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**NUTRITIONAL AND HORMONAL MODULATION
OF HUMAN MELANOMA PROGRESSION**

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ABBREVIATIONS

5-HETE: 5-Hydroxyeicosatetraenoic acid

5-LOX: 5-lipoxygenase

AA: arachidonic acid

AAAD: aromatic L-amino acid decarboxylase

ACTH: adrenocorticotrophic hormone

Akt: serin-threonin protein kinase

AMF: autocrine motility factor

AP1: activating protein 1

APAF1: apoptotic protease activating factor 1

AR: adrenergic receptors

ARE: antioxidant responsive element

ATP: adenosine triphosphate

AVP: arginin-vasopressin protein

BAD: Bcl-2-associated death promoter

Bcl2: B cells lymphoma 2

CA: catecholamines

cAMP: cyclic AMP

COX-2: cyclooxygenase type 2

CRH: corticotrophic hormone

CSF: colony stimulating factor

DA: dopamine

DAG: diacylglycerol

DBH: dopamine hydroxylase

DOPA: L-3,4-dihydroxyphenylalanin

E: epinephrine

ECM: extracellular matrix

EGF: endotelial growth factor

EMT: epithelial-mesenchymal transition

EPAC: exchange protein directly activated by cAMP

EPO: erythropoietin

ERK: extracellular-signal-regulated kinases

FADH: flavin adenine dinucleotide

FAK: focal adhesion protein

FGF: fibroblasts growth factor

FGFR: fibroblasts growth factor receptor

FH: fumarase

FIH: factor inhibiting HIF-1

FSP1: fibroblast specific protein-1

Gab 1: GRB2-associated-binding protein 1

G-CSF: granulocyte colony stimulating factor

GLUT1: glucose transporter 1

GLUT3: glucose transporter 3

GM-CSF: granulocyte-macrophage colony stimulating factor

Grb2: growth factor receptor-bound protein 2

GREs: glucocorticoids responsive elements

GSH: glutathione

GTP: guanosine triphosphate

HDF: human dermal fibroblasts

HGF: hepatocyte growth factor

HIF: hypoxia inducible factor

HPA axis: hypothalamic-pituitary-adrenal axis

HREs: hypoxia responsive elements

Hsp 90: heat shock protein 90

IFN- γ : interferon- γ

IFP: pressure of interstitial fluid

IGF: insulin-like growth factor

IL-1b, 4, 6, 8, 10, 12, 13, 23: interleukin 1b, 4, 6, 8, 10, 12, 13, 23.

IPAS: inhibitory PAS domain protein

JNK: N-terminal jun kinase

LDH-A: lactate dehydrogenase A

LPS: lipopolisaccharyde

LT: leucotriens

MAPK: mitogens-activated protein kinase

MCT-1, MCT-4: monocarboxylate transporter type 1 and 4

MF: myofibroblasts

MMPs: matrix metalloproteinase

NAC: N- acetyl cystein

NADH: nicotinamide adenin dinucleotide

NADPH: nicotinamide adenin dinucleotide phosphate

NE: norepinephrine

NF-kB: nuclear factor kB

NO: nitric oxide

Oct4: octamer-binding transcription factor 4

ODDD: oxygen-dependent degradation domain

PAF: platelet-activating factor

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor

PDK: pyruvate dehydrogenase kinase

PGD₂: prostaglandin D₂

PGH₂: prostaglandin H₂

PHD: prolil-hydroxylase

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

PKB/AKT: protein kinase B or AKT

PKC: protein kinase C

PKM2: pyruvate kinase isoform M2

PLC: phospholipase C

PNMT: phenylethanolamine n-methyltransferase

PTP: protein tyrosine phosphatase

pVHL: von Hippel Lindau protein

ROI: reactive oxygen intermediates

ROS: reactive oxygen species

RTK: tyrosine-kinase receptor

SDF-1: stromal derived factor 1

SDH: succinate dehydrogenase

SIRT: sirtuin

SOD: superoxyde dismutase

STAT: signal transducer and activator of transcription

TGF- β : transforming growth factor- β

TH: tyrosine hydroxylase

TNF- α : tumor necrosis factor α

TPA: 12-O-tetradecanoylphorbol-13-acetate

TRX: thioredoxin

U1, U2: uptake type 1 and 2

uPA: urinary plasminogen activator

uPAR: urinary plasminogen activator receptor

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

α -SMA: α -smooth muscle actin

INTRODUCTION

THE TUMOUR MICROENVIRONMENT

Tissues contain several cell types that work synergically in order to regulate normal physiology. These cells have positional identity so that their location and number are strictly defined. Cancer cells have lost these constrictions, but they keep creating a complex and continuative “cross-talk” with surrounding, non-malignant cells and/or with the extracellular architecture made of direct cell-to-cell contacts and paracrine/exocrine signals. These interactions are not static, but they evolve along with tumour progression. The tumour microenvironment can exert an inhibitory effect on malignant cells aggressiveness, but a state of constant alteration of microenvironment itself can become in turn strongly tumour-growth promoting. In fact, during the early stages of cancer development, the protective constraints of the microenvironment are overridden by conditions such as chronic inflammation and the local tissue microenvironment shifts to a growth-promoting state. In this light fibroblasts represent the key mediators in promoting tumour progression (Joyce e Pollard, 2009).

Fibroblasts

Fibroblasts are elongated cells, characterized by extensive cellular processes, with fusiform and tapered shape (Tarin and Croft 1969). They can be easily isolated from tissues and cultured *in vitro*. Their fusiform morphology makes them identifiable and, despite the paucity of specific markers, some molecules can be related to a fibroblastic phenotype, although none of these is exclusive of fibroblasts and/or expressed in all fibroblasts. Among these markers, the fibroblasts specific protein-1 (FSP-1) seems to provide the best specificity for the identifications of fibroblasts *in vivo*, but other markers can be considered site-specific, like desmin, a specific marker for skin fibroblasts.

A recent study has demonstrated high fibroblasts heterogeneity in mammals as they show completely different gene expression pattern in anatomically different tissues. This difference is evident from the specific secretion of extracellular matrix (ECM) factors, growth or differentiation factors. For example, fetal skin fibroblasts express

genes encoding for collagen type I and V, while those from lung tissues express different collagen patterns (Chang H. Y. et al. 2002).

Fibroblasts functions include ECM deposition, regulation of epithelial differentiation, wound healing and inflammatory mechanisms (Tomasek et al. 2002; Parsonage et al. 2005). According to that, fibroblasts produce several fibrillar ECM components like fibronectin and collagen type I, III and V (Tarin and Croft 1969; Tomasek et al. 2002). In particular, fibroblasts actively contribute to basal membrane formation through laminin and collagen type IV deposition. In addition, fibroblasts secrete proteases of the metalloproteinase (MMPs) family, which have a crucial role in regulating ECM turnover, and tissues homeostasis through growth factors production and regulation of epithelial cell-to-cell cross-talk (Simian et al. 2001; Chang et al. 2002).

Healthy fibroblasts are located within the ECM of connective tissue and constitutively express vimentin and FSP-1. As a result of specific environmental stimuli, fibroblasts may undergo an activated state, named “myofibroblastic”, which is characterized by the *de novo* expression of α -SMA protein, the actin isoform typical of smooth muscle cells, and the ability to synthesize large amounts of collagen and components of the ECM. Unlike normal fibroblasts which contains a well-developed rough endoplasmic reticulum, myofibroblasts are characterized by a large eucromatinic nucleus with two nucleoli, a prominent Golgi apparatus, and express microfilaments with dense bodies similar to those found in smooth muscle cells. In particular, myofibroblasts play a very important role in regulating tumour progression through three main mechanisms:

- Expression of ECM specific components;
- Activation of remodelling mechanisms in the granulose tissue and tension transmission to cancer cells through a Rho/Rho-kinase-dependent pathway;
- Cytokines secretion.

During tissutal lesion, different stimuli induce the activation of fibroblasts like growth factors such as the transforming-growth factor β (TGF- β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and b-fibroblasts-growth factor (bFGF), produced by both the same damaged epithelial cells and the infiltrated mononuclear cells, such as monocytes and macrophages (Kalluri and Zeisberg, 2006; Orimo and Weinberg, 2006). This activation can also occur through direct cell-cell interaction by binding with leukocytes through adhesion molecules like intercellular adhesion molecule-1 (ICAM1) and the vascular cell adhesion molecule-1 (VCAM1), or through reactive oxygen species (ROS) and complement C1 factor (Clayton et al. 1998) (Fig. 1).

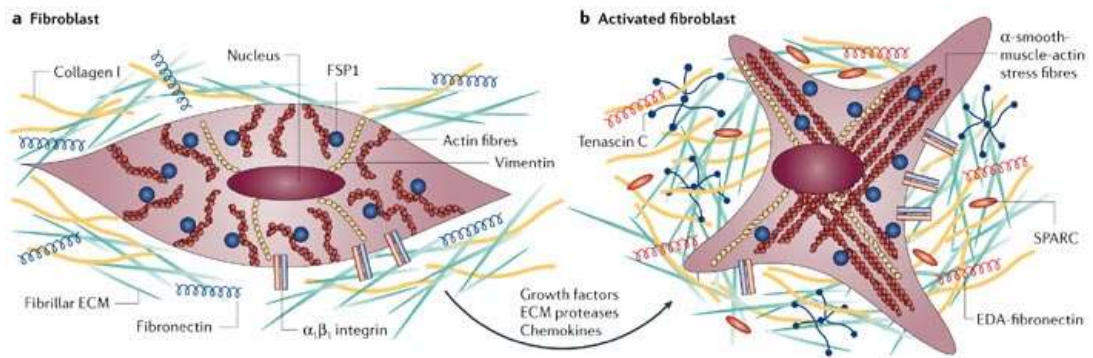


Fig. 1 Main characteristics of activated and not-activated fibroblasts.

Myofibroblasts are able to secrete higher levels of proteases that degrade the ECM, such as MMP-2, MMP-3, MMP-9, thereby increasing the turnover and altering the composition of the ECM. In addition, the activated form of fibroblasts produces growth factors like hepatocyte growth factor (HGF), insulin-like growth factor (IGF), nerve growth factor (NGF), WNT1, epidermal growth factor (EGF), and FGF-2, also promoting adjacent epithelial cells proliferation (Bhowmick et al. 2004).

Beside their expression in sclerotic tissue and sites of wound repair, activated fibroblasts act as modulators of the immune response through the secretion of cytokines, such as interleukin-1, and chemokines. In the case of wound repair, myofibroblasts return in the idle state once the stimulus is attenuated, while in fibrosis and cancer fibroblasts keep an activated state until the death of the surrounding tissue. In fact, fibroblasts isolated from a fibrotic tissue maintain their activated phenotype *in vitro*, as demonstrated by their continuous expression of ECM constituents, growth factors and cytokines. These observations show a self-maintaining autocrine and paracrine signaling that stimulates other fibroblasts. However, mechanisms underlying this continuous activation are not yet clearly understood (Strieter et al. 1989; Rollins et al. 1989).

In the early stages tumour is considered as a carcinoma *in situ* where tumour cells form a neoplastic lesion which spreads in the microenvironment of a target tissue, but remaining separate from the surrounding tissue as it turns out to be contained within the borders of basement membrane. Carcinoma in situ is associated with a stroma similar to

that observed during wound repair, and is therefore defined as "activated or reactive stroma" (Hanahan and Weinberg, 2000; Dvorak et al. 1984; Ronnov-Jessen et al., 1996).

