Ana Rita Lobato Cortesão Nobre

REGULATION OF *CDH3*/P-CADHERIN IN NORMAL AND TUMOUR EPITHELIAL TISSUES

Dissertação de Candidatura ao grau de Mestre em Oncologia, Especialização em Oncologia Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador - Doutora Joana Paredes
Categoria - Investigadora Principal e Professora Afiliada
Afiliação - Instituto de Patologia e Imunologia Molecular da Universidade do Porto e Faculdade de Medicina da Universidade do Porto.

Co-orientador - Doutor André Albergaria
Categoria - Investigador Auxiliar e Professor Afiliado
Afiliação - Instituto de Patologia e Imunologia Molecular da Universidade do Porto e Faculdade de Medicina da Universidade do Porto

AGRADECIMENTOS

A concretização desta tese não seria possível sem a ajuda de muitas pessoas às quais não posso deixar de agradecer, sendo no entanto impossível agradecer extensamente a cada uma delas.

À Joana Paredes, minha orientadora, agradeço primeiramente a oportunidade de integrar a "P-cadherin Team". Esta equipa reflete o teu elegante equilíbrio entre paixão contagiante pela Ciência e o lado humano das relações entre as pessoas que dela fazem parte. Obrigada pela confiança depositada em mim e pela oportunidade.

Ao André Albergaria, meu co-orientador, o meu obrigada pela disponibilidade, mas principalmente pela exigência e sentido crítico que me fazem sempre crescer. Foi um enorme prazer herdar o tema e paixão pela regulação.

À Ana, a minha chefinha, um profundo obrigado por todo o apoio, atenção e paciência. A tua rapidez e descontração são de causar inveja, mas a forma como alias tudo isso à simpatia e disponibilidade para ajudar toda a gente torna-se um incentivo ainda maior para aprender contigo. Acho que basta dizer que... até ao ginásio me fazes ir. Obrigada por tudo e espero nunca desiludir!

À Raquel Seruca, a leoa que rosna para as crias para as ensinar a crescer e sobreviver, mas que as defende com unhas e dentes perante factores externos. Obrigada pelo fervor e entusiasmo contagiante e pela oportunidade que me foi dada de vir para o Cancer Genetics.

Ao Professor Fernando Schmitt, um obrigada pelos ensinamentos de histologia e não só. Agradeço também os espíritos crítico e científico que me incutiu e que sei que me tornaram uma melhor profissional.

A todo o Cancer Genetics, obrigada pelo excelente ambiente de trabalho que me proporcionaram. Vocês aliam como ninguém trabalho, profissionalismo, exigência, bom humor e companheirismo.

Às maminhas, um muito obrigada pelo bom humor, palhaçadas e gargalhadas que imperam no "corredor da Mama". O nosso grupo tem, sem dúvida, uma dinâmica única: eu panico-me com os meus resultados, a Babi goza-me, a Mada e a Diana dizem "Ritinha, tem calma!" e o André "Realmente é muito estranho…". Até que chega a Ana, resolve tudo em segundos ou coloca nova hipótese e eu volto a arregaçar as mangas!

“Ai Babi, se as paredes tivessem ouvidos… Pior… se falassem!”, um muito obrigada pela tua amizade, conversas sem fim, conselhos, sinceridade e apoio a cada passo que temo dar. Desculpa mas não tive coragem de colocar aqui a tua foto como querias!

Ao Andrézinho, o meu defensor incondicional, um obrigada por manteres a ordem no meio da mulherada, uma tarefa difícil.

À Mada, a mulher com cara de menina, brincadeiras de criança mas “mente de gente grande”. As nossas gargalhadas combinadas foram a banda sonora de tardes inesquecíveis de trabalho, animação e parvoíce.

À Dianinha, obrigada pela doçura, o sorriso e o abraço amigos sempre presentes.

Ao Carlinhos, a primeira e única metástase descrita (por mim) de bom prognóstico.

Às não-maminhas mas igualmente amigas:

Patrícia, a Post-Duquesa com pulso firme mas coração de manteiga. Sim, eu tive a prova de que és uma comum mortal, talvez até um bocadinho mais pónei que o normal, com a frase inédita: “’Não sei se hei-de rir, se chorar!”.

Mafaldinha, a minha coleguinha de mestrado, que me trocou, e bem, por um PhD. Obrigada pela amizade e companheirismos e muito boa sorte, tu mereces tudo!

À Catarina Salgado, a minha companheira de “Fashion women in Science”. Beleza e bom gosto nunca foram antónimos de inteligência… E nós temos a função de nos apresentarmos sempre “Em Bom!”

À Inezocas, a “caçula” que cresceu e já põe as unhas de fora (quando necessário!).

E aos restantes compinchas Joaninha Carvalho, Daniel, Daniela e Adélia, obrigada pelos momentos de descontração, almoços animados, lanches e pausas “para fumar”.

Ao Sr. Oliveira, Sr. Mendes, Cátia, Zézinha e Sr. Mário um obrigada por cuidarem do IPATIMUP… e de nós!

Aos meus amigos, um muito obrigada por me mostrarem que é possível conciliar tudo, basta querermos. Obrigada André, pelo ouvido e abraço amigos. Obrigada Cátia, por me teres redireccionado e incentivado, mesmo na tua forma única. Vou ficar-te eternamente grata. Obrigada Sérgio pelo mimo e carinho. Obrigada Joana pelas conversas de tudo, sobre tudo. Obrigada Rita, Crispim e Ana. Eu cresci e cresço convosco e é um enorme prazer fazer parte da Família.

À Sílvia, Filipa e Fred, os exemplos vivos de que os amigos não precisam de estar perto para o serem. Obrigada pela vossa amizade.

Ao Ricardo, obrigada por me fazeres acreditar. Obrigada pela Paixão, Partilha, Parceria, Pactos, incansável Paciência, Parvoíces, Palhaçadas, Palermices, Pancas, Particularidades, Palavras, Pensamentos e a Promessa de que um dia vamos partilhar o Nobel. “Happiness only real when shared”.

Aos meus Papis e à minha irmã Jó agradeço o que sou e naquilo que me tornei, nada teria sido possível sem vocês. Um enorme obrigada por me apoiarem incondicionalmente, por acreditarem e me ensinarem que com trabalho e perseverança, tudo é recompensado.

Ao Duque, o meu fiel amigo!

Isto é também um pouco de cada uma destas pessoas, que me acompanharam não só no trabalho em si, mas na vida em geral. Obrigada a todas, com especial carinho a algumas delas.

ABSTRACT

P-cadherin is a cell-cell adhesion molecule, whose overexpression is associated with proliferative lesions of high histological grade, basal-like phenotype, decreased cell polarity and worse survival of breast cancer patients (1). Using *in vitro* models, it was showed that P-cadherin overexpression promotes invasive and migratory capacities (2, 3), as well as mediates cancer stem cell (CSC) activity (4). Nevertheless, little is known about *CDH3/P-cadherin* gene regulation in breast cancer.

With this project, our long term goal was to disclose molecular mechanisms regulating P-cadherin expression in breast cancer, as well as in normal epithelial tissues. Actually, we were able to show that *CDH3* gene is a direct transcriptional target of CCAAT/enhancer-binding protein β (C/EBPβ) and p63 transcription factors in breast cancer cells.

C/EBPβ is a transcription factor which share some of the mentioned features with P-cadherin, such as its overexpression in aggressive and proliferative tumours, with poor differentiation, basal-like phenotype and association with worse prognosis of breast cancer patients (5, 6). In fact, we have previously demonstrated that C/EBPβ was able to up-regulate *CDH3* promoter in breast cancer cells (7). In this study, we proved, by chromatin immunoprecipitation (ChIP) and site-directed mutagenesis, the direct link between C/EBPβ and *CDH3*/P-cadherin gene, as well as the co-localization of P-cadherin and C/EBPβ in the same tumour cells by immunohistochemistry. These results were corroborated by the increase of *CDH3* promoter activity and P-cadherin expression in human breast cancer cells, in response to C/EBPβ isoforms. We also demonstrated the isolated and distinct inducer rates of C/EBPβ isoforms and their synergetic effect in *CDH3* promoter.

P63 is also a transcription factor implicated in tumour formation and progression, with evidence for both tumour suppressive and oncogenic properties (8). Interestingly, Carrol *et al.* suggested that p63 plays an important role in the modulation of gene expression programs involved in cell adhesion (9) and, more recently, it was actually demonstrated that *CDH3*/P-cadherin gene is a transcriptional target of p63 in a human limb bud and hair follicle model (10).

Herein, concerning p63 isoforms, we have observed their ability to differently modulate the activity of *CDH3* promoter in breast cancer cells, being the truncated TAp63γ isoform the one which greater represses *CDH3* activity. Additionally, we observed a decreased of the P-cadherin induced functional proprieties, such as invasion and mammosphere formation efficiency, when cells were transfected with TAp63γ. However, we proved that this effect

was dependent on the p53 wild type or mutated status.

Overall, the results obtained under this project allowed us to understand the relationship between P-cadherin expression and the transcription factors C/EBPβ and p63, as well as their putative importance for the malignant phenotype of P-cadherin-overexpressing breast cancer cells. As ongoing and future work, we are trying to disclose if these mechanisms, or even new ones, regulating *CDH3*/P-cadherin expression in invasive carcinomas, are also important in the process of differentiation of normal epithelial tissues.

RESUMO

A P-caderina é uma molécula de adesão célula-célula, cuja sobre-expressão em carcinomas da mama se associa com lesões mais proliferativas, de alto grau histológico, com fenótipo do tipo basal e com um pior prognóstico para as doentes (1). Utilizando modelos *in vitro*, demonstrou-se que a P-caderina, quando sobre-expressa em células de cancro da mama, promove invasão e migração celular (2, 3), regulando também a actividade das células neoplásicas com propriedades estaminais (ou *cancer stem cells*) (4). No entanto, pouco se sabe acerca dos mecanismos moleculares que regulam a sua expressão ao nível da actividade do gene que a codifica (gene *CDH3*).

Com este projecto, tinhamos como principal objectivo identificar mecanismos moleculares importantes na regulação da expressão da P-caderina em cancro da mama, assim como em tecidos epiteliais normais. De facto, fomos capazes de demonstrar que o gene *CDH3* constitui um alvo directo dos factores de transcrição CCAAT/enhancer-binding protein β (C/EBPβ) e p63 em células de cancro da mama.

O factor de trancrição C/EBPβ partilha algumas das características de agressividade tumoral mencionadas para a P-caderina, uma vez que a sua sobre-expressão está também associada a tumores mais agressivos e proliferativos, pouco diferenciados, do sub-tipo basal e de pior prognóstico (5, 6). Neste trabalho pretendemos explorar a ligação entre C/EBPβ e o gene *CDH3*, uma vez que demonstrámos previamente que este promove a actividade do promotor desta caderina em células de cancro de mama (7). Com este intuito, recorremos a imunoprecipitação de cromatina (ChIP) e mutagénese dirigida para demonstrar a ligação da C/EBPβ ao promotor do gene *CDH3*. Por imunohistoquímica, vimos ainda a co-localização de C/EBPβ e P-caderina nas mesmas células tumorais. *In vitro,* observámos ainda um aumento da actividade do promotor *CDH3*, bem como dos níveis de P-caderina em resposta à transfecção das isoformas de C/EBPβ.

A p63 é também um factor de transcrição, descrito como estando envolvido na formação e progressão tumoral, possuindo uma actividade tanto oncogénica como supressora tumoral (8). Curiosamente, Carrol *et al.* publicou que a p63 tem um papel importante na modulação de genes envolvidos em adesão celular (9) e, em modelos de desenvolvimento, o gene *CDH3* foi descrito como alvo directo da p63 (10). Neste estudo, relativamente às diferentes isoformas de p63, observámos diferentes efeitos na actividade do promotor *CDH3*, sendo a isoforma TAp63γ a maior inibidora da actividade do promotor. De facto, quando transfectadas com TAp63γ, as células de cancro da mama apresentam um decréscimo de expressão de P-caderina, assim como consequente

diminuição das capacidades de invasão e formação de mamosferas, propriedades funcionais induzidas por esta caderina. No entanto, provámos ainda que este efeito é dependente do *status* da p53.

Em conclusão, os resultados obtidos neste trabalho revelaram-se importantes para clarificar a relação entre a expressão de P-caderina e os factores de transcrição C/EBPβ e p63, assim como a importância destes na malignidade em carcinomas de mama com sobre-expressão de P-caderina. Neste momento, estamos a estudar se os mecanismos moleculares que regulam a expressão de P-caderina em carcinomas da mama são também importantes no processo de diferenciação de tecidos epiteliais normais.

TABLE OF CONTENTS

Agradecimentos..... ii
Abstract..... v
Resumo..... vii
Table of Contents..... ix
Abbreviations List..... x
I. Introduction..... 1
1. Epithelial Cadherins..... 2
1.1. Gene Structure..... 2
1.2. Protein Structure and Function..... 3
1.3. Expression in Normal Tissues..... 5
2. Epithelial Cadherins in Cancer..... 7
2.1. P-cadherin in Breast Cancer..... 8
2.2. P-cadherin and Signalling Pathways..... 10
3. *CDH3*/P-cadherin Gene Regulation..... 13
3.1. Epigenetic Modulation of P-cadherin Expression..... 13
3.2. *CDH3* Transcriptional Regulation..... 14
II. Aims..... 16
III. C/EBPβ Isoforms as Transcriptional Regulators of *CDH3*/P-cadherin Gene.. 18
IV. *CDH3*/P-cadherin is Negatively Regulated by TAp63 in a p53-dependent Manner in Breast Cancer Cells..... 29
V. General Discussion..... 41
VI. Ongoing and Future Work..... 50
VII. References..... 54

ABBREVIATIONS LIST

AER – apical ectodermal ridge
BRCA – breast cancer associated gene
BS – binding site
C/EBP – CCAAT/enhancer-binding protein
CBD – catenin-binding domain
CDH – cadherin gene
ChIP - chromatin immunoprecipitation assay
CK – cytokeratin
CSC – cancer stem cell
Ctn - catenin
DBD – DNA binding domain
DCIS – ductal carcinoma *in situ*
DNA – deoxyribonucleic acid
EC - extracellular
E-cadherin – epithelial cadherin
ECM – extracellular matrix
EEM – ectodermal dysplasia, ectrodactyly, and macular dystrophy
EGFR – epidermal growth factor receptor
EMT – epithelial to mesenchymal transition
ER – oestrogen receptor
GSK – glycogen-synthase-kinase
HDAC – histone deacetylase
HER – human epidermal receptor
HJMD – hypotrichosis with juvenile macular dystrophy
HNSCC – head and neck squamous cell carcinoma
HRP – horse-radish peroxidase
IDC – invasive ductal carcinoma
ILC – invasive lobular carcinoma
JMD – juxtamembranar domain
KO – knock out
LAP – liver-enriched activating protein
LCIS – lobular carcinoma *in situ*
LIP – liver-enriched inhibitory protein
MFE – mammosphere formation efficiency

miRNA – micro ribonucleic acid
MMP – matrix metalloproteinase
mRNA – messenger ribonucleic acid
N-cadherin – neural cadherin
P-cadherin – placental cadherin
PCR – polymerase chain reaction
PgR – progesterone receptor
R-cadherin – retinal cadherin
RLU – relative light units
RNA - ribonucleic acid
SHFM – split hand/foot malformation
siRNA - small interfering ribonucleic acid
sP-cad – soluble P-cadherin
TA – transactivation domain
TDLU – terminal ductal-lobular unit
TSA – trichostatin A
TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling
WB – western blot
WT – wild type

CHAPTER I

INTRODUCTION

1. EPITHELIAL CADHERINS

The maintenance of adult tissue architecture mainly depends on the structural and functional integrity of cadherins, a superfamily of transmembrane glycoproteins that mediate calcium-dependent adhesion between neighbouring cells of all solid tissues of the organism. Cadherins have a crucial role in determining the epithelial phenotype, being involved in several processes such as cell polarity, cytoskeleton organization, differentiation and migration (11-14).

The epithelial cadherins, *CDH1*/E-(epithelial) and *CDH3*/P-(placental) cadherins, as the name says, have epithelium-specific expression and are preferentially located at intercellular junctions of adherent type, sharing a common basic structure, but with different molecular weights, specificity binding and tissue distribution (13, 15).

1.1. GENE STRUCTURE

E- and P-cadherin genes have been mapped to 16q22.1, being *CDH3* 32 kb upstream of *CDH1* (Figure I.1). Both display 16 exons and share a remarkable degree of conservation in intron positions, as well as a large intron after exon 2 (15). Additionally, both *CDH1* and *CDH3* genes harbour a 5′-located CpG island in their promoters (16, 17).

CDH1 transcription start site (TSS) is currently annotated at the coordinate 68,771,128 bp, the translation start site (ATG) 194 bp downstream of it (18) and its canonical promoter starts at least 125 bp upstream of the TSS and ends 27 bp downstream of it (19, 20). In this area there is no TATA box, but several regulatory elements such as GC boxes, E-boxes and a CAAT box are present (19, 21). Moreover, it was found, less than 500 bp upstream from the canonical ATG, an Alu repeat (AluJo) which may uncover putative new molecular mechanisms of gene regulation.

Concerning *CDH3* gene, its TSS 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 *CDH3* promoter, similarly to *CDH1*, exhibits no TATA box, neither an homologous sequence to the palindromic sequence E-pal, but includes a CAAT box, two putative AP2-binding motifs and a GC-rich region containing putative Sp1-binding sites, all highly conserved. An AluJo repeat was described, as well as in *CDH1*, ~700 bp upstream of the ATG and putatively encloses gene regulation or exonisation features (20, 22).

Interestingly, in the non-coding part of both *CDH1* and *CDH3* gene, it was described an intron, intron 2, with more than 63 Kb and 30 kb in length, respectively (23). This large

intron is a structurally conserved feature across mammals, which suggests the presence of common cis-regulatory elements, yet to be described.

Ensembl database currently describes four and two transcripts arising from the *CDH1* and *CDH3* gene locus, respectively (23).

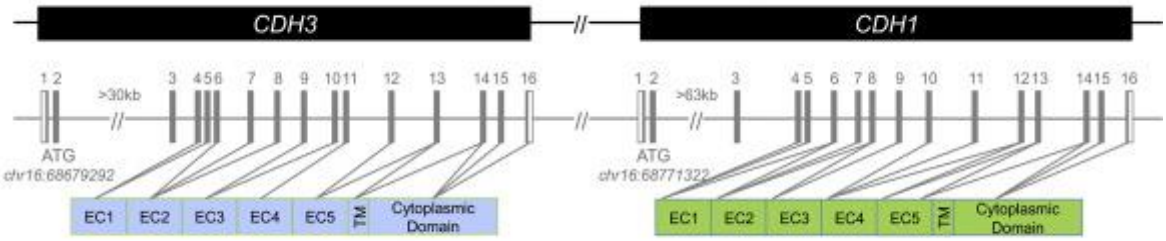


Figure I.1. Structure of the human *CDH3* and *CDH1* genes. The *CDH3* and *CDH1* genes are located on chromosome 16q22.1 and exhibit a lot of structural similarities. Adapted from Paredes J, 2012 (24).

1.2. PROTEIN STRUCTURE AND FUNCTION

The epithelial-calcium dependent cell–cell adhesion is achieved by the establishment of mainly, but not exclusively, homophilic interactions between two cadherin molecules of adjacent cells to form a homodimer (25, 26). Epithelial cadherin mature proteins are organised in three major structural domains: a large extracellular domain, a single transmembrane domain and a short intracellular/cytoplasmic domain (27) (Figure I.2).

The extracellular (EC) segment is composed by five tandemly repeated domains, known as cadherin motifs, which constitute a key element for their classification. These extracellular domains are sequences of 110 residues, commonly designated as EC1-EC5 (15, 27, 28). From the five EC domains, four are characteristic cadherin repeats, where is observed the highly conserved histidine, alanine and valine (HAV) tripeptide within the most N-terminal extracellular repeat (EC1), which plays a key role in the interaction between cadherins and consequent adhesive properties (29). The role of the other cadherin repeats (EC2-5) in the cell-cell interaction remains a matter of debate.

In the intercellular space, cadherin dimmers interact via their EC1 with cadherin dimers of the neighbouring cell, establishing a “zipper-like” structure at the level of the adherent junctions of epithelial cells. The correct conformation of cadherin molecules is stable only in presence of Ca^{2+}, whose binding with the extracellular portion of the polypeptide chain is a prerequisite for cadherin mediated cell-cell adhesion. Calcium binding sites consist in short highly conserved amino acid sequences, which are located between neighbouring EC repeats (15, 30, 31).

The intracellular domain of cadherins comprises about 150 aminoacid residues (29) and it is divided into a membrane proximal region, the juxtamembrane domain (JMD), and a

catenin-binding domain (CBD), which are known to be essential for cadherin function. It has been shown that the strength of cadherin interactions relies on the formation of complexes with catenins (ctn), which serve to link the cadherin cytoplasmic tail to the actin cytoskeleton (30, 32). The JMD is directly connected with p120-catenin (p120ctn) (31, 33, 34), while the cytoplasmic proteins, β- or γ-catenins, bind in a mutual-exclusive manner to the CBD and to α-catenin, which mediates interaction with the actin cytoskeleton (35-37).

The interactions of cadherins with the actin cytoskeleton are of no importance in the first steps of intercellular interaction, but are extremely important for contact stabilization and maturation, which leads to increased strength in cell-cell adhesion (38).

Interestingly, although the very similar aminoacid sequence between E-cadherin and P-cadherin (about 71% similarity in the extracellular domain, and 89% in the entire cytoplasmic tail), these harbour distinct patterns of tissue expression, as well as different biological functions (24).

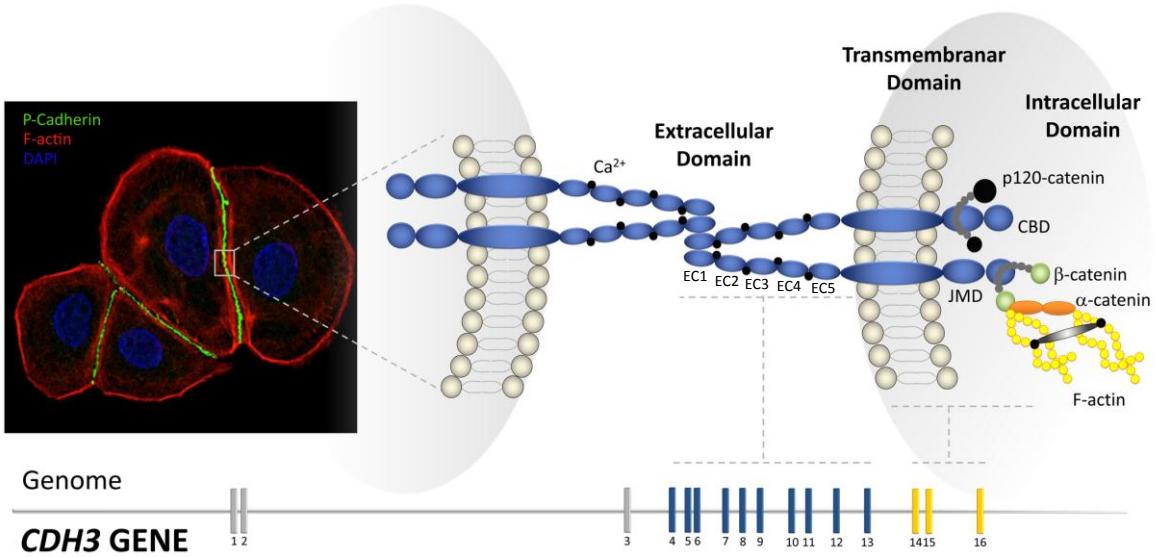


Figure I.2. *CDH3*/P-cadherin gene, protein structure and organization. Cadherins are organized in three major structural domains: an extracellular domain composed by 5 cadherin repeats (EC), a transmembrane domain and an intracellular domain, which links with actin cytoskeleton through catenins, establishing the cadherin/catenin complex. Adapted from Albergaria A, 2011 (1).

1.3. EXPRESSION IN NORMAL TISSUES

Epithelial cadherins play important roles in maintaining the structural integrity of adult epithelial tissues and are mainly involved in cell differentiation, as well as in tissue and organ development during embryogenesis (21, 39). In fact, E- and P-cadherin are the first adhesion molecules that are expressed in the embryo, and its proper development is assured only under conditions of tightly regulated cell-cell and cell-matrix interactions (40). Therefore, the majority of adult epithelial tissues, such as epidermis, breast, uterine cervix, prostate and lung, express E- and P-cadherin (Figure I.3). However, their distribution differ along the tissues: E-cadherin is usually found in epithelial differentiated cell layers, while P-cadherin expression is restricted to undifferentiated and proliferative cells, which are usually found in the basal layers of epithelial tissues (39, 41, 42).

The specific and well defined expression patterns of E- and P-cadherins within normal epithelial tissues (Figure I.3) suggest their distinct and complementary role in epithelial cell differentiation and development, where E-cadherin is essential to maintain the structural continuum of a differentiated epithelium (43, 44), which importance is clearly demonstrated by the lethality of E-cadherin KO-mice in early stages of embryogenesis (45); concerning P-cadherin, it seems to be necessary to restrain differentiation of proliferative cells of epithelial tissues and to wound regeneration (41), since P-cadherin-mediated signals are crucial to maintain the undifferentiated state of an epithelium. In contrast to E-cadherin, P-cadherin KO-mice are viable, but virgin females display precocious differentiation of the mammary gland (46).

Germline mutations of E-cadherin and P-cadherin are also significantly associated to human developmental defects. In the case of E-cadherin, germline mutations of its codifying gene lead to various aberrant transcripts, which have been associated to congenital midline malformations, namely for the craniofacial morphogenesis, such as lip and palate clefting (47). On the other hand, *CDH3* germline mutations were shown to cause P-cadherin functional inactivation, leading to developmental defects associated with two inherited syndromes in humans: 1) hypotrichosis with juvenile macular dystrophy (HJMD) (48, 49) and 2) ectodermal dysplasia, ectrodactyly, and macular dystrophy (EEM syndrome) (50). Both diseases are characterized by sparse hair and macular dystrophy of the retina, while only EEM syndrome shows split hand/foot malformation (SHFM). Mammary development or other epithelial bud structures do not present defects; however, it is known that during bud patterning, a special arrangement occurs, where cells change their interaction with their neighbours and break their attachments to the extracellular matrix (ECM), by a specific activation of some transcriptional programs (10).

