2013

Sofia de Almeida Santos de Castro e Abreu Bacterial protein azurin as a new candidate anticancer drug by decreasing cell adhesion through integrins

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“Science is never finished. It proceeds by successive approximations, edging closer and closer to a complete and accurate understanding of nature, but it is never fully there.”

Carl Sagan

“The scientist is not a person who gives the right answers; he's one who asks the right questions.”

Claude Lévi-Strauss

Acknowledgments

I would like to express my gratitude to my supervisor, Professor Arsénio Fialho (IST), who gave me this opportunity and orientated me in my Master's Thesis.

I want to thank Professor Carmen Alpoim to be my tutor at Departamento de Ciências da Vida (FCTUC).

I also want to thank "chefinho" Nuno Bernardes to guide me and teach me all the methods at the laboratory.

In addition, thanks to all BSRG for the help, the animated lunches, companionship during computer work and all the birthday chocolates cakes, in particular to 6.6.6 lab (Dalila, Gonçalo, Maria Inês, Sofia, Inês e Fátima).

Finally, I am thankful to Rodrigo for all the psychological support; and all my family and friends who are always there.

Thank you all. Obrigado.

Abstract

Azurin is a 14 kDa protein produced by *Pseudomonas aeruginosa* which has cytotoxicity activity towards human cancer cell lines. Azurin can enter preferentially into cancer cells, forming a complex with the tumour suppressor p53, stabilizing it and inducing apoptosis. The capacity of migration and invasion is due, in part, to the regulation of adhesion proteins, like P-cadherin. P-cadherin is over-expressed in 30% of breast cancers and it is a marker of poor survival. Therefore, P-cadherin is a potential therapeutic target in breast cancer. For that reason, azurin was used to target P-cadherin, decreasing its level in P-cadherin over-expressing breast cancer models and membranar localisation. However, the mechanism of action of azurin is not well known. On the other hand, lung cancer has similar signalling pathways associated with adhesion, and therefore, this work also focuses on the impact of azurin in lung cancer. Not much is known about how the stromal microenvironment at metastasis sites provides a suitable home to tumour cells. It is important to study the interaction between metastatic cells and ECM.

We treated different cancer cells models with azurin (50 μM and 100 μM): four breast cancer cell lines with distinct levels of P-cadherin expression and different invasive capacities (MCF-7/AZ.Mock, MCF-7/AZ.Pcad, SUM149 and BT-20) and one non-small cell lung cancer cell line (A549). We investigated the effect of azurin in cell adhesion with different ECM components (laminin-332, collagen type-I, fibronectin and collagen type-IV) and we also investigated integrin subunits (α_6, β_1 and β_4) expression by western blot. The azurin effects were also evaluated by others parameters, such as ROS measurement, immunocytochemistry, gelatine zymography to evaluate MMP-2 activity and invasion capacity.

Azurin decreased integrin subunits (α_6, β_1 and β_4) in all studied models (breast and lung cancer), more consistently in the integrin subunit β_1. Moreover, azurin decreased adhesion to ECM components, with more significance in collagen and laminin (breast cancer) and fibronectin (lung cancer), both main components in each cancer type. In BT-20 and A549, azurin decreased MMP-2 activity and the invasion through Matrigel™. All these results corroborate azurin as potential cancer therapeutic drug.

Funded project:

Bacterial protein azurin as a new candidate drug to treat poor-prognosis breast cancer, PTDC/EBB-BIO/100326/2008, PI: Arsénio M. Fialho

Collaboration:

This project is being pursued in collaboration with Dr. Joana Paredes and Dr. Raquel Seruca, IPATIMUP, Portugal.

Key-words: Adhesion, Azurin, Breast cancer, Integrin, Lung cancer, P-cadherin.

Resumo

A azurina é uma proteína de 14 kDa produzida por *Pseudomonas aeruginosa*, com actividade citotóxica em linhas celulares cancerígenas humanas. A azurina entra preferencialmente em células cancerígenas, formando um complexo com a proteína supressora tumoral p53, estabilizando-a e induzindo a apoptose. A capacidade de migração e invasão deve-se, em parte, à regulação de proteínas de adesão celular, como a P-caderina. A P-caderina é sobre-expressa em 30% dos cancros de mama e é um marcador de mau prognóstico. Por isso, a P-caderina é um potencial alvo terapêutico em cancro de mama. Por essa razão, a capacidade da azurina diminuir esta proteína em modelos de cancro de mama que sobre-expressam P-caderina foi testada, verificando-se que a azurina diminui a sua expressão proteica e localização membranar. No entanto, o mecanismo de acção da azurina não é ainda bem conhecido. Por outro lado, o cancro do pulmão tem vias de sinalização semelhantes associadas à adesão celular, e por isso, este trabalho também se foca no impacto da azurina no cancro de pulmão. Não se sabe muito sobre como o microambiente estromal em locais de metástase fornece um ambiente adequado para as células tumorais. É por isso importante estudar a interação entre células metastáticas e o meio extracelular.

Diferentes linhas celulares de cancro de mama com níveis distintos de expressão de P-caderina e diferentes capacidades invasivas (MCF-7/AZ.Mock, MCF-7/AZ.Pcad, SUM149, BT-20) e de cancro do pulmão (A549) foram tratadas com azurina (50μM e 100μM). O efeito de azurina na adesão celular com diferentes componentes do meio extracelular (laminina-332, colagénio do tipo-I, fibronectina e colagénio do tipo-IV) foi investigado, bem como a expressão de subunidades de integrina (α_6, β_1 e β_4) por western blot. Os efeitos da azurina também foram avaliados por outros parâmetros, tais como a medição de ROS, imunocitoquímica, zimografia de gelatina de modo a avaliar a actividade da MMP-2 e a capacidade de invasão.

A azurina diminui a expressão das subunidades de integrina (α_6, β_1 e β_4) em todos os modelos estudados (cancro de mama e de pulmão), de forma mais consistente na subunidade de integrina β_1. Além disso, a azurina diminui a adesão celular a componentes da matriz extracelular, principalmente em colagénio e laminina (cancro da mama) e fibronectina (cancro de pulmão), os componentes principais de cada tipo de cancro. Em BT-20 e A549, a azurina diminui a actividade da MMP-2 e, consequentemente, a capacidade de invasão. Estes resultados confirmam a azurina como um potencial fármaco terapêutico no cancro.

Projecto financiado:

Bacterial protein azurin as a new candidate drug to treat poor-prognosis breast cancer, PTDC/EBB-BIO/100326/2008, PI: Arsénio M. Fialho

Colaboração:

Este projeto está a ser executado em colaboração com Dr. Joana Paredes e Dr. Raquel Seruca, IPATIMUP, Portugal.

Palavras-chave: Adesão, Azurina, Cancro de mama, Cancro de pulmão, Integrina, P-caderina.

Index

Abbreviations

Activator protein (AP)
Adherent junction (AJ)
Atomic force microscopy (AFM)
B-cell lymphoma 2 (Bcl-2)
Cadherin repeat (EC)
Carcinoma-associated fibroblast (CAF)
Cell division control protein 42 homolog (Cdc42)
C-terminal regulatory domain (CTD)
C-terminal Src kinase (Csk)
Cyclin-A2 (*CCNA2*)
Cyclin-dependent kinase 2 (CDK2)
Deoxyribonucleic acid (DNA)
DNA-binding domain (DBD)
Epidermal growth factor (EGF)
Epidermal growth factor receptor (EGFR)
Extracellular matrix (ECM)
Extracellular-signal-regulated kinase (ERK)
Fas-associated phosphatase (FAP)
Fibroblast growth factor receptor (FGFR)
Focal adhesion (FA)
Focal adhesion kinase (FAK)
Hepatocyte growth factor (HGF)
Receptor tyrosine kinase en code by *ERBB2* gene (ERBB2)
Heat-inactivated fetal bovine serum (FBS)
Human epidermal growth factor receptor (HER)
Immunoglobulin (Ig)
Interleukin (IL)
Mitogen-activated protein (MAP)
Mitogen-activated protein kinase (MAPK)
Matrix metalloproteinase (MMP)
Mouse double minute 2 homolog (MDM2) also known as E3 ubiquitin-protein ligase
N-terminal domain (NTD)
Nicotinamide adenine dinucleotide phosphate (NADPH)
Non-small-cell lung cancer (NSCLC)
Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)
Oestrogen receptor (ER)
p53 up-regulated modulator of apoptosis (PUMA)
PEA3 site of MMP promoter (PEA3)

Phosphatidylinositol 3’-kinase (PI3K)

Proto-oncogene tyrosine-protein kinase (Src)

Proto-oncogene serine/threonine-protein kinase (Raf-1)

Pseudomonas aeruginosa (*P. aeruginosa*)

Ras-related C3 botulinum toxin substrate 1 (Rac1)

Ras-related protein 1 (Rap1)

Ras small GTPase family (Ras)

Reactive oxygen species (ROS)

Receptor tyrosine kinase (RTK)

Rho family of GTPases (Rho)

Tissue inhibitor of metalloproteinase (TIMP)

Tumour-associated macrophage (TAM)

Tumour necrosis factor (TNF)

Tyrosine protein kinase 5 (Fyn)

Vascular endothelial growth factor (VEGF)

List of figures

List of tables

This scientific work results in two articles:

Bernardes, N., Ribeiro, A. S., Abreu, S., Mota, B., Matos, R. G., Arraiano, C. M., Seruca, R., Paredes, J., Fialho, A. M. (2013). The bacterial protein azurin impairs invasion and FAK / Src signaling in P-cadherin over-expressing breast cancer cell models. *PlosOne*, *8*(7), 1–23.

Bernardes, N., Ribeiro, A. S., Abreu, S., Vieira, A. F., Carreto, L., Santos, M., Seruca, R., Paredes, J., Fialho, A. M. (2013). High-throughput molecular profiling of a over-expressing breast cancer model reveals new targets for the anti-cancer bacterial protein azurin. *submitted*, 1–43.

1. Azurin and cancer

1.1. Bacterial protein azurin

In 1890, William B. Coley described for the first time bacteria as anticancer agents. Not only live bacteria have applications in cancer therapies, but also bacteria-derived products (Bernardes *et al.*, 2010). *Pseudomonas aeruginosa* (*P. aeruginosa*) produces a potent virulence factor, called exotoxin A, that is a promising anticancer agent (Wolf & Elsässer-Beile, 2009). *P. aeruginosa* also produces at least two more cytotoxic proteins against cancer cells: cytochrome c_{551} and azurin (Figure 1) (Bernardes *et al.*, 2010).

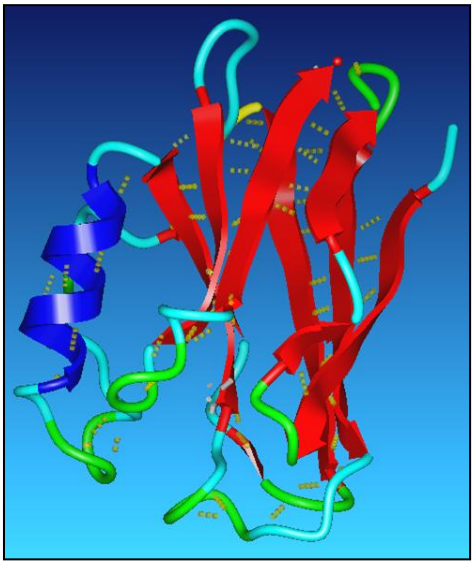

Figure 1: Three dimensions structure of azurin from *P. aeruginosa* (PDB_1JZG)

Azurin is a water soluble, low molecular weight copper-containing redox protein. The presence of a copper ion in the polypeptide chain contributes to azurin stability (Ramachandran *et al.*, 2011). This molecule, with 128 amino acids and 14 kDa, is involved in the electron transport chain (Bernardes *et al.*, 2010), during denitrification by *P. aeruginosa*. Azurin has very different targets and is designated as “anticapavi” agent (Fialho *et al.*, 2007), since other human diseases can be targeted by this protein.

Laz, a modified form of azurin, was characterized from *gonococci* and *meningococci*, such as *Neisseria meningitidis*, which can cause meningitis, an inflammation of the brain meninges. This surface-exposed Neisserial azurin has an extra epitope (39 amino acids) in the N-terminal called H.8 (Hong *et al.*, 2006; Yamada *et al.*, 2005). This lipidated epitope is responsible for entry in glioblastoma cells by penetrating blood-brain barrier, an ability that azurin does not possess (Hong *et al.*, 2006). H.8 epitope is important for the surface display of Laz but not to its cytotoxic capacity, suggesting an important role in disrupting entry barriers to glioblastoma cells in brain tumour (Fialho, Gupta, & Chakrabarty, 2008). Azurin and Laz interfere with the growth of parasite *Plasmodium falciparum*, in entry of HIV-1 virus in human cells and breast cancer

cells; and exert cytotoxicity in human cancer cells (Fialho *et al.*, 2007). The summary of mechanisms of action of azurin and Laz (induction of cell death, prevention of adhesion and invasion and growth suppression) is explained in Figure 2.

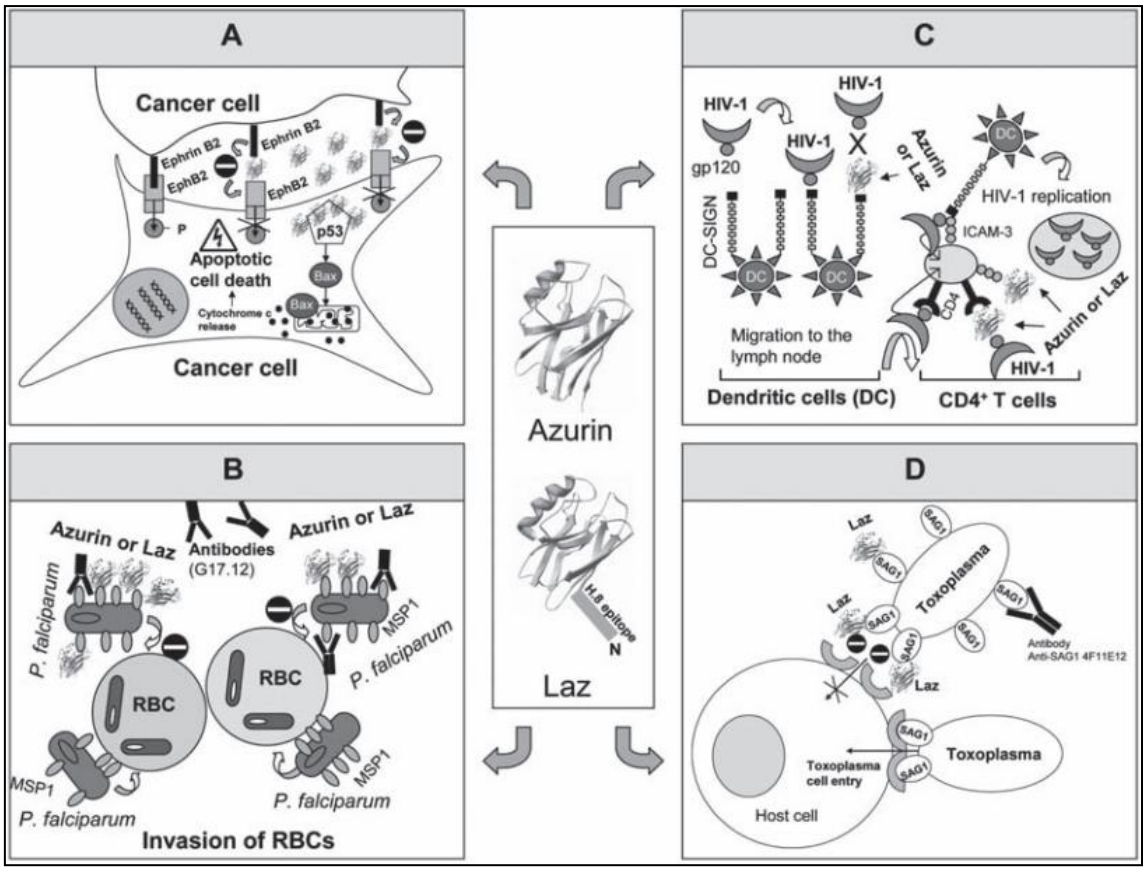

Figure 2: Azurin and Laz as novel drug candidates effective against cancers and infectious agents, such as HIV-1 virus, *P*. *falciparum* and *T*. *gondii* parasites. The mode of action in the induction of cell death, prevention of adhesion and invasion, and growth suppression. (Fialho *et al.*, 2008).

1.2. Azurin secretion by *P. aeruginosa* and entry in host cells

Azurin is secreted, out of the periplasmic space of *P. aeruginosa*, to the outside medium in an energy-independent manner, when *P. aeruginosa* cells are exposed to human cancer cells, but less when exposed to normal cells (Mahfouz *et al.*, 2007). Also, azurin enters preferentially in human cancer cells compared to normal cells. The preferential entry of azurin in cancer cells is mediated by the amino acids 50-77 of the protein, termed p28, which is the protein transport domain of azurin. p28 forms an extended amphipathic α-helix with both a hydrophobic amino acids (50-66) and hydrophilic amino acids (67-77) (Yamada *et al.*, 2005). The protein transduction domain p28 was further refined, by reducing the N-terminal to amino acids 50-67, called p18. p18 is the minimal fragment responsible for the preferential entry of azurin into

human cancer cells. The authors showed that the entry of p28 and p18 occurs mainly via a non-endocytic and without loss of membrane integrity (Taylor *et al.*, 2009).

Recently, the start-up company CDG Therapeutics has terminated phase I human clinical trials of p28 for its anticancer activity (www.clinicaltrials.gov, NCT00914914). This trial comprised 15 advanced-stage cancer patients with metastatic and solid tumours (7 melanoma, 4 colon, 2 sarcoma, 1 pancreatic, and 1 prostate) in patients where the tumours were no longer responding to conventional drugs (radiation therapy and temozolomide). When p28 was given intravenously, seven patients demonstrated stable disease, three patients showed partial regression, one complete response. Very little toxicity was seen even with the highest concentration of p28, and three surviving patients has been living disease-free for over 110, 140 and 158 weeks (Warso *et al.*, 2013).

1.3. Azurin action toward cancer cells

Two main effects of azurin's action to cancer cells have been described so far: one acts through p53/Bax and the other by the Eph receptors family. Azurin binds to the intracellular tumour suppressor p53, stabilizing it and leads to increased expression of pro-apoptotic protein Bax and Bax-dependent apoptosis in cancer cells (Figure 3) (Apiyo & Wittung-Stafshede, 2005; Yamada *et al.*, 2002a; 2004). It also binds to several Eph receptor tyrosine kinases (RTKs), a family of extracellular receptor proteins known to be up-regulated in many tumours; and this binding with EphB2 interferes in its phosphorylation at the tyrosine residue, resulting in inhibition of cell signalling and cancer growth (Figure 4) (Chaudhari *et al.*, 2007).

1.3.1. Azurin and p53 interaction

p53 is a major tumour suppressor protein that is mutated or inactivated in 50% of human cancers (Gabellieri *et al.*, 2011). The first evidence of azurin-p53 interaction came from glycerol gradient and column binding (Punj *et al.*, 2003). Later, isothermal titration calorimetry demonstrated that four azurin molecules bind per one monomer of p53 with a dissociation constant of 33±12 nM, presumably in the p53 N-terminal domain (NTD) (Apiyo & Wittung-Stafshede, 2005).

Azurin enters in cancer cells and form a complex with the tumour suppressor protein p53, thereby stabilizing this normally labile protein and enhancing its intracellular concentration (Fialho *et al.*, 2008). This stabilization of p53 was thought to be due to the binding of azurin close to the MDM2-binding site; consequently, MDM2 could not promote p53 ubiquitination. However, amino acids 19-26 of p53, the MDM2-binding site, were not detected as a preferred binding site for azurin, and, as a result of this, prevention of p53 degradation is through to occur through a MDM2-independent pathway (Yamada *et al.*, 2009). Azurin interacts with p53 at the level of the trans-activation domain, more exactly with amino acids 1-63 of p53 (Gabellieri *et al.*, 2011). Using p18 (amino acids 50-67), p18b (amino acids 60-77) and p12 (amino acids 66-77),

it was proved that the maximal binding of p53 occurs within amino acids 60-67 of azurin (Yamada *et al.*, 2009). However, this is a controversial issue and there are others opinions and suggestions to the binding site of azurin in p53 (Gabellieri *et al.*, 2011).

Some studies suggest that the complex formation with p53 and generation of reactive oxygen species (ROS), rather than azurin redox activity, were important in the cytotoxicity action of azurin (Fialho *et al.*, 2008). p53 is not only able to up-regulate pro-apoptotic genes such as *Bax*, *caspase-9*, and *PUMA* but also to repress some anti-apoptotic genes like *B-cell lymphoma 2 (Bcl-2)*. A ratio of Bax to Bcl-2 is important to the fate of the cell in response to death stimuli. The average ratio of Bax:Bcl-2 in breast cells treated with azurin was higher than in the untreated cells, meaning that azurin increases Bax and decreases Bcl-2 expression, activating the caspase-9, which in turn actives caspase-7, and consequently induce apoptosis (Figure 3) (Punj *et al.*, 2004). So, azurin applies part of its anticancer activity through induction of p53-mediated apoptosis (Yamada *et al.*, 2009). Moreover, the increase of p53 level also mediates various cellular responses including DNA damage (Ramachandran *et al.*, 2011) and G_2-M-arrest cells. This last event is triggered by the increase of p21 and p27, which in turn inactivates the CDK2-Cyclin A complex, causing the cell cycle arrest (Yamada *et al.*, 2009).

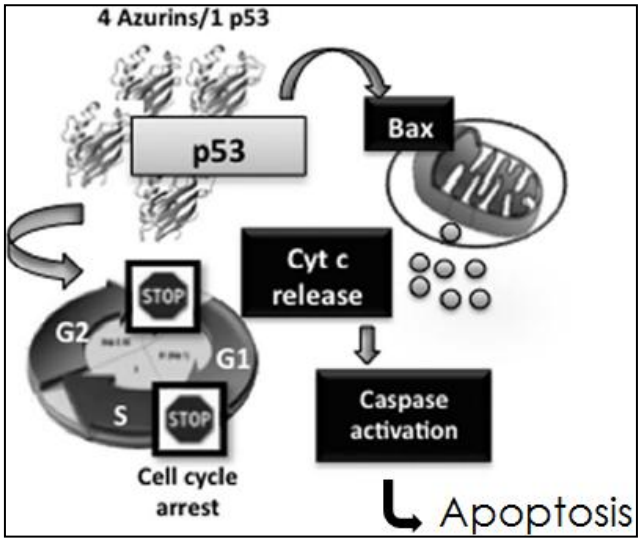

Figure 3: The mode of action of azurin mediated by p53 protein in the induction of apoptosis in breast cancer cells (Adapted from Bernardes *et al.*, 2010)

In breast cancer cells, it led to statistically significant regression of the tumours, without any apparent toxicity to normal cells, suggesting potential application of azurin in cancer therapy (Vasu Punj *et al.*, 2004; Yamada *et al.*, 2002a).