There are some key differences between normal and activated stroma: the first one contains a minimum number of fibroblasts associated with a physiological ECM (Ronnov-Jessen et al., 1996), while the second is associated with an increase of fibroblasts, capillaries and deposition of type I collagen and fibrin. In particular, activated vascular endothelial growth factor (VEGF), produced mainly by fibroblasts and inflammatory cells, represents a key molecule for the development of the stroma (Brown et al. , 1999). VEGF induces microvascular permeability, thus allowing the extravasation of plasma proteins such as fibrin, which attracts fibroblasts, endothelial cells and inflammatory cells (Senger et al. 1983; Dvorak et al. 1991; Brown et al. 1999). These cells produce ECM that is rich in fibronectin and type I collagen, both implicated in the development of tumour angiogenesis (Leung et al. 1989; Brown et al . 1999; Feng et al. 2000).

During tumour progression from carcinoma *in situ* to invasive carcinoma tumour cells invade the reactive stroma (Dvorak 1986; Ronnov-Jessen et al. 1996). Basement membrane and stroma are degraded, and myofibroblast come into direct contact with the tumour cells. Invasive cancer is usually associated with the expansion of tumour stroma and to an increase of the deposition of ECM, known as desmoplasia (Shekhar et al. 2003; Ronnov-Jessen et al. 1996; van Kempen et al. 2003; Schedin and Elias 2004). This phenomenon appears to be very similar to the changes that take place during fibrosis, but while fibrosis is associated with a decrease of vascularization (Brown et al. 1989), solid tumours are more vascularized than healthy tissues (Folkman et al. 1989). The desmoplastic stroma contains a larger amount of fibrillar collagen, fibronectin, proteoglycans and tenascin C (Ronnov-Jessen et al. 1996). In particular, tenascin C is absent in mature mammary glands, but is highly expressed in breast tumours (Chiquet - Ehrismann et al. 1986; Mackie et al. 1987) and correlated with increased invasiveness and a worse prognosis (Ishihara et al. 1995; Brunner et al. 2004; Mackie et al. 1987; De Wever and Mareel 2002).

The activated fibroblasts within the tumour stroma are called Cancer Associated fibroblasts (CAFs) (Barsky et al. 1984; De Wever and Mareel, 2002; Mueller and Fusenig, 2004) and appear as large mesenchymal cells with tapered shape and with similar characteristics to smooth muscle cells and fibroblasts. They were first identified

by immunocytochemistry using a combination of different markers such as α -SMA (α -smooth-muscle-actin), vimentin, desmin and fibroblast activation protein (FAP), a serine protease located on the cell surface of tumour stromal fibroblasts (Garin-Chesa et al. 1990; Lazard et al. 1993; Mueller and Fusenig 2004).

The presence of CAFs in the activated tumour stroma has been observed in various types of cancer like breast cancer (Chauhan et al. 2003), prostate cancer (Olumi et al. 1999; Mueller and Fusenig 2004) and skin tumours (Skobe and Fusenig 1998). In fact, it has been demonstrated *in vivo* that the combination of prostatic epithelial cells with healthy CAFs leads to a limited tumour growth that resembles a prostatic intraepithelial neoplasia, while injecting them with prostatic epithelial non-tumourigenic immortalized cells results in the formation of malignant tumours (Olumi et al., 1999; Cunha et al., 2003). CAFs induce tumourigenic alterations in epithelial cells and promote tumour progression through specific interactions with tumour cells, promoting their invasiveness if co-injected into mice models (Dimanche-Boitrel et al. 1994). CAFs are also able to express various growth factors and cytokines like IGF-1 and HGF that promote survival, migration and invasion of tumour cells (Li et al. 2003; De Wever et al. 2004; Lewis et al. 2004). Through expression of growth factors like VEGF or MCP-1, CAFs contribute to tumour microenvironment formation by activating tumour stroma through stimulation of angiogenesis and recruitment of inflammatory cells (Orimo A. et al., 2005; Erez N. et al., 2010). In addition, they also produce proteases such as MMPs able to degrade the ECM allowing the cancer cells to overcome the boundaries of the tissues and therefore to exit from the primary site of the tumour (Sternlicht et al. 1999; Boire et al. 2005). MMPs and other proteases act directly on the motility and invasiveness of cancer cells. This was demonstrated for MMP-3, also known as stromelysin-1, a MMP highly expressed in fibroblasts that cuts the extracellular domain of E-cadherin, thus promoting epithelial-mesenchymal transition (EMT) and increasing tumour cells invasiveness (Lochter et al. 1997) (Fig. 2).

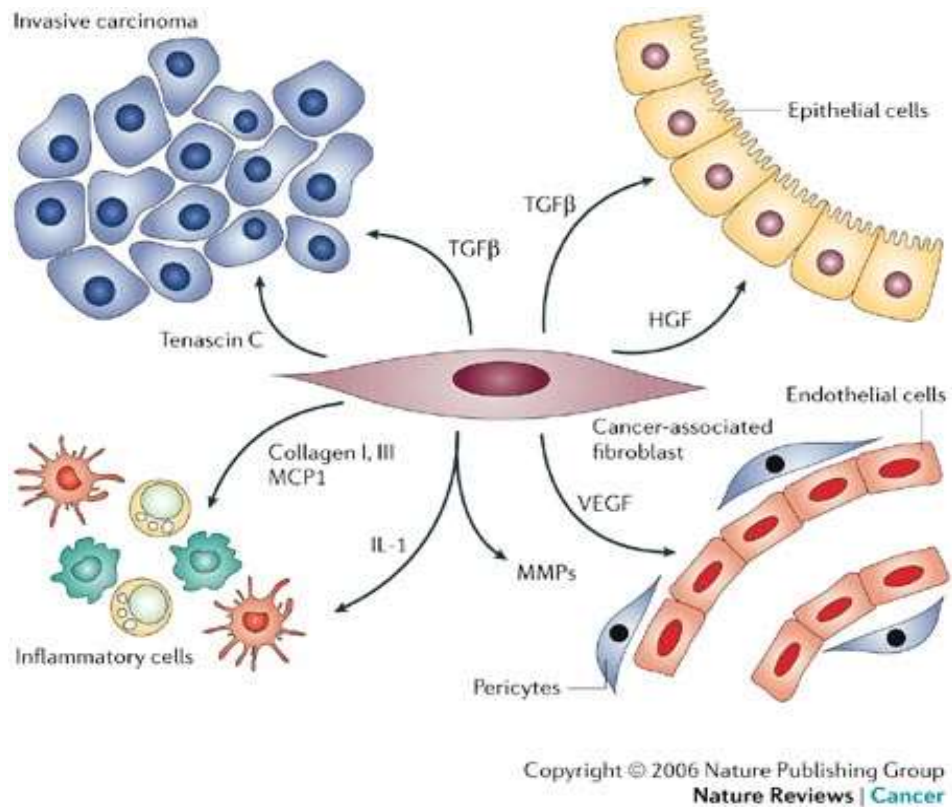


Fig. 2. Cross-talk between stromal fibroblasts and different types of cells of tumour microenvironment.

Tumour cells release growth factors such as TGF- β , PDGF and bFGF that mediate the activation of fibroblasts (Dvorak 1986; Elenbaas and Weinberg 2001). TGF- β is associated with an increase of fibrotic tissue, tumour progression and fibroblasts recruitment (Siegel and Massague 2003). In addition, TGF- β is the most important factor of the tumour microenvironment promoting EMT in tumour cells and leading fibroblasts to a CAF phenotype *in vitro* (Siegel and Massague 2003). In healthy tissue, TGF- β impairs growth of epithelium and normal development of malignant tumours (Hanahan and Weinberg 2000; Siegel and Massague 2003), therefore the TGF- β probably has a role as tumour suppressor (Akhurst 2002). For what fibroblasts is concerned, TGF- β facilitates interactions with epithelial cells that suppress the onset of cancer (Bhowmick et al. 2004). On the contrary, in advanced stages of tumour progression TGF- β progressively loses its anti-proliferative effects while facilitates EMT programme in tumour cells, thereby increasing invasiveness and metastatization (Siegel and Massague 2003). The signaling pathways that are involved in TGF- β -mediated EMT involve Smad proteins, in particular SMAD3, the phosphatidylinositol-

3-kinase-Akt and p38 MAPK pathways (Derynck et al., 2001; Kalluri and Neilson 2003). Also PDGF is correlated with tumour progression (Bronzert et al., 1987), although most of tumour cells do not express its receptor (Forsberg et al. 1993). Unlike TGF- β , PDGF induces fibroblasts proliferation, but not the acquisition of the activated phenotype associated to an excessive deposition of ECM (Shao et al. 2000). bFGF is another growth factor that stimulates the proliferation of fibroblasts, but not the expression of α -SMA (Armelin 1973; Ronnov - Jessen et al. 1996).

Since EMT is a phenomenon whereby epithelial cells lose their cell-cell contacts acquiring mesenchymal properties, it promotes the onset of a more invasive phenotype (Hay 1995). The EMT of healthy epithelial cells adjacent to tumour cells contributes to the development of CAFs (Kalluri and Neilson 2003). Recent studies on lung epithelial cells have provided evidence of transition of these cells to fibroblasts during pulmonary fibrosis development, suggesting that EMT is an important source of fibroblasts (Robin 1978). Furthermore it has been shown that exposure of cells to epithelial MMPs may lead to increased ROS cellular levels, which stimulate the transdifferentiation into myofibroblasts. These studies suggest that the increased expression of MMPs can stimulate fibrosis, tumourigenesis and tumour progression through induction of a specific EMT programme in which epithelial cells directly differentiate into activated myofibroblasts (Radisky et al . 2007).

It has been observed that the level of TGF- β on fibroblasts, through a PKC-dependent signaling, induces a significant increase in ROS levels that appear to be key secondary messengers of the signaling pathway triggered by TGF- β (Cat B. et al. 2006). According to that, treatment with antioxidants such as NAC or selenite causes an almost complete regression of the TGF- β signaling pathway, thereby blocking the expression of α -SMA and the transdifferentiation to myofibroblasts. One of the major effects induced by TGF- β stimulation is a significant increase in lipid hydroperoxides. Blocking this stimulation there is a strong inhibition of the TGF- β -induced fibroblasts activation, thus suggesting that lipid hydroperoxides are the main messengers responsible for the redox-dependent signal transduction triggered by TGF- β . It was also demonstrated that following treatment with TGF- β and subsequent fibroblasts activation there is significant gene expression changes, including induction of α -SMA and cytokines such as HGF, VEGF and interleukin 6 (IL-6). These changes are typical of myofibroblasts as they influence tumour cells invasive capacity (Cat B. et al. 2006).

It should be noted that, although it is widely accepted that CAFs assume a myofibroblastic phenotype, the *in vivo* mediators are not well understood yet (Kalluri and Zeinberg, 2006). Only some cytokines like TGF- β released by tumour cells have been associated with the differentiation of fibroblasts (Kalluri and Zeisberg 2006; Massague J. 2008). Also recent studies conducted in our laboratory have shown that IL-6, secreted by prostate carcinoma PC3 cells isolated from a bone metastasis of prostate carcinoma cells (PCa), promotes a particular phenotype named PCa-activated fibroblast (PCa-AF). In contrast to the TGF- β -dependent phenotype, these cells do not express α -SMA, but their activated state is confirmed by the expression of the FAP protein and production of ECM. The PCa-AFs strongly activate the process of EMT and therefore PC3 cells invasiveness as demonstrated by the fact that interruption of IL-6-mediated signaling between PC3 and fibroblasts impairs the process (Giannoni et al., 2010). According to our observations, production of IL-6 by tumour cells has been correlated to higher carcinomas aggressiveness (Royuela M et al., 2004; Chung LW et al., 2005; YN Niu et al., 2009).