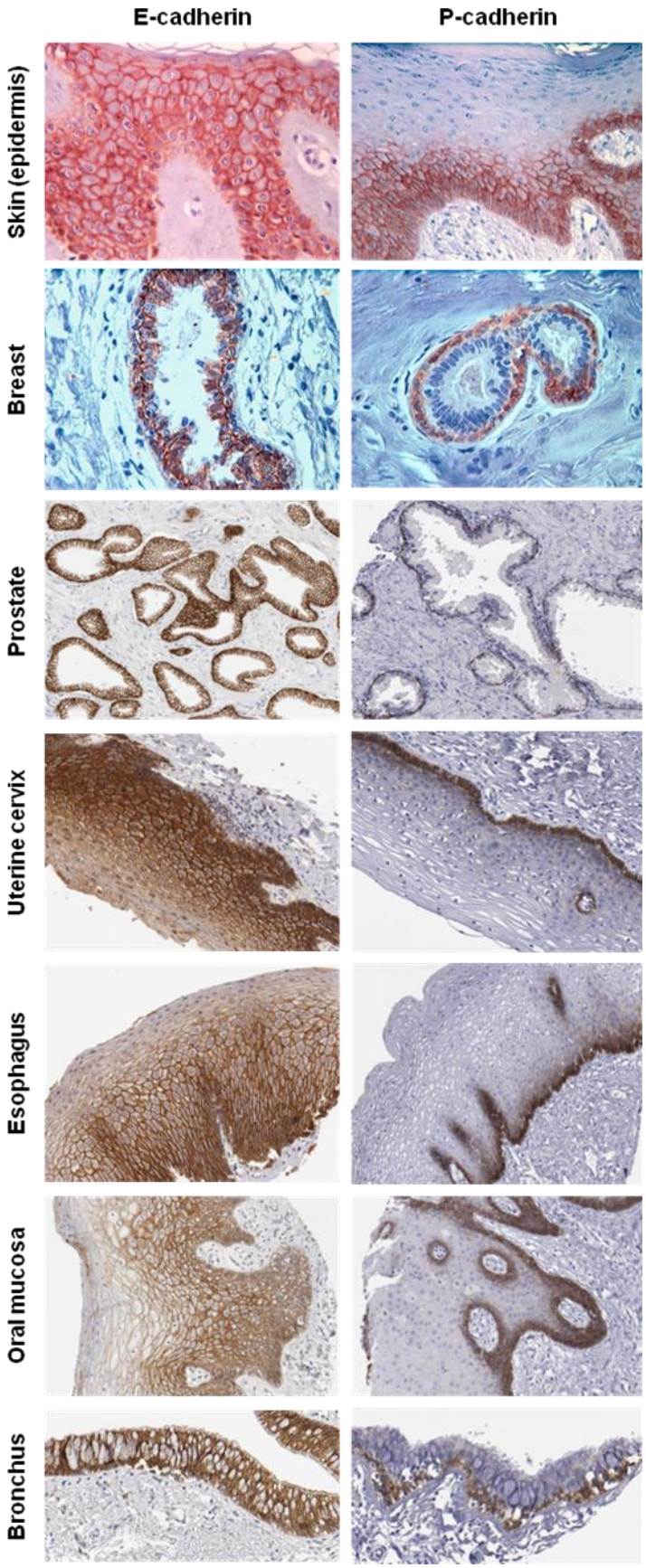


Figure I.3. E- and P-cadherin immunoexpression in normal epithelial tissues. E- and P-cadherin are co-expressed in cells of normal epithelial tissues that constitute the proliferative and undifferentiated niche of epithelial tissues, whereas differentiated epithelial cells only express E-cadherin.

2. EPITHELIAL CADHERINS IN CANCER

Loss of cell-cell adhesion is a hallmark of epithelial tumours, since it allows cells to detach from each other, resulting in the destruction of the histological architecture and, consequently, in cancer invasion (51). The majority of the studies implicating cadherins in carcinogenesis have been focused on E-cadherin, since it is the major cadherin expressed by epithelial cells. In 90% of all epithelial cancers, the turning point in cancer progression is mediated by E-cadherin dysfunction (52, 53). *In vitro* and *in vivo* studies showed that inhibition of E-cadherin function turned non-invasive epithelial and polarized cells into invasive cells, showing a role for this protein as an important suppressor of cell invasion. Indeed, decreased or loss of E-cadherin expression and/or function has already been described in most human carcinomas (26, 54), being associated to tumours with an increased infiltrative pattern of growth, including sporadic and hereditary diffuse gastric and lobular breast cancers (55, 56).

Moreover, in tumours characterized by loose cell-cell adhesion, structural alterations (mutations and deletions) of *CDH1* gene are found as initiating events (57-59), while epigenetic alterations (promoter methylation) or expression of E-cadherin repressors emerge as progression events in a wider range of advanced stage tumours (16, 60, 61). Furthermore, loss of E-cadherin has also been implicated in the induction of epithelial to mesenchymal transition (EMT), which frequently occurs during cancer invasion, and is considered the basis for the acquisition of metastatic capacity by cancer cells (62, 63). Despite the correlation between E-cadherin dysfunction and malignancy, E-cadherin is not lost in some epithelial tumours, being concomitantly co-expressed with other cadherin, like P-cadherin (64). Indeed, some studies have shown that the expression of an inappropriate cadherin in epithelial cells is another way to alter cell endogenous cadherin function (65-67). Mesenchymal cadherins, for example, like N- or R-cadherin (44, 68), can have a direct and dominant influence on the phenotype of epithelial cells, despite their continued expression of E-cadherin (69). Our group also showed that patients with invasive breast carcinomas co-expressing both E- and P-cadherin have as poor prognosis as those with carcinomas lacking both E- and P-cadherin, which suggests that P-cadherin could be a modulator of E-cadherin function in cancer cells that do not harbour structural E-cadherin gene alterations (64, 70).

In fact, the role played by P-cadherin in carcinogenesis is still a matter of debate. However, aberrant expression of P-cadherin has been described in several solid tumours such as breast (22, 42, 71, 72), gastric (73), endometrial (74), ovarian (75), prostate (76, 77), pancreatic, colorectal, and bladder carcinomas (78), as well as in basocellular and

squamous carcinomas of the skin (79). In all these tumours, P-cadherin was preferentially expressed in invasive rather than in *in situ* lesions, showing that its aberrant expression could be a useful marker of invasion capacity of tumour cells, as well as a general marker of poorly differentiated tumours with aggressive clinical behaviour and a novel tumour-associated antigen (78). More specifically, increased P-cadherin expression is a poor prognosis factor for breast cancer patients, being presently mainly in triple-negative basal-like tumours (ER, PgR and HER-2 negative), which still lack an efficient targeted therapy.

2.1. P-CADHERIN IN BREAST CANCER

The role of E-cadherin in breast carcinogenesis has been extensively studied and results suggested a correlation between its loss or reduced expression and cancer progression (16, 51, 80). In contrast, overexpression of P-cadherin has been associated to carcinogenesis, increased tumour cell motility, migration, invasion and metastasis (42, 81) in this cancer model.

P-cadherin is *de novo* expressed in 20% to 40% of invasive breast carcinomas, as well as in 25% of ductal carcinomas *in situ* (DCIS), being reported as a marker of poor prognosis in breast cancer. In fact, P-cadherin-positive carcinomas are significantly associated with short-term overall survival, as well as with distant and loco-regional relapse-free interval (22, 72, 82, 83), being considered a valuable prognostic factor. Overexpression of P-cadherin has also been associated with high histological grade tumours, as well as with well-established markers and biological parameters of poor prognosis, like the expression of Ki-67, epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), vimentin, p53, and HER-2, high proliferation rates (MIB-1) and mitotic index and decreased cell differentiation (22, 72, 82, 84). P-cadherin expression is also inversely related with age at diagnosis, hormonal receptors (ER and PgR), and Bcl-2 expression (22, 72, 82, 83). Besides these strong associations, transgenic mice overexpressing *CDH3*/P-cadherin in the luminal epithelial layer of the mammary gland showed normal morphogenesis, architecture, lactation and involution, with no spontaneous formation of mammary tumours (46). Nevertheless, it was demonstrated a significant increased shedding of soluble P-cadherin in nipple aspirate fluids from women with breast cancer when compared with healthy subjects or with women with pre-cancer conditions, suggesting its possible release via a proteolytic processing in cancer cells (85).

According with this, we have showed that one of the mechanisms underlying the increased invasive capacity of P-cadherin-overexpressing breast cancer cells is mediated by the secretion of MMPs (or metalloproteases), which are able to degrade the extracellular matrix (ECM) during invasion (2, 3); in addition, these proteases also cleave

P-cadherin extracellular domain, producing the soluble P-cadherin fragment, which needs to be inhibited to block cell invasion (3). Interestingly, this invasive phenotype mediated by P-cadherin was seemingly dependent on the concomitant expression of wild-type E-cadherin: in cell models where P-cadherin showed an invasion promoter function, E-cadherin was also expressed (2, 81, 86, 87); contrarily, in models expressing only P-cadherin, this protein was described as an invasion suppressor (88-90). This dual functional role of P-cadherin was recently clarified, since we have been able to show that P-cadherin expression disrupts the normal invasive suppressor function of E-cadherin (70) by the destabilization of the normal cadherin/catenin complex (64); in fact, once in the cytoplasm, p120ctn can inhibit RhoA and activate other Rho GTPases, Rac1 and Cdc42, altering the actin cytoskeleton polymerization and promoting cell migration and motility, as well as an increased invasive and tumourigenic potential (70, 81). These results highlight the dual role of P-cadherin, either as an adhesion molecule, when expressed alone, or as an invasion promoter and poor prognosis marker when co-expressed with E-cadherin, reinforcing the importance of P-cadherin as a prognostic factor in breast cancer and suggesting that its overexpression is an alternative mechanism for cancer progression and invasion in E-cadherin-positive breast carcinomas. Therapeutically, this knowledge supports the development of anti-P-cadherin strategies to control highly aggressive breast carcinomas co-expressing both cadherins.

Another clinical challenge in breast cancer research is to fight against resistance to current therapies, leading to recurrence and metastasis, features associated with cancer stem cells (CSC). CSCs have inherent ability to form a hierarchy, survive as circulating tumour cells and to form micrometastasis, remaining quiescent in distant sites for a long period. CSCs are also able to proliferate, originating more stem-like cells, which exhibit resistance to current therapies (91-94). Targeting CSCs, in combination with current therapies, is the forthcoming goal in cancer treatment. Interestingly, P-cadherin has a crucial role in mediating cancer stem cell (CSC) activity in breast cancer, since P-cadherin enriched populations were also enriched for anchorage independent survival (mammosphere forming efficiency, MFE), as well as for the expression of CD24, CD44, CD49f and ALDEFLUOR^{bright}, already described as CSC markers. P-cadherin also conferred resistance to X-ray induced DNA damage, supporting a role for this molecule in the maintenance of another CSC property (4). Hence, the strategy of directing therapies to the breast CSCs, by specifically targeting P-cadherin, could potentially help to eradicate CSCs.

2.2. P-CADHERIN AND SIGNALLING PATHWAYS

Cadherins role in carcinogenesis and tumour progression seems not to lie only on their adhesive function, but also depends on their interaction with other molecules, such as cytoskeletal components, integrins, growth-factor receptors, and signalling pathways (61). This signalling-structural network is evident in the stabilization of the cadherin/catenin complex as a regulatory mechanism for oncogenic signalling pathways, which guide cell fate decisions through the modulation of specific genes at the transcriptional level and consequent regulation of several crucial cellular processes as proliferation, survival, polarization, differentiation, shape and migration (Figure I.4).

Although E-cadherin-induced signalling pathways have been extensively studied in cancer, little is known about the signalling pathways activated by P-cadherin. As a cadherin and cell-cell adhesion molecule, P-cadherin is expected to share common signalling pathways with other cadherins; however, by microarray gene expression profiling of a breast cancer cell line (MDA-MB-231, negative for E- and P-cadherins) transfected with E- and P-cadherin, it has been revealed that both cadherins share common signalling pathways but 40 genes belonging to a wide range of biological functions were differentially modified by the expression of either cadherin type. These differentially modified pathways include signal transduction and growth factors (VEGFC, FGFR4), cell cycle (CCNA2), cell adhesion and ECM (CDH4, COL12A1), or cytokines and inflammation (IL24), among others (89).

One of the molecules that has been several times referred has having a specific role in signalling related to P-cadherin is p120ctn. In fact, our group described the association between breast carcinomas co-expressing E- and P-cadherin, p120ctn cytoplasmic localization and poor patient survival (64), due to P-cadherin's interference in normal binding of p120ctn to E-cadherin (70). Indeed, transfection of HEK293T cells with several mutants of P-cadherin showed that only the ones with altered JMD were not able to induce cell invasion in *in vitro* cell models, which showed that the pro-invasive activity of P-cadherin requires the JMD of its cytoplasmic tail (2). Moreover, it was described that induced cell migration by P-cadherin expression was due to activation of Rho GTPases, Rac1 and Cdc42, through the accumulation of p120ctn in the cytoplasm (81). In pancreatic and ovarian cancer cell models, it was also reported that p120ctn signalling mediated by P-cadherin expression also lead to increased activity levels of Rac1 and Cdc42 (81, 87). This connection between cadherins and Rho GTPases is made by p120ctn and highlight the role of P-cadherin and the assembly of the adhesion complex in the organization of the cytoskeleton, with great consequences on cell behaviour (95-98).

Furthermore, p120ctn pathway has been also shown to intermediate P-cadherin cooperation with insulin-like growth factor-1 receptor promoting metastatic signalling of gonadotropin-releasing hormone in ovarian cancer (87). Another study has shown that p120ctn and P-cadherin, but not E-cadherin, regulate cell motility and invasion of DU145 prostate cancer cells (99).

P-cadherin regulatory role in cell migration was also related with the expression of the non-muscle myosin II-B isoform, an ATP-dependent molecular motor protein that can interact with and contract filamentous actin (F-actin) (100), another evidence of the coordinated cross-talk between adhesion molecules and cellular migration-related proteins. In addition, the role of P-cadherin in breast cancer cell invasion were clarified when we found that the presence of P-cadherin, in an E-cadherin positive cellular background, is able to provoke the secretion of pro-invasive factors, such as MMP-1 and MMP-2, leading to P-cadherin ectodomain cleavage (sP-cad) which induces a pro-invasive activity by itself (3).

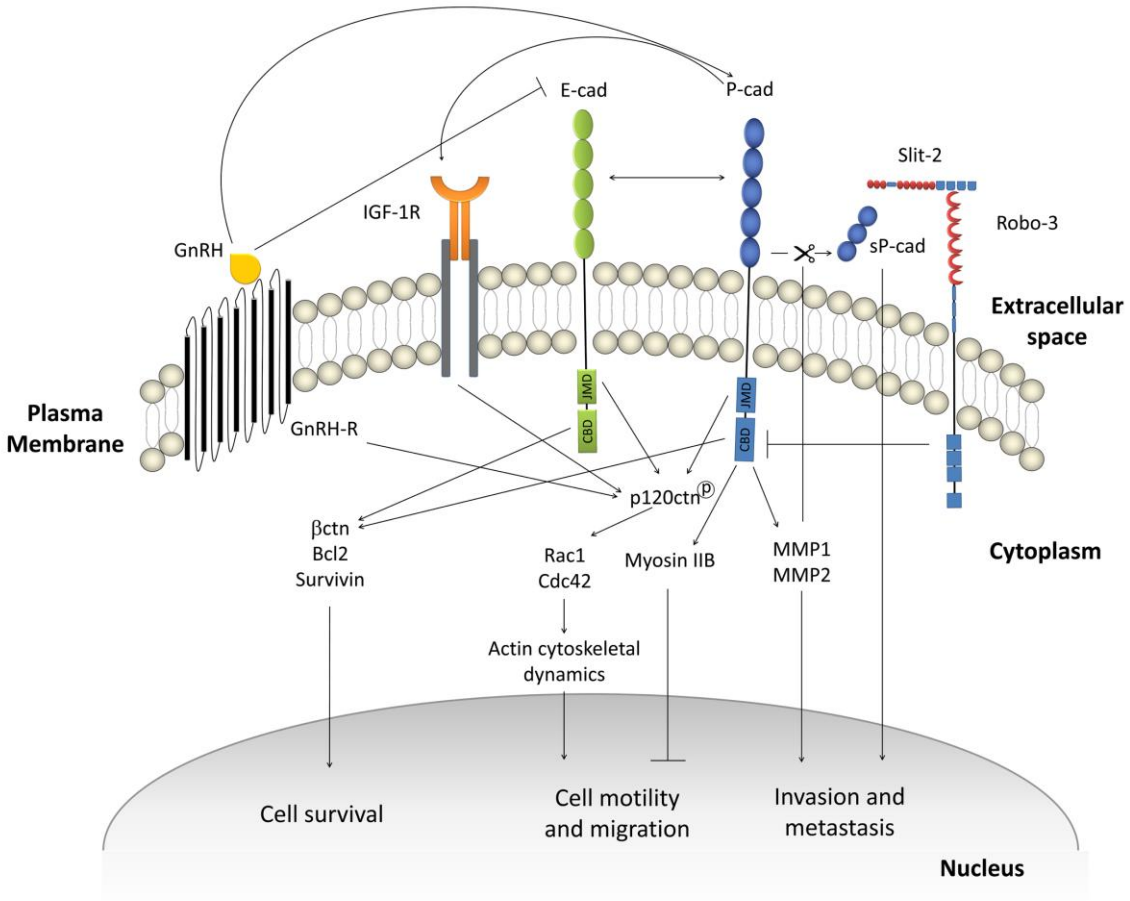


Figure I.4. Signalling pathways regulated by P-cadherin expression. P-cadherin signals are transduced by many intracellular signalling pathways, which ultimately result in alterations of the cancer cells survival, as well as cell migration and invasion capacity. Adapted from Albergaria A, 2011 (1).

In an oral squamous cancer cell model, which is deficient for classical cadherins, the induced-P-cadherin overexpression led to a gain of an epithelial-like morphology, with Snail translocation to the cytoplasm. It has been described that glycogen-synthase-kinase-3β (GSK-3β) bound to Snail, as well as that an increase in activated GSK-3β phosphorylates Snail, leading to its cytoplasmic translocation (88). In the same model, it was also showed that Slit-2, a secreted ECM glycoprotein that acts as a molecular guidance signal in cellular migration, facilitates the interaction of P-cadherin with Robo-3, its receptor, inhibiting cell migration (101).

Another signalling pathway that is known to affect both gene expression and cell migration is the Wnt signalling pathway, a powerful regulator of cell proliferation and differentiation. In fact, β-catenin, a central player in Wnt signalling, is directly involved in both gene transcription and cell adhesion due to its transcriptional activation of CDH3/P-cadherin and by also being one of the players that constitute cadherin-catenin complexes (102, 103). Whenever β-catenin is not bound to cadherins and is free in the cytoplasm, it is rapidly phosphorylated and degraded by ubiquitin-proteosome pathway (102, 104).

Finally, it is important to highlight that the effect of cadherins on the overall gene expression program of cancer cells is highly dependent on the cellular type and the biological context.

3. *CDH3*/P-CADHERIN GENE REGULATION

Regulation of cadherin-mediated adhesion seems to be a very dynamic, elegant and complex net of mechanisms and players, which underlie the dynamics of the adhesive interaction between cells. Although the described evidences that the expression of inappropriate cadherins can result from growth factors and hormones stimulation in the tumour microenvironment or from changes in the promoter regions of cadherins and transcriptional and post-translational regulation, specific data concerning *CDH3* gene regulation is still very limited.

3.1. EPIGENETIC MODULATION OF P-CADHERIN EXPRESSION

Epigenetic alterations, resulting in the loss of tumour suppression genes, are frequently involved in tumour development and progression. Moreover, aberrant DNA methylation or acetylation in tumour cells have been described as a trigger of improper cadherin expression, which is a well-established mechanism of E-cadherin loss of expression (12).
Concerning *CDH3* gene, P-cadherin negative epithelial/luminal normal breast cells are consistently methylated in a specific region of the *CDH3* gene promoter, whereas in breast cancer, it was established the existence of a significant correlation between P-cadherin overexpression and hypomethylation of this same specific promoter region (22). The important regulatory role of DNA methylation in the expression of this protein is also evident in other cancer models. Analyzing the *CDH3* promoter revealed that it was hypomethylated in colonic aberrant crypt foci, in colorectal cancer and, occasionally, in the normal epithelium adjacent to cancer, inducing P-cadherin expression; however, in pancreatic and melanoma cancer models, *CDH3* gene was shown to be silenced by aberrant methylation (105-107). Furthermore, demethylation of the *CDH3* gene was found in 69% of primary gastric carcinomas and was significantly associated with increased TNM stage, implying that the aberrant demethylation of *CDH3* is a frequent event in gastric carcinomas, (108).
Interestingly, not only methylation, but also other epigenetic events may regulate the *CDH3* gene promoter. Such claim is sustained by its genomic structure, like the enrichment in CpG islands, as well as the attributed DNA hypersensitive sites. In fact, an up-regulation of *CDH3* promoter activity and P-cadherin expression was observed by our group in cells treated with the histone deacetylases (HDAC) inhibitor Trichostatin A (TSA), showing that chromatin-activating modifications play an important role in the modulation of this gene (7).

3.2. *CDH3* TRANSCRIPTIONAL REGULATION

The pivotal molecular mechanism involved in *CDH3*/P-cadherin deregulation is mainly occurring at the promoter region of the gene and not by structural alterations of its coding sequences. An example of it is the regulation of P-cadherin expression by transcriptional factors: ERα (2), BRCA1 and c-Myc (109) as repressors and β-catenin (110), C/EBPβ (7) and p63 (10) as activators (Figure I.5).

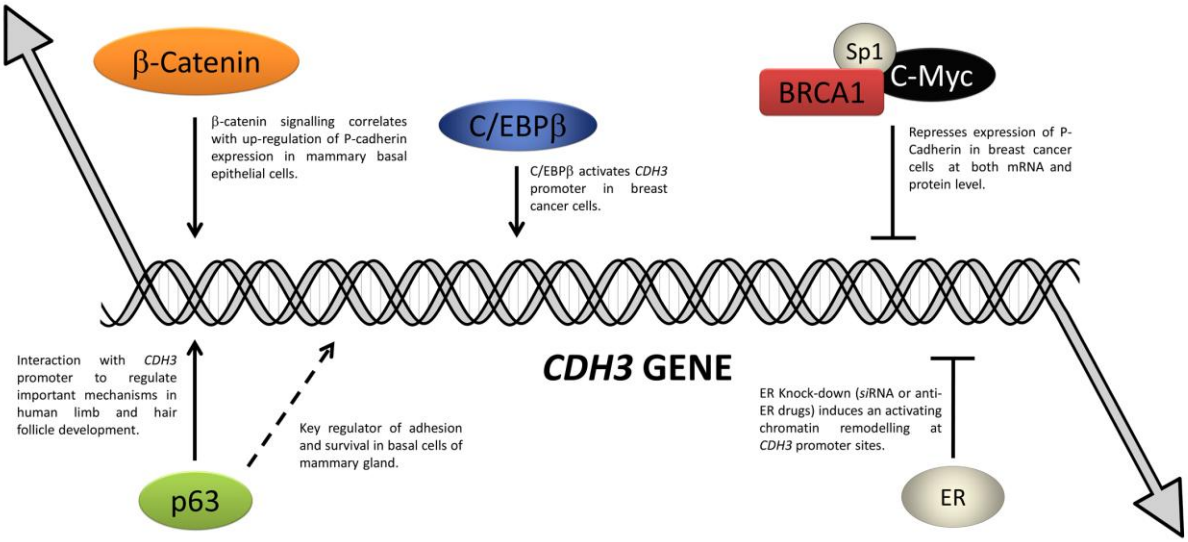


Figure I.5. Described transcriptional regulators of *CDH3*/P-cadherin promoter gene. It has been shown that β-catenin, p63 and C/EBPβ are transcriptional activators of *CDH3* promoter, inducing its expression at the mRNA and protein level. In contrast, estrogen receptor (ER) and BRCA1/c-Myc/Sp1 complex act as transcriptional repressors of *CDH3* promoter gene. Adapted from Albergaria A, 2011 (1).

Since P-cadherin overexpressed breast tumours were essentially ER (estrogen receptor) negative, our group explored the link between ER signalling and the regulation of P-cadherin expression in breast cancer. In 2004, it was described that the lack of ERα signalling is responsible for the P-cadherin *de novo* expression, categorizing *CDH3* as a putative oestrogen-repressed gene. In fact, it was also showed that the anti-oestrogen ICI 182,780 is able to increase *CDH3* promoter activity, mRNA and protein levels in a time and dose dependent manner (2). Furthermore, this anti-ER drug induces a chromatin structural remodelling, eventually allowing the binding of nearby transcriptions factors (7). In fact, upon endocrine therapy resistance and disease progression, there's usually an acquired increased invasive phenotype of ER-positive breast cancer cells that may be due to this genomic de-repression effect.

The expression profiling of *BRCA1*-deficient hereditary tumours has identified a pattern of gene expression similar to basal-like breast tumours (111, 112). Thus, as a gene associated with the basal-like phenotype in breast cancer, the mRNA and protein levels of

CDH3/P-cadherin gene were shown to be also transcriptionally repressed by functional BRCA1 protein in breast cancer cell lines. This repression is achieved after the formation of *BRCA1* and *c-Myc* repressor complex (BRCA1-c-Myc complex) on the promoters of specific basal genes, including *CDH3* gene, and represent a potential mechanism to explain the observed overexpression of key basal markers in *BRCA1*-deficient tumours (109). Actually, in breast carcinomas, it has been shown that P-cadherin expression is strongly associated with the presence of *BRCA1* mutations (113), which means that this repression does not occur in breast cancer cells with a BRCA1 mutation and *CDH3* gene is codified and expressed.

Conversely, *in vitro* and *in vivo* studies also have shown that β-catenin activates *CDH3* promoter leading to overexpression of P-cadherin in basal mammary epithelial cells. In fact, it was shown that activation of β-catenin signalling correlates with up-regulation of *CDH3* promoter and P-cadherin expression, as well as downregulation of endogenous β-catenin levels inhibited *CDH3* promoter activity (110).

Another putative transcription factor of P-cadherin is CCAAT/enhancer-binding protein β (C/EBPβ), which was demonstrated as able to up-regulate *CDH3* promoter in breast cancer cells. Moreover, the expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and both linked with a worse prognosis of breast cancer patients (7).

Carroll and collaborators demonstrated the importance of a classical transcription factor in the regulation of cell adhesion programmes in epithelial cells. This study showed that a p53 family related factor, p63, is a key regulator of adhesion and survival in basal cells of the mammary gland, showing that several cell adhesion-associated genes were downregulated due to p63 expression, which also led to detachment between mammary epithelial cells (9). This involvement of p63 in cell adhesion mechanisms was finally linked with *CDH3* gene in developmental models, where P-cadherin has been described as a direct p63 transcriptional target, interplaying a crucial role in human limb bud and hair follicle development (10).

