been made during the past decade, providing the development of new SERMs, such Raloxifene, which has lower toxicity, decreases breast cancer incidence and has no oestrogen-like action on the uterus (54).

Whether a synthetic drug acts as an oestrogen or anti-oestrogen on a specific gene may be dictated by the particular ensemble of the ER subtype, by the receptor interacting proteins or other transcription factors, or by specific elements within the promoter of oestrogen-regulated genes. Alterations in these other factors may also play a role in the

resistance to hormonal therapies. Ablative endocrine therapies, such as aromatase inhibitors, have recently been shown to be superior to tamoxifen and are being incorporated into first line therapy of advanced disease (10, 55). Aromatase inhibitors inhibit the tumour growth by lowering the systemic oestrogen concentration, due to the blockage of peripheral production of oestrogen in adipose tissue and in the tumour itself (Figure 4C). Most importantly, aromatase inhibitors are effective even in postmenopausal women with low oestrogen concentrations – probably due to the tumour ability of the tumour to become hypersensitive to oestrogen, after prolonged oestrogen deprivation (55). Clinically interesting, a report showed that aromatase inhibitors achieved a higher response rate than tamoxifen in ER-positive breast cancer patients (56), especially when these tumours also express high levels of the oncogene HER2, which is known to increase tamoxifen resistance (57). A recent study also demonstrated that overall survival was prolonged for patients who switched from tamoxifen to aromatase inhibitors therapy (49), and that these compounds showed superiority over tamoxifen in the neoadjuvant setting, even challenging chemotherapy with regard to response in selected group of patients (58-59).

Despite the potential to exploit the selective agonist pharmacology of SERMs, the elimination of partial agonist also has been regarded as a highly desirable goal, in order to improve clinical efficacy. In the last decade, the development of a specific or “pure” antioestrogen with high affinity for ER and without any agonist effects was initiated with the purpose to provide increased benefits over tamoxifen in the treatment of patients with oestrogen dependent disease. The search for such drug revealed a compound with the appropriate effect, known as ICI 182,780 (ICI) (50, 60-61). This compound, commercially known as Faslodex® or Fulvestrant, is a steroidal pure antioestrogen with a similar binding affinity for ER, when compared to that of estradiol, and much greater than that of tamoxifen (60-61). Partial tamoxifen agonistic effects have been attributed to the fact that one of the activation domains (N-terminal AF-1) of ER remains active in the tamoxifen-ER complex (62) (Figure 4B). In contrast, ICI, due to multiple changes in the relative orientation of the major ligand-dependent ER transcriptional activation domain AF-2, attenuates the ability of ER to activate or inhibit transcription in a ligand-dependent or independent manner *in vivo*, contributing to the oestrogen action blockade (63) (Figure 4D). These changes include

impaired dimerization, increased turnover, and disrupted nuclear localization (50, 53, 62, 64-65), leading not only to ER malfunctioning, but also to its reduced cellular levels and half-life, impairing the transcription of ER-regulated genes (60). The molecular basis for this increased turnover is not completely clear, but is accompanied by a block in nucleo-cytoplasmic shuttling. In the presence of ICI, nuclear ER re-shuttling does not occur and degradation takes place (66).

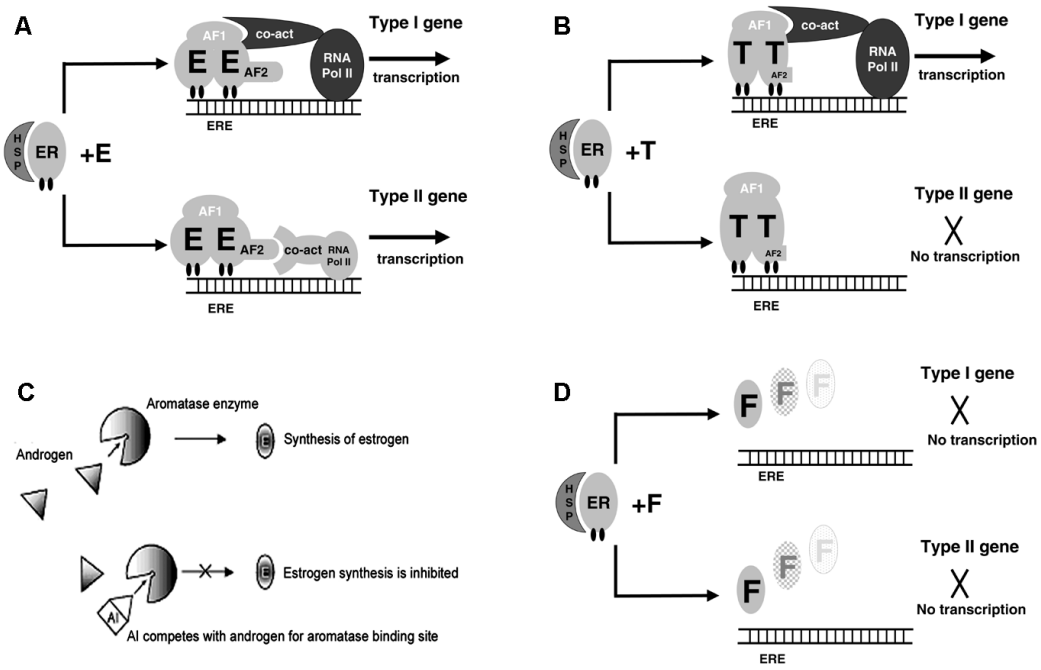

Figure 4. Molecular effects of oestradiol and anti-oestrogen compounds. Shown are the molecular effects of **(A)** oestradiol, **(B)** the selective oestrogen receptor modulator (SERM) tamoxifen, **(C)** the aromatase inhibitors and **(D)** the steroidal pure antioestrogen fulvestrant on ER signalling. As shown in panel A, oestradiol binding to ER leads to loss of heat shock proteins (HSPs), dimerization and phosphorylation of receptors, with conformational change leading to coactivator activation at both AF1 and AF2 sites; a full agonist effect is seen. In panel B, SERM (tamoxifen) binding to ER leads to loss of HSPs, dimerization and phosphorylation of receptors, but with different specific conformational changes, leading to coactivator activation at AF1 only, and not at AF2 sites; therefore, a partial agonist effect is seen. In panel C, aromatase inhibitors compete with androgen for the aromatase enzyme binding site, preventing the conversion of androgen to oestrogen in post-menopausal woman. In panel D, fulvestrant binding to ER leads to loss of HSPs, but impair receptor dimerization due to altered conformational changes. Thus, receptor degradation is enhanced with no activation at AF1 or AF2 sites; no agonist effect is seen. [Adapted from Jonhston SR, 2005 (69) and Morris & Wakeling, 2002 (61)].

Clinically, fulvestrant is the subject of much ongoing research, which utilises knowledge of its novel mechanism and pharmacokinetic profile, in order to optimise clinical efficacy and explore new roles, including first line use in advanced breast cancer or in combination with existing agents (63, 67). It has been reported that fulvestrant is an effective and well-tolerated drug for treatment of metastatic oestrogen-sensitive breast

cancer (67), as it has been suggested as a suitable therapeutic option in extensively pre-treated patients with HER2 and hormone receptor-positive advanced breast cancer (65). Most importantly, it has been reported that it offers potential advantages over other oestrogen target therapies, since no uterine pathologies, such as increase of uterine volume or endometrial growth, have been observed after 3 months of fulvestrant treatment in postmenopausal women with metastatic breast cancer, previously exposed to tamoxifen and aromatase inhibitors (68).

Endocrine therapy resistance is one of the main challenges in the treatment of ER-positive breast cancer patients (69). Resistance mechanisms to these therapies appear to be related to a cross-talk between ER and growth factor-signalling cascades that regulate cell survival, cell death and differentiation (70-71). Some studies have demonstrated that the PgR-negative status of ER-positive tumours may reflect altered growth factor receptor signalling, giving some explanation to the lower response rates to tamoxifen of these carcinomas when compared to cancers typed PgR-positive (72). It has been hypothesized that endocrine therapy response depends on HER2 expression. Despite the still conflicting conclusion about this, such studies are bringing forward a new window of opportunity that relays on the association of endocrine therapies with specific inhibitors of important signalling pathways, like PI3K or MAPK, as a strategy to overcome endocrine resistance in ER-positive breast cancer (69). A recent study in endocrine resistant breast cancer cell lines showed that PI3K/Akt or MAPK pathways inhibition was sufficient to reverse both tamoxifen or fulvestrant resistance (69). Furthermore, regardless the ERα status, a decreased activation of PI3K/Akt, increased ERK, and IGF-1R pathways inactivation have been reported as cellular responses to antioestrogen treatment (71), highlighting a new and promising prospect to delay the onset of anti-hormonal resistance, thereby significantly improving patient's survival.

1.3.2 – ErbB2/HER2

The protein encoded by ERBB2 gene (or HER2/neu) is a transmembrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor (EGFR), although without ligand-binding domain. At the cellular level, HER2 is transactivated by

EGFR upon binding of its ligand EGF, resulting in the formation of EGFR/HER2 heterodimers. However, formation of other heterodimers, such as HER2/HER3 and HER2/HER4, are also likely to occur. This heterodimerization between HER2 and the other receptors of the family allows the participation of HER2 in signal transduction, frequently leading not only to a loss of G1-S checkpoint, but also to the disruption of the delicate balance between cell survival and death signals (6, 73). It has been estimated that 15-30% of breast cancers overexpresses HER2 gene (6). Patients with primary HER2-positive disease have a higher risk of recurrence and death; however, the main importance of HER2 is as a predictor of response to agents that target this transmembrane protein (21, 74). The approval, in 1998, of monoclonal antibodies targeting the extracellular domain of the HER2 protein, such as trastuzumab, broadened the scope of targeted therapy and marked the first of many steps towards improved understanding of breast cancer biology (6). One of trastuzumab's mechanisms of action is via antibody-dependent cellular toxicity; the activation of natural killer cells initiates lysis of cancer cells that are bound to the therapeutic antibody. Most importantly, trastuzumab inhibits the PI3K pathway, which is activated by overexpression of HER2, reducing PTEN phosphorylation and increasing AKT dephosphorylation and, therefore, increasing cell death (74-76).

The wide range of function mechanisms of trastuzumab give rise to various mechanisms of resistance due to the cross-talk of HER2 with other extracellular domains of HER proteins, resulting in incomplete inhibition and lateral activation of proliferative pathways (77-78). This motivated the development of receptor tyrosine-kinase inhibitors (RTKi); small molecules, such as lapatinib, that compete for binding sites on intracellular portions of HER1 (EGFR) and HER2, targeting the downstream ERK1-2 pathways, which regulate cell proliferation, and the PI3K/AKT pathway, that regulates cell survival (79).

1.4 - Gene Expression Profiles of Breast Cancer

Human breast carcinomas represent a heterogeneous group of tumours, which are diverse in their natural history, their outcome, and their responsiveness to treatment. In fact, patients with identical tumour types and stage of disease can present different responses to therapy and different overall outcomes. This problem challenges the current

classification system and stem from the inability to take into account biological prognostic determinants (80-82). More recent developed techniques, that examine the DNA, RNA, and proteins of carcinomas in a global way, have provided a framework for new molecular classifications of invasive carcinomas (NOS) (21). With a high throughput and parallel analysis of thousands of genes, the advent of microarray technology and gene expression profiling has allowed the linkage of molecular expression profiles to clinical patient's outcomes and responses to therapy, generating a tool to better tailor treatment strategies to specific subgroups of patients, whose tumours have particular molecular aberrations (83). Microarray-based gene expression profiling led to a working model for a breast cancer molecular taxonomy, where clusters of genes, with coherent expression patterns, could be related to specific features of biological variation among tumour samples; for example, variations in proliferation rates and activation of specific signal transduction pathways (84). Another important implication is that genetic profiling may lead to the identification of new therapeutic targets (10, 27). Recent cDNA and tissue microarrays studies have showed that breast tumours can be classified into specific molecular subtypes, distinguished by differences in their gene expression patterns, providing a distinctive portrait for each tumour and the basis for an improved breast cancer molecular taxonomy (27, 82, 85-86). As expected, the majority of the studies generally separate the tumours into those that are clinically described as ER-positive and those that are ER-negative. Thus, using unsupervised clustering, they could already distinguish to some extent between "good prognosis" and "poor prognosis" tumours. The breast cancer molecular classification distinguishes three major subtypes: the ER-positive/luminal-like subtype, a gene expression cluster characteristic of the luminal cells and anchored by a cluster of transcription factors that include ER; the ER-negative/basal-like subtype, comprising tumours that express basal cell markers; and the HER2-overexpressing subtype, usually associated with gene amplification of the HER2 proto-oncogene (Figure 5). These studies have largely contributed to understanding the complex behaviour of certain types of breast cancer.

1.4.1 – ER-Positive / Luminal-Like Tumours

The ER-positive/luminal-like molecular subtype, which represents the largest group of invasive tumours (NOS), consists in all tumours characterized by relatively high expression of genes that are known to be expressed by luminal epithelial cells (87). The "good prognosis signature" of this subtype is dominated by dozens of genes under the control of ER and other ER-associated genes, such the one which codifies for the oestrogen-regulated protein LIV-1, the transcription factors hepatocyte nuclear factor-3α HNF3A (or FOXA1), trefoil factor 3 (TFF3), X-box-binding protein (XBP1), and GATA-binding protein 3 (GATA-3) (87) (Figure 5). These cancers, mostly occurring in postmenopausal women, are generally HER2-negative (luminal A), well- or moderately differentiated, slow growing and well-responsive to endocrine treatments. Conversely, only a small percentage of luminal-like tumours usually respond to standard chemotherapy (21).

1.4.2 – ER-Negative / Basal-Like Tumours

ER-negative/basal-like breast tumours represent one of the most intriguing subtypes, since comprises a small proportion of cancers which exhibit a basal/myoepithelial phenotype, defined by immunohistochemical positivity for myoepithelial markers (molecules normally seen in the basal/myoepithelial compartment of the normal breast) (88-89). Hence, these tumours, that failed to express ER and most of the other genes that are usually co-expressed with it (27), express basal keratins, p63, P-cadherin, laminin and integrin-β4 (90-91) (Figure 5). By strict definition basal-like cancers are a subgroup of ER-PgR-HER2 "triple negative" carcinomas (92-93). This cluster also encompasses the expression of many genes involved in cell cycle, invasion, metastasis and angiogenesis (81), which might explain the association of these tumours with a poor prognosis signature, with high proliferative and metastatic potential, high grade tumours and short patient survival (21). Basal-like cancers are of particular interest because of their distinct genetic features (21). When these breast carcinoma immunoprofiles were compared to familial and sporadic origin, we could observe that basal tumours were mostly associated with familial cases (90). Actually, it has been described that tumours from *BRCA1*-mutated carriers share an immunohistochemical profile very similar to that from sporadic basal-type carcinomas (high-grade, ER-negative, PgR-

negative, HER2-negative), a finding recently confirmed by the analysis of the referred basal molecular markers (CK14 and CK17, P-cadherin, p63, and EGFR) expression (94-95). These results led to the assumption that this genotype strongly predisposes to the basal-like tumour subtype. Based on these results, Foulkes *et al.* have hypothesized that the wild-type *BRCA1* key function is to act as a stem-cell regulator, besides promoting the differentiation towards glandular epithelium in the normal breast. In *BRCA1* mutated tumours, this transition has failed or was not completed, and basal-cell phenotype gene expression was retained (96-98). In a recent study by Lim and colleagues, it was observed that the expression of certain basal markers such as CK14 in *BRCA1*-associated tumors was also consistent with luminal progenitor cells having an altered differentiation program. This group showed that the delineation of specific epithelial cell types within the human mammary hierarchy, unexpectedly revealed an aberrant luminal progenitor cell population in *BRCA1* mutation carriers, suggesting it may serve as a cellular target for oncogenic events (99).

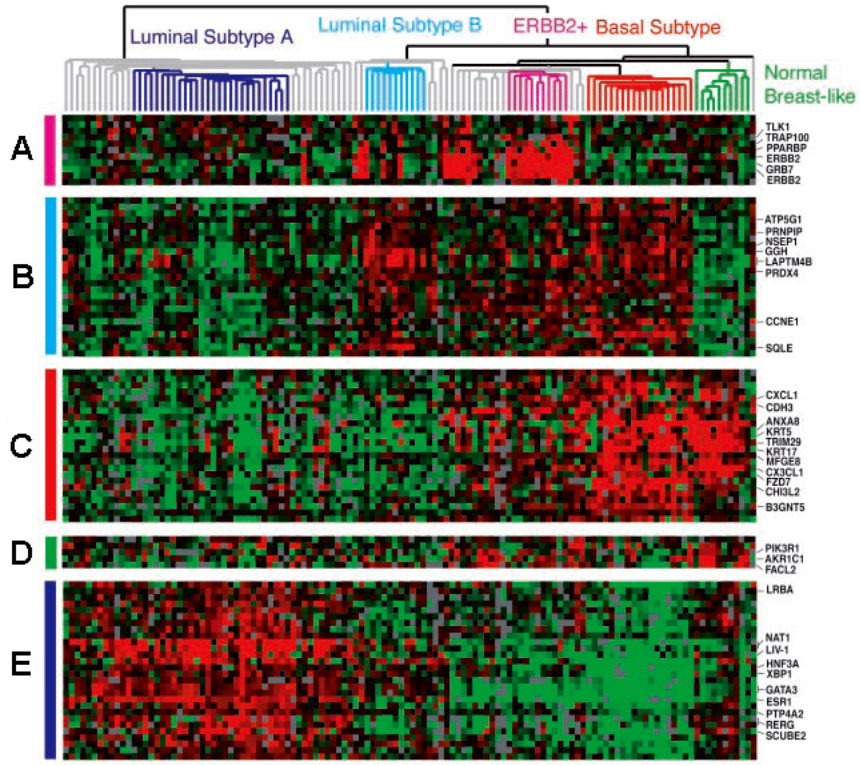

Figure 5. Hierarchical clustering of 115 tumour tissues and 7 non-malignant tissues using the "intrinsic" gene set. **(*A*)** Gene cluster showing the *ERBB2* oncogene and other coexpressed genes. **(*B*)** Gene cluster associated with luminal subtype B. **(*C*)** Gene cluster associated with the basal-like subtype. **(*D*)** A gene cluster relevant for the normal breast-like group. **(*E*)** Cluster of genes including the oestrogen receptor (*ESR1*), highly expressed in luminal subtype A tumours. [Adapted from Sorlie T *et al.*, 2003 (100)].

1.4.3 – ER-Negative / HER2-Overexpressing Tumours

HER2-overexpressing subtype comprises ER-negative carcinomas that overexpress HER2/*neu* protein (Figure 5). In more than 90% of HER2-positive cancers, overexpression is due to amplification of a DNA segment including the gene ERBB2 and several others in the same 17q21-22 amplicon, such as GRB7 and MLN64, which dominate the gene signature of this group (27, 86-87, 101). In rare cases, HER2/*neu* protein overexpression may occur as a result of mechanisms other than gene amplification (102). These cancers are usually poorly differentiated, with high proliferation rates and associated with a high frequency of brain metastasis (21).

1.4.4 - Other Expression Profiles

Over the last years, successive extended data sets allowed the separation of the ER-positive/luminal-like tumours into two distinct subgroups: luminal A and luminal B. While luminal A tumours are those anchored by the high expression of ER-associated genes, the smaller group of tumours, designated as luminal B, showed low to moderate expression of the luminal-specific genes including the ER cluster, but with a high expression of a novel set of genes whose coordinated function is still unknown. Expression of this cluster of genes is a feature that is shared with the basal-like and HER2-positive subtypes and associated with poor outcome (84, 87).

Some authors suggest the existence of another group of tumours, which may represent between 6-10% of NOS cancers, where the gene expression pattern is typified by the high expression of genes characteristic of basal epithelial cells and also from non-epithelial cell types, but with low expression of luminal epithelial genes. These tumours have been clustered into a subtype name “normal-like tumours” but, until now, it is unclear whether these tumours represent poorly sampled tumour tissues or a really distinct and clinically important group (21, 87). A recent study from Weigelt *et al.*, where an agreement analysis has been done between three microarray-based single sample predictors for the whole classification system, and also for the five molecular subtypes individually in each cohort, proposed that normal-like tumours could be an artefact derived from analysis of tumour specimens with a high proportion of normal tissue contamination (83).

Described recently, a potential new breast cancer molecular subtype, termed as "claudin-low", was identified and characterized by the low expression of genes involved in tight junctions and cell-cell adhesion, including claudins 3, 4 and 7, occludin and E-cadherin. These human tumours were also characterized by the expression of many epithelial-to-mesenchymal-transition (EMT)-associated genes, a comparatively high expression of lymphocyte and endothelial markers and a $CD44^{+}/CD24^{-/low}$-cancer stem cell signature (103-104).

1.5 - Regulation of Gene Expression in Breast Cancer

It is convenient to distinguish between genetic and epigenetic mechanisms that regulate gene expression; the former is based directly on the information encoded in the DNA sequence, while the latter, on those processes necessary for the manifestation of this information (105). In other words, epigenetics refers to information inheritance based on gene expression levels, while genetics refers to the information transmitted on the basis of gene sequence itself (106). However, genetic and epigenetic phenomena are almost indefinable interlinked and interdependent, contributing for an integrated approach which helps in the true appreciation of how the genome operates, in both health and diseases, such as cancer. Regulation of gene expression through binding of transcription factors to target DNA sequences illustrates particularly well the blending of genetics and epigenetics to deliver spatio-temporal gene expression patterns that are not predictable by considering either component alone (107-108). In fact, transcription factors, chromatin and chromatin-modifying enzymes are key components in a complex network through which the genome interacts with its environment. For many transcription factors, binding motifs are found adjacent to the promoter regions of a large proportion of genes, requiring mechanisms that confer binding specificity in any given cell type. These include association of the factor with other proteins and chromatin packing at the binding sequences, in order to inhibit or facilitate the binding. The functional consequences of transcription factor binding are frequently dependent on protein modifying enzymes, particularly those that alter lysine methylation at selected histone residues (108). A classical example of this targeting of transcription factors to selected sites is the well known MYC transcription factor involved in breast cancer. Evidence that MYC binding occurs in chromatin regions, enriched in specific

histone modifications (109), raised numerous questions concerning 1) if the enriched modifications simply reflect a generally “open” and accessible chromatin conformation, allowing the binding, or 2) if specific modifications serve as recognition signals bound by guide proteins directly linked to MYC itself and, thereby, targeting it to regions enriched in such signals. In one way or another, in breast development, as well as in breast carcinogenesis, the imbalance and deregulation of the mechanisms related with known important signalling pathways (e.g. PI3K, MAPK or ER signalling) are indubitably linked with the prior control of transcription factor modulation and associated epigenetic events.

1.5.1 - Transcription Factors

The actions of major transcription factor families are selective at several levels to govern the expression of sub-transcriptomes that are phenotypically related. The flexibility of transcriptional control includes the exact choice of the target sequence, the timing, the amplitude and magnitude of transcription and the integration with other transcriptional programs and signal transduction events. In breast cancer, the dexterity of targeting and regulation is blunted and, instead, transcription factors become limited to specific sub-transcriptomes, for example, those associated with blockage of programmed cell death and progression through the cell cycle, invasiveness potential and, ultimately, stimulation of cell migration (108).

1.5.1.1 - Steroid Hormone Receptors (SHR)

Perhaps the clearest examples of loss of transcriptional plasticity and the evolution of an unresponsive transcriptome in malignancy are found among the members of the nuclear receptor superfamily. These transcription factors dimerize and form a single network to regulate self-renewal and homeostasis in a number of epithelial systems, where breast is highlighted (108). Nuclear/steroid hormone receptors are members of the abovementioned family of nuclear receptors. They are ligand-activated transcription factors and sensors for growth factor-initiated signalling pathways, controlling the expression of target genes in hormonally regulated tissues (110-111). Steroid hormones control proliferation and survival

of breast epithelial cells. This activity has been so far attributed to the interaction of steroids with their cognate receptors and the consequent regulation of gene transcription (112) (Figure 6).

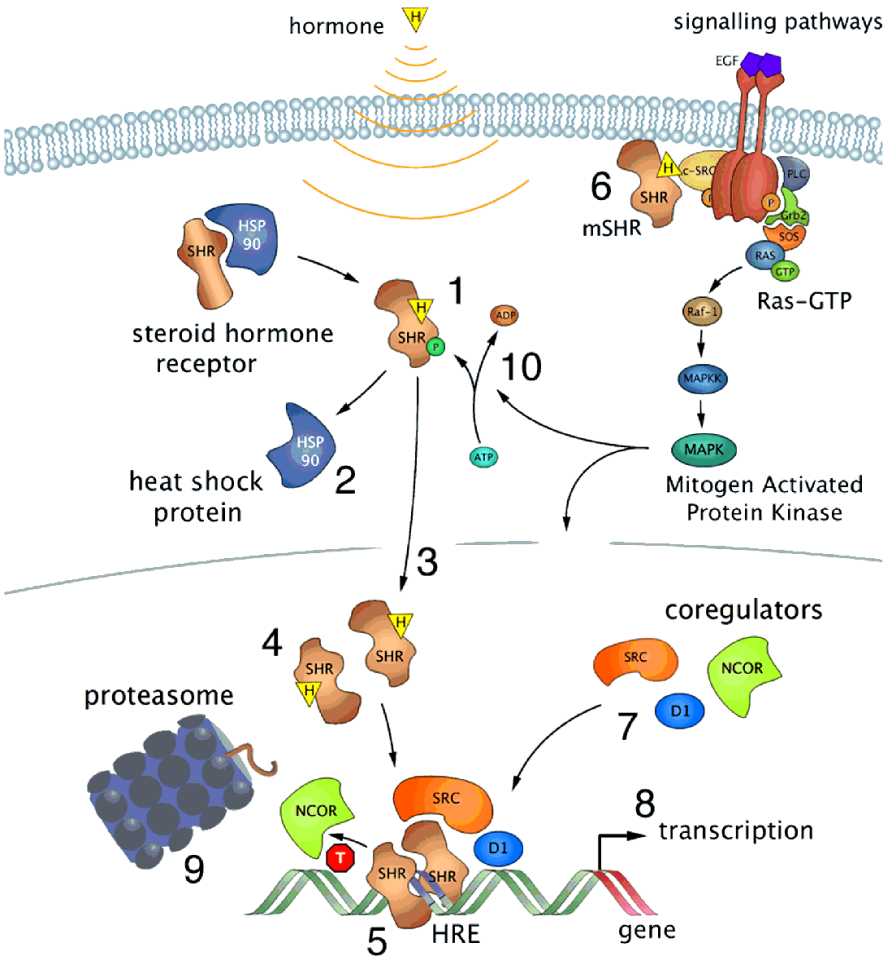

Figure 6. Steroid Hormone Receptors (SHR) act as hormone dependent nuclear transcription factors. Upon entering the cell by passive diffusion, the hormone (H) binds the receptor, which is subsequently released from heat shock proteins, and translocates to the nucleus. There, the receptor dimerizes, binds specific sequences in the DNA, called Hormone Responsive Elements or HREs, and recruits a number of coregulators that facilitate gene transcription. **(1)** hormone binding, **(2)** chaperone release, **(3)** nuclear translocation, **(4)** receptor dimerization, **(5)** DNA binding, **(6)** putative membrane-bound receptors, **(7)** coregulator recruitment, **(8)** transcription, **(9)** proteasomal degradation, **(10)** modulation by cellular signalling pathways [Adapted from Griekspoon, A *et al.*, 2007 (116)].

Oestrogens are probably the best studied steroid hormones which, acting through specific hormone receptors – ER, assume an important role as regulators of breast cancer growth. In fact, oestrogens have been found to control several key G1 phase cell cycle regulators, such as cyclin D1, MYC, CDK2, CDK4 and CDK inhibitors (6, 113). Target cell

response to oestrogens is dictated by the presence or absence of ER. In the absence of steroid hormone, monomeric inactive ER lies in the cell nucleus bound to heat-shock proteins (114), which are a requisite not only for the proper protein folding and assembly of competent stable heterocomplexes to ligand binding, but also to connect ER to protein trafficking systems (111). Due to its steroidal nature, oestrogens are able to diffuse passively through the plasma and nuclear membranes and to bind to its receptor (114). Upon oestrogen binding to the ER ligand-binding domain (LBD), a series of molecular modifications occur, including conformational changes (activation), phosphorylations, dissociation from the chaperone proteins and ER dimerizarion (115). Stimulation of target gene expression in response to oestrogen is thought to be mediated by two distinct pathways: the "genomic" or the "non-genomic" (114) (Figure 6).

The genomic pathway of ER action, also called nuclear-initiated steroid signalling (NISS), involves two different mechanisms: 1) a classical pathway, where the activated ER dimer binds to discrete DNA sequences, termed oestrogen responsive elements – EREs, which are located in regulatory DNA promoter regions of oestrogen-regulated genes, directly interacting with coactivator proteins (e.g. AIB1 and GRIP1) and components of the RNA polymerase II transcription initiation complex, and resulting in enhanced transcription of genes, such as complement 3 and PS2 (114, 117-118); and 2) a non-classical pathway, which do not require the direct binding of ER to DNA (119-120). In fact, ER can influence the transcription of genes lacking ERE sequences and regulated by other transcription factors that bind to alternative regulatory DNA sequences. The promoter elements involved in this response include AP-1 sites, cyclic AMP-response elements (CREs) and Sp1 sites, which directly bind Jun/Fos (121), c-Jun/ATF-2 (122) and Sp1 (123), respectively. ER interacts with these transcription factors, stabilizing their direct binding to DNA, and thus enhancing the transcription of target genes such as cyclin D1 or IGF-R1 (124-125). It can also occur that some ER-target genes, like VEGF gene, have both ERE and non-classical sites in their regulatory region. VEGF transcription can be induced by oestrogen, due to a variant ERE or through Sp1 sites, according to the cellular context (126-127). Moreover, some transcriptional-induction synergies can be achieved by ERE half-sites and other regulatory elements within a promoter region, as is the case of PgR gene, which displays combinations of half ERE upstream of Sp1 binding sites; ER can induce PgR expression by directly binding

to the half-site and by indirectly binding with proteins bound to Sp1 sites (128). Regardless if the induction is via ERE, or indirectly via non-classical sites, the oestrogen recruitment is able to induce gene transcription only if the important ER regions AF1 and AF2, responsible for transcriptional activity, are activated. It is important to highlight that if either AF1 or AF2 is dominant, oestrogen behaves as an agonist on gene transcription (114). AF2 activation is totally dependent on the ligand. The binding of oestrogen to ER induces the LBD rearrangement, uncovering AF2 region, serving as a binding surface for proteins that will act as coactivators, such as nuclear-receptor coactivators (NCoA 1,2 or 3), also known as steroid receptor coactivators (SRC) and translation initiation factor 2 (TIF2) (129). Binding of these molecules to AF2 region form large complexes that recruit histone-acethyltransferases (HAT), enzymes that cause the chromatin decompactation required for gene transcription. In contrast, corespressor proteins also can bind to AF2 region, such as nuclear-receptor corespressor 1 and 2 (NCoR), reducing ER-driven transcription by histone deacetylases (HDACs) recruitment to the promoter sites. This leads to chromatin condensation and decreased rate of transcriptional initiation (130-131). In contrast with AF2, AF1 activity is ligand independent. In the absence of oestrogen, AF1 is activated by phosphorylation, following the activation of MAPK and PI3K/AKT pathways. These different kinase pathways are triggered by growth factors receptors, such as EGFR or IGF-R1 (132).