While wild-type azurin bound to the N-terminal of p53, a mutant azurin (M44KM64E azurin, where two hydrophobic amino acids were replaced by two polar amino acids within the hydrophobic patch) formed a different complex with p53, affecting p53's oligomerization. The wild-type azurin induces apoptosis but little inhibition of cell cycle progression in J774 while the M44KM64E mutant causes the reverse effect (Yamada *et al.*, 2004), demonstrating how azurin,

based on its hydrophobicity, modulates the nature of p53 complex formation and its transcriptional specificity in mammalian cells (Fialho *et al.*, 2008).

In azurin, amino acids Met-44 and Met-64, located in a hydrophobic patch of the protein, is important for the interactions with p53 and their substitutions resulted in altered complex formation with p53 (Yamada *et al.*, 2004; 2002b). But only Met-64 is present in the p53-binding site of p28 (Yamada *et al.*, 2009). By atomic force microscopy (AFM), p53 was immobilized in a gold substrate and azurin was tethered in the AFM tip. The results confirmed the interaction between both proteins with an estimated dissociation constant of 6 μM, lower than that estimated before. The complex forms between azurin and p53 DNA-binding domain (DBD) was investigated. p53 DBD acquires a β-sandwich fold, formed by two antiparallel β-sheets (S_1 and S_{11}), with 4 and 5 strands, respectively (s_1,s_2,s_3,s_8 and s_4,s_6,s_7,s_9,s_{10}). This structure is a scaffold for two large loops, L_2 and L_3, and a loop-sheet-helix (L_I-S_{III}-H_2). Using Zdock docking program, and appealing to some characteristics, the best model of azurin has been chose. It involves L_1 loop (maybe the most flexible region capable of a strong structural adaptation) and strands s_7 and s_8 in the p53 DBD binding interface, and the residues in the hydrophobic patch of azurin. This flexibility of L_1 loop can be one of the reasons for the azurin-induced stability of p53 (Chen & Weng, 2002; De Grandis *et al.*, 2007; Taranta *et al.*, 2009).

The NTD and C-terminal regulatory domain (CTD) are predominantly unstructured under normal conditions. The NTD is organized in an α-helix, H_I, and two turns assuming another α-helix structure, H_{II} and H_{III}, linked by a fragment of 30-32 residues. Using Zdock docking program, and appealing to some characteristics, the best model of azurin has been chose. It involves the helices H_{II} and H_{III} of p53, that have a strong adaptation to the azurin shape, increasing packing between both proteins, through numerous and favourable Van der Waals interactions. Comparing the two best models (DBD p53-azurin and NTD p53-azurin), the latter has the best result (lower free energy). For a more detailed review see (Bernardes *et al.*, 2010).

1.3.2. Azurin and Eph receptor interaction

Eph RTKs, like EphB2, are the family of 14 extracellular receptors which bind to ephrins, like ephrinB2, known to initiate cell signalling leading to cancer growth (Fialho *et al.*, 2008). Eph-ephrin interaction induces a series of cellular signalling processes, like proliferation, migration, invasion and angiogenesis. Some Eph receptors and ephrin ligands are up-regulated in some tumours, like the case of EphB2 which is up-regulated in glioblastoma, hepatocellular carcinoma, gastrointestinal and renal carcinomas, and prostate, lung and ovarian cancers. Azurin has structural similarities with the ephrinB2 ectodomain and has high values of interaction with receptor EphB2 and also EphA6, EphA4 and EphA7. In particular, the fragment amino acids 88-113 of azurin, coincident with the G-H loop of ephrinB2-Fc, had a high affinity for binding with EphB2 (12 nM), leading to the highest inhibition of cancer growth. This suggests that azurin interferes with the EphB2-ephrinB2 binding. Consequently, this interaction could be

a way of blocking the signalling process, antagonizing the Eph-ephrin mediated tumour progression (Figure 4) (Chaudhari *et al.*, 2007).

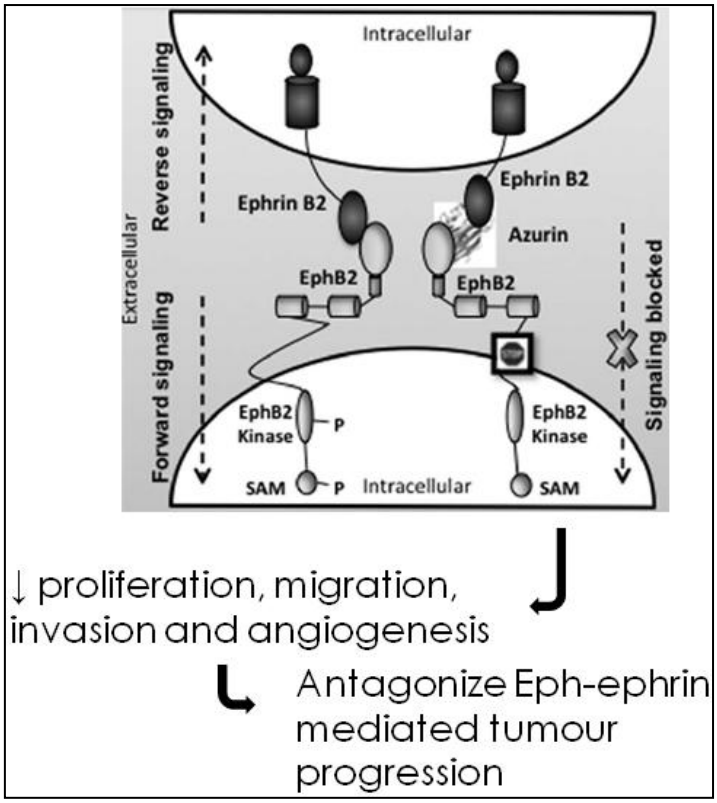

Figure 4: The mode of action of azurin in the induction of growth inhibition in breast cancer cells (Adapted from Bernardes *et al.*, 2010)

1.4. Azurin as a potential breast cancer therapeutic drug

Azurin and p28 are potential breast cancer therapeutics. There are many reasons that support this idea. Firstly, both enter preferentially in cancer cells, rather than normal cells. Secondly, the four exposed loop regions are believed to be involved in its bindings with other proteins. Consequently, the most interesting characteristic of azurin is its ability to bind various unrelated mammalian proteins relevant in cancer, conferring on it the property of a natural scaffold protein. This also allows the blockage of different signalling cascades that promote cancer cell growth, survival and/or invasion. Thirdly, bacterial protein azurin is inexpensive to produce (Bernardes *et al.*, 2010), because it can be easily hyper-expressed in *E. coli* (Fialho *et al.*, 2008). Fourthly, because azurin is able to bind to multi-target, it is hard to acquire resistance. Fifthly, *in vitro* and *in vivo* assays reveal that azurin induces little side effects (Bernardes *et al.*, 2010). Sixthly, azurin has a hydrophobic patch and is water soluble which should help in its tissue penetration and clearance from the blood stream. Finally, as a bacterial protein, azurin could be susceptible to immune attack, but preliminary evidence indicates that azurin has low immunogenicity, due to the fact that azurin, a scaffold protein, is a non-antibody recognized protein (Fialho *et al.*, 2008).

The ability of a single bacterial protein, azurin, to interfere in the growth of cancer, is an interesting example of a potential drug candidate that can target multiple unrelated targets, interfering in multiple steps in the disease progression. Also, the ability of p28 to act as a vehicle to carry cargo proteins inside cancer cells and the ability of azurin to bind many different proteins, due to its unique structure features, makes azurin a potentially important natural scaffold protein for therapeutic purposes (Bernardes *et al.*, 2010).

2. Tumour microenvironment, cancer cell adhesion and invasion

2.1. Normal mammary structure

2.1.1. Mammary epithelium

The mammary gland is a complex interactive network of cells that leads to a proper development and functioning. All the interactions with the microenvironment can influence and modify the proliferation, survival, polarity, differentiation and invasive capacity of mammary epithelial cells (Polyak & Kalluri, 2010). Normal mammary gland contains epithelium and stroma. Mammary epithelium is composed by an inner layer of luminal epithelial cells and outer layer of myoepithelial cells (Muschler & Streuli, 2010), which is in contact with the basement membrane, a physical barrier separating the epithelial and the stromal compartments (Polyak & Kalluri, 2010).

Mammary epithelium also is source of stem and progenitor cells. There are two models that explain the heterogeneity in tumour: “cancer stem cell” model and “clonal evolution” model. The first one defends that accumulation of random mutations in stem cells leads to cancer stem cells that in turn leads to tumour progression and recurrence. The second one defends that any cells (differentiated or undifferentiated) can accumulate mutations leading to tumour formation. Breast cancer is enriched in undifferentiated cancer cells that are more aggressive and metastatic. Integrin subunits α_6 (also known by CD49f) and integrin subunits β_1 (also known by CD29) are highly expressed in normal stem cells (and low level of CD24). Altogether, those markers are able to regenerate a mammary gland. The integrin patterns of cancer stem cells is low CD24, high CD29 and low CD61; and cancer progenitor cells is high CD24, low CD29 and high CD61. Knowing that, it is important to develop inhibitor of specific integrins, allowing to impair self-renewal and differentiation of cancer stem cells (Pontier & Muller, 2009) (Figure 5).

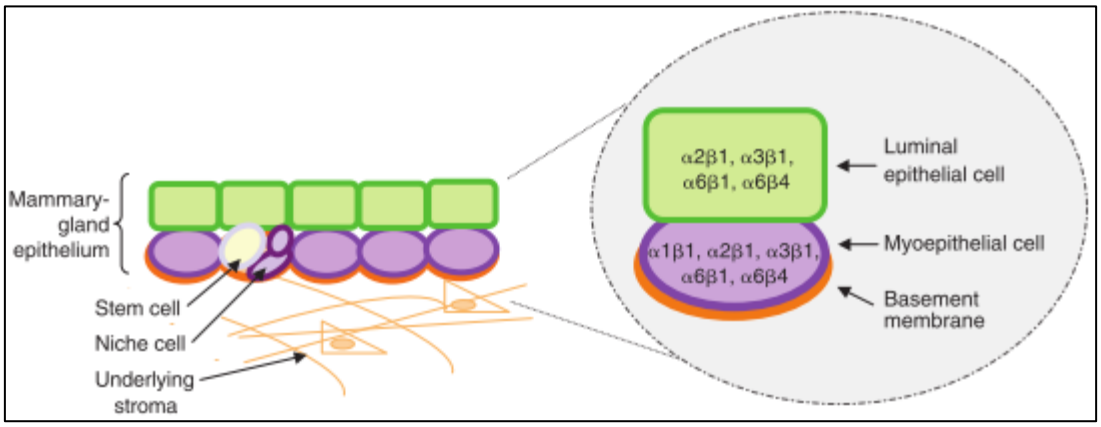

Figure 5: Schematic representation of the structure of the mammary epithelium and the different integrin heterodimers expressed in luminal epithelial cells and myoepithelial cells (Pontier & Muller, 2009)

2.1.2. Stroma

Stroma is composed by fibrous connective tissues (Muschler & Streuli, 2010), ECM components (collagen type-I, -III and -IV, fibronectin, laminin, proteoglycans) and a variety of cell types like inflammatory/immune cells, endothelial cells and fibroblasts (Gangadhara *et al.*, 2012); it provides nutrients, blood supply and immune defences (Muschler & Streuli, 2010). The ECM is a thick sheet of glycoproteins and proteoglycans, with laminin around (like laminin-111, -322, -511 and -521) and some cross-linked with collagen type-IV fibrils (Guo & Giancotti, 2004). Stromal changes may take place first leading to transformation of epithelial cells (invasion of stroma) or transformed epithelia may activate stromal cells in a paracrine way (Gangadhara *et al.*, 2012).

2.1.3. Basement membrane

Basement membrane, beyond myoepithelial cells, is in contact with endothelium of the vasculature and adipocytes. Basement membrane interacts with mammary epithelial cells through integrins, like receptors for collagen ($\alpha_1\beta_1$ and $\alpha_2\beta_1$), laminin-111, -511, -521 ($\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$), laminin-322 ($\alpha_3\beta_1$ and $\alpha_6\beta_4$), fibronectin ($\alpha_4\beta_1$, $\alpha_5\beta_1$ and β_3) and vitronectin ($\alpha_5\beta_1$ and β_3) (Muschler & Streuli, 2010). Cancer cells ted to loss integrins that adhere to the basement membrane and maintain or over-express integrins that promote survival, migration, proliferations, invasion and metastasis (Guo & Giancotti, 2004).

The luminal epithelial cells have sialomusin, epithelial specific antigen and occluding in the apical membrane; and integrin subunit β_4 on the basolateral membrane. This apical-basal polarity is observed in luminal epithelial cells grown in Matrigel^{TM} but not in collagen type-I (Gudjonsson *et al.*, 2002). However, co-culture with normal myoepithelial cells restores luminal epithelial cell polarity even in collagen cultures, in part mediated by laminin-1 secreted by the myoepithelial cells. So, cells grown in three-dimension environment can produce unique components that sometimes mimic the *in vivo* conditions (Polyak & Kalluri, 2010). Signalling for epithelial polarity is one of the basement membrane's role in tumour suppression, so it gatekeeper function is determinant of cancer progression (Muschler & Streuli, 2010).

2.2. The importance of cancer cell invasion in cancer progression

Invasion is a hallmark of malignant cancer cells. In order to invade, cancer cells must first disrupt pre-existing adhesion to other cells, dynamically reorganize their interactions with ECM, up-regulate matrix metalloproteinases (MMPs) and alter their cytoskeleton organization enhancing their motility (Guarino, 2010). Cancer cells use the same genetic programs, mediated by the same transcription factors, as healthy cells do, which become activated at the wrong time (Leber & Efferth, 2009). Also, with aging, the stroma/cell microenvironment changes progressively, accumulating enough damage to cause epithelial cells deregulations even in the

absence of genetic damage. In breast, the myriad of genetic changes cause tumour development, often remaining as ductal carcinoma *in situ*. Cells become invasive when gain the capacity to compromise the integrity of the basement membrane or the myoepithelial layer, allowing the luminal cells to contact with the stromal ECM components, such as collagen type-I. This new environmental signals lead to aberrant polarity, up-regulation of MMPs, invasion and metastasis (Bissell *et al.*, 2011) (Figure 6).

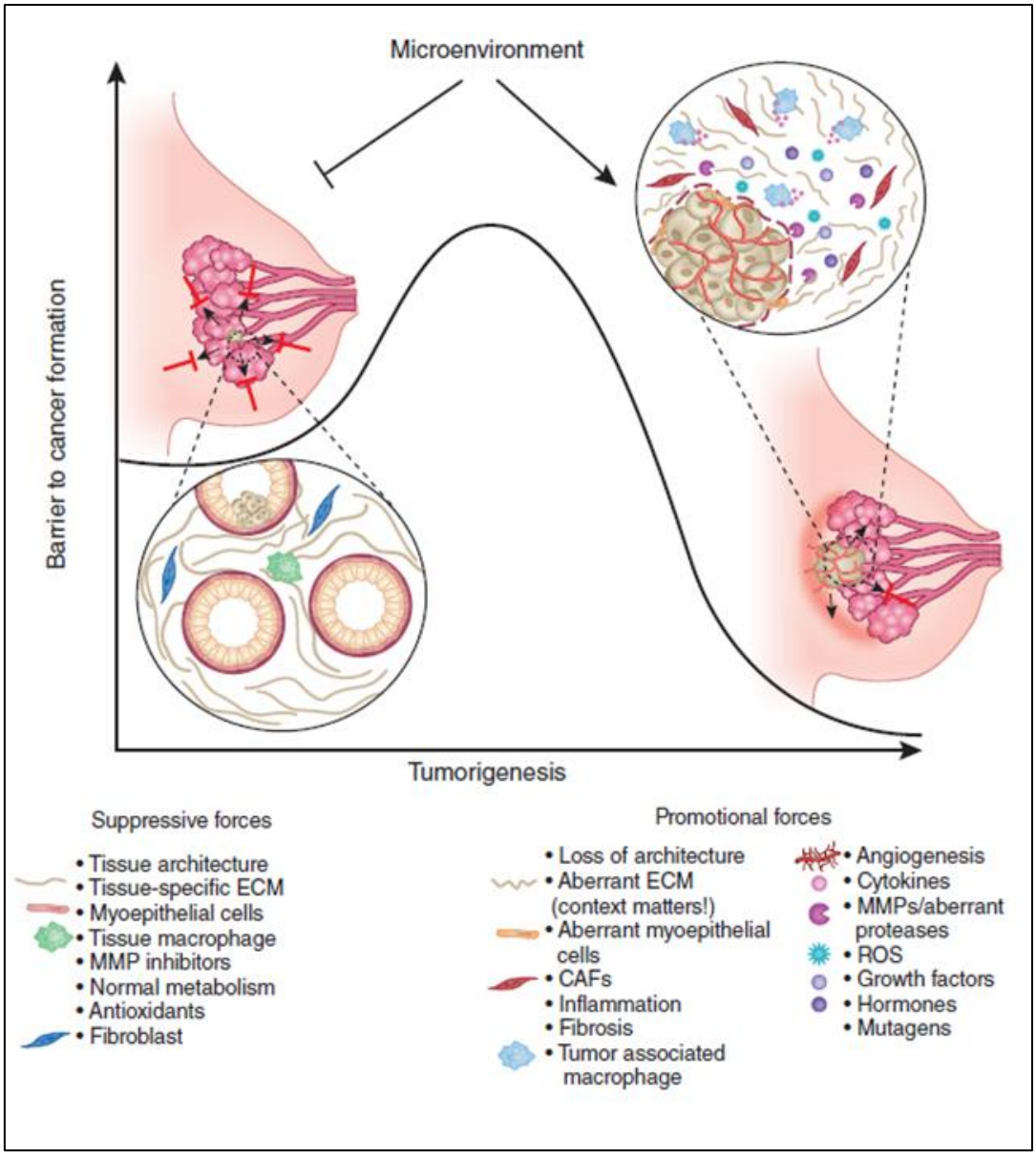

Figure 6: Normal mammary microenvironment to breast tumour microenvironment. The normal tissue microenvironment acts as a barrier to tumourigenesis in normal tissue homeostasis conditions, exerting suppressive forces to prevent tumourigenesis (bottom left in graph). But the microenvironment can also be permissive to tumour growth. The combination of mutagens, inflammation, growth factors and other tissue-associated promotional forces can breach the barrier, allowing tumour formation which may result in cancer development (top right). Adapted from Bissell *et al.*, 2011.

2.3. Invasive breast cancer through ECM remodelling

Human mammary epithelial cells *in vivo* express several integrins; however they are altered in the majority of human breast carcinomas, suggesting that deregulation of integrin expression may be an important parameter in breast tumourigenesis. A study reveals that deregulation of integrins in MDA-MB-435 is more severe in metastatic cells, suggesting a correlation between deregulation of integrin expression and aggressive tumour behaviour (Howlett *et al.*, 1995).

To cross the basement membrane, tumour cells itself changes proprieties like increasing matrix degrading enzymes (MMP), altering cell adhesion (integrins), fluctuating receptor-facilitated laminins assembly and laminins endocytosis (example: loss of laminins-111), and ECM signalling mechanisms. Basement membrane also suffers remodelling, allowing invasive sites (Muschler & Streuli, 2010). MMPs degrade the basement membrane, cell-cell and cell-matrix adhesion, and activate growth factors and inactivated MMPs. (Gangadhara *et al.*, 2012). Microenvironment has a huge role to drive cancer progression. Normal myoepithelial cells have the opposite function, they secrete inhibitors of ECM-degrading proteases, for instant, in the breast cancer case, maspin (Muschler & Streuli, 2010). Maspin can inhibit integrin subunits α_2, α_4 (can promote indirectly MMP-2 production), α_6 and α_v (inhibiting malignant capacity) but controversy induce integrin subunit α_5 (Koistinen & Heino, 2000).

After crossed the basement membrane, tumour cells are exposed to a different matrix, proteases and cytokines; leading to the increase secretion of matrix components, such collagen and hyaluronan and increase lysyl oxidase activity. All this imparts distinct biochemical and mechanical influences, which can foster malignancy and metastasis (Muschler & Streuli, 2010).

To acquire an invasion phenotype, cells suffer changes like loss of cadherin-dependent intercellular adhesion, epithelial-mesenchymal transition, and a partial degradation and remodelling of the ECM. Integrins binding to ECM components is implicated in cell growth, survival, adhesion, migration, invasion and tumour metastasis. In ductal carcinoma, an increase in MMP-2 and MMP-9 expression had been noted, similarly, in the stroma around pre-invasive lesions in MMP-1, -2, -3, -9 and -11. At genetic level, MMP-1, -11 -12 and -13 genes are up-regulated and related with poor prognosis (Gangadhara *et al.*, 2012).

2.4. The association between cell adhesion and the invasive phenotype - cadherins and integrins

In humans, the cellular response to molecular messengers synthesized and released by neighbour cells is critical to regulate cell growth, survival and differentiation. Adhesion receptors link cells to their surroundings, either other cells or the ECM, and, concomitantly, mediate information flow into the cells by activating the same signaling pathways as do growth factor receptors (Ivaska & Heino, 2011). Epithelial cells connect to their neighbourhood through diverse intercellular adhesion complexes which include adherent junctions (anchored to cortical actin and microtubules), tight junctions and desmosomes. Also, cells attach to the underlying

basement membrane through other adhesion complexes, namely focal adhesions (FAs, connection to the basement membrane) and hesmidesmosomes. Adhesion events are mainly mediated by cadherins and integrins which are transmembrane glycoproteins that play an important role in the physiological balance of epithelial cells (Epifano & Perez-moreno, 2012). Cancer cells typically develop alterations, indeed, expression of genes encoding cell-to-cell and cell-to-ECM adhesion molecules is altered in some aggressive carcinomas (Hanahan & Weinberg, 2011).