CHAPTER II

AIMS

Based on evidences that:

1. P-cadherin is expressed in a specific niche of epithelial tissues, undifferentiated and proliferative cells, but overexpressed in 20 to 40% of the invasive breast carcinomas;
2. P-cadherin overexpression is associated with malignancy, poor patient prognosis and tumour aggressive behaviour.

Our main aim is **to reveal the molecular mechanisms regulating P-cadherin expression in breast cancer, as well as in normal epithelial tissues**. In order to achieve this goal, the following specific topics were addressed:

1. To validate two putative *CDH3*/P-cadherin transcriptional regulators and to clarify their relationship with P-cadherin expression, malignant phenotype and more proliferative and aggressive breast cancer.
   1.1. C/EBPβ (Described in Chapter III)
   1.2. p63 (Described in Chapter IV)

2. To disclose new mechanisms that regulate *CDH3*/P-cadherin expression in invasive carcinomas and evaluate if the same mechanisms are also important in the process of differentiation of normal epithelial tissues (Described in Chapter VI).

CHAPTER III

C/EBPβ ISOFORMS AS TRANSCRIPTIONAL REGULATORS OF *CDH3*/P-CADHERIN GENE

CONTRIBUTION OF THE AUTHORS

AA, CR and ARN carried out the majority of the experimental work; AA did the mutagenesis assays, CR the ChIP experiments and ARN all the *in vitro* studies. ASR and BS provided assistance in data analysis and interpretation. Finally, JCM, RS, JP and FS were involved in study design and interpretation and AA in the manuscript production. All authors had final approval of the submitted version.

OPEN ACCESS Freely available online

PLOS ONE

CCAAT/Enhancer Binding Protein β (C/EBPβ) Isoforms as Transcriptional Regulators of the Pro-Invasive *CDH3*/P-Cadherin Gene in Human Breast Cancer Cells

André Albergaria[1,2☯], Carlos Resende[1☯], Ana Rita Nobre[1,3], Ana Sofia Ribeiro[1], Bárbara Sousa[1,3], José Carlos Machado[1,2], Raquel Seruca[1,2], Joana Paredes[1,2], Fernando Schmitt[1,2]*

1 Cancer Genetics Group, Institute of Molecular Pathology and Immunology of Porto University (IPATIMUP), Porto, Portugal, **2** Department of Pathology, Medical Faculty of Porto University, Porto, Portugal, **3** Institute of Biomedical Sciences of Abel Salazar (ICBAS), Porto, Portugal

Abstract

P-cadherin is a cell-cell adhesion molecule codified by the *CDH3* gene, which expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. In breast cancer, P-cadherin is frequently overexpressed in high-grade tumours and is a well-established indicator of aggressive tumour behaviour and poor patient prognosis. However, till now, the mechanisms controlling *CDH3* gene activation have been poorly explored. Since we recently described the existence of several CCAAT/Enhancer Binding Protein β (C/EBPβ) transcription factor binding sites at the *CDH3* promoter, the aim of this study was to assess if the distinct C/EBPβ isoforms were directly involved in the transcriptional activation of the *CDH3* gene in breast cancer cells. DNA-protein interactions, mutation analysis and luciferase reporter assay studies have been performed. We demonstrated that C/EBPβ is co-expressed with P-cadherin in breast cancer cells and all the three isoforms function as transcriptional regulators of the *CDH3* gene, directly interacting with specific regions of its promoter. Interestingly, this transcriptional activation was only reflected at the P-cadherin protein level concerning the LIP isoform. Taken together, our data show that *CDH3* is a newly defined transcriptional target gene of C/EBPβ isoforms in breast cancer, and we also identified the binding sites that are relevant for this activation.

Citation: Albergaria A, Resende C, Nobre AR, Ribeiro AS, Sousa B, et al. (2013) CCAAT/Enhancer Binding Protein β (C/EBPβ) Isoforms as Transcriptional Regulators of the Pro-Invasive *CDH3*/P-Cadherin Gene in Human Breast Cancer Cells. PLoS ONE 8(2): e55749. doi:10.1371/journal.pone.0055749

Editor: Olivier de Wever, Ghent University, Belgium

Received July 13, 2012; **Accepted** December 31, 2012; **Published** February 6, 2013

Funding: This work was supported by research grants (SFRH/BPD/73247/2010 to A.A., SFRH/BD/46504/2008 to C.R., SFRH/BPD/75705/2011 to A.S.R. and SFRH/BD/69353/2010 to B.S.), from the Portuguese Foundation for Science and Technology (FCT). J.P. is supported by Programa Ciência 2007 (POPH - QREN - Tipology 4.2). Institute of Molecular Pathology and Immunology of Porto University (IPATIMUP) is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: fschmitt@ipatimup.pt

☯ These authors contributed equally to this work.

Introduction

The molecular changes that occur during breast cancer progression, which include the amplification/overexpression of transcription factors, can disrupt the delicate balance between cell proliferation, differentiation and apoptosis. C/EBPβ is one of those transcription factors, which has been implicated in cell cycle regulation, playing an important role in mammary gland development and oncogene-induced breast tumorigenesis [1–4]. Encoded by an intronless gene, C/EBPβ is expressed as distinct protein isoforms, which can accomplish distinct biological and regulatory functions, ultimately leading to gene transactivation [5]. The longer C/EBPβ proteins (liver-enriched transcriptional activating proteins, LAP1 and LAP2) regulate proliferation and differentiation of many cell types [6]; the shorter protein product (liver-enriched transcriptional inhibitory protein, LIP) lacks the transactivation domain and acts mainly as a dominant-negative [7]. AS LAP isoforms, LIP also binds to the consensus sequences within genomic DNA, sometimes even with a higher affinity than the other C/EBPβ isoforms [6,7]. In fact, LIP inhibits the transcriptional activity of LAPs by competing for the same consensus binding sites or by forming inactive heterodimers with them. However, some emerging evidence suggest that LIP can also act as a transcriptional activator in some cellular contexts [5].

In breast, C/EBPβ most likely contributes to tumorigenesis through significant elevations in the LIP:LAP ratio, mostly observed in ER-negative, highly proliferative and metastatic mammary tumours, usually associated with a poor patient prognosis [8]. Indeed, LIP isoform overexpression has been associated to a lack of contact inhibition, resulting in proliferation and *foci* formation in epithelial breast cancer cell lines [9]. It has been hypothesized that aberrant expression of C/EBPβ-LIP isoform may contribute to an increased growth rate and result in a more proliferative and aggressive breast carcinoma.

P-cadherin, a classical cadherin encoded by the *CDH3* gene [10], has been explored by our group for several years and has been also extensively associated with breast tumour aggressiveness. This protein was found to be aberrantly expressed in 20–40% of invasive ductal carcinomas, being strongly associated with proliferative lesions of high histological grade, decreased cell

polarity and poor patient survival [11–16]. At the *in vitro* level, we demonstrated that P-cadherin overexpression induces invasion [14], motility and migration of wild-type E-cadherin expressing breast cancer cells, through the secretion of pro-invasive factors, such as matrix metalloproteinase (MMP)-1 and MMP-2 [17]. In fact, P-cadherin-associated functions in breast cancer have been widely studied, which reflects the growing importance of this cadherin in human breast cancer biology and prognosis.

However, the mechanisms controlling its overexpression in breast cancer have only recently started to be unrevealed [11,18]. In non-cancer models, *CDH3* promoter was shown to be genetically regulated through direct binding of transcription factors, such as p63 [19] and β-catenin [20]. Gorski and collaborators also demonstrated that BRCA1 and c-Myc form a repressor complex on *CDH3* promoter and on other promoters of specific basal genes, representing a potential mechanism to explain the overexpression of key basal markers in BRCA1-deficient breast tumours [21]. Additionally, we established a direct link between P-cadherin overexpression and the lack of oestrogen receptor (ER)-signalling in breast cancer cells, categorizing *CDH3* as a putative ER-repressed gene [14]. In 2010, we described a regulatory mechanism whereby a selective ER-downregulator is able to up-regulate P-cadherin expression in MCF-7/AZ breast cancer cells through chromatin remodelling at *CDH3* promoter level [18]. This epigenetic process was accomplished by the induction of high levels of the active chromatin mark H3K4me2 and a consequent de-repression of the *CDH3* promoter, which exposed a high number of putative C/EBPβ transcription binding sites [18]. The induction of *CDH3* promoter activity by C/EBPβ was also confirmed by reporter assays, as well as its expression association with worse prognosis of breast cancer patients [18].

However, since the mechanistic link and the consequent transcriptional regulatory relevance of C/EBPβ proteins on *CDH3* gene were not demonstrated, in the present study we revealed that C/EBPβ isoforms are indeed transcriptional regulators of P-cadherin, directly interacting with conserved and specific regions of the *CDH3* promoter. Interestingly, we show that this transcriptional activation is reflected in the P-cadherin protein levels, especially for the LIP isoform. We conclude that *CDH3* is a newly defined transcriptional target gene of C/EBPβ in breast cancer.

Materials and Methods

Antibodies

The following primary anti-human antibodies were used for Western Blot and/or Immunohistochemistry against: P-cadherin (BD Transduction Biosciences, Lexington, KY), C/EBPβ (Santa Cruz Biotechnology, CA), β-actin (Santa Cruz Biotechnology) and β-tubulin (Sigma-Aldrich, St. Louis, NO). Technical conditions are described in Table S1 (Supporting Information). Anti-mouse and anti-goat horseradish peroxidase-conjugated secondary antibodies were used for WB [HRP-conjugated, dilutions: 1:2000] (Santa Cruz Biotechnology). For chromatin immnunoprecipitation (ChIP) assays, the following antibodies were used: anti-C/EBPβ (C-19, Santa Cruz Biotechnology), and two control IgGs (Active Motif, CA and Santa Cruz Biotechnology).

Promoter Vectors and cDNA Constructs

The pLENTI-C/EBPβ expression vectors (C/EBPβ-LAP1, C/EBPβ-LAP2 and C/EBPβ-LIP) were generated according to the human *CEBPB* nucleotide sequence obtained from Ensembl and Pubmed databases. Oligonucleotide primer sequences for LAP1, LAP2, and LIP isoforms are listed in Table S2 (see Supporting Information).

CEBPB cDNA was obtained from total RNA extracted from the gastric cancer cell line AGS, and amplified for each *CEBPB* isoform using HotStart Taq DNA Polymerase (Qiagen, Cambridge, MA). Amplification was performed for 35 cycles as follows: denaturation at 95°C for 1 minute, annealing at 60°C for LAP1 and LAP2 and 58°C for LIP for 1 minute, and extension at 68°C for 2 minutes per cycle. PCR products for each isoform were separated by electrophoresis in a 1.5% agarose gel and bands were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Beaconsfield, UK). To validate the isoforms nucleotide sequence, amplified products were purified through Sepharose (GE Healthcare, Waukesha, WI) and sequenced on both strands on an ABI Prism 3100 automated sequencer (Perkin-Elmer). PCR products were inserted into the mammalian expression vector pLENTI6/V5 Directional (Invitrogen, Ltd, Paisley, UK), using manufacturer instructions, and incorporated into chemically competent TOP10 *E. coli* (Invitrogen). Transformed bacteria were grown overnight in ampicillin-supplemented LB-Agar (Applichem, Germany). Plasmid DNA from transformed *E. coli* cells was sequenced to check the orientation and nucleotide sequence for each *CEBPB* isoform.

The human full-length *CDH3*-luciferase vector was generated by our group, as previously described [18]. Normalization pRL-CMV Renilla Luciferase Control Reporter Vector was purchased to Promega (Promega Corporation, Madison, WI).

Immunohistochemistry

Double immunostaining for C/EBPβ and P-cadherin was performed in 3 μm sections of 23 formalin-fixed paraffin-embedded (FFPE) invasive breast carcinomas that have previously showed strong expression of both proteins, in order to illustrate their consistent cellular co-localization. Standard immunohistochemistry was performed as previously described [16]. For the reaction, we used the Envision G2 Double-stain (DakoCytomation, Glostrup, Denmark), according to manufacturer instructions. Specific conditions used for C/EBPβ and P-cadherin are listed in Table S1. FFPE sections from normal breast gland, skin or normal gastric mucosa were used as positive controls for C/EBPβ and P-cadherin. Negative controls were performed by replacing the primary antibody with PBS/non-immune serum.

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 (National Regulative Law number 12/2005 – I Serie-A, n°. 18–26th January, 2005).

Cell Culture

Human breast cancer cell line MCF-7/AZ was kindly provided by Prof. Marc Mareel (Ghent University, Belgium) [22], while BT-20 cells were purchased to American Type Culture Collection (ATCC, Manassas, VA). Cell lines were routinely maintained at 37°C, 5% CO_2, in the following media (Invitrogen): 50% DMEM/50% HamF12 (MCF-7/AZ), or only DMEM (BT-20). All media contained 10% of heat-inactivated foetal bovine serum (Greiner Bio-one, Wemmel, Belgium), 100 IU/mL penicillin and 100 mg/mL streptomycin (Invitrogen).

Transient Transfection

For gene reporter assays, cells were grown in 96-well plates to 60–70% confluence and transfection was done using the liposome-mediated FuGENE 6 transfection reagent (Roche Diagnostic GmbH, Mannheim, Germany), prepared according to the

manufacturer's instructions. A ratio of FuGENE/DNA of 3:1 was used. For protein expression assays, cells were grown in 6-well plates to 60% confluence. Transient transfections of C/EBPβ expression vectors were done using Lipofectamine 2000 (Invitrogen), with a ratio of Lipofectamine/DNA of 3:1 and prepared according to the manufacturer's instructions.

For knock-down assays, cells were transiently transfected at 60% confluence with specific siRNA for C/EBPβ (100 nM, FlexiTube siRNA – Hs_C/EBPβ 5-Qiagen) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's procedure. Maximum C/EBPβ knock-down was achieved after 48 h of incubation. A siRNA with no homology to any gene was also used as a negative control.

CDH3-luciferase Reporter Gene Analysis

Cells were co-transfected with pGL3-*CDH3*/luc promoter vector (20 ng) and with pRL-CMV Renilla vector (5 ng). For promoter analysis, 24 hours after transfection, cells were washed twice in PBS-cold and lysed for firefly/Renilla luciferase assays, using the Luclite Reporter Gene Assay System (Perkin Elmer), according to the manufacturer. Luciferase bioluminescence from Renilla was measured using native coelenterazine 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 and pGL3/luc-Control (pLUC) vector (Promega) were included as controls in all *CDH3*-reporter assays. Luminescence was read using the Wallac/Perkin Elmer-1450-028 Trilux Microbeta (Perkin Elmer) plate reader, and the results are shown as a mean of relative light units (RLU), which was calculated by the ratio between the luminescence signal emitted from luciferase and the luminescence signal obtained by the Renilla (normalization).

Western Blot

Cells were lysed and the concentration of total protein was determined by Bradford quantification. Western Blot was performed as earlier described [17,18]. For MCF-7/AZ cell line, due to its lower expression of P-cadherin, 50 μg of total protein lysate has been loaded; for BT-20, due to its P-cadherin overexpression, the gel loading was done only with 20 μg of protein lysate. Membranes were incubated with primary antibodies according to the conditions described in Table S1.

Site-Directed Mutagenesis

All the C/EBPβ binding sites mutations in *CDH3* promoter were performed in order to impair the binding of any predicted transcription factor: bioinformatic prediction tools were used to blast all point mutated sequences. To introduce point mutations in the *CDH3* promoter region, the QuickChange Site-directed Mutagenesis Protocol (Stratagene, Cedar Creek, USA) was followed, and the oligos used are listed in Table S2. The PCR cycles were set as follows: 95°C for 30 seconds; 16 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 5 minutes. Following PCR reaction, products were incubated with DpnI (1 hour at 37°C) and transformed into *E-coli* competent cells (Stratagene). All mutated plasmids were checked by sequencing and primer sequences are also listed in Table S2.

Chromatin Immunoprecipitation (ChIP) Assay

For chromatin immunoprecipitation of the endogenous *CDH3* promoter regions in MCF-7/AZ cells, the ChIP-IT^{TM} kit (Active Motif) was used and the assay was performed according with the manufacturer's procedures. Briefly, cells (4.5×10^7) were fixed with 1% formaldehyde in culture medium for 10 minutes. Fixation was stopped by incubating the cells for 5 minutes with a 1× Glycine Stop-Fix Solution, homogenized and centrifuged. The cell-pellets were resuspended in a shearing buffer and sonicated into chromatin fragments of 200–1500 bp in length. To reduce non-specific background, sonication-sheared lysates were pre-cleared with Protein G beads. The sheared chromatin lysates were incubated with 5 μg of C/EBPβ antibody or with a control rabbit IgG, overnight at 4°C, and immunoprecipitated with Protein G beads (2 hours at 4°C). The precipitated DNA-protein complex was washed 7 times, eluted, incubated for 8 hours at 65°C in a reverse cross-link buffer, and digested with proteinase K for 2 hours at 42°C. The resultant DNA was purified, resuspended in DEPC H_2O and quantified by real-time qPCR amplification. The PCR primers sequences used in this amplification are listed in Table S2.

For chromatin Immunoprecipitation in BT-20 cells and in an invasive breast carcinoma highly positive for P-cadherin and C/EBPβ, the Magna ChIP G Kit (Millipore) was used, according to manufacturer's protocol. Basically, the essential steps applied for BT-20 cells were the same as the ones used for MCF-7/AZ cells, differing only in the use of protein G magnetic beads instead of non-magnetic beads for simplicity of use. However, for the tumour sample, some alterations in the basic protocol were employed. Briefly, the tumour sample, that was frozen at −80°C since surgical extraction, was thawed and immediately fixed in 1% formaldehyde for 25 minutes, followed by the addition of 1× glycine solution for 5 minutes, washed in 1× PBS twice, frozen in liquid nitrogen, and finally pulverized mechanically. The following steps were the same used for breast cancer cell lines.

Statistical Analysis

Data are expressed as mean values of at least three independent experiments ± s.d. Student's t-tests were used to determine statistically significant differences ($*P<0.05$).

Results

P-cadherin is co-expressed with C/EBPβ and is regulated by this transcription factor in breast cancer cells

Using a large cohort of invasive breast carcinomas, the expression of C/EBPβ was previously demonstrated to be significantly associated with P-cadherin expression in about 60% of the cases [18]; however, the cellular co-expression of these two proteins was not verified. Thus, based on the hypothesis that C/EBPβ directly activates the *CDH3* gene promoter, a double immunostaining was performed in all invasive breast carcinomas that previously showed strong positivity for both proteins. As represented in Figure 1A, C/EBPβ expression was found in the nuclei of the same cells that were expressing P-cadherin at the cell membrane, pointing for a putative functional relationship between both proteins.

Based on these results, two different breast cancer cell models were used to demonstrate if P-cadherin expression could be affected by C/EBPβ: 1) MCF-7/AZ, which is an ER+/luminal type breast cancer cell line expressing moderate levels of P-cadherin, and 2) BT-20, an ER-negative/basal-like breast cancer cell line, highly positive for P-cadherin [17]. The siRNA mediated-knock-down of C/EBPβ induced a significant downregulation of all C/EBPβ isoforms (LAP1, LAP2 and LIP) in both cell lines. Interestingly, P-cadherin expression was also affected by the reduction of C/EBPβ isoforms, being this effect more pronounced in MCF-7/AZ cells (Figure 1B). According with these results, and

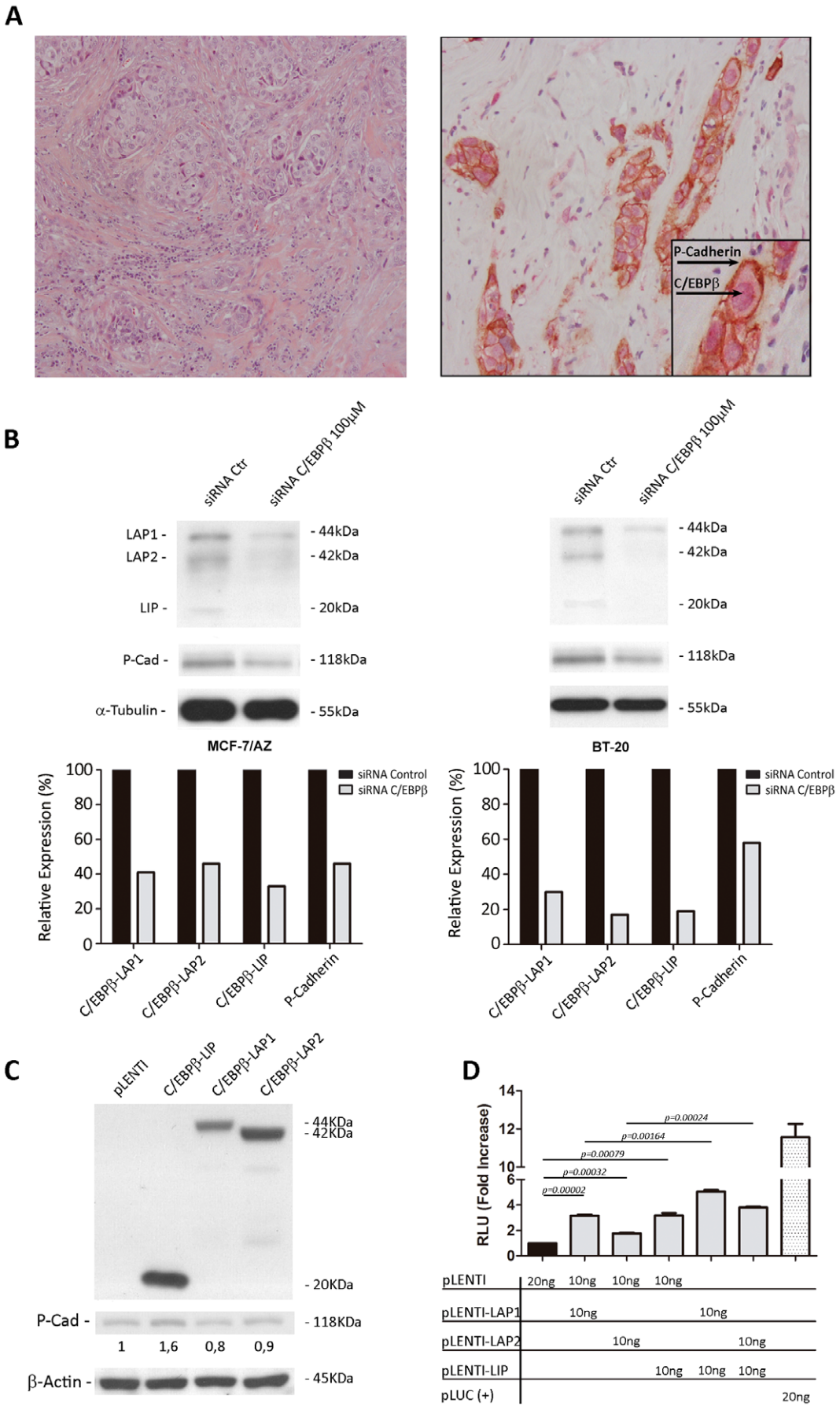

A
P-Cadherin
C/EBPβ
B
siRNA Ctr
siRNA C/EBPβ 100μM
LAP1 -
LAP2 -
LIP -
P-Cad -
α-Tubulin -
- 44kDa
- 42kDa
- 20kDa
- 118kDa
- 55kDa
MCF-7/AZ
BT-20
Relative Expression (%)
siRNA Control
siRNA C/EBPβ
C/EBPβ-LAP1
C/EBPβ-LAP2
C/EBPβ-LIP
P-Cadherin
C
pLENTI
C/EBPβ-LIP
C/EBPβ-LAP1
C/EBPβ-LAP2
- 44KDa
- 42KDa
- 20KDa
P-Cad -
- 118KDa
1
1,6
0,8
0,9
β-Actin -
- 45KDa
D
RLU (Fold Increase)
p=0.00024
p=0.00164
p=0.00079
p=0.00032
p=0.00002
pLENTI
pLENTI-LAP1
pLENTI-LAP2
pLENTI-LIP
pLUC (+)
20ng
10ng

Figure 1. Association and regulatory interplay between C/EBPβ and *CDH3*/P-cadherin expression in breast cancer cells. A) Double immunostaining for C/EBPβ and P-cadherin of an invasive breast carcinoma specimen (basal-like carcinoma, histological grade III), where it can be observed C/EBPβ expression in the nuclei and P-cadherin at the cell membrane of tumour cells (magnification ×200 and ×400-inset); a haematoxylin-eosin staining of this same case is shown to ascertain tissue integrity (magnification ×100); **B**) Using C/EBPβ-targeted siRNA, a consequent reduction of P-cadherin protein levels was observed in both MCF-7/AZ and BT-20 breast cancer cell lines; **C**) MCF-7/AZ cells transiently transfected with the different C/EBPβ isoforms (LAP1, LAP2 and LIP) displayed upregulation of P-cadherin protein levels only after induction of the C/EBPβ-LIP isoform; **D**) Luciferase reporter assays performed in cells transfected with the different C/EBPβ isoforms showed that the promoter activation induced by LIP and LAP1 isoforms was significantly greater compared with the activation induced by LAP2. The co-transfection of both LIP and each LAP1 or LAP2 induced the activation of the *CDH3* promoter in an additive manner.
doi:10.1371/journal.pone.0055749.g001

in order to decipher which C/EBPβ isoform was more relevant for P-cadherin activation, the expression of LAP1, LAP2 and LIP was induced in both breast cancer cell lines. As shown in Figure 1C, only C/EBPβ-LIP isoform was able to induce P-cadherin expression in more than 1.5-fold increase in MCF-7/AZ cells, while the remaining isoforms did not produce valuable effects on P-cadherin expression. This result was not found for BT-20 cells, probably due to their high basal levels of P-cadherin expression (data not shown).

Interestingly, in a previous study performed by our group, we found that the *CDH3*/P-cadherin promoter activation induced by the LIP isoform was significantly greater compared with the activation induced by LAP1 and LAP2 [18]. However, in the present study, this same experiment has been performed and, although the same significant result was observed at the promoter level for LIP ($p = 0.00079$), the *CDH3* promoter was also strongly and significantly activated by LAP1 ($p = 0.00002$) and less prominently, but also in a significant way, by LAP2 ($p = 0.00032$) (Figure 1D). Nevertheless, since it has been described that LIP can function as a dominant negative inhibitor of both LAP's activity [5], we decided to co-transfect both LIP and each LAP1 or LAP2 , in order to study their combined effect on *CDH3* promoter activity. The results showed that there is a significant increased activation of the promoter with any of the combinations compared with LAP1 or LAP2 alone, demonstrating that there is an additive effect of both isoforms ($p = 0.00164$ and $p = 0.00024$, respectively) on *CDH3* promoter activation, when added to LIP.