In addition to the above described nuclear effects, there are ER functions that can occur very quickly in the cell (within seconds or minutes) and are initially independent of gene transcription. This rapid non-genomic mechanism of action is mediated by the membrane associated ER, by the so called membrane-initiated steroid signalling (MISS) pathway (133). At the membrane, ER associates with caveolin rafts and, upon oestrogen binding, interact directly with adaptor proteins, like SRC, p85 subunit of PI3K and G-proteins, resulting in the activation of growth factor receptors, such as EGFR, IGFR, HER2, and cytoplasmatic kinases like MAPKs, PI3K, AKT and mTOR (134-136). In turn, cytoplasmatic kinases can phosphorylate ER and its coregulators, resulting in the activation of nuclear ER-driven transcription (137). It is suggested thought, that the ER non-genomic and genomic activities are complementary and even synergistic (114).

1.5.1.2 - Forkhead-Box Proteins (FOX)

Forkhead box (Fox) proteins are a family of evolutionarily conserved transcription factors, defined by a common DNA-binding domain termed the Forkhead box or winged-helix domain (138). Despite the highly conserved Forkhead box DBD, Fox protein regulation and function vary significantly between sub-families, arising in part from sequence variations outside the DBD. In fact, based on forkhead box domain, the forkhead genes are grouped into 19 subclasses of FOX genes (139).

Fox protein family members are important for a wide spectrum of biological processes, including metabolism, development, differentiation, proliferation, apoptosis and invasion (138). Although the first mammalian forkhead type proteins to be identified were the FOXA class of factors, forkhead O transcription factors (FOXO) is one of the largest and more important subgroups of forkhead family members (138-139). Nevertheless, several Fox subfamilies such as FOXO, FOXM, FOXP, FOXC and FOXA have been linked to tumorigenesis and progression of several cancers, working as crucial regulatory proteins, which can act as tumour suppressor genes or as oncogenes. Indeed, Fox proteins can both activate and repress gene expression through the recruitment of cofactors or corepressors, primarily HDACs. In addition, Fox proteins interact extensively with other factors, such as p53 and ER, to modulate gene transcription. A growing body of evidence suggest that Fox transcription factors may represent direct targets and/or indirect effectors for cancer therapeutic intervention (138).

Fox factors are regulated by multiple layers of post-transcriptional modifications, including phosphorylation, acetylation and ubiquitylation, which determine their cellular localization and consequent functional activity. Thus, nuclear Fox proteins act as transcriptional regulators, whereas cytoplasmic Fox proteins are inactive and often subject to proteossomal degradation. The shuttling of Fox proteins between nuclear and cytoplasmatic compartment is driven by interaction with exportin/importin accessory proteins (138).

In breast cancer, it has been described that essentially FOXO, FOXM, FOXA and FOXC proteins assume an important role in crucial tumorigenic signalling pathways, in therapeutic responses and as tumour behaviour markers. Concerning FOXOs, activation of cell survival

pathways, such as PI3K/AKT/IKK or Ras/MAPK, are known to phosphorylate these transcription factors, leading to their binding to 14-3-3 protein, nuclear exclusion and degradation. Consequently, this cytoplasmatic shuttling and FOXO's inactivation result in the suppression of FOXOs transcriptional activity. Perturbation of nuclear FOXOs function leads to deregulated cell proliferation and accumulation of DNA damage, since they are crucial to maintain cells in check, controlling genes related with apoptosis and cell cycle arrest, such as $p27^{kip1}$, Bim, FasL, GADD45α and cyclin D (139-142) (Figure 7).

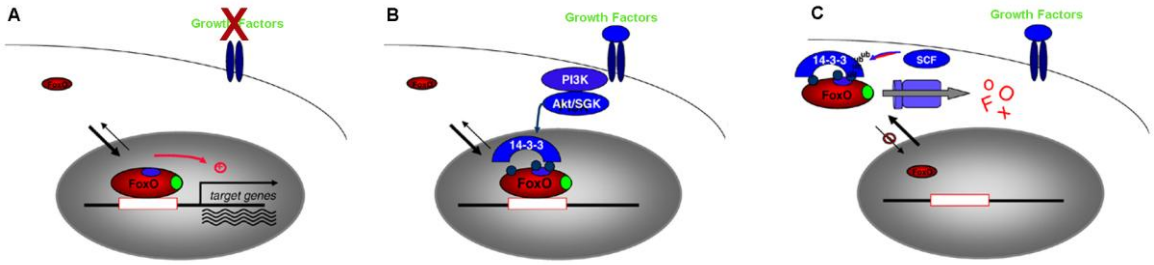

Figure 7. Model for the sequential inhibition of FOXO transcription factors in response to growth factors. FOXO factors are constantly shuttled between the cytoplasm and the nucleus. (**A**) In the absence of growth factors, FOXO are mostly localized in the nucleus, activating their target genes. (**B**) Activation of the PI3K–Akt/SGK pathway by growth factors, triggers the phosphorylation of FOXO in the nucleus, the binding of the 14-3-3 and the release of FOXO from their DNA-binding sites, leading to FOXO nuclear export. (**C**) In the cytoplasm, phosphorylated FOXO is degraded by proteasome-dependent degradation. [Adapted from Calnan & Brunet, 2008 (143)].

Growing evidence has demonstrated FOXOs as *bona fide* tumour suppressor genes. Therefore, the inhibition of FOXOs transcriptional activity by kinase pathways is associated with cell transformation, tumour progression and angiogenesis (144-147). FOXO3a overexpression has been shown to inhibit breast cancer cells growth *in vitro* and tumour size *in vivo*, and its cytoplasmatic localization correlates with poor patient survival (146). Since AKT negatively controls the activity of FOXO3a, inducing its phosphorylation and consequently degradation, it has been demonstrated that AKT inhibition leads to dephosphorylation and nuclear localization of FOXO3a, resulting in the activation of its downstream targets (148). Hence, inhibition of EGFR family members by breast cancer clinically used drugs, such as paclitaxel, imatinib, doxorubicin, lapatinib or trastuzumab, effectively up-regulates active FOXO3a and its targets, FasL, Bim and p27, by reducing AKT activity (139, 149-150). Interestingly, reinforcing the close impact of FOXO3a in breast

cancer, there are studies showing that FOXO3a is a key intracellular regulator of ERα gene transcription and expression (151-152). It was demonstrated that the widely described down-regulation of ERα induced by the activation of PI3K/AKT pathway (153-155) is mediated through the nuclear exportation and consequent inactivation of FOXO3a (151). These findings established an important cross-talk between the kinase pathways, the ERα function and the expression of FOXO3a, showing that the treatment with agents that inhibit HER2/PI3K/AKT kinase signalling, enhances FOXO3a activity and elevate the level of ERα expression in breast cancer cells. Interestingly, using gefitinib-sensitive breast cancer cell lines, we showed that gefitinib targets the transcription factor FOXO3a to mediate cell cycle arrest predominantly at G0-G1 phase and cell death. This mechanism is associated with FOXO3a dephosphorylation at AKT sites and its nuclear translocation (156) (see PAPER III in Appendix section). Curiously, it has been suggested that the sensitivity to cytotoxic drugs is mediated by some FOXOs, which may differ according to different cellular growth displayed by different tissues. In endometrial cancer, for example, we demonstrated that FOXO1, another member of FOXO's family, serves as a tumour suppressor involved in normal growth control, maintenance of genomic stability and limiting proliferation of endometrial cancer cells; however, we showed that its induction and activation confers resistance to paclitaxel (157) (see PAPER IV in Appendix section).

In contrast to FOXOs, where tumour development is associated with inactivation by phosphorylation, and consequent nuclear export, FOXM factors are activated by phosphorylation (158). FOXM1 is ubiquitously expressed in proliferating cells and a key regulator of both G1/S and G2/M phases of the cell cycle. This transcription factor is localised mainly at the cytoplasm, being phosphorylated and translocated to the nucleus upon activation of the Raf/MEK/MAPK, before cells entry in G2/M phase (158-160). Consistent with its role in promoting proliferation, elevated expression of FOXM1 has recently been reported in human breast cancer (159, 161). FOXM1 down-regulation inhibited the growth of breast cancer cell lines and its silencing resulted in the down-regulation of MMP-2, MMP-9, uPA and VEGF. Interestingly, induced FOXM1 overexpression in breast cancer cells expressing low levels of this transcription factor, resulted in increased cell proliferation, migration and invasion (162). Very recently, *in vitro* data have demonstrated the FOXM1 protein binding to the ESR1 promoter, thus leading to

upregulation of ERα mRNA and protein levels, demonstrating that FOXM1 is a physiological regulator of ERα expression in breast cancer cell lines (160). Based on these findings, inhibition of FOXM1 has been seen as a novel therapeutic approach for the treatment of aggressive breast cancer (159, 162).

Concerning FOXC transcription factors, little is known about these and about its downstream targets. However, recent evidence has pointed that the expression of FOXC2 is significantly correlated with the highly aggressive basal-like subtype of human breast cancers, suggesting a crucial role in promoting invasion and metastasis (163). Moreover, it has been shown that FOXC2 is critical for tumour development and for the formation of tumour blood vessels (angiogenesis) and with increased metastatic capabilities of breast cancer cells (164). Interestingly, FOXC2 specifically promotes mesenchymal differentiation during EMT and may serve as a key mediator to orchestrate the mesenchymal component of the EMT program (163).

Deregulation of other FOX proteins may be more associated with specific cancers, where they have an important role during development or tissue homeostasis. This is the case of FOXA1, where its deregulation is particularly associated with breast cancer due to its strong interaction with ERα (138). ERα binding to chromatin requires co-factors that can assist in defining the locations that ERα can bind in the genome. FOXA1 (also known as HNF3α) is a pioneer factor that has been receiving considerable attention, since it interacts with *cis*-regulatory regions of heterochromatin, enhancing the ERα interaction to chromatin, which is required for subsequent gene expression (165-167). In fact, FOXA1 can mimic histone proteins to enable binding to chromatin, and has been shown to be essential for changes in chromatin structure, for example, during oestrogen induction of the cyclin D1 gene (124). Recently, Carroll and colleagues described several robust data demonstrating the requirement of FOXA1 for optimal expression of nearly 50% of ERα-regulated genes and oestrogen-induced proliferation (165-166) (Figure 8A).

FOXA1 can bind to the promoters of more than 100 genes associated with metabolic processes, regulation of signalling pathways and cell cycle (168-169). Some studies have shown that FOXA1 can act either as a growth stimulator/activator or as a repressor. As a stimulator, FOXA1 binds to chromatinised DNA and opens the chromatin, enhancing the

binding of ERα to its target genes (170), which suggests a growth-promoting role for this forkhead protein (166, 170). In breast cancer, however, a growth inhibitory function has been attributed to FOXA1, since its overexpression can also block the metastatic progression by influencing the expression of the BRCA1-associated cell-cycle inhibitor p27 and promoting E-cadherin expression (171-172). Recent studies suggest FOXA1 as a favourable prognostic factor and as a predictor of better survival in breast cancer, with potential relevance in the subclassification of luminal/ER-positive tumours into two subgroups with different biological behaviour and prognosis, the luminal A and the luminal B (86). In breast cancers, expression of FOXA1 and ERα correlate exceptionally well, and they constitute two of the genes that define the luminal breast cancer signature (27, 86). Loss of FOXA1 is associated with potential for endocrine independent proliferation and survival, endocrine resistance and tumour progression (138). Indeed, the lack of expression of FOXA1 through methylation or inactivation of its putative activator, GATA-3, lead with loss of expression of ERα and formation of mammary tumours (173) (Figure 8B).

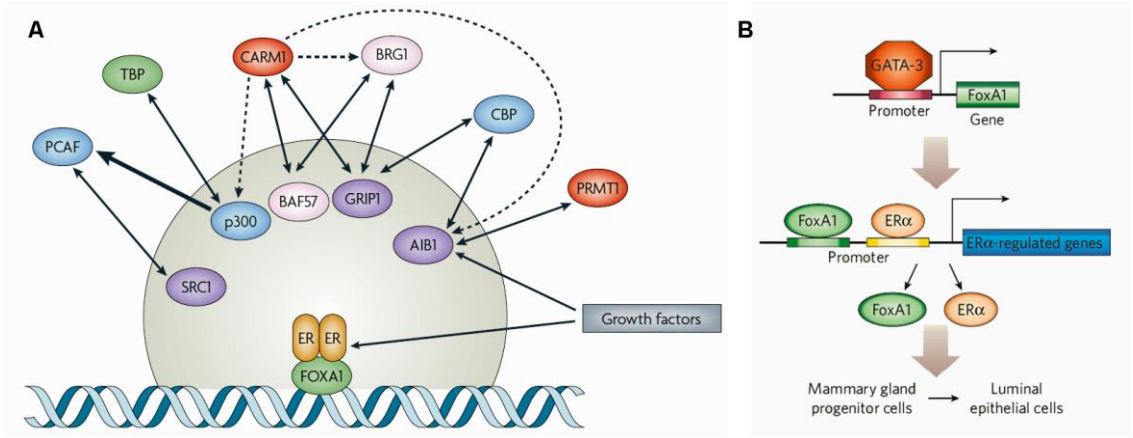

Figure 8. Interaction between FOXA1, GATA-3 and ERα and associated co-factors: **(A)** Pioneer factors, such as FOXA1, function to identify where in the chromatin ERα binds. A large number of co-factors can associate with the ERα complex to either bring in other co-factors, regulate the protein structure of ERα or co-factors, or to directly modify the chromatin structure. The activity of ERα and AIB1 are also regulated by growth-factor-signalling pathways. All of these interactions between ERα and co-factors determine the transcriptional activity of the target gene. **(B)** GATA-3 binds to the regulatory region (promoter) of the gene encoding FOXA1 and possibly activates its expression. Perturbation of this pathway as a result of decreased GATA-3 expression, may contribute to breast tumours. [Adapted from Green K & Carroll J, 2007 (174) and Tong Q & Hotamisligil S, 2007 (175)].

Interestingly, along with FOXA1, GATA3 is also one of the few genes that define ERα-positive luminal breast cancers. Both GATA3 and FOXA1 are oestrogen-regulated genes, and both are essential co-factors for oestrogen-mediated cell-cycle progression (174, 176). As

FOXA1 may also be a downstream effector of GATA-3, it may be a bridge between GATA-3 and ER pathways (173), controlling and regulating the biology of luminal mammary cells, breast cancer progression and behaviour.

1.5.1.3 - CCAAT/Enhancer-Binding Proteins (C/EBP)

CCAAT/enhancer binding proteins (C/EBPs) are a family of leucine zipper transcription factors, that bind as homodimers and heterodimers to sequence-specific regions in order to regulate gene transcription. The C/EBP family members have important roles in the control of cellular proliferation, differentiation and apoptosis, metabolism, survival, inflammation and transformation, oncogene-induced senescence and tumorigenesis (177-180). These transcription factors contain an amino-terminal transactivation domain and a basic DNA-binding region, immediately adjacent to the highly conserved carboxyl-terminal leucine-rich dimerization domain. C/EBPs must dimerize to bind DNA and dimerization can occur within a C/EBP family, between different C/EBP family members, between different groups of leucine zipper proteins (180), or even with other transcription factors of the AP-1, NFκB and RB families (181). Therefore, due to this flexibility to form different dimers and also due to the type of post-translational modification, the transactivation potential of each C/EBP isoform can be quite different, resulting in a myriad of regulatory effects on gene expression (182).

In breast, C/EBPs play a pivotal role in controlling growth and differentiation of the mammary gland and, among the six C/EBP genes identified so far (C/EBPα, -β, -δ, -ε, -γ, -ζ), the encoded intron-less genes C/EBPα, C/EBPβ and C/EBPδ are the most thoroughly studied in rodent and human mammary tissue, since they are temporally expressed to coordinately control mammary growth, differentiation and programmed cell death (180). In terms of function in mammary gland, C/EBPα and -δ protein levels are thought to be more related with differentiation and development. Experimental data have shown that C/EBPα proteins repress proliferation and induce differentiation in epithelial cells (183). Moreover, it was found that this protein isoform is expressed in high levels during lactation and at lower levels during involution in the rat mammary gland. C/EBPδ protein expression levels are similarly low in the involuted gland, but high expression has been observed during

pregnancy and on late lactation (180). In contrast, C/EBPβ is detected in whole cell extracts of the virgin gland and readily detectable throughout lactation and involution, revealing a more complex and function-related role in breast, other than differentiation and development. In fact, C/EBPβ can be translated into several distinct protein isoforms, whose expression is regulated by the alternative use of several in frame translation start sites. This leads with distinct biological and regulatory functions, since it modulates the binding affinity to bind to DNA and thus to transactivate gene expression (182). For example, the longer C/EBPβ proteins, which arise from usage of the first two AUG codons (liver-enriched transcriptional activating proteins, LAP) support proliferation and repress differentiation of many cell types (184); however, the shorter protein product, which arises by usage of the third start codon or by post-translational proteolytic cleavage (liver-enriched transcriptional inhibitory protein, LIP), lacks the transactivation domain and acts as a dominant-negative repressor in experimental systems (185). Nevertheless, LIP isoforms bind to the consensus sequences within the DNA, with higher affinity than other C/EBP proteins (184-185). LAP1 and LAP2, both with N-terminal activation domain, are transcriptional activators and are associated with differentiation, being the shorter LAP1 isoform a weaker activator than LAP2, which is able to transactivate important genes, such as cyclin D1 promoter (186). However, these two isoforms seem to have unique actions and that in specific cellular context the LAP1:LAP2 ratio may be important for regulation of gene expression (182). In contrast with the LAP isoforms, LIP can function to inhibit the transcriptional activity of other C/EBPs, by competing for C/EBP consensus binding sites or by forming inactive heterodimers with other C/EBPs, as a dominant negative. However, emerging evidence suggest that LIP can act as a transcriptional activator in some cellular contexts; then, the mechanism might include the interactions of LIP with other, non-C/EBP transcription factors, such as glucocorticoid receptor, NF-κB or PgR (182). Unlike C/EBPα, C/EBPβ most likely contributes to tumorigenesis through significant elevations in the LIP:LAP ratio, mostly observed in ER-negative, aneuploid, highly proliferative and metastatic mammary tumours that are associated with a poor prognosis (181). An increase in the LIP:LAP ratio has also been linked to a TGFβ-dependent cytostatic response in metastatic breast cancer cells. Interestingly, forced expression of LAP2 in cells expressing elevated LIP, through a mechanism which involves the association with FOXOs and consequent repression of Myc,

restored the TGFβ cytostatic response and lead to a significant reduction in the proliferative activity of metastatic cells (187). LIP isoform overexpression also leads to a lack of contact inhibition, resulting in proliferation and *foci* formation in epithelial breast cancer cell lines (188). Importantly, LIP expression is restricted to the mammary tumours and is not detected in preneoplastic lesions, and evaluation of its expression has been suggested as a prognostic marker for patients with breast cancer (189-190). The importance of C/EBPs in breast cancer is still highlighted by their interaction with receptor tyrosine kinases. In general, EGFR signalling leads to increased C/EBPβ-LIP protein expression, resulting in an increased LIP/LAP ratio, which contribute to the mitogenic effects of ErbB signalling by promoting proliferation and a more aggressive disease state (191).

1.5.2 - Epigenetics

Cancer is an epigenetic disease at the same level that it can be considered a genetic disease (106). The key mechanisms that underlie target gene activation and repression by modulating the accessibility of transcriptional activation complexes to target gene *loci* are the chromatin and nucleossomal remodeling and DNA methylation (173). These epigenetic processes, which occur at the chromosomal level in transformed cells, mutually interact with each other, in order to modulate the chromatin structure to form euchromatin or heterochromatin and, in turn, activate or silence gene expression (192). Alterations in the expression of key genes through aberrant epigenetic regulation lead to initiation, promotion and maintenance of carcinogenesis, and is even implicated in the generation of drug resistance (192-195). Changes in DNA methylation include global hypomethylation and focal hypermethylation. Global hypomethylation is linked to genomic instability and activation of oncogene expression (196). By contrast, gene-locus-specific hypermethylation can lead to the transcriptional silencing of tumour suppressor genes (193-194, 197). In addition to DNA methylation, post-translational histone modifications are another epigenetically regulated mechanism that can modulate chromatin structure to control gene expression (193, 195, 198). DNA methylation can, however, be associated with some specific types of histones modifications that can cooperatively affect chromatin structure to silence gene expression (195, 199) (Figure 9).

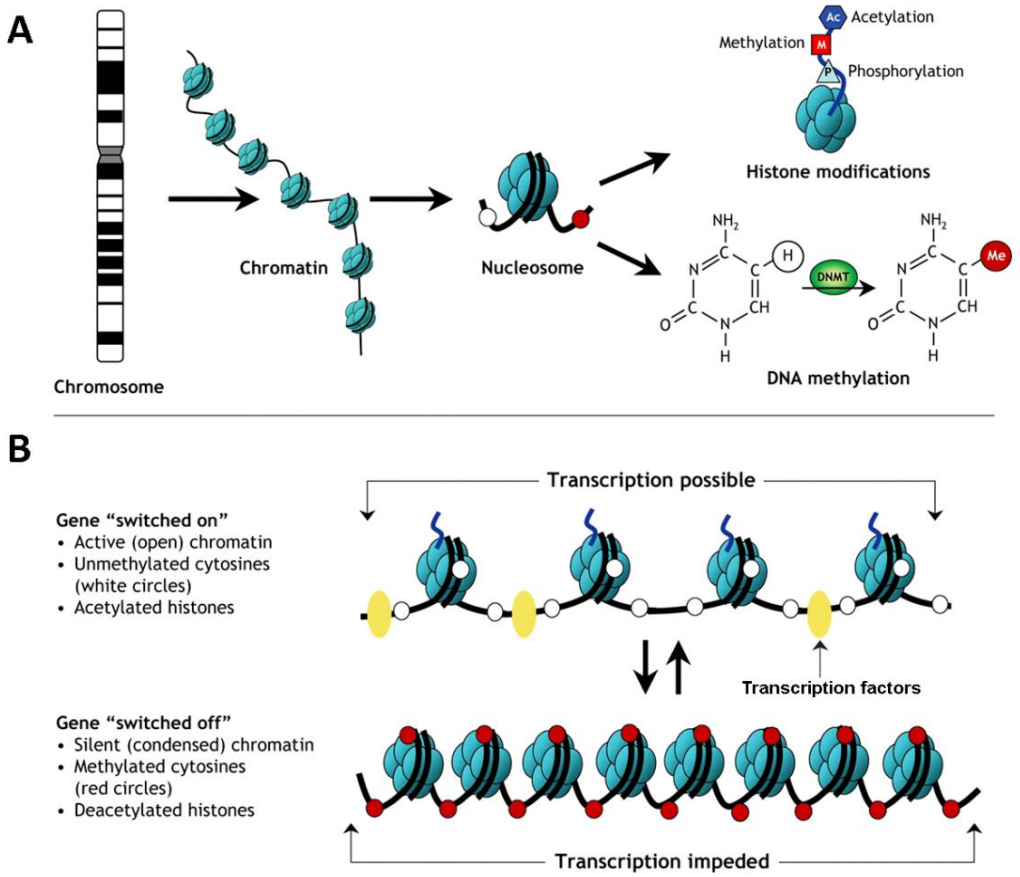

Figure 9. Epigenetic mechanisms. There are two primary and interconnected epigenetic mechanisms - DNA methylation and covalent modification of histones. **(A)** Schematic of epigenetic modifications. Strands of DNA are wrapped around histone octamers, forming nucleosomes, which are organized into chromatin, the building block of the chromosome. Reversible and site-specific histone modifications occur at multiple sites through acetylation, methylation and phosphorylation. DNA methylation occurs at 5-position of cytosine residues, in a reaction catalyzed by DNA methyltransferases (DNMTs). Together, these modifications provide a unique epigenetic signature that regulates chromatin organization and gene expression. **(B)** Schematic of the reversible changes in chromatin organization that influence gene expression: genes are expressed (switched on) when the chromatin is open (active), and they are inactivated (switched off) when the chromatin is condensed (silent). White circles = unmethylated cytosines; red circles = methylated cytosines [Adapted from Collaborative Research Centre, Münster University].

It was already demonstrated that epigenetic changes are one of the main driving mechanisms leading to breast cancer and that both hypermethylation and specific histone modifications events are essential in maintaining transcriptionally repressive chromatin by forming suppressive complexes at the DNA level (200). Moreover, it was recently described significant changes in histone modification levels in the course of tumour progression from normal breast epithelium to *in situ* and invasive ductal carcinoma (201), reinforcing the importance of epigenetic mechanisms in breast carcinogenesis.

1.5.2.1 – DNA Methylation

DNA methylation is a heritable epigenetic change, that alters gene expression and results from the activity of a family of DNA methyltransferase (DNMT) enzymes, which catalyse the addition of a methyl group to cytosine residues at CpG dinucleotide (adjacent cytosine and guanine nucleotides in DNA) (192, 202). The distribution of CpG dinucleotides is not random, and some of them cluster together to form CpG-rich DNA regions called CpG islands, normally located in the upstream promoter and exon 1 region (203). CpG-island-containing gene promoters are usually unmethylated in normal cells to maintain euchromatic structure, which is the transcriptionally active chromatin conformation, allowing gene expression. However, during cancer development, hypermethylation at CpG-island-containing gene promoters occur in order to inactivate gene transcription, by changing open euchromatic structure to compact heterochromatic structure (193, 195, 197, 199). Hypermethylated genes identified from breast neoplasms form a long list, and their biological functions encompass cell cycle regulation ($p16^{INK4A}$, $p14^{ARF}$, 14-3-3σ, cyclin D2, $p57^{KIP2}$), apoptosis (APC, HOXA5), DNA repair (GSTP1, MGMT, BRCA1), hormone receptors (ERα and PgR), cell adhesion (CDH1, APC, TIMP3), angiogenesis (maspin) and cellular growth-inhibitory signalling (RARβII, TGFβII, SOCS1, WIF1) (192). Besides hypermethylation of gene-associated CpG islands, hypomethylation of repetitive genomic DNA has also been identified as a specific feature in breast cancers (196, 204). Although less well studied, several lines of investigation indicate that the global hypomethylation indentified in cancer cells might contribute to structural changes in chromosomes, aberrant activation of proto-oncogenes expression and increased mutagenesis (197, 204-206). Genomic hypomethylation in breast cancer genes has been known to correlate with some clinical features, such as disease stage, tumour size and histological grade (207), as it is the case of *CDH3* gene (208) (see PAPER I in Appendix section). Some proto-oncogenes, implicated in proliferation and metastasis (e.g., N-cadherin, ID4, annexin A4, β-catenin and WNT11 genes), have been found to be upregulated in breast cancer through the hypomethylation of their promoters (209-210) .

1.5.2.2 - Acetylation and Chromatin Remodelling

The condensation of eukaryotic DNA in arrays of nucleossomes, folded into higher-order chromatin fibres, influences several aspects of DNA metabolism (211). Knowledge of how chromatin structure is organized and maintained is crucial to understand the origins of epigenetic alterations in cancer. The basic building block of chromatin - the nucleossome, is formed by an octamer of histone proteins, namely H1, H2A, H2B, H3 and H4 (192). Histone N-terminal tails, protruding from the nucleossomes, are subject to a variety of covalent modifications in a dynamic and reversible manner (212). These modifications include acetylation, methylation, phosphorylation, ubiquitylation and sumoylation, which are critically implicated in the regulation of chromatin structure and gene expression. Each histone modification is a unique mark to show the status of chromatin structure (active or repressive), defining the accessibility of the DNA to the transcription machinery (192, 213). Acetylation of histone lysines, the best characterized modification of chromatin structure (214), has been known to be associated with open chromatin structure and active transcription; methylation of these residues is associated with either active or repressive states of chromatin architecture and transcription is dependent on the modified site (198, 215). Thus, the promoters of transcription-active genes are associated with active histone marks, such as acetylation at lysine 9 (K9) of H3 as well as K5, K8, K12 and K16 of H4 and methylation at K4 of H3 (H3K4me), which are involved in a loosening of chromatin structure (euchromatic state) (195, 215). In contrast, repressive histone marks, including mono-, di- and tri-methylation of histone H3 lysine 9 (H3K9), H3K27 and H4K20, that are implicated in initiating and maintaining closed chromatin structure (heterochromatic state) (193, 198, 204). Bidirectional enzymatic machineries modify chromatin and the dynamics of histone acetylation is balanced by the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs), while histone methylation is done by histone methyltransferases (HMTs) and histone demethylases. Some of these histone modification enzymes have been recognized as components of nucleossomal remodelling complexes, which work together to regulate chromatin structure and gene expression (195, 198, 216-217).

Acetylation of lysine residues in histone tails by HATs decreases electrostatic interactions between the negatively charged DNA and the basic lysine residues, resulting in decondensation of chromatin and enhanced transcription, presumably due to the increased

accessibility of nucleossomal DNA to transcription factors (214, 218). Importantly, HATs cannot bind to target gene promoters directly but are recruited by DNA-bound transcription factors (214). Concerning HDACs, their action is to restore a positive charge to lysine residues in the amino tail of histones, leading to the compaction of chromatin, which is refractory to transcription (219). The 18 human HDACs known are subdivided in classes, where the class I HDACs (HDAC-1, -2, -3 and -8) are generally localized in the nucleus and associate with various transcriptional corepressors (e.g. NCoR, SMRT or Sin3A) and cofactors to form protein complexes (220-221). In addition to histones, many non-histone proteins have been identified to be substrates of HDACs, such as proteins involved in transcription (p53, E2F1, STAT1, STAT3, GATA1 and NF-κB), hormone receptors (AR and ERα), cytoskeletal structure (α-tubulin), WNT signalling (β-catenin) and heat shock/chaperon response proteins (HSP90) (222-225). In fact, this broad spectrum of HDAC substrates reveal the complexity of HDAC functions to regulate gene expression (192).