Adhesion receptors cooperate to coordinate response to the signals derived from other cells and from the microenvironment. This crosstalk response allows cells to rapidly respond to biochemical or mechanical inputs that are transformed in intracellular signals regulating cell behaviour (Figure 7). De-regulations in this coordination may result in chronic activation of the stroma (such as secretion of soluble factors, cytokines, chemokines, MMPs and changes in ECM composition). When this occurs, the signaling loops of cells may become permanently activated with gain of migratory and invasive capacities.

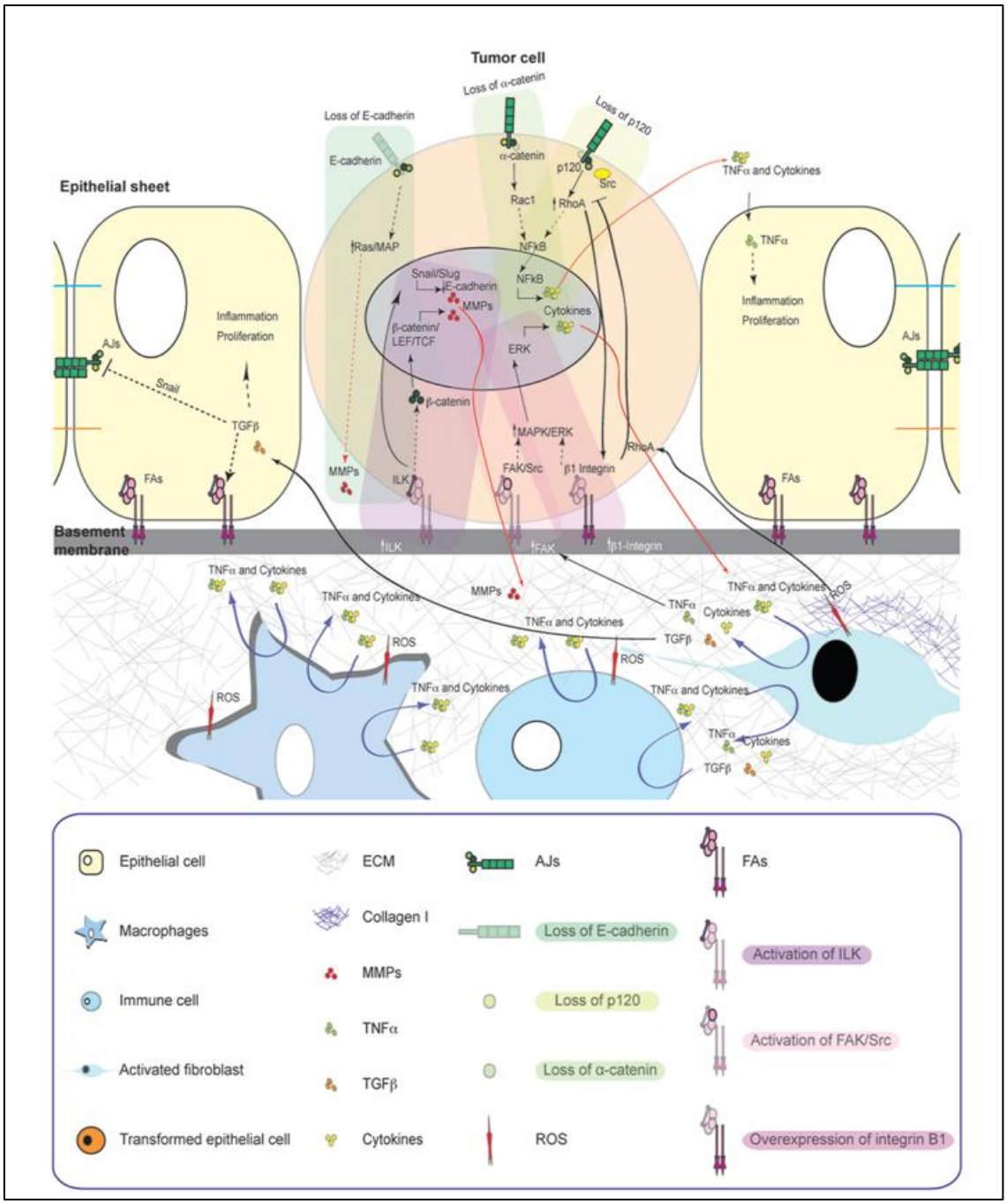

Figure 7: Coordinated regulation of FAs and AJs in epithelial cells is involved in the crosstalk of epithelial cells between themselves and with the stroma. FAs and AJs share downstream signaling molecules, including Rho GTPases and Src, and interactions with actomyosin cytoskeleton, which contribute to the coordination of their adhesive network. During tumourigenesis coordination crosstalk between FAs and AJs in epithelial cells is impaired, and may result in the chronic activation of the stroma (e.g., secretion of soluble factors, cytokines, chemokines, MMPs and changes in ECM composition). These can generate perpetuating signaling loops without a clear endpoint that, if unresolved, may lead to further epithelial transformation with gain of migratory and invasive characteristics (Epifano and Perez-Moreno 2012).

2.5. Alterations in interactions and mechanisms during breast cancer progression: integrins and cancer

The normal mammary microenvironment is capable of reverse the malignant phenotype of breast cancer cells, suggesting that cancer cells need an abnormal microenvironment to progress (Polyak & Kalluri, 2010). Tumour microenvironment is increasingly recognized as a major regulator of carcinogenesis (Place, *et al.* 2011).

The integrin family are transmembrane glycoprotein receptors that mediate cell-cell and cell-matrix adhesion, forming focal adhesions that contact with ECM ligands by the long extracellular domain (i.e. fibronectin, laminin, vitronectin and collagen). Integrins are the major receptors for the environment of the cell (Pontier & Muller, 2009). Integrins recognize some specific sequences like RGD and related sequences, found in ECM. Disintregins (originally found in viper venoms) block integrins' functions. They are composed by RGD sequences and act as an inhibitor of platelet aggregation (required component of metastasis) and adhesion. In addition, integrins regulate not only adhesion, but also cell proliferation, differentiation, survival and gene expression. Integrins have a role during various cancer stages such as malignant transformation, tumour growth and progression, invasion and metastasis and apoptosis (Mizejewski, 1999).

Integrins which are composed by heterodimers of one of 18 α-chains and one of 8 β-chains, accounting at least 24 combinations, where 12 contain integrin subunit β_1 (Koistinen & Heino, 2000). Those combinations have specific tissue distribution and specific and non-redundant functions as shown by their specificity for ECM ligands (Lahlou & Muller, 2011). Integrins have a large extracellular domain (N-terminal), a transmembrane domain, and an intercellular domain (C-terminal). Integrin subunit α_1 is the smaller intracellular domain (less that 40 amino acids) and integrin subunit β_4 the bigger one (1018 amino acids) (Koistinen & Heino, 2000).

The whole Integrin subunit α has 150-200 kDa, is composed by a heavy and light chain with disulphide bond and seven N-terminal (60 amino acids) forming b-propeller. This structure, with β-chain, is the ligand binding domain and it is need to stabilize the active conformation of integrin receptor. The α-chains exhibit four repeat amino acid segments believed to bind calcium (Ca^{2+}) and possibly other divalent cations such as Mg^{2+} and Mn^{2+}. These Ca^{2+} binding regions associated with amino acids 100–200 on the β-chain (bI-like domain) form the ligand binding site, a cation-dependent process. The N-terminal half of the integrin α-chain is folded into a β-sheet propeller motif that contains seven weak amino acid sequence repeats (Mizejewski, 1999).

The β-subunits have 90-110 kDa (except β_4 with 210 kDa) (Koistinen & Heino, 2000) and exhibit at least four cysteine-rich repeats (C-terminal), in linear juxtaposition, that stabilize the large extracellular amino terminal loop (Mizejewski, 1999).

The secondary structure is thought to be arranged in a geometric configuration around a central axis with Mg^{2+} ions bound to the upper faces of the propeller and Ca^{2+} ions bound to the

lower faces. The intracellular domains of both the α- and β-chains are short (except β_4) following their transmembrane insertion. The short β-cytoplasmic tails is capable of binding to cytoskeleton-associated proteins that link the integrins to the actin cytoskeleton system, through actin, vinculin, talin and paxillin. A calreticulin association is known with α-chains that regulate the calcium transmembrane channel influx (Mizejewski, 1999).

2.6. Integrins patterns

Integrins, when over-expressed in tumour cells, contribute to cancer progression and metastasis by increasing cell migration, invasion, proliferation, survival and tumour angiogenesis, through an intracellular and extracellular signalling involving crosstalk between RTK or G-protein-coupled receptor and integrins; but also involving integrin endocytosis and recycling (Lahlou & Muller, 2011). The effects of ECM on cells are mainly mediated by integrins by transmitting mechanical and chemical signals. Changes in adhesion signalling and integrins patterns are crucial to invasion process (Guo & Giancotti, 2004).

The binding of integrins and ligands are controlled by a mechanism that need a receptor clustering alone, or ligand occupancy plus receptor clustering, or clustering, ligand occupancy, and tyrine kinase activation. This process also needs an outside-in signalling (bidirectional signalling) and conformational changes in the chains, leading to an affinity modulation for the ligand. Moreover, adhesion plaques are formed at the cell membrane that serve as focal points for recruitment of proteins (talin, veniculin, paxillin, etc.) to provide cascade interfaces for actin, G-proteins, calcium-binding proteins, MAP and tyrosine kinases (Src family) and transcription factors, such NF-κB (Mizejewski, 1999).

Integrins are involved in all stages of metastasis: migratory behaviour, invasion and colonization of target tissues. To be able to migrate, metastatic cells need to have the ability to generate locomotors forces, capacity to breach vessel walls, ability to navigate through the dense collagen tissue surrounding tumours, ability to degrade the extracellular matrix (ECM) and squeeze through the interstitial spaces, described as amoeboid type, colonize foreign microenvironments, and dynamic interaction with changing microenvironment. For those reasons, integrin-mediated adhesion had a critical role in metastasis process. Integrins interact with ECM components, like collagen and laminin, for structural and functional integrity, but also promote growth factor receptors, leading to the activation of downstream signalling pathways, such cell cycle progression and oncogenic transformation (White & Muller, 2007).

Malignant transformation is characterized by disruption of cytoskeleton organization, decreased adhesion (by alteration in cell adhesion receptors) and altered adhesion-dependent responses. Studies reveal that different tumours have different patterns of integrins type and distribution (Table 1). Reduced levels of integrin subunit α_5, α_3, and α_2 expression have been reported in carcinomas, whereas increased levels of $\alpha_6\beta_4$ appear in head, neck, and skin tumours. Both quantitative and qualitative alterations in integrin cell surface patterns have been observed *in vitro* and *in vivo*. In turn, this altered integrin expression may have a role in invasion

and metastasis process. For instant, $\alpha_5\beta_1$ is correlated with low levels of transformation in certain tumours (i.e. ovary cancer cells) and $\alpha_v\beta_3$ is associated with high transformation. In human malignant mammary tumour progression, $\alpha_3\beta_1$ is present in non-neoplastic and fibroadenomas but were low or absent in invasive mammary carcinomas. Also, $\alpha_2\beta_1$ is high express in normal breast tissue (Mizejewski, 1999) (to maintain the differentiation of cell phenotype) and low $\alpha_2\beta_1$ level is found in breast adenocarcinoma (in collagen type-I, it promote MMP-1 by PKC-z and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathways, and MMP-13 by activation of p38) (Koistinen & Heino, 2000), like low level of $\alpha_5\beta_1$ and $\alpha_v\beta_3$. In contrast, $\alpha_6\beta_4$ is up-regulated in breast tumour, and it is an indicator of poor prognosis and metastatic potential. In breast cancer, $\alpha_6\beta_4$ is reduced in primary site and constant level at metastatic sites (Mizejewski, 1999).

The luminal epithelial cells of the human breast express the laminin and/or collagen integrin receptors: $\alpha_1\beta_1$ (mainly in collagen type-IV), $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ (mainly in laminin) (Weaver *et al.*, 1996), although the fibronectin express also $\alpha_5\beta_1$ and $\alpha_4\beta_1$ (Koistinen & Heino, 2000). $\alpha_2\beta_1$ and $\alpha_3\beta_1$ have a basolateral expression, while α_6 and β_4 are present where cells interact with the basement membrane at the basal surface. A study reveals that high integrin subunit α_6 level is correlated with decreased patient survival, more aggressive tumour phenotype (Weaver *et al.*, 1996) and increase migratory potential (Koistinen & Heino, 2000); moreover a dominant-negative integrin subunit β_4 is correlated with decreased metastatic potential. Until now, there are no studies revealing the importance of alterations in the adherens junction cell–cell adhesion system and relation to alterations in cell–ECM or trophic factors (Weaver *et al.*, 1996) (Table 1).

Table 1: Integrins in cancer progression (Cheresh & Desgrosellier, 2010)

Tumour type	Integrins expressed	Associated phenotypes
Melanoma	αvβ3 and α5β1	Vertical growth phase [35,172–174] and lymph node metastasis[173,175]
Breast	α6β4 and αvβ3	Increased tumour size and grade[176], and decreased survival[177] (α6β4). Increased bone metastasis[36–38,64] (αvβ3)
Prostate	αvβ3	Increased bone metastasis[39]
Pancreatic	αvβ3	Lymph node metastasis[40]
Ovarian	α4β1 and αvβ3	Increased peritoneal metastasis[178] (α4β1) and tumour proliferation[179] (αvβ3)
Cervical	αvβ3 and αvβ6	Decreased patient survival[41,180]
Glioblastoma	αvβ3 and αvβ5	Both are expressed at the tumour–normal tissue margin and have a possible role in invasion[181]
Non-small-cell lung carcinoma	α5β1	Decreased survival in patients with lymph node-negative tumours[182]
Colon	αvβ6	Reduced patient survival[109]

2.7. Integrins signalling in breast cancer progression

2.7.1. PI3K pathway

Integrin, localize in the tips of the forward reaching invasive structures, suggesting a traction, locomotion and migration role. Integrins clustering at focal adhesion sites allows regulation of actin polymeration and cytoskeleton rearrangement, mediated by Rho family including Rho, Rac and Cell division control protein 42 homolog (Cdc42) (White & Muller, 2007). Furthermore, Cdc42 and Rac1 could be the link between PI3K and integrin, like $\alpha_3\beta_1$ that are involved in cell migration/invasion in breast cancer by regulating MMP-2 production. $\alpha_3\beta_1$ also have correlation with metastatic capacity by increasing MMP-9 activity (Koistinen & Heino, 2000) (Figure 8). Epidermal growth factor (EGF) receptors interact with β_1-itegrins, resulting in an increase auto-phosphorylation of EGF and consequent activation of pathways. The migratory and invasive proprieties depend on local gradients of chemotactic growth factors such HGF and EGF (White & Muller, 2007) (Figures 8 and 9).

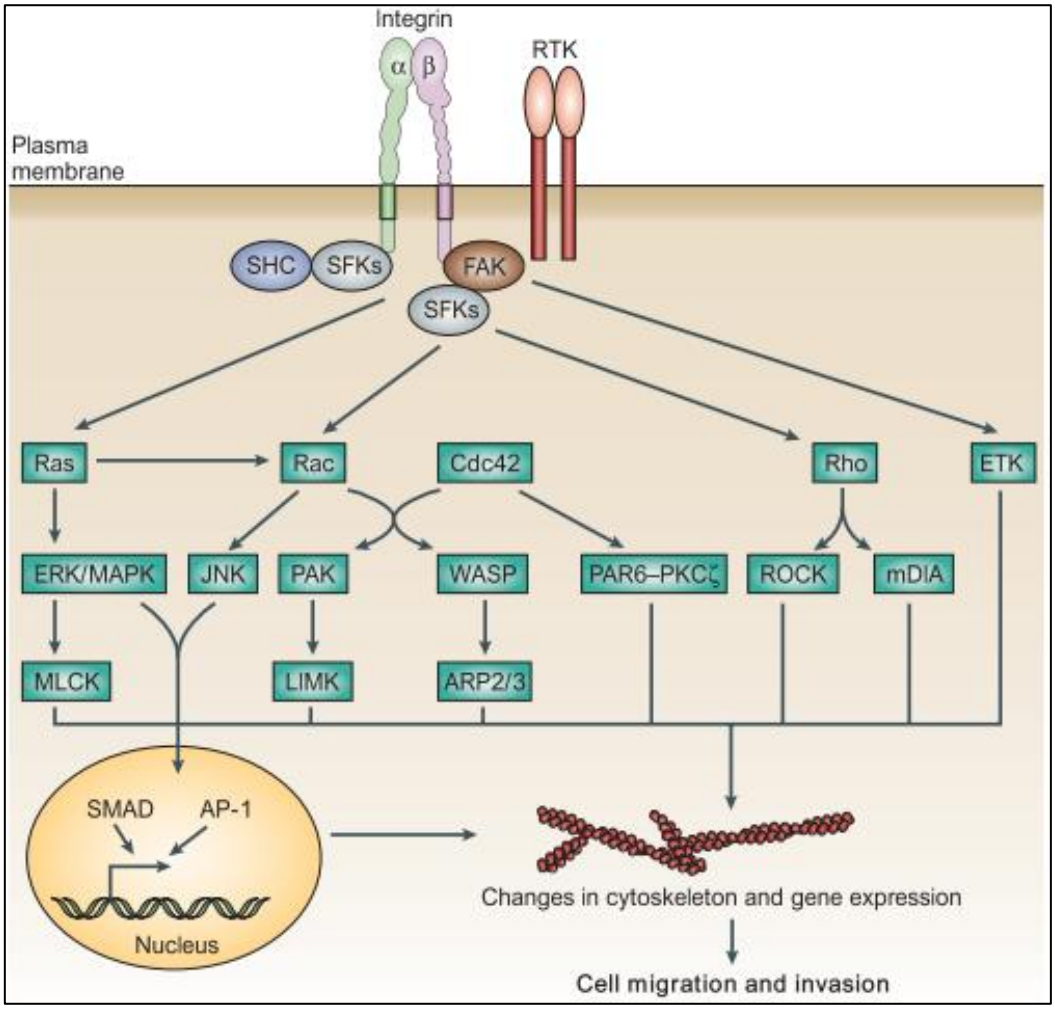

Figure 8: Integrin-receptor-tyrosine-kinase signalling induces cell migration and invasion (Guo & Giancotti, 2004)

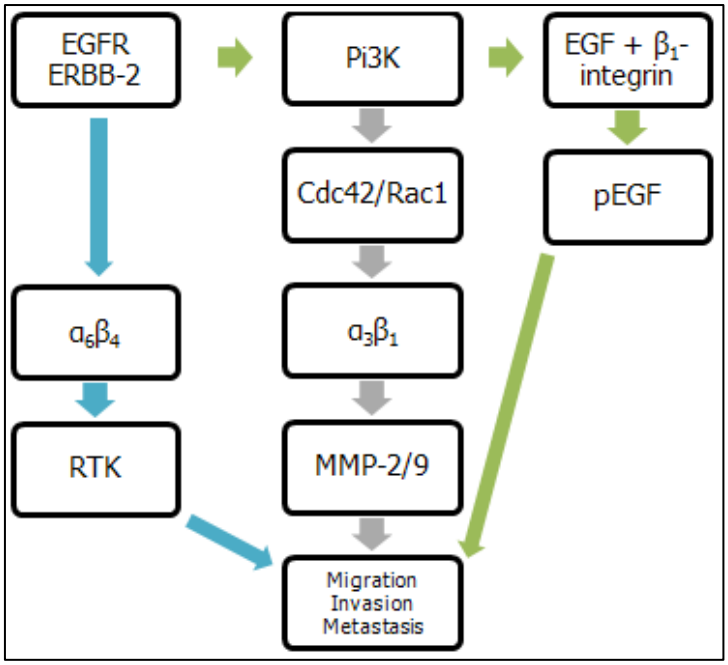

Figure 9: Integrin pathways leading to tumour progression

2.7.2. EGFR and ERBB-2 pathway

Moreover, epidermal growth factor receptor (EGFR), as well as ERBB-2, up-regulates integrin subunit β_1 function and breast cancer progression, via PI3K. ERBB-2 also down-regulate integrin subunit α_6 and may contribute to fibronectin-depend invasion (Koistinen & Heino, 2000). A study reveals that $\alpha_6\beta_4$ cooperates with EGFR and ERBB2, amplifying genes encoding RTK, and consequently promoting carcinoma growth (Guo & Giancotti, 2004) (Figure 9).

2.7.3. FAK pathway

The activation of focal adhesion kinase (FAK) requires both ligand binding to integrins (integrin subunits β_1 β_2 and β_3) and intact cytoskeleton (binding to paxillin, talin and maybe to vinculin). These binding leads to FAK auto-phosphorylation, in turn it is a binding site for kinases like Csk, Fyn and Src; consequently it may induce MAPK/ERK/JNK pathway to promote MMPs production (Koistinen & Heino, 2000) (Figure 10).

The switch of type and frequency of integrins is dependent of microenvironment. ECM remodelling involves alteration in integrins expression, which regulates FAK/Src family kinase activation and the cross-talk with soluble growth factor receptors and cytokines. FAK activation is present in invasive breast cancer, so Src family members could be a target to suppress tumour cell migration (Gangadhara *et al.*, 2012). Integrin subunit β_1 blocking attenuates EGF signalling and cell cycle progression. Also, blocking integrin subunits β_1 or β_4, or FAK or Src, impair tumourigenesis. For example, blocking $\alpha_5\beta_1$ and $\alpha_v\beta_3$ receptors impair the growth and

metastasis of invasive human breast cancer (White & Muller, 2007) by inhibition of MMP-9 and cell adhesion. $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are involved in bone cancer, in metastatic potential and migration; and cell adhesion, invasion and proliferation, respectively (Koistinen & Heino, 2000). A study reveal that tumour expressing $\alpha_v\beta_3$, normally not found in normal mammary tissue, also have up-regulated MMPs (White & Muller, 2007). $\alpha_v\beta_3$ can associate with uPAR, inducing the conversion of plasminogen in plasmin, that can degrade ECM components, directly or indirectly by activation of MMPs (Guo & Giancotti, 2004). Integrin expression is also associated with invasion process, by MMPs. A good therapeutic strategy could be inhibition of integrin, to reduce MMP activation and consequently reduce invasion behaviour (White & Muller, 2007).