It has been also demonstrated that CAFs are composed by a mixed population of both α -SMA positive fibroblasts (MF), probably activated by a TGF- β -dependent mechanism, and PCa-AF, α -SMA negative fibroblasts activated by an IL-6-dependent-mechanism, which suggests a coexistence *in vivo* of phenotypes reliable to both myofibroblasts and PCa-AFs. These data lead to the hypothesis that different populations of CAFs have specific roles in tumour growth. Both types of fibroblasts are able to induce EMT in PC3 cells and a greater ability invasive, involving different pathways like urinary plasminogen activator (uPA/uPAR) for the phenotype MF and the MMPs for the phenotype PCa-AFs (Giannoni et al., 2010). Thus endothelial cells lose cell-to-cell contact and acquire mesenchymal properties, thereby showing greater invasive and metastatic abilities (Hay 1995; Thiery 2006).

Tumour microenvironment and cancer progression

A raising number of evidences have demonstrated a strong, positive correlation between cancer and tissue microenvironment. Tumour progression is considered as the result of constant communication between cancer cells and the surrounding stroma, also called “tumour reactive stroma”, composed by a specific ECM and several heterotypic cells like, proinflammatory cells (cancer-associated macrophages, CAMs), endothelial cells and pericytes. This interplay has a fundamental role in different aspects of tumour

biology. Tumour stroma composition can vary among different kind of tumours and it is not directly correlated to its aggressiveness. Anyway, recent studies *in vivo* have shown that tumour-derived stromal fibroblasts are crucial for breast cancer development (Dvorak et al., 1983; Kuperwasser et al., 2004).

As previously stated, this “tumour reactive stroma” can also be morphologically defined as “a desmoplastic response”, referring to growth of dense connective tissue rich in fibroblasts, proinflammatory and immune cells which produce large amounts of collagen, fibronectin, proteoglycans and tenascin C (Ronnov-Jessen et al., 1996) (Fig. 3). A desmoplastic microenvironment can support key cancer mechanisms like development of new blood and lymphatic vessels in order to favor cancer growth and dissemination (Folkman 2003).

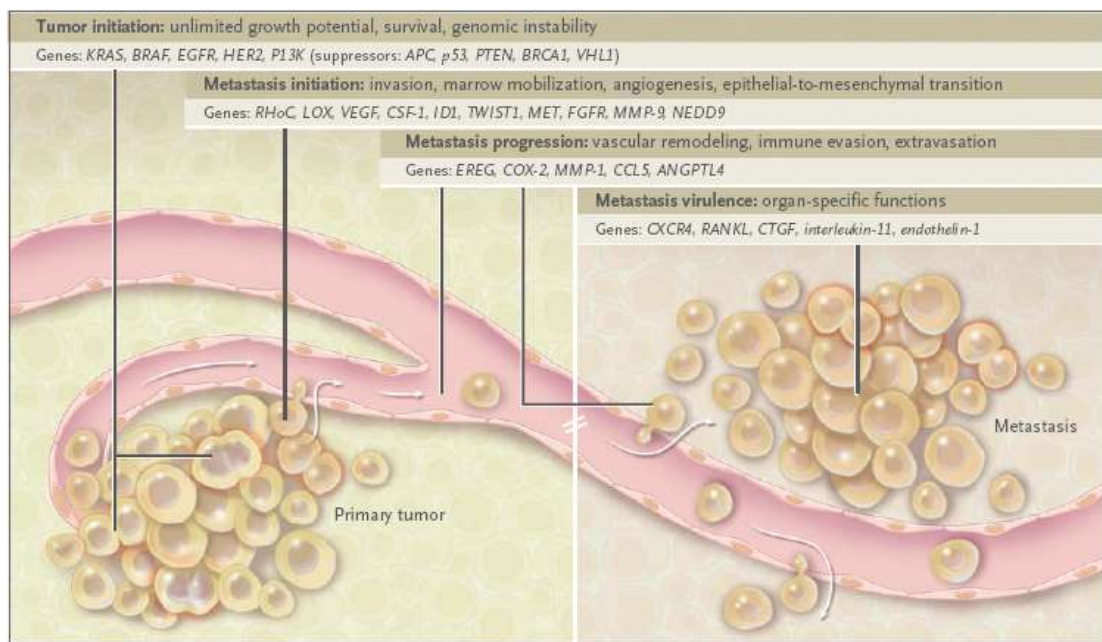


Fig. 3 Cancer development: a multi-step process which requires activation of the surrounding stroma.

Cancer cells create an hospitable microenvironment producing stroma-modulating factors like bFGF, PDGF, EGF, colony-stimulating factor (CSF) and TGF- β . These factors activate surrounding cells like fibroblasts, smooth muscle cells and adipocytes, both in a paracrine and autocrine way, which in turn produce new cytokines, growth factors and proteolytic enzymes responsible of ECM remodelling and cancer cells

invasion (Coussens and Werb, 2002; Bergers and Benjamin, 2003; Stetler-Stevenson and Yu 2001; Mueller et al. 2003).

The mechanism of ECM degradation also induces unmasking of proteins cryptic domains (Kalluri 2003). For example, matrix MMPs can partially degrade molecules on cells surface and soluble factors during ECM remodelling in order to create new activated factors with pro-migratory and proangiogenic abilities (Brinckerhoff and Matrisian 2002; McCawley and Matrisian 2001; Egeblad and Werb 2002). In particular, cancer aggressiveness is often positively correlated to expression levels of MMP-1 and MMP-9. Cancer cells co-cultured with stromal cells and injected in murine models express larger amounts of MMP-1 and MMP-9 in comparison with benign cancer cells (Borchers et al. 1997; Airola e Fusenig 2001; Egeblad e Werb 2002).

The proteolytic fragments produced by cancer cells proteases can also modulate proliferation, survival and migration of surrounding endothelial cells. In particular, the action of several types of MMPs like MMP-3, MMP-7, MMP-9, MMP-12 can generate angiostatin from the amino-terminal part of plasminogen. Other anti-angiogenic factors are endostatin, proteolytic fragment from collagen type VIII reducing VEGF-mediated growth and motility of endothelial cells, and tumstatin, generated from collagen IV digestion, which is an endothelial proapoptotic factor (Dong et al. 1997; Cornelius et al. 1998 O'Reilly et al. 1997; Marneros e Olsen 2001). In conclusion, the regulation of proteases homeostasis by cancer cells represents a crucial step for tumour angiogenic ability, which results from a continuative balance between pro and antiangiogenic signals.

Cancer cells also produce proinflammatory cytokines in order to recruit hematopoietic cells like lymphocytes, monocytes/macrophages and neutrophils nearby the tumour mass (Bachmeier et al. 2000; Coussens e Werb 2002). CSF-1 induces growth and differentiation of mononucleated cells like macrophages and is highly expressed in breast, ovarian and prostate cancers (Kacinski 1995). Loss of CSF-1 expression prevents macrophages recruitment by epithelial cancer cells and reduces tumour growth and metastatization. (Lin et al. 2001).

Recent studies have demonstrated the importance of hematopoietic factors like granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) which promote mobilization of monocyte/macrophages and neutrophils from bloodstream and surrounding tissues. Growing proinflammatory signals can in turn induce recruitment of endothelial cells progenitors, thereby

promoting angiogenesis into the tumour burden. Neutrophils ease this process remodelling the ECM through secretion of MMP-9 and MMP-13. Also macrophages express several types of proteases like the uPA and MMPs which generate pro-angiogenic molecules from ECM degradation like VEGF (Obermueller et al. 2004; Carmeliet e Jain 2000) (Fig. 4).

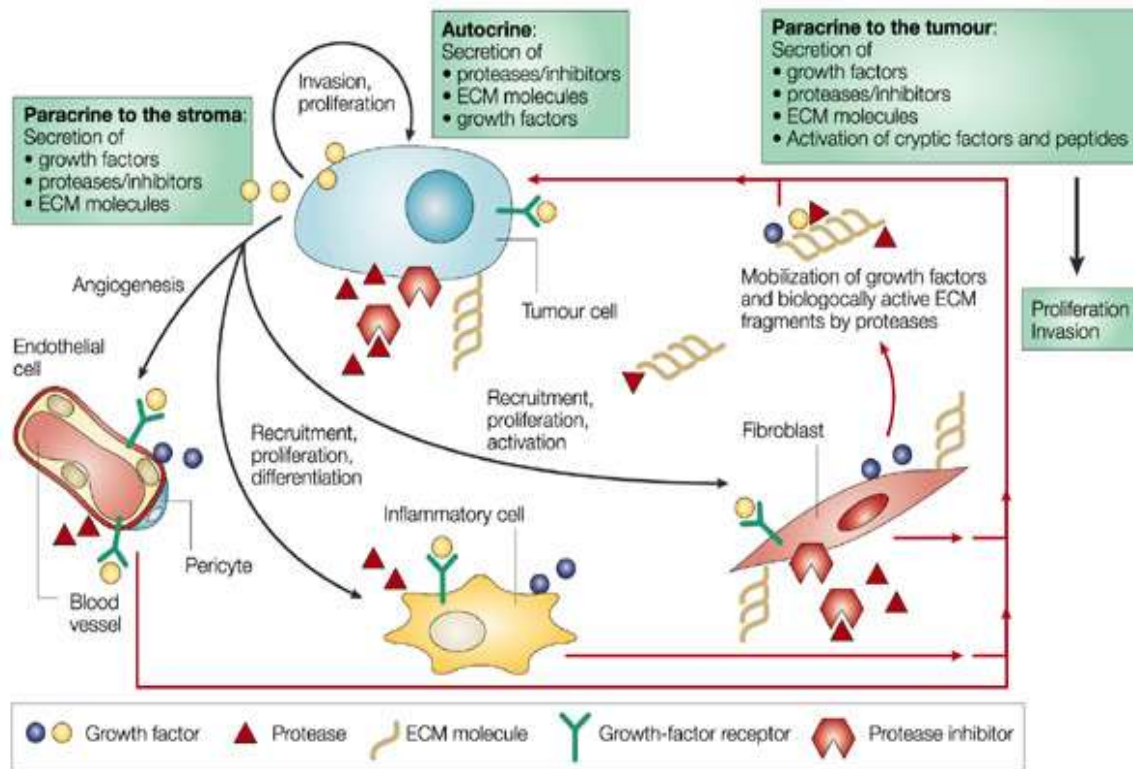


Fig. 4 The interplay between tumour reactive stroma and endothelial cells.

In vivo studies have shown that blocking antibodies against VEGF receptor 2 (VEGFR2) impairs angiogenesis and cancer invasive spur, inducing a regression from a high malignant cancer to a pre-malignant, not invasive phenotype. This process is strongly correlated to stabilization of the tumour stroma which turns into a normal connective tissue with new epithelial structures, like intact membranes basements and cell-to-cell junctions, and well-organised collagen fibres nearby the tumour. Normalization of tumour stroma is probably correlated to impairment of MMPs activity. As a result the reduced turnover of laminin, fibronectin and collagen type I and IV promotes their accumulation and correct reorganization, thereby reducing angiogenesis

and almost completely reverting cancer progression. (Skobe et al 1998; Vajkoczy et al. 2000) (Fig. 5).