But, while histone lysine acetylation alters directly the chromatin structure by charge ablation, other histone modifications present binding sites for effector proteins tethering to histone marks in a modification and context-specific fashion to generate chromatin changes (226). Histone lysine methylation has received lot of interest, as it marks both active and inactive chromatin, playing a key role in regulation of transcription. So far, histone methylation is the major mark for recruitment of chromatin modifying machineries (212, 226). The dimethyl- and trimethyl-H3K4 modifications, highly conserved epigenetic marks associated with actively transcribed loci and euchromatin (active modification marks) (227), have been reported to be catalyzed by the *Trithorax* group of HMTs (e.g. SET1) (228), a know group of effectors that have long been implicated in the transcriptional activation of developmental regulatory genes and whose actions are balanced by the opposing effects of the Polycomb group (PcG) factors (229). In breast cancer, global histone modification analysis has revealed histone modification profiles that have been correlated with prognosis. As an example, while H4K16 acetylation is generally reduced or absent, suggesting that this alteration may represent an early event of breast cancer, moderate to low levels of lysine methylation (H3K4me2 and H3K4me3) has been associated with breast carcinomas of poorer prognosis (230).

Much of the research efforts to date have concentrated on the identification of epigenetic altered genes implicated in breast tumorigenesis. Since ERα is a central gene in breast development and function, but also in breast carcinogenesis, this gene has been the target of multiple studies concerning its regulation and its potential in the epigenetic setting. Some of these studies have been shown that abnormal exposure to oestrogen or oestrogenic chemicals induces epigenetic alterations in breast progenitor cells, which have been previously implicated in breast cancer (192, 231). Although aberrant activation of oestrogen signalling can lead to tumour-associated alterations in the epigenome of breast progenitor cells, approximately 30% of diagnosed breast cancer cases lack oestrogen signalling due to loss or downregulation of ERα, also subject to epigenetic silencing (232). How ERα-negative breast cancer cells acquire more aggressive properties after loss of oestrogen signalling is a very important issue in the field of breast cancer research. A robust link between ERα signalling and acetylation and chromatin remodelling is given by the study of Leu *et al*., where ERα signalling abrogation by small-interfering RNA-mediated knock down resulted in epigenetic inactivation of ERα targets, which initiate by the recruitment of PcG repressors and HDACs to their promoters, and then progressively followed by DNA methylation (233). Their results suggest that epigenetic regulation of ERα target genes is required for establishing ERα-independent growth and other characteristics of ER-negative breast cancer cells. Many other genes, involved in breast carcinogenesis, are targets or important actors of chromatin remodelling processes. The breast cancer susceptibility gene *BRCA1* displayed hypoacetylated and condensed chromatin in its proximal promoter region in hereditary breast cancer and chromatin remodelling is a common element for this multifunctional protein in processes such as DNA repair, DNA replication and transcription. *BRCA1* interacts with components of several chromatin remodelling complexes, making this gene both a participant in, and a target of, epigenetic regulation in breast cancer (234). Also the function of *BRCA2* has been linked to the presence of HAT activity (235). In several studies, the cyclin-dependent kinase inhibitor $p21^{WAF/CIP}$ is found to be up-regulated after treatment of breast cancer cell lines with HDAC inhibitors, resulting from histone hyperacetylation specifically associated with Sp1 sites (236-237). Similarly, the actin filament-binding protein gelsolin is upregulated by the treatment with HDAC inhibitors and this epigenetic induction of p21 and gelsolin lead with reduced proliferation and cell cycle

arrest and with alterations in cell morphology (238), respectively. Finally, the c-myc oncogene, which is amplified and overexpressed in some human breast cancers, is transcriptionally regulated by modulation of chromatin structure at its *locus* (239).

Recent advances in genome-scale technologies aimed at revealing epigenetic alterations in breast cancer, and the current progress in translating this profiling knowledge has contributed to diagnosis, prognosis (192) and to new promising anticancer drugs as epigenome-modulating agents (214). In fact, several *in vitro* studies have reported that HDAC inhibitors are able to inhibit proliferation of breast cancer cells, regulate ER expression and activity, increase the anti-proliferative activity of anti-oestrogens in ERα breast cancer cell lines and restore this activity in ERα-negative or anti-oestrogen resistant cells (240-241).

1.6 - CADHERINS

Epithelial cell-cell junctions provide tissue integrity and promote cell polarity (242). The junctional complex comprises tight junctions, adherens junctions and desmossomes (243). The adherens junctions play a pivotal role in regulating the activity of the entire junctional complex, and the major adhesion molecules in the adherens junctions are the cadherins (243-244). The so called classical cadherins, such as E-cadherin, N-cadherin and P-cadherin, are the best characterized subgroup of adhesion proteins (245). They are the transmembrane component of the adherens junction and are composed by three domains: 1) an extracellular domain responsible for homotypic cadherin-cadherin interaction which mediate calcium-dependent cell-cell adhesion, 2) a single pass transmembrane domain, and 3) a highly conserved cytoplasmatic domain that, through intracellular catenins, is linked to actin filaments and thus serves to connect the cell surface to the cytoskeleton (246). The cadherin-catenin complexes constitute the main building block of the adherens junctions, which are assembled by direct binding between extracellular domains of cadherins (247). The cytoplasmatic domain of cadherins interacts with p120-catenin and β-catenins, the former, playing an important role in the delivery and retention of cadherins at the adherens junctions, and the last providing a connection to α-catenin, which is required for the actin-

dependent clustering of cadherin-catenin complexes to form the adherens junctions (248) (Figure 10).

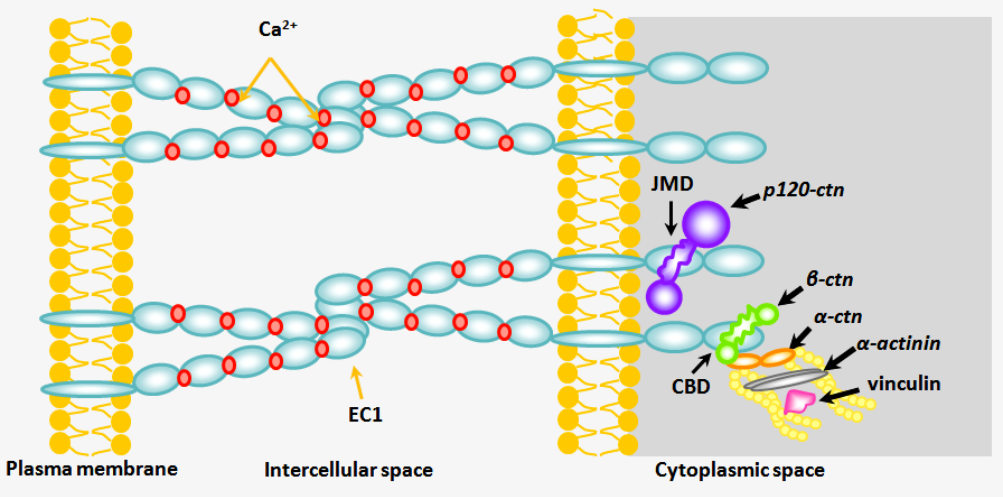

Figure 10. Schematic representation of the classical cadherin-catenin complex. Classical cadherins (blue), which mediate calcium-dependent (red) intercellular adhesion, are composed by an extracellular domain, a transmembrane domain and a cytoplasmic domain. This last domain comprises a juxtamembrane domain (JMD), which binds p120-catenin (violet), and a catenin-binding domain (CBD), which binds β-catenin (green), which in turns binds α-catenin (orange). Both α-catenin, α-actinin (grey) and vinculin (pink) establish a direct link between the cadherin-catenin complex and the actin cytoskeleton (yellow) [Adapted from Paredes J *et al.*, 2007 (249)].

It has been demonstrated that these cadherin-catenin complexes are not only the structural elements stabilizing adhesive contacts, but also the important signalling centres that may function as biosensors of the external cellular microenvironment (250). Because of the importance of cadherins to cell recognition, adhesion and signalling, disruption of cadherin function has significant implications for the development and behaviour of tumours (246). In fact, maintenance of normal epithelial cellular architecture is frequently altered in tumour progression. In a multistep process, termed EMT, tumour cells progressively downregulate their normal cell–cell adhesion epithelial-specific proteins, such as E-cadherin, and express *de novo* mesenchymal adhesion molecules, such as N-cadherin (251). This cadherin switch leads to the inhibition of cell–cell contacts and elicits active signals, which support tumour cell migration, invasion and metastatic dissemination (252).

1.6.1. *CDH3*/P-Cadherin in Breast Cancer

The vast majority of the studies implicating cadherins in tumorigenesis and invasion have focused on E-cadherin, since this is the major cadherin involved in epithelial adherent

junctions, and most importantly, many epithelium-derived cancer cells have loose E-cadherin expression. The adherens junctions function to maintain the normal phenotype of epithelial cells and is responsible for the strong cell-cell adhesion that promotes epithelial polarity and prevents epithelial cells from migrating away from their appropriate location (246). During embryogenesis, the critical importance of E-cadherin to normal development and tissue function is demonstrated by the lethality of E-cadherin gene knockout in mice in the very early stages (253). Extensively studied over the last years, the gene encoding E-cadherin (*CDH1*) was one of the first to be considered as an invasion suppressor gene (252, 254-255) and this notion was strongly supported by immunohistochemical changes in E-cadherin expression and location in several types of human cancers (256-258). A common finding was a positive correlation between disturbance of E-cadherin function/expression and decreased differentiation and higher aggressiveness of the tumours (259).

In normal breast tissue, E-cadherin is expressed by the luminal epithelial cells and is found concentrated at cell-cell borders. As is the case of other carcinomas, breast cancer cells can exhibit reduced or missing E-cadherin expression or function (246), that could be attributed to multiple mechanisms, including complete or partial gene deletion, inactivation by promoter methylation, and chromatin rearrangements (260). In diffuse gastric cancers and lobular breast cancers, E-cadherin inactivation was associated with somatic point mutations of the *CDH1* gene, as well as with LOH, promoter hypermethylation or overexpression of transcriptional repressors (261-262). However, in some epithelial tumours, E-cadherin is not lost, and is expressed concomitantly with other cadherin, such as P-cadherin. Interestingly, invasive breast carcinomas co-expressing E- and P-cadherin show a worse patient survival when compared to carcinomas with loss of E-cadherin expression (263).

P-cadherin (or placental cadherin) was the third classical cadherin to be indentified and its encoding gene is identified as *CDH3*. Although far less well characterized than is *CDH1*, they share 66% of gene homology. Particularly, the sequence of the upstream 5' region of the P-cadherin gene exhibits structural similarities to the 5' region of the *CDH1* gene. *CDH3* is currently annotated to chromosome 16q22.1, a region that comprises a cluster of several cadherin genes, just 32 kb upstream of the gene encoding the human E-cadherin (264-266). Briefly characterizing *CDH3* gene structure, the TSS of *CDH3* is currently

annotated to the coordinate 68,678,739 bp on the forward strand and the ATG is found 553 bp downstream of the TSS. The P-cadherin promoter exhibits no TATA-box, while including a CAAT-box, two E-boxes, two putative AP2-binding motifs and a GC-rich region containing putative SP1-binding sites. Similarly to *CDH1*, an *AluJb* repeat is found ~700 bp upstream of the ATG putatively enclosing gene regulation or exonization features (266).

Mutations in the *CDH3* gene have been reported to be responsible for congenital hypotrichosis associated with juvenile macular dystrophy, a rare autosomal-recessive disorder characterized by abnormal growth of scalp hair, followed by progressive macular retinal degeneration that leads to early blindness (267). P-cadherin is transiently expressed in various tissues during development and its expression is limited to adult tissues at cell-cell boundaries. Unlike E-cadherin, which is broadly distributed in all epithelial tissues, P-cadherin exhibits a singular pattern of expression, co-localizing partially with E-cadherin and being restricted to the basal proliferative cell layer of the majority of stratified epithelia. Additionally, it has been proposed that its expression is correlated with undifferentiation and proliferation status in these type of tissues (268-270) and that its expression is crucial for orderly progression of terminal differentiation of the epidermis (270). In mammary gland, P-cadherin-mediated adhesion or signals derived from its cell-cell interaction are important determinants of mammary gland growth control (271). Moreover, in normal adult non-lactating tissue, spatially selective expression of E-cadherin, which is expressed by both luminal and myoepithelial cells, and P-cadherin, restrictedly found in myoepithelial and cap cells, appears to be crucial for mammary gland differentiation (272). P-cadherin protein is still expressed in the lactating mammary gland tissue, and high levels of an 80kDa soluble P-cadherin in human milk have been found, raising the suggestion that this protein may be a secreted protein rather than an adhesion protein (273).

P-cadherin mutations are infrequent in human tumours; however, P-cadherin has been shown to be overexpressed in several solid tumours, including breast (90-91, 208, 249, 263, 272, 274-275), ovarian (276), endometrial (277), prostate (278), colon, gastric (279), pancreatic and bladder cancer (280). In contrast with what has been observed for E-cadherin, P-cadherin is frequently upregulated in tumours. Additionally, P-cadherin was shown to have a role in promoting cell migration in several cancer models. Taniuchi *et al.* showed that a pancreatic cancer cell line, transfected with wild-type P-cadherin, migrated

faster than the cells without this molecule (281). In breast cancer model, we have shown that P-cadherin overexpression by breast cancer cells promotes their invasive and migratory capacity (249, 282-283) (see PAPERS II and VI in Appendix section). Additionally, the expression of this protein induces the secretion of MMPs, which will be responsible by the cleavage of its extracellular domain, giving rise to a soluble 80 kDa form of P-cadherin (sP-cad). Overexpression of exogenous P-cadherin is able to promote single cell motility, inducing an increase in the number of moving cells and speed when compared with cells with low levels of this protein. We proved that this fragment is a pro-invasive factor, which needs to be inhibited in order to render cancer cells non-invasive (283). Moreover, P-cadherin is able to induce phenotypic changes involving alterations in cell polarity and leading edge morphology, formation of membrane protrusions, as well as increase of their cytoplasmic area, which usually is characteristic from cells with a motile behavior. Indeed, P-cadherin-overexpressing cells not only showed increased single cell motility, but also increased directional cell migration, as well as, invasion capacity through the matrigel (283) (see PAPER VI in Appendix section).

1.6.1.1 - Diagnostic and Prognostic Relevance

In human breast cancer, P-cadherin has been found to be aberrantly expressed in a subset of carcinomas. Based on microarray technology, this molecule is essentially expressed in those breast lesions termed basal-like carcinomas (27), which are characterized by their negativity for ER, PgR and HER2 (triple-negative) and by their association with poor prognosis (284). With the development of P-cadherin monoclonal antibodies, it was demonstrated that P-cadherin is expressed in around 30%-50% of invasive ductal carcinomas of the breast, being however infrequent in lobular carcinomas, where the loss of E-cadherin is the major hallmark (275). We and others have reported that the membranous P-cadherin expression is strongly associated with proliferative lesions of high histological grade, decreased cell polarity, poor patient survival over short-term follow up and lower disease-free survival (249, 272, 274-275, 285-286) (see PAPERS I and II in Appendix section). Besides the association of P-cadherin expression with hormonal-negative phenotype of breast cancer, the expression of this protein also associates with the expression of EGFR, p53 expression, high proliferative rates (MIB-1), high mitotic index and decreased cell

differentiation (208, 272, 275, 285); altogether being biological conditions strongly associated with malignant behaviour and poor outcome for breast cancer patients. However, P-cadherin expression has been shown to be not related with tumour size, lymph node metastasis and angiogenesis (208, 272, 275, 287-288). Interestingly, aberrant P-cadherin expression identifies a subgroup of high-grade ductal carcinoma *in situ*, which lacks ER expression, has high proliferation rates and nuclear pleomorphism (274). Still, by immunohistochemistry, we have found that this protein, together with other well described basal markers, such as CK5 constitutes one of the most useful adjunctive markers for distinguishing the precursor basal-like lesions of DCIS (91). In addition, we still found that P-cadherin antibody has a high sensitivity to identify invasive basal-like carcinomas, compared with the one demonstrated for the "gold-standard" pair CK5/EGFR (see PAPER VII in Appendix section). Overall, these findings largely contributed to recognize P-cadherin expression as a good indicator of poor prognosis in breast cancer patients (208, 249, 272, 275, 286).

1.6.2 - *CDH3*/P-Cadherin Regulation in Breast Cancer

Signalling pathways or other cellular mechanisms that are involved in the regulation of cadherin-mediated adhesion are thought to underlie the dynamics of the adhesive interactions between cells (255, 289). Additionally, it is known that the expression of an inappropriate cadherin can result from growth factors and hormones stimulation in the tumour environment, as well as from changes in the promoter regions of cadherin encoding genes (246). However, although P-cadherin associated functions in breast cancer, as well as its role in the molecular pathology setting have been extensively studied, little is known about the signalling pathways and mechanisms involved in the regulation of P-cadherin expression in breast cancer cells.

1.6.2.1 - P-Cadherin Transcriptional and Post-Translational Regulation

Data concerning the *CDH3* promoter regulation by transcription factors is still very limited. Recently, it was demonstrated that *CDH3* gene is a direct transcriptional target of

p63 (290) and this evidence is supported by a study from Carrol *et al.*, where it is suggested that p63 play an import role in the regulation of gene expression programs involved in cell adhesion (291). In addition, in a study using basal mammary epithelial cells, it was shown that β-catenin is associated with *CDH3* promoter and activates its expression independently of LEF/TCF in a cell-type specific-manner. Down-regulation of endogenous β-catenin levels, by RNA interference technique, inhibited P-cadherin promoter activity. *In vivo*, in mammary gland and skin of mutant mice, activation of β-catenin signalling correlates with up-regulation of P-cadherin expression, suggesting that β-catenin-dependent modulation of P-cadherin expression can contribute to the establishment of the basal phenotype (292).

In 2004, our group explored the link between ERα signalling and the regulation of P-cadherin expression in breast cancer cell lines, since we found that breast tumours positive for P-cadherin expression were essentially ERα negative. Taking support in what was previously suggested by Gamallo and co-workers for invasive breast cancer, and by Soler and colleagues in prostate cancer, we postulated that P-cadherin could, in fact, be related to a phenotype that is insensitive to circulating hormones. Indeed, in our study, we showed that P-cadherin *de novo* expression was a result from a lack of ERα signalling, induced by the pure anti-oestrogen ICI and counteracted by E2 (282). In fact, cell lines treated with ICI showed a two to three-fold increase of P-cadherin mRNA and protein levels in a time and dose dependent manner (282). Importantly, these findings established that the lack of ERα signalling is responsible for the increase in P-cadherin, categorizing *CDH3* as a putative oestrogen-repressed gene.

P-cadherin post-translational regulation mechanisms are largely unexplored. However, as it has been described for others classical cadherins (293), p120-catenin (p120ctn) has demonstrated a key role in P-cadherin control at protein level (294-295). Our group has shown that P-cadherin is unable to induce cancer cell invasion when its juxtamembrane domain (JMD) is not present or when p120ctn binding-domain is mutated, suggesting an important role for this catenin in the P-cadherin function (263). In fact, the JMD binding to p120ctn has been already implicated in the stable maintenance of endogenous cadherins (294-295). In a pancreatic cell model, Taniuchi *et al*. demonstrated that overexpression of P-cadherin was strongly associated with cytoplasmic accumulation of p120ctn and cadherin

switching (N-cadherin to P-cadherin) in pancreatic cancer cells (PDAC cells), suggesting that alteration of p120ctn cell distribution may be due to its binding strength, conformation or different affinity for each classical cadherin. In this model, cytoplasmic p120ctn was able to activate Rho-family GTPases, Rac1 and Cdc42, promoting cell migration and motility (281). In accordance to this, very recently was confirmed by us that P-cadherin^{-}/E-cadherin^{+} breast carcinomas generally maintain p120ctn expression at the membrane, while tumours expressing P-cadherin exhibit cytoplasmic p120ctn immunostaining, especially those also co-expressing E-cadherin. Thus, the assumption that the P-cadherin-induced invasion is achieved as a result of competition for p120ctn, through destabilization of anti-invasive cadherin/catenin complexes has been considered (263). Alternatively, P-cadherin could simply generate a specific pro-invasive signal via its JMD, possibly with the recruitment of other protein binding to P-cadherin, resulting in the activation of pathways that overcome the suppressive signals mediated by endogenous cadherins (263). It is interesting to highlight, that, in a clinical setting, a poor prognosis in breast cancer patients was found to be associated with the cytoplasmic accumulation of p120-catenin and co-expression of P- and E-cadherin (263).

1.6.2.2 - *CDH3*/P-Cadherin Epigenetic Modulation

Changes in the promoter regions of cadherin encoding genes can trigger abnormal expression of these important adhesion molecules, some of them associated with cancer. A classic example is the silenced expression of E-cadherin in gastric diffuse cancer and as well as in lobular carcinoma of the breast, which in both cases, is due to changes in DNA methylation or chromatin acetylation events at *CDH1* gene (246).

The epigenetic deregulation of P-cadherin was recently demonstrated in other cancer models. Sato *et al.* identified *CDH3* gene promoter to be aberrantly methylated in 20% of pancreatic cancers, but not in normal pancreatic epithelia, which displayed an unmethylated promoter pattern (296). Similar results were obtained in prostate cancer cell lines, although without finding an association between loss of expression of P-cadherin with promoter methylation status of *CDH3 in vivo* (297). In another study, although conducted restrictedly in cell lines, evidences showed that P-cadherin gene seems to be silenced by methylation in

melanoma cells (298). *CDH3* was also shown to be epigenetically deregulated in colorectal cancer; however, in contrast with the pancreatic model, the *CDH3* promoter was found hypomethylated in colonic aberrant crypt foci, in colorectal cancer, and, occasionally, in the normal epithelium adjacent to cancer. This hypomethylation pattern was also associated with the induction of P-cadherin expression in the neoplastic colon. Additionally, the epigenetic demethylation of the P-cadherin promoter in the human intestine permits its ectopic expression very early in the colorectal adenoma-carcinoma sequence and persists during invasive cancer (299). Similarly, demethylation of the *CDH3* gene was recently detected in 25 out of the 36 (69%) primary gastric carcinomas and was significantly associated with increasing TNM stage (p=0.0261), suggesting that the aberrant demethylation of *CDH3* is also a frequent event in gastric carcinomas (300). Very recently, a study using in a large series of gastric tumours, showed that there is a correlation between P-cadherin expression and *CDH3* promoter hypomethylation, and also that in nonneoplastic gastric mucosa P-cadherin expression was silenced. Additionally, they also demonstrated the existence of a prognostic value for the methylation pattern of *CDH3* promoter in gastric tumours (301).

In breast cancer, a complete lack of data concerning *CDH3* promoter methylation pattern was observed until 2005, when we published a study where we evaluated the P-cadherin promoter methylation as the putative molecular mechanism underlying the overexpression of P-cadherin in a subset of highly aggressive breast carcinomas (208) (see PAPER I in Appendix section). As a previous approach, and in order to assess if *CDH3* promoter would be prone to be regulated by methylation, breast cancer cell lines displaying low levels of P-cadherin were treated with 5-Aza-2'-deoxycytidine demethylating agent (5-Aza-dC) and increased P-cadherin mRNA and protein expression levels were observed in comparison with the untreated control cells. Additionally, P-cadherin promoter methylation was analyzed in five cases of normal breast tissue, from which only epithelial cells were microdissected. In these, methylation of P-cadherin gene was found in all the cases analyzed, because none of the samples showed the presence of unmethylated alleles. Indeed, these results were highly correlated with P-cadherin expression because normal breast epithelial cells are negative for this protein. Conversely, in a large series of invasive breast carcinoma samples, methylation of P-cadherin gene was found in 58% and when

these results were correlated with P-cadherin expression, a statistically significant association was found between these variables: 71% of P-cadherin-negative cases were methylated, whereas 65% of positive cases were unmethylated. These results established for the first time the existence of a significant correlation between P-cadherin overexpression and hypomethylation of a specific region of *CDH3* gene promoter, suggesting an important regulatory role for cytosine methylation in the aberrant expression of P-cadherin in breast cancer (208) (see PAPER I in Appendix section).

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2. Expression of FOXA1 and GATA-3 in Breast Cancer: The Prognostic Significance in Hormone Receptor-Negative Tumours

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Expression of FOXA1 and GATA-3 in breast cancer: the prognostic significance in hormone receptor-negative tumours

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Abstract

Introduction The expression of additional genes, other than oestrogen receptor (ER), may be important to the hormone-responsive phenotype of breast cancer. Microarray analyses have revealed that forkhead box A1 (FOXA1) and GATA binding protein 3 (GATA-3) are expressed in close association with ERα, both encoding for transcription factors with a potential involvement in the ERα-mediated action in breast cancer. The purpose of this study was to explore if the expression of FOXA1 and GATA-3 may provide an opportunity to stratify subsets of patients that could have better outcome, among the ERα-negative/poor prognosis breast cancer group.

Methods We evaluate FOXA1 and GATA-3 expression in 249 breast carcinomas by immunohistochemistry, associating it with breast cancer molecular markers, clinicopathological features and patient's survival. The clinicopathological features and immunohistochemical markers of the tumours were compared using the chi-square test and ANOVA. Disease-free survival was analysed through Kaplan–Meier survival curves and Cox regression.

Results FOXA1 expression was demonstrated in 42% of invasive carcinomas, while GATA-3 was detected in 48% of the cases. FOXA1 expression was inversely associated with tumour size, Nottingham Prognostic Index, histological grade, lymph vascular invasion, lymph node stage and human epidermal growth factor receptor-2 (HER-2) overexpression, while GATA-3 expression showed inverse association with histological grade and HER-2. Both FOXA1 and GATA-3 were directly associated with ERα and progesterone receptor. Among FOXA1-positive tumours, 83.1% are comprised in the luminal A subtype, similar to GATA-3 where 87.7% of positive tumours were classified within this molecular subtype. In the subset of ERα-negative patients, those who were FOXA1-negative had a 3.61-fold increased risk of breast cancer recurrence when compared with the FOXA1-positive.

Conclusions FOXA1 was a significant predictor of good outcome in breast cancer, whereas GATA-3 was an important luminal marker. The expression of FOXA1 may be used for risk stratification among ERα-negative patients.

CK: cytokeratin; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; FOXA-1: forkhead box A1; GATA-3: GATA binding protein 3; HER-2: human epidermal growth factor receptor 2; PBS: phosphate-buffered saline; P-cadherin: placental cadherin; PR: progesterone receptor.

Introduction

The expression of oestrogen receptor (ER) is an important prognostic and predictive factor in breast cancer and has relevant implications for the biology of this type of carcinomas. Patients with tumours that express ER have a longer disease-free interval and overall survival than patients with tumours lacking ER expression [1].

According to international treatment guidelines for early breast cancer, patients with ERα and/or progesterone receptor (PR) expression should receive an adjuvant endocrine therapy, since their expression is associated with higher response rates to anti-hormonal treatment [2]. However, the association between ERα expression and hormonal responsiveness is far from perfect, since approximately 30% of ER-positive tumours do not respond to hormonal treatment and 5 to 15% of ER-negative tumours curiously respond to endocrine therapy [3].

In order to overcome and explore this unpredictable breast tumour behaviour, numerous studies, based on cDNA microarrays, have shown that the gene expression profile in breast cancer can provide molecular phenotypes that identify distinct tumour subclasses [4-6], patient survival prediction [5-7], and differences in tumour biology or clinical features. The molecular classification of breast cancers distinguishes three major subtypes: the ER-positive/luminal-like subtype, a gene expression cluster characteristic of the luminal cells and anchored by a cluster of transcription factors that include ER; the basal-like subtype, comprising tumours that express basal cell markers (namely keratin 5, keratin 14, integrin β_4 and laminin); and the human epidermal growth factor receptor-2 (HER-2)-overexpressing subtype, usually associated with gene amplification of the HER-2 proto-oncogene and high expression of several genes in the ERBB2 amplicon at 17q22.24 [4,5,8]. These studies have largely contributed to understanding the complex behaviour of certain types of breast cancer, including the ones that respond better to endocrine therapies, regardless of ER expression.

Oestrogen plays an important role in the regulation of growth, proliferation and differentiation of mammary epithelium. The action of oestrogen is mediated through the ER, which functions as an oestrogen-activated transcription factor. The expression of an additional set of genes that is not part of the canonical oestradiol-response pathway may also be essential in clarifying the hormone-responsive phenotype, since intrinsic differences in the list of transcription factors bound to the *ER* gene promoter have been described [9].

Additionally, the distinct behaviour observed between ER-positive luminal subtypes A and B (a subgroup of tumours with low to moderate expression of the luminal-specific genes including the ER cluster) may in part be due to the influence of additional factors, including transcriptional factors, co-activators and co-repressors modulating ER activity [10], which can also be explored towards a therapeutic purpose.

In 2004 Lacroix and Leclercq compiled considerable extensive data describing the strong association and cross-talk between ERα, forkhead box A1 (FOXA1) and GATA binding protein 3 (GATA-3) [11]. In most of these studies, GATA-3 and FOXA1 have been highlighted within the ERα pathway in the luminal A subtype [4-6,12]; FOXA1, a forkhead family transcription factor, has been receiving considerable attention, since it interacts with *cis*-regulatory regions of heterochromatin, enhancing the interaction of ERα with DNA [13]. Carroll and colleagues recently described several robust data demonstrating the requirement of FOXA1 for optimal expression of nearly 50% of ERα-regulated genes and oestrogen-induced proliferation [13,14].

FOXA1 is expressed in the liver, pancreas, bladder, prostate, colon and lung, as well as in the mammary gland, and can bind to the promoters of more than 100 genes associated with metabolic processes, regulation of signalling pathways and cell cycle [15-17]. Some studies have shown that FOXA1 can act either as a growth stimulator/activator or as a repressor. As a stimulator, FOXA1 binds to chromatinised DNA and opens the chromatin, enhancing binding of ERα to its target genes [18] – which suggests a growth-promoting role for this forkhead protein [14,18]. In breast cancer, however, FOXA1 overexpression can also block the metastatic progression by influencing the expression of the BRCA1-associated cell-cycle inhibitor p27 and promoting E-cadherin expression [19,20]. Recent studies also suggest FOXA1 as a favourable prognostic factor in breast cancer, with potential relevance in the subclassification of luminal/ER-positive tumours into two subgroups with different biological behaviour and prognosis, the luminal A and the luminal B [5].

FOXA1 and ERα have been explored as potential participants involved in mammary tumours together with another gene, GATA-3 [21,22], which regulates the lineage determination and differentiation of many cells types. In the breast, GATA-3 plays a central role in luminal epithelia differentiation and the subsequent formation of the ductal tree of differentiated epithelial cells [23], suggesting that this protein might be involved in breast tumorigenesis [24].

Meta-analysis of four microarray datasets indicated that GATA-3 was a strong predictor of clinical outcome in breast tumours and is among the best predictors of ER-positive status [4,9,25-27]. Among all of the molecular subgroups of breast cancer, the luminal A subtype has a relatively favourable outcome and the highest GATA-3 and ERα expression levels, compared with luminal B and basal-like breast carcinomas [24].