C/EBPβ physically interacts with endogenous *CDH3* gene promoter in breast cancer cells

Since the three C/EBPβ isoforms were able to transactivate the 1.8 Kb *CDH3* promoter gene construct (Figure 1D), we decided to evaluate in detail the sequence of this putative regulatory region using distinct bioinformatic tools, which can predict for the binding of specific transcription factors. Four concordant C/EBPβ-putative binding sites were identified within the first 1400 nucleotides. Interestingly, we found that there is a high degree of conservation of these predicted C/EBPβ binding sites between humans and other primates within the *CDH3* promoter (Figure 2A), and the left panel of Figure 2B shows their relative localization.

In fact, in order to demonstrate if there was a physical interaction between C/EBPβ proteins and *CDH3* promoter in these specific binding sites, ChIP has been performed in MCF-7/AZ breast cancer cells. Indeed, The results showed that there was an enrichment (relative to *input*) of the *CDH3* DNA-amplified fragments precipitated with the C/EBPβ antibody in all binding sites (Figure 2B, right panel), demonstrating that C/EBPβ transcription factors directly bind to the selected regions within the *CDH3* promoter.

This same experiment has been performed in BT-20 breast cancer cells, as well as in a frozen primary basal-like breast carcinoma, which was selected for being highly positive for P-cadherin and C/EBPβ expression. Interestingly, we could confirm the results, since there was precipitation with the C/EBPβ antibody in all the binding sites studied, in both cells and primary tumour (Figure 2C). Moreover, in BT-20 cells, which overexpress P-cadherin, the binding in all sites was very strong compared with the one found in MCF-7/AZ breast cancer cells.

C/EBPβ binding sites are important for *CDH3* gene activity and are selectively activated by the different C/EBPβ isoforms

In order to evaluate the importance of the aforementioned binding sites to the *CDH3* gene activation, as well as the specificity of the different C/EBPβ isoforms to the *CDH3* promoter, point mutations were introduced in the specific C/EBPβ binding sequences. Figure 3A illustrates the *CDH3* point mutations and their position within the C/EBPβ binding sites in relation to the wild-type *CDH3* promoter.

Interestingly, when MCF-7/AZ cells were transfected with the *CDH3* promoter containing point mutations at the binding sites 1 and 4 (*CDH3*-BS1 and BS4), there was a statistically significant alteration in *CDH3* promoter activity related to the wild-type promoter sequence (Figure 3B). In contrast, the activity of the *CDH3* promoter was not affected by the mutation introduced at the BS3 site, and only slightly affected by the introduced mutation at the binding site 2 (BS2). These results were mostly confirmed in BT-20 cells, especially for the BS4 mutation, located at the transcription start site region of the *CDH3* promoter, which also significantly induced its activity (Figure 3B). Although not significant, the reduction on *CDH3* promoter activity observed with the BS1 mutant was also found in BT-20 cells, suggesting that this distal C/EBPβ binding site is also important to *CDH3* gene transcriptional activation. In addition, the BS2 mutant significantly reduced *CDH3* promoter activity in BT-20 cells, showing that this is also a crucial site for the activation of P-cadherin transcription in this model. Finally, we could not find any effect of BS3 mutation in *CDH3* promoter activity also in BT-20 cells, proving that this site is not relevant for its regulation.

Since the distinct C/EBPβ isoforms have been documented has having different functions in cancer gene activation and in a cell-specific context, we co-transfected LAP1, LAP2 and LIP together with the different mutants of *CDH3* promoter in both breast cancer cell lines. The results demonstrated that distal *CDH3*-BS1 and BS2 are significantly important for the induced promoter activity mediated by all C/EBPβ isoforms. In contrast, BS3 did not play a significant role in C/EBPβ-mediated *CDH3* promoter activity, since mutations in this specific region were not important to impair the activation of *CDH3* gene mediated by any of the distinct isoforms. Similar results were observed concerning BS4, which did not reveal to be important for *CDH3* promoter activity mediated by LAP1, LAP2 or LIP isoforms. Finally, although not significant, the same trend was observed with BT-20 cells, proving that BS1 and BS2 are most likely the binding sites where all C/EBPβ isoforms bind to induce P-cadherin transcription in breast cancer.

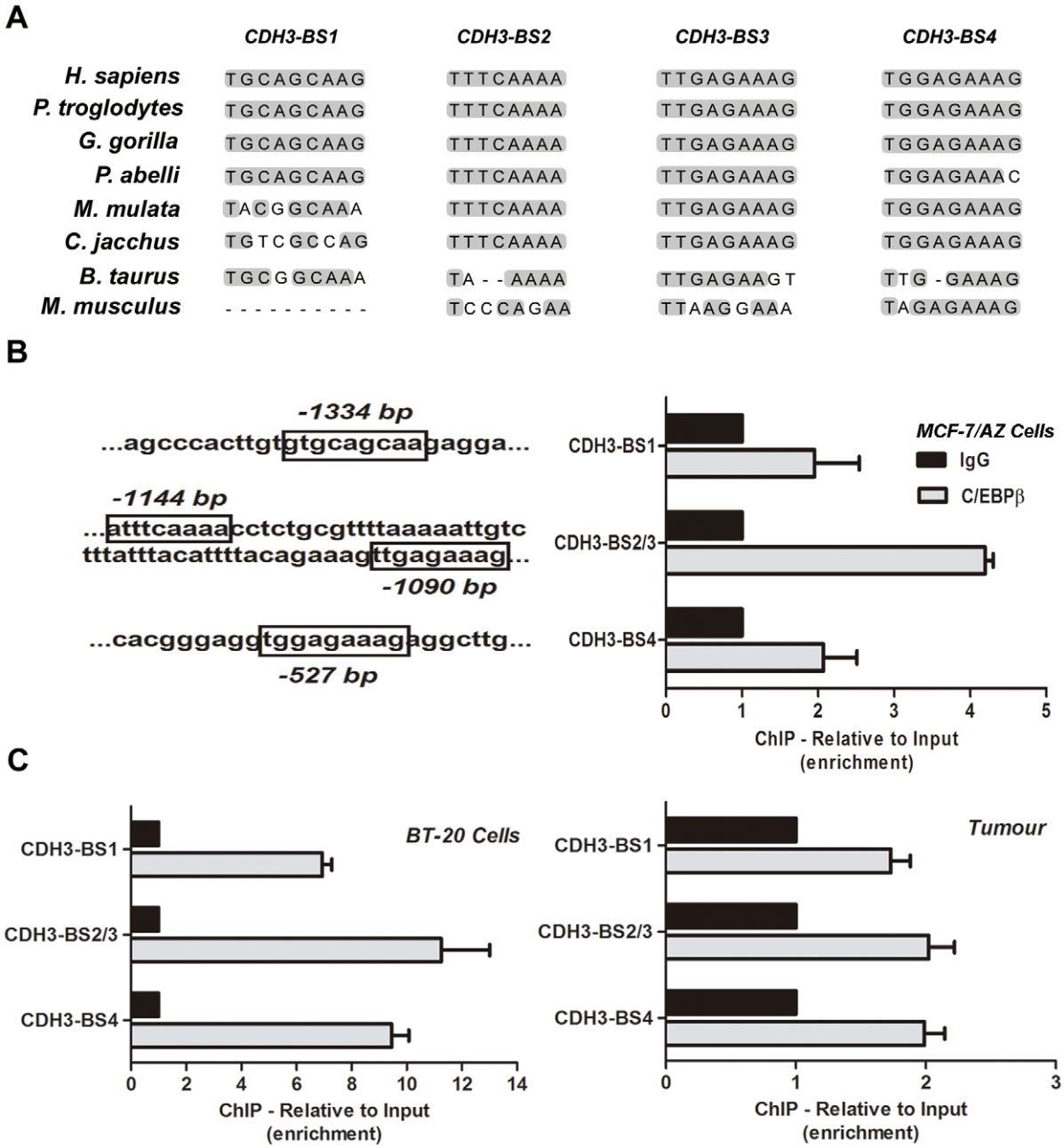


Figure 2. C/EBPβ physical interaction with the *CDH3* gene promoter. A) Putative C/EBPβ-binding sites within the *CDH3* gene promoter, where it can be observed their degree of conservation between human and other primates. Grey regions represent total sequence conservation in comparison with human sequence; **B**) Proximal regulatory region of *CDH3* promoter displaying the relative localization of the predicted C/EBPβ binding sites (left panel). The right panel illustrates the enrichment (relative to input) of the *CDH3* promoter DNA-amplified fragments precipitated from DNA-protein complexes obtained by ChIP in MCF-7/AZ breast cancer cells. **C**) ChIP experiment performed in BT-20 breast cancer cells and on a frozen primary breast tumour, highly positive for P-cadherin and C/EBPβ expression, also showed the same enrichment pattern for all the putative binding sites.
doi:10.1371/journal.pone.0055749.g002

Discussion

P-cadherin has been receiving a growing interest in the last years, since its overexpression is significantly associated with high histological grade breast tumours and with short-term patient overall survival [11,23–25]. The important association between P-cadherin expression and well-established markers correlated to breast cancer poor prognosis, such as high levels of Ki-67, epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), vimentin, p53 and HER2, has been also largely documented [11]. Although P-cadherin has been detected as altered in distinct tumour models, its effective role in the carcinogenesis process remains discussible, since it behaves differently depending on the studied cancer cell context [26]. If in some models P-cadherin has been suggested to act as an invasion suppressor, such as in colorectal cancer [27] or in melanoma [28], in several other models, including breast cancer, P-cadherin behaves as an

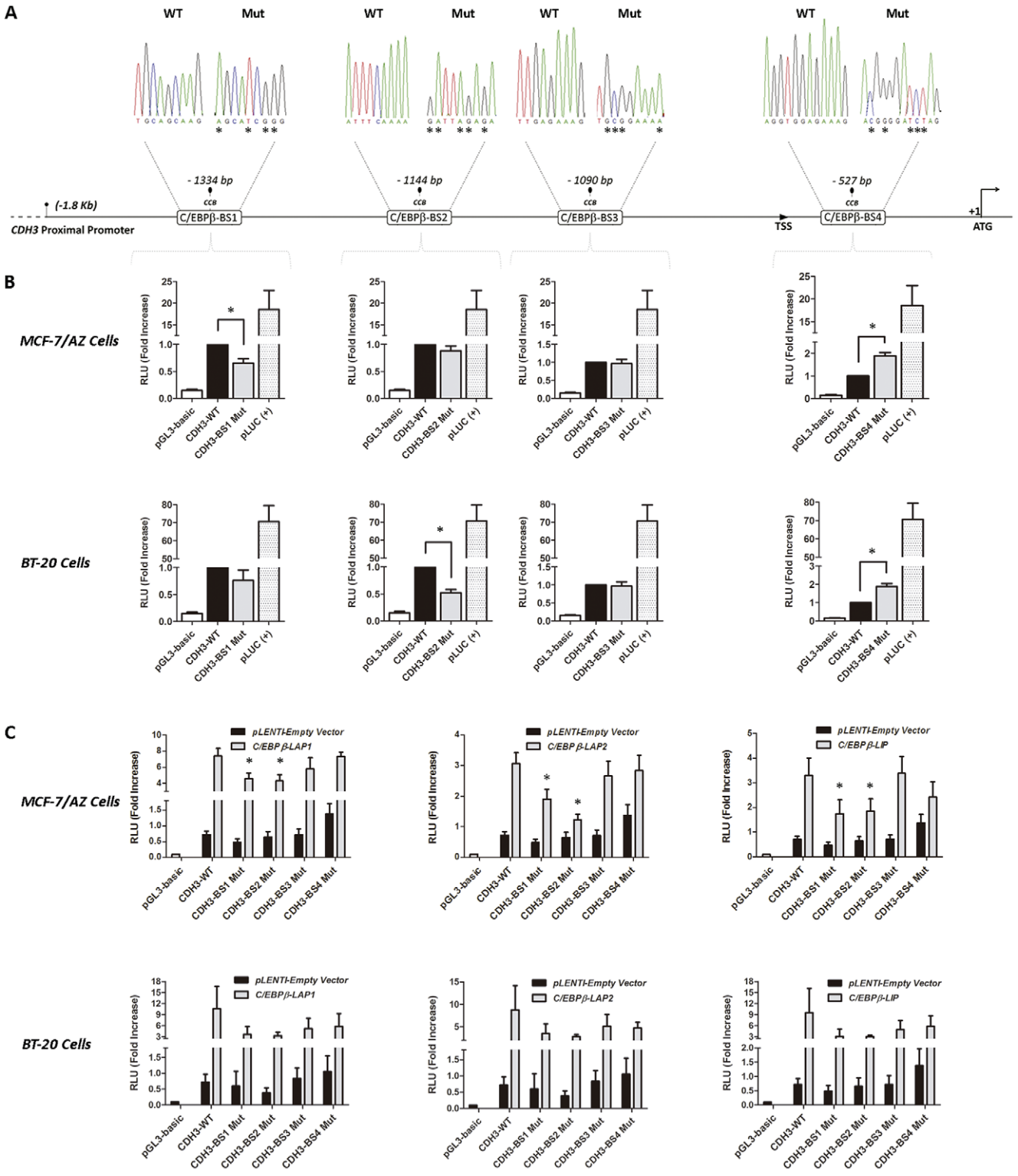


Figure 3. Relevance of C/EBPβ-isoforms and their putative binding sites in the activation of the *CDH3* gene. A) Schematic representation of the wild-type and mutated *CDH3* promoter; **B**) *CDH3*-Luciferase Reporter Assays performed with each of the mutations introduced at C/EBPβ binding sites demonstrating that *CDH3*-BS1, BS2 and BS4 are the most important for the activity of *CDH3* promoter in both MCF-7/AZ and BT-20 breast cancer cells; *p-value<0.05; **C**) *CDH3*-Luciferase Reporter Assays upon co-transfection of LAP1, LAP2 and LIP C/EBPβ isoforms, showing the relevance of specific C/EBPβ isoforms across *CDH3* promoter binding sites in both MCF-7/AZ and BT-20 breast cancer cells. *CDH3*-BS1 and BS2, but not BS3 and BS4, are responsive to all C/EBPβ isoforms; *p-value<0.05.
doi:10.1371/journal.pone.0055749.g003

oncogene, inducing increased tumour cell motility and invasiveness when aberrantly overexpressed [12–14,27,29–31].

However, data concerning *CDH3* gene regulation in breast cancer is still very limited. The induction of *CDH3* promoter activity in breast cancer cells was recently described by our group to be putatively linked to the transcription factor C/EBPβ, as well as P-cadherin and C/EBPβ expression have been reported to be highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients [18]. In fact, the expression of C/EBPβ shares interesting biologic and functional features with the ones attributed to P-cadherin expression. Similarly to what has been described concerning C/EBPβ biology, P-cadherin is involved in homeostatic processes, such as cell differentiation, development and embryogenesis [32]. We have recently found that P-cadherin enriched cell populations show enhanced mammosphere forming efficiency (MFE), as well as increased expression of CD24, CD44 and CD49f, already described as normal or cancer stem cell markers. These results allowed to link P-cadherin expression to the luminal progenitor phenotype of the normal breast hierarchy and established an indirect effect of P-cadherin in stem cell biology [33]. Interestingly, these findings come along with observations that C/EBPβ regulates stem cell activity and specifies luminal cell fate in the mammary gland, categorizing C/EBPβ as one of the several critical transcription factors that specifies mammary stem cells fate during mammary gland development [34]. In a breast cancer biology setting, another interesting finding is related to the fact that P-cadherin, like C/EBPβ, is not mutated in breast tumours, but its overexpression has been widely described in a subset of aggressive breast cancers [5]. Importantly, at a clinicopathological level, some C/EBPβ isoforms, especially C/EBPβ-LIP, correlates with an ER-negative breast cancer phenotype, highly proliferative and high grade lesions and poor patient outcome [8,35]. All these characteristics overlap with the ones observed in highly malignant breast tumours overexpressing P-cadherin.

The present work demonstrates for the first time that P-cadherin and C/EBPβ co-localize in the same breast cancer cells, and that there is a physical interaction between this transcription factor and *CDH3* gene promoter. Herein, in addition to the identification of the promoter binding sites that are relevant for the transcriptional modulation of *CDH3* gene activity by C/EBPβ, we still tested the relevance of the different C/EBPβ isoforms along the *CDH3* promoter.

In fact, we show that C/EBPβ-LIP is the only isoform capable to significantly induce P-cadherin protein expression, confirming in a way the results obtained in our previous study, where a significant activation of the promoter was only revealed for LIP, although LAP1 and LAP2 were also able to activate the promoter. However, in this study, we found that *CDH3* gene is also significantly responsive to LAP1 and slightly to LAP2 isoform at the promoter level. These significant results were probably due to improved transfection efficiencies; however, although LAP1 and LAP2 are activating the gene promoter, supporting the classical knowledge described for these isoforms as transcriptional activators, this might not imply that these isoforms induce functional activity through protein synthesis. In fact, it has been largely discussed that the functionally transactivation potential of each C/EBPβ isoform can be highly modulated, since this ability strongly depends not only on dimer composition formed by C/EBPs, but specially on the partner proteins and responsive elements found in target gene promoters [5]. The fact that LIP activates *CDH3* promoter, leading to protein synthesis, reinforces the emerging evidence that LIP acts as a transcriptional activator of gene expression, challenging the long-standing concept that LIP fashionably functions as a dominant-negative isoform [5]. We also observed that LAP2 was the C/EBPβ isoform that activated *CDH3* promoter in a less extent, which is apparently surprising in light that LAP2 isoform is considered to be the most transcriptionally active C/EBPβ isoform [5]. However, it is also known that, in transformed cancer cells, an increase in LIP expression leads to a reduction in LAP2 activity and, therefore, impair its mediated transcription potential [36].

A novel observation also obtained in this study was the existence of interaction between C/EBPβ proteins to the conserved regions of the *CDH3* gene promoter, identified as C/EBPβ responsive elements. The ChIP results, obtained from the DNA region containing both BS2 and BS3 binding sites, revealed a cumulative increased C/EBPβ antibody-precipitated DNA when compared to individual BS1 and BS4, reinforcing the existence of bounding complexes. This was denoted for both MCF-7/AZ and BT-20 breast cancer cell lines and also for the basal-like tumour studied by *in vivo* ChIP.

Concerning the impact of C/EBPβ binding sites to the *CDH3* promoter activity, we found that BS1, BS2 and BS4 were the most relevant ones, while BS3 was not responsible for the modulation of the *CDH3* promoter. A detailed analysis of the *CDH3* promoter using the Ensemble ENCODE Project, revealed two DNAse Hypersensitive (DHS) sites located around BS1 and BS4 specific sequences, confirming an increased regulatory activity on these specific regions.

Interestingly, one of the most curious effects was the one found at BS4, which is located at the transcription start site region of *CDH3* promoter. In contrast with the distal sites, binding impairment at BS4 significantly induced the activity of *CDH3* promoter. In a first approach, we may hypothesize that specific C/EBPβ proteins are regulating negatively the activity of the promoter through that specific binding site and, upon mutation, this repression is released. However, since we did not find a significant effect mediated by LAP1, LAP2 or LIP when BS4 was mutated, we believe that other factors not C/EBPβ-related are responsible for the negative regulation in this binding site, or the mutation introduced in BS4 generated a sequence which allowed the binding of a transcription factor that is able to activate the *CDH3* gene promoter. Additionally, it is also interesting to note that, although the BS2 mutation did not create a significant decrease in *CDH3* promoter activity in MCF-7/AZ cells, this binding site is important to LAP2-mediated activation, indicating that it may not be endogenously active in these breast cancer cells, but probably highly active in BT-20 cells.

In conclusion, this study contributes to clarify the individual role of C/EBPβ proteins in breast cancer-related *CDH3*/P-cadherin gene, as well as to expand the limited characterization of the mechanisms and players that regulate this pro-invasive protein in breast cancer.

Supporting Information

Table S1 Conditions of the primary antibodies.
(PDF)

Table S2 Primers sequences used in the different assays.
(PDF)

Author Contributions

Conceived and designed the experiments: AA CR JP JCM RS FS. Performed the experiments: AA CR BS ARN ASR. Analyzed the data: AA JP FS. Contributed reagents/materials/analysis tools: AA CR JCM JP. Wrote the paper: AA JP FS.

References

1. Poli V (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem 273: 29279–29282.
2. Sebastian T, Johnson PF (2006) Stop and go: anti-proliferative and mitogenic functions of the transcription factor C/EBPbeta. Cell Cycle 5: 953–957.
3. Zahnow CA (2002) CCAAT/enhancer binding proteins in normal mammary development and breast cancer. Breast Cancer Res 4: 113–121.
4. Zahnow CA, Cardiff RD, Laucirica R, Medina D, Rosen JM (2001) A role for CCAAT/enhancer binding protein beta-liver-enriched inhibitory protein in mammary epithelial cell proliferation. Cancer Res 61: 261–269.
5. Zahnow CA (2009) CCAAT/enhancer-binding protein beta: its role in breast cancer and associations with receptor tyrosine kinases. Expert Rev Mol Med 11: e12.
6. Lekstrom-Himes J, Xanthopoulos KG (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem 273: 28545–28548.
7. Xiong W, Hsieh CC, Kurtz AJ, Rabek JP, Papaconstantinou J (2001) Regulation of CCAAT/enhancer-binding protein-beta isoform synthesis by alternative translational initiation at multiple AUG start sites. Nucleic Acids Res 29: 3087–3098.
8. Milde-Langosch K, Loning T, Bamberger AM (2003) Expression of the CCAAT/enhancer-binding proteins C/EBPalpha, C/EBPbeta and C/EBP-delta in breast cancer: correlations with clinicopathologic parameters and cell-cycle regulatory proteins. Breast Cancer Res Treat 79: 175–185.
9. Calkhoven CF, Muller C, Leutz A (2000) Translational control of C/EBPalpha and C/EBPbeta isoform expression. Genes Dev 14: 1920–1932.
10. Takeichi M (1988) The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development 102: 639–655.
11. Paredes J, Albergaria A, Oliveira JT, Jeronimo C, Milanezi F, et al. (2005) P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. Clin Cancer Res 11: 5869–5877.
12. Paredes J, Correia AL, Ribeiro AS, Albergaria A, Milanezi F, et al. (2007) P-cadherin expression in breast cancer: a review. Breast Cancer Res 9: 214.
13. Paredes J, Lopes N, Milanezi F, Schmitt FC (2007) P-cadherin and cytokeratin 5: useful adjunct markers to distinguish basal-like ductal carcinomas in situ. Virchows Arch 450: 73–80.
14. Paredes J, Stove C, Stove V, Milanezi F, Van Marck V, et al. (2004) P-cadherin is up-regulated by the antiestrogen ICI 182,780 and promotes invasion of human breast cancer cells. Cancer Res 64: 8309–8317.
15. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. (2000) Molecular portraits of human breast tumours. Nature 406: 747–752.
16. Sousa B, Paredes J, Milanezi F, Lopes N, Martins D, et al. (2010) P-cadherin, vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. Histol Histopathol 25: 963–974.
17. Ribeiro AS, Albergaria A, Sousa B, Correia AL, Bracke M, et al. (2010) Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. Oncogene 29: 392–402.
18. Albergaria A, Ribeiro AS, Pinho S, Milanezi F, Carneiro V, et al. (2010) ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBP{beta} in CDH3 gene activation. Hum Mol Genet 19: 2554–2566.
19. Shimomura Y, Wajid M, Shapiro L, Christiano AM (2008) P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle. Development 135: 743–753.
20. Faraldo MM, Teuliere J, Deugnier MA, Birchmeier W, Huelsken J, et al. (2007) beta-Catenin regulates P-cadherin expression in mammary basal epithelial cells. FEBS Lett 581: 831–836.
21. Gorski JJ, James CR, Quinn JE, Stewart GE, Staunton KC, et al. (2010) BRCA1 transcriptionally regulates genes associated with the basal-like phenotype in breast cancer. Breast Cancer Res Treat 122: 721–731.
22. Bracke ME, Van Larebeke NA, Vyncke BM, Mareel MM (1991) Retinoic acid modulates both invasion and plasma membrane ruffling of MCF-7 human mammary carcinoma cells in vitro. Br J Cancer 63: 867–872.
23. Gamallo C, Moreno-Bueno G, Sarrio D, Calero F, Hardisson D, et al. (2001) The prognostic significance of P-cadherin in infiltrating ductal breast carcinoma. Mod Pathol 14: 650–654.
24. Peralta Soler A, Knudsen KA, Salazar H, Han AC, Keshgegian AA (1999) P-cadherin expression in breast carcinoma indicates poor survival. Cancer 86: 1263–1272.
25. Turashvili G, McKinney SE, Goktepe O, Leung SC, Huntsman DG, et al. (2011) P-cadherin expression as a prognostic biomarker in a 3992 case tissue microarray series of breast cancer. Mod Pathol 24: 64–81.
26. Albergaria A, Ribeiro AS, Vieira AF, Sousa B, Nobre AR, et al. (2011) P-cadherin role in normal breast development and cancer. Int J Dev Biol 55: 811–822.
27. Van Marck V, Stove C, Jacobs K, Van den Eynden G, Bracke M (2011) P-cadherin in adhesion and invasion: opposite roles in colon and bladder carcinoma. Int J Cancer 128: 1031–1044.
28. Van Marck V, Stove C, Van Den Bossche K, Stove V, Paredes J, et al. (2005) P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. Cancer Res 65: 8774–8783.
29. Cheung LW, Leung PC, Wong AS (2010) Cadherin switching and activation of p120 catenin signaling are mediators of gonadotropin-releasing hormone to promote tumor cell migration and invasion in ovarian cancer. Oncogene 29: 2427–2440.
30. Mandeville JA, Silva Neto B, Vanni AJ, Smith GL, Rieger-Christ KM, et al. (2008) P-cadherin as a prognostic indicator and a modulator of migratory behaviour in bladder carcinoma cells. BJU Int 102: 1707–1714.
31. Taniuchi K, Nakagawa H, Hosokawa M, Nakamura T, Eguchi H, et al. (2005) Overexpressed P-cadherin/CDH3 promotes motility of pancreatic cancer cells by interacting with p120ctn and activating rho-family GTPases. Cancer Res 65: 3092–3099.
32. Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, et al. (1997) Precocious mammary gland development in P-cadherin-deficient mice. J Cell Biol 139: 1025–1032.
33. Vieira AF, Ricardo S, Ablett MP, Dionísio MR, Mendes N, et al. (2012) P-cadherin is co-expressed with CD44 and CD49f and mediates stem cell properties in basal-like breast cancer. Stem Cells.
34. LaMarca HL, Visbal AP, Creighton CJ, Liu H, Zhang Y, et al. (2010) CCAAT/enhancer binding protein beta regulates stem cell activity and specifies luminal cell fate in the mammary gland. Stem Cells 28: 535–544.
35. Zahnow CA, Younes P, Laucirica R, Rosen JM (1997) Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. J Natl Cancer Inst 89: 1887–1891.
36. Gomis RR, Alarcon C, Nadal C, Van Poznak C, Massague J (2006) C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. Cancer Cell 10: 203–214.