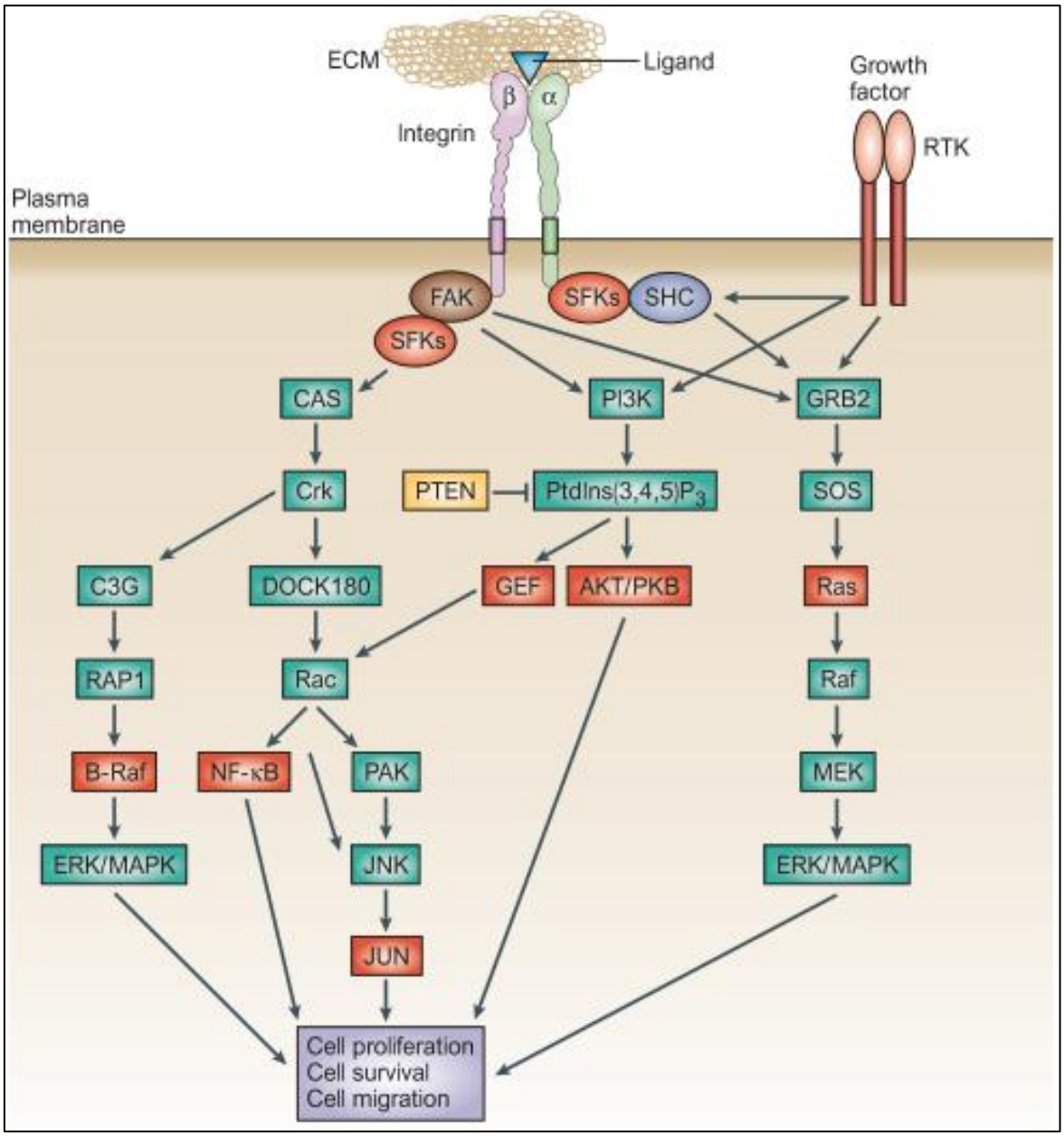

Figure 10: Integrin signalling (Guo & Giancotti, 2004)

2.7.4. Integrins, MMPs and mechanisms

One study reveals that β_4-shRNA decreases integrin subunit α_6, concluding that $\alpha_6\beta_4$ increase tumour survival and decrease apoptosis (via vascular endothelial growth factor expression – VEGF) and p53-dependet caspase-3 (Lipscomb *et al.*, 2005). These transformations involve increase phosphorylation and glycosylation of integrins, and decreased affinity. However, it is important to remain in mind that, although all this alteration, some integrins still maintain their normal expression during malignant transformation, tumour progression and metastasis (Mizejewski, 1999). $\alpha_v\beta_3$ co-localized with MMP-2 in melanoma cells that facilitate tumour cell invasion. $\alpha_v\beta_3$ not only induce MMP-2 but also promotes inhibition of inhibitor of MMP-2. Integrin subunit β_1 and $\alpha_6\beta_4$ also promote MMP-2 production (Koistinen & Heino, 2000). Others examples are: $\alpha_4\beta_1$ that initiates the growth and spread; $\alpha_5\beta_1$ and $\alpha_v\beta_3$ that are expressed in advanced tumour and metastases, suggesting that integrins may have prognostic value; increased $\alpha_4\beta_1$ together with decreased $\alpha_6\beta_1$ that is correlated with metastases; $\alpha_v\beta_3$ and $\alpha_v\beta_5$ that are implicated in neo-vascularisation; $\alpha_6\beta_4$ that induces p21 cyclin-dependent kinases (Mizejewski, 1999) and apoptosis via p53 (inhibiting malignant capacity). However, if p53 is mutated (inactive form), $\alpha_6\beta_4$ facilitate cancer progression and invasion through PI3Ks (Koistinen & Heino, 2000). Anoikis is a process in which normal cells died by apoptosis after matrix detachment (Mizejewski, 1999). However, cancer cells are relatively resistant to anoikis (Guo & Giancotti, 2004). Anoikis can be distinguished from necrosis by cell/nuclear morphology, inter-nucleosomal DNA cleavage, nuclear lamina cleavage and loss of *Bcl-2*. $\alpha_5\beta_1$ prevent apoptosis of cells attached to fibronectin by activating the Bcl-2 pathway (anti-apoptotic) (Mizejewski, 1999). Also $\alpha_5\beta_1$ induces MMP expression by binding to PEA3- and activator protein-1 (AP-1) sites of MMP promoter (Koistinen & Heino, 2000). p63 confer resistance to anoikis through integrin subunit β_4. Also NF-κB confer resistance to apoptosis through integrin subunit β_4 (Pontier & Muller, 2009).

Anti- Tumour necrosis factor-α (TNF-α) inhibit $\alpha_5\beta_1$ and MMP-9, but not MMP-2. It is possible that this inhibition take place by Raf-1 and mitogen-activated protein kinases pathway or Ras/MAP kinases. Also NF-κB, Specificity Protein-1, and AP-1 may be involved at least in TNF-α-related induction of MMP-9 expression; due to the fact that in breast cancer cells over-express Bcl-2, increase NF-κB-dependent transcriptional MMP-9 activity, TIMP-1 and TIMP-2 (Koistinen & Heino, 2000).

2.8. Integrins, cadherins and ROS

Adhesions have two major roles in migration: traction and signalling. Focal adhesion is a cluster of integrin receptors, associated with complexes of signalling and proteins linked to cytoskeleton, giving structural and signalling functions. During migration, nascent adhesions are formed at the leading edge and integrin-mediated adhesions are dissembled at rear-end,

allowing cellular movement. This turnover of focal adhesions is regulated by FAK and Src (Huttenlocher & Horwitz, 2011).

Integrins are the major cell-(ECM adhesion receptors and cadherins are cell-cell adhesion receptors. For that reason, it is though that there is a molecular crosstalk between them. Small GTPases of the Ras and Rho family, Src, FAK and phosphatidylinositol 3'-kinase (PI3K) are some example of this crosstalk. Another example is Rap1 that acts as a turnabout for endosome signalling and membrane traffic to delivery integrins and cadherins. It was suggest that ROS (like free radicals and peroxides –superoxide anion and hydrogen peroxide–) also play a role in the modulation of this crosstalk. Activation of redox signalling at integrin-mediated cell-matrix adhesion sites induces assembly of focal adhesions, but also, in turn, integrins induce ROS burst by promoting changes in mitochondrial metabolic/redox function. Contrary, activation of redox signalling at cadherin-mediated cell-cell junctions induces disassembly of adherent junctions. ROS are involved in the redox-dependent regulation of multiple signal transduction pathways, including cell adhesion, migration, proliferation, differentiation, and survival. However, ROS at high levels (production of ROS is higher that cellular antioxidant mechanisms), cause cellular damage through oxidative stress. Caveolae/lipid rafts, focal adhesions and cell-cell contacts promote NADPH oxidases, allowing ROS production and activation of specific redox signalling events (Goitre *et al.*, 2012).

2.9. Tumour microenvironment and integrins in lung cancer

The interactions of cancer cells with components of their tumour microenvironment are bi-directional and are crucial for cancer progression. When associated with cancers, mesenchymal stromal cells are often called carcinoma-associated fibroblasts (CAFs) and monocytes/macrophages are referred to as tumour-associated macrophages (TAM). CAFs display a greater ability than normal fibroblasts to enhance the tumourigenicity in non-small-cell lung cancer (NSCLC), over-expressing genes involved in TGF-β signaling, focal adhesion, and the MAPK signaling pathway (Saintigny & Burger, 2012). Up-regulated genes in TAMs (EGF, COX-2, MMP-9, uPA, VEGF, HGF) contributed to suitable microenvironments for lung cancer invasion and metastasis. The increase of invasiveness was also correlated MMP-9. Anti-uPA and anti-MMP-9, but not anti-VEGF monoclonal antibodies, can inhibit TAM-induced invasion (R. Wang *et al.*, 2011).

Lung epithelial cells adhere to a basement membrane, rich in laminin-332, where the main integrin receptors are $\alpha_6\beta_4$ and $\alpha_3\beta_1$. Silencing $\alpha_5\beta_1$ integrin, the major fibronectin receptor, impairs the mitogenic effect of nicotine on lung cancer cells. Increase $\alpha_5\beta_1$ level is correlated with lymph node metastasis in NSCLCs. $\alpha_v\beta_6$ integrin is also a negative prognostic factor for the survival of NSCLC patients. $\alpha_v\beta_6$, similarly to $\alpha_5\beta_1$, enhances ability to adhere, migrate, and invade the fibronectin-rich matrix that surrounds NSCLCs, through activation TGFβ signaling (Caccavari *et al.*, 2009).

There are different tumour environment therapies approaches in NSCLC. Target hypoxic cells in lung cancer is one approach (example: tirapazamine), but hypoxia decreases therapy response. Another approach is to alter microenvironment (and consequently some factors like VEGF and HIF-α) of NSCLC to impair hypoxia. EGFR is over-expresses in 80% of NSCLC. EGFR activation leads to the activation of multiple intracellular signaling pathway including the Ras and Akt pathways. And the PI3K pathway plays a key role in controlling cell proliferation, growth and survival, is activated in many cancers. For that reason, inhibitors of the EGFR/PI3K/Akt pathway (example: monoclonal antibody cetuximab, or small molecule tyrosine kinase inhibitors gefitinib and erlotinib) is another approach that "normalize" tumour vessels, allowing for increased chemotherapy delivery or improved oxygenation and radiosensitivity (Graves, Maity, & Le, 2010).

2.10. Tumour microenvironment therapies

Currently, there are different approaches to tumour microenvironment therapies: aromatase inhibitors, angiogenesis-modulating agents, inhibitors of HER family receptors, VEGF inhibitors, MMP inhibitors, antibodies targeting FAP, c-Met antagonists and multi-targeted RTK inhibitors, bisphosphonates, denosumab and microenvironmental reprogramming. This last hypothesis is an over-expression of histidine-rich glycoprotein that induces normalization of TAMs (which convert M2 pro-tumour phenotype to M1 anti-tumour phenotype) and blood vessel structure; and consequently decreases tumour growth and increases sensitivity to chemotherapy. All the therapies are based on chemotherapy, metronomic therapy (low doses of chemotherapy with low side effects) or epigenetic therapy (Place, *et al.* 2011).

Four classes of integrin inhibitors are currently in preclinical and clinical development: monoclonal antibodies (example: Vitaxin/Abegrin; MedImmune, Gaithersburg, MD), synthetic peptides containing an RGD sequence (example: Cilengitide; Merck KGaA, Darmstadt, Germany), non-RGD antagonists (such as ATN-161, inhibitor of integrin $\alpha_5\beta_1$), and general integrin-targeted therapeutics (Danhier, Le Breton, & Préat, 2012).

Vitaxin is an humanized monoclonal antibody to the integrin $\alpha_v\beta_3$., that after clinical trial phase I concluded that it is well tolerated with little or no toxicity (Gutheil *et al.*, 2000). However, the absence of objective disease responses seen in clinical trial II was attributed to limitations of affinity and stability *in vivo*. After affinity improvement, Abegrin appears and follows clinical trial I and II, unfortunately, treatment alone or in combination did not significantly impact overall survival. c7E3 (abcixmab) is approved by the U.S. Food and Drug Administration and recognizes $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$. It has an anti-angiogenic and antitumour activities, but also anti-metastatic activity by preventing the adhesion (Millard *et al.*, 2011).

Cilengitide, RGD antagonist, is currently in clinical phase III for treatment of glioblastomas and in phase II for several other tumours. This drug is anti-angiogenic and inhibits integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ (Mas-moruno, Rechenmacher, & Kessler, 2010).

ATN-161 has antiangiogenic effects by inhibition of $\alpha_5\beta_1$. ATN-161 inhibited VEGF-induced migration and capillary tube formation *in vitro* and *in vivo* (Wang *et al.*, 2011). Another inhibitor of $\alpha_5\beta_1$ is volociximab. Clinical trial phase I showed that 8 patients have partial response and 17 had stable disease; concluding that volociximab combined with carboplatin and paclitaxel was generally well-tolerated and showed preliminary evidence of efficacy in advanced NSCLC (Besse *et al.*, 2013). Additional Phase II and III trials involving volociximab as a are currently underway for the treatment of metastatic melanoma, non-small cell lung cancer and peritoneal cancer (Millard *et al.*, 2011).

Nowadays, there are three U.S. Food and Drug Administration approved therapeutics targeting $\alpha_{IIb}\beta_3$ (abciximab, eptifibatide and tirofiban) and one α_4 antagonist (natalizumab) (Millard *et al.*, 2011) (Table 2).

Table 2: Integrin inhibitors (Chen, Alexander, & Wayne, 2012)

LEC integrins	Matrix ligands	Current inhibitors FDA approved or in clinical trials
$\alpha1\beta1$, $\alpha2\beta1$	Collagens	None
$\alpha5\beta1$	Fibronectin	Volociximab PF-04605412 JSM6427
$\alpha v\beta3$, $\alpha v\beta5$	Fibronectin (RGD), Osteopontin, Vitronectin, Fibrinogen Fibrillin	Cilengitide, CNTO95 EMD525797 IMGN388
$\alpha9\beta1$	Fibronectin (EDA), Osteopontin, Tenascin-C, VEGF-A/C/D	None
$\alpha4\beta1$	Fibronectin (CS1), Osteopontin, Emillin-1	Natalizumab Vedolizumab ELND002
$\alpha6\beta1$	Laminin, Netrin-4	None

3. Breast and lung cancer

3.1. Cancer incidence

Breast and lung cancer incidence is increasing in women, with an estimated 232,340 and 110,110 new cases; and estimated 39,620 and 71,220 deaths worldwide, respectively, making it both the most commons types of cancer affecting women (Figure 11) (American Cancer Society, 2013). It is also known that 10% of women with breast cancer develop a second, and women with breast cancer have a 3- to 7-fold increased relative risk of cancer developing in the opposite breast (Richie & Swanson, 2003).

Estimated New Cases		Estimated Deaths	
Male	**Female**	**Male**	**Female**
Prostate 238,590 (28%)	Breast 232,340 (29%)	Lung & bronchus 87,260 (28%)	Lung & bronchus 72,220 (26%)
Lung & bronchus 118,080 (14%)	Lung & bronchus 110,110 (14%)	Prostate 29,720 (10%)	Breast 39,620 (14%)
Colon & rectum 73,680 (9%)	Colon & rectum 69,140 (9%)	Colon & rectum 26,300 (9%)	Colon & rectum 24,530 (9%)
Urinary bladder 54,610 (6%)	Uterine corpus 49,560 (6%)	Pancreas 19,480 (6%)	Pancreas 18,980 (7%)
Melanoma of the skin 45,060 (5%)	Thyroid 45,310 (6%)	Liver & intrahepatic bile duct 14,890 (5%)	Ovary 14,030 (5%)
Kidney & renal pelvis 40,430 (5%)	Non-Hodgkin lymphoma 32,140 (4%)	Leukemia 13,660 (4%)	Leukemia 10,060 (4%)
Non-Hodgkin lymphoma 37,600 (4%)	Melanoma of the skin 31,630 (4%)	Esophagus 12,220 (4%)	Non-Hodgkin lymphoma 8,430 (3%)
Oral cavity & pharynx 29,620 (3%)	Kidney & renal pelvis 24,720 (3%)	Urinary bladder 10,820 (4%)	Uterine corpus 8,190 (3%)
Leukemia 27,880 (3%)	Pancreas 22,480 (3%)	Non-Hodgkin lymphoma 10,590 (3%)	Liver & intrahepatic bile duct 6,780 (2%)
Pancreas 22,740 (3%)	Ovary 22,240 (3%)	Kidney & renal pelvis 8,780 (3%)	Brain & other nervous system 6,150 (2%)
All sites 854,790 (100%)	All sites 805,500 (100%)	All sites 306,920 (100%)	All sites 273,430 (100%)

Figure 11: Estimated new cancer cases and deaths worldwide for leading cancer sites (American Cancer Society, 2013)

Some studies reveal that 95% of breast cancers are carcinomas, meaning that they arise from breast epithelial elements. There are two groups: *in situ* carcinomas (arise in ductal or lobular epithelium) and invasive or infiltrating carcinomas (potential for metastases) (Richie & Swanson, 2003). Invasive ductal carcinoma is the most common morphological subtype, representing 80% of the invasive breast cancers (Sandhu *et al.*, 2010).

3.2. P-cadherin in breast cancer

3.2.1. Molecular characterization

The cadherin superfamily is composed by classical cadherins (main components of cell-cell adhesion), by non-classical cadherin (like desmosomal cadherins) and by proto-cadherins

(implicated in neuronal plasticity) (Paredes *et al.*, 2012). Classical cadherins are calcium-dependent cell-cell adhesion proteins, localized in adhesion-type junctions (Paredes *et al.*, 2007), including *CDH1*/E-cadherin (epithelial), *CDH2*/N-cadherin (neuronal), *CDH3*/P-cadherin (placental) and *CDH4*/R-cadherin (retinal) (Albergaria *et al.*, 2011).

As all classical cadherins, P-cadherin is a transmembranar glycoprotein with 118 kDa (Figure 12). These classical cadherins promote mainly homotypic interactions between cadherins of the same type, forming homodimers (Paredes *et al.*, 2007, 2012).

The extracellular domain is composed by five cadherin repeats (EC), which are sequences of 110 residues, designated EC1-EC5. The EC1 is the most important for the adhesion role. The normal conformation of P-cadherin is only stable in the presence of calcium which is required to the cell-cell adhesion function. Calcium-binding sites are conserved sequences and are located between neighbouring EC repeats (Paredes *et al.*, 2012). The extracellular domain creates lateral dimmers (Albergaria *et al.*, 2011) by the amino-terminal domain, a zipper-like structure between neighbour cells (Figure 12) (Paredes *et al.*, 2007).

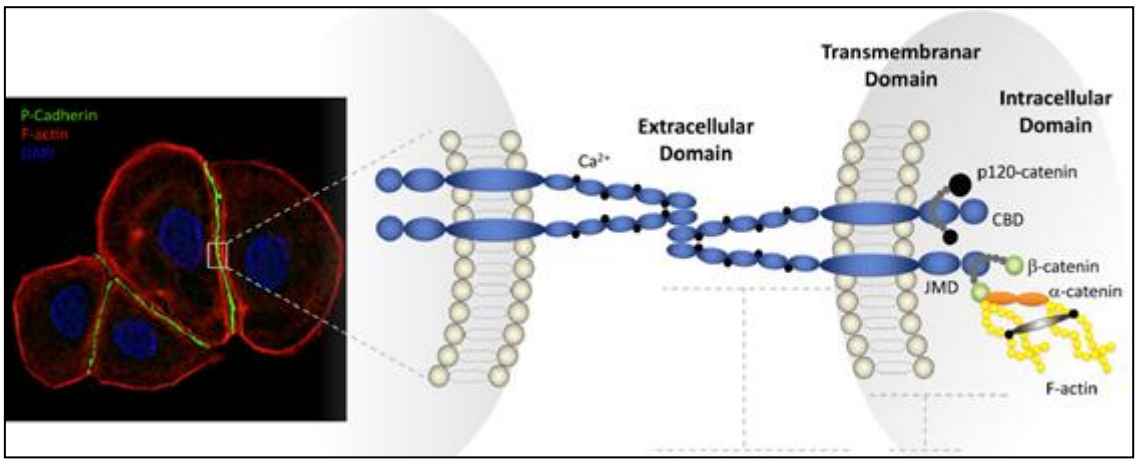

Figure 12: Schematic representation of the structural components of the P-cadherin adhesive junction (Albergaria *et al.*, 2011).

For an entire review: Paredes *et al.*, 2005, 2007, 2012 and Albergaria *et al.*, 2011.

3.2.2. P-cadherin over-expression

Cadherins affect tumourigenesis and tumour behaviour for the reason of their role in invasion and migration. P-cadherin is frequently found in breast, gastric, endometrial, colorectal and pancreatic carcinomas (Albergaria *et al.*, 2011). With the development of new antibodies, 30% to 50% of invasive ductal carcinoma were identify P-cadherin positive, but not in lobular type (Paredes *et al.*, 2007). In other studies, P-cadherin was described in 20% to 40% of invasive breast carcinoma and in 25% of ductal carcinoma *in situ* (Albergaria *et al.*, 2011).