As a result of all of these extensive studies underlying GATA-3 and ERα in mammary epithelia, it has been clear that GATA-3 is a crucial regulator of tumour differentiation and suppressor of tumour dissemination [22]. It has been also suggested that these functions in mammary luminal cells may be linked by transcriptional regulators, whereas FOXA1 appears as a candidate gene, which is necessary for the transcriptional activity of ERα and its binding to oestrogen-responsive elements in target gene promoters [13,18]. As FOXA1 may also be a downstream effector of GATA-3, it may be a bridge between GATA-3 and ER pathways [22], controlling and regulating the biology of luminal mammary cells, breast cancer progression and behaviour.

Based on this intricate and functional complex between FOXA1 and GATA-3 in breast cancer biology, it is reasonable to consider that these transcription factors, in addition to ERα, are important in establishing and clarifying the hormone-responsive phenotype and prognosis in breast cancer. This gene set may therefore be used as a diagnostic tool for more accurate determination of ERα status, in the decision on endocrine therapeutic strategies, as well as in the assessment of breast cancer patient's outcome.

In the present study we provide an immunohistochemical approach studying FOXA1 and GATA-3 expression, in order to predict the tumour behaviour of breast cancer patients. In the whole series, we verified that patients harbouring FOXA1-positive tumours show a better disease-free survival. Interestingly, and for the first time, we also found the same power of risk stratification among the ERα-negative breast cancer patients, demonstrating the clinical importance of this biomarker in breast cancer molecular classification and prognosis. These results show that FOXA1 and ERα should be used together in order to subclassify breast carcinomas and to predict the outcome of breast cancer patients.

Materials and methods

Patient selection

A series of 249 cases of primary operable invasive breast carcinomas were retrieved from the files of the Department of Pathology, Hospital do Divino Espírito Santo, Azores, Portugal and from the Federal University of Santa Catarina, Florianopolis – SC, Brazil. These samples were obtained from patients with age ranging from 30 to 89 years. All of the formalin-fixed paraffin-embedded histological sections were reviewed by three pathologists (VC, FS and FM) and the diagnoses were confirmed as follows: 208 invasive ductal carcinomas, seven invasive lobular carcinomas, three mixed breast carcinomas, three tubular breast carcinomas, eight medullary breast carcinomas and 20 invasive breast carcinomas of other special histological types. These tumours have been fully characterized for clinical and pathological features – namely, age, tumour size, histological type, lymph nodes invasion, tumour grade, Nottingham Prognostic Index, ERα, PR and HER-2 status. The patients' clinical and pathological characteristics are summarized in Table 1.

Follow-up information was available for 218 cases, ranging from a minimum of 2 months to a maximum of 129 months (median 32 months). The disease-free survival data interval was evaluated and defined as the time from the date of surgery to the date of breast-cancer-derived relapse/metastasis. Owing to the short follow-up of the studied series and the consequent limited number of death events, overall survival was not analysed.

The present study was conducted under the national regulative law for the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

Tissue microarray construction and immunohistochemical analysis

Representative areas of different lesions were carefully selected on haematoxylin and eosin-stained sections and were marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and were precisely deposited into a recipient paraffin block using a tissue microarray workstation (tissue microarray builder ab1802; Abcam, Cambridge, UK) as described elsewhere [28,29]. In each tissue microarray block, non-neoplastic breast tissue cores were also included as controls.

Immunohistochemistry was performed in 3 μm formalin-fixed, paraffin-embedded sections. Expression for the most commonly used breast cancer markers – namely, HER-2, ER, PR, P-cadherin, epidermal growth factor receptor (EGFR), vimentin and basal cytokeratins (CK5, CK14) – was assessed. The immunohistochemistry technique was performed using an Envision Detection System (DAKO Cytomation Envision System HRP; DAKO Corporation, Carpinteria, CA, USA) or the classical streptavidin – avidin – biotin complex method according to the manufacturer's instructions. Imunohistochemistry assay conditions and antibodies specifications were based on previously published studies from our group [28-30]. Immunoreactivity for ERα, PR, P-cadherin, CK5, CK14, EGFR, vimentin and HER-2 was classified by estimating the percentage of tumour cells showing characteristic staining, in accordance with previous work [28-30].

Expression of FOXA1 was analysed using a mouse monoclonal antibody (clone 2F83, ab40868; AbCam), as well as GATA-3 expression (clone H-63-31, Sc-268; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Sections were deparaffinized with xylene and rehydrated in a series of decreasing concentration of ethanol solutions. Heat-induced epitope retrieval was carried out in 10 mM citrate buffer (sodium citrate) (pH 6) or in 1 mM ethylenediamine tetraacetic acid buffer (pH 8) (LabVision Corporation, Fremont, CA, USA), in a 98°C

Table 1

Patient characteristics and tumour parameters

Variable	Data
Age at diagnosis (years)	
Mean and standard deviation	57 ± 14.2
Range	59 (minimum 30; maximum 89)
Tumour size (mm)	
Mean and standard deviation	32 ± 21 mm
Range	145 (minimum 5; maximum 150)
Lymphovascular invasion	
Present	111 (44.6)
Absent	111 (44.6)
Not assessed	27 (10.8)
Lymph node sage	
Negative	111 (44.6)
1 to 3 lymph nodes	57 (22.9)
>3 lymph nodes	54 (21.7)
Not assessed	27 (10.8)
Tumour grade	
Grade I	51 (20.5)
Grade II	116 (46.6)
Grade III	82 (32.9)
Histology	
Invasive ductal carcinoma (not otherwise specified)	208 (83.5)
Invasive lobular carcinoma	7 (2.8)
Mixed	3 (1.2)
Tubular	3 (1.2)
Medullary	8 (3.3)
Other special types	20 (8.0)
Nottingham Prognostic Index	
<3.4	46 (18.5)
3.4 to 5.4	106 (42.6)
>5.4	55 (22.0)
Not assessed	42 (16.9)
Oestrogen receptor-α status	
Positive	141 (56.6)
Negative	107 (43.0)
Unknown	1 (0.4)
Progesterone receptor status	
Positive	89 (35.8)
Negative	154 (61.8)
Unknown	6 (2.4)
Human epidermal growth factor receptor 2 status	
Positive	42 (16.9)
Negative	201 (80.7)
Unknown	6 (2.4)

Data presented as *n* (%) unless stated otherwise.

water bath, for 14 and 20 minutes for FOXA1 and GATA-3, respectively. After cooling retrieval solutions for at least 30 minutes at room temperature, the slides were treated for 10 minutes with 3% H_2O_2 in methanol, in order to block endogenous peroxidase. Slides were incubated with monoclonal antibodies for FOXA1 (1:450) and GATA-3 (1:100) for 2 hours at room temperature and were labelled with the Envision Detection System from DAKO. Colour reaction product was developed with 3,3'-diaminobenzidine, tetrahydrochloride (DAB plus; DAKO Glostrup, Denmark) as a substrate, and nuclear contrast was achieved with haematoxylin/ammoniacal water counterstaining. Formalin-fixed, paraffin-embedded sections from normal breast gland were used as FOXA1 and GATA-3 positive controls. Negative controls were performed by replacing the primary antibody with PBS/nonimmune mouse serum.

The scoring method used for FOXA1 and GATA-3 expression was based on a semi-quantitative scoring system previously described by Thorat and colleagues, where the cutoff value for FOXA1 positivity was validated [31]. In this scoring system, the percentage of staining was categorized as: 0 = no nuclear expression; 1 = 1 to 10% positive tumour nuclei; 2 = 11 to 20%; and so on until a maximum score of 10 = 91 to 100% positive tumour nuclei. The intensity was scored as: 1+ = weak staining; 2+ = moderate staining; and 3+ = strong staining. The numeric final score was generated by the multiplication product of percentage and intensity of nuclear expression (scoring = percentage × intensity) [10,32]. Based on this semiquantitative scoring system, scores between 0 and 3 were classified as negative, and scores ≥ 4 to a maximum of 30 were considered positive.

Statistical analysis

Statistical analysis was performed using Stata™, version 9.2 software (StataCorp, College Station, TX, USA). Continuous variables were presented as the mean ± standard deviation, and categorical variables were presented as the number (percentage). The clinicopathological features and immunohistochemical markers of the tumours were compared across groups of expression of FOXA1 and GATA-3 using analysis of variance and the chi-square test, respectively, for continuous and categorical variables.

Survival curves were estimated by the Kaplan – Meier method using the log-rank test to assess significant differences for disease-free patient survival. A maximum cutoff value of 60 months (5 years) was considered, since this is the expected clinical time for breast cancer recurrence. Cox regression models were fitted to estimate hazard ratios and the corresponding 95% confidence interval for the classical prognostic factors, FOXA1 and GATA-3. In all analyses, a significant level of 5% was considered.

Results

FOXA1 and GATA-3 expression in normal and malignant breast tissues

From the total 249 cases, only cases with clear and restricted nuclear expression for FOXA1 and GATA-3 were selected for immunohistochemistry classification. Three representative cases were selected to build a panel, illustrated in Figure 1, comprising a classical example of the following molecular subtypes of breast cancer: luminal A subtype (Figure 1, L1 to L7), basal-like subtype (Figure 1, B1 to B7) and HER-2-overexpressing subtype (Figure 1, H1 to H7). Strong immunoexpression of FOXA1 and GATA-3 in the nuclei of malignant cells, as well as in some luminal epithelial cells from adjacent normal ducts, is shown in Figure 1 (L3 and L4). FOXA1 was positive (score ≥ 4) in the nuclei of 42% (93 out of 224) of the invasive carcinomas, while GATA-3 was detected in 48% (97 out of 204) of the cases.

Association between FOXA1 and GATA-3 expression and clinicopathological features and biological markers

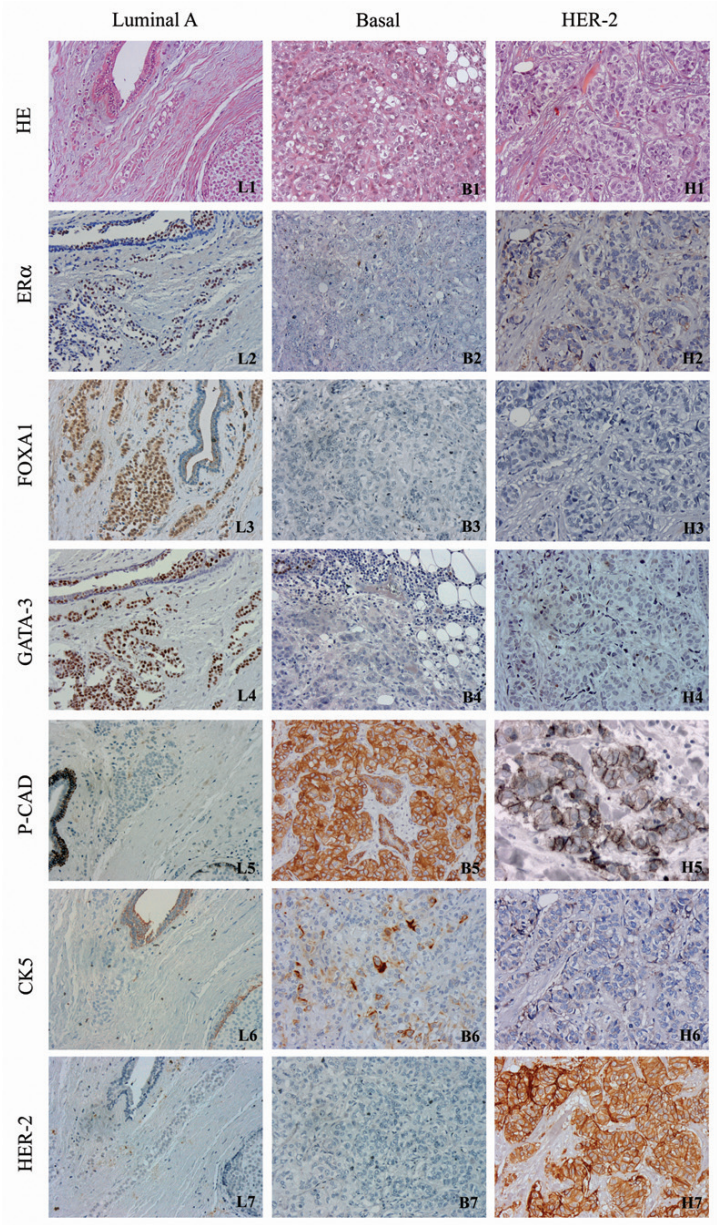
The expression of FOXA1 was inversely associated with tumour size ($P = 0.005$), Nottingham Prognostic Index ($P = 0.002$), histological grade ($P = 0.001$), vascular invasion ($P = 0.012$), lymph node stage ($P = 0.022$) and HER-2 overexpression ($P = 0.017$), and was directly associated with ERα expression ($P < 0.0001$) and PR expression ($P < 0.0001$). GATA-3 expression showed an inverse association with histological grade ($P = 0.013$) and HER-2 overexpression ($P < 0.0001$), and a direct association with ERα expression ($P < 0.001$) and PR expression ($P < 0.001$) (Table 2).

When we compared the expression of FOXA1 and GATA-3 with the molecular subtype, we found that 83.1% and 87.7% of FOXA1 and GATA-3, respectively, were comprised in the luminal A subtype ($P < 0.0001$) (Table 3 and Figure 1, L1 to L7). Basal-like subtype tumours were negative for FOXA1 (Figure 1, B3) and for GATA-3 (Figure 1, B4) in 85.7% and 84.6% of the cases, respectively (Table 3).

The immunohistochemical evaluation of FOXA1 and GATA-3 in breast tumour samples revealed that in 201 of interpretable cases a very significant direct association between the expression of FOXA1 and GATA-3 was observed ($P < 0.0001$) (Table 3).

On the evaluation of these two transcription factors with other important immunohistochemical markers in breast cancer, we found a strong inverse association with basal-like phenotype markers – namely, CK14 ($P = 0.007$, $P = 0.0002$), CK5 ($P = 0.027$, $P < 0.0001$), vimentin ($P = 0.003$, $P = 0.0006$) and P-cadherin ($P = 0.012$, $P < 0.0001$) for FOXA1 and GATA-3, respectively. GATA-3, but not FOXA1, showed an interesting inverse association with EGFR ($P = 0.001$) (Table 3).

Figure 1



Immunohistochemistry panel showing differential expression pattern of FOXA1 and GATA-3. An example of luminal A **(L1 to L7)**, basal-like **(B1 to B7)** and human epidermal growth factor receptor 2 (HER-2)-overexpressing **(H1 to H7)** invasive breast tumours. Expression of the most commonly used breast cancer markers is also illustrated for comparison with the forkhead box A1 (FOXA1) and GATA binding protein 3 (GATA-3) expression. (L1, B1, H1) Haematoxylin-eosin stainings from each of the selected core cases. (L3, L4) Strong and restricted nuclear expression of FOXA1 and GATA-3 in the normal breast duct (internal control) and in the luminal A invasive tumour (grade II). (B3, B4) Negative expression of FOXA1 and GATA-3 in basal subtype tumour (grade III). (H3, H4) HER-2-overexpressing tumour showing negativity for FOXA1 and GATA-3 expression (grade III). All microscopy images are at 40× magnification. ER, oestrogen receptor; P-CAD, P-cadherin; CK, cytokeratin.

Survival and patient outcome analysis

Kaplan–Meier survival curves demonstrate that patients with FOXA1-positive breast carcinomas showed a significant difference towards the longer disease-free survival time ($P < 0.001$; Figure 2a). Although these are no statistically significant differences in survival according to GATA-3 expression ($P = 0.055$; Figure 2b), the positivity for this marker is also associated with a better outcome for breast cancer patients.

As previously demonstrated in other studies, univariate Cox proportional hazard analysis showed that the tumour size, lymph node stage, tumour grade, as well as the expression of ER, PR and HER-2 were significant predictors for disease-free

Table 2

Association between FOXA1 and GATA3 expression and the clinicopathological features of the infiltrative breast carcinomas

Variable	*N*	FOXA1-negative (%)	FOXA1-positive (%)	*P* value	*n*	GATA3-negative (%)	GATA3-positive (%)	*P* value
Tumour size (mm)	209	35.2 ± 24.0 (126)	26.8 ± 15.7 (83)	0.005	191	34.4 ± 25.2 (100)	28.6 ± 16.7 (91)	0.064
Lymphovascular invasion	203				185			
Present	97	67 (69.1)	30 (30.9)	0.012	92	49 (53.3)	43 (46.7)	0.829
Absent	106	55 (51.9)	51 (48.1)		93	51 (54.8)	42 (45.2)	
Lymph node stage	203				185			
Negative	106	55 (51.9)	51 (48.1)	0.022	93	51 (54.8)	42 (45.2)	0.166
1 to 3 lymph nodes	49	31 (63.3)	18 (36.7)		46	20 (43.5)	26 (56.5)	
>3 lymph nodes	48	36 (75)	12 (25)		46	29 (63)	17 (37)	
Grade	224				204			
Grade I	44	25 (56.8)	19 (43.2)	0.001	40	24 (60)	16 (40)	0.013
Grade II	105	50 (47.6)	55 (52.4)		96	40 (41.7)	56 (58.3)	
Grade III	75	56 (74.7)	19 (25.3)		68	43 (63.2)	25 (36.8)	
Histology	224				204			
IDC	188	113 (60.1)	75 (39.9)	0.119	171	91 (53.2)	80 (46.8)	0.104
ILC	6	2 (33.3)	4 (66.7)		6	0 (0)	6 (100)	
Tubular	1	1 (100)	0 (0)		1	0 (0)	1 (100)	
Medullary	7	5 (71.4)	2 (28.6)		7	5 (71.4)	2 (28.6)	
Other	19	7 (36.8)	12 (63.2)		16	9 (56.2)	7 (43.8)	
Mixed	3	3 (100)	0 (0)		3	2 (66.7)	1 (33.3)	
Nottingham Prognostic Index	190				172			
<3.4	44	18 (40.9)	26 (59.1)	0.002	40	19 (47.5)	21 (52.5)	0.293
3.4 to 5.4	96	59 (61.5)	37 (38.5)		86	44 (51.2)	42 (48.8)	
>5.4	50	38 (76)	12 (24)		46	29 (63)	17 (37)	
ERα	224				204			
Positive	133	60 (45.1)	73 (54.9)	<0.0001	122	41 (33.6)	81 (66.4)	<0.0001
Negative	91	71 (78)	20 (22)		82	66 (80.9)	16 (19.5)	
PR	223				204			
Positive	83	33 (39.7)	50 (60.3)	<0.0001	74	22 (29.7)	52 (70.3)	<0.0001
Negative	140	98 (70)	42 (30)		130	85 (65.4)	45 (34.6)	
HER-2	220				201			
Positive	35	27 (77.1)	8 (22.9)	0.017	34	29 (85.3)	5 (14.7)	<0.0001
Negative	185	103 (55.7)	82 (44.3)		167	77 (46.1)	90 (53.9)	

FOXA-1, forkhead box A1; GATA-3, GATA binding protein 3; IDC, invasive ductal carcinoma (not otherwise specified); ILC, invasive lobular carcinoma; ERα, oestrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

Table 3

Association between FOXA1 and GATA3 expression and the immunohistochemical markers in infiltrative breast carcinomas

Variable	*N*	FOXA1-negative (%)	FOXA1-positive (%)	*P* value	*n*	GATA3-negative (%)	GATA3-positive (%)	*P* value
EGFR	223				203			
Positive	13	10 (7.6)	3 (3.3)	0.171	11	11 (10.4)	0 (0)	0.001
Negative	210	121 (92.4)	89 (96.7)		192	95 (89.6)	97 (100)	
P-cadherin	220				202			
Positive	75	53 (40.8)	22 (24.4)	0.012	71	52 (49.1)	19 (19.8)	<0.0001
Negative	145	77 (59.2)	68 (75.6)		131	54 (50.9)	77 (80.2)	
Cytokeratin 5	224				204			
Positive	50	36 (27.5)	14 (15)	0.027	48	39 (36.4)	9 (9.3)	<0.0001
Negative	174	95 (72.5)	79 (85)		156	68 (63.6)	88 (90.7)	
Cytokeratin 14	219				201			
Positive	14	13 (10.1)	1 (1.5)	0.007	14	14 (13.3)	0 (0)	0.0002
Negative	205	116 (89.9)	89 (98.9)		187	91 (86.7)	96 (100)	
Vimentin	203				194			
Positive	34	28 (23.1)	6 (7.3)	0.003	32	26 (25)	6 (6.7)	0.0006
Negative	169	93 (76.9)	76 (92.7)		162	78 (75)	84 (93.3)	
FOXA1	-	-	-	-	201			
Positive	-	-	-		82	16 (15.4)	66 (68)	<0.0001
Negative	-	-	-		119	88 (84.6)	31 (32)	
GATA-3	201				-	-	-	-
Positive	97	31 (26.1)	66 (80.5)	<0.0001	-	-	-	
Negative	104	88 (73.9)	16 (19.5)		-	-	-	
Subtype	202				187			
Luminal A	125	56 (47)	69 (83.1)	<0.0001	114	36 (36.7)	78 (87.7)	<0.0001
Luminal B	8	4 (3.4)	4 (4.8)		8	5 (5.1)	3 (3.4)	
HER-2	27	23 (19.3)	4 (4.8)		26	24 (24.5)	2 (2.2)	
Basal	42	36 (30.3)	6 (7.3)		39	33 (33.7)	6 (6.7)	

FOXA-1, forkhead box A1; GATA-3, GATA binding protein 3; EGFR, epidermal growth factor receptor; HER-2, human epidermal growth factor receptor 2.

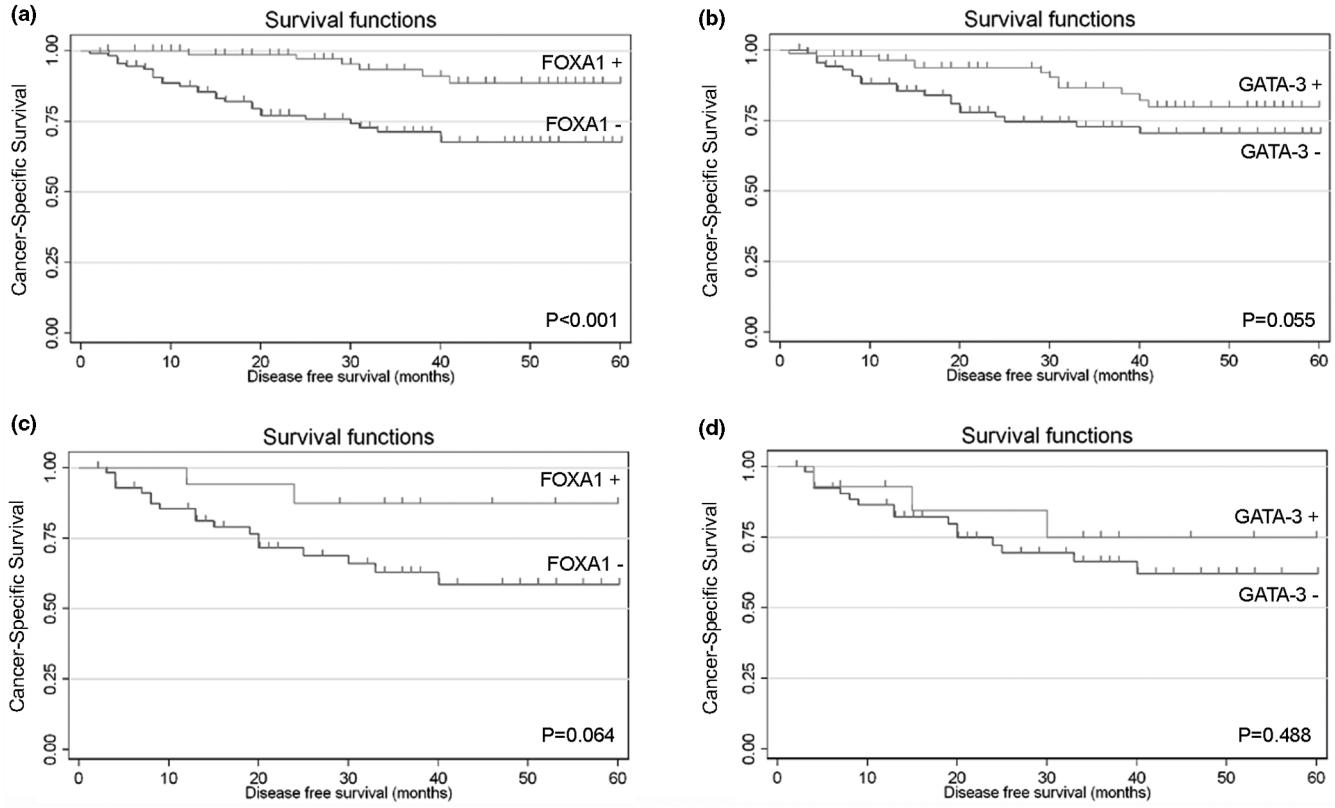
survival. In accordance with the trend shown by the Kaplan–Meier curves, the expression of FOXA1 was also a significant predictor for disease-free survival, showing that negative cases carry a fourfold increased risk of recurrence (hazard ratio = 4.25, 95% confidence interval = 1.76 to 10.28) when compared with the positive ones. In contrast, GATA-3 expression was revealed not to be important as a predictive marker for better outcome in this series (hazard ratio = 1.97, 95% confidence interval = 0.96 to 4.01) (Table 4).

Prognostic significance of FOXA1 and GATA-3 expression in ER-negative breast cancer

Several studies have shown that FOXA1 and GATA-3 expression are strong predictors of better clinical outcome in breast tumours and are among the best predictors of ERα-positive status [9-12,26,31,33,34]. Since FOXA1 and GATA-3 show an intrinsic high correlation between themselves and with ERα status, however, the prognostic and predictive value of these markers may simply reflect this high expression association. A cohort of ERα-negative patients was therefore studied in order to evaluate the predictive importance of FOXA1 and GATA-3 expression in this subset of breast carcinomas.

When the association analysis was performed in the subset of ERα-negative patients, FOXA1 and GATA-3 failed to show any significant association with the studied clinicopathological features. Analysing the association of these transcription factors with the immunohistochemical biomarkers in breast can-

Figure 2



Kaplan–Meier survival curves for disease-free survival. **(a)** Survival functions for forkhead box A1 (FOXA1) in the whole breast cancer patient series ($P < 0.001$). **(b)** Survival functions for GATA binding protein 3 (GATA-3) in the whole breast cancer patient series ($P = 0.055$). **(c)** Survival functions for FOXA1 in the oestrogen receptor α-negative breast cancer patient cohort ($P = 0.064$). **(d)** Survival functions for GATA-3 in the oestrogen receptor α-negative breast cancer patient cohort ($P = 0.488$).

cer, no significant associations were found concerning FOXA1 expression. GATA-3 negativity, however, showed significant association with P-cadherin and CK14 expression. Although not statistically significant, we also observed a trend towards the association of GATA-3 with the other studied basal-like phenotype markers (namely, EGFR, CK5 and vimentin), where the majority of the negative cases for GATA-3 are positive for those proteins (Table 5).

In this subset of ERα-negative patients, however, an association between loss of FOXA1 expression and worst disease-free survival was found ($P = 0.064$), in contrast with GATA-3 expression ($P = 0.488$) (Figure 2c and 2d, respectively). Moreover, in order to quantify the risk of these survival associations, univariate analysis was performed for FOXA1 and GATA-3 as well as for the classical prognostic factors in breast cancer. In line with the Kaplan–Meier curves, GATA-3 negativity does not account for an increased risk of recurrence in ERα-negative tumours. FOXA1 expression, however, is able to stratify this relative risk among this subset of carcinomas, since its loss accounts for a 3.61-fold increased risk for breast cancer recurrence (Table 6). These results suggest a protective role for this forkhead protein in this poor-outcome breast cancer subgroup.

Additionally, the multivariate Cox hazard analysis, with models including tumour size and lymph vascular invasion, demonstrates the independent value of FOXA1 expression as a predictor of patient outcome in ERα-negative tumours. FOXA1 negativity is strongly related to breast cancer recurrence, this association being very close to statistical significance (FOXA1-negative vs. FOXA1-positive: hazard ratio = 7.02, 95% confidence interval = 0.92 to 53.37; $P = 0.060$). This analysis also confirmed that GATA-3 expression is not an important predictor of breast cancer recurrence in ERα-negative carcinomas (GATA-3-negative vs. GATA-3-positive patients: hazard ratio = 1.46, 95% confidence interval = 0.40 to 5.29; $P = 0.559$).

Discussion

Several studies of global gene expression revealed high levels of FOXA1 often associated with the expression of ERα [6,35,36]. In addition, other gene whose expression has been highly correlated with ERα in breast cancer encodes the transcription factor GATA-3 [6,11,26,27]. Indeed, FOXA1, GATA-

Table 4

Univariate Cox proportional hazard analysis (disease-free survival) in the whole breast cancer series

Variable	Evaluation	Hazard ratio (95% confidence interval)	*P* value
Tumour size	T1 (≤ 2 mm)	1	
	T2 (2 < T ≤ 5 mm)	1.57 (0.70 to 3.51)	0.265
	T3 (>5 mm)	3.12 (1.16 to 8.41)	0.024
Lymph node stage	Negative	1	
	1 to 3 lymph nodes	0.56 (0.20 to 1.56)	0.272
	>3 lymph nodes	2.68 (1.30 to 5.49)	0.007
Tumour grade	Grade I	1	
	Grade II	2.60 (0.58 to 11.56)	0.208
	Grade III	7.65 (1.80 to 32.48)	0.006
ER expression	ER-positive	1	
	ER-negative	2.94 (1.52 to 5.57)	0.001
PR expression	PR-positive	1	
	PR-negative	2.16 (1.04 to 4.46)	0.038
HER-2/neu expression	HER-2/neu-negative	1	
	HER-2/neu-positive	2.47 (1.19 to 5.09)	0.014
FOXA1 expression	FOXA1-positive	1	
	FOXA1-negative	4.25 (1.76 to 10.28)	0.001
GATA-3 expression	GATA-3-positive	1	
	GATA-3-negative	1.97 (0.96 to 4.01)	0.061

ER, oestrogen receptor; FOXA-1, forkhead box A1; GATA-3, GATA binding protein 3; HER-2, human epidermal growth factor receptor 2; PR, progesterone receptor.

3 and ERα form a transcriptional circuit required for growth, differentiation and hormonal dependency of the lineage of mammary luminal cells [22,37]. Previous work using immunohistochemistry has shown that the expression of FOXA1 [10,31,33] and of GATA-3 [9,34,37] is in close association with ERα expression in breast cancer, highlighting their prognostic and predictive value in this malignancy. In fact, since these three proteins are components of a transcriptional network that dictates the phenotype of hormonal-dependent breast cancer [37], the study of their expression would improve our understanding of the ERα, FOXA1 and GATA-3 relationship in breast cancer patients.