CHAPTER IV

CDH3/P-CADHERIN IS NEGATIVELY REGULATED BY TAP63 IN A P53-DEPENDENT MANNER IN BREAST CANCER CELLS

CONTRIBUTION OF THE AUTHORS

ARN carried out the experimental work and ASR and AFV provided assistance in data analysis and interpretation. BP, RS, FS and JP were involved in study design and interpretation and AA in the manuscript production. All authors had final approval of the submitted version.

CDH3/P-cadherin is negatively regulated by TAp63 in a p53-dependent manner in breast cancer cells. Effects on P-cadherin-mediated invasion and self-renewal

André Albergaria[1,2], Ana Rita Nobre[1,3], Ana Sofia Ribeiro[1], André Filipe Vieira[1], Bernardo Peixoto[4], Raquel Seruca[1], Fernando Schmitt[1,2], Joana Paredes[1,2,#]

[1]Cancer Genetics Group, Institute of Molecular Pathology and Immunology of Porto University (IPATIMUP).
[2]Department of Pathology, Medical Faculty of University of Porto, Alameda Prof. Hernâni Monteiro, Porto, Portugal.
[3]Institute of Biomedical Sciences of Abel Salazar (ICBAS), Porto, Portugal
[4]Departament of Mathematics and Bioinformatics of the University of Azores

Abstract

P-cadherin is a cell-cell adhesion molecule codified by the *CDH3* gene, whose expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. In breast cancer, P-cadherin is frequently overexpressed in high-grade tumours, being a well-established indicator of poor patient prognosis and has been reported as an important inducer of cancer cell migration and invasion. P-cadherin also confers stem cell features to breast tumorigenic cells that could be linked to the aggressive behavior of basal-like breast cancers. P-cadherin has been associated with already described stem cell markers, such as p63, which was recently demonstrated to transcriptionally regulate *CDH3* in a context of the developmental biology. In fact, the parallelism between p63 and P-cadherin interestingly involves the cancer and the developmental setting. In cancer, however, the relationship between p63 and P-cadherin was only explored in a pathological perspective.
We demonstrate that TAp63 isoforms transcriptionally represses *CDH3* promoter, downregulating P-cadherin protein expression in MCF7/AZ breast cancer cells. This repression is functionally reflected on P-cadherin-induced breast cancer cellular invasion and mammosphere-forming efficiency. Interestingly, we also observed that this effect of TAp63 isoform on *CDH3*/P-cadherin was not replicated in cells harboring p53 mutations, and that the induction of p53 hotspot mutations on p53 wild-type cells restored *CDH3* promoter activation. These results suggest that the repressive effect of TAP63γ isoform onto *CDH3* promoter is disabled by the p53 mutants. The validation of these observations in human breast cancer samples revealed that breast tumours expressing TAp63γ isoform, but harboring some type of known pathogenic p53 mutations were positive for P-cadherin expression, while the only case negative for P-cadherin expression was the one where no p53 mutations were detected. Taken together, our data reveal previously unknown molecular functions of TAp63γ isoform on *CDH3*/P-cadherin where TAp63γ is able to repress *CDH3* promoter activity and P-cadherin expression levels, being this regulation dependent of p53 mutational status.

Keywords: p63 isoforms, *CDH3*/P-cadherin, p53 mutations, breast cancer.

[#]To whom requests for printings should be addressed, at Institute of Molecular Pathology and Immunology of Porto University (IPATIMUP), Rua Dr. Roberto Frias s/n 4200-465 Porto, Portugal. Phone: 00351 225570700; Fax: 00351 225570799; E-mail: jparedes@ipatimup.pt

Introduction

P-cadherin is a cell-cell adhesion molecule codified by the *CDH3* gene, whose expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. In breast cancer, P-cadherin is frequently overexpressed in high-grade tumours, being a well-established indicator of poor patient prognosis and it has been reported as an important inducer of cancer cell migration and invasion. P-cadherin also confers stem cell features to breast tumorigenic cells that could be linked to the aggressive behavior of basal-like breast cancers. P-cadherin has been associated with already described stem cell markers, such as p63, which was recently demonstrated to transcriptionally regulate *CDH3* in a context of the developmental biology. In fact, the parallelism between p63 and P-cadherin interestingly involves the cancer and the developmental setting. In cancer, however, the relationship between p63 and P-cadherin was only explored in a pathological perspective, as their expression is associated between each other and with basal-like phenotype in breast cancer [1-5].
P63 is distinct from its homologue p53 in that its role as a tumour suppressor is controversial, an issue complicated

by the existence of two classes of p63 isoforms [6]. In fact, P63 expression is driven by two alternative promoters, resulting in a full-length TA isoform or an N-terminally truncated ΔN form that lacks the transactivation domain. Furthermore, ΔNp63 and TAp63 transcripts can be spliced to yield α, β, γ, δ or ε variants with alternative C-termini. Not only do these isoforms and variants have different expression patterns according to cell type and differentiation status, they have been reported to possess different and sometimes opposing functions, which challenges the understanding of p63 function [7]. In addition, a recent study has shown that endogenous p63 proteins binds to thousands of gene promoter target sites [8], a fact that, combined with a multiplicity of transcripts and protein products of which the literature has perceived a wide range of functions (often with conflicting results and interpretations), is emblematic of a gene that is among the most complex in human physiology [9]. The wide variety of targets that are changed in response to p63 include transcriptions factors, a large number of adhesion molecules, and a functionally diverse set of signaling molecules, making p63 a gene that may be directly affecting nearly 7% of the coding genes in the genome [10].

P63 is expressed in basal layers of proliferative tissues and, similarly to P-cadherin, p63 is expressed in basal layers and transiently expressed in various tissues during development. P-cadherin and p63 appear to be crucial for mammary gland differentiation and, as previously described, basal-like breast cancers typically express these basal cells markers. Another feature shared between P-cadherin and p63 is that their expression is associated with undifferentiated and proliferative status of these tissues, being crucial for orderly progression of terminal differentiation of the epidermis [11-13].

Shimomura observed that mutations in the p63 gene, as in *CDH3*/P-cadherin gene, result in hypotrichosis with juvenile macular dystrophy (HJMD) and split hand/foot malformation (SHFM), and that the expression patterns of p63 and P-cadherin overlap in the hair follicle placode and apical ectodermal ridge (AER). To clarify this relationship, they performed promoter assays and ChIP, which revealed that p63 interacts directly with two distinct regions of the *CDH3* promoter [14].

Moreover, in 2006, there was a first work linking p63 and regulation of gene expression programs involved in cell adhesion, which was published by Carroll and her group [15]. A number of genes regulated by TAp63 support the notion that p63 is involved in tight transcriptional control of epithelial differentiation, cell adhesion, and tumorigenesis via cell cycle arrest, apoptosis, and other cellular functions [16]. This was recently confirmed by a gene profiling microarray supporting TAp63γ as a potent transcriptional regulator of gene expression [16].

The mechanisms controlling *CDH3* gene activation has only recently start to be explored. The evidences linking p63 and P-cadherin, especially connecting them at the development and differentiation level, together with the transcriptional differences between p63 isoforms, which confer to this gene, the ability to induce opposite effects on target genes, led us to study whether *CDH3*/P-cadherin was a target of p63 isoforms in breast cancer and especially if the transcriptional control over *CDH3* was differently exerted by the different p63 isoforms. The P-cadherin induced effects in breast cancer cells was also evaluated in the regulatory background exerted by p63 isoforms.

Our data reveal previously unknown molecular functions of TAp63γ isoforms on *CDH3*/P-cadherin where TAp63γ is able to repress *CDH3* promoter activity and P-cadherin expression levels, being this regulation dependent of p53 mutational status.

Material and Methods

Cell Culture and transfections

Human breast cancer cell lines were obtained as described: BT20, MDA-MB-468, MCF10A (ATCC, USA), SUM149 (Dr. Stephen Ethier, University of Michigan, USA), MCF7/AZ (Prof. Dr. Marc Mareel, Laboratory of Experimental Cancerology - Ghent University, Belgium) and MCF7/AZ.Mock and MCF7/AZ.Pcad were retrovirally stable transduced as described earlier [17].

Cells were routinely maintained at 37˚C and 5% CO_2, MCF10A cells were cultured in DMEM/F12 (1:1), supplemented with 5% heat inactivated horse serum (Invitrogen Ltd, UK), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml EGF, 100 ng/ml cholera toxin (Sigma-Aldrich, USA). The other cell lines were grown in (Invitrogen Ltd, UK): DMEM (BT20 and MDA-MB-468) and DMEM/F12 (1:1) (SUM149 and MCF7/AZ) supplemented with 10% heat-inactivated fetal bovine serum (PAA, USA), 100 IU/ml penicillin and, 100 μg/ml streptomycin (Invitrogen Ltd, UK). SUM149 medium was supplemented with 5 μg/ml insulin and 1 μg/ml hydrocortisone (Sigma-Aldrich, USA).

To perform luciferase reporter gene assays, MCF7/AZ cells were growth in 96-well plates to 60% confluence and transfection was performed using XtremeGENE 6 transfection reagent (Roche), according to the manufacturer's instructions. The cells were incubated in supplemented medium with the transfectant mix for 24 hours.

For all the other techniques, cells were growth in 25 cm^2 flasks to 60% confluence and transiently transfected using 3 μg of cDNA and 9 μL of lipofectamine 2000 (Invitrogen Ltd, UK), according to the manufacturer's instructions. The cells were incubated in supplemented medium with the transfectant mix for 6 hours.

Promoter and Expression Vectors

pcDNA3-p63 and pCB6-p53 expression vectors were kindly given by Prof. Frank McKeon (Harvard Medical School) and by Prof Karen Voudsen (Beatson Institute for Cancer Research), respectively. Super-competent *E.coli* cells (TOP10, Invitrogen Ltd, UK) were transformed by

heat shock and selected in LB-Agar (AppliChem, GER) supplemented with ampicillin. *CDH3*-luciferase vector was generated as described in Albergaria A, 2010 [18]. Plasmid DNA was extracted with JETstar 2.0 - Plasmid Purification kit (GenoMed, POR), quantified and saved at -20ºC.

***CDH3*-Luciferase Reporter Gene Assay**

MCF7/AZ cells were co-transfected with the human full-length pGL3-*CDH3*/luc promoter vector and with pCMV-Renilla luciferase construct (Promega, USA), for normalization of transfection efficiency. For promoter analysis, 16 hours 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, UK), according to the manufacturer's instructions. Luciferase bioluminescence from Renilla was measured using native coelenteranzine substrate reagent (Lux Biotechnology, UK). Individual transfection experiments were repeated at least three times and in quadruplicate per transfection condition. Empty pGL3-basic (E1751) and pGL3-Control (pLUC) vectors (E1741), both from Promega, were included as controls in all luciferase reporter assays. Luminescence was then read using the Wallac/Perkin Elmer-1450-028 Trilux Microbeta (Perkin Elmer, UK) plate reader, and the results are shown as mean of relative light units (RLU).

Western Blotting (WB)

After transfection and incubation, cells were lysed with catenin lysis buffer and concentration of total protein was determined by Bradford quantification. The proteins, separated in polyacrilamide gel, were transferred to nitrocellulose membranes which were incubated for 1 hour in milk buffer 5%. The following primary antibodies were used: P-cadherin (mouse, clone 56; BD Biosciences, USA), p63 (mouse, clone 4A4; Neomarkers, USA) and β-actin (goat, clone I19; Santa Cruz Biotechnologies, USA). All reactions were revealed with luminol and hydrogen peroxide, which react with HRP labelled to secondary antibodies (Santa Cruz Biotechnologies, USA). Blots were exposed to autoradiographic film and quantificatified using Quantity One software (Bio-Rad, USA). The experiments selected to show are representative ones.

Matrigel Invasion Assay

Matrigel invasion assay was performed using 8 µm pore size BD BioCoat™ Matrigel Invasion Chambers (BD Biosiences, USA). In the upper compartment of the chamber, $5x10^4$ transfected cells were added, whereas in the lower compartment, only fresh supplemented medium was present. After 48 hours of incubation at 37°C, the upper surface of the filter was cleared from non-invasive cells with a cotton swab and washed with PBS. The remaining (invasive) cells, which were attached to the lower surface of the filter, were fixed with cold methanol and stained and mounted with vectashield containing 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI) (Vector Laboratories, Inc., Burlingame, USA). Invasive cells were scored by counting the cells in the filter with a fluorescence microscope (Leica DM 2000), at 200X of magnification.

Mammosphere Formation Efficiency Assay

Monolayer transfected cells were enzymatically detached with trypsin-EDTA (Sigma-Aldrich, USA), manually disaggregated with a 25-gauge needle to a single-cell suspension and resuspended in cold PBS. Cells were plated at 750/cm^2 in nonadherent culture conditions, in wells coated with 1.2% poly(2-hydroxyethylmethacrylate)/95%ethanol (Sigma-Aldrich, USA). Cells were grown for 5 days, in DMEM/F12 containing B27 supplement, 500 ng/mL hydrochortisone, 10 µg/mL insulin, 20 ng/mL hEGF and 1% of Penicilin/streptomycin. Mammosphere forming efficiency (MFE) was calculated as the number of mammospheres (≥50 µm) formed, divided by the cell number plated, being expressed as a percentage.

BrdU Proliferation Assay

BrdU incorporation assay was performed to evaluate cell proliferation. Briefly, transfected cells were cultured in coverslips, incubated with BrdU for 1 hour and fixed with 4% paraformaldehyde, for 30 minutes. Cells were first treated with HCl 2M for 20 minutes and then incubated with anti-BrdU primary antibody (Dako Cytomation, USA) for 1 hour, and with anti-mouse FITC secondary antibody, for 30 minutes. The samples were mounted with vectashield containing DAPI (Vector Laboratories, Inc., USA) and the percentage of proliferating cells was calculated.

Apoptosis Assay

TUNEL assay was performed to evaluate apoptosis. After transfection, the cell medium was collected and cells were enzymatically detached with trypsin-EDTA (Sigma-Aldrich, USA). All the cells were added to the initial medium and it was centrifuged at 2000 rpm, for 10 minutes, twice. The cells were fixed with 4% paraformaldehyde, for 15 minutes, and another centrifugation was performed (2000 rpm, 10 minutes). The pellet was re-suspended and each sample was added to the wells of the cytospin, and centrifuged at 500 rpm, for 5 minutes. The slides were incubated for 2 minutes at 4ºC with a permeabilization solution (0.1% Triton X-100 in 0.1% Sodium Citrate) and with TUNEL-reaction mix (Roche), at 37ºC for 1 hour. Finally, cells were washed in the dark and mounted with vectashield mounting medium with DAPI (Vector Laboratories, Inc., USA).

Tissue Samples

Eight frozen cases of primary operable invasive breast carcinomas were used to assess the RNA expression of TAp63 and P-cadherin and also to obtain DNA to analyze the p53 mutational status. After selection and isolation of the core area of the tumour samples by a trained

pathologist, the samples were kept in RNAlater and conserved at -80ºC, ensuring an high quality nucleic acids. The cases were obtained from the Unit of Genetics and Molecular Pathology of the Hospital of Divino Espirito Santo, Azores, Portugal, under patient informed consent process and with ethical approval by the Hospital Ethical Commission. 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.

DNA isolation and p53 mutational analysis

DNA was isolated using an Invisorb Spin Tissue Mini Kit (Invitek), according to the protocol provided by the manufacturers. Purity and concentration was determined in a ND-1000 spectrometer (Nanodrop). All the samples were examined for mutations in all p53 exons covering all the coding sequences of the p53 gene. PCR amplifications were performed by multiplex according to QIAGEN® Multiplex PCR Handbook (Qiagen, Cambridge, MA) and using a panel of primers described in the Table S1 (Supplementary data).

RNA Isolation and RT-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 and half microgram of RNA per sample was reverse-transcribed to synthesize cDNA, using SuperScript II reverse-transcriptase (Invitrogen) according to the manufacturer's instructions. P-cadherin cDNA was amplified using the sense primer 5'ACGAAGACACAAGAGAGATTGG and the antisense primer 5'CGATGATGGAGATGTTCATGG, while TAp63 cDNA was amplified using the sense primer 5'AAGATGGTGCGACAAACAAG and the antisense primer 5'AGAGAGCATCGAAGGTGGAG. PCRs were done using the Qiagen Taq PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were done with an initial denaturation at 95ºC for 5 minutes; 30 cycles of 95ºC for 30 seconds (denaturation), 55ºC (P-cadherin) or 62ºC (TAp63) for 45 seconds (annealing) and 72ºC for 2 minutes (elongation); followed by a final extension at 72ºC for 10 minutes. Negative controls without cDNA were used for all sets of PCRs, as well as, positive controls (one breast tumour P-cadherin postitive and a testicule sample as TAp63-positive tissue). The products were analysed on a 1% agarose gel and compared to a 100-bp DNA ladder (Fermentas).

Statistical Analysis

Data are expressed as mean values of at least three independent experiments ± SEM. Student's t-tests were used to determine statistically significant differences (*p<0.05; **p<0.01).

Results

TAp63 isoforms transcriptionally represses *CDH3* at the promoter level and downregulates P-cadherin protein expression in MCF7/AZ breast cancer cells.

The only study showing *CDH3* as a transcriptional target gene of p63 was published in 2008 by Shimomura and colleagues. Although in a context of the developmental biology, this work was the first one demonstrating the deregulated *CDH3* promoter activity mediated by the presence of TAp63 isoforms [14].

In order to decipher the effect of the different p63 isoforms on *CDH3* promoter activity in breast cancer cells we co-transfected MCF7/AZ cells with different p63 isoforms and with pGL3-*CDH3*/luc promoter vector. As shown in Figure 1A, both TAp63α and γ isoforms are able to reduce the basal promoter activation of *CDH3*. The *CDH3*-luciferase reporter assay also shown that ΔNp63 isoforms deregulates *CDH3* promoter, although not statistically significant and not in a comparable pattern between both

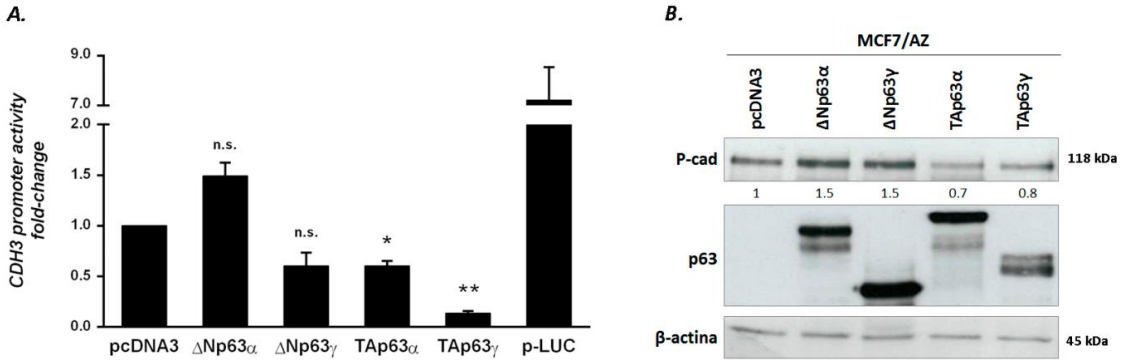


Figure 1. Induction of p63 isoforms differently modulates *CDH3*/P-cadherin in breast cancer cells. MCF7/AZ cells were transfected with isolated isoforms of p63 and *CDH3* promoter activity and protein levels where assessed. A. *CDH3* luciferase gene reporter assays showed that TAp63 isoforms possess a repressive activity in *CDH3* promoter, a feature also shared by p63γ. Thus, TAp63γ leads to the greater decrease when compared to the endogenous promoter activity (pcDNA3). **B.** The opposite effects of ΔNp63 and TAp63 isoforms are even more drastic in protein levels. After 72 hours of transfection with ΔNp63 isoforms, it was observed an 1,5-fold increase in P-cadherin levels. In contrast, cells transfected with TAp63 isoforms have a decrease in P-cadherin expression.

COOH-terminus α and γ isoforms. Nevertheless, γ isoform is also able to induce a considerable reduction on *CDH3* promoter expression. Concerning TA-mediated regulation of *CDH3* promoter it is interesting to observe that in addition to the same effect on transcriptional repression, while TAp63α isoform induces a repression of about 40% on *CDH3* promoter, TAp63γ isoform almost fully knockdown it's basal/control activity within the cells (Figure 1A). At the protein level, the opposite effects of ΔNp63 and TAp63 isoforms on the regulation of P-cadherin are very clear. While ΔNp63 isoforms induces a 1,5-fold increase in P-cadherin levels, the general transcriptional repressive effects of TAp63 isoforms on P-cadherin expression is also replicated in accordance with the results obtain in luciferase reporter assay (Figure 1B).

TAp63 counteracts the P-cadherin-induced invasion and mammosphere-forming efficiency in breast cancer cells.

It has been largely described the pro-invasive potential that overexpression of P-cadherin exerts in MCF-7/AZ breast cancer cells [17,19]. More recently, our group also demonstrated that P-cadherin mediates stem cell properties, namely by conferring increased self-renewal ability. In these previous studies we showed that stable transduction of P-cadherin in MCF-7/AZ cells (MCF-7/AZ.Pcad) led to an increase in matrigel invasion capacity as well as of mammosphere formation efficiency (MFE) when compared with the mock breast cancer cell line. These two effects of P-cadherin on invasion potential and on MFE in MCF-7/AZ are herein reinforced in Figure 2A and 2B. MCF-7/AZ.Pcad cells show a ~4-fold increase capacity to invade matrigel (A) when compared with the control/empty vector cells (MCF-7/AZ.Mock), an inductive effect that is also observed on the mammosphere formation efficiency (B).

The evidence that TAp63γ isoform has the ability to heavily repress the transcriptional activity of *CDH3* promoter with consequences on the reduction of the expression levels of P-cadherin, led us to study the cellular effects mediated by the overexpression of TAp63γ in cells stably transfected with P-cadherin (MCF-7/AZ.Pcad), and therefore, with increased invasion and self-renewal potential. Figure 2C show that, similarly with what we have observed in MCF-7/AZ cells, in MCF7/AZ.Pcad cells, TAp63γ was able to downregulated the expression levels of P-cadherin in about 50% compared with the cells transfected with empty vector. Most importantly, this reduction on the expression levels of P-cadherin in P-cadherin-induced cells, is reflected at the functional level as we demonstrated that TAp63γ counteracts the P-cadherin-induced invasion and mammosphere-forming efficiency in breast cancer cells. The Figure 2D show that, upon transfection of TAp63γ isoform, the invasion rate of MCF-7/AZ.Pcad cells was severely reduced in about 70%, when compared with the cells transfected with empty vector. A decrease of about 20% on the mammosphere-forming efficiency was also detected on the P-cadherin-induced cells when transiently transfected with TAp63γ (Figure 2E). In order to exclude that differences observed in the abovementioned functional assays were due to apoptosis or altered cell proliferation, TUNEL and BrdU proliferation assays were performed and no significant differences on apoptosis nor in proliferation index were detected in MCF-7/AZ.Pcad cells when transfected with TAp63γ (Figured 2F and 2G).

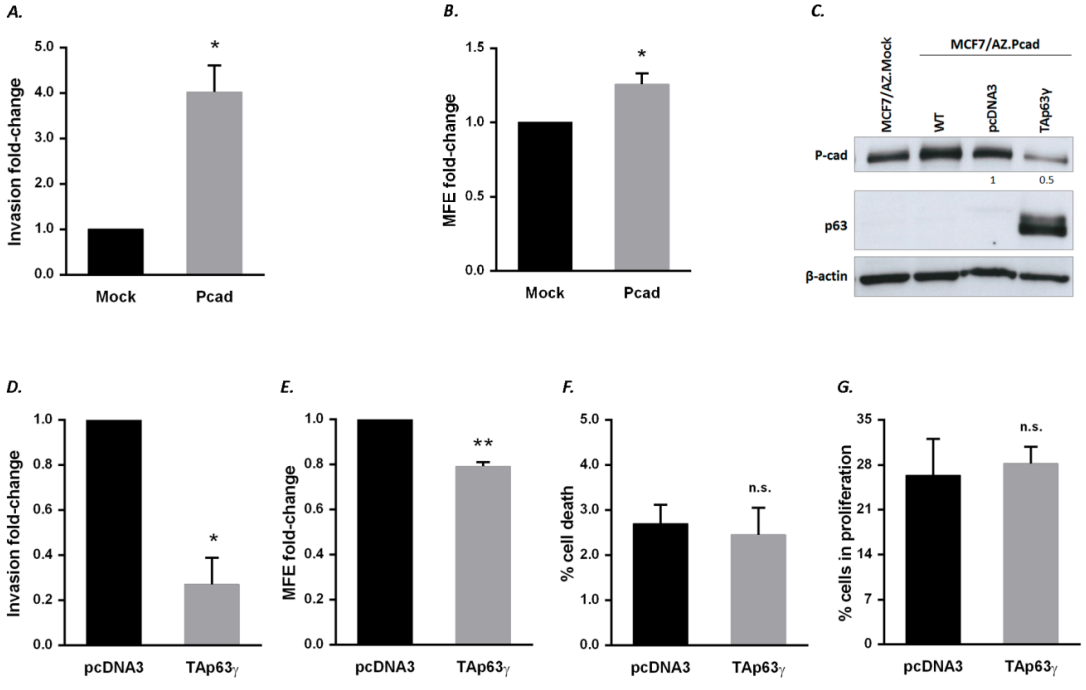


Figure 2. TAp63γ counteracts the P-cadherin-induced functional properties. A-B. In a breast cancer cell model with induction of P-cadherin overexpression (MCF-7/AZ.Pcad cells), there is an increase of ~4-fold matrigel invasion capacity (A), as well as, an increase in

mammosphere formation efficiency (B) compared with control cells with empty vector (MCF7/AZ.Mock). **C-E.** Transfection of TAp63γ isoform abrogates P-cadherin induced overexpression in MCF-7/AZ.Pcad cells (C), and consequent P-cadherin-induced functional properties, such as invasion (D) and mammosphere formation efficiency (E). **F-G.** TUNEL and BrdU proliferation assay was performed to exclude that differences observed in functional assays were due to altered cell proliferation. No significant differences were observed in the percentage of cell death (F), neither proliferation (G) when cells were transfected with TAP63γ isoform.