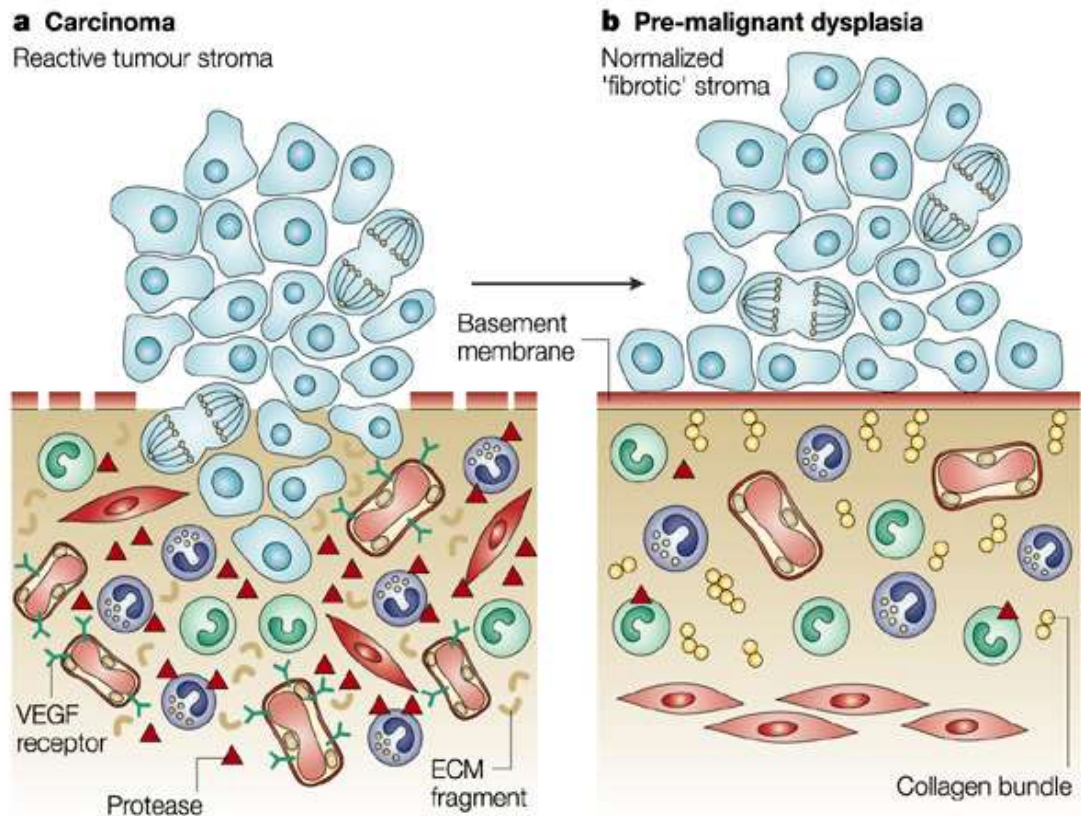


Fig. 5 Tumour-activated stroma normalization induces tumour regression.

In the light of these experimental evidences, tumour microenvironment could be considered a promising therapeutic target because tumour stroma cells are genetically more stable than cancer cells and show less ability in developing drug resistance (Kerbel 1997; Sporn e Suh 2000). For example, the inhibition of cytokine production by inflammatory cells after administration of non-steroidal drugs impairs the risk of developing colon and breast cancer (Ricchi et al. 2003). In addition, MMP inhibitors are proving to be promising antitumour agents in the clinic. Inactivation of MMPs by administration of synthetic MMP inhibitors may prevent initiation of MMP activation cascades and excessive ECM degradation. On the other side, these small molecule inhibitors also exhibit unpleasant side effects due to their broad substrate specificity. The lack of highly specific MMP inhibitors for individual MMPs makes it difficult to understand the role of individual MMPs. In addition, cancer cells can also avoid

farmacological treatment adopting an ameboid, MMPs-independent motogenic program (Stetler-Stevenson e Yu 2001).

The tumour stroma can also act on cancer cells as a mutagenic factor itself. Genomic instability is a commonly observed feature of tumours. Most investigations addressing the mechanism of tumour progression have focused on the genetic factors that may play a role. Growing evidence now suggests that, in addition to these endogenous factors, the exogenous environment within solid tumours may by itself be mutagenic and constitute a significant source of genetic instability. The tumour microenvironment is characterized by regions of fluctuating hypoxia, low pH, and nutrient deprivation. Each of these microenvironmental factors has been shown to cause severe disturbance in cell metabolism and physiology. Both in vivo and in vitro data demonstrate that exposure of tumour cells to adverse conditions can directly cause mutations, contributing to genetic instability (Yuan and Glazer 1998).

MELANOMA

Definition

Melanoma is the typical malignant tumour of melanocytes, cells that secrete a dark pigment, melanin, which is responsible for the color of skin. They are predominantly located in the epidermal basal layer, but are also found in other parts of the body, like the bowel, the oral cavity, the inner ear, the meninges and the eyes, so melanoma can originate in any part of the body that contains melanocytes. Cutaneous melanoma represents only the 3-5% of skin cancers, but it causes the majority (75%) of deaths related to skin cancer because of its high invasive and metastatic ability (Boring et al., 1991).

Etiology

The earliest stage of melanoma starts when the melanocytes begin to grow out of control. Melanocytes are found between the outer layer of the skin (the epidermis) and the next layer (the dermis). This early stage of the disease is called the radial growth phase, and the tumour is less than 1mm thick. Because the cancer cells have not yet reached the skin blood vessels, it is very unlikely that this early-stage cancer will spread to other parts of the body. If the melanoma is detected at this stage, then it can usually be completely removed with surgery. When the tumour cells start to move vertically up into the epidermis and into the papillary dermis, the behaviour of the cells changes dramatically

The next step in the evolution is the invasive radial growth phase, when individual cells start to acquire invasive potential, thereby leading to cancer spreading.

The following step in the process is the invasive melanoma called the vertical growth phase (VGP). The tumour attains invasive potential so it can grow into the surrounding tissue and can spread into distant tissues through blood or lymph vessels. The tumour thickness is usually more than 1 mm, and the tumour involves the deeper parts of the dermis (Fig. 6).

During the VGP phase, the host elicits an immunological reaction against the tumour, which can be visible by the presence and activity of tumour infiltrating lymphocytes (TILs). These cells sometimes completely destroy the primary tumour and this phase is called regression, which is the latest stage of the melanoma development.

In certain cases, the primary tumour is completely destroyed and only the metastatic tumour is discovered.

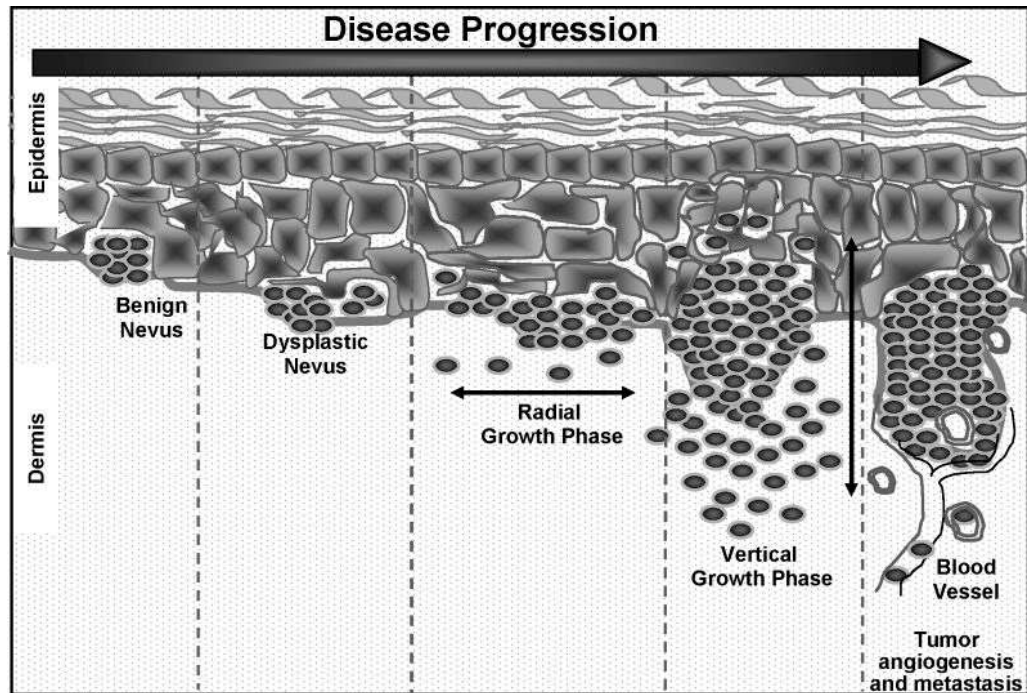


Fig. 6 Malignant progression of cutaneous melanoma.

Incidence

Many studies have reported increasing incidence rates of cutaneous melanoma during the last fifty years and this rapid increase in incidence has led to the concept of “epidemic melanoma”, thereby becoming a real sanitary emergency. The most affected populations are represented by Australians (40/100.000 inhabitants) and populations of New Zealand and Northern Europe. Also in Italy melanoma incidence has been rising 5-7% every year, with an incidence of 9,97/100.000 for men and 8,24/100.000 for women.

Mortality

Every year almost 41.000 deaths for cancer worldwide are related to skin melanoma and 1400 of them occur in Italy. A white male living in industrialized countries has 1/100 possibility to develop skin melanoma during his life. In addition, in the last decades melanoma shows higher mortality among young and middle-aged people. In particular, in the past five years, in Italy the number of deaths clearly related to skin melanoma were 3178 in males and 2807 in females, with almost a doubled mortality incidence in northern regions compared to southern ones.

Prevalence

The prevalence proportion is the proportion of people found to have the condition with the total number of people studied and it is correlated to both frequency and prognosis of pathology. In Italy the prevalence of skin melanoma is 48,4/100.000 inhabitants for women and 101,6/100.000 inhabitants for men. The discrepancy between the sexes is related to the highest incidence and better survival for women. Inside the country, prevalence is three-fold higher in Veneto and Emilia if compared with southern regions.

Risk factors

Both endogenous and exogenous risk factors for skin melanoma have been identified.

Endogenous risk factors

1. Number of naevi: number of naevi is the most important susceptibility factor, independent from naevi dimension and distribution. In fact, skin melanoma develops from a congenital or acquired naevus in the 22-57% of all cases. In addition, the number of naevi is related to major risk of developing melanoma from healthy skin.
2. Presence of atypical/dysplastic naevi: a single dysplastic naevus is correlated to a two-fold higher risk to develop melanoma, while presence of ten or more naevi results in a twelve-fold higher risk.
3. Familiarity: frequency of familial malignant melanoma varies from 5 to 10% of total cases. Subjects with close-related parents (parents, brothers) affected by melanoma are at risk of developing malignant melanoma. The risk augments with the rising number of related affected.
4. Previous melanoma: almost 11% of subjects with melanoma develop a new melanoma in the following five years. In addition, other melanomas can show up during the lifetime.
5. Adulthood: melanoma in children and adolescents is very rare, even if its frequency is rising in the past decades. During adulthood the frequency rises rapidly.

Other risk factors are presence of various solar lentigos, fair and red-headed people and individuals with blistering or peeling sunburns. Different phototypes seem to modulate the cancerogenic effects of ultraviolet rays (UV).

Exogenous risk factors

Development of skin melanoma seem to be related to intense but intermittent sun exposure, specially in usually non photo-exposed cutaneous areas. In addition, exposure and sunburn during childhood becomes a more important risk factor.