In the present study the staining pattern of FOXA1 and GATA-3 in normal breast tissue is strikingly similar to that of ERα, which suggests the same cellular co-localization of these three cross-functional proteins. In the studied series, the expression of FOXA1 was inversely associated with clinicopathological features – namely, with tumour size, tumour grade, Nottingham Prognostic Index, lymph vascular invasion, lymph node stage and HER-2 overexpression – while its expression was directly associated with ERα, PR and the luminal A subtype. Thorat and colleagues, in a recent published study of 139 cases, did not demonstrate a significant association with tumour size, lymph node status or HER-2 [31]. Moreover, these authors also found an inverse association between FOXA1 and basal-like phenotype markers (namely, CK5 and CK14). Importantly, in our study we reinforced this inverse association between FOXA1 expression and the expression of P-cadherin or vimentin.

The requirement of this forkhead for optimal expression of at least 50% of ERα-regulated genes and oestrogen-induced proliferation was recently described [13], and our and other results may just represent the strong regulatory interdependency between ERα and FOXA1. Since ERα is one of the central genes for the regulation of growth/proliferation of mammary epithelia, and for the hormone-responsive phenotype of breast tumours [9], FOXA1 appears an important biological-regulatory factor with prognostic consequences in this setting. In fact, in the present study, FOXA1 expression was shown to be an important predictor of disease-free survival, in addition to the robust association with clinicopathological features. Interestingly, univariate analysis showed that the evaluation of FOXA1 expression has an important value in the assessment of the prognostic risk for breast cancer patient

Table 5

Association between FOXA1 and GATA3 expression, clinicopathological features and immunohistochemical markers in ER-negative cohort

Variable	*n*	FOXA1-negative (%)	FOXA1-positive (%)	*P* value	*n*	GATA3-negative (%)	GATA3-positive (%)	*P* value
Tumour size	80	36.7 ± 24.2 (65)	36.6 ± 24.8 (15)	0.987	74	34.46 ± 23.6 (60)	43.4 ± 27.4 (14)	0.229
Lymphovascular invasion	83				78			
Present	35	29 (82.8)	6 (17.2)	0.391	35	29 (82.8)	6 (17.2)	0.867
Absent	48	36 (75)	12 (25)		43	35 (81.4)	8 (18.6)	
Lymph node stage	83				78			
Negative	48	36 (75)	12 (25)	0.456	43	35 (81.4)	8 (18.6)	0.609
1 to 3 lymph nodes	12	11 (91.7)	1 (8.3)		12	11 (91.7)	1 (8.3)	
>3 lymph nodes	23	18 (78.3)	5 (21.7)		23	18 (78.3)	5 (21.7)	
Grade	87				80			
Grade I	8	6 (75)	2 (25)	0.166	8	8 (100)	0 (0)	0.366
Grade II	33	23 (69.7)	10 (30.3)		28	22 (78.6)	6 (21.4)	
Grade III	46	40 (86.9)	6 (13.1)		44	36 (81.8)	8 (18.2)	
Nottingham Prognostic Index	76				71			
<3.4	11	9 (81.8)	2 (18.2)	0.982	10	9 (90)	1 (10)	0.615
3.4 to 5.4	34	27 (79.4)	7 (20.6)		32	26 (81.3)	6 (18.7)	
>5.4	31	25 (80.6)	6 (19.4)		29	22 (75.8)	7 (24.2)	
HER-2	85				79			
Positive	26	23 (88.5)	3 (11.5)	0.254	26	24 (92.3)	2 (7.7)	0.102
Negative	59	46 (77.9)	13 (22.1)		53	41 (77.4)	12 (22.6)	
EGFR	86				80			
Positive	12	10 (83.3)	2 (16.7)	0.771	11	11 (100)	0 (0)	0.100
Negative	74	59 (79.7)	15 (20.3)		69	55 (79.7)	14 (20.3)	
P-cadherin	87				80			
Positive	53	45 (84.9)	8 (15.1)	0.107	52	47 (90.4)	5 (9.6)	0.011
Negative	34	24 (70.6)	10 (29.4)		28	19 (67.8)	9 (32.2)	
Cytokeratin 5	87				80			
Positive	33	28 (84.8)	5 (15.2)	0.318	32	29 (90.6)	3 (9.4)	0.118
Negative	54	41 (75.9)	13 (24.1)		48	37 (77.1)	11 (22.9)	
Cytokeratin 14	85				78			
Positive	14	13 (92.8)	1 (7.2)	0.188	14	14 (100)	0 (0)	0.053
Negative	71	55 (77.5)	16 (22.5)		64	50 (78.1)	14 (21.9)	
Vimentin	82				75			
Positive	27	25 (92.6)	2 (7.4)	0.074	25	24 (96)	1 (4)	0.064
Negative	55	42 (76.4)	13 (23.6)		50	40 (80)	10 (20)	
FOXA1	-				78			
Positive	-	-	-	-	15	6 (40)	9 (60)	<0.0001
Negative	-	-	-		63	58 (92.1)	5 (7.9)	
GATA-3	78				-			
Positive	14	5 (35.7)	9 (64.3)	<0.0001	-	-	-	-
Negative	64	58 (90.6)	6 (9.4)		-	-	-	

ER, oestrogen receptor; EGFR, epidermal growth factor receptor; FOXA-1, forkhead box A1; GATA-3, GATA binding protein 3; HER-2, human epidermal growth factor receptor 2.

Table 6

Univariate Cox proportional hazard analysis (disease-free survival) in the oestrogen-receptor-negative cohort

Variable	Evaluation	Hazard ratio (95% confidence interval)	*P* value
Tumour size	T1 (≤ 2 mm)	1	
	T2 (2 < T ≤ 5 mm)	1.45 (0.40 to 5.21)	0.567
	T3 (>5 mm)	3.57 (0.88 to 14.4)	0.073
Lymph node stage	Negative	1	
	1 to 3 lymph nodes	0.47 (0.10 to 2.16)	0.338
	>3 lymph nodes	2.49 (1.02 to 6.03)	0.044
Tumour grade	Grade I	[a]	
	Grade II	1	
	Grade III	2.85 (1.05 to 7.57)	0.040
HER-2/neu expression	HER-2/neu-negative	1	
	HER-2/neu-positive	2.04 (0.90 to 4.61)	0.086
FOXA1 expression	FOXA1-positive	1	
	FOXA1-negative	3.61 (0.83 to 15.60)	0.086
GATA-3 expression	GATA-3-positive	1	
	GATA-3-negative	1.53 (0.44 to 5.28)	0.495

FOXA-1, forkhead box A1; GATA-3, GATA binding protein 3; HER-2, human epidermal growth factor receptor 2. [a]There were no oestrogen-receptor-negative cases classified as grade I among the patients with available follow-up information.

recurrence, with a magnitude of association similar to the observed for the classical prognostic factors, such as tumour size and lymph node stage, tumour grade, and ER and HER-2 expression. This finding is in line with previously published works, where both Badve and colleagues and Habashy and colleagues also demonstrated that FOXA1 expression is able to significantly predict a better survival for breast cancer patients [10,33], although the multivariate analysis showed that it is not an independent prognostic marker, exactly as shown for ER. These studies still suggest that ERα/FOXA1-expressing cells, after acquiring tumorigenicity, may promote selective clonal expansion, resulting in a specific subtype of breast cancer – the luminal subtype A. Thorat and colleagues also suggested that FOXA1 immunohistochemistry may be used as a marker for tumours pertaining to luminal subtype A breast cancer, which has an exceptionally good prognosis [31].

In contrast to FOXA1, GATA-3 failed the association with most of the clinicopathological features – the exception being an inverse association with HER-2 expression and tumour histological grade, although it was also directly associated with ERα and PR expression, as well as with tumours from the luminal A subtype. These results are partially in line with previous work from Mehra and colleagues, which found that low levels of GATA-3 expression were associated with higher tumour histological grade, positive lymph nodes, larger tumour size, negative ER expression and HER-2 overexpression [24]. In the present study we could not find an association between GATA-3 expression and lymph node status, in agreement with a recent cohort study from Voduc and colleagues comprising more than 3,000 invasive breast cancers [34]. Regarding the association between GATA-3 and ERα, 66% of the cases co-expressed these markers – which is a larger percentage than those previously described by Mehra and colleagues (46%) and by Voduc and colleagues (39%) [24,34].

Through the analysis of Kaplan–Meier survival curves it was not possible to demonstrate a significant association between GATA-3 expression and disease-free survival in this breast cancer series, which is in accordance with data from the large cohort study of Voduc and colleagues [34]. The univariate analysis confirmed this observation, although there is an association between the positivity for this marker and the better outcome for breast cancer patients.

Interestingly, the strength of the inverse association that was observed between GATA-3 and basal-like markers – namely, CK5, CK14, vimentin, EGFR and P-cadherin – suggests that GATA-3 can be important for the differentiation state of the malignant cells, where its presence, together with other differentiation involved partners, may drive the luminal profile of a malignant cell population within the tumour. Actually, this growth and differentiation role for GATA-3 in normal mammary

epithelial cells has been already widely described [9,22,34,38]. Moreover, GATA-3-induced genes were found in the luminal cluster of gene expression studies, highlighting its putative ability to maintain a luminal differentiated phenotype [34].

In the past, several studies have shown that FOXA1 expression and GATA-3 expression are among the best predictors of ERα-positive status [9-12,26,31,33,34]. Additionally, some reports have proven that FOXA1 expression is able to significantly differentiate patients with a better survival within the luminal A subgroup, or even within the ERα-positive cohort (including luminal A and luminal B subtypes) [10,31]. These authors claim that FOXA1 can serve as a clinical marker for the luminal A subtype, and that its prognostic ability in these low-risk breast cancers may prove to be useful in clinical treatment decisions. In contrast, Habashy and colleagues did not find any clinical relevance in the immunohistochemical assessment of FOXA1 in breast cancer routine practice [33], since it was not able to stratify ER-positive (luminal-like) tumours into clinically significant subgroups.

Although never assessed, the difference between these studies can be possibly due to the endocrine and chemotherapy administered to the different series of patients, which can block the ERα-associated pathways and confound interpretation of the results. Moreover, since ERα, FOXA1 and GATA-3 show an intrinsic high correlation between themselves, the prognostic and predictive values of these markers may simply reflect this high expression association and the described biological interactions.

In order to study whether there was a prognostic value for the expression of these two transcription factors in the absence of ERα expression, we therefore decided to perform an exploratory subgroup analysis in a cohort of ERα-negative patients. The aim was to test, for the first time, the possible utility of FOXA1 and/or GATA-3 as classifiers for breast cancer recurrence in this high-risk subset of patients, revealing a stratification of ER-negative tumours with different biological behaviours. Interestingly, only FOXA1-positive expression showed a clear protective effect for breast cancer relapse in this cohort of patients with poor prognosis. Patients with loss of FOXA1 tumour expression showed an increased risk for breast cancer recurrence compared with the patients that were positive for this marker. The relative risk estimate was higher than that calculated for HER-2 positivity, which is a well-known prognostic factor in hormone-independent breast carcinomas. Moreover, the multivariate analysis, including the tumour size and lymph node status, demonstrated the independent value of FOXA1 as a predictor of patient outcome in ERα-negative tumours.

In conclusion, our results confirmed the strong association between ERα and FOXA1 in breast cancer and confirmed the role of FOXA1 as a significant breast cancer predictor of good outcome in univariate analysis, directly associated with luminal A and inversely associated with basal-like subtype of breast cancer. GATA-3 was neither a predictor for breast cancer disease-free survival nor a prognostic marker, but was shown to be an important and robust luminal differentiation marker, even stronger than FOXA1. Based on these findings, the expression assessment of FOXA1 and GATA-3 in breast cancer patients can provide important clinical information – not only regarding the favourable prognostic nature and tumour behaviour, but the expression can also constitute an important tool to define and assess the luminal A subtype in breast cancer. We demonstrated that FOXA1 expression also has an important role as breast cancer predictor of good outcome in ER-negative breast carcinomas.

Based on our results, we can consider that the expression of FOXA1, as an ER-associated gene, may be important to the hormone-responsive phenotype of breast cancer, regardless of the tumour ER status. The absence of FOXA1 in luminal/ER-positive breast cancer patients may contribute to identify the 30% of ER-positive tumours that are not hormone responsive. Additionally, because of the known cross-talk and functional network between FOXA1 and the regulation of ERα and its downstream targets, the expression of FOXA1 in ER-negative breast cancer patients may represent the existence of an alternative oestradiol-independent response pathway, which may allow the 5 to 15% of ER-negative tumours to become responsive to endocrine-driven therapies. The clinical implication of these findings requires a larger prospective cohort, especially to evaluate the value of FOXA1 in the therapeutic response setting. Nevertheless, the current study already represents an important step forward in the overview the ER-negative type of tumours, with putative future benefit for staging and treatment of these patients.

Conclusions

Current challenges in oncology include prediction of tumour behaviour and selection of effective therapy for individual treatment based on molecular targets. In breast cancer, ERα expression alone has been used to guide systemic therapy and to estimate patient prognosis. Not all ER-positive carcinomas, however, show comparable prognosis or react similarly to anti-hormonal therapy, and some ER-negative tumours curiously respond to therapy. This clinical evidence demonstrates that breast carcinomas are extremely heterogeneous, emphasizing the need for improving the molecular classification within tumours to better predict their clinical behaviour and the patient's response to current therapies.

The identification of transcription factors that control the ERα pathway provide an opportunity to identify specific subsets of patients that will have a good prognosis, as well as who will benefit from endocrine treatment. In the present work, we studied FOXA1 and GATA-3 expression in order to evaluate

whether the proteins would predict the recurrence behaviour of breast cancer patients. We verified that patients harbouring FOXA1-positive and ER-negative tumours show a better disease-free survival, demonstrating the clinical importance of these two biomarkers in breast cancer molecular classification and prognosis. The analyses showed that FOXA1 and ERα should be used together in order to subclassify breast carcinomas and to predict the outcome of breast cancer patients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AA, BS, DV and NLo carried out all the immunoassays and general laboratory work. FM, VC and FS were the pathologists who revised and classified all cases. NLu, JB and SC performed the statistical analysis. AA and JP contributed to the conception and design, analysis and interpretation of data. AA, JP, FM, EWL and FS were involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

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3. ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBPβ in *CDH3* gene activation

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ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBPβ in *CDH3* gene activation

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***CDH3*/P-cadherin is a classical cadherin. Overexpression of which has been associated with proliferative lesions of high histological grade, decreased cell polarity and poor survival of patients with breast cancer. *In vitro* studies showed that it can be up-regulated by ICI 182,780, suggesting that the lack of ERα signalling is responsible for the aberrant P-cadherin overexpression and for its role in inducing breast cancer cell invasion and migration. However, the mechanism by which ER-signalling inhibition leads to P-cadherin expression is still unknown. The aim of this study was to explore the molecular mechanism linking the ERα-signalling and P-cadherin-regulated expression in breast cancer cell lines. This study showed that ICI 182,780 is able to increase *CDH3* promoter activity, inducing high levels of the active chromatin mark H3 lysine 4 dimethylation. We also observed, for the first time, that the transcription factor C/EBPβ is able to up-regulate *CDH3* promoter activity in breast cancer cells. Moreover, we showed that the expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients. This study demonstrates the existence of an epigenetic regulation by which ICI 182,780 up-regulates P-cadherin expression in MCF-7/AZ breast cancer cells through chromatin remodelling at *CDH3* promoter, bringing forward the growing evidence that ERα signalling-abrogation by anti-oestrogens is able to induce the expression of ERα-repressed genes which, in the appropriate cell biology context, may contribute to a breast cancer cell invasion phenotype.**

***CDH3* GenBank accession no. NT_010498.**

INTRODUCTION

Classical cadherins, such as E-, N-, and P-cadherin, are the major structural components of the adherens junctions in many tissues (1). This superfamilly of transmembrane glycoproteins is responsible for calcium-dependent cell–cell adhesion, mediating specific homophilic protein interactions through their extracellular domain and being intracellularly

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linked to the actin cytoskeleton (2). Previous studies showed the involvement of classical cadherins in many biological processes, such as cell recognition, cell signalling, morphogenesis and tumour development (1).

Among these, P-cadherin has extensively been studied by our and other groups, where it has currently been recognized as an important biomarker in breast cancer. In human breast carcinomas, which represent a heterogeneous group of tumours, diverse in behaviour, outcome and response to therapy, P-cadherin was found to be aberrantly expressed in 30–50% of invasive ductal carcinomas of the breast, being strongly associated with proliferative lesions of high histological grade, decreased cell polarity and poor survival of patients over short-term follow-up (3–7). In addition, the expression of P-cadherin, together with other well-described basal markers, such as cytokeratin (CK)5, constitutes one of the most useful adjunctive markers to distinguish basal-like carcinomas of the breast (8).

At the *in vitro* level, our group demonstrated that P-cadherin plays an important role in cell invasion induction through its juxtamembrane domain (5), and that its overexpression induces motility and migration in wild-type E-cadherin breast cancer cell lines, through the secretion of pro-invasive factors, such as matrix metalloproteinase (MMP)-1 and MMP-2. These recent findings revealed the mechanism underlying this *in vitro* invasion behaviour induced by overexpression of P-cadherin and most likely associated with the poor prognosis of breast tumours (9,10).

However, although P-cadherin-associated functions in breast cancer have been extensively studied, the mechanisms controlling P-cadherin overexpression are still unclear.

It is known that the expression of an inappropriate cadherin can result from growth factors and hormones stimulation in the tumour environment, as well as from changes in the promoter regions of cadherin-encoding genes (11). In non-cancer models, *CDH3* promoter was shown to be genetically regulated through direct binding of transcription factors, such as p63 (12) and β-catenin (13).

In 2005, we have reported a significant association between P-cadherin overexpression and the hypomethylation of a specific region of *CDH3* promoter, suggesting an important regulatory role for CpG DNA methylation in the regulation of P-cadherin expression in breast cancer. Interestingly, the study of normal P-cadherin-negative epithelial/luminal cells revealed consistent hypermethylation at this same promoter region (3). The epigenetic regulation of *CDH3*/P-cadherin gene was recently demonstrated in other cancer models, like pancreatic and colorectal carcinomas, as well as in melanomas (14–17).

Indeed, one of our current aims is to find upstream regulators and identify the epigenetic mechanisms that are involved in P-cadherin overexpression in breast cancer cells. In this study, we explored the link between ER-signalling and P-cadherin-regulated expression in breast cancer cell lines, since P-cadherin-positive tumours are essentially ER negative. In fact, our group found that abnormal P-cadherin expression results from a lack of ER-α signalling (5), since treatment of breast cancer cells with the pure anti-oestrogen ICI 182,780 (ICI, Fulvestrant) induced a 2–3-fold increase of P-cadherin protein and *CDH3* mRNA levels in a time- and dose-dependent manner, being this effect counteracted by 17β-oestradiol (E2) (18). Taken together, these previous findings suggested that the lack of ER-α signalling was responsible for the increase of P-cadherin, categorizing *CDH3* as an oestrogen-repressed gene. However, until now, it remained to be determined whether the induction of the *CDH3* gene was due to an epigenetic effect of the anti-oestrogens at *CDH3* promoter level and/or if it would require the prior induction of other genes/proteins.

Herein, we describe the epigenetic remodelling induced by the anti-oestrogen ICI, which leads to higher levels of the active chromatin mark H3 lysine 4 dimethylation (H3K4me2) at *CDH3* promoter sites. We demonstrated in this study that when ER-positive breast cancer cells are treated with ICI, specific transcription sites of the *CDH3* promoter become exposed to putative transcription regulators that, if located nearby, can induce the inappropriate expression of P-cadherin protein. Moreover, we observed, for the first time, that expression of the transcription factor C/EBPβ is able to directly activate P-cadherin promoter and its transcription in breast cancer cells. We further supported our *in vitro* results, showing that the expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients.

RESULTS

ERα signalling pathway inhibition induces the transcription and up-regulation of the pro-invasive *CDH3*/P-cadherin in breast cancer cells

P-cadherin expression is tightly regulated by ERα-signalling pathway in breast cancer cells (5). In MCF-7/AZ cells, P-cadherin protein and mRNA expression levels were up-regulated after the treatment with anti-oestrogen ICI and down-regulated by oestradiol (Fig. 1A and B). However, until now, the molecular mechanism leading to increased levels of P-cadherin by ICI was never determined. In this study, we tested whether the ICI-induced P-cadherin overexpression was due to a molecular effect at the *CDH3* promoter level, as a consequence of ERα-signalling pathway deregulation.

To address if ERα-signalling pathway was able to regulate P-cadherin expression levels through *CDH3* promoter activation/repression, a luciferase reporter gene assay was performed in ERα-positive MCF-7/AZ breast cancer cells. The full-length *CDH3* gene promoter was cloned at pGL3-basic vector, as well as *PS2/TFF1* promoter, which is a well-known direct oestrogen-responsive gene, here used as a positive control. Cells were transiently transfected with the pGL3-basic empty vector, *CDH3* or *PS2/TFF1* promoter vector, and treated with E2 or with the anti-oestrogen ICI. As a negative control, cells were treated with ethanol (drug vehicle). As expected, *PS2/TFF1* promoter was strongly activated by E2 and inhibited by the pure anti-oestrogen in the hormonal-dependent MCF-7/AZ breast cancer cells (Fig. 1C). Concerning P-cadherin, we found that ICI significantly increased *CDH3* promoter activity, whereas E2 repressed it (Fig. 1C). These effects mediated by ICI and E2 were detected at mRNA and protein level. Although these differences were not as evident as the ones observed for the positive control *PS2/TFF1* gene, the results were statistically significant. pGL3–control (pLUC) containing a modified coding region for firefly luciferase, optimized for monitoring

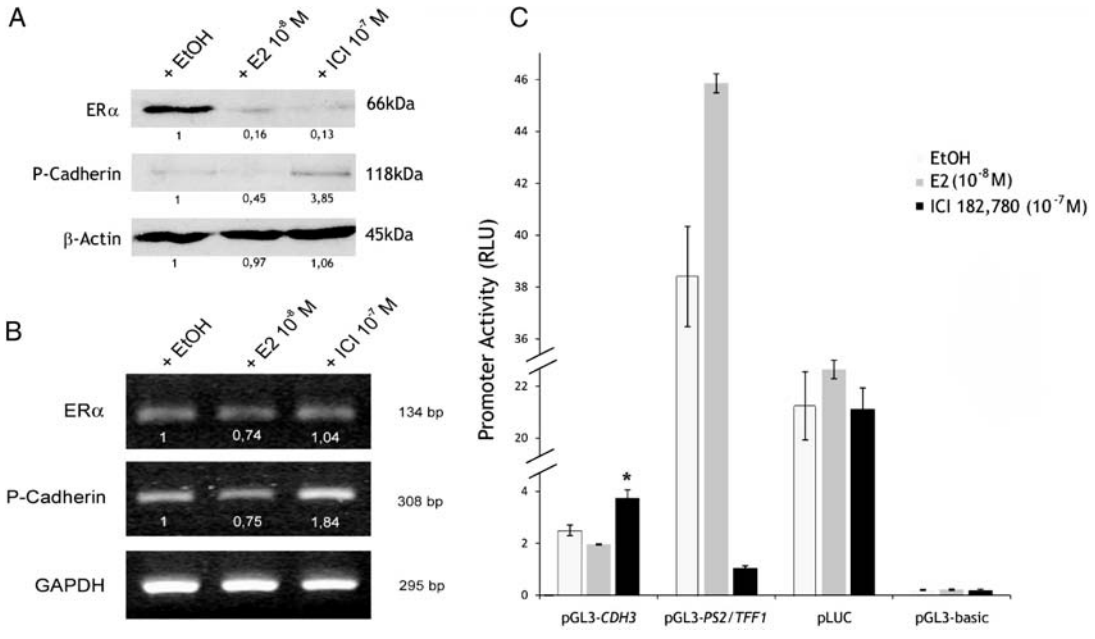

Figure 1. Regulation of P-cadherin expression by the anti-oestrogen ICI 182,780 in MCF-7/AZ breast cancer cells. MCF-7/AZ cells were treated with the indicated concentrations of ICI or 17β-estradiol (E2) for 24 h. (**A**) ERα protein levels of MCF-7/AZ cells treated with E2 or ICI were both decreased, whereas the P-cadherin protein expression was 3.8-fold increased after ICI treatment, relative to the ethanol control treatment. In contrast, P-cadherin expression was reduced in ∼55% when cells were treated with E2. Immunostaining for anti-β-actin was done to control for equal loading. (**B**) mRNA levels of cells treated with E2 or ICI showed no alteration concerning ERα expression, but a marked increased in P-cadherin levels is showed after treatment with ICI. A slight reduction of ∼25% in P-cadherin expression is observed after treatment with E2. GAPDH housekeeping gene amplification was used as a control. (**C**) Luciferase reporter assay quantification was done using relative light units—RLU (relative to *renilla*). ICI significantly induced *CDH3* gene promoter activity when cells were treated with the pure anti-oestrogen ($^*P < 0.001$), while E2 slightly induced down-regulation of the promoter activity. Oestrogen-responsive PS2/TFF1 gene promoter vector showed the efficiency and activity of both treatments, namely been activated in the presence of E2 and highly repressed by the presence of ICI. As a negative control, the pGL3-empty vector showed no activity with any of the treatments.

transcriptional activity in transfected eukaryotic cells, was used as positive luciferase assay control. As expected, pLUC– control activity was high and similar in all the treatment conditions. pGL3-basic empty vector did not show any activity with or without treatments.

Similarly, breast cancer cells were transiently transfected with ERα-siRNA, in order to test if the up-regulation of P-cadherin expression could be indeed attributed to specific ERα degradation, or if the ICI-mediated P-cadherin induction could be due to a secondary effect not related to the ERα-signalling pathway. As can be seen in Figure 2A and B, the siRNA for ERα also induced an increased P-cadherin expression at the mRNA and protein level. Overall, we showed that the inhibition of ERα-signalling pathway, by ICI or by ERα-siRNA, induces the transcription and up-regulation of the pro-invasive *CDH3*/P-cadherin in breast cancer cells.

The anti-oestrogen ICI 182,780 up-regulated P-cadherin expression is associated with high levels of the active chromatin mark H3K4me2 at *CDH3* promoter regulatory regions

On the basis of the above-mentioned results, we also aimed to understand the molecular mechanism by which ICI is able to increase the transcription of P-cadherin gene.

Epigenetic mechanisms were already described as induced by ICI (19), which most probably can affect *CDH3* transcription. In previous works, we showed that *CDH3* promoter is able to be regulated by methylation (3), but ICI did not caused any significant change in the methylation pattern of *CDH3* promoter (data not shown). However, it was previously described that pure anti-oestrogen ICI can induce gene transcription through releasing HDACs and ERα from Sp1 sites in ERα-repressed genes (19). Varshochi *et al.* (19) demonstrated that, in the presence of ICI, ERα and HDACs are dissociated from Sp1, resulting in an increased histone acetylation and de-repression of the $p21^{Waf1}$ promoter and expression induction. However, for *CDH3*gene, which promoter is enriched in Sp1 and ERα binding sites, ICI-induced histone acetylation changes were never studied.

In order to access whether *CDH3* promoter is prone to be regulated by acetylation mechanisms, cells transfected with the full-length *CDH3* gene promoter were treated with increased doses of Trichostatin A (TSA), a known histone deacetylase (HDAC) inhibitor. The *CDH3* promoter transfected into MCF-7/AZ breast cancer cells showed a significant dose-dependent activation after treatment with 0.05 μM TSA and with 0.1 μM TSA for 12 h, compared with the activation from the cells treated only with the vehicle [dimethyl sulfoxide (DMSO) (Fig. 3A)]. The increase in *CDH3* promoter activation is also reflected at P-cadherin protein levels (Fig. 3B), indicating that *CDH3* promoter is sensitive to chromatin alterations and that these alterations affect P-cadherin expression. No alterations in ERα expression levels were observed after TSA treatment.

In order to address which type of chromatin modifications could be induced by ICI in *CDH3* promoter potentially

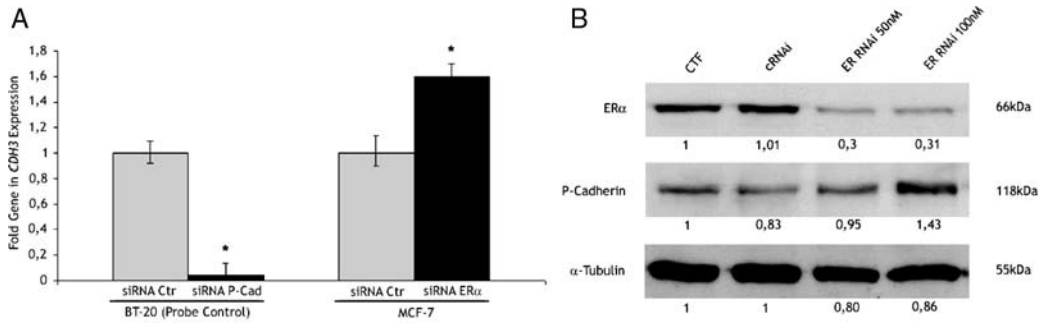

Figure 2. Regulation of P-cadherin expression by the siRNA for ERα in MCF-7/AZ breast cancer cells. (**A**) Real-time PCR showed that cells transfected with siRNA for ERα show a significant increase in P-cadherin expression at the mRNA level. P-cadherin high expressing breast cancer cells BT-20 were used as detection sensitivity control for P-cadherin probe, when P-cadherin is inhibited with a specific siRNA ($^*P < 0.005$). (**B**) At the protein level, the up-regulation of P-cadherin was observed when cells were transfected with an ERα siRNA concentration of 100 nM. The knock-down of ERα expression was observed at both ERα siRNA concentrations of 50 and 100 nM.