TAp63γ represses *CDH3* promoter activity and P-cadherin expression levels in a p53 dependent manner.

Whole-genome DNA microarrays have identified and clustered different breast cancer cell lines in distinct molecular subtypes. In this context, the expression of P-cadherin is heterogeneous across distinct breast cancer (BC) cells lines molecular profiles, being however, more predominantly presented in basal-like molecular subtype cell lines and in normal-like BC cells, such as MCF10A cells. Herein, we demonstrated that TAp63γ is able to abrogate *CDH3*/P-cadherin at the transcriptional and expression level in a P-cadherin low-expressing MCF-7/AZ cell line (luminal subtype), as well as in a P-cadherin-induced cell line, MCF-/AZ.Pcad. In order to test the effect exerted by TAp63γ in a panel of P-cadherin-overexpressing BC cell lines, we transiently transfected the TAp63γ isoform in MCF10A, BT20, SUM149 and in MDA-MB-468 cells. Interestingly, we observed that the repressive effect of TAp63γ isoform over P-cadherin expression was not replicated in the basal-like cells. In another hand, a moderate reduction of P-cadherin was detected in the P-cadherin-overexpressing MCF10A cells in the presence of TAp63γ isoform, a result in line with the one found in the luminal cells. A feature that has been described to be shared among these basal-like cells but not in the MCF10A and the luminal breast cancer cell lines, MCF7/AZ is the p53 mutation status and, in fact, the three basal-like cell lines that we studied herein were already described to harbor p53 mutations [20]. Based on these findings and on the large amount of evidence demonstrating the binding and inhibition of TAp63 activity by p53 mutants [21-23], we hypothesized that the mutational status of p53 in the basal-like cells interferes with TAp63γ, abrogating its repressive effect over P-cadherin expression. As such, the Figure 3A shows that in MCF10A, a p53 wild-type BC cell line [24,25], the transfection of TAp63γ leads to a decrease in P-cadherin expression, while in mutant-p53 cell lines (BT20, SUM149 and MDA-MB-468), P-cadherin expression is not affected by TAp63γ transfection (Figure 3A). With the purpose of testing this hypothesis at the transcriptional level, we used MCF7/AZ cells, a cell line displaying a p53 wild-type genetic background, which was co-transfected with *CDH3*-luciferase reporter and with TAP63γ isoform. Under this *CDH3* promoter-repressed condition, two different hotspot p53 mutations (R175H and R273H) were induced in order to evaluate whether these mutations were able to restore *CDH3* promoter activation. In fact, Figure 3B show that the two hotspot p53 mutations, specifically described as having a strong effect on the inhibition of TAP63γ [7,21,22,26-28], reestablished *CDH3* promoter activity, demonstrating that the repressive effect of TAP63γ isoform onto *CDH3* promoter was abrogated by the p53 mutants.

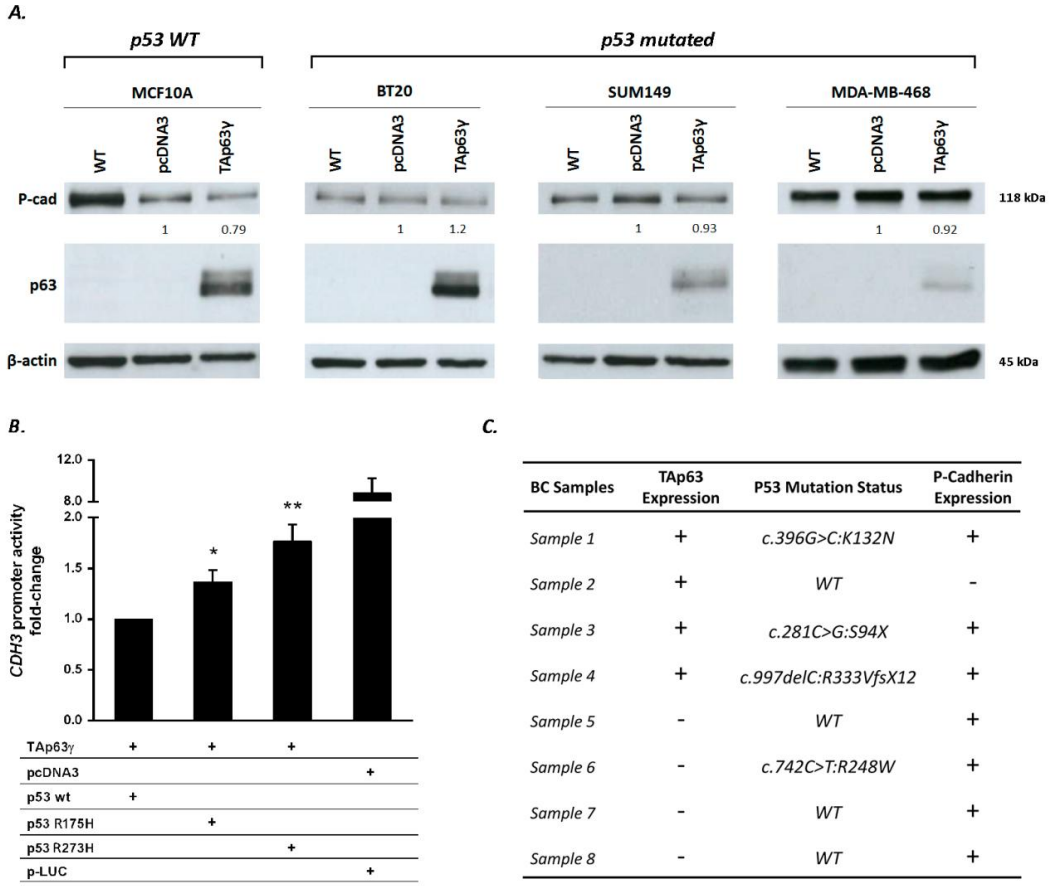
In order to validate these observations in human breast cancer samples, we analyzed the mutational status of p53 in a panel of 8 human breast cancer frozen specimens: 4 samples displaying high levels of TAP63γ RNA expression and 4 samples where no RNA expression of TAp63γ was detected. We then associate this p53 mutational status and TAp63γ RNA expression with P-cadherin RNA levels. It is also important to state that P-cadherin RNA expression detected on these cases were in accordance to previously P-cadherin protein levels detected by immunohistochemistry. Figure 3C shows that in breast tumours expressing TAp63γ isoform, the only case negative for P-cadherin expression was the one where no p53 mutations were detected. By contrast, in all the cases displaying some type of known pathogenic p53 mutations, the expression of P-cadherin was positive. On the other hand, tumour samples negative for TAp63γ, were P-cadherin positive, irrespectively of the p53 status (Figure 3C).

Taken together, these findings led us to conclude that TAp63γ is able to represses *CDH3* promoter activity and P-cadherin expression levels, but this regulation is dependent of p53 mutational status (Figure 4).

Discussion

The shared phenotypes and patterns of expression that has been described to P-cadherin and P63 sparked our interest in studying the regulatory and functional relationship between this potent transcriptional regulator of adhesion programme, p63 [15]; and this largely known cadherin associated with malignant breast cancer phenotype P-cadherin [29-33]. The actual challenge was to know the p63 isoform molecular targets and different functions since p63 gene generates transcripts encoding proteins with or without a N-terminal transactivation domain, TAp63 and ΔNp63, respectively. Additionally, both transcripts can be alternatively spliced to generate proteins with different C-termini α, β, γ, δ or ε [34]. The aim of this work was to characterize the different regulatory effects of the different p63 isoforms in *CDH3* gene and on the expression of P-cadherin and its cellular functions.

As p63 can come in different flavors, in cancer, it has been implicated in tumour formation and progression, acting as an oncogene or a tumour suppressor depending on the cellular context.



BC Samples	TAp63 Expression	P53 Mutation Status	P-Cadherin Expression
Sample 1	+	*c.396G>C:K132N*	+
Sample 2	+	*WT*	-
Sample 3	+	*c.281C>G:S94X*	+
Sample 4	+	*c.997delC:R333VfsX12*	+
Sample 5	-	*WT*	+
Sample 6	-	*c.742C>T:R248W*	+
Sample 7	-	*WT*	+
Sample 8	-	*WT*	+

Figure 3. TAp63γ represses *CDH3*/P-cadherin in a p53 dependent manner. A. In wild type-p53 breast cancer cell lines (MCF10A), the transfection of TAp63γ leads to a decrease in P-cadherin expression, while in mutant-p53 cell lines (BT20, SUM149 and MDA-MB-468), P-cadherin expression is not affected by Tap63γ transfection. **B.** In the p53-wt MCF7/AZ cells, it was observed a recovery of *CDH3* promoter activity when cells were co-transfected with TAp63γ and with the p53 hotspot mutations, R175H and R273H, compared with simple transfection of TAp63γ. **C.** In breast tumours expressing TAp63γ isoform, the only case displaying no p53 mutations were the case also negative for P-cadherin, while all the cases displaying known pathogenic p53 mutations expressed P-cadherin. In the other hand, tumour samples negative for TAp63γ, were P-cadherin positive, independently of the p53 status.

Some findings that support p63 as an oncogene are: p63 is very rarely mutated in cancer [35], but it has been shown to be overexpressed in many tumours, especially in squamous cell carcinoma of head and neck cancers [36], lung [37], cutaneous [38], uterine [39] and breast cancer [40,41]. Furthermore, many groups using human squamous cell carcinomas demonstrated that these tumours actually overexpress the ΔNp63 isoforms [42,43], which are the isoforms generally associated with oncogenic activity. Convergently, Senoo et al., also demonstrated that primary human skin cancers showed a loss of TAp63 while normal skin retained its expression [43]. In head and neck squamous cell carcinoma (HNSCC), although ΔNp63α, β and γ isoforms are present, ΔNp63α is the predominant isoform expressed and is overexpressed in tumours compared with matched normal tissue specimens, which suggests that ΔNp63α plays an anti-differentiation and anti-apoptotic role in the mucosal epithelium, possibly playing a key role in the formation of HNSCC [44]. ΔNp63 isoforms were also shown to inactivate p53 gene, and Rocco et al. suggested that the advantage of having ΔNp63 overexpression is the ΔNp63α ability to repress the induction of apoptosis. Additionally, it was shown that ΔNp63 can act as a dominant negative to inhibit p53, TAp63 and TAp73 transactivation and consequent apoptosis [45,46]. Moreover, p63 is hypothesized to play an important role in maintaining the epidermal stem cell population, as well as, in maintaining the proliferative capacity of epithelial stem cells [34].

Nevertheless, all these do not exclude the possibility that p63 can also act as tumour-suppressor, as it has been suggested by other findings.

Given the structural similarity between p63 and p53, it has been hypothesized that p63 acts as a sensor to DNA damage. Indeed, multiple studies have shown that p63 can induce apoptosis being upregulated in cells that have been treated with DNA damaging agents. The most potent inducer of apoptosis is the TAp63γ isoform, which contains the transactivation domain and lacks the inhibitory domain present in the alpha isoforms [47,48]. Data not confirmed by our model, in which we do not have

variations in TUNEL assay when cells where transfected with TAp63γ isoform.

Many studies were done showing that the knockdown of p63 lead to a loss of cell adhesion, cellular arrest, invasion, and metastasis, which are important steps in tumour progression [15,26,49]. Hu M et al., in 2008, suggest that p63 is required for myoepithelial cell differentiation and that the elimination of it results in loss of myoephithelial cells and progression to invasion [49]. Additionally, it has been reported that TAp63α is inhibited in metastatic cells, as well as, restoration of TAp63α function impairs lamellipodia formation and TGFβ-induced migration in vitro and severely opposes metastatic dissemination of injected aggressive breast and skin cancer cells in immunodeficient mice [26,27]. Taken together, these studies indicate that TAp63 bears tumour progression and metastasis suppressive properties, ideas that fit with our results in which we show a decrease of invasion and mammosphere formation efficiency when cells were transfected with TAp63γ isoform, a functional effect of P-cadherin repression.

Noticeably, this equilibrium is altered upon p53 mutation, the best-understood mechanism by which TAp63 activity can be attenuated. Indeed, mutation of p53, one of the most frequent lesions in human cancers, does not necessarily lead to loss of p53. In contrast, hot-spot mutations hitting the p53 DNA-binding domain often cause expression of a stable, yet transcriptional deficient mutant-p53 protein, which is able to form a complex with p63, limiting p63 transcriptional activity [26,27]. Although molecularly speculative, in our models we observed that in wild type-p53 breast cancer cell lines, we have a repression of *CDH3*/P-cadherin by TAp63γ, while in mutant-p53 cell lines, P-cadherin expression is not affected by Tap63γ transfection, probably by the formation of the mut-p53-TAp63 complex, which impairs the TAp63 activity as transcription factor. This was corroborated by the recovery of *CDH3* promoter activity when cells are transfected both with TAp63γ and mutant-p53, compared with cells only transfected with TAp63γ.

Together, our data demonstrate that TAp63γ represses *CDH3*, limiting P-cadherin induced aggressive behaviour, in a p53 dependent manner.

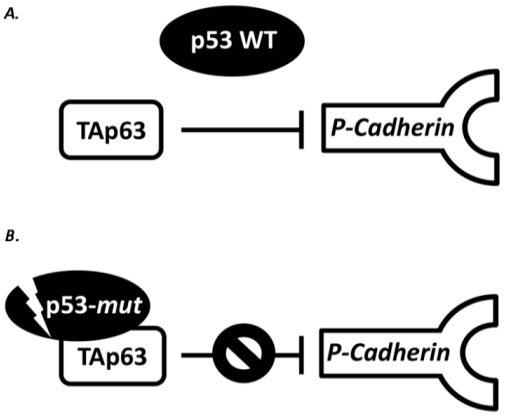


Figure 4. Schematic representation of the molecular link between P-cadherin, p63 and p53. In wild type-p53 context, *CDH3*/P-cadherin is repressed by TAp63γ. However, in the presence of mutant-p53, P-cadherin expression is no longer affected by TAp63γ, probably due to the formation of the mut-p53-TAp63 complexes, which impair TAp63γ to repress its target.

References

1. Matos I, Dufloth R, Alvarenga M, Zeferino LC, Schmitt F (2005) p63, cytokeratin 5, and P-cadherin: three molecular markers to distinguish basal phenotype in breast carcinomas. Virchows Arch 447: 688-694.

2. Paredes J, Lopes N, Milanezi F, Schmitt FC (2007) P-cadherin and cytokeratin 5: useful adjunct markers to distinguish basal-like ductal carcinomas in situ. Virchows Arch 450: 73-80.

3. Rakha EA, Ellis IO (2009) Triple-negative/basal-like breast cancer: review. Pathology 41: 40-47.

4. Coelho L, Chaves E, Vasconcelos S, Fonteles M, De Sousa F, et al. (2010) [Attention deficit hyperactivity disorder (ADHD) in children: neurobiological aspects, diagnosis and therapeutic approach]. Acta Med Port 23: 689-696.

5. Sousa B, Paredes J, Milanezi F, Lopes N, Martins D, et al. (2010) P-cadherin, vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. Histol Histopathol 25: 963-974.

6. Rizzardi K, Landberg K, Nilsson L, Ljung K, Sundas-Larsson A (2011) TFL2/LHP1 is involved in auxin biosynthesis through positive regulation of YUCCA genes. Plant J 65: 897-906.

7. Montonen J, Landberg R, Kamal-Eldin A, Aman P, Knueppel S, et al. (2010) Reliability of fasting plasma alkylresorcinol concentrations measured 4 months apart. Eur J Clin Nutr 64: 698-703.

8. Kronblad A, Jirstrom K, Ryden L, Nordenskjold B, Landberg G (2006) Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response. Int J Cancer 118: 2609-2616.

9. Crum CP, McKeon FD (2010) p63 in epithelial survival, germ cell surveillance, and neoplasia. Annu Rev Pathol 5: 349-371.

10. Mehra R, Varambally S, Ding L, Shen R, Sabel MS, et al. (2005) Identification of GATA3 as a breast cancer prognostic marker by global gene expression meta-analysis. Cancer Res 65: 11259-11264.

11. Hines MD, Jin HC, Wheelock MJ, Jensen PJ (1999) Inhibition of cadherin function differentially affects markers of terminal differentiation in cultured human keratinocytes. J Cell Sci 112: 4569-4579.
12. Hirai Y, Nose A, Kobayashi S, Takeichi M (1989) Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. I. Lung epithelial morphogenesis. Development 105: 263-270.
13. Wakita H, Shirahama S, Furukawa F (1998) Distinct P-cadherin expression in cultured normal human keratinocytes and squamous cell carcinoma cell lines. Microsc Res Tech 43: 218-223.
14. Shimomura Y, Wajid M, Shapiro L, Christiano AM (2008) P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle. Development 135: 743-753.
15. Carroll DK, Carroll JS, Leong CO, Cheng F, Brown M, et al. (2006) p63 regulates an adhesion programme and cell survival in epithelial cells. Nat Cell Biol 8: 551-561.
16. Kok M, Holm-Wigerup C, Hauptmann M, Michalides R, Stal O, et al. (2009) Estrogen receptor-alpha phosphorylation at serine-118 and tamoxifen response in breast cancer. J Natl Cancer Inst 101: 1725-1729.
17. Paredes J, Stove C, Stove V, Milanezi F, Van Marck V, et al. (2004) P-cadherin is up-regulated by the antiestrogen ICI 182,780 and promotes invasion of human breast cancer cells. Cancer Res 64: 8309-8317.
18. Albergaria A, Ribeiro AS, Pinho S, Milanezi F, Carneiro V, et al. (2010) ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBP{beta} in CDH3 gene activation. Hum Mol Genet.
19. Ribeiro AS, Albergaria A, Sousa B, Correia AL, Bracke M, et al. (2010) Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. Oncogene 29: 392-402.
20. Butt S, Borgquist S, Garne JP, Landberg G, Tengrup I, et al. (2009) Parity in relation to survival following breast cancer. Eur J Surg Oncol 35: 702-708.
21. Berglund P, Stighall M, Jirstrom K, Ryden L, Ferno M, et al. (2008) Cyclin E confers a prognostic value in premenopausal breast cancer patients with tumours exhibiting an infiltrative growth pattern. J Clin Pathol 61: 184-191.
22. Landberg R, Kamal-Eldin A, Andersson R, Aman P (2006) Alkylresorcinol content and homologue composition in durum wheat (Triticum durum) kernels and pasta products. J Agric Food Chem 54: 3012-3014.
23. Svensson Mansson S, Reis-Filho J, Landberg G (2007) Transcriptional upregulation and unmethylation of the promoter region of p16 in invasive basal cell carcinoma cells and partial co-localization with the gamma 2 chain of laminin-332. J Pathol 212: 102-111.
24. Landberg R, Kamal-Eldin A, Andersson A, Vessby B, Aman P (2008) Alkylresorcinols as biomarkers of whole-grain wheat and rye intake: plasma concentration and intake estimated from dietary records. Am J Clin Nutr 87: 832-838.
25. Borgquist S, Holm C, Stendahl M, Anagnostaki L, Landberg G, et al. (2008) Oestrogen receptors alpha and beta show different associations to clinicopathological parameters and their co-expression might predict a better response to endocrine treatment in breast cancer. J Clin Pathol 61: 197-203.
26. Berthelsen AK, Dobbs J, Kjellen E, Landberg T, Moller TR, et al. (2007) What's new in target volume definition for radiologists in ICRU Report 71? How can the ICRU volume definitions be integrated in clinical practice? Cancer Imaging 7: 104-116.
27. Howell A, Landberg G, Bergh J (2009) Breast tumour stroma is a prognostic indicator and target for therapy. Breast Cancer Res 11 Suppl 3: S16.
28. Ryden L, Landberg G, Stal O, Nordenskjold B, Ferno M, et al. (2008) HER2 status in hormone receptor positive premenopausal primary breast cancer adds prognostic, but not tamoxifen treatment predictive, information. Breast Cancer Res Treat 109: 351-357.
29. Paredes J, Albergaria A, Oliveira JT, Jeronimo C, Milanezi F, et al. (2005) P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. Clin Cancer Res 11: 5869-5877.
30. Paredes J, Correia AL, Ribeiro AS, Albergaria A, Milanezi F, et al. (2007) P-cadherin expression in breast cancer: a review. Breast Cancer Res 9: 214.
31. Paredes J, Correia AL, Ribeiro AS, Milanezi F, Cameselle-Teijeiro J, et al. (2008) Breast carcinomas that co-express E- and P-cadherin are associated with p120-catenin cytoplasmic localisation and poor patient survival. J Clin Pathol 61: 856-862.
32. Paredes J, Milanezi F, Reis-Filho JS, Leitao D, Athanazio D, et al. (2002) Aberrant P-cadherin expression: is it associated with estrogen-independent growth in breast cancer? Pathol Res Pract 198: 795-801.
33. Paredes J, Milanezi F, Viegas L, Amendoeira I, Schmitt F (2002) P-cadherin expression is associated with high-grade ductal carcinoma in situ of the breast. Virchows Archiv 440: 16-21.
34. Westfall MD, Pietenpol JA (2004) p63: Molecular complexity in development and cancer. Carcinogenesis 25: 857-864.
35. Ryden L, Jirstrom K, Bendahl PO, Ferno M, Nordenskjold B, et al. (2005) Tumor-specific expression of vascular endothelial growth factor receptor 2 but not vascular endothelial growth factor or human epidermal growth factor receptor 2 is associated with impaired response to adjuvant tamoxifen in premenopausal breast cancer. J Clin Oncol 23: 4695-4704.
36. Ekberg J, Holm C, Jalili S, Richter J, Anagnostaki L, et al. (2005) Expression of cyclin A1 and cell cycle proteins in hematopoietic cells and acute myeloid leukemia and links to patient outcome. Eur J Haematol 75: 106-115.
37. Stighall M, Manetopoulos C, Axelson H, Landberg G (2005) High ID2 protein expression correlates with a favourable prognosis in patients with primary breast

cancer and reduces cellular invasiveness of breast cancer cells. Int J Cancer 115: 403-411.
38. Bjartell A, Paju A, Zhang WM, Gadaleanu V, Hansson J, et al. (2005) Expression of tumor-associated trypsinogens (TAT-1 and TAT-2) in prostate cancer. Prostate 64: 29-39.
39. Kapur A, Carson PL, Eberhard J, Goodsitt MM, Thomenius K, et al. (2004) Combination of digital mammography with semi-automated 3D breast ultrasound. Technol Cancer Res Treat 3: 325-334.
40. Reis-Filho JS, Albergaria A, Milanezi F, Amendoeira I, Schmitt FC (2002) Naked nuclei revisited: p63 Immunoexpression. Diagn Cytopathol 27: 135-138.
41. Stendahl M, Kronblad A, Ryden L, Emdin S, Bengtsson NO, et al. (2004) Cyclin D1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients. Br J Cancer 90: 1942-1948.
42. Landberg T, Mailhot JD, Brainerd EL (2003) Lung ventilation during treadmill locomotion in a terrestrial turtle, Terrapene carolina. J Exp Biol 206: 3391-3404.
43. Helczynska K, Kronblad A, Jogi A, Nilsson E, Beckman S, et al. (2003) Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma in situ. Cancer Res 63: 1441-1444.
44. Hedberg Y, Davoodi E, Ljungberg B, Roos G, Landberg G (2002) Cyclin E and p27 protein content in human renal cell carcinoma: clinical outcome and associations with cyclin D. Int J Cancer 102: 601-607.
45. Landberg G, Ostlund H, Nielsen NH, Roos G, Emdin S, et al. (2001) Downregulation of the potential suppressor gene IGFBP-rP1 in human breast cancer is associated with inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation in estrogen receptor negative tumors. Oncogene 20: 3497-3505.
46. Azizi E, Landberg T (2002) Effects of metamorphosis on the aquatic escape response of the two-lined salamander (Eurycea bislineata). J Exp Biol 205: 841-849.
47. Ljungberg B, Bozoky B, Kovacs G, Stattin P, Farrelly E, et al. (2001) p53 expression in correlation to clinical outcome in patients with renal cell carcinoma. Scand J Urol Nephrol 35: 15-20.
48. Rexhepaj E, Jirstrom K, O'Connor DP, O'Brien SL, Landberg G, et al. (2010) Validation of cytoplasmic-to-nuclear ratio of survivin as an indicator of improved prognosis in breast cancer. BMC Cancer 10: 639.
49. Kadar L, Albertsson M, Areberg J, Landberg T, Mattsson S (2000) The prognostic value of body protein in patients with lung cancer. Ann N Y Acad Sci 904: 584-591.

CHAPTER V

GENERAL DISCUSSION

Clinically, during cancer progression, the most important processes to determine patient prognosis is the presence of cancer cell invasion and metastasis. In an ideal scenario, biomarkers should be used to distinguish lesions with high probability to invade and to develop clinically relevant metastases from those that will remain indolent. In breast cancer, our group has shown that P-cadherin overexpression is a poor prognostic marker due to its ability to induce *in vitro* invasive capacity to cancer cells by the induction of MMPs secretion to the extracellular matrix (ECM) (1, 2). These enzymes will degrade the ECM, as well as will cleave the extracellular domain of full-length P-cadherin, which generates a soluble fragment (sP-cad) with a key role in the induction of cell invasion.

Regarding gene regulation of P-cadherin expression, several signalling pathways and cellular mechanisms have been already described has being involved: promoter methylation (3), ER-α (1) and BRCA1 expression (4) as P-cadherin repressors; and, on the other hand, β-catenin (5), C/EBPβ (6) and p63 (7) as putative activators. Nevertheless, the molecular mechanisms underlying P-cadherin *de novo* expression in breast cancer are still far from being well recognized.

The work presented throughout this thesis addressed the validation of two putative transcriptional factors, C/EBPβ and p63, in the regulation of P-cadherin expression in breast cancer cells.