Several hypotheses appear to try to explain the aberrant presence of P-cadherin in breast cancer. One theory is that P-cadherin should be involved in the proliferative process; however

the presence of this protein did not change the proliferative rate, indicating that P-cadherin is not directly involved in cell cycle (Paredes *et al.*, 2007). Although others studies reveal the opposite (Paredes *et al.*, 2012). Another theory is that P-cadherin should be an oncofetal protein member, based on the fact that P-cadherin is highly expressed in embryogenesis and neoplasias but weakly expressed in adult tissues; however there is no confirmation of this hypothesis. A third theory is that P-cadherin expression could be related to a histogenetic origin in cap cells, due to the fact that caps cells have a high migration capacity without oestrogen receptors and with the ability to differentiate into myoepithelial cells, suggesting that they could be responsible for the development of P-cadherin positive breast cancer cells. Another idea is that P-cadherin is mis-expressed, following epithelial transformation; this lead to the alteration of the behaviour of the tumour cells and consequently contribute to the poor survival of women with P-cadherin positive breast cancers (Paredes *et al.*, 2007).

P-cadherin expression is correlated with high histological grade tumour, lack of oestrogen and progesterone receptors, increased aggressiveness (Knudsen & Wheelock, 2005), Bcl-2 low expression, short-term overall and disease-specific survival, short-term relapse-free survival, increased motility (Albergaria *et al.*, 2011), nuclear pleomorphism and decreased cell polarity (Paredes *et al.*, 2007). In addition, P-cadherin is positively associated with Nottingham prognostic index, p53, Her2, lymph node stage, antigen Ki-67 (associated with cell proliferation), recurrence, distant metastasis, invasion (Liu *et al.*, 2012), high proliferative rate (*MIB-1* gene), high mitotic index and decreased cell differentiation (Paredes *et al.*, 2007). For all these reasons, P-cadherin is considered a marker of poor prognosis (Albergaria *et al.*, 2011).

P-cadherin is also a basal-like marker (Figure 13) like cytokeratins (CK5/6, CK14, CK17), vimentin, αB-crystalline, caveolins 1/2 and EGFR (Albergaria *et al.*, 2011).

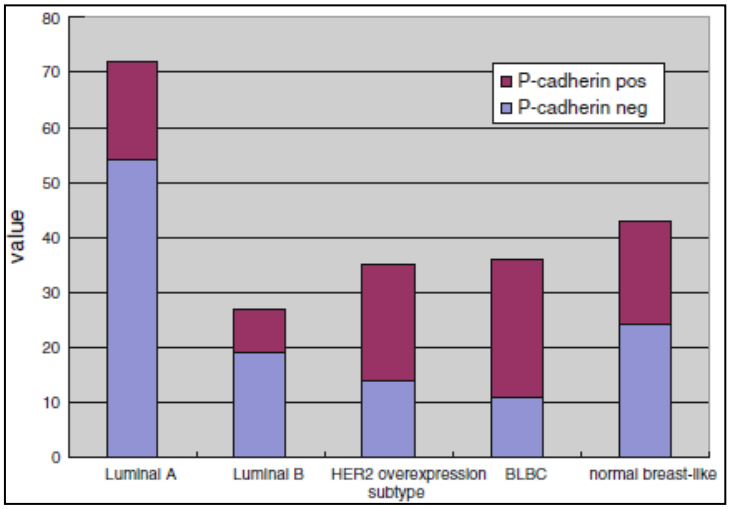

Figure 13: Expression of P-cadherin in all molecular subtypes of breast cancer, mainly in basal-like type (Liu *et al.*, 2012)

3.2.3. P-cadherin and invasion of breast cancer cells

Local invasion and distant metastasis are the later stage of carcinomas progress. Those processes depend on the cell-cell and cell-matrix interactions. The disruption of these adhesions leads to motility, invasion and metastasis of tumour cells (Albergaria *et al.*, 2011).

The role of P-cadherin in the carcinogenic process is still controversial, since it depends on cancer cell model studied. For instance, P-cadherin acts like a tumour suppressor gene in malignant melanoma, in which there is a gradual loss of P-cadherin, allowing cells to invade and migrate. In colorectal cancer cell line and melanomas, it is suggest that P-cadherin have an anti-invasion and pro-adhesion role. However, in breast cancer, P-cadherin expression increase and enhance cell invasion and tumour aggressiveness (Paredes *et al.*, 2007). *CDH3* gene acts as an oncogene and consequently P-cadherin increased tumour cell motility, directional cell migration and invasiveness (Albergaria *et al.*, 2011).

Some studies reveal that the lost of E-cadherin leads to an up-regulation of N- and P-cadherin, a process known by cadherin switching. This switch is, in part, responsible for the tumour cell invasion, metastasis and, in some cases, the promotion of epithelial-to-mesenchymal transition. The cadherin switch from E- to P-cadherin is common during embryo development and some reports describe it during tumour progression. Indeed, some breast cancer models maintain the E-cadherin expression and the abnormal P-cadherin expression. Although, it is though that P-cadherin only is functional when the cell system already express an endogenous and functional cadherin, like E-cadherin in breast cancer. This suggest that P-cadherin interact with E-cadherin and promote the disruption between E-cadherin and β-/p120-catenin, a negative signal to tumour cell growth and invasion (Albergaria *et al.*, 2011).

Besides the role of cadherin in cell polarity, cadherin are important to cell-cell adhesion. In breast cancer, cadherin level is normally altered. For instance P-cadherin over-expressing, in an E-cadherin wild-type model, is correlated with poor survival and high aggressiveness. P-cadherin promote invasion and migration, by increasing of MMP-1/-2, which in turn cleave P-cadherin (soluble P-cadherin) and increase invasion (Ribeiro *et al.*, 2010).

In addition, P-cadherin also regulate an overall genetic program of breast cancer cells, like genes involved in signal transduction, in growth factors (*VEGF-C)* and Fibroblast growth factor receptor 4 (*FGFR4*), in cell cycle [Cyclin-A2 (*CCNA2*)], in metalloproteinases (*MMP-1/-2*), in cytokines and inflammation [Interleukin-24 (*IL-24*)] (Albergaria *et al.*, 2011).

3.2.4. P-cadherin as a potential therapeutic target

P-cadherin plays a role in the cancer cell survival, invasiveness and metastatic potential. For that reason, *CDH3*/P-cadherin is a possible target for immunotherapy of breast cancer, as a novel tumour-associated antigen, meaning that was strongly expressed in tumour cells but not in normal cells. P-cadherin silencing in breast cancer cells in nude mouse inhibit *in vivo* tumour growth. Recently, a monoclonal antibody anti-P-cadherin PF-03732010 shows an anti-tumour

and anti-metastatic activity in different cancer models with no side effects in mice. It also shows no affinity to other cadherins, decreased Ki-67, increased caspase-3 expression (Albergaria *et al.*, 2011), and suppressed β-catenin, Cyclin D1, vimentin, Bcl-2 and survivin expression (Paredes *et al.*, 2012). The next step should be developing a reproducible method to quantify P-cadherin in human tumours (Albergaria *et al.*, 2011).

P-cadherin over-expression occurs in about 30% of all breast carcinomas, leading to invasion and migration. Three breast cancer cell lines (MCF7.AZ/Mock, MCF7.AZ/Mock and SUM149) were treated with azurin to evaluate P-cadherin level. This study concluded that P-cadherin protein level decreases 30-50% in MCF-7/AZ.Pcad and SUM149, but the levels of E-cadherin remain unaltered. Azurin is able to decrease P-cadherin level (protein level and not mRNA level) leading to a tumour less aggressive. Also, azurin decrease invasion and MMP-2 activity; and decrease the phosphorylation levels of both FAK and Src proteins. Azurin could possibly be considered a therapeutic tool to treat over-expressing P-cadherin in a wild type E-cadherin context, via FAK/Src signaling (Bernardes *et al.*, 2013).

4. Objectives and thesis outline

P-cadherin over-expression in breast cancer is correlated with poor prognosis (Paredes *et al.*, 2012). As previously showed, azurin, a bacterial protein, decreases P-cadherin protein level (an adhesion protein) in an E-cadherin wild type model of breast cancer. For that reason, azurin is a potential breast cancer drug and P-cadherin is a potential therapeutic target. Also, azurin decreases the phosphorylation level of FAK and Src (Bernardes *et al.*, 2013), a down-stream signalling of integrins that lead to migration and invasion.

Lung cancer, in particularly NSCLC, has similar signalling involved in adhesion as in breast cancer. To try to prove a general impact of azurin in cancer, we will use both models.

Previously, our group has performed a microarray analysis of MCF7/AZ.Pcad cell line treated with azurin (100µM) during 48h. The results were then analysed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) and software One of main categories enriched in genes with decreased expression in treated cells compared to normal cells was biological adhesion, accounting for the biological processes of cell-to-cell and cell-to-matrix adhesion. To confirm this information, a functional validation was done in three P-cadherin over-expressing breast cancer cell lines: MCF7/AZ.Pcad, SUM149 and BT-20 and also in one lung cancer cell line A549, to confirm if this was one possible mode of action towards different cancer types. Thus, we decided to investigate the role of azurin in interfering with the capacity of cancer cells to adhere to several ECM components, by performing adhesion assays to different ECM components (laminin-332, collagen type-I, fibronectin and collagen type-IV). Also, as integrins are major receptors in this process, we went to investigate the expression of these proteins by western blot, using different biological matrices (collagen type-I matrix and Matrigel^{TM}). As previously proved for other models, we also performed a gelatine zymography to determine MMP-2 activity and Matrigel^{TM} invasion assays to evaluate the impact of azurin in the invasion process in different cancer models.

5. Materials and methods

5.1. Cell lines and cell cultures

Four human breast cancer cell models have been used in this study: MCF7/AZ [kindly provided by Doctor Joana Paredes (IPATIMUP) in the context of a collaborative ongoing project; MCF7/AZ.Mock and MCF7/AZ.Pcad were stably transduced with empty vector (control) or *CDH3*/P-cadherin cDNA, respectively], BT-20 and SUM-149 [kindly provided by Prof. Stephen Ethier (University of 161 Michigan, MI, USA), constitutively express high levels of P-cadherin]; one lung cancer cell line A-549; and mouse macrophages J774.

MCF7/AZ.Pcad, MCF7/AZ.Mock and BT-20 were routinely maintained in DMEM, SUM149 in DMEM-F12 (1:1 v/v) and A-549 in F-12 (Gibco, Invitrogen Ltd, Paisley, UK); supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 100 IU/mL penicillin and 100 mg/mL streptomycin (Invitrogen). SUM-149 medium was supplemented with 1 µg/mL hydrocortisone and 5 µg/mL insulin (Sigma-Aldrich-Aldrich, St. Louis, MO, USA). J774 was maintained in DMEM (Gibco, Invitrogen Ltd, Paisley, UK) supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin (Invitrogen). Cells were grown at 37ºC in a humidified chamber containing 5% CO_2 (Binder CO_2 incubator C150).

5.2. Bacteria growth, over-expression, extraction and purification of azurin

To perform the pre-inoculum, 100 mL of LB medium with 100 µL of 150 µg/mL ampicillin was inoculated over-night with *Escherichia coli* SURE (cloned with the plasmid pWH844, containing the gene *azu*, from *Pseudomonas aeruginosa* PAO 1, responsible for the synthesis of azurin) (Bernardes *et al.*, 2013) at 37 ºC in an agitator at 250 rpm. The following day, the inoculum was made with the pre-inoculum at OD_{640} 0.1 in 1 L of SB medium (3.2% tryptone, 2% yeast extract and 0.5% NaCl) with 150 µg/mL ampicillin at 30-37 ºC in an agitator at 250 rpm. The culture was grown until OD_{640} 0.6-0.8, and protein expression was induced with 0.2 mM IPTG. The culture was grown during 5 hours at 30-37 ºC in an agitator at 250 rpm. Cells were collected after centrifuge the culture at 8000 rpm at 4ºC during 10 minutes (Beckman J2-MC Centrifuge), and the pellets were ressuspended in 15 mL of START buffer (10 mM imidazole, 0.2 mM sodium phosphate, 0.5 M NaCl, pH 7.4) and stored at -80 ºC until purification.

For purification, cells were sonicated (Branson Sonifier Sound Enclosure) and centrifuged at 17600 g at 4º C during 5 minutes (B. Braun Sigma-Aldrich 2K15). To remove debris, the supernatant was again centrifuged during 1 hour. Protein was purified in a histidine affinity column (HisTrap™ FF, GE Healthcare) and eluated with increased concentrations of imidazole (20-500 mM). Azurin is eluted with 100-200 mM of imidazole. Afterwards, buffer was exchanged to PBS in ÄKTA system (ÄKTA Prime, Amersham Biosciences) with a desalting column (HiPrep™ 26/10 Desalting, GE Healthcare), following the manufacturer's instructions. After injection of the sample and elution, protein was collected and centrifuged in a 3 kDa cut-off

column (Amicon Ultra Centrifugal Filter, Ultracel 3K, Milipore) at 5000 rpm at 4 ºC (Eppendorf Centrifuge 5804R), to concentrate the sample. Protein was then passed through a detoxing column (Detoxi-Gel™ Endotoxin Removing Column, Thermo Scientific) to remove endotoxins from *E. coli* host strain; and centrifuged again in a falcon with a 3 kDa cut-off to concentrate. The concentration was calculated after reading the absorbance at 280 nm, and using Beer-Lambert equation, where ε(280)=9.1 x 10^3 $M^{-1}.cm^{-1}$ [Abs - absorbance, ε - extinction coefficient ($M^{-1}.cm^{-1}$), Γ coverage ($mol.cm^{-2}$) and [azurin] – concentration of azurin] (van Amsterdam *et al.*, 2002).

To verify any contamination, test spot (two spot with 10µL of azurin in a LB agar plate) was performed over-night at 37 ºC. Azurin was kept at 4 ºC until further use.

5.3. Protein extraction and western blot

Matrix coating [collagen type-I matrix (08-115, Millipode) or Matrigel™ (BD Matrigel™ Basement Membrane Matrix, BD Biosciences)] was performed in 6-well plate (200 µL/well). After 2 hours at 37 ºC, cells were plated with 5x10^5 cells (MCF7/AZ.Pcad and MCF7/AZ.Mock) or 7.5x10^5 cells (SUM-149 and BT-20). The following day, cells were treated with azurin (50-100 µM) in complete medium.

Cancer cells untreated or treated with azurin were washed twice with PBS, lysed in 100 µL of catenin lysis buffer (1% Triton X-100, 1 % Nonidet-P40 in deionized PBS) with 1:100 phosphatases inhibitor (Cocktail 3, Sigma-Aldrich) and 1:7 proteases inhibitor (Roche Diagnostics GmbH, Germany) and after 10 minutes at 4 ºC were scratched). The lysates were collected, vortexed three times and centrifuged at 14,000 rpm at 4 ºC for 10 minutes (B.Braun Sigma-Aldrich 2K15) and quantified by BCA method (BioRad Protein Assay). 20 µg of total protein per sample were prepared, denatured at 95ºC during 5 minutes, and then separated by electrophoresis in a SDS-PAGE (Table 3).

Table 3: SDS-PAGE

	Gel Resolving 8%	Gel Resolving 15%	Gel Stacking 5%
H_2O	2.3 mL	850 µL	1.35 mL
30% Acrilamide	1.35 mL	1.9 mL	335 µL
Tris	1.25 mL (1.5 M)	950 µL (1.5 M)	250 µL (1 M)
10 % SDS	50 µL	34 µL	20 µL
APS	50 µL	34 µL	20 µL
TEMED	3 µL	1.5 µL	2 µL

Gels were transferred onto nitrocellulose membranes (RTA Transfer Kit, BioRad), using Trans-Blot Turbo Transfer System (BioRad), following manufacturer's instructions. After blocking the non-specific binding sites for 1 hour with 5% (w/v) non-fat dry milk in PBS-tween-20 (0.5% v/v), the membranes were incubated in a agitator overnight at 4 ºC with different primary

antibodies (anti-E-cadherin [HECD 1, Sigma-Aldrich], anti-azurin [AB0048-200 SicGen] and anti-actin [sc-1616, Santa Cruz Biotecnology] diluted 1:1000 in 5% non-fat milk; anti-P-cadherin [clone 56, BD Transduction Laboratories] diluted 1:500, and anti-integrins [α_6, sc-13542, β_1, sc-18887; β_4, sc-6629, Santa Cruz Biotecnology] diluted 1:200).

The membranes were washed three times with PBS-tween-20 (0.5% v/v) for 5 minutes and probed with the appropriated secondary antibody, conjugated with horseradish peroxidase [anti-mouse (sc-2005, Santa Cruz Biotecnology) for cadherins and integrins, and anti-goat (sc-2354, Santa Cruz Biotecnology) for azurin and actin, diluted 1:2000 in 0.5% PBS tween-20] at room temperature for 1 hour, in an agitator. After washed, the membranes were developed by adding ECL substrates (Pierce) and capture the chemiluminescence by Fusion Solo (Vilber Lourmat) equipment. The band intensity was measured using ImageJ and results are present as the ratio between the signal intensities in azurin treated samples to untreated cells. The protein levels were normalized by the respective actin level.

5.4. Adhesion assay to ECM components

Cells were plated in 6-well plates with $5x10^5$ cells (MCF7/AZ.Mock and MCF7/AZ.Pcad) or $7.5x10^5$ cells (SUM 149 and A-549) and left to adhere. The following day, cells were treated with azurin in complete medium supplemented with 10% FBS. For each cell line, three conditions were analyzed: 0 µM (control), 50 µM and 100 µM of azurin.

Different proteins from the ECM [laminin-332 (Sigma-Aldrich), collagen type-I (Millipode) and -IV (Sigma-Aldrich), and fibronectin (Sigma-Aldrich)] at 5 µg/mL diluted in sterile PBS were coated in a 96-well plates (over-night at 4 ºC); and BSA 0.5% and plastic were used as controls. Before addition of cells, plates were washed three times with sterile PBS containing PenStrep (Invitrogen) and non-specific binding sites were blocked with 0.5% BSA during 2 hours at 37 ºC.

Azurin treated and control cells were washed twice with sterile PBS, collected with trypsine, and ressuspended in complete medium supplemented with 10% FBS. After centrifuged at 1200 rpm during 5 minutes (Eppendorf Centrifuge 5702R), cells were washed twice with PBS, ressuspend in simple medium. Cells (100 µL at the density of 10^6 cells/mL) were plated in the 96-well coating plates and left to adhere to the different ECM components, during 30 minutes at room temperature. Cells were washed three times with PBS to remove non-adherent cells, attached cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet (0.25 mM crystal violet, 20% ethanol, 56.3 mM ammonium oxalate) during 10 minutes at room temperature. After washed excessive dye twice with PBS, the dye was dissolved in 200 µL of 100% ethanol. The absorbance was read at 570 nm to quantify crystal violet staining. The analysis of the adhesion assay was made using control absorbance as 100% of staining, meaning 100% of adhesion.

5.5. Immunocytochemistry

MCF7/AZ.Pcad cells were seeded on a round glass coverslip in 24-well plates with $5x10^4$ cells and left to adhere in a CO_2 incubator at 37ºC. The following day, cells were treated with azurin in complete medium. Three conditions were analysed: 0 µM (control), 50 µM and 100 µM of azurin.

After 48 hours, coverslips were rinsed with PBS three times. For fixation, cells in coverslips were immersed in 4% formaldehyde for 20 minutes at room temperature. After wash three times in PBS, cells in coverslips were immersed in ammonium chloride (50 mM in PBS) for 10 minutes at room temperature. After washed three times in PBS, cells in coverslips were immersed in 0.2% Triton X-100 (Sigma) for 5 minutes at room temperature, to achieve permeabilization. For immunostaining, cells in coverslips were blocked with 5% BSA in PBS at room temperature during 30 minutes. Without washing, BSA excess was removed and cells were incubated with primary antibody (1:50 anti-integrins) during 1-2 hours, in the dark at room temperature, wash three times in PBS and incubated in 1:500 secondary antibody (Alexa Fluor 488 anti-mouse or anti-goat, Invitrogen) during 1 hour, in the dark at room temperature. After washed three times in PBS, cells in coverslips were mounted with Vectashield with DAPI (Vector Inc., Burlingame, CA, USA) and observed in confocal microscope (Zeiss).

5.6. ROS measurement

Cells were seeded $2x10^4$/well (MCF7/AZ.Pcad and MCF7/AZ.Mock) or $5x10^4$/well (SUM 149 and J774) in phenol-red free medium in 96-well black plates for 24 hours at 37 ºC and 5% CO_2. The following day, the medium was aspirated and cells were washed twice in PBS. Cells were treated with azurin in DMEM phenol-red free medium (Gibco, Invitrogen Ltd, Paisley, UK) during 24 hours (SUM 149 and J774) or 48 hours (MCF7/AZ.Pcad and MCF7/AZ.Mock). After washed twice with PBS, cells were exposed to 2',7'-diclorodihydrofluorescein di-acetate (*DCFH-DA, 10 µM*) during 30 minutes at 37 ºC and 5% CO_2. DCFH-DA was aspirated and cells were washed twice with PBS. Finally, 200 µL/well of PBS were added and fluorescence intensity was read (Software SoftMax Pro 6.1) with excitation wavelength of 485 nm and emission wavelength of 535 nm (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices).

5.7. Gelatine zymography

Cells were seeded in collagen type-I in 6-well plates. Cell conditioned media were collected and centrifuged at 1200 rpm at 4 ºC during 5 minutes (Eppendorf Centrifuge 5702R) to remove cell's contaminations. Equal volumes of samples were prepared and added to zymography buffer (0.25 M Tris, 10% SDS, 4% sucrose, 0.03% bromophenol blue, pH 6.8). After perform an electrophoresis using a 10% polyacrylamide gel containing 0.1% gelatine (Sigma-Aldrich) at 80 V, the gel was washed twice in 2% Triton X-100 (Sigma-Aldrich) during 30 minutes at room

temperature to remove SDS. The gel was left over-night (~16 hours) in gelatine reaction buffer (0.2 M NaCl, 4 mM $CaCl_2$, 50 mM Tris, 1% Triton X-100, pH 7.4) at 37ºC in a gently agitation (60 rpm). Gelatine reaction buffer was discarded and stained with Coomassie Blue Staining Solution [0.1% Coomassie Blue R250 in 10% acetic acid solution and 40% (v/v) methanol] during 30 minutes. After destained (20% methanol and 10% acetic acid), both active and inactive forms of the MMP-2 can be visualized (white band on a blue background), according to the molecular weight.