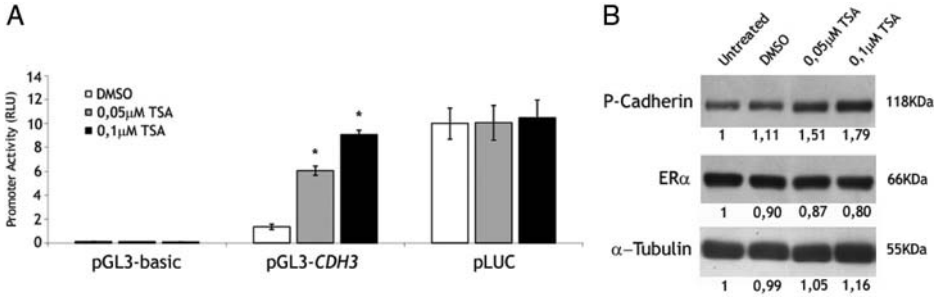

Figure 3. Regulation of *CDH3* promoter and P-cadherin expression by epigenetic activating mechanisms. (**A**) MCF-7/AZ cells transiently transfected with *CDH3* promoter vector were treated with sequential concentrations of TSA during 12 h. An increase of promoter activation was observed when cells were treated with gradual of TSA concentrations ($^*P < 0.0001$). (**B**) The western blot also showed a gradual increased of P-cadherin protein levels without alteration of ERα protein levels.

leading to increased mRNA and protein overexpression, chromatin immunoprecipitation (ChIP) assays were performed using specific antibodies to identify conventional histone-activating (H3K4me2, H3K9ac and H3ac) or repressive marks (H3K27me3 and H4K20me3) within the *CDH3* gene promoter region. We studied two different DNA promoter regions (a distal Region 1 and a proximal Region 2) that were selected according to CpG islands enrichment and to the attributed DNA hypersensitive (DHS) sites within the *CDH3* gene promoter sequence. On the basis of the already described effect of ICI in ERα-repressed genes, predicted ERα and Sp1-binding sites were also considered to select these studied promoter regions (Fig. 4A). No significant alterations in the levels of activating or repressive histones marks were detected at *CDH3* Promoter Region 1 after treatment with ICI (Fig. 4B). However, anti-oestrogen ICI-treatment induced a strong enrichment in H3K4me2 levels, a mark for transcription activation at the proximal *CDH3* promoter Region 2, while neither active H3K9ac and H3ac or repressive H3K27me3 and H4K20me3 marks showed alterations (Fig. 4C).

Altogether, it is suggested that the proximal *CDH3* Promoter Region 2, which is closer to the transcriptional start site (TSS) and ATG, is prone to epigenetic regulation under ICI-treatment, in order to become transcriptionally active.

The C/EBPβ transcription factor activates *CDH3* promoter in breast cancer cells

H3K4me2 is an epigenetic mark which is frequently enriched at regions surrounding known TSSs (20,21). Therefore, we decided to analyse which transcription factors were better represented within a sequence region flanking 250 bp up- and downstream the *CDH3* Promoter Region 2, overlapping with the first nucleotides of the TSS. Combining data from three transcription factors bioinformatic tools (Genomatix, TFSearch and TESS), the transcription factor CCAAT/enhancer-binding protein (C/EBPβ) was predominantly present at this studied region, being this frequency validated by at least two of the three predictive software resources. Accordingly, five putative C/EBPβ-binding sites were found around and comprising the *CDH3* Promoter Region 2 (Fig. 5A), turning this transcription factor as a putative candidate for playing a novel regulatory role on *CDH3* promoter activation in breast cancer cells.

C/EBPβ is a well-known transcription factor and a key regulator of epithelial cell growth, proliferation and differentiation of the mammary gland (22,23). C/EBPβ is expressed in several distinct protein isoforms [liver-enriched transcriptional activating protein (LAP1, LAP2) and liver-enriched transcriptional inhibitory protein (LIP)] that harbour particular regulatory functions (24–26). On the basis of this knowledge,

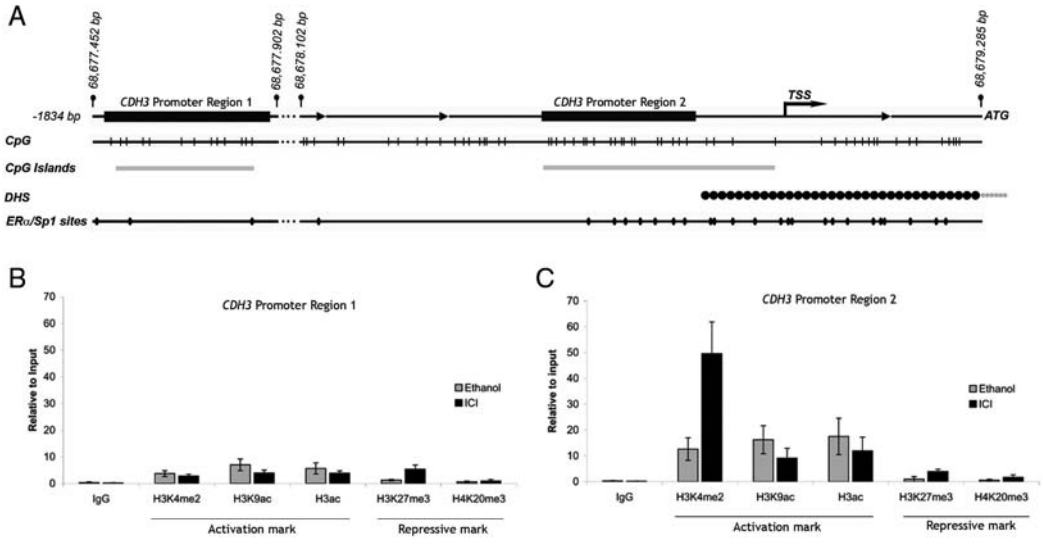

Figure 4. Chromatin immunoprecipitation (ChIP) analysis of histone modifications at *CDH3* promoter after treatment with the pure anti-oestrogen ICI. (**A**) Representation of the *CDH3* promoter structure showing epigenetic regulatory regions (CpG islands and DHS—DNAase hypersensitive sites) and putative ERα/Sp1 sites, predicted by bioinformatic tools (Genomatix, TESS and TFSearch). Transcription start site (TSS) and the analysed distal *CDH3* Promoter Region 1 and proximal *CDH3* Promoter Region 2 are also illustrated. (**B**) and (**C**) ICI-mediated induction of histone activating (H3K4me2, H3K9ac and H3ac) and repressive (H3K27me3 and H4K20me3) marks in *CDH3* Promoter Region 1 and 2, respectively. In promoter Region 1, weak pronounced chromatin alterations were detected after treatment with ICI. In contrast, significant levels of enrichment for H3K4me2 were observed in promoter Region 2, induced by the anti-oestrogen treatment (C).

we decided to test the relevance of these different C/EBPβ isoforms in *CDH3* promoter activation, as well as their relevance in *CDH3* expression at mRNA level.

By luciferase gene reporter assay, using the cloned *CDH3* promoter, different C/EBPβ cDNAs vectors, codifying for a particular C/EBPβ isoform (LAP1, LAP2, or LIP) were independently co-transfected in a cDNA amount titration basis (5, 10 and 20 ng of cDNA) into MCF-7/AZ cells. Luciferase readouts revealed that *CDH3* promoter was gradually activated by the three isoforms, although the promoter activation induced by the C/EBPβ-LIP isoforms was significantly greater compared with the activation induced by LAP1 and LAP2. *CDH3* promoter activation observed with the lowest co-transfected amount of C/EBPβ-LIP (5 ng) was higher when compared with the highest amount of LAP1 and LAP2 isoforms (20 ng) co-transfected into MCF-7/AZ cells. Most importantly, promoter activation was raised when titration was done using higher amounts of C/EBPβ-LIP isoform (Fig. 5B). This result shows that among the three C/EBPβ isoforms with known regulatory functions in breast cancer, *in vitro*, the LIP isoform is the most important in *CDH3* promoter activation.

In order to test this hypothesis, MCF-7/AZ cells were co-transfected with *CDH3* promoter and with C/EBPβ-LIP vector isoform and treated with ICI/control ethanol (EtOH) and luciferase activity was measured. This same experiment was repeated in order to evaluate *CDH3* mRNA expression levels by real-time PCR. The luciferase reporter assay showed that further than the demonstrated activation of *CDH3* promoter by C/EBPβ-LIP isoform, the treatment with ICI provoked a significant synergistic effect towards the activation of *CDH3* promoter (Fig. 5C). The same trend was also observed at the mRNA levels, although not statistically significant (Fig. 5D). In order to see if the induction of P-cadherin expression by ICI would coincide with C/EBPβ nuclear accumulation, immunofluorescence for this transcription factor was performed. Interestingly, we could observe that C/EBPβ is highly expressed at the nuclei of MCF-7/AZ breast cancer cells, independently if these are treated with ICI or EtOH (Fig. 5E).

All these results allowed us to conclude that ICI is able to actively remodel the chromatin at *CDH3* promoter, which permit to expose the C/EBPβ-binding sites. Since C/EBPβ is available in the nuclei of these cells, it will promote P-cadherin transcription and consequent expression.

C/EBPβ is associated with P-cadherin expression and with features of poor prognosis in human breast carcinomas

Similar to what we have previously described for P-cadherin expression, high levels of C/EBPβ have also been associated with tumour progression and as an indicative of an unfavourable prognosis in breast cancer. Most importantly, for the shorter isoform LIP, correlations with ER-negative and poorly differentiated phenotype were previously demonstrated (27,28). Taking into account these results and our *in vitro* data, we decided to perform an immunohistochemical characterization of C/EBPβ expression in a series of 249 invasive primary breast carcinomas previously characterized for P-cadherin expression profile.

From the total 249 cases, only the ones with clear nuclear expression of C/EBPβ were selected for immunohistochemistry classification. Strong immunoexpression of C/EBPβ is observed in the nuclei of luminal epithelial cells from adjacent normal ducts, as showed in Figure 6A. In parallel with what is found for P-cadherin expression in normal epithelial gland (Fig. 6A), C/EBPβ is also expressed in the vast majority of

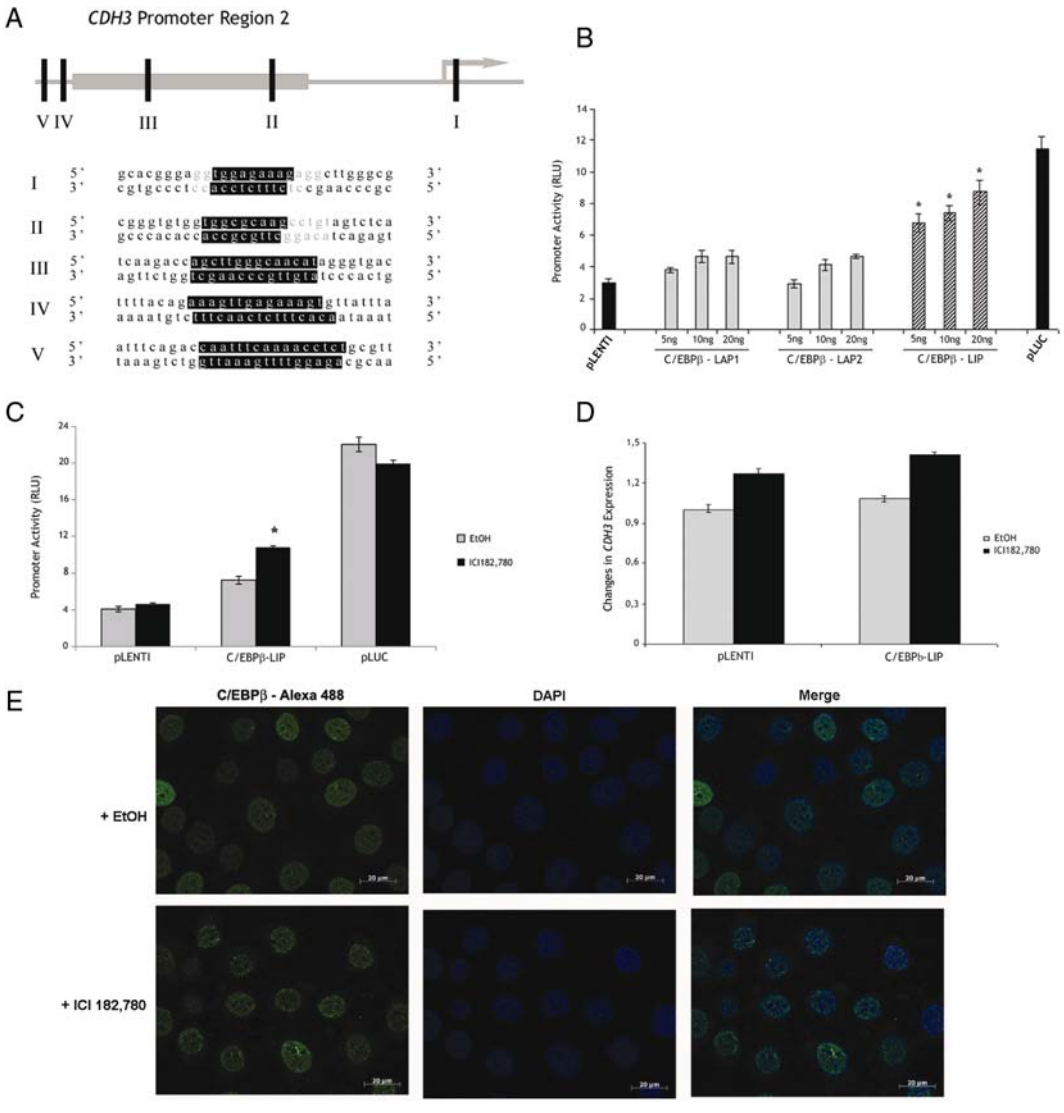

Figure 5. Activation of *CDH3* promoter and P-cadherin mRNA levels by the transcription factor C/EBPβ in MCF-7/AZ cells. (**A**) Proximal *CDH3* promoter region showing five C/EBPβ-binding sites with their predicted sequences based on transcription factors bioinformatic tools. The DNA sequence inside the black area represents the concordant sequence that was validated by at least two of the three bioinformatic tools used (high score), whereas the grey sequence area results from the prediction of a single web tool out of the three used (low score). There is one putative C/EBPβ-binding site at the *CDH3* TSS region and another two inside the studied promoter region 2. Two high-scored C/EBPβ-binding sites are also localized immediately before the limits of the established proximal *CDH3* promoter region. (**B**) *CDH3* luciferase reporter assay where MCF-7/AZ cell were transfected with different amounts (5, 10 and 20 ng) of C/EBPβ cDNA isoforms (LAP1, LAP2 and LIP). Comparative with the pLENTI empty vector, the C/EBPβ−LIP isoform significantly activates the *CDH3* promoter in a dose-dependent manner ($^*P < 0.001$). (**C**) *CDH3* promoter activation by the C/EBPβ−LIP isoform in response to ICI treatment in MCF-7/AZ cells, where the anti-oestrogen induced a synergistic effect with C/EBPβ−LIP towards the activation of *CDH3* promoter ($^*P < 0{,}001$). (**D**) Real-time PCR analysis of P-cadherin mRNA expression levels after ICI treatment. P-cadherin mRNA was up-regulated not only by the transfection of C/EBPβ−LIP isoform, but also by an ICI-mediated synergistic effect. (**E**) MCF-7/AZ cells show nuclear expression of C/EBPβ (Alexa 488—green), treated with ethanol or ICI. The DAPI staining (blue) confirms the nuclei localization of C/EBPβ (see the merge image). All the figures show a ×630 magnification. The white line in each picture represents a 20 μm scale.

the myoepithelial/basal cells. In positive tumour samples, C/EBPβ expression was restricted to the nuclei of malignant cells, while P-cadherin presented its typical membranous staining (Fig. 6B). C/EBPβ was positive (>10% of positive cells) in the nuclei of 43% (86 of 198) of the invasive carcinomas, whereas P-cadherin was present in 33% (64 of 194).

Importantly, C/EBPβ was significantly associated with P-cadherin expression ($P = 0.004$), with nearly 60% of co-expression of these two proteins (Table 1). When we compared the expression of C/EBPβ with the molecular subtype, we found that while 60% of C/EBPβ-negative cases were comprised in the luminal A subtype, basal-like subtype carcinomas expressed C/EBPβ in ∼74% of the cases (Table 1). On the basis of these results, we showed that the expression of C/EBPβ strongly associates with aggressive behaviour features as high proliferation rates, poor differentiation and basal-like phenotype. Furthermore, the expression of C/EBPβ was associated with high histological

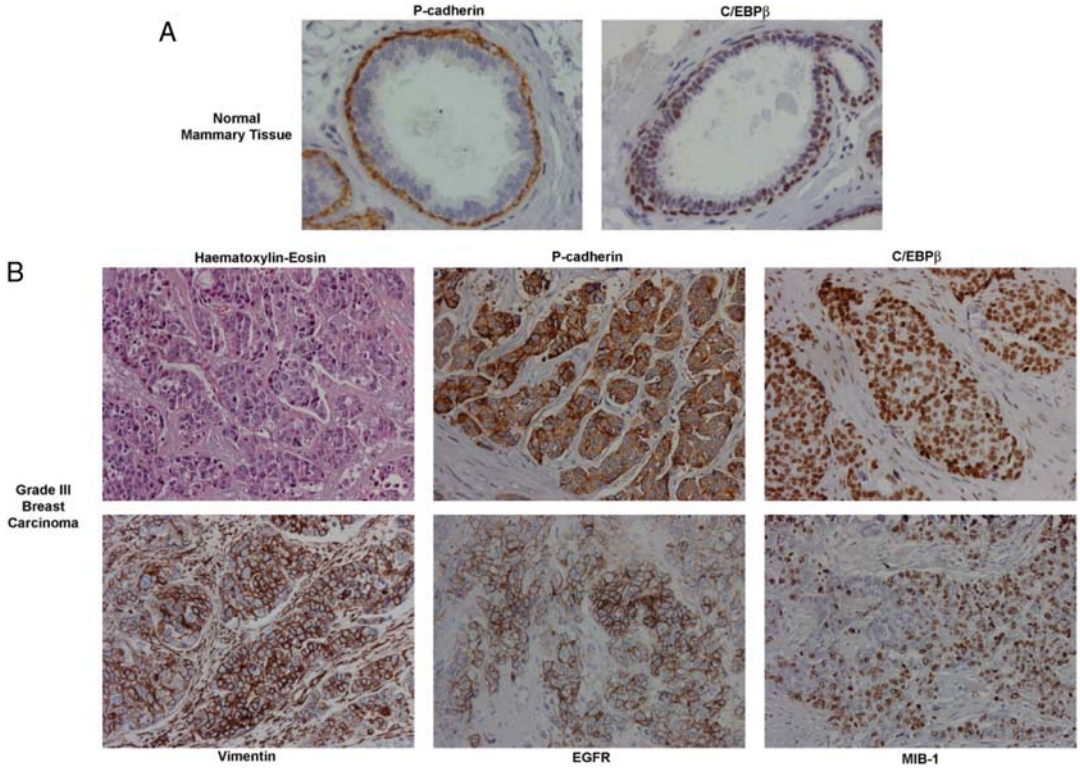

Figure 6. (**A**) Immunohistochemistry representation of the expression pattern of C/EBPβ and P-cadherin in normal breast tissue. P-cadherin expression is restricted to myoepithelial cell layer, whereas nuclear expression of C/EBPβ is seen in myoepithelial cells of a normal breast duct and in scattered normal luminal cells (magnification ×400). (**B**) An immunohistochemical panel of a grade III invasive breast carcinoma (Haematoxylin–eosin staining), showing positive membrane expression for P-cadherin and EGFR, membranar/cytoplasmic expression for vimentin and nuclear expression for C/EBPβ and MIB-1 (magnification ×200).

grade ($P = 0.002$), but no association was found with tumour size or regional lymph node involvement. We also found a strong association of C/EBPβ with breast cancer markers of aggressive phenotype, namely CK14 ($P = 0.015$), vimentin ($P = 0.001$), high proliferative index-MIB-1 ($P < 0.0001$) and EGFR ($P = 0.002$), where 90% of the cases that expressed EGFR were also positive for C/EBPβ. Figure 6B shows an example of a high-grade invasive carcinoma, which was concomitantly positive for P-cadherin, C/EBPβ, vimentin, EGFR and MIB-1 expression.

In summary, we demonstrated, for the first time, an association of the expression of this transcription factor with the expression of P-cadherin, a pro-invasive and migration inducer protein, which also constitutes an important marker of poor prognosis and aggressive basal-like phenotype in breast carcinomas.

DISCUSSION

Over the last years, we and others have been describing the association of P-cadherin expression with malignant behaviour, poor prognosis and short survival in breast cancer (3,6,7,29,30). Recent findings have contributed to the elucidation of P-cadherin function in breast tumour cell biology (10), but the expression regulation of this protein in breast cancer has poorly been explored. Previously, we reported a significant association between P-cadherin overexpression and *CDH3* hypomethylation, suggesting an important regulatory role of epigenetic events in the regulation of P-cadherin expression in breast cancer (3). Moreover, we demonstrated that the abrogation of ERα-signalling pathway, caused by the pure anti-oestrogen ICI, was responsible for the increase of P-cadherin protein and mRNA expression, pointing *CDH3* as an oestrogen-repressed gene (5). However, the mechanisms by which epigenetic events and ERα-signalling inhibition leads to P-cadherin expression and to aggressive tumour behaviour are still unknown.

In the present study, we identified, for the first time, the existence of an epigenetic regulation by which ICI up-regulates P-cadherin expression in MCF-7/AZ breast cancer cells through chromatin remodelling at *CDH3* promoter. After treatment of this ERα-positive breast cancer cell line with the oestrogen antagonist ICI, an important histone-activating mark (H3K4me2) was enriched at the proximal region of the *CDH3*/P-cadherin promoter.

Previous studies showed that chromatin structural remodelling and nuclear entropy can be induced by the treatment of breast cells with anti-oestrogens such as ICI (31). In fact, and although it was initially believed that anti-oestrogens function merely by competing with endogenous oestrogens for receptor binding, recent studies also demonstrated that ICI and tamoxifen can induce distinct conformational changes in ER, implying that the ligand-bound ER can recruit specific co-regulators to modulate different gene promoters, thereby regulating gene expression (32,33).

Table 1. Association of C/EBPβ expression with clinicopathological features and immunohistochemical markers in invasive breast carcinomas

Variables	No.	C/EBPβ negative (%)	C/EBPβ positive (%)	*P*-value
Tumour size	187			
<15 mm	16	11 (68.7)	5 (31.3)	0.288
≥15 mm	171	94 (55.0)	77 (45.0)	
LNI	180			
Present	91	55 (60.4)	36 (39.6)	0.138
Absent	89	44 (49.4)	45 (50.6)	
Tumour grade	198			
GI	42	31 (73.8)	11 (26.2)	0.002
GII	90	54 (60.0)	36 (40.0)	
GIII	66	27 (40.9)	39 (59.1)	
NPI	169			
NPI < 3.4	38	26 (68.4)	12 (31.6)	0.067
3.4 ≤ NPI ≤ 5.4	85	39 (45.9)	46 (54.1)	
NPI > 5.4	46	25 (54.3)	21 (45.6)	
EGFR	197			
Positive	10	1 (10.0)	9 (90.0)	0.002
Negative	187	111 (59.4)	76 (40.6)	
P-Cadherin	194			
Positive	64	27 (42.2)	37 (57.8)	0.004
Negative	130	83 (63.8)	47 (36.2)	
CK5	198			
Positive	41	22 (53.7)	19 (46.3)	0.673
Negative	157	90 (57.3)	67 (42.7)	
CK14	193			
Positive	10	2 (20.0)	8 (80.0)	0.015
Negative	183	107 (58.5)	76 (41.5)	
Vimentin	178			
Positive	25	7 (28.0)	18 (72.0)	0.001
Negative	153	95 (62.0)	58 (38.0)	
MIB-1	197			
<10	94	70 (74.5)	24 (25.5)	<0.0001
10–20	20	9 (45.0)	11 (55.0)	
>20	83	33 (39.8)	50 (60.2)	
Subtype	178			
Luminal	122	74 (60.7)	48 (39.3)	0.001
HER-2	22	14 (63.7)	8 (36.3)	
Basal	34	9 (26.5)	25 (73.5)	

Data presented as n (%) unless stated otherwise.
C/EBPβ, CCAAT/enhancer-binding protein beta; LNI, lymph node involvement; NPI, Nottingham prognostic index; EGFR, epidermal growth receptor; P-cadherin, placental cadherin; CK, cytokeratin; MIB-1, mindbomb homolog 1; HER-2, human epidermal growth factor receptor 2.

A study in 2005 showed that ICI can induce transcription of the ERα-repressed gene $p21^{Waf1}$, through the dissociation of HDACs and ERα from Sp1 sites and therefore, resulting in increased histone acetylation and de-repression of the $p21^{Waf1}$ promoter (19). In fact, the authors not only found that the proximal Sp1 sites are crucial in mediating the promoter's response to ICI, but also that HDAC inhibition by TSA leads to $p21^{Waf1}$ promoter activity (19). Further than the fact that most of the proximal promoter regions are generally important to gene transcription regulation, the studied *CDH3* proximal Region 2, which showed an ICI-induced enrichment for the active histone mark H3K4me2, additionally displays a CpG island and a DNaseI hypersensitive site (DHS) region, overlapping with the TSS. As described by the ENCODE Project Consortium, the aggregate signal of histone modifications is mainly attributable to active TSS region, in particular those near CpG islands and DHS, both genomic regions thought to be enriched for regulatory information (34). Importantly, after a prediction analysis of the proximal *CDH3* promoter, comprising a region from the TSS to the ATG, we observed that this promoter area was remarkably enriched in Sp1 sites, having also a significant number of ERα coupled with those (Fig. 4A). Hence, if a repression complex, mediated by ERα and HDACs at Sp1 sites, is able to be released by the treatment with ICI and therefore enhancing the gene transcription, the characteristics of the proximal *CDH3* promoter, together with the H3K4me2 enrichment in Region 2, strongly suggest that this chromatin de-repression mechanism plays an important role in the ICI-induced promoter transcriptional activation. Reinforcing this, we also observed an up-regulation of *CDH3* promoter activity and P-cadherin protein expression in cells treated with TSA, showing that chromatin-activating modifications are indeed important to the modulation of this gene.

The most prominent activating mark found within the *CDH3* promoter Region 2 was H3K4me2, which is the histone modification better correlated with DHS regions and chromatin accessibility (20,21,34), as well as with active gene transcription (35).

Herein, we further investigated which transcription factors were strongly represented within *CDH3* promoter Region 2, TSS and DHS region. We have found that putative C/EBPβ-binding sites were predominantly present within this region. Furthermore, we tested the ability of C/EBPβ to transactivate P-cadherin protein and mRNA expression, as well as *CDH3* promoter, demonstrating, for the first time, that, among the three different C/EBPβ isoforms, C/EBPβ-LIP was the most relevant in a P-cadherin expression activation setting. C/EBPβ proteins are transcription factors which regulate cellular proliferation, differentiation and apoptosis in mammary gland (24). However, like P-cadherin, C/EBPβ is not mutated in breast tumours, but its overexpression has widely been described in a subset of aggressive breast cancer (25). Interestingly, transgenic and overexpression studies showed that C/EBPβ–LIP induces proliferation in mammary epithelial cells and that a C/EBPβ–LIP-initiated growth cascade may play a role in the development of breast cancer (24,26). At a clinicopathological level, LIP isoform correlates with an ER-negative breast cancer phenotype, high proliferative index and histological grade, aneuploidy and poor differentiation. These findings are not only suggestive of the involvement of C/EBPβ–LIP in tumour progression and indicative of an unfavourable patient prognosis (27), but also show that its expression should be evaluated as a prognostic marker for breast cancer patients (28). Remarkably, breast carcinomas expressing C/EBPβ–LIP displays the unfavourable clinicopathological features described for the aggressive breast tumours overexpressing P-cadherin. Additionally, we also observed an association of C/EBPβ with aggressive markers, namely EGFR, CK14 and vimentin expression, as well with basal-like molecular phenotype. Thus, based in our results, it is tempting to consider that, under conditions of ICI-mediated increased chromatin accessibility, C/EBPβ, and most likely the LIP isoform, can play a role in the activation of *CDH3* promoter towards a typical P-cadherin-related aggressive tumour phenotype.

Our results are also of clinical relevance since there is growing evidence that selective ER modulators, such as

tamoxifen or ICI (Fulvestrant), are able to induce expression of genes which, in the appropriate cell context, may contribute to adverse cell phenotype, in part by inducing breast cancer cell invasion (36). Although anti-oestrogens have been the mainstay of therapy in patients with ERα-positive breast cancer and have provided significant improvements in survival, their benefits are limited by tumour recurrence in a significant proportion of initially drug-responsive breast cancer patients due to acquired anti-oestrogen resistance (36). Therefore, it is tempting to assume that one of the important mechanisms by which this endocrine resistance occur should be the inappropriate activation of ER-repressed genes at late stages of long-course endocrine therapeutic regimens.

To date, mechanistic studies have revealed important roles for growth factor signalling pathways, such as those regulated EGFR and HER2, as contributors to endocrine resistance (37–39). Similarly, in ER-positive breast cancer cells, tamoxifen has been reported to increase the expression of poor prognosis markers in breast cancer patients [14-3-3σ (40)], as well as of signalling elements frequently linked to tumour migration and invasion (MAPK, FAK and Src) (41,42).

The present study highlights that, in ERα-positive breast cancer cells, the anti-oestrogen ICI is able to induce the expression of *CDH3* gene, leading to P-cadherin overexpression, which is described as a pro-invasive protein in breast cancer. These data, together with other studies, contribute to clarify the ability of selective ER modulators and steroidal anti-oestrogens, like fulvestrant, to induce expression of genes normally repressed by oestrogen/ER signalling, and thus, playing an important role in the capacity of breast cancer cells to evade their growth inhibitory effects (37,43). It is important to know which signalling pathways are activated in anti-oestrogen resistant breast cancer, in order to find new and effective therapeutic targets to use in this setting. In the future, it would be interesting to study if P-cadherin can be a good biomarker in this group of breast tumour recurrences that occur upon endocrine therapy.

MATERIALS AND METHODS

Antibodies and chemicals

The following primary anti-human antibodies were used for western blot (WB), immunohistochemistry (IHC) and immunofluorescence (IF) against: P-cadherin [mouse monoclonal, clone 56; BD Transduction Biosciences, Lexington, KY, USA; dilutions: 1:250 (WB) and 1:50 (IHC)], C/EBPβ [mouse monoclonal, clone H7, Santa Cruz Biotechnologies, CA, USA; dilutions: 1:500 (WB) and 1:100 (IHC, IF)], β-actin [goat monoclonal, I-19; Santa Cruz Biotechnologies; dilutions: 1:1000 (WB)], ERα [mouse monoclonal, NCL-L-ER-6F11; Novocastra, Newcastle; dilutions: 1:50 (WB) and 1:200 (IHC)]. Anti-mouse and anti-goat horseradish peroxidase-conjugated secondary antibodies were also used for WB [HRP-conjungated, Santa Cruz Biotechnologies; dilutions: 1:2000]. For chromatin immnunoprecipitation (ChIP) assays, the following antibodies were used: anti-acetyl-H3K9 antibody (07–352; Upstate Biotechnology, Lake Placid, NY, USA), anti-acetyl-H3 antibody (17–615; Upstate), anti-dimethyl-H3K4 antibody (07–030; Upstate), anti-trimethyl-H3K27 antibody (07–449; Upstate), anti-trimethyl-H4K20 antibody (ab9053; Abcam plc, Cambridge, UK) and rabbit anti-mouse-IgG antiserum (M7023; Sigma-Aldrich, Bornem, Germany).

ICI 182,780 Imperial Chemical Industries (ICI), 17β-oestradiol (E2) and Trichostatin A (TSA) were all purchased from Sigma. ICI and E2 were dissolved in 100% ethanol (EtOH) while TSA was dissolved in DMSO.