The UV rays are the leading cause of melanoma (Iarc 1997). It has been demonstrated that this type of cancer is more common in decreasing latitudes and rising altitudes. In particular, UV-B rays are the more carcinogenic to: 1) direct damage to DNA, 2) damage to mechanisms of DNA repair, 3) partial suppression of cell-mediated immunity. UV-A rays, once considered harmless, increase the effect of UV-B and act as co-carcinogenic agents. The risks arising from a history of sunburn are significant for both those experienced in childhood, adolescence, or any age (Elwood and Jopson 1997).

As for the ionizing radiation, the onset of melanoma depends on the total dose accumulated and time of accumulation (radiologists, airline pilots, radio-treated patients).

Clinical aspects

Signs and symptoms

Early signs of melanoma are changes to the shape or color of existing moles or, in the case of nodular melanoma, the appearance of a new mole on the skin. At later stages, the mole may ulcerate or bleed (Fiddler 1995). Early signs of melanoma are summarized by the mnemonic "ABCDE" staging (Friedman et al., 1985):

- Asymmetry
- Borders (irregular)
- Color (variegated)
- Diameter (greater than 6 mm)
- Evolving over time

These classifications do not, however, apply to the most dangerous form of melanoma, nodular melanoma, which has its own classifications:

- Elevated above the skin surface
- Firm to the touch
- Growing

Metastatic melanoma may cause nonspecific paraneoplastic symptoms, including loss of appetite, nausea, vomiting and fatigue. Metastasis of early melanoma is possible, but relatively rare: less than 1/5 of early diagnosed melanomas become metastatic and brain metastasis represent the most typical result of metastatic melanoma. It can also spread to the liver, bones, abdomen or distant lymph nodes. A recent new method of melanoma detection is called the "ugly duckling sign" and it is commonly used because it is a simple diagnostic procedure and it is highly effective in detecting melanoma. Simply, correlation of common characteristics of a person's skin lesion is made. Lesions which greatly deviate from the common characteristics are labeled as an "Ugly Duckling", and further professional exam is required (Mascaro et al., 1998) (Fig. 7).

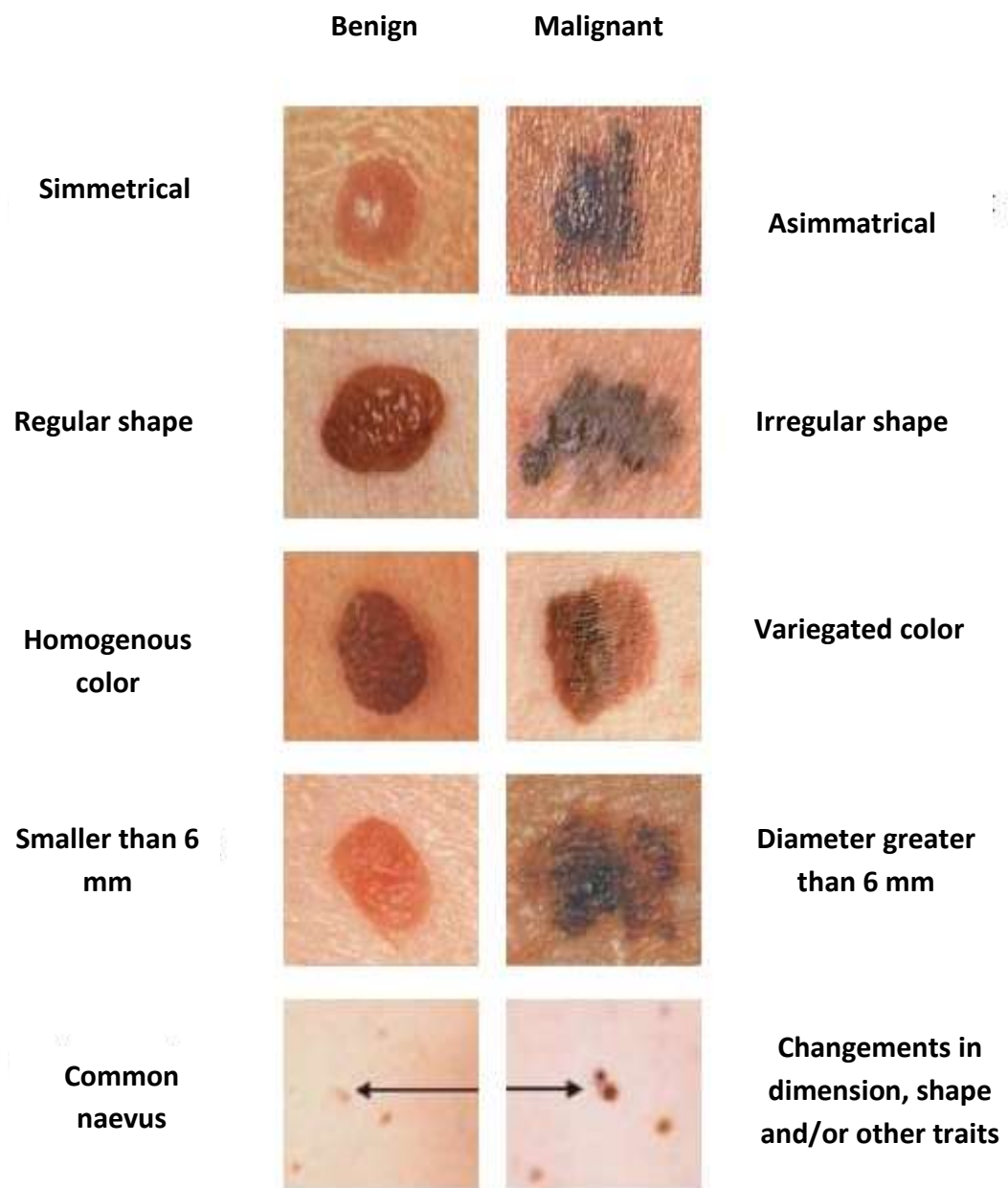


Fig. 7 Pictures and main characteristics of normal naevi and malignant melanoma.

Classification

Microscopically melanoma appears as a not-limited, asymmetrical lesion, consisting of proliferation of melanocytes with atypical cytological characteristics, with a tendency to migrate to the more superficial layers of the epidermis. Melanoma in situ, which is located only into the epidermis and in the epithelium of skin appendages, can be distinguished as follows: lentigo maligna type, superficial spreading type, acral lentiginous type, uveal melanoma and nodular type (James et al., 2006).

- **Lentigo maligna melanoma** is a melanoma that has evolved from a lentigo maligna. It is usually found as a darkly pigmented raised papule, arising from a patch of irregularly pigmented flat brown to dark brown lesion of sun exposed skin of the face or arms in an elderly patient. Lentigo maligna is the non-invasive skin growth and it is often considered as a melanoma-in-situ or a precursor to melanomas. Once a lentigo maligna becomes a lentigo maligna melanoma, it is considered and treated as if it were an invasive melanoma.
- **Superficial spreading melanoma**, also known as "Superficially spreading melanoma (SSM)", is usually characterized as the most common form of cutaneous melanoma in Caucasians (60-70% of total melanomas). The average age at diagnosis is in the fifth decade, and it tends to occur on sun-exposed skin, especially on the backs of males and lower limbs of females. This disease commonly evolves from a precursor lesion, usually a dysplastic nevus, but it can also arise from previously healthy skin. A prolonged radial growth phase, where the lesion remains thin, may eventually be followed by a vertical growth phase where the lesion becomes thick and nodular. As the risk of spread varies with the thickness, early SSM is more frequently cured than late nodular melanoma.
- **Acral lentiginous melanoma** is a kind of lentiginous skin cancer. Acral lentiginous melanoma is observed on palms, soles, under the nails and in the oral mucosa. Unlike the commonest forms of melanoma, acral lentiginous melanoma does not seem to be directly connected to sun exposure. In fact, it can occur on non hair-bearing surfaces of the body which may or may not be exposed to sunlight, but is also found on mucous membranes. It is the most common form of melanoma diagnosed amongst Asian and Black ethnic groups. The average age at diagnosis is between sixty and seventy years. However, the melanoma can also occur in Caucasians and in young people. Typical signs of

acral lentiginous melanoma include longitudinal tan, black, or brown streak on a finger or toe nail, pigmentation of proximal nail fold and areas of dark pigmentation on palms of hands or soles of feet.

- **Uveal melanoma** is a cancer of the eye involving iris, ciliary body, or choroid, collectively referred to as the uvea and tumours start from the melanocytes that reside in the uvea and that are responsible for the color of the eye. These melanocytes are distinct from the retinal pigment epithelium cells underlying the retina that do not form melanomas. Several clinical and pathological prognostic factors have been identified that are associated with higher risk of metastasis of uveal melanomas. These include large tumour size, ciliary body involvement, presence of orange pigment overlying the tumour, and older patient age. Several histological and cytological factors are associated with higher risk of metastasis, thereby including presence/extent of cells with epithelioid morphology, looping extracellular matrix patterns, increased infiltration of several types of immune cells, as well as staining with several immunohistochemical markers.
- **Nodular melanoma (NM)** is the most aggressive form of melanoma and grows more rapidly in thickness than in diameter. Instead of arising from a pre-existing mole, it may appear in a spot where a lesion did not previously exist. Because of these characteristics the prognosis is often worse because it takes longer for a person to be aware of the symptoms. NM is most often darkly pigmented, even if some NM lesions can be light brown, multicolored or even colorless (non-pigmented). A light-colored or non-pigmented NM lesion may escape detection because the appearance is not alarming, however an ulcerated and/or bleeding lesion is common. A particular variant of nodular melanoma, of viral origin, is represented by the polypoid melanoma. The microscopic hallmarks are dome-shaped at low power, thin or normal epidermis, dermal nodule of melanocytes with a 'pushing' growth pattern and no "radial growth phase".

Histopathological factors and prognosis

Features that are evaluated as affecting prognosis are tumour thickness in millimeters (Breslow's depth), depth related to skin structures (Clark's levels), type of melanoma, presence of ulceration, presence of lymphatic/perineural invasion, presence of tumour-

infiltrating lymphocytes, location of lesion, presence of satellite lesions, and presence of regional or distant metastasis (Day et al, 1981). Certain types of melanoma have worse prognoses, but this is explained by their thickness. Interestingly, less invasive melanomas, even with lymphnode metastases, carry a better prognosis than deep melanomas without regional metastasis at time of staging. Local recurrences tend to behave similarly to a primary unless they are at the site of a wide local excision, since these recurrences tend to indicate lymphatic invasion. When melanomas have spread to the lymphnodes, one of the most important factors is the number of nodes with malignancy. Extent of malignancy within a node is also important; micrometastases in which malignancy is only microscopic have a more favorable prognosis than macrometastases. Macrometastases in which malignancy is clinically apparent as the cancer completely replaces a node, have a far worse prognosis, and if nodes are matted or if there is extracapsular extension, the prognosis is progressively worse. When distant metastasis are detected, the cancer is generally considered incurable. The five year survival rate is less than 10% and the median survival is 6 to 12 months. Metastases to skin and lungs have a better prognosis, while metastases to brain, bone and liver are associated with a worse prognosis.