C/EBPβ is one out of six members of a family of leucine zipper transcription factors (C/EBPs, CCAAT/enhancer-binding proteins), which have important roles in cellular proliferation, differentiation, survival, apoptosis, metabolism, inflammation, transformation, and oncogene-induced senescence and tumorigenesis (8). As well as P-cadherin, C/EBPβ is not mutated in breast tumours, but its overexpression strongly associates with aggressive behaviour neoplastic features, such as ER-negative tumours, with poorly differentiated phenotype, high proliferation rates, basal-like phenotype and worse prognosis of breast cancer patients (6, 9). Importantly, C/EBPβ was also significantly associated with P-cadherin expression in breast carcinomas, with nearly 60% of co-expression of both proteins (6). This association was corroborated in the present work by the co-expression of both proteins in the same cells, with the presence of C/EBPβ in the nuclei and P-cadherin at the cellular membrane (Chapter III). Additionally, it is known that aberrant expression of C/EBPβ can lead to cancer progression and multidrug resistance; however, the real role of each individual isoform remains to be determined (8). The larger C/EBPβ proteins, LAP 1 and 2 (liver-enriched transcriptional activating proteins), support

proliferation and repress differentiation of many cell types (10). On the other hand, the smaller protein product, LIP (liver-enriched transcriptional inhibitory protein), lacks the transactivation domain and, thus, it was initially believed as acting only as a dominant negative repressor (11, 12). However, it is interesting that in some cellular contexts, evidences emerged to support a role for LIP as a transcriptional activator of gene expression (8). In fact, in the present study (Chapter III), we showed that C/EBPβ-LIP leads to the greater increase of *CDH3* promoter activity comparing with the others isoforms, which fits both with the idea that LIP can actually act as a transcriptional activator and that its induced growth cascade may play a role in the development of breast cancer (12). Moreover, C/EBPβ-LIP not only acts as a transcriptional activator of *CDH3*, but also has a synergistic effect in the presence of C/EBPβ-LAP isoforms, contradicting the theory that C/EBPβ-LIP acts as a dominant negative repressor in this context. Although the molecular mechanism is unclear, C/EBPβ-LAP2 was considered the most transcriptional active isoform of C/EBPβ (8), data that is also not confirmed in our models. In fact, we demonstrated separate and distinct effects of C/EBPβ-LAP isoforms, but with C/EBPβ-LAP 1 appearing as a higher activator than LAP2. This controversy about LAP1 and LAP2 is extended to normal and neoplastic breast tissue, in which the same authors described both isoforms as expressed in non-malignant human mammary cells, such as MCF10A cells (13), and in breast tumours (14, 15), while others have shown that LAP1 is predominantly expressed in normal mammary cells, whereas LAP2 is restricted to dividing cells in both normal and neoplastic mammary epithelial cells (16). Moreover, it was shown that the overexpression of LAP2 in MCF10A cells leads to epithelial–mesenchymal transition and transformation (17).

Together, our data demonstrate that *CDH3*/P-cadherin is a direct transcriptional target of C/EBPβ. Furthermore, the results corroborate the idea that there are different roles and powers for each C/EBPβ isoform and an already described importance for the LIP:LAP ratio in the regulation of gene expression in developmental models, as well as in breast tumours (9, 14, 15). Increased LIP:LAP ratio have been associated with oestrogen-receptor-negative, aneuploid, highly proliferative and poor prognosis breast tumours (9, 14), as well as to a defective transforming growth factor β (TGF-β)-dependent cytostatic response in metastatic breast cancer cells. Interestingly, it has been demonstrated that the overexpression of LAP2 allows a decreased LIP:LAP ratio and the TGF-β cytostatic response, which significantly reduced the proliferative activity of metastatic cells. In contrast, an increased LIP expression antagonises LAP2 activity and the high LIP:LAP ratio favours the inactivation of p15/INK4b by FoxO-Smad complex, repression of c-Myc

by an E2F4/5-Smad, and the consequent proliferative behaviour of metastatic breast cancer cells (15). Curiously, c-Myc is an important transcriptional repressor of *CDH3* gene, when in a complex with BRCA1 (4), leading to decreased levels of P-cadherin mRNA and protein. However, c-Myc has also been described as required for β-catenin-mediated mammary stem cell amplification and tumorigenesis (18), which suggests that it has probably a dual role in *CDH3* regulation, being a repressor in complex with BRCA1, but a required element in the activation of *CDH3* by β-catenin. Additionally, Myc is also an ERα target, as well as P-cadherin (1, 6), and there is a significant overlap between ERα-negative tumours and the expression of Myc-regulated genes associated with the control of proliferation (19).

p63, another *CDH3* transcription factor, is expressed, as P-cadherin, in basal layers of proliferative tissues and transiently expressed in various tissues during development, suggesting that both proteins have a crucial role in mammary gland differentiation and, as previously described, in basal-like breast cancers, which also express these two basal cell markers. Another feature shared between these proteins is the association between their expression and the undifferentiated and proliferative status of epithelial tissues, being for example crucial for the terminal differentiation of the epidermis (20-22). Indeed, Shimomura observed that the expression patterns of p63 and P-cadherin also overlap in the hair follicle placode and apical ectodermal ridge (AER) and that mutations in the *p63* and *CDH3* genes also result in hypotrichosis with juvenile macular dystrophy (HJMD) and split hand/foot malformation (SHFM). To clarify this relationship, they performed promoter assays and ChIP, which revealed that p63 interacts directly with two distinct regions of the *CDH3* promoter (7). Moreover, in 2006, a link between p63 and the regulation of gene expression programs involved in cell adhesion was first published by Carroll and her group (23). These publications, as well as the shared phenotypes and patterns of expression, sparked our interest in the relationship between p63 and P-cadherin; however, the actual challenge was to know the role of p63 isoforms, since *p63* gene generates transcripts encoding proteins with or without the N-terminal transactivation domain, TAp63 and ΔNp63, respectively. Additionally, both transcripts can be alternatively spliced to generate proteins with different C-termini α, β, γ, δ or ε. (24). Thus, the special effort of the second part of this work was to clarify the different effects of the different p63 isoforms in the regulation of the *CDH3* gene.

Depending on the cellular context, and respective isoform, p63 has been implicated in tumour formation and progression, acting as an oncogene or as a tumour suppressor. Some findings that support p63 as an oncogene are: p63 is very rarely mutated in cancer (25), but it has been shown to be overexpressed in many tumours, especially in

squamous cell carcinoma of head and neck cancers (26), lung (27), cutaneous (28), uterine (29, 30) and breast cancer (31, 32). Furthermore, ΔNp63 isoforms, which are the ones generally associated with oncogenic activity, are actually overexpressed in these tumours (33-35). Accordingly, it was also demonstrated that primary human skin cancers showed a loss of TAp63, while normal skin retained its expression (35).

In head and neck squamous cell carcinoma (HNSCC), although ΔNp63α, β and γ isoforms are present, ΔNp63α is the predominant isoform expressed and is overexpressed in tumours compared with matched normal tissue specimens, suggesting that it has a role as an anti-differentiation and anti-apoptotic isoform in the mucosal epithelium, possibly playing a key role in the formation of HNSCC (36). ΔNp63 isoforms were also shown to inactivate *p53* gene and Rocco *et al*. suggested that the advantage of having ΔNp63 overexpression is the ΔNp63α ability to repress the induction of apoptosis by inhibiting the ability of p73 to transactivate NOXA and PUMA, known apoptosis inducers. Additionally, it was shown that ΔNp63 can act as a dominant negative to inhibit p53, TAp63 and TAp73 transactivation and consequent apoptosis (37, 38). It is also hypothesized that p63 has an important role in maintaining the epidermal stem cell population and their proliferative capacity (24).

Nevertheless, a tumour-suppressor role for p63 cannot be excluded by our results and it has actually been suggested by other findings. Multiple studies have shown that p63 can induce apoptosis, being upregulated in cells that have been treated with DNA damaging agents, a role that has been hinted by the structural similarity between p63 and p53. This, it has been hypothesized that p63 could act as a sensor to DNA damage. The most potent inducer of apoptosis is the TAp63γ isoform, which contains the transactivation domain and lacks the inhibitory domain present in the alpha isoforms (39, 40). However, this data was not confirmed by our model (Chapter IV), as we do not observe variations in TUNEL assay in cells transfected with TAp63γ isoform.

Several studies demonstrated that the knockdown of p63 lead to a loss of important features in tumour progression, such as loss of cell adhesion, cellular arrest, invasion, and metastasis (23, 41, 42). Hu *et al*., in 2008, suggested that p63 is required for myoepithelial cell differentiation, since without its expression, there is a loss of myoephithelial cells and progression to invasion ensues (42). Additionally, it has been reported that TAp63α is inhibited in metastatic cells, and restoring its function impairs lamellipodia formation and TGFβ-induced migration *in vitro*. In addition, it severely opposes metastatic dissemination of injected aggressive breast and skin cancer cells in immunodeficient mice (41, 43). Taken together, these studies indicate that TAp63 bears tumour progression and metastasis suppressive properties, ideas that fit with our results in which we show a

decrease of invasion and mammosphere formation efficiency when cells were transfected with TAp63γ isoform, a functional effect of P-cadherin repression (Chapter IV).

Notably, this equilibrium is altered upon p53 mutation, the best-understood mechanism by which TAp63 activity can be attenuated. Indeed, p53 mutations, one of the most frequent genetic alterations diagnosed in human cancers, does not necessarily lead to loss of p53 expression. In contrast, hot-spot mutations hitting the p53 DNA-binding domain often cause expression of a stable, yet transcriptional deficient mutant-p53 protein, which is able to form a complex with p63, limiting its transcriptional activity, and leading to increased invasion, migration and metastasis capacities (41, 43). Although molecularly speculative, we have observed that in wild type-p53 breast cancer cell lines there is a repression of *CDH3*/P-cadherin by TAp63γ, while in mutant-p53 cell lines, P-cadherin expression is not affected by TAp63γ transfection, probably by the formation of the mut-p53-TAp63 complex, which impairs the TAp63 activity as a transcription factor. This result was corroborated by the recovery of *CDH3* promoter activity when cells were transfected both with TAp63γ and mutant-p53, compared with cells only transfected with TAp63γ. Together, our data demonstrate that TAp63γ represses the transcription of the *CDH3* gene, limiting P-cadherin induced aggressive behaviour in a p53 dependent manner.

Finally, there is evidence of additional signalling pathways involving p63, p53 and P-cadherin. β-catenin, a critical activator of *CDH3* gene and P-cadherin expression, is degraded in normal cells due to p53, which facilitates the degradation of ΔNp63 and seems to activate GSK3β. In contrast, in cancer, mutant p53 fails to downregulate ΔNp63, which binds B56α, inhibiting GS3Kβ and decreasing phosphorylation levels of β-catenin, leading to induced nuclear accumulation of β-catenin and activation of β-catenin-dependent transcription of genes (44, 45). In addition, the transcriptional upregulation of ΔNp63 proteins is also critical for BRCA1 suppressor function and defects in BRCA1-ΔNp63 signalling are key events in the pathogenesis of basal-like breast cancer (40), a molecular subtype of breast cancer with association with P-cadherin overexpression. Actually, in breast carcinomas, it has been shown that P-cadherin expression is strongly associated with basal-like tumours, as well as, with the presence of *BRCA1* mutations (46).

Taken all together, mechanisms by which P-cadherin becomes *de novo* expressed in breast cancer are now clearer. However, many questions remain open because all the above mentioned transcriptional factors were already described as being expressed in normal epithelial tissues and having a role in development and cell differentiation, which led us to pursue P-cadherin in the same context and, possibly, find a role on it. The existence of stem cells (SCs) has been demonstrated in various adult tissues including

brain, bone marrow and peripheral blood, muscle, skin, breast, lung, kidney, liver, pancreas and thyroid gland (47-59). However, genes that contribute to the SC phenotype and cell differentiation still need to be elucidated. Nevertheless, gene expression studies of niche-resident cells have revealed a number of SC markers and regulators of epidermal SC maintenance, differentiation and lineage commitment (60), including same regulators of *CDH3*/P-cadherin: C/EBPβ (61), p63 (62), β-catenin (18) and c-Myc (63). Interestingly, it has also been proposed that tumours contain rare stem-like cells called cancer stem cells (CSC), or tumour initiating cells, characterized by self-renewing capacity, low proliferation rates, ability to differentiate into proliferating tumour cells and the ability to withstand cancer therapy (64, 65). Serial transplantation of CSCs gave rise to heterogeneous tumours with tissue specific cell types of the parental tumour, suggesting that only this small subpopulation of neoplastic cells with stem-like capacities promote the maintenance and development of the tumour. CSCs have also been suggested as cause of tumour reappearance after initially successful treatment, which probably target the bulk of neoplastic cells, but do not eradicate CSCs (66). ΔNp63α has been already described as an inductor of breast cancer stem cell phenotype, since the overexpression of ΔNp63 in MCF7 breast cancer cells increased the percentage of the $CD44^{+}/CD24^{-}$ breast CSC subpopulation and led to increased cancer cell proliferation, clonogenicity (increased colony formation ability in soft agar), anchorage-independent growth (ability to grow into mammospheres) and the incidence of tumour xenografts formed *in vivo* (67). In addition, ΔNp63α over-expressing cells were more drug resistant, suggesting that ΔNp63α might be a tumour-initiating transcription factor in breast cancer and proposing ΔNp63 as one of the possible markers of CSCs in epithelial tissues (67). Furthermore, in normal tissues, the functional alterations in ΔNp63 described in both human and mouse models underscore the dependence of basal cells of the skin and derived appendages on this gene for normal function (68, 69). Moreover, ΔNp63α is selectively expressed at high levels in the basal cells of stratified and glandular epithelia and its expression decreases with cellular differentiation (39, 70, 71). Presumably, the stem cell compartments exist within the basal population (72), thus p63 predominates within a selected subset of epithelial tissues, signifying a unique population of stem cells. Supporting this hypothesis, Mills *et al.* observed a complete absence of stratified epidermis in $p63^{-/-}$ mice (68); however, Yang *et al.* observed stratified, but disrupted, epidermis in $p63^{-/-}$ mice (69), suggesting that the embryonic epidermis of $p63^{-/-}$ mice undergoes an unusual process of non-regenerative differentiation. Thus, p63 is proposed to be critical for the proliferation and maintenance of the epithelial progenitor cell populations that give rise to the differentiated stratified epithelial cells, rather than for the differentiation process itself.

Another player in this controversial issue is p53. Described as “guardian of the genome” (73), p53 also takes part in the p53/p63/p73 “orchestra of isoforms to harmonise cell differentiation and response to stress” (74). Germline deletion of p53 in mice with critically short telomeres spares damaged stem cells from apoptosis and protracts their survival (75, 76). Additionally, the skin of such p53-deleted mice displays improved wound healing and hair growth, apparently due to increased numbers of epidermal SCs (77); and in a mouse model of HER2-overexpressing breast cancer, cultured $p53^{-/-}$ mammospheres were found enriched for CSCs due to loss of p53 control over asymmetric cell division (78). Similarly, p53-deficient mouse hematopoietic SCs have improved repopulation capacity in transplantation assays (79), as well as p53-deficient human hematopoietic SCs better resist radiation-induced apoptosis (80). However, and surprisingly, upon repeated *in vivo* expansion without acute genotoxic insult, these cells actually display reduced self-renewal capacity, apparently due to persistent accumulation of renewal, by maintaining rigorous genome integrity and quality control (80).

The deletion of p53 also confers an advantage to mutant cells by increasing their proliferation rate. By contrast, the absence of c-Myc limits cell propagation. Moreover, lower levels of p53 and higher levels of c-Myc make cells highly competitive, with a growth or survival advantage (81, 82). Additionally, c-Myc is required for *β*-catenin-mediated mammary stem cell amplification (18), another central player in the epidermal lineage selection. Activation of *β*-catenin signalling pushes cells towards hair follicle differentiation (83), while deleting *β*-catenin in adult stem and progenitor cells directs differentiating cells to adopt an interfollicular epidermal fate (84, 85). Although *c-Myc* is a downstream target of *β*-catenin (86), c-Myc independently influences epidermal cell proliferation, migration and lineage commitment (63). Mice with ectopic targeted c-Myc activity in the basal undifferentiated epidermis exhibit larger sebaceous glands, hair loss and epidermal hyperplasia (87-90). Furthermore, Myc has an essential non-redundant function in the maintenance of the self-renewing multipotent stem cell population responsible for the regenerative capacity of the mammary epithelium and is required downstream from ovarian hormones, for the control of mammary stem and progenitor cell functions (91).

Also, in a normal breast model, C/EBPβ-deficient mammary epithelial cells showed an decreased mammosphere formation efficiency, impaired repopulation ability and decreased outgrowth potential, premature mammary epithelial cells senescence and increased differentiated luminal cells (61), revealing the importance of C/EBPβ in mammary gland development, repopulation activity and luminal cell lineage commitment.

In summary, C/EBPβ, p63, β-catenin and c-Myc pathways are interconnected and usually implicated in both cancer and normal stem cells. Additionally, basal layers of normal

epithelial tissues, the niche of proliferative and undifferentiated cells, express C/EBPβ, p63, β-catenin and c-Myc, as well as, P-cadherin, and during differentiation, these markers are lost. Therefore, it is likely that P-cadherin, a marker of poor prognosis in breast cancer, inductor of CSC properties and common player in all the above mentioned pathways, could also have a role in normal epithelial stem cell maintenance and restraining differentiation.

CHAPTER VI

ONGOING AND FUTURE WORK

It is now clear that *CDH3*/P-cadherin's regulation is a tightly regulated multi-factorial mechanism. Therefore, it is mandatory to explore different regulatory mechanisms which may be modulating the expression of this protein. Thus, the second aim of this project was **to disclose new mechanisms that regulate *CDH3*/P-cadherin expression in invasive carcinomas and evaluate if the same mechanisms are also important in the process of differentiation of normal epithelial tissues.** In order to address this aim, the following tasks are being performed:

1. **Identification of putative miRNAs underlying P-cadherin *de novo* expression in breast cancer;**

miRNAs are small non-coding RNA molecules recognized as a class of biological regulators, acting mainly in a combinatorial regulation mechanism. A given miRNA may have multiple different mRNA targets, and a given target might similarly be targeted by multiple miRNAs (195, 196). Moreover, different sets of miRNAs are found in different cell types and tissues (197), and aberrant expression and dysregulation of miRNAs has been already implicated in numerous diseases, including cancer (198-201). Therefore, we decided to study if miRNAs could be also responsible for *CDH3*/P-cadherin regulation, since there is no knownledge concerning miRNA profiles in this context. In order to achieve this goal, our initial strategy was to perform miRNA microarrays in breast cancer models where we could modulate P-cadherin expression levels: BT20 cell line, which overexpress P-cadherin, with and without siRNA for *CDH3*; and MCF7/AZ cell line, retrovirally stable transduced to encode P-cadherin (MCF7/AZ.Pcad cell line) (2)). RNA extraction was already performed with mirVana - miRNA Isolation Kit (Ambion, USA), according to the manufacturer's instructions, and the differential expression of *CDH3*/P-cadherin was confirmed by real-time PCR (Figure VI.1).

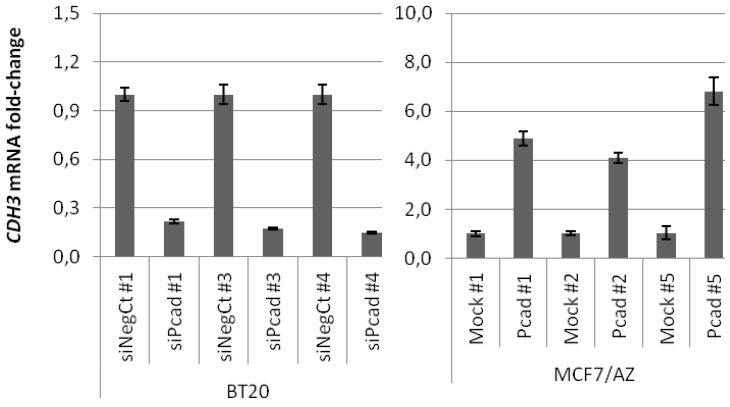


Figure VI.1. Analysis of CDH3 mRNA levels by real-time PCR to validate the P-cadherin modulation in our breast cancer cell models. GAPDH mRNA levels were analysed and used as loading control.

Subsequently, a DNase treatment (Invitrogen, USA) and an Agilent Human miRNA Microarray will be performed, in collaboration with Prof. Manuel Santos from Universidade de Aveiro (ongoing work). In the data analysis, a special attention will be given to miRNAs targeting the 3'-UTR of P-cadherin and/or its transcription regulators. Additionally, a second miRNA profiling will be performed in a small series of P-cadherin positive and negative tumours, in order to validate the results.

At the end of this task, we expect to reveal the specific miRNAs signatures associated with P-cadherin expression in breast cancer, as well as the specific miRNAs that suppress transcriptional inhibitors of P-cadherin mRNA, allowing the overexpression of this protein in this disease.

2. Evaluation of molecular mechanisms regulating *CDH3*/P-cadherin expression in breast cancer and in normal epithelial tissues.

Although P-cadherin expression and its disturbance are significant during tumourigenesis, its expression in normal tissues is "niche-specific" (1, 42). P-cadherin is expressed in developing embryos, undifferentiated and proliferative cells in adult epithelial tissues (41), and has a critical role, not only to form cell-cell interactions, but also to promote cell sorting and cell-signalling events that regulate normal development and differentiation. Bearing this in mind, it is also emergent to understand P-cadherin's regulation in normal epithelial tissues.

In order to evaluate all the described mechanisms of *CDH3* regulation in normal epithelial tissues, as well as to verify if these are associated with the differential expression of P-cadherin in the distinct components of these tissues, we are optimizing an *in situ* hybridization of small mRNA molecules assay (202) (Figure VI.2), in collaboration with Dr. Ola Soderberg from Uppsala University, Sweden. This methodology will allow us to simultaneous genotype transcripts, measure the relative mRNA and evaluate the tissue-specific transcript variants. All these can be performed directly in both fixed cells and tissues, and the signals are amplified with a padlock-rolling circle amplification method (202). At the end of this task, we expect to obtain common, as well as distinct, mechanisms modulating P-cadherin expression in breast cancer and during differentiation of normal epithelia.

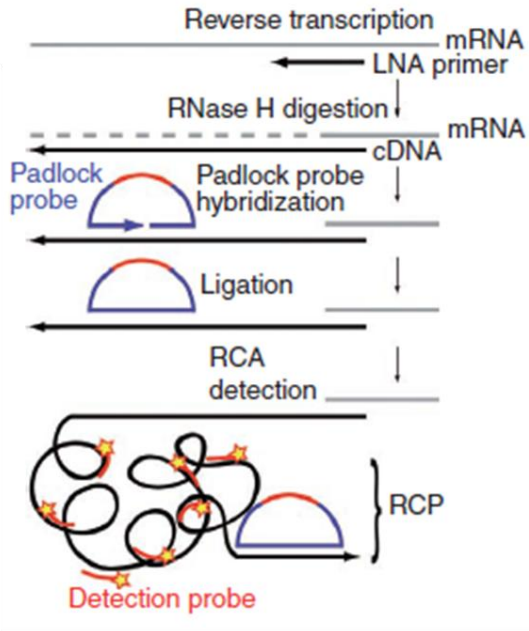


Figure VI.2. Schematic representation of the *in situ* hybridization of small mRNA molecules methodology. cDNA is created using locked nucleic acid (LNA)-modified primers and is probed after degradation of mRNA by RNase H. RCPs are identified through hybridization of fluorescent detection probes. Adapted from Larsson *et al.*, 2010 (202).

CHAPTER VII

REFERENCES

1. Albergaria A*, et al.* (2011) P-cadherin role in normal breast development and cancer. *The International Journal of Developmental Biology* 55(7-9):811-822.
2. Paredes J*, et al.* (2004) P-cadherin is up-regulated by the antiestrogen ICI 182,780 and promotes invasion of human breast cancer cells. *Cancer Res* 64(22):8309-8317.
3. Ribeiro AS*, et al.* (2010) Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. *Oncogene* 29:392-402.
4. Vieira AF*, et al.* (2012) P-cadherin is coexpressed with CD44 and CD49f and mediates stem cell properties in basal-like breast cancer. *Stem Cells* 30(5):854-864.
5. Milde-Langosch K, Loning T, & Bamberger AM (2003) Expression of the CCAAT/enhancer-binding proteins C/EBPalpha, C/EBPbeta and C/EBPdelta in breast cancer: correlations with clinicopathologic parameters and cell-cycle regulatory proteins. *Breast Cancer Res Treat* 79(2):175-185.
6. Zahnow CA (2002) CCAAT/enhancer binding proteins in normal mammary development and breast cancer. *Breast Cancer Res* 4(3):113-121.
7. Albergaria A*, et al.* (2010) ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBP{beta} in CDH3 gene activation. *Hum Mol Genet* 25(8):963-974.
8. Crum CP & McKeon FD (2010) p63 in epithelial survival, germ cell surveillance, and neoplasia. *Annu Rev Pathol* 5:349-371.
9. Carroll DK*, et al.* (2006) p63 regulates an adhesion programme and cell survival in epithelial cells. *Nat Cell Biol* 8(6):551-561.
10. Shimomura Y, Wajid M, Shapiro L, & Christiano AM (2008) P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle. *Development* 135(4):743-753.
11. Wheelock MJ & Johnson KR (2003) Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* 19:207-235.
12. Wheelock MJ, Soler AP, & Knudsen KA (2001) Cadherin junctions in mammary tumors. *J Mammary Gland Biol Neoplasia* 6(3):275-285.
13. Wheelock M, Shintani Y, Maeda M, Fukumoto Y, & Johnson K (2008) Cadherin switching. *Journal of Cell Science* 121:727-735.
14. Foty RA & Steinberg MS (2004) Cadherin-mediated cell-cell adhesion and tissue segregation in relation to malignancy. *Int. J. Dev. Biol.* 48:397-409.
15. Yagi T & Takeichi M (2000) Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev* 14(10):1169-1180.
16. Berx G*, et al.* (1995) Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1). *Genomics* 26(2):281-289.
17. Jarrard DF*, et al.* (1997) P-Cadherin is a basal cell-specific epithelial marker that is not expressed in prostate cancer. *Clin Cancer Res* 3(11):2121-2128.
18. Hubbard TJ*, et al.* (2009) Ensembl 2009. *Nucleic Acids Res* 37(Database issue):D690-697.
19. van Roy F & Berx G (2008) The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci* 65(23):3756-3788.
20. Faraldo ML & Cano A (1993) The 5' flanking sequences of the mouse P-cadherin gene. Homologies to 5' sequences of the E-cadherin gene and identification of a first 215 base-pair intron. *J Mol Biol* 231(3):935-941.
21. Stemmler MP (2008) Cadherins in development and cancer. *Mol Biosyst* 4(8):835-850.
22. Paredes J*, et al.* (2005) P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. *Clin Cancer Res* 11(16):5869-5877.
23. Sakharkar KR & Chow VT (2004) PPD - Proteome Profile Database. *In silico biology* 4(2):219-223.
24. Paredes J*, et al.* (2012) Epithelial E- and P-cadherins: role and clinical significance in cancer. *Biochim Biophys Acta* 1826(2):297-311.