Cell culture, transfection and treatment conditions

The human breast cancer cell line MCF7/AZ was kindly given by Prof. Marc Mareel (Laboratory of Experimental Cancerology, Ghent University, Belgium), whereas BT-20 breast cancer cell line was purchased from American Type Culture Collection—ATCC (Manassas, VA, USA). Cell lines were cultured in growth media consisting of Dulbecco's modified Eagle's medium [DMEM (Invitrogen Ltd, Paisley, UK)], supplemented with 10% heat inactivated fetal bovine serum (Invitrogen), 100 IU/mL penicillin and 100 μg/ml of streptomycin (Invitrogen), at 37°C. MCF/AZ cells were grown at 10% CO_2 and BT-20 cells at 5% CO_2 controlled atmosphere.

For transient transfections, reagents were used as described subsequently. For gene reporter assays, MCF-7/AZ cells were grown in 96-well plates to 60–70% confluence and transfection was achieved using the liposome-mediated FuGENE 6 transfection reagent (Roche Diagnostic GmbH, Mannheim, Germany), prepared according to the manufacturer's instructions. These transient transfections used a charge ratio (FuGENE/DNA) of 3:1 where equal amounts (20 ng) of *CDH3* promoter vector, as well as from the expression vector, were added together with 5 ng of pCMV-Renilla normalization vector. For RNA or protein expression assays, MCF-7/AZ cells were grown in 6-well plates to 70–80% confluence. Transient transfections of 1 μg of C/EBPβ expression vector were done using Lipofectamine 2000 (Invitrogen), using a ratio (Lipofectamine/DNA) of 3:1 prepared according to the manufacture's instruction.

Whenever not specified, cells treatments with ICI were carried out for 24 h at a final concentration of 0.1 μM (10^{-7} M), while treatments with E2 were performed at a final concentration of 0.01 μM (10^{-8} M) for 24 h. In treatments with TSA, cell measurements were done after 12 h of incubation at the final concentration of 0.05 μM or 0.1 μM.

Promoter vectors and cDNA constructs

The human pLENTI-C/EBPβ expression vectors (C/EBPβ-LAP1, C/EBPβ-LAP2 and C/EBPβ-LIP) were kindly provided by Dr. Peter Gott (Institute of Anthropology and Human Genetics, Tübingen, Germany). To generate the full-length *CDH3*-luciferase vector, a 2.1 kb 5′ untranslated region of human *CDH3* gene (GenBank accession no. NT_010498) was generated by PCR, using a *Pfu* DNA Polymerase (MBI Fermentas, Burlington, Canada), the sense primer (5′-TGCTAGGCCTGAGAGAGCAAG-3′) and antisense primer (5′-CCTTCCGGGACTCCCTTG-3′). The PCR product was subcloned into a TOPO Cloning TA vector (Invitrogen) and then transferred to a pGL3-luciferase reporter plasmid (Promega, Corporation, Madison, WI, USA), after

digestion of both recipient vector and PCR fragment with *Kpn*I and *Nco*I restriction enzymes (MBI Fermentas). Ligation was performed using T4 ligase enzyme (New England Biolabs, Ipswich, MA, USA), and a pGL3/*CDH3*-luciferase reporter full-length vector (positions from −1834 to +1 ATG site), framed with ATG/firefly luciferase cDNA from the pGL3-luciferase reporter plasmid, was generated. Direct sequencing (ABI, Perkin-Elmer, Foster City, CA, USA) was performed to confirm the cloning frame and integrity of the promoter.

CDH3-luciferase reporter gene assay

MCF-7/AZ cells were co-transfected with the human full-length pGL3-*CDH3*/*luc* promoter vector and with pCMV-Renilla luciferase construct (Promega), for normalization of transfection efficiency. For promoter analysis, 24 h after transfection, cells were washed twice in PBS-cold and then harvested and lysed for firefly/Renilla luciferase assays using the Luclite Reporter Gene Assay System (Perkin Elmer, Beaconsfield, UK), according to the manufacturer's instructions. Luciferase bioluminescence from Renilla was measured using native coelenteranzine substrate reagent (Lux Biotechnology, Edinburgh, UK). Individual transfection experiments were repeated at least three times and in quadruplicate per transfection condition. Empty pGL3-basic vector (E1751) and pGL3-Control (pLUC) vector (E1741), both from Promega, were included as controls in all *CDH3*-reporter assays. Luminescence was then read using the Wallac/Perkin Elmer-1450-028 Trilux Microbeta (Perkin Elmer) plate reader, and the results are shown as mean of relative light units (RLU).

Protein extraction and WB analysis

Protein lysates were prepared from cultured cells, using catenin lysis buffer [1% (v/v) Triton X-100 and 1% (v/v) NP-40 (Sigma) in deionized phosphate-buffered saline (PBS)] supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmbH, Germany). Cells were washed twice with PBS and were allowed to lyse in 500 µl of catenin lysis buffer for 10 min, at 4°C. Cell lysates were mixed with a vortex for three times and centrifuged at 20 000*g* at 4°C, during 10 min. Supernatants were collected and protein concentration was determined using the Bradford assay (BioRad Protein Assay kit). Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 5 min at 95°C. Samples were separated by SDS–PAGE, and proteins were transferred into nitrocellulose membranes [Amersham Hybond enhanced chemiluminescence (ECL)] at 130 V for 1 h. For immunostaining, membranes were blocked with 5% (w/v) non-fat dry milk in PBS containing 0.5% (v/v) Tween-20. These were subsequently incubated with primary antibodies, during ∼1–2 h, followed by four 5 min washes in PBS/Tween-20 (PBS-T). Then the membranes are incubated with horseradish peroxidase-conjugated secondary antibodies, during 45 min. Proteins were detected using ECL reagent (Amersham), as a substrate, and blots were exposed to an autoradiographic film. Quantification of WBs was performed using Quantity One software (BioRad), and the ones selected to show are representative experiments.

Immunofluorescence

Briefly, MCF-7/AZ cells were cultured on glass coverslips, and fixed with cold methanol for 10 min on ice. After fixation, cells were permeabilized with 1% Triton X-100 in PBS for 5 min, at room temperature. Non-specific binding was blocked by cell treatment with PBS containing 3% BSA, for 30 min, at room temperature. Cells were then stained with the primary antibody for C/EBPβ (Santa Cruz), during 1 h, at 1:100 dilution. After PBS washes, cells were incubated, for 1 h, with the rabbit polyclonal secondary antibody, conjugated with Alexa 488 (Invitrogen), at 1:500 dilution. After a wash with PBS, each sample was mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) containing 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI). The C/EBPβ and DAPI dual cell staining was observed with a Zeiss microscope (Imager Z1) with apotome, and images were acquired using the Axiovision software.

ChIP assay

Exponentially growing MCF-7/AZ cells were treated with ICI for 24 h and fixed with 1% formaldehyde (37°C, 10 min). The reaction was stopped with addition of glycine to a final concentration of 0.125 M. Whole-cell fixed lysed extracts were prepared for use in ChIP assays as described previously (44). Briefly, fragmented chromatin to an average size of 300–1000 bp was incubated (4°C, 1 h) with 30 µl of blocked protein-A-agarose beads (Sigma) on a rotating wheel. Pre-cleared chromatin (150 µg) was immunoprecipitated (4°C, overnight) with 10 µl anti-acetyl-H3K9 antibody, 10 µl anti-acetyl-H3 antibody, 5 µl anti-dimethyl-H3K4 antibody, 5 µl anti-trimethyl-H3K27 antibody, 5 µl anti-trimethyl-H4K20 antibody and 2 µl of a rabbit anti-mouse-IgG antiserum as a negative control. After elution of immune complexes, DNA was resuspended in 100 µl of TE (10 mM Tris, 1 mM EDTA at pH 8.0) solution. Quantification of precipitated DNA was performed using real-time qPCR amplification carried out on a Chromo4 DNA engine (Biorad), using SYBR green jumpstart PCR master mix (Sigma) and 0.3 µM of the following primers: distal promoter region 1, forward: 5′-CAGGTTAGCCCTGGAAGGTCAA-3′; reverse: 5′-TGAGATGGAGTCTCACTGTCGTCC-3′; proximal promoter region 2, forward 5′-CTGTGAAATGGAAG AAGCGGTC-3′, reverse 5′-GCTGGTCTTGAACTTCTGGA CTC-3′. The amount of DNA precipitated by each antibody was normalized against 1 in 10 of the starting input material.

Gene silencing with small interfering RNAs (siRNAs)

MCF-7/AZ and BT-20 cells in 2 ml of culture medium were transfected with siRNA for ERα (50 nM and 100 nM Hs_ESR1, GW Validated siRNA, Qiagen, Cambridge, MA, USA) or for P-Cadherin (50 nM, Hs_CDH3_6, GW Validated siRNA, Qiagen). Transfection was carried out at starvation conditions for 6 h after which appropriated culture, medium was added to the cells. After 48 h, the cells were harvested

for RNA isolation or protein extraction for real-time PCR or WB analysis.

RNA isolation and real-time PCR

RNA was isolated using a Qiagen RNAeasy extraction kit (Qiagen), according to the protocol provided by the manufacturers and concentration was determined in a ND-1000 spectrometer (Nanodrop). One microgram of RNA per sample was used to synthesize cDNA, using reverse-transcriptase RT (Invitrogen). *CDH3* TaqMan probe (HS00354998_m1, Applied Biosystems, Foster City, CA, USA) was used to specifically recognize *CDH3* fragments, which were amplified through 40 cycles (Applied Biosystems 7000). Relative *CDH3* gene expression was determined by its normalization with *GAPDH* expression, using a Human *GAPDH* endogenous control (NM_002046.3, Applied Biosystems).

Patient selection

A series of 249 cases of primary operable invasive breast carcinomas were retrieved from the files of the Department of Pathology, Hospital of Divino Espírito Santo, Azores, Portugal and from the Federal University of Santa Catarina, Florianópolis-SC, Brazil. These samples were obtained from patients with age ranging from 30 to 89 years old. All the formalin-fixed paraffin-embedded histological sections were reviewed by three pathologists (V.C., F.S. and F.M.) and the diagnoses were confirmed as follows: 208 invasive ductal carcinomas, 7 invasive lobular carcinomas, 3 mixed, 3 tubular, 8 medullary and 20 invasive breast carcinomas of other special histological types. These tumours have been fully characterized for the clinical and pathological features, namely tumour size, lymph nodes invasion, tumour grade, Nottingham Prognostic Index (NPI) and for the following breast cancer markers: ERα, PR, HER-2, EGFR, P-cadherin, CK5, CK14, vimentin and MIB-1, as well as classified for breast cancer subtype (4,7,8,45,46).

This study was conducted under the national regulative law for the usage of human biological specimens, where the samples are delinked from their donor's identification and are exclusively available for retrospective research purposes.

Immunohistochemistry analysis

IHC was performed in 3 μm formalin-fixed paraffin-embedded sections. The IHC technique was performed using an Envision Detection System (DAKO Cytomation Envision System HRP, DAKO Corporation, Carpinteria, CA, USA) or the classical streptavidin–avidin–biotin complex (SABC) method according with the manufacturer's instructions.

Expression of C/EBPβ was analysed using a mouse monoclonal antibody. Sections were deparaffinised with xylene and rehydrated in a series of decreasing concentrations of ethanol solutions. Heat-induced epitope retrieval was carried out in 10 mM citrate buffer (sodium citrate) (pH 6) (LabVision Corporation, Fremont, CA, USA), in a 98°C water bath, for 30 min. After cooling retrieval solutions, for at least 30 min at room temperature, the slides were treated for 10 min with 3% H_2O_2 in methanol, in order to block endogenous peroxidase. Slides were incubated overnight at 4°C with monoclonal antibody for C/EBPβ and then labelled with the Envision Detection System from DAKO. Colour reaction product was developed with 3,3′-diaminobenzidine, tetrahydrochloride (DAB plus, DAKO Glostrup, Denmark) as a substrate, and nuclear contrast was achieved with haematoxylin/ammoniacal water counterstaining. Formalin-fixed paraffin-embedded sections from normal breast gland, skin or normal gastric mucosa were used as positive controls. Also, negative controls were performed by replacing the primary antibody with PBS/non-immune mouse serum.

Immunostained slides were reviewed by two pathologists (F.M. and F.S.) and cases with >10% of nuclei-stained positive cells for C/EBPβ were considered positive.

Statistical analysis

C/EBPβ immunoexpression associations were analysed using StatView, version 5.0 software (SAS Institute, Inc.). Continuous variables were presented as mean ± standard deviation (SD), and categorical variables were presented as number (%). The clinicopathological features and immunohistochemical markers of the tumours were compared across groups of expression of C/EBPβ using ANOVA and the chi-square test, respectively, for continuous and categorical variables.

For luciferase reporter gene analysis, independent quadruplicate measurements per each analysed variable were performed and RLU were compared between variables using Student's *t*-test. This same statistical method was also used to assess the variations in RNA expression.

WEB RESOURCES

URLs for *CDH3*-related data presented in this article and accession numbers used were the following:

(1) GenBank, http://ncbi.nlm.nih.gov/GenBank, NM_001793.
(2) Ensemble,http://www.ensembl.org/Homo_sapiens/Location/Overview?r=16:68677452-68679285
(3) New England Biolabs Cutter, http://tools.neb.com/NEBcutter2/index.php.
(4) Transcription Element Search System (TESS), http://www.cbil.upenn.edu/cgi-bin/tess/tess.
(5) CpG Island Searcher, http://cpgislands.usc.edu/cpg
(6) TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH.html.
(7) Genomatix, http://www.genomatix.de.

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4. General Discussion

4. General Discussion

The work presented throughout this thesis addressed two major hot topics in breast oncology research. Herein, we highlight the relevance of transcription factors in breast cancer as potential tools for prognosis and predictors of patient outcome, as well as, markers for therapeutic response and sensitivity. Additionally, we provide innovative data concerning *CDH3*/P-cadherin regulation, pushing the borders of the knowledge about the significance of epigenetic regulation in initiating breast carcinogenesis, promoting tumorigenic phenotypes, and assisting in the development of drug resistance to anti-oestrogens.

4.1 - The Broad Relevance of Transcription Factors in Cancer

Transcription is the key step in the regulation of gene expression and involves several distinct dynamic events. The basal transcription machinery and regulatory components are recruited to their responsive elements on target genes, and active interactions of transcription factors with chromatin – and with each other – play an important role in initiation and elongation of this cellular mechanism. The dynamic nature of transcription factor-binding event is not only a fundamental property of the transcription machinery, but it also emerges as an important modulator of physiological processes, such cell differentiation and embryo development (1).

The human genome encodes approximately 3000 transcription factors, regulating 23.000 genes spread over 3300Mb of DNA, in contrast with *Caenorhabditis elegans* which encodes just 600 transcription factors, regulating 19.000 genes spread over 100Mb of DNA (2-3). However, completion of the human genome sequence revealed just 4000 more genes over *C. elegans* (4), albeit the complexity of Humans. Viewed in this manner, it seems that the number of transcription factors relate more closely to complexity than with gene number. Reinforcing this, human genome accounts for about 700.000 responsive elements for just 3000 transcription factors, establishing a new important issue that is the selective binding site sequence recognition (3). Nevertheless, it is the interplay and combinations between transcription factors and co-regulators, at specific timings of living cells that govern

the differences in the gene expression levels (2). The convergence over recent years between the complementary fields of signal transduction and gene regulation has allowed a more complete and integrated view concerning the biodiversity of the mechanisms that control gene transcription (5-6). Since transcription factors lie at the heart of almost every fundamental development and homeostatic organism process – including DNA replication and repair, cell growth and division, control of apoptosis and cellular differentiation – it is not surprising that inherited or acquired defects in structure and function of these factors contribute to human carcinogenesis. Historically, cancer researchers have been elucidating the mechanisms that govern gene expression, by analyzing the function of proteins commonly overexpressed in transformed and malignant cells. Hence, roles were ascribed to several major groups of human transcription factors and a pleiotropic view of the biology of transcription factor families has emerged (7).

There are some transcription factor families that are frequently associated with cancer. Nuclear receptors (like ER), the MYC, the p53 and the AP-1 transcription factor families are among the most commonly studied in the context of human malignancies (7). Many of the well studied proto-oncogenes and tumour suppressor genes are known to encode proteins which, alone or in complex with other factors, act as transcriptional regulators. Defects in ER, MYC, Rb and p53 contribute to a common growth deregulatory mechanism that drives the uncontrolled cellular proliferation underlying breast tumours and are known to be transcription factors of clinical importance. Among these, c-MYC and its associated proteins activate the expression of a diverse range of genes with important relevance for the aggressive and poor-outcome signature of breast cancer (8-10). As a transcription factor, recent results suggest that MYC binding is influenced by the chromatin package conformation at potential binding sites, and particularly by patterns of histone modifications (11). Indeed, MYC was found to bind in regions enriched for several histone modifications generally associated with active chromatin such as H3K4me3, which seems to pinpoint the accessibility of MYC to gene promoter binding sites (12). At *in vitro* level, MYC ectopic expression attenuates responses to anti-oestrogen treatment, being suggested that oestrogen regulates cell growth mainly via MYC (9, 13). Importantly, some studies suggest that MYC oncogene activity may be necessary for the translocation of poor-outcome human breast tumours to distant sites, since functional inactivation of MYC in human breast cancer

cells specifically inhibits distant metastasis *in vivo* and invasive behaviour *in vitro* (10). c-MYC represents, therefore, a classical example of a transcription factor that, not only acts as a cell growth regulator, but also as a prognostic and predictive factor in breast cancer.

However, one of the most revealing examples of how transcription factor function can be regulated by multiple players, including binding of proteins, ligand and chromatin-modifying enzymes, comes from studies on the ERα. The kinetics and transcriptional signatures of ERα under different conditions have been extensively studied, largely owing to its vital role in the initiation, progression and therapy of breast cancer (7). In the absence of ligand, ERα can bind to ERE, as part of large transcriptionally repressive complexes, containing corepressor proteins maintaining a locally condensed chromatin structure (14). Prior to entry of oestradiol into cells, the epigenome is essentially primed for particular responses; combinations of specific histone modifications dictate where pioneer factors and transcription factors will bind and thus which genes are activated (15). This equilibrium is altered upon ligand addition, through the recruitment of transcription factors and their associated chromatin remodelling enzymes, allowing relaxation of the local chromatin and binding of further factors (16). In the particular framework where the nuclear receptor ERα functions as an oestrogen-activated transcription factor, the crucial pioneer factor which allows and signalizes its binding to response elements is FOXA1. Studies have shown that siRNA mediated knock-down of FOXA1 prevents ERα binding to responsive elements and alters DNase I sensitivity at the promoters of certain classes of ERα target genes. Conversely, E2-induced gene expression is significantly inhibited in the absence of FOXA1 (17-18). FOXA1 binds with high specificity to certain genomic consensus sequences in order to define the locations where ERα can bind within the chromatin and, similarly with what is observed for c-MYC, the occupied sites are significantly enriched in H3K4me1 and H3K4me2 activation marks (15). Interestingly, knock-down of FOXA1 does not alter the levels of these histone modifications, indicating that these are already present in DNA prior to FOXA1 binding, presumably to localize preferential recruitment of transcription factors (15, 17). Carroll *et al*. recently described several robust data demonstrating the requirement of FOXA1 for optimal expression of nearly 50% of ERα-regulated genes (19), highlighting the importance of this ERα pioneer factor as the defining cofactor that determines the subset of responsive elements with which ERα can associate (20). The identification of FOXA1 as an

important transcription factor was originated from experiments showing that it could modulate chromatin structure around some genes such as ALB (albumin) (21). However, it is actually known that it can bind to the promoters of more than 100 genes associated with several cellular mechanisms, such as metabolic processes, regulation of signalling pathways and cell cycle (22-24).

Despite the widely reported relevance of FOXA1 as a transcription factor necessary for the transcriptional activity of ERα, the expression of this forkhead box protein has been shown to be a potential candidate tool in breast cancer molecular pathology. Indeed, recent works have been demonstrating that FOXA1 expression is able to significantly predict a better survival for breast cancer patients (25-26), and that its assessment may be used as a marker for tumours pertaining to luminal A breast cancer, which have an exceptionally good prognosis (27). In our study, involving 249 breast cancer patients (see Chapter 2), we have found FOXA1 expression as a significant predictor of good outcome in breast cancer, being inversely associated with tumour size, Nottingham Prognostic Index, histological grade, lymph vascular invasion, lymph node stage and human epidermal growth factor receptor-2 (HER-2) overexpression. However, because the biological strong regulatory interdependency between ERα and FOXA1, the prognostic and predictive value of FOXA1 could simply reflect the high expression association and the above mentioned biological interplay between these two luminal markers. Thus, we further conducted an exploratory subgroup analysis in a cohort of ERα-negative patients, in order to evaluate whether there was a prognostic value for the expression of FOXA1 in the absence of ERα. In fact, the presence of ERα correlates with increased disease-free survival and an overall better prognosis compared to breast cancers that lack ERα, which are characterized by a more aggressive phenotype and a poor prognosis. Importantly, ERα-positive breast cancers respond to endocrine therapies, like tamoxifen, whereas ERα-negative tumours are resistant to endocrine therapies (28). In our work, the aim was to test, for the first time, if FOXA1 would be able to stratify the high-risk subset of ERα-negative patients in two different groups of biological behaviour. Herein, we showed that patients with loss of FOXA1 tumour expression showed an increased risk for breast cancer recurrence and that it constitutes an important and independent predictor of patient outcome in ERα-negative tumours (see Chapter 2). Based on our results, we consider that the expression of FOXA1 is able to stratify the risk of breast cancer recurrence

among hormonal-negative patients, demonstrating the clinical importance of this transcription factor in breast cancer molecular classification and prognosis. Supporting these findings at the biological level, it is very logical to consider that the presence of FOXA1 in ERα-negative breast carcinomas represents an increase in patient disease-free survival, since this expression probably confers 1) some biological growth inhibitory potential on cancer cells, or 2) increases the response efficiency to breast cancer treatment, therefore, delaying the recurrence hit. Analyzing the first assumption, it is important to retain that FOXA1 is a transcription factor and, as so, it can bind to the promoters of a myriad of genes associated with important signalling pathways, being the ERα-signalling pathway one of the classical examples. However, this transcription factor has been recently described to be important for the activation and expression of the cell cycle inhibitor $p27^{kip1}$ in breast cancer cell models. Importantly, this p27 induction is achieved by interaction of FOXA1 with BRCA1, since co-transfection of these two transcription factors resulted in a synergistic activation on the $p27^{Kip1}$ promoter (29). In this interesting study from Williamson *et al*., it was shown that co-transfection of FOXA1 and BRCA1 resulted in a greater amount of each protein, compared to transfection of each expression vector alone, and that the half-life of FOXA1 was increased when co-expressed with BRCA1 (29). Even so, FOXA1 is capable of p27 activation, either alone or in conjunction with BRCA1 (29). The tumour suppressor BRCA1 is probably the best studied gene in breast cancer and its role in regulating the expression of many genes implicated in cell cycle regulation (p21, p27, cyclin B1) and DNA repair are well established (29-30). Most importantly, a robust study from Liu *et al.*, showed that FOXA1 also plays an important role in the upregulation of the E-Cadherin gene (*CDH1*) promoter through its binding to the E-cadherin regulatory sequence (31). The activation of these well known tumour suppressor genes by FOXA1, in cooperation with p300 and AML1, led to the expression of E-cadherin and consequent reduction on motility of ERα-negative metastatic breast cancer cells. Notably, it was suggested that FOXA1 positively regulates the expression of E-cadherin through chromatin modulation at *CDH1* promoter level, and thus, allowing the coactivators binding to this promoter (31). Overall, it seems plausible that, in ERα-negative patients harbouring FOXA1 expression, this protein would provide a "compensatory growth-inhibitory effect", which is translated by the FOXA1-induced activation of important tumour suppressor genes, such as p21, p27 or E-cadherin. This would lead to a reduction, not only

on the cell cycle errors and DNA damages associated with tumorigenesis, but also in increasing the cellular adhesive potential towards a lessening of the metastatic behaviour.

Although speculative in the context of our study, it is tempting to suggest that FOXA1, as an ERα-associated gene, might be important to the hormone-responsive phenotype of breast cancer, regardless of the tumour ERα status. ERα expression alone has been used to guide systemic therapy and to estimate breast cancer patient prognosis. However, not all ER-positive carcinomas show comparable prognosis or react similarly to anti-hormonal therapy (30%), whereas some ER-negative tumours (5-15%) curiously respond to endocrine therapy (32). Taking in account the previously described cross-talk and functional network between FOXA1 and the regulation of ERα and its downstream targets, especially in what concerns the chromatin changes produced by FOXA1 as a signal for ERα binding, it is likely that, in the absence of ERα, the chromatin marks associated with FOXA1 would be occupied by coregulators complexes which, in an oestradiol-independent response pathway, would still activate some ERα-target genes. Thus, if this might enlighten a mechanism by which those 5 to 15% of ERα-negative tumours become responsive to endocrine-driven therapies, it also brings up the crucial biological role of FOXA1, as well as other transcription factors and coregulators, in a setting of those 30% of ERα-positive tumours that are not hormone-responsive. In other words, although ERα is likely to be essential to hormone response, the expression of an additional set of genes that are not part of the oestradiol-response pathway may be also important in defining the hormone-responsive phenotype in ER-negative breast cancer or, at least, in delaying the recurrence of the disease. A classical example of this hypothesis in breast cancer are the AP2 transcription factors, which control a set of genes that are commonly expressed in association with ER and contribute to the hormone response via oestrogen-independent signalling pathways (33). Curiously, Doane *et al*. identified an ER/PgR-negative breast cancer subset characterized by a hormonally regulated transcriptional program (34). In this very robust study, this subset of hormonal-negative malignant lesions, which were named as “ER-negative class A” tumours, was characterized by a hormonally regulated transcription program and response to androgen. More importantly, they display a paradoxical expression of genes known to be either direct targets of ER, responsive to oestrogen, or typically expressed in ER+ breast cancer. FOXA1 was one of these genes identified in this hormonal-responsive ER-negative subset of

tumours (34). Interestingly, authors suggested that ER-negative class A breast cancers bear a much closer molecular relationship to ER-positive breast cancers than to the basal subtype, despite the shared ER-negative phenotype. Moreover, authors ascribe part of this hormonally regulated transcriptional program to the presence of androgen receptor (AR) within these ER-negative tumours, speculating that this receptor may act in concert with other signal transduction pathways, in order to modulate the molecular events associated with this special ER-negative subset and contributing to its phenotype (34). In fact, AR expression is well known in ER+ breast cancer and has been associated with ER-negative breast tumours with apocrine histological features (35-36).

Another transcription factor gene, which is not oestradiol-responsive but contributes to the ERα associated breast cancer phenotype, is GATA-3. In fact, a striking association between ERα and GATA-3 expression in breast carcinomas has been demonstrated, indicating an important functional role of this transcription factor in hormone-responsive breast cancers (37). Additionally, GATA-3 has emerged recently as a strong predictor of clinical outcome in human luminal breast cancer, and its fundamental role in maintaining the differentiation and adhesion of the luminal cells has been extensively explored (38). Experimental data showed that induced expression of GATA-3, in GATA-3-negative undifferentiated breast cancer cells, is sufficient to induce differentiation and inhibit tumour dissemination in a mouse model. In fact, GATA-3 defines a distinct class of cancer genes that are differentiation factors, which affect the malignant phenotype by enforcing differentiation, rather than conventional tumour suppressor genes (38). As a transcription factor, GATA-3 works in a very similar fashion as FOXA1; GATA-3 binds to a consensus DNA sequence at gene promoters, to directly activate or repress expression, by recruiting chromatin remodelling complexes to remodel gene loci (39-40). Indeed, FOXA1, GATA-3 and ERα form a transcriptional circuit required for growth, differentiation and hormonal dependency of mammary luminal cells, where FOXA1 appears as a downstream target of GATA-3 (41). Since this intricate functional complex has been shown to be important for breast cancer progression and behaviour, we included GATA-3 in our study, in order to assess at which level this transcription factor could be important as a prognostic factor in breast cancer (see Chapter 2). Herein, besides the strong association found with the “good prognosis signature” of the luminal tumours, GATA-3 expression failed the association with

most of the clinicopathological features and did not demonstrate a significant association with disease-free survival. Indeed, GATA-3 was neither a predictor for breast cancer survival nor a prognostic marker, but was shown to be an important and robust luminal differentiation marker, even stronger than FOXA1. Interestingly, a very strong inverse association was observed with the basal-like markers, namely, CK5, CK14, vimentin, EGFR and P-cadherin (see Chapter 2), suggesting that GATA-3 can be important for the differentiation state of the malignant cells, rather than acting as a tumour suppressor gene, as actually was recently suggested by other authors (38). Thus, the presence of GATA-3 expression, together with other differentiation partners, may drive the luminal profile of a malignant cell population within the tumour.

In conclusion, transcription factors are determinants of human complexity and are crucial in cancer (1, 7). The majority of oncogenic signalling pathways converge on sets of transcription factors, which ultimately control gene expression patterns, resulting in tumour-related processes. However, under normal physiological conditions, whole sets of genes, with similar functions, are regulated by highly specific, tightly regulated upstream transcriptional regulators; in cancer, aberrant activation of these transcription factors lead to deregulated expression of multiple gene sets associated with tumour development and progression (42). Over the last years, transcription factors have been attracting growing attention as new biological tools with clinical, pathological and therapeutic potential. In fact, because activation or inactivation of these proteins drive the cellular gene expression pattern and consequent cell functions in a tightly regulated manner, they represent not only important markers of tumour behaviour with pathological usefulness, but they also constitute highly desirable and logical points of therapeutic interference in cancer development, progression and in treatment-response prediction.

4.2 – P-cadherin Overexpression and its Regulation by Anti-Oestrogens in Breast Cancer

In contrast to E-cadherin, P-cadherin expression is usually related to tumourigenic properties, enhancing cell invasion and tumour aggressiveness, and indicating a worse prognosis in breast cancer patients (43-45). P-cadherin overexpression is able to identify a subgroup of lesions with a more aggressive behaviour (43-49). Indeed, it is possible to see

that there is a significant association between P-cadherin overexpression and the increased invasion capacity and directional migration of breast cancer cells that maintain the expression of wild-type E-cadherin (47, 50). Additionally, using *in vitro* cell models, we found for the first time that overexpression of exogenous P-cadherin is able to promote single cell motility, inducing an increase in the number and speed of moving cells, when compared with cells with low levels of this protein (50). This behaviour was shown to be directly dependent on P-cadherin, as when overexpressing cells were treated with a P-cadherin blocking antibody or transfected with a siRNA to inhibit P-cadherin transcripts, there was an inhibition of both migration and invasion effects (50). Interestingly, other authors have recently identified *CDH3* as one of the genes involved in the regulation of breast cell migration using a siRNA approach (51). Usually characterized as an hallmark of the cellular motile behaviour, P-cadherin is also able to induce phenotypic changes involving alterations in cell polarity, leading to edge morphology, formation of membrane protrusions, as well as increase of their cytoplasmic area (50). As such, there is an association between P-cadherin expression and actin cytoskeleton reorganization, which suggest that P-cadherin has a role in the mechanism that integrates localized and transient signalling events and, therefore, regulating the cellular architecture changes that are needed to promote cell migration and invasion (50). Moreover, P-cadherin induces the secretion of factors that facilitate cell invasion of non-invasive MCF-7/AZ cells, that we identified as being active forms of MMP-1 and MMP-2 (50).