5.8. Matrigel™ invasion assay

Matrigel™ invasion assay was performed using BD Biocoat Matrigel™ Invasion Chambers with 8 micron pore size PET membrane with a thin Matrigel™ layer (BD Biosciences), following manufacturer's instructions. Briefly, chambers were pre-incubated with serum-free medium during 2 hours at 37 ºC. In the upper compartment of the chamber $2x10^4$ (A-549) or $2.5x10^4$ (BT-20) cells were added in completed medium and in the lower compartment only complete medium. After 48h (A-549) or 24h (BT-20) at 37ºC, non-invasive cells were cleared chambers were washed with PBS (four times). Cells were fixed in cold methanol during 10 minutes at 4ºC. Invasive cells attached to the lower surface were stained with DAPI and counted under the microscope (Zeiss). Invasion index is express compared with control (untreated).

5.9. Statistical analysis

For *in vitro* experiments, at least one independent replicate were performed (n=1 to 4 sample/experiment). Experiment performed once was considered preliminary results ($:preliminary results). All p-values were calculated using Student's t-test (two-tailed distribution, two-sample equal variance). Values of $p<0.05$ were considered statistically significant (*:$p<0.05$)

6. Results

Azurin decreases adhesion of breast cancer cell lines to ECM components

As described in the objectives section, after a microarrays analysis of MCF7.AZ/Pcad cell line treated with azurin (100µm, 48h), biological, cell-cell and cell-matrix adhesion were groups that were enriched in the genes down-regulated by azurin (Bernardes *et al.*, 2013). To verify those alterations induced by azurin, we performed adhesion assays to different ECM components (laminin-332, collagen type-I, fibronectin and collagen type-IV), using BSA and plastic coating as controls. Cells were treated with azurin in standard plastic culture, and left to adhere during 30 minutes to the different components. Afterwards, adhesion was quantified by the violet-crystal method.

As we can see in Figure 14, in the MCF7.AZ/Pcad cell line, azurin decreased the adhesion to ECM components, when compared with untreated cells. This effect is more marked after 48h treatment compared with 24h treatment; and also in laminin-332 or collagen type-I (20% less of adhesion) and fibronectin (10% less of adhesion).

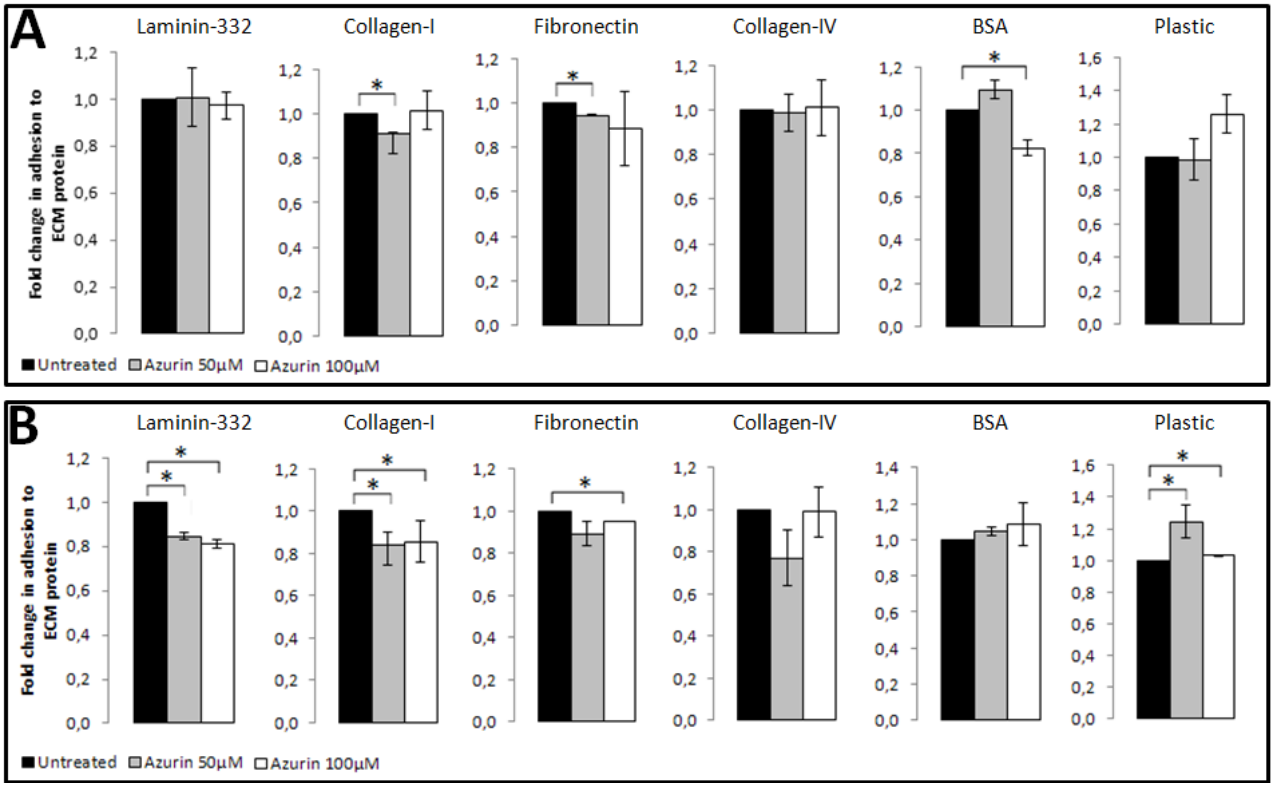

Figure 14: Adhesion assays in breast cancer cells (MCF7.AZ/Pcad). MCF7.AZ/Pcad treated with azurin during 24h (A) and 48h (B); all were let to adhere during 30min in different ECM components (*: $p<0.05$).

In the MCF7.AZ/Mock, this effect (in laminin-332 or collagen type-I) is also observed but only with the higher concentration of azurin and only after 48h of treatment.

Since it had already been demonstrated that azurin can also lead to a decrease in the invasion and P-cadherin levels in SUM149 breast cancer cell model (Bernardes *et al.*, 2013), we also performed adhesion assays in this breast cancer cell line (Figure 15), showing also that azurin decreased cell-matrix adhesion, mainly in laminin-332 and collagen type-IV (in a dose-dependent manner), with a decreased of 20-30% and 10-15%, respectively. A decreased was also observed in fibronectin (less 10% of adhesion) but only with significantly with the lower concentration of azurin.

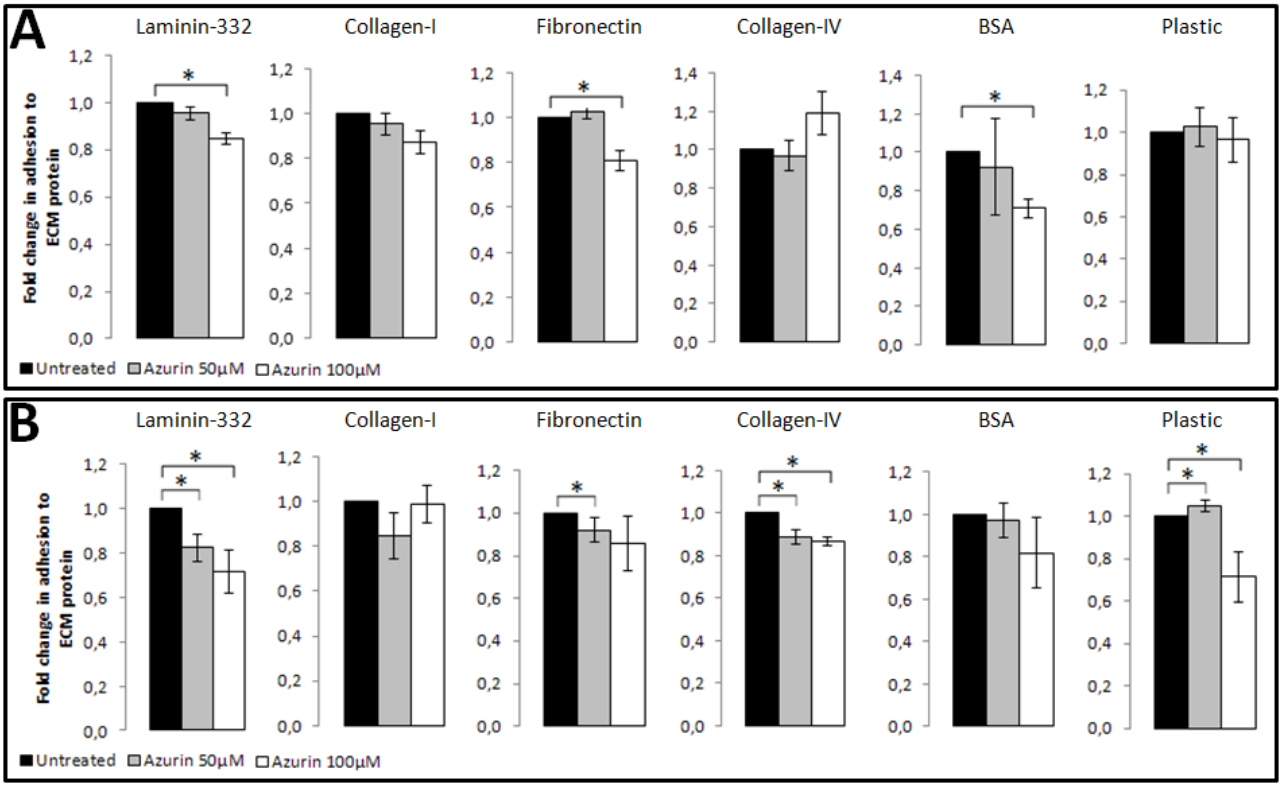

Figure 15: Adhesion assays in breast cancer cell lines (MCF.AZ/Mock and SUM149). MCF.AZ/Mock treated with azurin during 48h (A) and SUM149 treated with azurin during 24h (B); all were let to adhere during 30min in different ECM components (*: $p<0.05$).

Together these results suggest that azurin decreases the adhesion of cancer cells to ECM components, in P-cadherin over-expressing breast cancer, mainly in laminin-332 and collagen matrices.

Azurin alters integrins expression in breast cancer cell lines

The ability of azurin to decrease cell adhesion to ECM components could be dependent on alterations in integrin receptors. Furthermore, it has been demonstrated that in basal-like breast cancers, P-cadherin is co-expressed with CD49f (integrin subunit α_6) (Vieira *et al.*, 2012). For those reasons, we performed Western blot analysis to identify possible alterations of relevant integrin subunits. We looked to the expression of anti- integrin subunits α_6-, β_1- and β_4 due to the fact that β_1-intergin is one of the most common integrin subunits present in heterodimers, and subunits α_6 and β_4 are more specific to laminin and the mostly expressed in normal mammary tissue. Both integrins $\alpha_6\beta_4$, $\alpha_6\beta_1$ have been previously associated with breast cancer migration and invasion (Koistinen & Heino, 2000; Mizejewski, 1999).

In MCF7.AZ/Pcad cell line, results about integrin subunit α_6 are irregular. In plastic conditions, azurin treatments seem to maintain or even increase integrin subunit α_6 (Figure 16). However, in collagen type-I and Matrigel^{TM} matrices, azurin decreases the expression integrin subunit α_6 about 30% related to untreated protein levels. This reinforces the importance of mimic the real tumour microenvironment. Also, integrin subunits β_1 and β_4 are decreased by azurin when cells were cultured in collagen type-I and Matrigel^{TM} matrices: 40-60% in plastic and 50% in Matrigel^{TM} in integrin subunit β_1 level, and 25% in plastic and 20-40% in collagen type-I in integrin subunit β_4 level. However, in plastic conditions, in the lower concentration of azurin, we observed an increase in integrin subunit β_1 level, which was not observed in a higher concentration, which could be due to an experimental error or to a different mechanism that cells use to compensate the entry of azurin, that was no longer tolerated to the higher concentration, as observed in cells cultured on top of the protein matrices used.

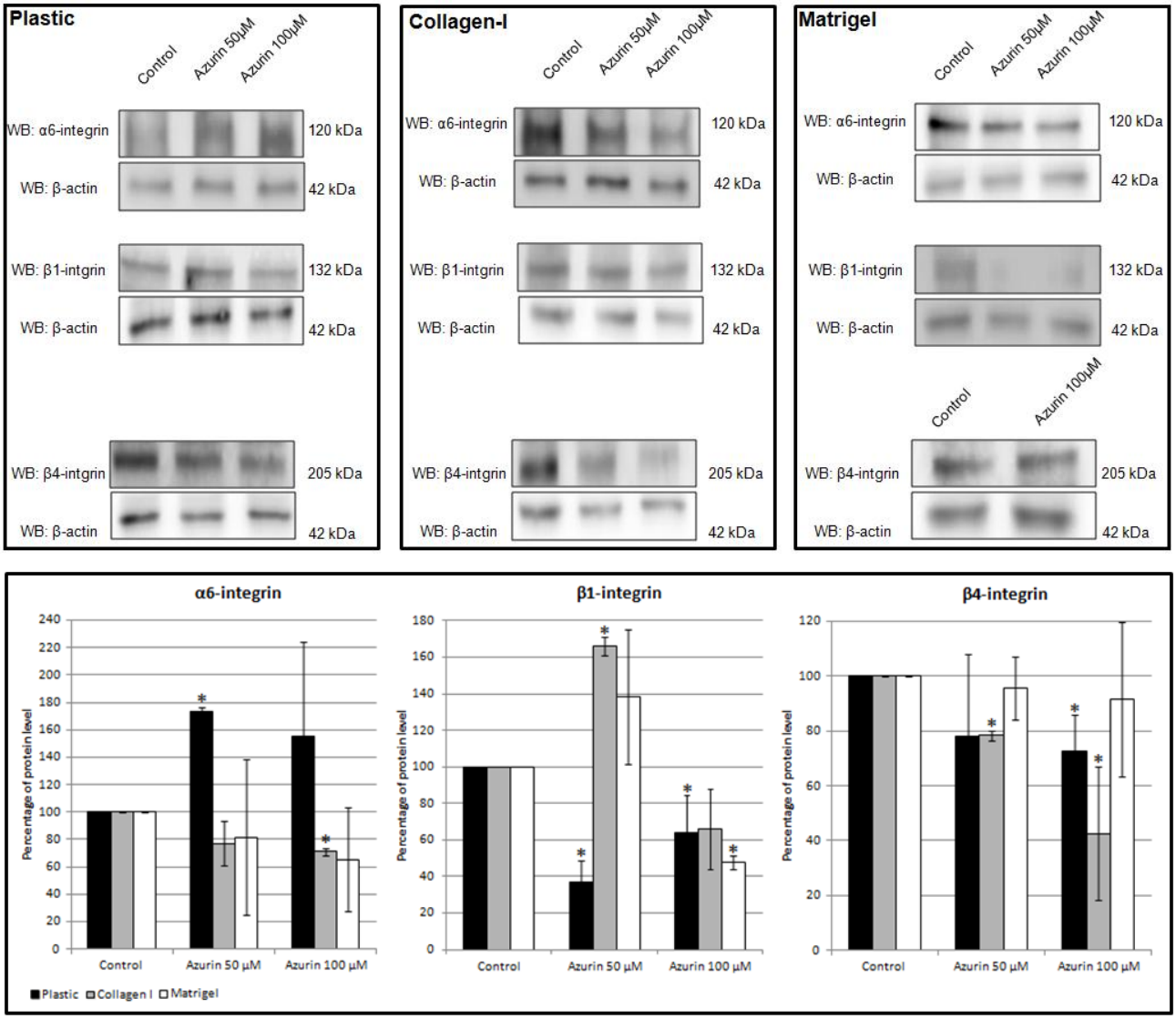

Figure 16: Azurin decreases protein expression of integrins (MCF-7/AZ.Pcad). MCF-7/AZ.Pcad were treated with azurin (50µM and 100µM) during 48h in plastic conditions, collagen type-I matrix or Matrigel™ (*: $p<0.05$).

By immunocytochemistry it was possible to verify that azurin decreases integrin subunits levels and membrane localizations, using MCF-7/AZ.Pcad cell line (Figure 17). In untreated cells, integrin subunits α_6 and β_1 are localized in the membrane of the cell, however, after treatments with azurin, integrins are no longer in membrane and it seem to be compartmentalised inside the cells.

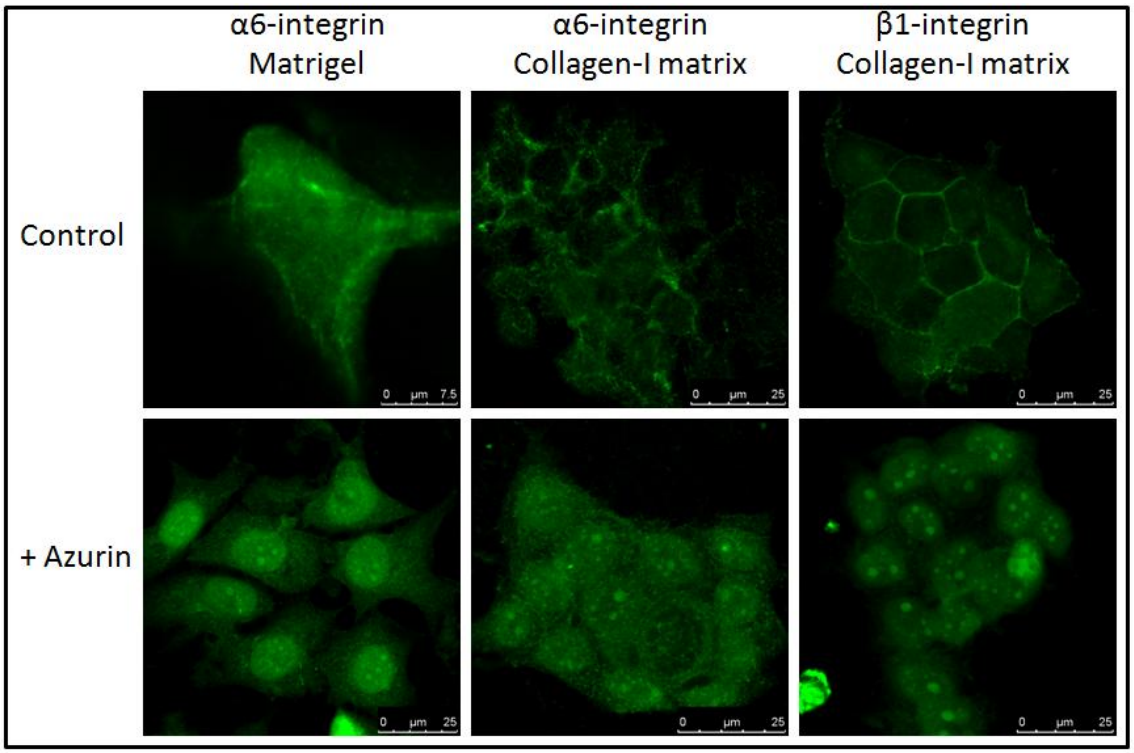

Figure 17: Azurin decreases integrin subunits and re-localized in MCF-7/AZ.Pcad cell line.

In SUM149 cell line (Figure 18), integrin subunit α_6 levels decreases significantly in plastic (45-80%), in collagen type-I (30-40%) and Matrigel™ (40%). integrin subunit β_1 levels also decreases, about 20-60% in plastic and 50% in Matrigel™. integrin subunit β_4 level decrease 50% in plastic and seems to decrease in collagen type-I. However, integrin subunit β_4 level increases 50% in Matrigel™.

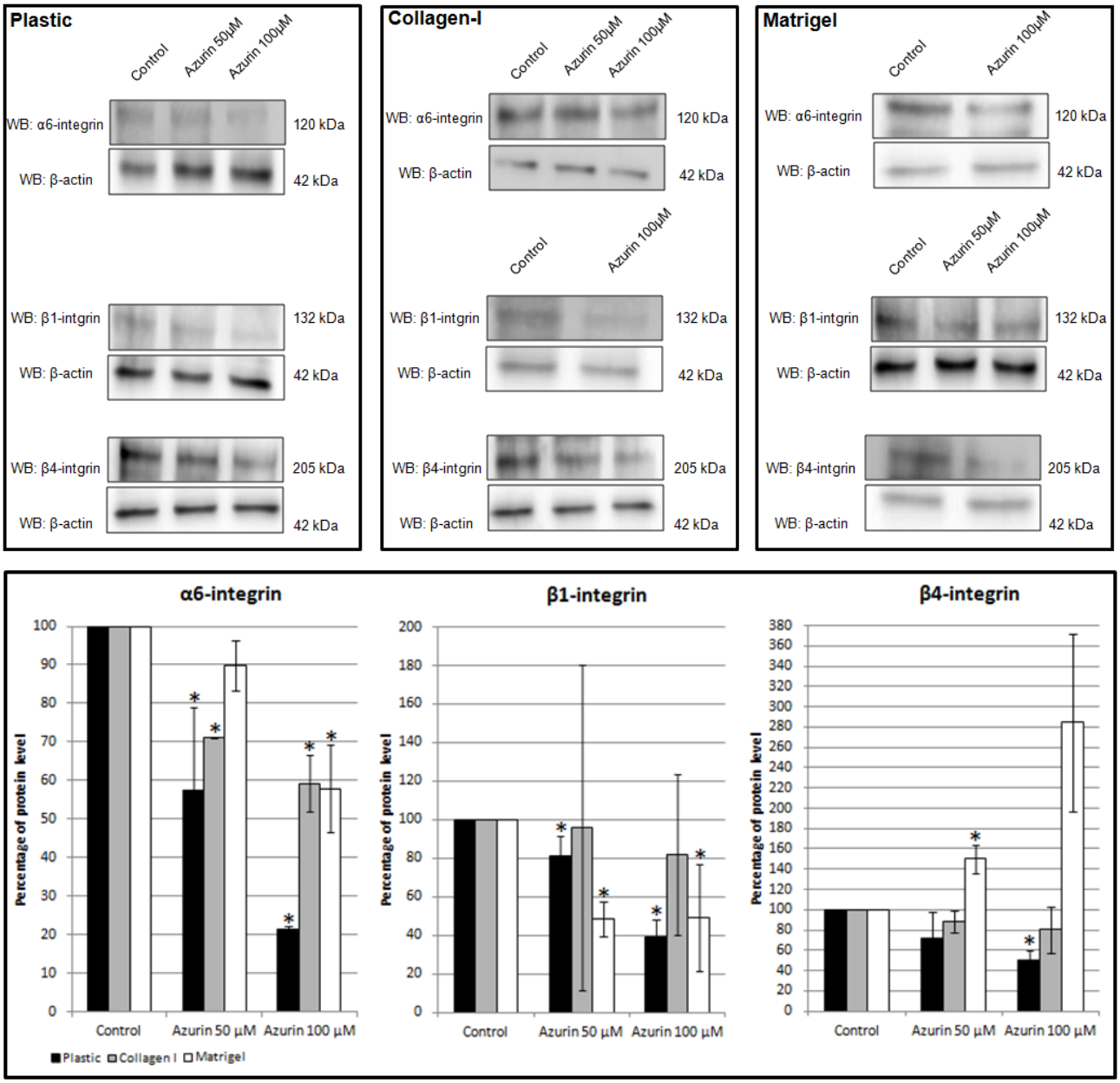

Figure 18: Azurin decreases protein expression of integrins (SUM149). SUM149 were treated with azurin (50µM and 100µM) during 24h in plastic, collagen type-I matrix or Matrigel™ (*: $p<0.05$).