25. Pignatelli M & Bodmer WF (1988) Genetics and biochemistry of collagen binding-triggered glandular differentiation in a human colon carcinoma cell line. *Proc Natl Acad Sci U S A* 85(15):5561-5565.
26. Takeichi M (1993) Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 5(5):806-811.
27. Takeichi M (1995) Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* 7(5):619-627.
28. Takeichi M (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251(5000):1451-1455.
29. Blaschuk OW, Sullivan R, David S, & Pouliot Y (1990) Identification of a cadherin cell adhesion recognition sequence. *Dev Biol* 139(1):227-229.
30. Shapiro L*, et al.* (1995) Structural basis of cell-cell adhesion by cadherins. *Nature* 374:327-337.
31. Ozawa M, Ringwald M, & Kemler R (1990) Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc Natl Acad Sci U S A* 87(11):4246-4250.
32. Conacci-Sorrell M, Zhurinsky J, & Ben-Ze'ev A (2002) The cadherin-catenin adhesion system in signaling and cancer. *J Clin Invest* 109(8):987-991.
33. Ozawa M & Ohkubo T (2001) Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. *J Cell Sci* 114(Pt 3):503-512.
34. Yap AS, Niessen CM, & Gumbiner BM (1998) The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J Cell Biol* 141(3):779-789.
35. Aberle H*, et al.* (1994) Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J Cell Sci* 107 (Pt 12):3655-3663.
36. Hulsken J, Birchmeier W, & Behrens J (1994) E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *J Cell Biol* 127(6 Pt 2):2061-2069.
37. Pokutta S & Weis WI (2000) Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Mol Cell* 5(3):533-543.
38. Green KJ, Getsios S, Troyanovsky S, & Godsel LM (2010) Intercellular Junction Assembly, Dynamics, and Homeostasis. *Cold Spring Harbor Perspectives in Biology* 2(2):a000125.
39. Lourenço SV, Kamibeppu L, Fernandes JD, Sotto MN, & Nico MMS (2008) Relationship of adhesion molecules expression with epithelial differentiation markers during fetal skin development. *Journal of Cutaneous Pathology* 35(8):731-737.
40. Takeichi M (1988) The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* 102(4):639-655.
41. Koizumi M, Matsuzaki T, & Ihara S (2005) Expression of P-cadherin distinct from that of E-cadherin in re-epithelialization in neonatal rat skin. *Development, growth & differentiation* 47(2):75-85.
42. Paredes J*, et al.* (2007) P-cadherin expression in breast cancer: a review. *Breast Cancer Res* 9(5):214.
43. Cavallaro U & Christofori G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer* 4(2):118-132.
44. Gumbiner BM (2005) Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* 6(8):622-634.
45. Larue L, Ohsugi M, Hirchenhain J, & Kemler R (1994) E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc Natl Acad Sci U S A* 91(17):8263-8267.
46. Radice GL, Sauer CL, Kostetskii I, Peralta Soler A, & Knudsen KA (2003) Inappropriate P-cadherin expression in the mouse mammary epithelium is compatible with normal mammary gland function. *Differentiation* 71(6):361-373.

47. Frebourg T*, et al.* (2006) Cleft lip/palate and CDH1/E-cadherin mutations in families with hereditary diffuse gastric cancer. *J Med Genet* 43(2):138-142.
48. Shimomura Y, Wajid M, Kurban M, & Christiano AM (2010) Splice site mutations in the P-cadherin gene underlie hypotrichosis with juvenile macular dystrophy. *Dermatology* 220(3):208-212.
49. Sprecher E*, et al.* (2001) Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin. *Nat Genet* 29(2):134-136.
50. Kjaer KW*, et al.* (2005) Distinct CDH3 mutations cause ectodermal dysplasia, ectrodactyly, macular dystrophy (EEM syndrome). *J Med Genet* 42(4):292-298.
51. Frixen UH*, et al.* (1991) E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 113(1):173-185.
52. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, & van Roy F (1991) Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 66(1):107-119.
53. Van Aken E, De Wever O, Correia da Rocha AS, & Mareel M (2001) Defective E-cadherin/catenin complexes in human cancer. *Virchows Arch* 439(6):725-751.
54. Birchmeier W & Behrens J (1994) Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* 1198(1):11-26.
55. De Leeuw WJ*, et al.* (1997) Simultaneous loss of E-cadherin and catenins in invasive lobular breast cancer and lobular carcinoma in situ. *J Pathol* 183(4):404-411.
56. Oliveira C, Seruca R, & Carneiro F (2009) Hereditary gastric cancer. *Best practice & research. Clinical gastroenterology* 23(2):147-157.
57. Oliveira C*, et al.* (2009) Germline CDH1 deletions in hereditary diffuse gastric cancer families. *Hum Mol Genet* 18(9):1545-1555.
58. Oliveira C, Seruca R, & Carneiro F (2006) Genetics, pathology, and clinics of familial gastric cancer. *International journal of surgical pathology* 14(1):21-33.
59. Pinheiro H*, et al.* (2010) Allele-specific CDH1 downregulation and hereditary diffuse gastric cancer. *Hum Mol Genet* 19(5):943-952.
60. Risinger JI, Berchuck A, Kohler MF, & Boyd J (1994) Mutations of the E-cadherin gene in human gynecologic cancers. *Nat Genet* 7(1):98-102.
61. Onder TT*, et al.* (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 68(10):3645-3654.
62. Christofori G & Semb H (1999) The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci* 24(2):73-76.
63. Thiery JP, Acloque H, Huang RY, & Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* 139(5):871-890.
64. Paredes J*, et al.* (2008) Breast carcinomas that co-express E- and P-cadherin are associated with p120-catenin cytoplasmic localisation and poor patient survival. *J Clin Pathol* 61(7):856-862.
65. Hazan RB, Qiao R, Keren R, Badano I, & Suyama K (2004) Cadherin switch in tumor progression. *Ann N Y Acad Sci* 1014:155-163.
66. Maeda M*, et al.* (2006) Expression of inappropriate cadherins by epithelial tumor cells promotes endocytosis and degradation of E-cadherin via competition for p120(ctn). *Oncogene* 25(33):4595-4604.
67. Nieman MT, Prudoff RS, Johnson KR, & Wheelock MJ (1999) N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J Cell Biol* 147(3):631-644.
68. Leckband D & Prakasam A (2006) Mechanism and dynamics of cadherin adhesion. *Annual review of biomedical engineering* 8:259-287.
69. Fujimori T & Takeichi M (1993) Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. *Mol Biol Cell* 4(1):37-47.

70. Ribeiro AS*, et al.* (2013) P-cadherin functional role is dependent on E-cadherin cellular context: a proof of concept using the breast cancer model. *J Pathol* 229(5):705-718.
71. Palacios J*, et al.* (1995) Anomalous expression of P-cadherin in breast carcinoma. Correlation with E-cadherin expression and pathological features. *Am J Pathol* 146(3):605-612.
72. Peralta Soler A, Knudsen KA, Salazar H, Han AC, & Keshgegian AA (1999) P-cadherin expression in breast carcinoma indicates poor survival. *Cancer* 86(7):1263-1272.
73. Shimoyama Y & Hirohashi S (1991) Expression of E- and P-cadherin in gastric carcinomas. *Cancer Res* 51(8):2185-2192.
74. Moreno-Bueno G*, et al.* (2003) Abnormalities of E- and P-cadherin and catenin (beta-, gamma-catenin, and p120ctn) expression in endometrial cancer and endometrial atypical hyperplasia. *J Pathol* 199(4):471-478.
75. Patel IS, Madan P, Getsios S, Bertrand MA, & MacCalman CD (2003) Cadherin switching in ovarian cancer progression. *Int J Cancer* 106(2):172-177.
76. Arenas MI, Romo E, Royuela M, Fraile B, & Paniagua R (2000) E-, N- and P-cadherin, and alpha-, beta- and gamma-catenin protein expression in normal, hyperplastic and carcinomatous human prostate. *Histochem J* 32(11):659-667.
77. Soler AP*, et al.* (1997) Expression of P-cadherin identifies prostate-specific-antigen-negative cells in epithelial tissues of male sexual accessory organs and in prostatic carcinomas. Implications for prostate cancer biology. *Am J Pathol* 151(2):471-478.
78. Imai K*, et al.* (2008) Identification of a novel tumor-associated antigen, cadherin 3/P-cadherin, as a possible target for immunotherapy of pancreatic, gastric, and colorectal cancers. *Clin Cancer Res* 14(20):6487-6495.
79. Wakita H, Shirahama S, & Furukawa F (1998) Distinct P-cadherin expression in cultured normal human keratinocytes and squamous cell carcinoma cell lines. *Microsc Res Tech* 43(3):218-223.
80. Oka H*, et al.* (1993) Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res* 53(7):1696-1701.
81. Taniuchi K*, et al.* (2005) Overexpressed P-cadherin/CDH3 promotes motility of pancreatic cancer cells by interacting with p120ctn and activating rho-family GTPases. *Cancer Res* 65(8):3092-3099.
82. Gamallo C*, et al.* (2001) The prognostic significance of P-cadherin in infiltrating ductal breast carcinoma. *Mod Pathol* 14(7):650-654.
83. Turashvili G*, et al.* (2011) P-cadherin expression as a prognostic biomarker in a 3992 case tissue microarray series of breast cancer. *Mod Pathol* 24(1):64-81.
84. Sousa B*, et al.* (2010) P-cadherin, vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. *Histol Histopathol* 25(8):963-974.
85. Mannello F, Tonti GA, Medda V, Pederzoli A, & Sauter ER (2008) Increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer. *Cancer Sci* 99(11):2160-2169.
86. Ben Hamida A*, et al.* (2008) Markers of subtypes in inflammatory breast cancer studied by immunohistochemistry: prominent expression of P-cadherin. *BMC Cancer* 8:28.
87. Cheung LW*, et al.* (2011) P-cadherin cooperates with insulin-like growth factor-1 receptor to promote metastatic signaling of gonadotropin-releasing hormone in ovarian cancer via p120 catenin. *Oncogene* 30(26):2964-2974.
88. Bauer K, Dowejko A, Bosserhoff AK, Reichert TE, & Bauer RJ (2009) P-cadherin induces an epithelial-like phenotype in oral squamous cell carcinoma by GSK-3beta-mediated Snail phosphorylation. *Carcinogenesis* 30(10):1781-1788.
89. Sarrio D*, et al.* (2009) Functional characterization of E- and P-cadherin in invasive breast cancer cells. *BMC Cancer* 9:74.

90. Van Marck V*, et al.* (2005) P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. *Cancer Res* 65(19):8774-8783.
91. Visvader JE (2011) Cells of origin in cancer. *Nature* 469(7330):314-322.
92. Mani SA*, et al.* (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133(4):704-715.
93. Morel AP*, et al.* (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* 3(8):e2888.
94. Ablett MP, Singh JK, & Clarke RB (2012) Stem cells in breast tumours: are they ready for the clinic? *Eur J Cancer* 48(14):2104-2116.
95. Braga V (2000) Epithelial cell shape: cadherins and small GTPases. *Exp Cell Res* 261(1):83-90.
96. Braga VM (1999) Small GTPases and regulation of cadherin dependent cell-cell adhesion. *Mol Pathol* 52(4):197-202.
97. Braga VM, Betson M, Li X, & Lamarche-Vane N (2000) Activation of the small GTPase Rac is sufficient to disrupt cadherin-dependent cell-cell adhesion in normal human keratinocytes. *Mol Biol Cell* 11(11):3703-3721.
98. Fukata M & Kaibuchi K (2001) Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nat Rev Mol Cell Biol* 2(12):887-897.
99. Kumper S & Ridley AJ (2010) p120ctn and P-cadherin but not E-cadherin regulate cell motility and invasion of DU145 prostate cancer cells. *PLoS One* 5(7):e11801.
100. Jacobs K*, et al.* (2010) P-cadherin counteracts myosin II-B function: implications in melanoma progression. *Mol Cancer* 9:255.
101. Jallal H*, et al.* (2007) A Src/Abl kinase inhibitor, SKI-606, blocks breast cancer invasion, growth, and metastasis in vitro and in vivo. *Cancer Res* 67(4):1580-1588.
102. Meniel V & Clarke AR (2003) Wnt-cadherin connections in normal and neoplastic mammary epithelium. *J Mammary Gland Biol Neoplasia* 8(4):435-447.
103. Nelson WJ & Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303(5663):1483-1487.
104. Behrens J*, et al.* (1996) Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382(6592):638-642.
105. Sato N*, et al.* (2003) Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res* 63(13):3735-3742.
106. Sato N*, et al.* (2003) Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res* 63(14):4158-4166.
107. Tsutsumida A*, et al.* (2004) Epigenetic silencing of E- and P-cadherin gene expression in human melanoma cell lines. *Int J Oncol* 25(5):1415-1421.
108. Milicic A*, et al.* (2008) Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo. *Cancer Res* 68(19):7760-7768.
109. Gorski JJ*, et al.* (2010) BRCA1 transcriptionally regulates genes associated with the basal-like phenotype in breast cancer. *Breast Cancer Res Treat* 122(3):721-731.
110. Faraldo MM*, et al.* (2007) beta-Catenin regulates P-cadherin expression in mammary basal epithelial cells. *FEBS Lett* 581(5):831-836.
111. Palacios J*, et al.* (2003) Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to BRCA1 or BRCA2 mutations: differences from breast carcinomas arising in BRCA1 and BRCA2 mutation carriers. *Clin Cancer Res* 9(10 Pt 1):3606-3614.
112. Palacios J*, et al.* (2005) Phenotypic characterization of BRCA1 and BRCA2 tumors based in a tissue microarray study with 37 immunohistochemical markers. *Breast Cancer Res Treat* 90(1):5-14.
113. Arnes JB*, et al.* (2005) Placental cadherin and the basal epithelial phenotype of BRCA1-related breast cancer. *Clin Cancer Res* 11(11):4003-4011.

114. Hager GL, McNally JG, & Misteli T (2009) Transcription dynamics. *Mol Cell* 35(6):741-753.
115. Thorne JL, Campbell MJ, & Turner BM (2009) Transcription factors, chromatin and cancer. *Int J Biochem Cell Biol* 41(1):164-175.
116. Zahnow CA (2009) CCAAT/enhancer-binding protein beta: its role in breast cancer and associations with receptor tyrosine kinases. *Expert Rev Mol Med* 11:e12.
117. Lekstrom-Himes J & Xanthopoulos KG (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J Biol Chem* 273(44):28545-28548.
118. Xiong W, Hsieh CC, Kurtz AJ, Rabek JP, & Papaconstantinou J (2001) Regulation of CCAAT/enhancer-binding protein-beta isoform synthesis by alternative translational initiation at multiple AUG start sites. *Nucleic Acids Res* 29(14):3087-3098.
119. Baldwin BR, Timchenko NA, & Zahnow CA (2004) Epidermal growth factor receptor stimulation activates the RNA binding protein CUG-BP1 and increases expression of C/EBPbeta-LIP in mammary epithelial cells. *Mol Cell Biol* 24(9):3682-3691.
120. Zahnow CA, Younes P, Laucirica R, & Rosen JM (1997) Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. *J Natl Cancer Inst* 89(24):1887-1891.
121. Gomis RR, Alarcon C, Nadal C, Van Poznak C, & Massague J (2006) C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* 10(3):203-214.
122. Eaton EM, Hanlon M, Bundy L, & Sealy L (2001) Characterization of C/EBPbeta isoforms in normal versus neoplastic mammary epithelial cells. *J Cell Physiol* 189(1):91-105.
123. Bundy LM & Sealy L (2003) CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. *Oncogene* 22(6):869-883.
124. Musgrove EA*, et al.* (2008) Identification of functional networks of estrogen- and c-Myc-responsive genes and their relationship to response to tamoxifen therapy in breast cancer. *PLoS One* 3(8):e2987.
125. Moumen M*, et al.* (2013) Myc is required for beta-catenin-mediated mammary stem cell amplification and tumorigenesis. *Mol Cancer* 12(1):132.
126. Hines MD, Jin HC, Wheelock MJ, & Jensen PJ (1999) Inhibition of cadherin function differentially affects markers of terminal differentiation in cultured human keratinocytes. *J Cell Sci* 112:4569-4579.
127. Hirai Y, Nose A, Kobayashi S, & Takeichi M (1989) Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. I. Lung epithelial morphogenesis. *Development* 105(2):263-270.
128. Westfall MD & Pietenpol JA (2004) p63: Molecular complexity in development and cancer. *Carcinogenesis* 25(6):857-864.
129. Mills AA (2006) p63: oncogene or tumor suppressor? *Curr Opin Genet Dev* 16(1):38-44.
130. Yamaguchi K*, et al.* (2000) Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma. *Int J Cancer* 86(5):684-689.
131. Uramoto H*, et al.* (2006) Expression of the p53 family in lung cancer. *Anticancer Res* 26(3A):1785-1790.
132. Takeuchi Y, Tamura A, Kamiya M, Fukuda T, & Ishikawa O (2005) Immunohistochemical analyses of p63 expression in cutaneous tumours. *Br J Dermatol* 153(6):1230-1232.
133. Quade BJ*, et al.* (2001) Expression of the p53 homologue p63 in early cervical neoplasia. *Gynecol Oncol* 80(1):24-29.
134. Lin Z*, et al.* (2006) DeltaNp63 protein expression in uterine cervical and endometrial cancers. *J Cancer Res Clin Oncol* 132(12):811-816.
135. Reis-Filho JS, Albergaria A, Milanezi F, Amendoeira I, & Schmitt FC (2002) Naked nuclei revisited: p63 Immunoexpression. *Diagn Cytopathol* 27(3):135-138.
136. Wang X*, et al.* (2002) p63 expression in normal, hyperplastic and malignant breast tissues. *Breast Cancer* 9(3):216-219.

137. Hibi K*, et al.* (2000) AIS is an oncogene amplified in squamous cell carcinoma. *Proc Natl Acad Sci U S A* 97(10):5462-5467.
138. Crook T, Nicholls JM, Brooks L, O'Nions J, & Allday MJ (2000) High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? *Oncogene* 19(30):3439-3444.
139. Senoo M*, et al.* (2001) Transcriptional dysregulation of the p73L / p63 / p51 / p40 / KET gene in human squamous cell carcinomas: expression of Delta Np73L, a novel dominant-negative isoform, and loss of expression of the potential tumour suppressor p51. *Br J Cancer* 84(9):1235-1241.
140. Sniezek JC, Matheny KE, Westfall MD, & Pietenpol JA (2004) Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. *Laryngoscope* 114(12):2063-2072.
141. Liefer KM*, et al.* (2000) Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res* 60(15):4016-4020.
142. Ratovitski EA*, et al.* (2001) p53 associates with and targets Delta Np63 into a protein degradation pathway. *Proc Natl Acad Sci U S A* 98(4):1817-1822.
143. Yang A*, et al.* (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2(3):305-316.
144. Buckley NE*, et al.* (2011) The DeltaNp63 proteins are key allies of BRCA1 in the prevention of basal-like breast cancer. *Cancer Res* 71(5):1933-1944.
145. Adorno M*, et al.* (2009) A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* 137(1):87-98.
146. Hu M*, et al.* (2008) Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell* 13(5):394-406.
147. Muller PA*, et al.* (2009) Mutant p53 drives invasion by promoting integrin recycling. *Cell* 139(7):1327-1341.
148. Patturajan M*, et al.* (2002) DeltaNp63 induces beta-catenin nuclear accumulation and signaling. *Cancer Cell* 1(4):369-379.
149. Melino G (2011) p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ* 18(9):1487-1499.
150. Ma DK, Bonaguidi MA, Ming GL, & Song H (2009) Adult neural stem cells in the mammalian central nervous system. *Cell Res* 19(6):672-682.
151. Gibelli B, El-Fattah A, Giugliano G, Proh M, & Grosso E (2009) Thyroid stem cells--danger or resource? *Acta otorhinolaryngologica Italica : organo ufficiale della Societa italiana di otorinolaringologia e chirurgia cervico-facciale* 29(6):290-295.
152. Wu X, Wang S, Chen B, & An X (2010) Muscle-derived stem cells: isolation, characterization, differentiation, and application in cell and gene therapy. *Cell Tissue Res* 340(3):549-567.
153. Snyder JC, Teisanu RM, & Stripp BR (2009) Endogenous lung stem cells and contribution to disease. *J Pathol* 217(2):254-264.
154. Little MH & Bertram JF (2009) Is there such a thing as a renal stem cell? *J Am Soc Nephrol* 20(10):2112-2117.
155. Pincelli C & Marconi A (2010) Keratinocyte stem cells: friends and foes. *J Cell Physiol* 225(2):310-315.
156. Katsumoto K, Shiraki N, Miki R, & Kume S (2010) Embryonic and adult stem cell systems in mammals: ontology and regulation. *Development, growth & differentiation* 52(1):115-129.
157. Petersen OW & Polyak K (2010) Stem cells in the human breast. *Cold Spring Harb Perspect Biol* 2(5):a003160.
158. Ratajczak MZ, Zuba-Surma EK, Machalinski B, & Kucia M (2007) Bone-marrow-derived stem cells--our key to longevity? *Journal of applied genetics* 48(4):307-319.

159. Beltrami AP*, et al.* (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114(6):763-776.
160. Tumbar T*, et al.* (2004) Defining the epithelial stem cell niche in skin. *Science* 303(5656):359-363.
161. Collins CA & Partridge TA (2005) Self-renewal of the adult skeletal muscle satellite cell. *Cell Cycle* 4(10):1338-1341.
162. Herrera MB*, et al.* (2006) Isolation and characterization of a stem cell population from adult human liver. *Stem Cells* 24(12):2840-2850.
163. Ambler CA & Maatta A (2009) Epidermal stem cells: location, potential and contribution to cancer. *J Pathol* 217(2):206-216.
164. LaMarca HL*, et al.* (2010) CCAAT/enhancer binding protein beta regulates stem cell activity and specifies luminal cell fate in the mammary gland. *Stem Cells* 28(3):535-544.
165. Koster MI, Kim S, Mills AA, DeMayo FJ, & Roop DR (2004) p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* 18(2):126-131.
166. Watt FM, Frye M, & Benitah SA (2008) MYC in mammalian epidermis: how can an oncogene stimulate differentiation? *Nat Rev Cancer* 8(3):234-242.
167. Reya T, Morrison SJ, Clarke MF, & Weissman IL (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414(6859):105-111.
168. Tan BT, Park CY, Ailles LE, & Weissman IL (2006) The cancer stem cell hypothesis: a work in progress. *Lab Invest* 86(12):1203-1207.
169. Schatton T, Frank NY, & Frank MH (2009) Identification and targeting of cancer stem cells. *Bioessays* 31(10):1038-1049.
170. Du Z*, et al.* (2010) Overexpression of DeltaNp63alpha induces a stem cell phenotype in MCF7 breast carcinoma cell line through the Notch pathway. *Cancer Sci* 101(11):2417-2424.
171. Mills AA*, et al.* (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398(6729):708-713.
172. Yang A*, et al.* (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398(6729):714-718.
173. Nylander K*, et al.* (2002) Differential expression of p63 isoforms in normal tissues and neoplastic cells. *J Pathol* 198(4):417-427.
174. Reis-Filho JS, Torio B, Albergaria A, & Schmitt FC (2002) p63 expression in normal skin and usual cutaneous carcinomas. *J Cutan Pathol* 29(9):517-523.
175. Watt FM (2001) Stem cell fate and patterning in mammalian epidermis. *Curr Opin Genet Dev* 11(4):410-417.
176. Lane DP (1992) Cancer. p53, guardian of the genome. *Nature* 358(6381):15-16.
177. Murray-Zmijewski F, Lane DP, & Bourdon JC (2006) p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ* 13(6):962-972.
178. Begus-Nahrmann Y*, et al.* (2009) p53 deletion impairs clearance of chromosomal-instable stem cells in aging telomere-dysfunctional mice. *Nat Genet* 41(10):1138-1143.
179. Chin L*, et al.* (1999) p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* 97(4):527-538.
180. Flores I & Blasco MA (2009) A p53-dependent response limits epidermal stem cell functionality and organismal size in mice with short telomeres. *PLoS One* 4(3):e4934.
181. Cicalese A*, et al.* (2009) The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* 138(6):1083-1095.
182. Bondar T & Medzhitov R (2010) p53-mediated hematopoietic stem and progenitor cell competition. *Cell Stem Cell* 6(4):309-322.
183. Milyavsky M*, et al.* (2010) A distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* 7(2):186-197.

184. Vivarelli S, Wagstaff L, & Piddini E (2012) Cell wars: regulation of cell survival and proliferation by cell competition. *Essays in biochemistry* 53:69-82.
185. de Beco S, Ziosi M, & Johnston LA (2012) New frontiers in cell competition. *Dev Dyn* 241(5):831-841.
186. Lo Celso C, Prowse DM, & Watt FM (2004) Transient activation of beta-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. *Development* 131(8):1787-1799.
187. Huelsken J, Vogel R, Erdmann B, Cotsarelis G, & Birchmeier W (2001) beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* 105(4):533-545.
188. Lowry WE*, et al.* (2005) Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. *Genes Dev* 19(13):1596-1611.
189. Honeycutt KA & Roop DR (2004) c-Myc and epidermal stem cell fate determination. *J Dermatol* 31(5):368-375.
190. Arnold I & Watt FM (2001) c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. *Curr Biol* 11(8):558-568.
191. Frye M, Gardner C, Li ER, Arnold I, & Watt FM (2003) Evidence that Myc activation depletes the epidermal stem cell compartment by modulating adhesive interactions with the local microenvironment. *Development* 130(12):2793-2808.
192. Koster MI, Huntzinger KA, & Roop DR (2002) Epidermal differentiation: transgenic/knockout mouse models reveal genes involved in stem cell fate decisions and commitment to differentiation. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* 7(1):41-45.
193. Waikel RL, Kawachi Y, Waikel PA, Wang XJ, & Roop DR (2001) Deregulated expression of c-Myc depletes epidermal stem cells. *Nat Genet* 28(2):165-168.
194. Moumen M*, et al.* (2012) The proto-oncogene Myc is essential for mammary stem cell function. *Stem Cells* 30(6):1246-1254.
195. Chen K & Rajewsky N (2007) The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet* 8(2):93-103.
196. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136(2):215-233.
197. Lagos-Quintana M*, et al.* (2002) Identification of tissue-specific microRNAs from mouse. *Curr Biol* 12(9):735-739.
198. Mraz M & Pospisilova S (2012) MicroRNAs in chronic lymphocytic leukemia: from causality to associations and back. *Expert review of hematology* 5(6):579-581.
199. He L*, et al.* (2005) A microRNA polycistron as a potential human oncogene. *Nature* 435(7043):828-833.
200. Lu J*, et al.* (2005) MicroRNA expression profiles classify human cancers. *Nature* 435(7043):834-838.
201. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, & Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435(7043):839-843.
202. Larsson C, Grundberg I, Soderberg O, & Nilsson M (2010) In situ detection and genotyping of individual mRNA molecules. *Nat Methods* 7(5):395-397.