Clark's level is a related staging system, used with Breslow's depth, which describes the level of anatomical invasion of the melanoma in the skin (Balch et al., 2001) (Fig. 8). Five anatomical levels are recognized, and higher levels have worsening prognostic implications. These levels are:

- Level 1 : Melanoma confined to the epidermis (melanoma in situ)
- Level 2 : Invasion into the papillary dermis
- Level 3 : Invasion to the junction of the papillary and reticular dermis
- Level 4 : Invasion into the reticular dermis
- Level 5 : Invasion into the subcutaneous fat

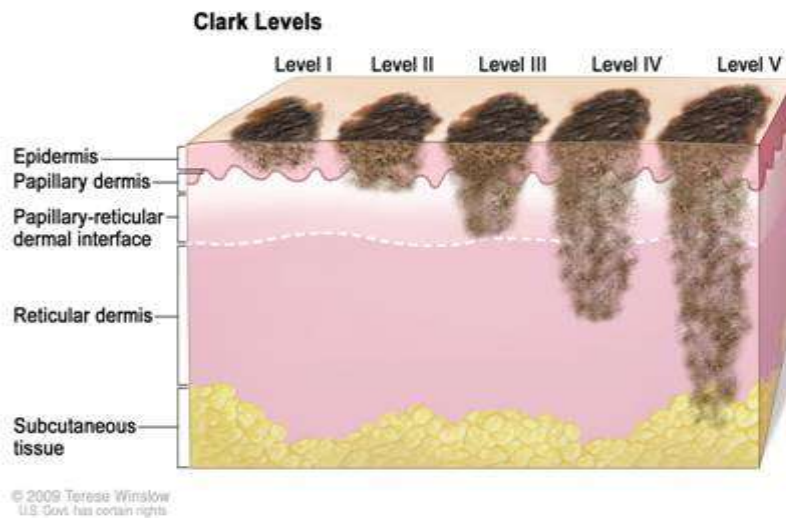


Fig. 8 Clark's levels.

Medical treatment

Surgical treatment is the therapy of choice for the primary melanoma. The surgical procedure involves the removal of the primary tumour with part of surrounding healthy skin, reaching the muscle tissue. Currently, for patients with cutaneous melanoma thicker than or equal to 1 mm, in combination with surgical excision of the primary lesion, the sentinel lymph node biopsy is performed, which is proved to be highly sensitive in identifying clinically occult lymph node metastases. Therapy for metastatic melanoma includes surgical radicalization of the lesion, use of chemotherapy or combined protocols, or the use of radiation therapy for head can not be reached by the drugs. Currently therapies are being tested with so-called "vaccines" which exploit the biological response of the individual appropriately stimulated to slow the progression of the disease.

Biological and genetic aspects

Melanoma is usually caused by DNA damage from UV light from the sun, but UV light from sunbedscan also contributes to the disease.

A number of rare mutations, which often run in families, are known to greatly increase one's susceptibility to melanoma. Several different genes have been identified as increasing the risk of developing melanoma. Some rare genes have a relatively high risk of causing melanoma; some more common genes, such as a gene called MC1R that

causes red hair, have a relatively low risk. Genetic testing can be used to determine whether a person has one of the currently known mutations.

One class of mutations affects gene CDKN2A. An alternative reading frame mutation in this gene leads to the destabilization of p53, which is involved in apoptosis and its mutations are detected in over the 50% of human cancers. Another mutation in the same gene results in a loss of function of the inhibitor of CDK4, a cyclin-dependent kinase that promotes cell division. Mutations that cause the skin condition xeroderma pigmentosum (XP) also seriously predispose one to melanoma. Scattered throughout the genome, these mutations reduce a cell's ability to repair DNA. Both CDKN2A and XP mutations are *highly penetrant*, meaning that the chances of a person carrying the mutation to express the phenotype is very high (Golstein e Tucker, 2005).

Familial melanoma is genetically heterogeneous, and loci for familial melanoma have been identified on the chromosome arms 1p, 9p and 12q. Multiple genetic events have been related to the pathogenesis of melanoma. The multiple tumour suppressor 1 (CDKN2A/MTS1) gene encodes p16INK4a, low-molecular weight protein inhibitor of cyclin-dependent protein kinases (CDKs), which has been localised to the p21 region of human chromosome 9.

Other mutations confer lower risk, but are more prevalent in the population. People with mutations in the melanocortin receptor 1 (MC1-R) gene, for example, are two to four times more likely to develop melanoma than those with two wild-type copies of the gene. MC1-R mutations are very common; in fact, all people with red hair have a mutated copy of the gene. Also mutation of the MDM-2 SNP309 gene is associated with increased risk of melanoma in younger women.

Other mutations affect genes regulating cell proliferation like Neuroblastoma RAS viral (v-ras) oncogene homolog (N-ras), V-raf murine sarcoma viral oncogene homolog B1 (BRAF) and Phosphatase and Tensin homolog (PTEN) genes. Pathogenically N-ras and BRAF mutations seem to occur in early cancer development, while alterations in PTEN gene are related to a late-stage development (Davies et al., 2002; Curtin et al., 2005; Haluska et al., 2006).

STRESS HORMONES: EPINEPHRINE AND NOREPINEPHRINE

Definition of stress

Stress is commonly defined as the general reaction of the organism in order to respond to endogenous and exogenous stimuli that can alter or affect the homeostasis of the organism itself. The term stress has been first introduced in medicine by Hans Selye in 1936 (Selye 1936). Selye began to study on stress while trying to isolate sex hormones in animals. By inoculating some toxic compounds in guinea pigs he could observe that in animals occurred a common, almost “syndromic” reaction characterized by i) adrenal hypertrophy , ii) atrophy of the thymus and lymph glands , iii) gastric ulcerations. He further noticed that the same biological reaction, characterized by the common state of hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis (see next paragraph), it appeared in all the experimental animals regardless of the type of stimulus, called "stressors". Selye defined the set of systemic reactions of the body resulting from prolonged exposure to stressors as "general adaptation syndrome" (GAS). During the following years, many and more complex research on the "new" concept of stress started. In 1975, Mason demonstrated through research conducted on monkeys and then humans that adrenocortical activation was not merely the result of exposure to the stressor itself, but was specifically triggered by emotional reaction to stimuli. The emotional arousal was characterized by the activation of the structures of the limbic system (SL) that, as confirmed by following studies, represents the place of coordination and control of stress reactions. The structures of the SL project abundantly to the hypothalamic-pituitary axis. Therefore, the emotional arousal that is induced by the stimulus is processed at the level of SL and occurs both at a biological/somatic level, with autonomic and endocrine changes, and at a psychological/behavioral level with sequences of motor behavior collectively known as the "flight or fight response" (Mason 1975).

In particular Selye identified three basic phases: **alarm reaction phase**, **resistance or adaptation phase**, and **exhaustion**, which follow one another in the body during each stress reaction and collectively called "general adaptation syndrome" (GAS). GAS syndrome is therefore a defense mechanism by which the body struggles to overcome the difficulties to return as soon as possible to its normal operating balance.

The adrenal gland

The adrenal glands are equal endocrine glands, pyramid-shaped (about $2 \times 6 \times 1$ cm, weight 4-6 g), located in the retroperitoneum superior to the kidneys, from which are separated by an adipose capsule and renal fascia. Each adrenal gland is composed of two structurally and functionally different parts, the adrenal cortex and the inner medulla where the latter is localized internally and is completely surrounded by the cortex. While the medullary part is nerve-derived, the cortex is organized in cellular wrapped cords (**zona glomerulosa**), then assume a radial course toward the medulla (**zona fasciculata**) and finally come together to form a coarse mesh (**zona reticularis**) (fig. 9).

The outermost layer, the zona glomerulosa, is the main site for production of mineralocorticoids, mainly aldosterone, which is largely responsible for the long-term regulation of blood pressure. Aldosterone's effects are on the distal convoluted tubule and collecting duct of the kidney where it causes increased reabsorption of sodium and increased excretion of both potassium by principal cells and hydrogen ions by intercalated cells of the collecting duct. Sodium retention is also a response of the distal colon, and sweat glands to aldosterone receptor stimulation. The major stimulus to produce aldosterone is represented by angiotensin II. Angiotensin II is stimulated by the juxtaglomerular cells when renal blood pressure drops below 90 mmHg.

Situated between the glomerulosa and reticularis, the zona fasciculata is responsible for producing glucocorticoids such as 11-deoxycorticosterone, corticosterone, and cortisol. Cortisol is the main glucocorticoid under normal conditions and its actions include mobilization of fats, proteins, and carbohydrates, but it does not increase under starvation conditions. Additionally, cortisol enhances the activity of other hormones including glucagon and catecholamines. Several factors, included emotional and physical stressors, influence production of cortisol in response to adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH is secreted from corticotrope cells expressed within the anterior lobe of the pituitary gland in response to the corticotropin-releasing hormone (CRH), released by the hypothalamus. ACTH acts by binding to cell surface ACTH receptors, which are located primarily on adrenocortical cells of the adrenal cortex, thereby influencing steroid hormone secretion by both rapid short-term mechanisms that take place within minutes and slower long-term actions.

In order to regulate the secretion of ACTH, many substances secreted within this axis exhibit both slow/intermediate and fast feedback-loop activities. According to that,

glucocorticoids secreted from the adrenal cortex work to inhibit CRH secretion by the hypothalamus, which in turn decreases anterior pituitary secretion of ACTH.

The most inner cortical layer, the zona reticularis, produces androgens, mainly dehydroepiandrosterone (DHEA), DEHA sulfate (DEHA-S) and androstenedione, which is the molecular precursor to testosterone.

The adrenal medulla is the core of the adrenal gland, and is surrounded by the adrenal cortex. The chromaffin cells of the medulla, named for their characteristic brown staining with chromic acid salts, are the body's main source of the circulating catecholamines (CA). In particular, they secrete approximately 20% norepinephrine (NE) and 80% epinephrine (E). To carry out its part of this response, the adrenal medulla receives input from the sympathetic nervous system through preganglionic fibers originating in the thoracic spinal cord from T5–T11. Because it is innervated by preganglionic nerve fibers, the adrenal medulla can be considered as a specialized sympathetic ganglion. Unlike other sympathetic ganglia, however, the adrenal medulla lacks distinct synapses and releases its secretions directly into the blood. Cortisol also promotes CA synthesis in the medulla. Produced in the cortex, cortisol reaches the adrenal medulla and, at high levels, the hormone can promote the upregulation of enzyme phenylethanolamine-N-methyltransferase (PNMT), thereby increasing CA synthesis and secretion (Bertagna 2006; Cotran et al. 1999; Cotran et al. 2000).

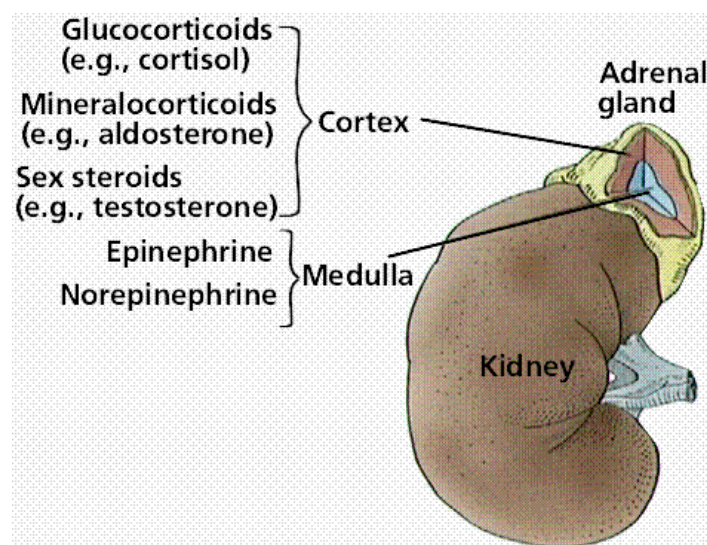


Fig. 9 Anatomic structure of the adrenal gland and hormones produced.