Together these results suggest that azurin decreased these integrin subunits levels. However, in MCF7.AZ/Pcad cell line integrin subunit α_6 seems to increase and in SUM 149 integrin subunit β_4 level seems to increase. In both breast cancer cell lines with P-cadherin over-expression, integrin subunit β_1 level decrease, suggesting that it is very important in breast cancer progression.

Azurin alters ROS production

ROS, and consequently oxidative stress and apoptosis via p53, may regulate integrin-mediated cellular response (ex: cell adhesion and migration). Caveolae/lipid rafts, focal adhesions and cell-cell contacts can target and activate NADPH oxidases, leading to ROS production and redox signalling. But also, integrin activation, in turn, increases ROS production (Goitre *et al.*, 2012). Using N-acetylcysteine, an anti-oxidant, hydrogen peroxide, which is a ROS, decreased and, consequently, also cell adhesion, proving that ROS production has a role in the signalling cascade triggered by integrins during cell-ECM interactions. Also, ROS production is mediated in part by the up-regulation of FAK (a pathway that induces cell adhesion) (Chiarugi *et al.*, 2003). ROS regulation could be a potential target to cancer therapy through impairment of cell adhesion receptors (Goitre *et al.*, 2012).

For that reason, ROS production was measured in the absence/presence of azurin (Figure 19). MCF7/AZ cell lines were exposed during 48h; and SUM149 and J774 during 24h (time of treatment depended on the cell line and the observed P-cadherin alteration (Bernardes *et al.*, 2013). As a positive control we used macrophages J774, where it had been previously showed that azurin induces ROS production (Yamada *et al.*, 2002b). As seen in the previous sections, azurin can decrease cell-matrix adhesion and integrin levels, suggesting that the decrease of ROS production (and oxidative stress) observed in MCF7.AZ/Pcad and MCF7.AZ/Mock is through NADPH oxidases inactivation. In fact, in the referred microarray analysis indicate a reduced expression of NADPH and others genes related with oxidative stress (unpublished data).

Because we observed in both MCF7.AZ a significantly decrease of ROS production, it is possible that ROS decreased is independent of P-cadherin. Also, as previously showed, azurin decreased FAK/Scr signalling (Bernardes *et al.*, 2013), so it may be possible that azurin acts ROS-dependent manner in MCF7.AZ mediated by FAK/Src signalling. However, SUM149 showed an increase of ROS production with azurin, suggesting that azurin can induce different pathways of actions. Probably, due to the fact that MCF7 breast cancer cell line is basal-like and SUM149 breast cancer cell line is luminal-like. To deepen, ROS measurement should be done in BT-20 (another basal-like breast cancer cell line) and A549 (lung cancer cell line).

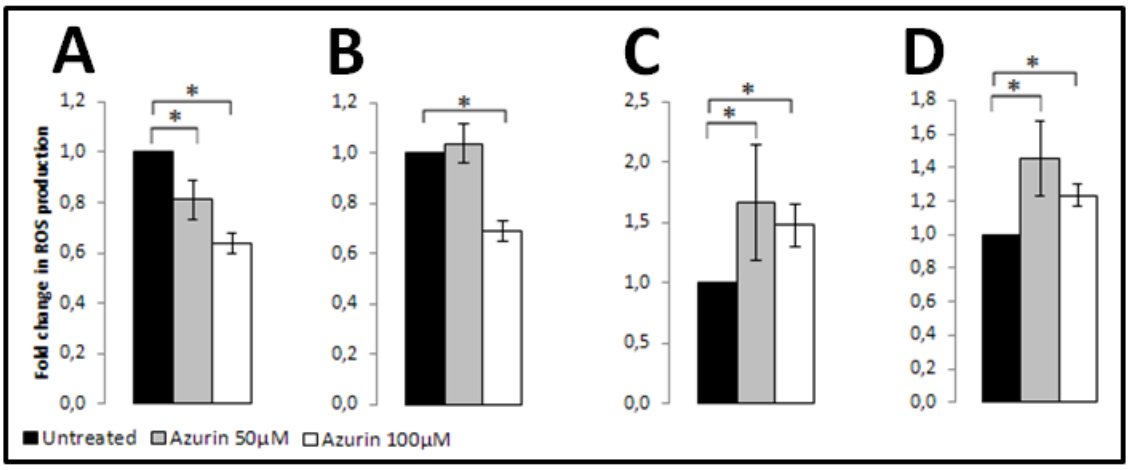

Figure 19: ROS measurement in breast cancer cells. MCF7.AZ/Pcad (A) and MCF.AZ/Mock (B) treated with azurin during 48h, SUM149 (C) and J774 (D) treated with azurin during 24h (D) (*: $p<0.05$).

Azurin alters integrins expression, invasion capacity and MMP activity in BT-20 cell line

To validate these results, we used another P-cadherin over-expressing breast cancer cell line, BT-20. This cell line was previously assessed for the anticancer activity of azurin, and cells were treated plastic growth conditions, however, in that conditions, no alterations were found in P-cadherin levels upon treatment. However, growing cells in a collagen type-I matrix or Matrigel™, azurin had an impact at this protein expression levels as well as at integrin subunits (Figure 20).

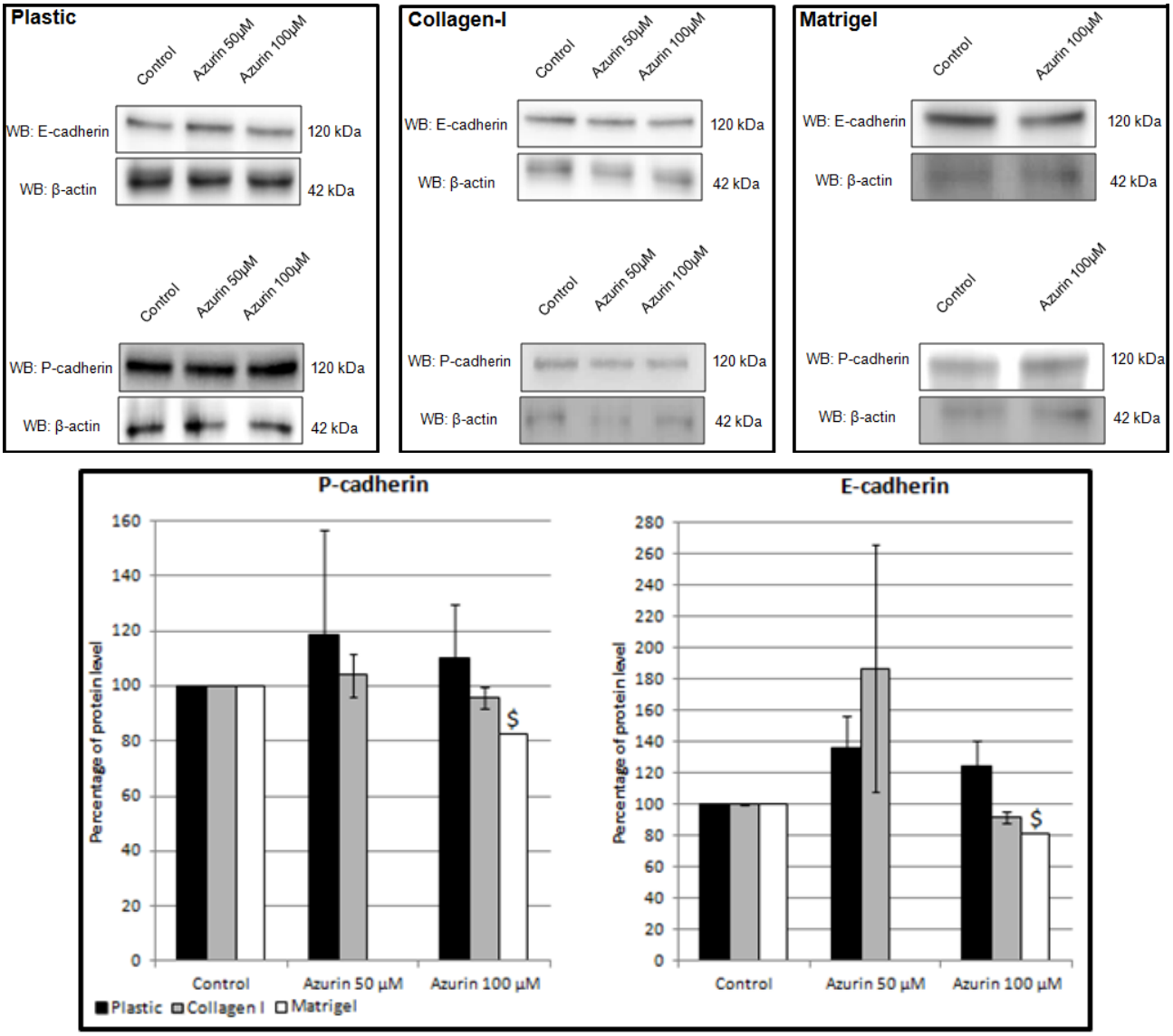

Figure 20: Azurin decreases P-cadherin in ECM components but not plastic (BT-20). BT-20 were treated with azurin (50μM and 100μM) during 24h in plastic conditions, collagen type-I matrix or Matrigel™ ($: preliminary results).

As seen in Figure 21, on BT-20 breast cancer cell line, integrin subunit α_6 levels decreased in collagen type-I and Matrigel™ matrices; integrin subunit β_1 level decreases 50% with the

higher concentration of azurin used (100μM); and integrin subunit β_4 levels increase in collagen type-I and decrease 20% in Matrigel™. The result more significant and consistent is the decrease in integrin subunit β_1 level.

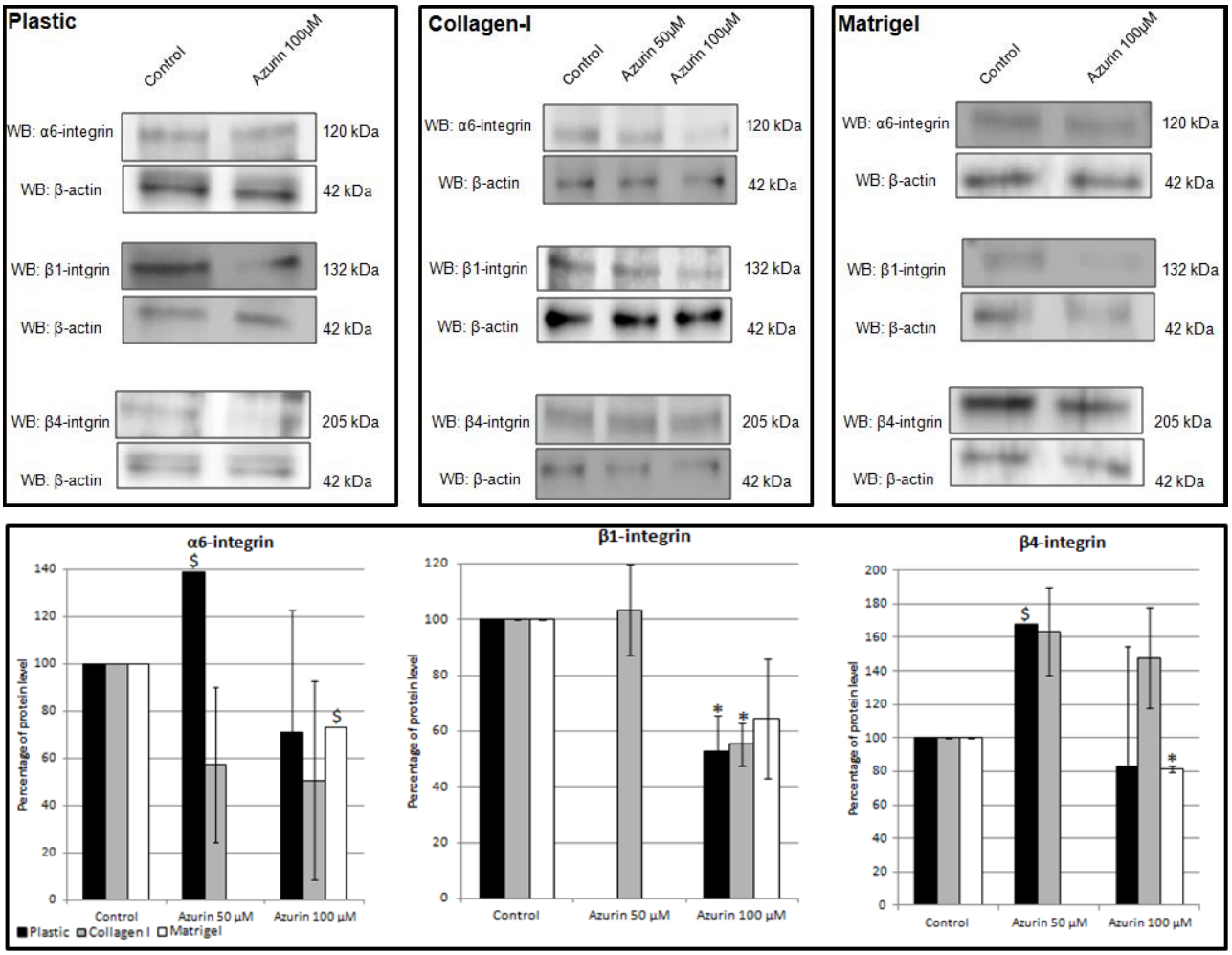

Figure 21: Azurin decreases protein expression of integrins (BT-20). BT-20 were treated with azurin (50μM and 100μM) during 24h in plastic conditions, collagen type-I matrix or Matrigel™ (*: $p<0.05$; $: preliminary results).

It has been previously demonstrated that P-cadherin-induced invasion is mediated, at least in part, by the secretion of MMP-1/-2 to the extracellular media (Ribeiro *et al.*, 2010). In MCF-7/AZ.Pcad and SUM149 cells, azurin induce a decrease in MMP-2 activity (Bernardes *et al.*, 2013). In order to perform that analysis, cells are gown in a collagen type-I matrix, to maximize MMPs secretion. Taking our previous results into account, we also analyzed if in BT-20 cell line, azurin also produced the same effect. Indeed, also, azurin decreases MMP-2 activity in BT-20 (Figure 22).

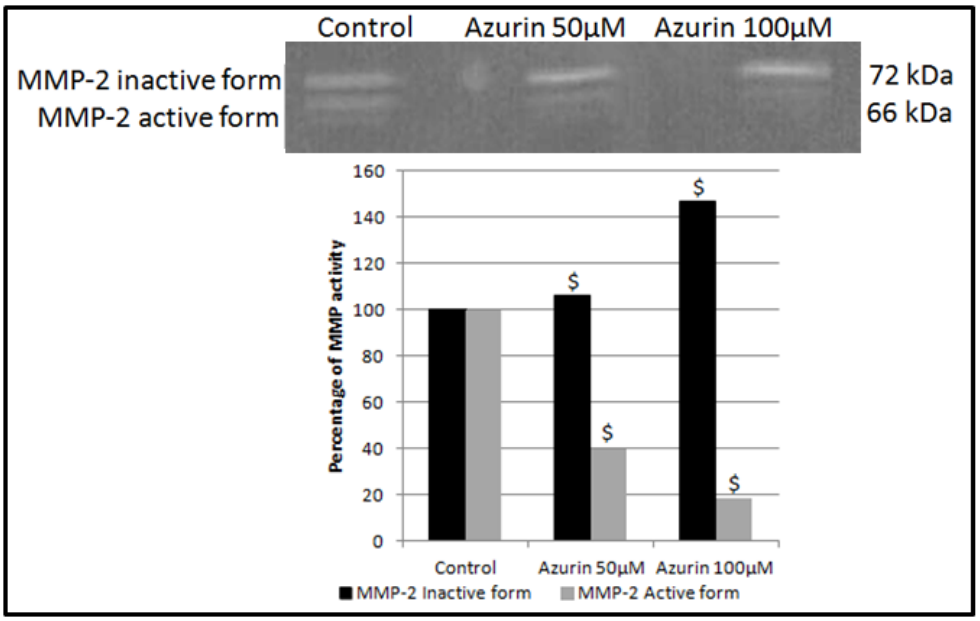

Figure 22: Gelatine zymography (BT-20). BT-20 cells grown in collagen type-I matrix, conditioned medium were used to observed MMPs activity ($: preliminary results).

BT-20 cell line is also a breast cancer cell line over-expressing P-cadherin, like MCF7-AZ/Pcad and SUM149, and in all three integrin subunit β_1 level decrease, reinforcing the importance of integrin subunit β_1 in cancer progression, like in invasion.

Beyond the decrease in MMP-2 activity, our group has also showed that azurin decreases invasion of P-cadherin over-expressing breast cancer cells (Figure 23). With the lower concentration of azurin (50µM), the invasion capacity of BT-20 decreased 20% and about 45% when cells were treated with 100µM. This is consistent with invasion results in MCF-7/AZ.Pcad and SUM149 cells, with a reduction of 66% and 44% of invasion, respectively (Bernardes *et al.*, 2013).

Together these results indicate that azurin in BT-20 decrease integrins (involved in adhesion), invasion capacity and MMP activity (involved in migration).

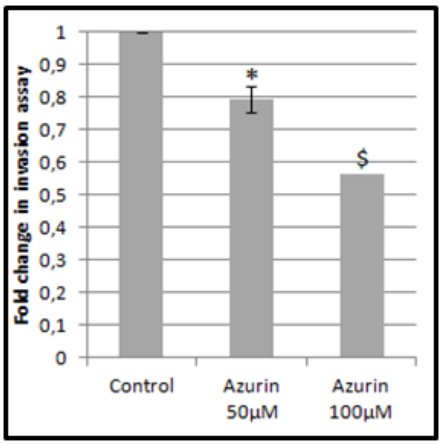

Figure 23: Invasion assay in Matrigel™ (BT-20). BT-20 cells were treated with azurin during 48h (*: $p<0.05$; $: preliminary results).

Azurin alters adhesion to ECM components, invasion and integrins expression in A549 lung cancer cell line

To identify some general impact of azurin in other cancer cell models, A549 non-small cell lung cancer was treated with azurin during 48h to evaluate some of the effects identified in the previous models. A decrease, in a depend-dose manner, in adhesion to ECM components was observed in all matrices, with statistical significance in collagen type-I (decrease of 20-60%), in fibronectin (decrease of 60%) and in collagen type-IV (decrease of 30%) (Figure 24).

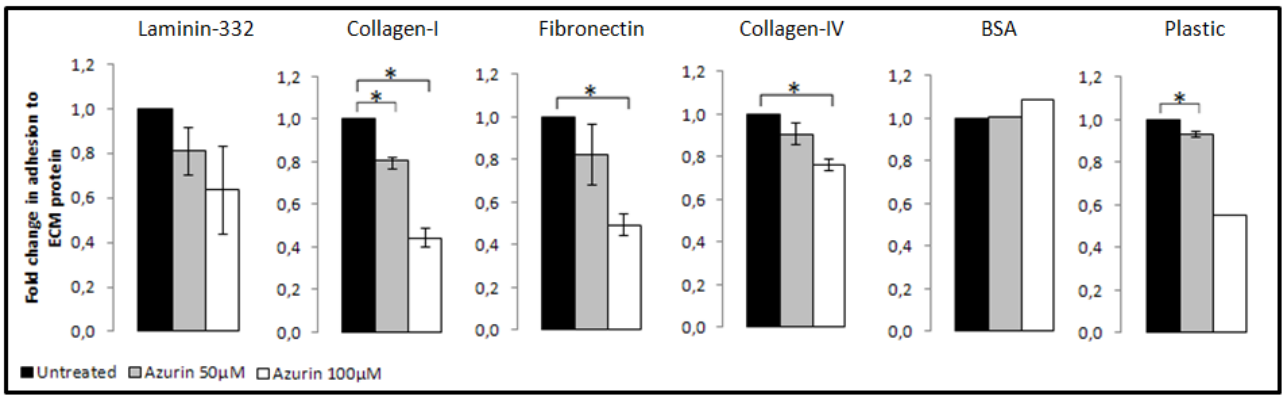

Figure 24: Azurin decreases adhesion in different ECM components (A549). A549 lung cancer cell line were treated with azurin during 48h and let to adhere during 30min in different ECM components (*: $p<0.05$).

We analyzed by western blot some of the relevant protein in cell-cell adhesion (E-cadherin) and cell-to-matrix adhesion (integrin subunit β_1) (Figure 25). A549 exhibits high E-cadherin expression but no detectable P-cadherin expression (Zhang *et al.*, 2010). For that reason, we only evaluate E-cadherin expression.

We observed an increase in E-cadherin levels upon azurin treatment and a decrease of integrin subunit β_1 level (cell-matrix adhesion). In this cell line, increased integrin subunit β_1 is correlated with decreased overall survival and recurrence-free survival (Yao *et al.*, 2007). A study reveals that lung metastasis in integrin subunit β_1 deficient mice revealed a two-fold reduction in the number of mice that developed metastasis and a six-fold reduction in the number of metastasis; and also a reduction of cell survival and angiogenic infiltration. Understanding the role and influence of each specific heterodimer in mammary tumourigenesis might be essential to develop a more selective therapeutic approach like of integrin subunit β_1 inhibitor (Lahlou & Muller, 2011).