The *in vitro* functions assigned by P-cadherin are in agreement with what has been described in invasive primary breast tumours. In clinical terms, P-cadherin-overexpressing tumours present high proliferative index, high histological grade, decreased cell polarity, aggressive behaviour and worse patient survival (43-46), reinforcing the clinical relevance of P-cadherin expression in the diagnosis and prognosis of patients with aggressive mammary carcinomas, especially those carrying node-negative breast cancers (43, 45, 47, 49). Very recently, a large study comprising 4.444 breast tumour samples confirmed the value of P-cadherin expression as a marker of poor prognosis, associated with high-grade tumour subtypes of breast cancer (52). Interestingly, P-cadherin is also a marker for basal-like breast cancers, including metaplastic breast carcinomas, and is strongly associated with the presence of *BRCA1* mutations and lack of ERα-signalling (47).

Recent works have allowed the clarification of P-cadherin function in breast cancer cells. However, the expression regulation of this protein has been poorly elucidated. The only mechanism known to be important as having a regulatory role on P-cadherin overexpression in breast cancer was described by our group, in a study where we demonstrated that hypomethylation of *CDH3* promoter was an epigenetic event associated with P-cadherin expression (43). Additionally, in a previous work, we reported a connection between ERα-signalling pathway and overexpression of P-cadherin in breast cancer cells. As such, we demonstrated that the abrogation of ERα-signalling pathway, caused by the pure anti-oestrogen ICI, led to the increase of P-cadherin protein and mRNA expression, pointing *CDH3* as an oestrogen-repressed gene (49). Since ERα is a key regulator of proliferation and differentiation in mammary epithelia cells, as well as a crucial prognostic indicator and therapeutic target in breast cancer (53), the finding that its blockage/degradation, through the usage of anti-oestrogen therapy, was associated with the up-regulation of a pro-invasive protein such as P-cadherin, represents a challenging biological and clinical issue.

Notably, breast cancer research has demonstrated that, although it was initially believed that anti-oestrogens would function merely by competing with endogenous oestrogens for receptor binding, anti-oestrogens can also interfere with a plethora of crucial cellular pathways. Indeed, at the epigenetic level, previous studies showed that chromatin structural remodelling and nuclear entropy can be induced by the treatment of breast cells with anti-oestrogens, such as ICI (54). The role of ICI in provoking elevated density (hyperplastic) of all epithelial structures in mice mammary gland development, as well as the finding that it can inhibit BKCa channels in vascular endothelial cells via a mechanism unrelated to its anti-oestrogenic activity (55), exemplifies how heterogeneous can be the non-ER-related mechanisms mediated by anti-oestrogens in cells (56). Depending of the anti-oestrogens type, ERα levels can be modulated differently. While tamoxifen increases ERα levels through its accumulation in the cytoplasm, ICI drastically decreases ERα levels (57). However, anti-oestrogens are able to modify and modulate other important pathways not directly related with ERα-signalling within the cell. It has been reported that tamoxifen and ICI can induce growth inhibitory effects via the sustained activation/inactivation of signalling pathways that regulate cell survival, cell death and differentiation in the absence of ERα (57). Indeed, levels of protein phosphorylation of the PI3K/Akt, ERK and IGF-1R

pathways suggested that the anti-tumour activity of tamoxifen and ICI is unlikely to be mediated solely by ERα. Particularly in this pathway, antioestrogen treatment modulates PI3K/Akt and increased ERK1/2 activation, irrespective of ERα expression (57). At the clinical level, it is important to highlight that oestrogen is known to up-regulate PI3K/Akt, and constitutive activation of Akt is associated with resistance to anti-oestrogens both *in vitro* and *in vivo* (58-60). Noteworthy, in a study where hormone-responsive MCF-7 cells were long-term exposed to ICI treatment, insensitivity to the growth-arrest capabilities of ICI, as defined by altered growth characteristics and an apparently resistant phenotype, was witnessed (61). The most significant observation with this ICI-resistant breast cancer cells was that their adaptation to growth in the steroid-deprived environment, and the resultant reduction of effective ERE-signalling mechanisms, was reflected on an acquired increased expression of a number of components involved in the EGFR/MAPK signalling pathway (61). This upregulation of both EGFR protein and mRNA levels in ER-positive breast cancer cells as a consequence of effective suppression of ER-mediated signalling after anti-oestrogen treatment or ERα deprivation has been supported in the literature (62-63). Curiously, and unlike wild-type cells, MCF-7 cells treated with ICI are also strongly growth inhibited by physiological doses of an EGFR-specific tyrosine kinase inhibitor (61). However, it remains probable that components of other signalling pathways in these cells have also been altered in response to ICI exposure and that the identified changes in the growth and phenotype of these cells may, in part, reflect these changes also (61).

In a different anti-oestrogen-modulated mechanism type, a study conducted by Lam *et al*., showed that the treatment of MCF-7 cells with anti-oestrogens led to the increase of Bcl-2 protein, known by its anti-apoptotic activities (64). Interestingly, this treatment, and consequent overexpression of Bcl-2, led to the junction dissociation and redistribution of junctional components (E-cadherin and ZO-1) to the cytoplasm in breast cancer cells (57). Similarly, a recent study also showed that treatment of ER-positive breast cancer cells with ICI led to the activation of the NFkB signalling pathway and up-regulation of Bcl-2. More importantly, increasing Bcl-2 levels by exogenous expression or by ICI treatment, decreased E-cadherin-mediated adhesion and induced an epithelial-to-mesenchymal-transition (EMT) (65). Based on this, it is tempting to consider that the effect of ICI in breast cancer cells is able to provoke changes on the membranar cadherins balance, by internalizing and

redistributing some cadherins in the cytoplasm and keeping others at their surface. Doing this, cells may compromise some adhesion properties with their neighbours and, therefore, acquire motile and invasive properties. In fact, our group showed that ICI treatment of MCF-7/AZ cells lead to an up-regulation of P-cadherin expression, but also to a decrease on cell-cell adhesion, with associated promotion of *in vitro* invasion (49). But, while P-cadherin-induced cells migrate faster than controls in wound healing migration assay, ICI-treated cells did not, although inducing invasion. This can be explained by the finding that ICI up-regulates additional pro-invasive genes, such as MMP-2 and -9 (49). Altogether, these findings concerning ICI-mediated upregulation of genes, such Bcl-2, NFkB, ERK1/2, and induction of MMPs, represent, in a manner, some genetic alterations that are induced by anti-oestrogens treatment in breast cancer cells which, depending of the cell type and its molecular background, may induce cell invasion and/or migration. Supporting this, it has been demonstrated that acquired resistance to ICI (commercially known as Fulvestrant) is an ERα-independent phenomenon, which uses pronounced up-regulation of multiple growth-stimulatory pathways to establish autocrine-regulated proliferation (66).

Exploring the results represented here, together with previous studies conducted by us, we identified *CDH3*/P-cadherin as a highly oestrogen-repressed gene, since ERα gene silencing by siRNA or treatment with anti-oestrogen ICI, led to overexpression of P-cadherin, suggesting a repression abrogation. Indeed, in breast cancer cells, ERα is a master regulator of transcriptional stimulation and repression. Yet, although the mechanisms by which agonistic-bound ERα elicits repression are poorly understood (67-68), the roles of ERα in hormone-induced repression have been attracting growing attention in breast cancer, as exemplified by some studies. In MCF-7 cells, the breast cancer and salivary gland expression (BASE) gene is repressed by oestrogen in an ERα-dependent manner. Similarly with what we have found, these authors showed that the cell treatment with ICI induced BASE transcription and increased mRNA levels (67). Also in MCF-7 cells, it was recently demonstrated that ERα was required for gene transcriptional repression of early target genes which is released by the treatment with ICI (68). NFAT3, a transcription factor involved in breast cancer, was also showed to be repressed at the transcriptional level by ERα through phosphorylation and dephosphorylation of ERα events (69). Using breast cancer cell lines, Cvoro *et al.* demonstrated that ERα, in the presence of E2 and recruiting

the GRIP co-repressor, acts as a repressor of TNFα (70). Interestingly, CD24, a gene which has recently generating considerable attention in tumour biology due to its role as a potential breast cancer stem cell marker, as well as concerning its function in cell adhesion and metastatic tumour spread, was described as an oestrogen-repressed gene. Additionally, this repression was a direct transcriptional effect depending of ERα and HDACs (71). In fact, in this study, and similarly with what demonstrated for *CDH3*/P-cadherin, treatment of MCF-7 cells with HDAC inhibitor Trichostatin A (TSA) completely abolished the gene transcription repression, reinforcing the existence of a repressive complex ERα-HDAC with relevant transcriptional inhibitory functions. The same repressive complex was also demonstrated to be important in the transcriptional repression of SLUG (one of the genes involved in EMT), pointing it as an oestrogen-repressive gene in MCF-7 cells (72). The exposure of MCF-7 cells to ICI also induced a significant up-regulation of KAI1 gene transcription, a tumour growth suppressor which is inactive in ERα-positive breast cancer cells (73). An elegant study, from 2005, showed that ICI can induce gene transcription through releasing HDACs and ERα from Sp1 sites in ERα-repressed genes. Varshochi and colleagues demonstrated that, in the presence of ICI, ERα and HDACs are dissociated from Sp1 sites, resulting in an increased histone acetylation and de-repression of the $p21^{Waf1}$ gene promoter, with consequent protein expression induction of this cyclin kinase inhibitor (74). Still, the authors found that the promoter activation induced by ICI was significantly reduced, when the proximal region of the $p21^{Waf1}$ promoter, containing six putative Sp1 binding sites, was deleted; this suggested that the proximal Sp1 sites are crucial in mediating the promoter's response to ICI, probably because these are preferable sites for ER binding. Similarly, when the cells were treated with TSA, the induction of $p21^{Waf1}$ promoter activity was observed, probably due to the disruption of the epigenetic repressive system formed by ERα and HDAC recruitment to Sp1 sites (74). A very similar work was performed, with the aim of understanding the mechanism by which cyclin G2 is also repressed by oestrogen in ER-positive breast cancer cells, leading to its rapidly down-regulation (75). They showed that ligand-activated ERα is recruited to cyclin G2 regulatory region, which is followed by dismissal of RNA polymerase II and recruitment of a complex containing N-CoR and HDACs. Additionally, these authors showed that ER-repressive binding occurred in a GC-rich region that interact with ERα and Sp1 proteins, and also that the half-ERE, as well as the Sp1 sites,

are determinants for the recruitment of ERα to the cyclin G2 promoter (75). In fact, GC-rich regions are known to be involved in ER-mediated gene promoter repression, where interplay of ERα with members of the Sp1 family of transcriptional factors seems to occur (75). Two common molecular features of all the abovementioned repressive mechanisms involving HDACs is the presence of Sp1 transcriptions factors, coupled or not with ERα proteins, as well as the presence of Sp1 sites in regulatory regions of repressed-target genes. Actually, the requirement of specific Sp1 sites for histone deacetylase-mediated repression and the interaction between Sp1-HDAC complexes with components of the cognate transcriptional regulators that bind to DNA, has been showed to be determinant for repression of several genes such as, as an example, the TGFβII, in pancreatic cells (76-77). These Sp1 sites are also important in mediating the transcriptional responsiveness to HDAC inhibitors, such as TSA or SAHA, in pancreatic (77) and in MCF-7 breast cancer cells (78), respectively.

In our study, the prediction analysis of the proximal *CDH3* promoter, revealed a promoter region remarkably enriched in ERα sites or half-sites, together with abundant Sp1 sites localised closer to the former ERs (see Chapter 3). Moreover, and similarly with what was observed for the ER-repressive binding in cyclin G2, the *CDH3* proximal promoter Region 2, which showed an ICI-induced enrichment for active histone mark, also displays a GC-rich region, reinforcing this promoter area as preferential for the described repressive interactions between ERα, Sp1 and HDAC proteins. Hence, in accordance with our suggested model, a repression complex, mediated by ERα and HDACs at Sp1 sites (Figure 11 A), is able to be released by the treatment with ICI, enhancing *CDH3* gene transcription through a mechanism similar to several other models here described. If so, the characteristics of the proximal *CDH3* promoter strongly suggests that this chromatin de-repression mechanism, driven by the H3K4me2 enrichment in Region 2, plays an important role in the ICI-induced promoter transcriptional activation and P-cadherin overexpression (Figure 11 B). Reinforcing this, our results are also corroborated by the findings of other studies described above, in such a manner that an up-regulation of *CDH3* promoter activity and P-cadherin protein expression in cells was observed when these were treated with HDAC inhibitors such TSA. In our model of ER-mediated repression, we suggest not only that Sp1-HDAC complexes are playing a role in mediating the transcriptional responsiveness of *CDH3* gene promoter to

HDAC inhibitors, but also, that chromatin activating modifications are indeed important to the modulation of this gene, in response to ICI treatment (Figure 11).

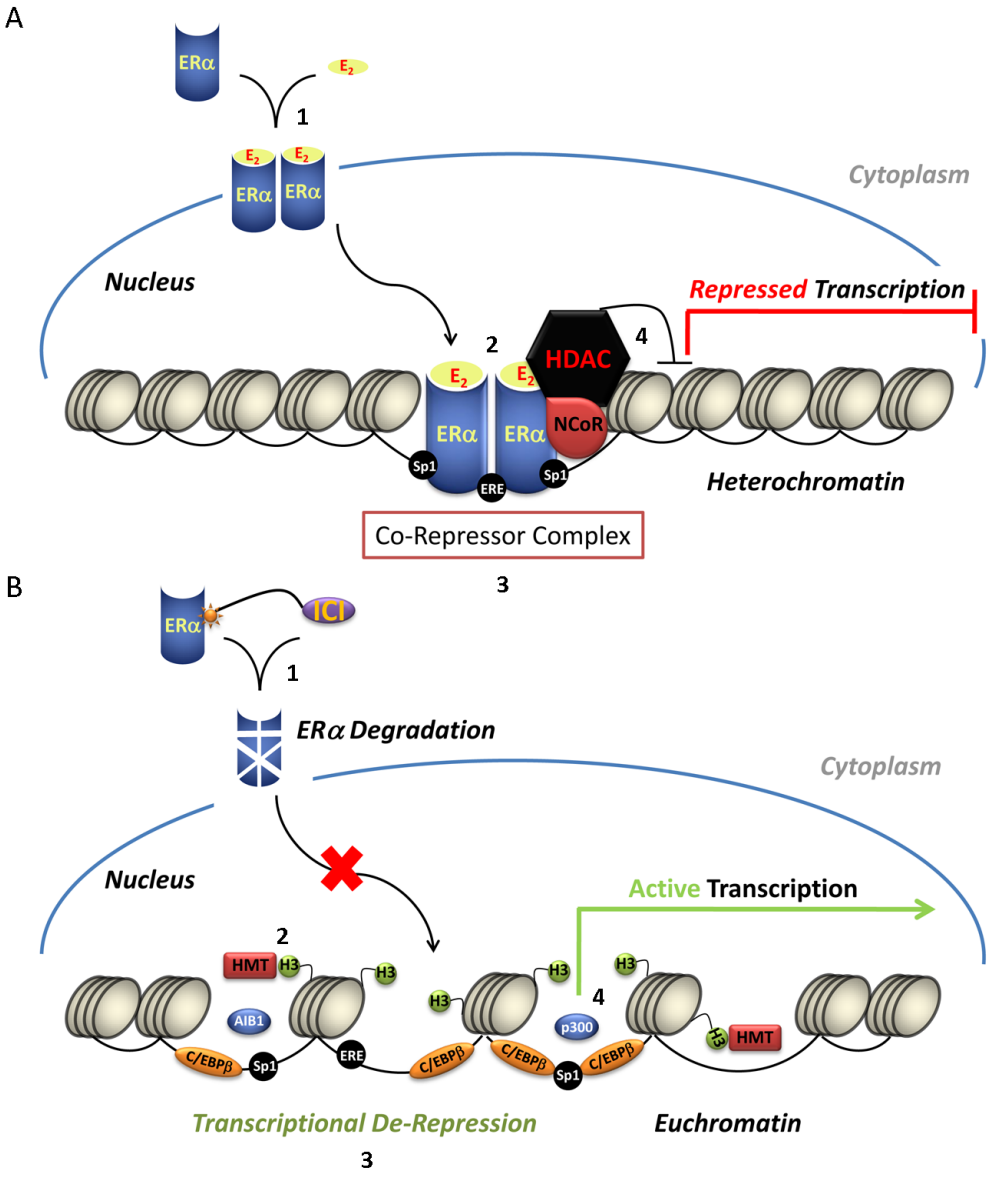

Figure 11. A proposed model for oestradiol-occupied ERα repression of the *CDH3* promoter in MCF-7 breast cancer cells. **(A)** Oestrogen receptor alpha, upon binding of E2 **(1)**, is recruited by Sp1 to the half-ERE-containing regions **(2)** and actively represses the *CDH3* promoter by recruiting a HDAC-containing co-repressor complex **(3)**, which causes release of RNA polymerase II **(4)**. Formation of this complex leads to hypo-acetylation of the N-terminal histone tails, which causes stabilization of the nucleossome structure, limiting accessibility to the basal transcriptional machinery and thus suppressing *CDH3* transcription. This action requires ERα binding to DNA and the presence of Sp1 at the promoter site. **(B)** Upon degradation of ERα by the pure anti-oestrogen ICI at the cytoplasm compartment **(1)**, ERα is no longer able to be shuttled to the nucleus and bind to DNA. ICI increases the RNA polymerase II occupancy by inducing an activating chromatin remodelling. Histone methyltransferases (HMT) are recruited to the transcriptional machinery inducing histone modifications marks, such as H3K4me2 **(2)**, leading to transcriptional de-repression **(3)** and exposure of binding sites. Sp1 factors can recruit activators (e.g. AIB1 and p300) forming a transcriptional activating complex at *CDH3* promoter, thus enabling a permissive chromatin conformation and gene transcription **(4)**. The abundant presence of C/EBPβ in the nucleus of MCF-7 cells enables its binding to exposed C/EBPβ binding sites, increasing *CDH3* promoter activation.

Gene expression can be epigenetically regulated at several levels, including the position of the gene in the nuclear space or through the conformation of chromatin at the genetic locus. Histone modifications are known to be altered in cancer cells and loss of selected histone acetylation and methylation marks has recently been shown to predict patient outcome in human carcinomas (79-80). These modifications generate a combinatory histone code that demarcates chromatin regions for transcription activation or repression (81). Although the "epigenetic" code is not fully understood, specific marks are associated with transcriptional active gene promoters, whereas others modifications are linked to repressed chromatin (81-82). In the present study (see Chapter 3), we identified for the first time the existence of a chromatin remodelling at *CDH3* promoter level induced by the treatment of MCF-7/AZ breast cancer cells with ICI. This epigenetic event was characterised by the occurrence of an important histone activating mark (H3K4me2) enrichment at the proximal region of the *CDH3/*P-cadherin promoter. Accordingly with several recent studies, histone H3K4 di-methylation is a post-translational modification that is localized to punctuate sites near the transcription start sites (83-84), being now well accepted as a fingerprint of active euchromatic regions of transcribed genes (82, 85-87). In a very interesting study, recently published, frequency of histone modifications were compared with clinical profiles in a large series of breast cancer samples. Here, these epigenetic alterations were clustered in 3 groups displaying distinct patterns of breast cancer and highly significant correlation between histone modification status, tumour biomarker phenotypes and clinical outcome were found (80). The histone modification cluster comprising the H3K4me2 activation mark was remarkably associated with the poor prognostic classes of basal and HER2 breast cancer subtypes, as well as with poorer breast cancer specific survival and disease-free survival (80). Notably, the overexpression of P-cadherin is strongly associated, not only with the basal and HER2 phenotypes, but also with poorer survival in breast cancer (43-47, 49, 88). Overall, this study emphasizes the importance of studying and understanding histone modification patterns in the context of different phenotype classes of invasive breast tumours, in order to better define the biological nature and clinical behaviour, and eventually, inform possible therapeutic strategies (80).

Concerning anti-oestrogen therapies in breast cancer, it has been shown that interrupting ERα function by anti-oestrogens can result in epigenetic modifications of chromatin and altered gene expression, leading to endocrine resistance mechanisms (89-90). In fact, oestrogenic chemicals induce epigenetic alterations in breast progenitor cells, which have been previously implicated in breast cancer (91). Previously studies have found distinct gene expression and promoter DNA methylation profiles associated with acquired resistance to fulvestrant and tamoxifen. Regarding ICI (or fulvestrant), which constitutes the focus of part of the work discussed here, it was shown that a large number of signature genes of ERα-positive tumours were significantly down-regulated in MCF-7 cells treated with ICI, suggesting that acquired fulvestrant resistance is an ERα-independent phenomena that is coupled with the generation of ERα-negative phenotype (66). These interesting results were recently reinforced by Borley *et al*., in a study that highlights the effect of anti-oestrogens in inducing breast cancer cell invasion and migration (92). This may occur in a specific context of absence of good cell-cell contacts, perhaps through a process involving activation of Src kinase and loss of E-cadherin (92). More interestingly, activation of growth-promoting genes, such as EGFR/HER2, Notch or β-catenin due to promoter hypomethylation, was more frequently observed in anti-oestrogen-resistant cells compared with gene inactivation by hypermethylation, revealing an unexpected insight into the molecular changes associated with endocrine resistance (66). Curiously, it was also demonstrated that cells displaying hypomethylation of ERα target genes may be able to escape the detrimental effects of anti-oestrogens, establishing that hypomethylation plays a role in the development of anti-oestrogen resistance (66). Although not in a context of endocrine resistance, in the work presented here, we also provide data that is in line with these findings above, since we described that hypomethylation of the ER-repressed *CDH3* promoter was found in high grade invasive tumours expressing high levels of the pro-invasive P-cadherin. This *CDH3* promoter hypomethylated state was associated with poor prognosis and decreased breast cancer survival (see PAPER I in Appendix section) (43). Additionally, we demonstrated here that histone activation of regulatory regions of chromatin at *CDH3* promoter level, another form of activate transcription state, is induced by antioestrogen treatment, leading with expression of P-cadherin in breast cancer cells (see Chapter 3). The action of ICI on *CDH3* promoter observed in our study is supported by a

robust study published recently, where identification of ERα-binding sites in MCF-7 cells, as well the effect of tamoxifen and fulvestrant on these ERα-binding sites using ChIP-Seq was assessed. Interestingly, the authors showed that in E2-repressed genes, while tamoxifen acts as an agonist, downregulating these genes, fulvestrant (ICI) antagonizes the E2-induced repression and often increases the RNA polymerase II occupancy enabling transcription (93). These findings demonstrates that, not only both antagonists act differently on E2-induced and E2-repressed genes, but more importantly, they corroborate our findings in a way that they showed the ability of ICI to induce ER-mediated de-repression of ERα-binding sites within ERα-repressed genes.

Another finding described here involves the putative potential of the transcription factor, C/EBPβ, as a novel activator of *CDH3* promoter activity and P-cadherin expression in breast cancer cells. *CDH3* promoter Region 2, where ICI-mediated chromatin remodelling was observed, together with the surrounding regions, was identified has containing several putative C/EBPβ binding sites. Notably, besides being identified by several binding sites prediction tools, the presence of these C/EBPβ sites in genomic regions enriched in ERE has been supported. As such, in a study from 2009, it was demonstrated that both C/EBPβ and FOXA1 motifs are positively associated with ERE regions (93). *In vitro* studies confirmed the relevance of this transcription factor in *CDH3* promoter activation, as well as demonstrated its abundant expression in the nucleus of MCF-7 breast cancer cells (see Chapter 3). Based on this, we suggest that after ICI-mediated chromatin activation by ERα deprivation and transcription de-repression, C/EBPβ binding sites become exposed to the C/EBPβ transcription factors (Figure 11 B) which, being abundantly present in the nucleus, will bind to the DNA and activate *CDH3* transcription. We also showed that among the three C/EBPβ isoforms, truncated LIP was the most important in a *CDH3* promoter activation context. A promoter feature that reinforces the hypothesis of interaction of C/EBPβ–LIP with *CDH3* regulatory region is the extremely abundant Sp1 sites within the chromatin activated region. Indeed, as demonstrated by some authors, the presence of Sp1 sites within promoters is crucial for C/EBPβ–LIP-mediated transcription of several genes (e.g. PLAC1 and prolactin receptor gene) in breast cancer cell lines (94-96). In tumours, C/EBPβ–LIP isoform is only overexpressed in highly aggressive/proliferative, progesterone/oestrogen-receptor-negative breast malignancies, but not in pre-neoplastic or normal breast gland (97-98). This

association with poor prognosis and breast cancer aggressive features, such as tumour grade, proliferative index and EGFR expression, overlap with the ones found in tumours overexpressing P-cadherin. Interestingly, it seems that C/EBPβ-LIP is an isoform that characterize a breast cancer cell malignant status. It has been described that C/EBPβ-LIP isoform, and elevated LIP:LAP ratio, induces potent oncogenes, such as MYC, leading to increased malignant transformation in human breast cells, and more importantly, with lack of cell contact inhibition (99). Additionally, in a study where the mechanism by which the LIP isoform is translationally regulated in mammary epithelial cells was explored, it was showed that the transcription factor C/EBPβ-LIP is a downstream target of EGFR in those cells and that this LIP expression is controlled post-transcriptionally (100). Reinforcing this, in our study (see Chapter 3), we actually showed a strong association between the expression of EGFR and C/EBPβ in a patients setting. These observations have led to the hypothesis that overexpression of the C/EBPβ-LIP isoform in the mammary gland can result in epithelial cell proliferation that may render cells to become more susceptible to additional oncogenic hits, resulting in the stochastic formation of breast tumours and, additionally, alter cell fate by preventing the transcription of genes that control differentiation (97, 100-101). Another interesting involvement of C/EBPβ-LIP in breast cancer was raised by a study in which it was showed that a high level of LIP is linked to a loss of the TGFβ-dependent cytostatic responses in metastatic cells from breast cancer patients. Here, authors also suggested a mechanistic link for how high LIP:LAP ratio can contribute to an unfavourable evolution of breast cancers (102). Moreover, due to its described association with hormonal-negative breast tumours and with its contribution to an increased growth and proliferation rate (97), it has been suggested that C/EBPβ-LIP might indirectly contribute to drug resistance in breast cancer (103-104).

In conclusion, we showed that 1) ICI can facilitate the binding of the transcription factor C/EBPβ−LIP to *CDH3* promoter, 2) the C/EBPβ-LIP is able to activate the pro-invasive P-cadherin gene and that 3) C/EBPβ expression is highly associated with the pro-invasive P-cadherin expression and with basal-phenotype markers in breast tumour samples. Taken together, it is tempting to consider that the activation of *CDH3* by C/EBPβ, especially under long ICI-treatment regimens, might be one important step for acquired resistance to the anti-oestrogen and more importantly, for an increased invasiveness cell capacity. Moreover,

in this study, we established that the oestrogen-occupied ER can carry out a program of negative regulation which, along with the ability of these oestrogen-occupied ERs to work at other genes to enact hormone-induced positive regulation, permits an integrated pattern of gene regulation that underlies the ability of this sex-steroid hormone to control important genes and cellular activities in breast cancer. Because our studies and those of others indicate that repression of gene expression by oestrogen plays a central role (105-107), a greater understanding of the mechanisms and proteins involved in ER-mediated regulatory functions should provide new and important insights, as well as reveal potential therapeutic strategies. This study constitutes a step toward understanding the way in which the oestrogen-ER complex can attenuate gene activity, but also, contributes to elucidate how anti-oestrogenic drugs can up-regulate gene activity of targets involved in aggressive breast cancer cell behaviour.

Still, several lines of evidence point out the significance of epigenetic regulation in initiating breast carcinogenesis, promoting tumourigenic phenotypes, and assisting in the development of drug resistance to anti-oestrogens. How breast cancer cells acquire more aggressive properties after loss of oestrogen signalling, remains a very important issue in the field of breast cancer research.

4.3 – Concluding Remarks

Accumulation of genetic changes is commonly believed to promote cancer development. However, if this concept provides the basis of our knowledge of cancer progression, it cannot explain the heterogeneity in tumour cell growth, invasion or resistance to therapy. The role of transcriptions factors, as key entities for the most fundamental cell functions, has becoming studied as important tools to be used in clinical pathology. Modulation of transcription factor activity, through genetic or epigenetic processes, constitutes the tip of the spear of the cellular gene expression and cellular biology. In cancer, where those genetic and epigenetic mechanisms are someway disrupted, transcription factors expression naturally represents important markers of tumour behaviour with pathological value. Moreover, since they drive the crucial human oncogenic

pathways, their usefulness as targets for therapeutic interference in cancer development, progression and in treatment-response prediction, has been collecting expressive consideration. Some transcription factors, through their interaction with chromatin and as pioneer factors for protein binding to DNA, also play an important role in cancer epigenetic processes. Indeed, it is the recruitment of distinct remodelling proteins and regulation factors to responsive sites within the DNA, which determines normal and deregulated expression patterns and makes the linkage between genetic and epigenetic phenomena in carcinogenesis.

Taken together, the study presented here encloses the following major conclusions:

- There is a strong association between ERα, FOXA1 and GATA-3 expression in breast cancer.
- The transcription factor FOXA1 is a significant predictor of good outcome in breast cancer and may be used for risk stratification among ERα-negative patients.
- GATA-3 was neither a predictor for breast cancer disease-free survival nor a prognostic marker, but was shown to be an important and robust luminal differentiation marker, even stronger than FOXA1.
- The expression assessment of FOXA1 and GATA-3 can provide important clinical information – not only regarding the favourable prognostic nature and tumour behaviour, but can also constitute an important tool to define and assess the luminal A subtype in breast cancer.
- *CDH3* is an ERα-repressed gene which is up-regulated by the abrogation of ERα-signalling pathway.
- A chromatin remodelling event, provoked by the pure anti-oestrogen ICI 182,780, is able to increase *CDH3* promoter activity and P-cadherin expression in breast cancer cells, through the induction of high levels of the active histone mark H3K4me2.
- The transcription factor C/EBPβ is able to up-regulate *CDH3* promoter activity in breast cancer cells.
- The expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients.

4.4 - References

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5. Appendix

Paper I

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Paper VII

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