The periferic nervous system and the hypothalamic-pituitary-adrenal axis (HPA)

All vertebrates keep the correct homeostasis of their physiological mechanisms by constant interaction between two main control systems, the endocrine and the nervous system. The latter is anatomically divided into the *central nervous system*, which includes the brain and the spinal cord, and the *peripheral nervous system*, which comprehends all the nerves responsible for transmitting signals between the central nervous system and the body's glands, muscles and organs, collectively known as effector organs.

The peripheral nervous system is organized in 43 pairs of nerves divided into 12 pairs of cranial nerves and 31 pairs of spinal nerves. Each nerve is formed by nerve fibres including the axons of efferent and/or afferent neurons. Therefore, the peripheral nervous system is further divided into two main parts: *the efferent division*, which coordinates signals going from the central nervous system towards effector organs, and *the afferent division*, which transmits signals from peripheric receptors towards the central nervous system.

The efferent division is further organized into the *somatic nervous system*, which innervates voluntary skeleton muscles, and the *autonomic nervous system*, regulating not-voluntary smooth and cardiac muscles, glands and gastrointestinal neurons.

Physiological and anatomical differences allow to divide the autonomic nervous system into three further parts: *sympathetic*, *parasympathetic* and *enteric*. The sympathetic nerve fibers originate from the central nervous system, particularly from the thoracic and lumbar regions of the spinal cord, while parasympathetic nerves leave the central nervous system in the brain and in the sacral region of the spinal cord.

Together with the nervous system, the endocrine system acts in order to integrate and correctly coordinate the functions of the nervous system. Unlike the nervous system, the endocrine system acts, in general, more slowly, normally in a span of time ranging from 30 minutes to three hours, and more diffusely, as all cells of the body can be reached by hormonal signals, and primarily transimitting to peripheric organs and tissues.

In particular, the integration between these two systems is related to the HPA axis, where hypothalamus is both part of the central nervous sytem and “interface” between nervous system itself and the endocrine one. Subsequently, hypothalamus controls periferic endocrine functions through pituitary gland stimulation (Kvetnansky et al. 2009) (fig. 10).

In the light of these observations, hypothalamus represents the most important gland for elaboration of stress responses. In particular the paraventricular nucleus (PVN) is considered as critical for generation of neurophysiological stress responses. The axons of the parvocellular neurosecretory neurons of the PVN project to the median eminence, a neurohemal organ at the base of the brain, where their neurosecretory nerve terminals release their hormones. The median eminence contains fiber terminals from many hypothalamic neuroendocrine neurons, secreting different neurotransmitters or neuropeptides, including vasopressin (VPN), CRH, thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), dopamine (DA) and somatostatin into blood vessels in the hypothalamic-pituitary portal system. In particular, CRH and VPN act synergistically in order to stimulate ACTH secretion and glucocorticoids production from the adrenal cortex (Vale et al. 1981). Several studies have in fact demonstrated that there is a positive, biunivocal interaction between CRH and AVP where the secretion of one induces production of the other and viceversa (Lamberts et al. 1984).

In absence of stressful conditions, both CRH and AVP are secreted every 2-3 hours into the bloodstream following a pulsatile, circadian rhythm (Engler et al. 1989). In resting conditions these hormones are usually produced in early hours of the morning with consequent rise of ACTH and cortisol circulating levels. This pulsatile secretion can be disrupted during stressful events (Horrocks et al. 1990; Chrousos et al. 1998).

During a single, momentary stress there is a massive release of CRH and VPN, thereby leading to a more frequent secretion of ACTH and cortisol. According to the type of stress, other molecules like angiotensin II and various cytokines act as signaling mediators inducing exponential activation of the HPA axis (Holmes et al. 1986; Phillips 1987).

In this light glucocorticoids represent the real final effectors of the HPA axis, thereby directly contributing to regulation of body homeostasis and stress systemic response. At the same time they present a key role in regulating basal activity of HPA axis and in blocking the systemic stress response through a negative-feedback mechanism both on hypothalamus and pituitary gland. According to this mechanism of action, the stimulation of glucocorticoids on periferic organs is progressively reduced when the stressful event is no longer present (De Kloet et al. 1991).

Glucocorticoids mediate their biological effects through activation of cytoplasmatic receptors ubiquitously expressed. In their inactive form these receptors are complexed

with heat shock proteins (HSPs) that impair their binding to DNA. Following ligand binding the receptor show a conformational change and dissociates from HSP. The new receptor-ligand complex, in an homodimeric form, thereby migrates to the nucleus where it directly binds specific DNA sequences located in the target genes promoters and named glucocorticoids responsive elements (GREs) (Smith et al. 1993; Pratt 1990). The activated receptors also act through protein-protein interaction in order to regulate specific signaling pathways like the c-jun/c-fos and NF-kB mediated ones as positive regulators of genes involve in activation and proliferation of immune system cells (Scheinman et al. 1995)

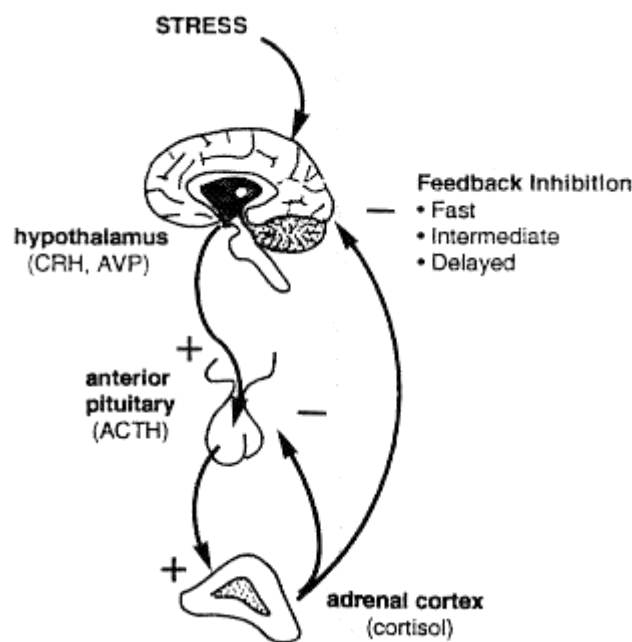


Fig. 10 The hypothalamic-pituitary-adrenal axis (HPA).

Cortisol and other glucocorticoids hormones exert both anabolic effects in the liver and anabolic effects in several peripheric tissues such as muscle, connectival, lymphoid and adipose tissues. In particular, glucocorticoids effects are:

- Inhibition of ACTH secretion. Cortisol inhibits ACTH secretion through a feedback inhibition exerted both at the hypothalamus and at the pituitary levels. In general all glucocorticoids inhibit ACTH secretion, and the more powerful is the glucocorticoid action, the greater is the degree of inhibition.
- Metabolic effects. Glucocorticoids stimulation increases liver glycogenolysis, while decreasing glucose uptake in muscle, adipose and other tissues, thereby inducing an increase in circulating glucose free levels. These actions synergize

in order to promote mobilization of energy sources, like amino acids, fatty acids and glycerol, from some tissues to provide energy substrates, particularly glucose, for others, notably the brain and heart, both characterized by high glucose consumption rate. The metabolic effects of glucocorticoids may be counterbalanced by those of other hormones, particularly insulin, the secretion of which is in turn stimulated by the rise in glucose circulating levels.

- Vascular reactivity. In addition to its effects on the organs and tissues directly involved in metabolic homeostasis, cortisol influences various other organs and systems. For example, cortisol maintains the responsiveness of vascular smooth muscle to CA and therefore participates in blood pressure regulation. In adrenal insufficiency, vascular smooth muscle becomes unresponsive to CA. The decreased responsiveness, together with the associated hypovolemia caused by mineralocorticoid deficiency, can result in severe hypotension (Chrousos 2007; Taché et al. 2007).
- Effects on central nervous system. Cortisol can directly modulate electrical activity of the neurons via type I and type II glucocorticoid receptors that are primarily expressed in limbic system and hippocampus. The ability of cortisol to decrease hippocampal volume as well as memory has also been demonstrated. Cortisol decreases REM sleep but increases both slow-wave sleep and time spent awake. Excessive concentrations of cortisol in blood can cause insomnia and strikingly increase or decrease mood. In addition, loss of sleep seems to be correlated to elevated circulating levels of IL-6 despite the reduced stimulation of IL-6 production exerted by catecholamines. This effect is probably related to reduced glucocorticoids-mediated inhibition (Vgontzas et al. 1999; Vgontzas et al. 2003).
- Effects on immune system and inflammatory responses. Various studies have demonstrated that cytokines and other umoral mediators of inflammatory response act as powerful activators of stress response from the central nervous system, thereby creating a molecular communication system between central nervous system and immune system. For example three of the most important proinflammatory cytokines, tumour necrosis factor α (TNF- α), interleukin-1b (IL-1b) and interleukin-6 (IL-6) directly stimulate HPA axis activity during chronic inflammatory diseases, both alone and/or through synergic actions (Chrousos 1995; Tsigos et al. 1997). Some of these positive effects are indirectly

related to stimulation of central and peripheral catecholaminergic signaling pathways. In particular activation of nociceptive, somatosensory and visceral peripheral nerve fibres induce secretion of CRH and CA through spinal ascending fibres (Chrousos 1995), thereby leading to a strong impairment of immune and inflammatory response as all cellular components are inhibited by glucocorticoids production. These hormones decrease differentiation and proliferation of local mast cells, stabilizes lysosomes and decreases production of platelet activating factor (PAF) and nitric oxide (NO). Glucocorticoids also suppress immune response by decreasing the number of circulating T-lymphocytes and by decreasing the production of interleukins and interferon- γ (IFN- γ) that are critical mediators of immune response (Chrousos 1995; Elenkov et al 1999). In addition, cortisol and all known glucocorticoids suppress synthesis and decrease the release of arachnidonic acid, the key precursor for a number of mediators of inflammation like prostaglandins (PGDs) and leucotrienes (LTs).

- Effects on stress. ACTH and cortisol secretion are increased by stressful stimuli including surgery, trauma, pain, infection, hypoglycemia and hemorrhage, thereby inducing an higher secretion of NE and E from adrenal medulla. In particular, locus ceruleus (LC) and other groups of cells secreting NE, collectively known as the LC/NE system, contribute in increasing autonomous and neuroendocrine stress responses through HPA axis activation. These observations show a biunivocal connection between CRH production and LC/NE system where CRH stimulate secretion of NE and viceversa (Taché 2007).

Biosynthesis of catecholamines

CA have been first characterized at the beginning of the twentieth century. CA are synthesized from the amino acid precursor L-tyrosine (Fig. 11). There are two primary sources of tyrosine, from the diet and from hydroxylation of the amino acid phenylalanine in the liver.

Upon entry into an adrenal chromaffin cell, sympathetic or brain catecholaminergic nerve terminals, tyrosine is converted to dihydroxyphenylalanine (DOPA) by the soluble cytoplasmic enzyme tyrosine hydroxylase (TH). TH is an iron-containing, bipterin-dependent aminoacid hydroxylase. It utilizes tyrosine, tetrahydrobiopterin (BH₄), and molecular oxygen to generate DOPA, dihydrobiopterin, and water. The cofactor BH₄ is resynthesized from dihydropterin by the enzyme dihydropteridine reductase. Since BH₄ is present in subsaturating levels, TH activity depends on its availability. In humans, several isoforms of TH can arise from alternative splicing of a single primary transcript. Primates exhibit only two of these isoforms, and lower animals have only one. The significance of these isoforms, the physiology of the alternative splicing, and whether it is affected by stress is not clear (Nagatsu 1991). TH activity is intricately regulated in the short and long term.