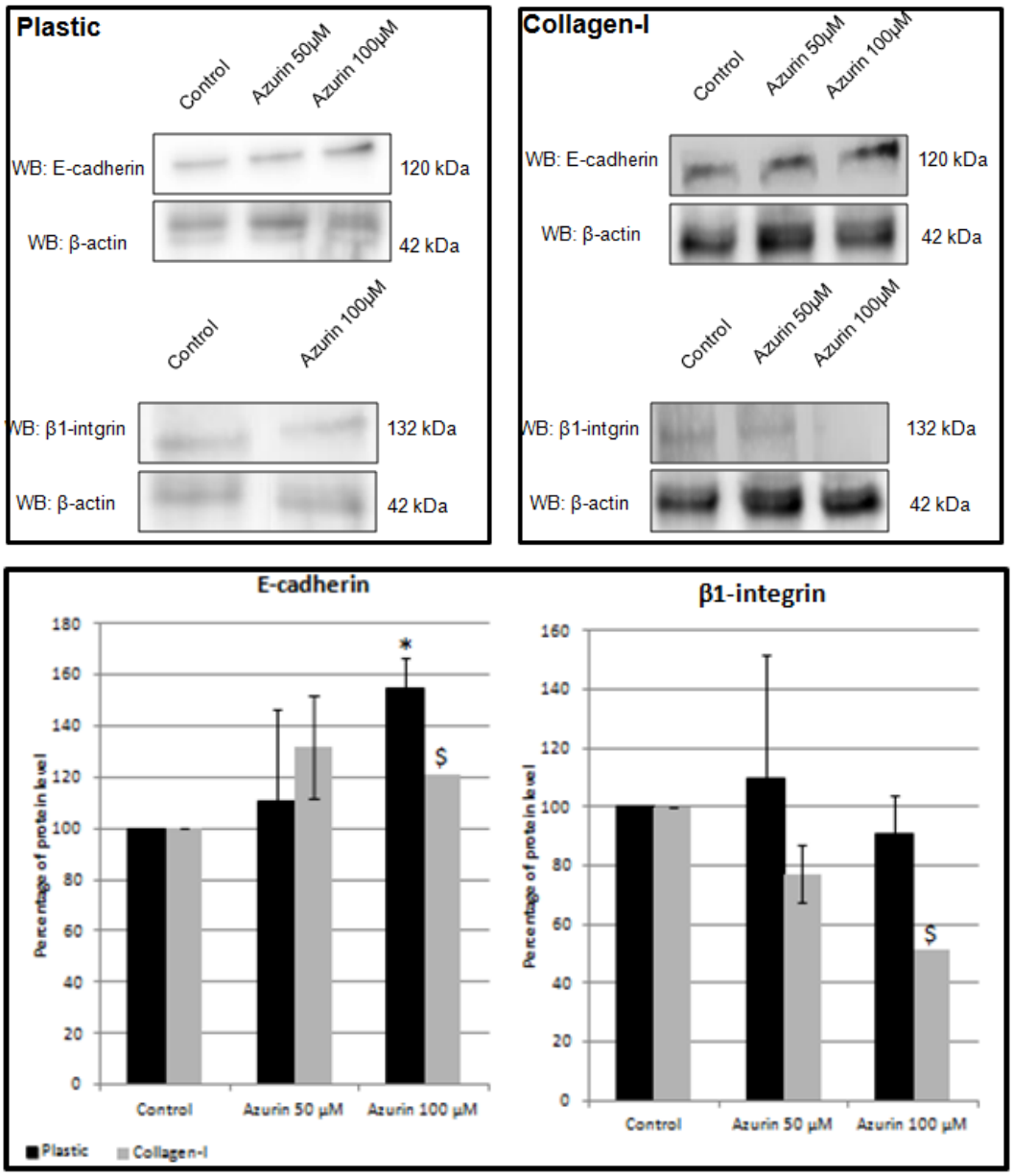

Figure 25: Azurin decreases protein expression of E-cadherin and integrin subunit β_1 (A549). A549 were treated with azurin (50μM and 100μM) during 48h in plastic conditions or collagen type-I matrix (*: $p<0.05$; $: preliminary results).

Also, and although preliminary, we analyzed the activity of MMP-2 and invasion through Matrigel™, as functional consequences of treating these cells with azurin (Figure 26). We observed an decrease of MMP-9 active form activity and a 25-40% decreased of invasion capacity was observed. Altogether, these results suggest that azurin has an impact in integrin subunit β_1, adhesion and invasion in non small cell lung cancer, suggesting that these phenomena are a broad line of action of this anticancer bacterial protein despite the tumour origin.

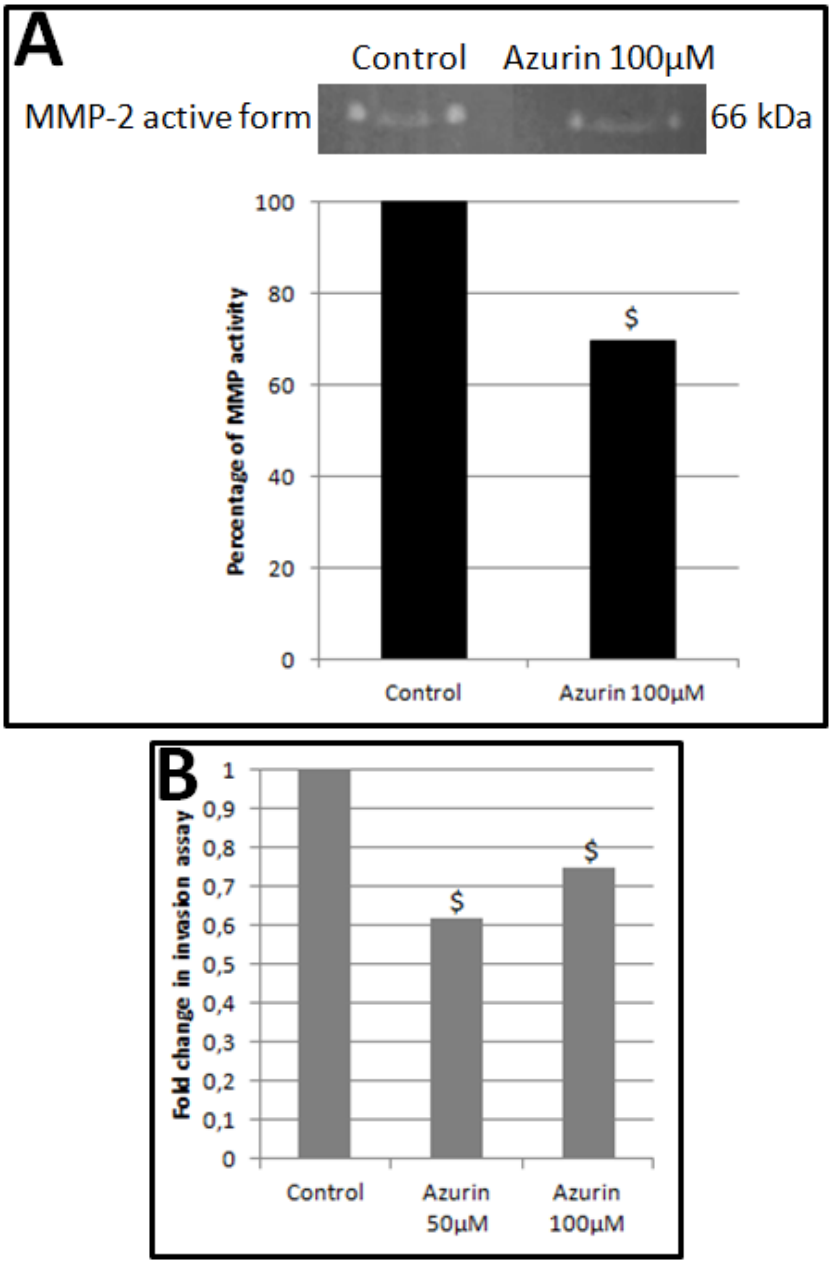

Figure 26: Azurin decreases MMP-2 activity and invasion capacity (A549). (A) Gelatine zymography, cells grown in collagen type-I matrix, conditioned medium were used to observed MMPs activity. (B) Invasion assay in Matrigel™, cells were treated with azurin during 48h. ($: preliminary results).

7. General discussion

P-cadherin expression in breast carcinomas is a marker of poor survival (Paredes *et al.*, 2007). On one hand, P-cadherin interferes with the normal invasive suppressive function of E-cadherin; on the other hand, there is no targeted therapy to this protein. For that reason, azurin was used to target P-cadherin, decreasing its level in P-cadherin over-expressing breast cancer models (Bernardes *et al.*, 2013). However, the mechanism of action of azurin is not well known. After treat MCF-7/AZ-Pcad cell line with azurin during 48 hours, a microarrays analysis was performed in which biological adhesion and cell-cell adhesion were two groups of genes that are down-regulated (Bernardes *et al.*, 2013, submitted).

Not much is known about how the stromal microenvironment at metastasis sites provides a suitable home to tumour cells. It is important to study the interaction between metastatic cells and niche cells, and between metastatic cells and ECM (Muschler & Streuli, 2010). The ECM of the basement membrane acts as a barrier and also as helper for cancer cells to migrate (Tsuruta *et al.*, 2008). For that reason, understanding the interaction with the microenvironment is extremely important.

In order to achieve a functional validation of the microarrays analysis, adhesion assays were performed to understand if azurin can alter cell adhesion to different ECM components (laminin-332, collagen type-I, fibronectin and collagen type-IV). For that, four breast cell lines expressing different level of P-cadherin were used: MCF-7/AZ.Mock and MCF-7/AZ.Pcad (p53 wild type), SUM149 and BT-20 (constitutively over-expresses P-cadherin and mutant p53) and A549 NCCLC cell line. In general, azurin decreased adhesion to all ECM components, but more significantly to collagen type-I and laminin (breast cancer) and fibronectin (lung cancer). In fact, in the mammary gland, the main ECM components found are laminin and collagen (Tsuruta *et al.*, 2008) and, in lung microenvironment, is fibronectin (Ritzenthaler, Han, & Roman, 2008).

Luminal cells contacting the stromal ECM, such as collagen type-I, is a feature known to lead to signalling into aberrant transformations, up-regulation of MMPs, invasion and metastasis (Bissell *et al.*, 2011). Targeting cell-matrix interaction could improve cancer therapy; such as matrix-degrading proteases inhibitors (target ECM modifications) and integrins inhibitors (target angiogenesis inhibition). Target integrins can enhance the responsiveness of breast tumour cells to radiation and Her-2 targeting. The targeting of cell-ECM interaction could be a standard component of the oncologist's therapeutic arsenal (Muschler & Streuli, 2010).

Because integrins are the main receptors in adhesion process and because CD49f (integrin subunit α_6) is co-expressed with P-cadherin (Vieira *et al.*, 2012), western blots were done to understand if azurin alters integrin subunits levels. In MCF-7/AZ.Pcad and SUM149, azurin decreased integrin subunits levels (α_6, β_1 and β_4). However, in MCF7.AZ/Pcad cell line integrin subunit α_6 seems to increase and in SUM 149 integrin subunit β_4 level seems to increase. In both P-cadherin over-expressing breast cancer cell lines, integrin subunit β_1 level decreased.

Also, in A549 lung cancer, integrin subunit β_1 levels decreased. In previous studies, shikonin, an active naphthoquinone, showed effective anti-cancer activity both *in vivo* and *in vitro*. Shikonin suppresses lung cancer adhesion, invasion and metastasis by inhibiting integrin subunit β_1 expression and the phosphorylation of ERK1/2, decreasing this signaling pathway (Wang *et al.*, 2013). So, maybe azurin in A549 cell line reduces integrin subunit β_1 level and adhesion via ERK. This hypothesis needs to be confirmed by western blot.

The expression levels of integrin subunits α_5, β_1 and β_3 predicted overall survival and disease free survival in NSCLC patients. For that, determining the integrin expression profile might serve as a tool in predicting the prognosis of individual patients (Dingemans *et al.*, 2010).

Together, these results suggest that different models with different characteristics and different microenvironment respond to azurin by the same signalling pathways through decreasing integrin subunit β_1. For that azurin could be a potential anti-invasion drug through decreasing integrin subunit β_1 levels.

Integrin subunit β_1 signalling has a crucial role for the focal adhesion kinase axis, due to the fact that integrin do not have enzymatic activity or actin binding domain. However, integrin subunit β_1 is able to bind to partners (ex: talin, tensin) that allows cytoskeleton remodelling and activation signalling cascade (cell adhesion and motility), but also to FAK, paxillin and Src that allows a scaffold function. FAK, that have a central role in integrin subunit β_1 signalling, is recruited by integrin subunit β_1, where two NXXY motifs in C-terminal are important, inducing its auto-phosphorylation on Y397 and consequently recruitment of c-Src. integrin subunit β_1 controls the expression of oestrogen receptor α, and in turn oestrogen and progesterone regulate $\alpha_5\beta_1$ expression. integrin subunits β_1 and α_6 are the only ones that are prove to be indispensable for appropriate mammary gland development (Lahlou & Muller, 2011).

In 3D culture and *in vivo*, it has been shown that anti-integrin subunit β_1 induces a dormant-like phenotype, impairing proliferation but with a reversible effect (White & Muller, 2007). Integrin subunit β_1, via uPA receptor and a complex containing FAK, in a fibronectin matrix induces cell proliferation through Ras-ERK pathway. Fibronectin:integrin:uPAR complex is required to reverse de dormant state. Curiously, integrins have two contradictory side, one in their role in dormancy (attenuating the cancer cell proliferation) and another by been a target to cancer therapy (White & Muller, 2007).

Nevertheless, down-regulation of integrins does not mean that these integrin subunits are unimportant to malignant phenotype of cancer cells, because optimal migration and invasion depend on ligand concentration, integrins expression and ligand-integrin affinity (Koistinen & Heino, 2000).

Recently, it was showed that dormant tumour cells may be resistant to chemotherapy and radiation. Integrin subunit β_1 regulates the switch from a dormant state to active proliferation and metastasis. Like ATN-161, volociximab, and JSM6427 target integrin subunit β_1 signaling to aim dormant cancer cells (Barkan & Chambers, 2011), maybe azurin act in this same way.

Our group has previously demonstrated that phosphorylated FAK and its partner Src were decreased P-cadherin breast cancer models upon azurin treatment, concomitantly with decreased invasion and P-cadherin levels. FAK and Src are important non-RTKs that can be activated by integrin engagement by the ECM (Bernardes *et al.*, 2013). So, azurin decreases integrin subunits levels, consequently it decreases phosphorylated FAK/ Src, that in turn decreases signaling, leading to a decreases of adhesion, invasion, migration and metastasis processes.

Several tumourigenic processes are mediated by MMPs, namely the breakdown of extracellular components, which accounts greatly to the ability of tumour cells to invade the surrounding tissues through an extensive matrix remodelling. MMPs also promote the release of bioactive molecules able to induce invasion, like the cleavage of laminin-5 γ2 chains by MMP-2, producing a fragment containing an epidermal growth factor (EGF)-like domain, which induces integrin signaling and cell migration. We assessed the activity of MMP-2, by gelatine zymography, of BT-20 breast cancer cell line and A549 lung cancer cell line treated with azurin and could observed a decrease in its activity. An effect also observed in other P-cadherin over-expressing breast cancer cell line (Bernardes *et al.*, 2013).

As previously showed, azurin decreased the invasion capacity in MCF-7/AZ.Pcad and SUM149 (Bernardes *et al.*, 2013). Curiously, the effects on cell invasion seem to be related with a specific decrease in P-cadherin protein. This specificity shown for azurin effect on cadherins is very interesting, since P-cadherin expression is correlated to increased cell motility, cell migration and invasion (Bernardes *et al.*, 2013, submitted). However, it is important to refer that this effect invasion capacity was observed not only in breast cancer cell models (MCF-7/AZ, SUM149 and BT-20) but in other distinct cancer cell model (A549 lung cancer), suggesting that azurin could be a potential therapeutic drug in different cancers, by its anti-invasion role, and consequently decreasing migration and metastasis processes.

The loss of integrin-mediated cell–ECM contact results in an apoptotic process termed anoikis (Giannoni *et al.*, 2008), and plays an essential role in the regulation of cancer cell metastasis (Rungtabnapa *et al.*, 2010). However, cancer cells are able to resist to anoikis (Mizejewski, 1999). NF-κB promote integrin subunit β_4 expression to mediate resistance to apoptosis and p63 induce integrin subunit β_4 expression to mediate resistance to anoikis, via STAT3 (Pontier & Muller, 2009). Another study proved that cancer cells over-expressed laminin and integrin subunit β_4, both promoting survival, leading to anoikis resistance too (Kim *et al.*, 2012). Also, cancer cells increases ROS level to promote survival and resistance to anoikis. Since azurin decreases integrin subunit β_4 levels and ROS in MCF-7/AZ.Pcad, maybe cancer cells are less resistance to apoptosis and anoikis. A hypothesis that indicates that azurin is a possible new therapeutic strategy.

8. Main conclusions and future perspectives

Azurin (50 μM and 100 μM) was used to treat breast cancer cell lines with distinct levels of P-cadherin expression and different invasive capacities (MCF-7/AZ.Mock, MCF-7/AZ.Pcad, SUM149, BT-20) and one non-small cell lung cancer (A549). We investigated the effect of azurin in cell adhesion to different ECM components (laminin-332, collagen type-I, fibronectin and collagen type-IV). We also quantified integrin subunits (α_6, β_1 and β_4) expression by Western blot. The azurin effects was measured by others parameters, such as ROS measurement, immunocytochemistry, gelatine zymography to evaluate MMP-2 activity and invasion capacity.

Azurin decreased integrin subunits (α_6, β_1 and β_4) in all studied models (breast and lung cancer), but more consistently integrin subunit β_1. Moreover, azurin decreases adhesion to all ECM components, but with more significance to collagen and laminin (breast cancer) and fibronectin (lung cancer), both main components in each cancer type. In BT-20 and A549, azurin decrease MMP-2 activity and consequently invasion capacity. All this is a functional validation of microarrays analysis performed in MCF-7/AZ.Pcad with azurin (100 μM, 48h) that corroborate that azurin is a potential cancer therapeutic drug.

Together, these results suggest that different models with different characteristics and different microenvironment respond to azurin by the same signalling pathways through decreasing integrin subunit β_1. For that azurin could be a potential anti-invasion drug through decreasing integrin subunit β_1 levels.

For following this particular work, in order to understand if azurin may have a general impact in integrins subunits in different cancer cells models, western blots in A549 lung cancer should be performed to investigate other subunits beyond integrin subunit β_1. Integrin subunit β_4 role in autophagy of lung adenocarcinoma cells is not clear. A study reveals that siRNA of β_4 increases dead cells and level of p53; suggesting that integrin β_4 is implicated in and associated with p53 in autophagy of lung cancer cells (He *et al.*, 2008). Also, immunocytochemistry should be done in all studies models to localize and visualise integrins subunits.

Azurin (or its derived peptide - p28) penetrates in cancer cells faster than in normal cells (Yamada *et al.*, 2005), by a mechanism that doesn't cause plasma membrane disruption but depends on some of its components. For example, it is known that cholesterol removal from the plasma membrane of cancer cells, using methyl-β-cyclodextrin, significantly reduced the azurin entry (Yamada *et al.*, 2009). After performing the microarray analysis of azurin treated breast cancer cells (MCF-7/AZ.Mock and MCF-7/AZ.Pcad) it was also revealed an up-regulation of genes associated vesicle transport and pathways associated with the lysosome, but also genes associated with endocytosis, membrane organization and endosome transport (Bernardes *et al.*, 2013). Protein degradation should be investigated to validate microarrays analysis, but also to identify the degradation pathway of integrins subunits.

Endocytosis and trafficking are also major mechanisms controlling signaling at the plasma membrane level. The mechanism by which azurin exerts its anti-cancer effects may depend on its route of cancer cell entry, disrupting caveolae and removing from the cell membrane selective receptors that may be over-activated. In cancer cells, the removal of functional receptors from cell surface and their targeting to lysosome was proven to be an important mechanism by which their permanent activation and consequent tumourigenesis is prevented, particularly to EGFR (Abella & Park, 2009). In anchorage-dependent cells, loss of integrin signaling stimulates caveolin-1 dependent internalization of lipid rafts (Rho GTPases, Erk, and PI3K) and transport to recycling endosomes, leading to a change in membrane organization (Norambuena & Schwartz, 2011). Caveolin-1 is a key protein involved in tumour metastasis. A study suggests that Cav-1 (down-regulated during cell detachment) plays a key role as a negative regulator of anoikis through ROS-dependent mechanism in human lung carcinoma (up-regulated during cell detachment) (Rungtabnapa *et al.*, 2010). Also, EGFR represents the main target for non-small cell lung cancer therapy, like A549. A study reveals that integrin subunit β_1-silenced cells show a defective activation of the EGFR signaling cascade, leading to decreased proliferation, migration and invasive behaviour. Integrin subunit β_1 silencing might represent an adjuvant approach to anti-EGFR therapy (Morello *et al.*, 2011). For those reasons, Cav-1 and EGFR level should be investigate by western blot (protein level) and qRT-PCR (mRNA level), in different cancer cells models and in different cancer cells models grown in different ECM components.

Cell migration involves cycles of cell-matrix adhesion/detachment that is regulated by integrin-based focal adhesions. Integrin subunit β_1 is internalized in a dynamin-dependent manner and it is need cholesterol and reduced lipid raft protein, caveolin-1. Furthermore, internalized integrin subunit β_1 is co-localized with lipid rafts marker and is via dynamin-dependent lipid raft-mediated pathway (Vassilieva *et al.*, 2008). The results suggest that azurin target integrin subunit β_1, so maybe azurin act via alteration of lipid raft.

To deepen the subject about the impact of azurin in A549, PI3K should be evaluated by western blot to better understand signalling associated with integrins; ROS measurement should be done to evaluate oxidative stress level; and treatment only with methyl-β-cyclodextrin (that disturb lipid raft) should be performed to investigate if lipid raft affects integrins level.

EGFR represents the main target for non-small cell lung cancer therapy, as its over-expression or constitutive activation contributes to malignancy and correlates with poor prognosis. Integrin subunit β_1 is required for propagating EGFR signaling. Silencing integrin subunit β_1 decreases EGFR signalling, increases sensitivity to cisplatin and gefitinib and consequently impairs migration and invasive behaviour (Morello *et al.*, 2011). Following this idea, synergetic potential of azurin with gefitinib (EGFR inhibitor used in NSCLC) should be assess.

This set of information will allow the better understanding the mechanism involved in azurin entry, but also accentuate the conclusions observed in breast cancer cell lines, to check an overall effect of azurin on cancer (independent of their origin), reinforcing the idea that azurin is a potential anti-invasive drug, by decreasing integrin subunit β_1 and consequently the signalling involved.

9. References

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