In the short term, TH enzymatic activity is regulated by feedback inhibition; thus TH is inhibited by catecholamines secretion (DOPA, NE, DA). TH is also regulated by allosteric regulation and direct protein phosphorylation. TH can be phosphorylated by a variety of kinases at several serines (positions 8, 19, 31, and 40) in the NH₂-terminal domain, but the role of this post-transcriptional modification has not been clarified yet.

On the other hand, in the medium to long term, TH can be regulated by enzyme stability, transcriptional regulation, RNA stability, alternative RNA splicing, and translational activity. Change in TH gene expression is a major mechanism whereby the catecholaminergic system responds to stress. For example, CREB is one of the mediators of the transcriptional response to stress: CREB is one of the transcription factors of the CREB/ ATF family that then binds as dimers to the cAMP response element (CRE) on a variety of genes, including TH. CREB phosphorylation on serine-133, necessary for its pro-transcriptional activation, is found elevated in the LC during exposure to single and repeated stressful events and this leads to increased TH expression (Nagatsu 1995).

Subsequently DOPA is converted into DA by a nonspecific enzyme, aromatic L-amino acid decarboxylase (AAAD) whose activity strongly depends upon availability of its

cofactor, pyridoxal phosphate. The most high DA-producing region in the nervous system is represented by the corpus striatum, which receives the projections from the substantia nigra and has a central role in regulating motor coordination. For example, in Parkinson's disease loss of dopaminergic neurons inside the substantia nigra lead to its peculiar motor disfunction.

Dopamine is then taken up from the cytoplasm into storage vesicles and converted into NE by dopamine hydroxylase (DBH), an enzyme found in soluble and membrane-bound forms within storage vesicles.

Both forms are encoded by the same mRNA. DBH activity utilizes Cu^{2+} , ascorbic acid, and O_2 . The NE is mainly synthesized in the adrenal medulla and the central and peripheral nervous system, where it is used by cells in the ganglia of the sympathetic nervous system, thereby showing that NE represents the main neurotransmitter in this section of the peripheral autonomic nervous system. It is also used in the locus coeruleus where it influences states of sleep and wakefulness, attention, and eating behavior.

NE is then converted into E by the soluble cytoplasmic enzyme phenylethanolamine *N*-methyltransferase (PNMT) that uses *S*-adenosyl-methionine as the cofactor and whose activity is inducible by glucocorticoids. PNMT is mainly localized in the adrenal medulla; however, sympathetically innervated organs and some brain areas are also able to synthesize small amounts of E. Separate populations of adrenal chromaffin cells contain NE and E as the final products of CA biosynthesis. PNMT gene expression was found also in some nonneuronal cells in the heart and skin (Ziegler et al. 1998).

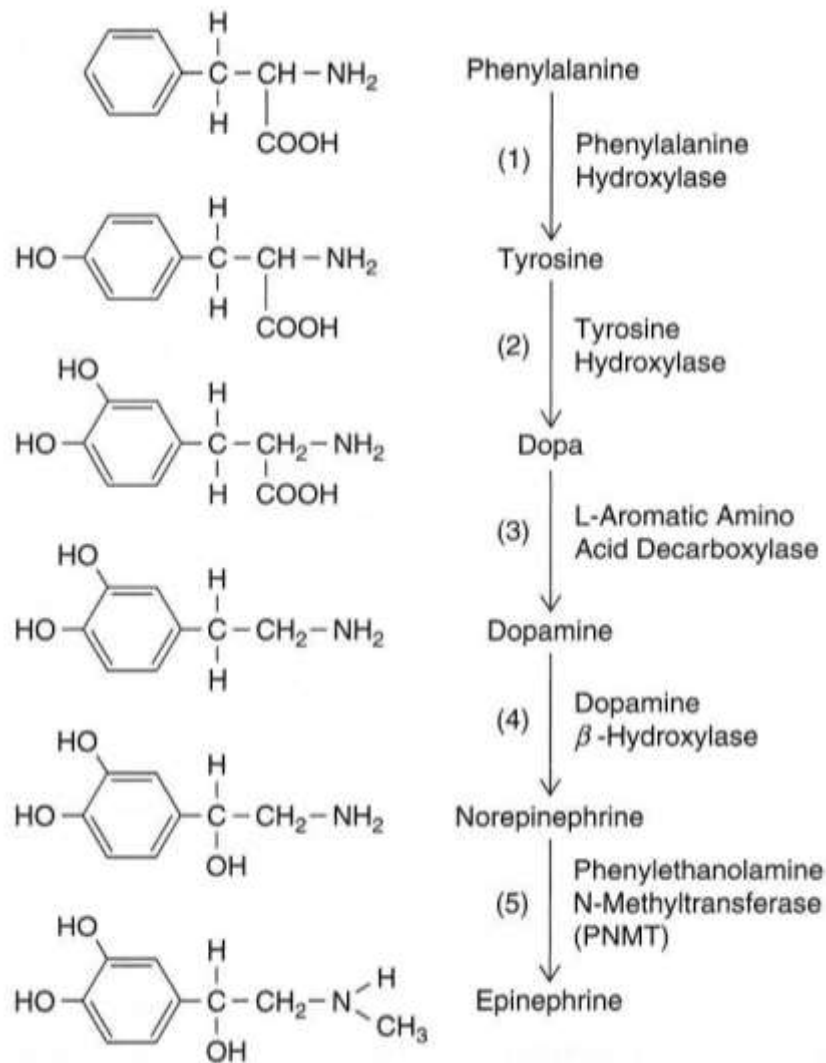


Fig. 11 Biosynthesis of catecholamines.

Release and action of catecholamines

The process of CA release is similar in the adrenal medulla and the sympathetic nerve endings (fig. 12). Acetylcholine released from sympathetic preganglionic nerve terminals binds to nicotinic cholinergic receptors and leads to a depolarization of cell membrane, resulting in an increase in membrane permeability to sodium. This initiates a series of events that lead to an increase in the influx of calcium. Then, CA storage vesicles fuse with the chromaffin or sympathetic neuronal cell membrane and release via exocytosis their contents of CA, together with chromogranins, other neuropeptides, ATP and a fraction of the soluble DBH, via exocytosis. However, the exact mechanism of Ca^{2+} evoked exocytosis is not clear (Young et al., 1998).

Currently, more than 30 biologically active substances have been localized in adrenal chromaffin, sympathetic neuronal, and brain catecholaminergic cells, and a number of

them are released following depolarization of the cell membrane. In addition, various biologically active neuropeptides are colocalized with acetylcholine within sympathetic preganglionic nerve terminals like substance P, neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP), and they all appear to act as neuromodulators or cotransmitters of cholinergic transmission (Young et al., 1998; De Diego et al., 2008). In particular, the VIP peptide has a key role in stimulating catecholamines release, while substance P modulates acetylcholine secretion. Finally, the neuropeptide NPY seems to also exert a trofic effect on sympathetic nerve terminations and has been recently described as the major factor responsible for stress-induced obesity (Zukovska et al., 1998; Kuo et al., 2007; Kuo et al., 2008). Another example is represented by protein sinexin, also named annexin VII, which exerts a role in mediating the signaling pathway responsible for vesicles fusion to neuronal cell membranes, while other proteins such as cathestatin have an inhibitory effect on catecholamines release (Pollard et al., 1998; Kennedy et al., 1998).

After release in the intersynaptic space, CA-mediated biological effects are quickly blocked through two main processes:

- Conversion of CA in biologically inactive products through enzymatic action of monoamine oxidase (MAO) in neuronal cells and catechol-O-methyltransferase (COMT) in non-neural cells.
- Re-uptake of active CA and formation of new cytoplasmic storage vesicles in both sympathetic and effectors neural cells.

MAO is a mitochondrial flavoprotein located in the outer membrane of presynaptic neurons that catalyzes deamination of amines, with production of aldehydes that are metabolized to carboxylic acids or alcohols. MAO-A subtype has a higher affinity for NE and E and is highly localized in brain neurons. MAO-B is responsible for degradation of DA. In sympathetic nerves, the aldehyde produced from NE and, to a less extent, from E by MAO-A is converted to dihydroxyphenylglycol (DHPG). Subsequent extraneuronal *O*-methylation leads to production of 3-methoxy-4-hydroxyphenylglycol (MHPG).

COMT is involved in the inactivation of the catecholamines through addition of a methyl group to the catecholamine, which is donated by S-adenosyl methionine. In particular, the enzyme catalyzes reactions of methylation of NE in normetanephrine and of E in metanephrine. COMT can also be found extracellularly, although extracellular

COMT plays a less significant role in the CNS than it does peripherally where is primarily expressed in the liver (Golan and Armen).

The mechanisms of neural uptake act synergistically with COMT and MAO enzymatic activity. In particular, neuronally released CA are inactivated by “uptake 1” (U1) in combination with enzymatic degradation by MAO, while circulating CA are inactivated by “uptake 2” (U2) and COMT-mediated enzymatic degradation (Axelrod et al., 1957; Axelrod et al., 1959).

Uptake 1 (U1) acts in order to recapture locally released NE or circulating NE to save it by intraneuronal storage for reuse. U1 requires energy and is a mechanism carrier-mediated, where the carrier involved moves CA against large concentration gradients. Since U1 acts as a first-order kinetic process, U1 increases in parallel with increase NE release during exposure to stressors. In fact, about 90% of released NE is reuptaken to neurons through two main transporters, NE transporter (NET) and dopamine transporter (DAT), while it seems to have a less important role in circulating E inactivation (Eisenhofer et al., 1990; Eisenhofer et al., 1991). In particular, NE is translocated by NET about twofold more effectively than E. This explains why sympathetic nerves take up NE more efficiently than E. Dopamine is a much better substrate for DAT than NE or E. NET and DAT-mediate transport is Na^+ and temperature-dependent and is characterized by high catecholamines affinity, but very low capacity. In brain, TH and DAT are considered characteristic markers for dopaminergic neurons. While DAT is mainly expressed in brain dopaminergic cells, neither NET nor DAT expression is restricted to central or peripheral noradrenergic neurons. Uptake of NE by NET also takes place in some extraneuronal cell types that express the same NET isoform that is expressed in noradrenergic neurons. Extraneuronal sites of NET expression are in the chromaffin cells of adrenal medulla, in lung and placental tissues, while extraneuronal DAT expression was found in the gastrointestinal tract, pancreas and kidney (Eisenhofer et al. 2001).

Extraneuronal uptake 2 (U2) is an active process of transport into non-neuronal cells. The extraneuronal monoamine transporter of U2 (EMT) has little stereospecificity and has very low affinity and specificity for CA. In particular, unlike U1, U2 favors E over NE and is not a Na^+ and Cl^- -dependent process. U2 is responsible for formation of CA metabolites in liver, kidney, and lung and is highly sensitive to inhibition by glucocorticoids. As a negative feedback control mechanism, U2 activity is strongly

impaired by CA *O*-methylated metabolites normetanephrine, metanephrine, and by corticosteroids. (Bonish 1980).

In addition, until now at least three catecholamines transporters located in non-neural sites have been described and named cationic organic transporters (OCT)-1, 2 e 3, all characterized by high specificity for DA transport (Eisenhofer et al. 2001).

Subsequently reuptaken CA are stored into new cytoplasmic vesicles through action of the monoamin vesicular transporter (VMAT). Two isoforms of this transporter have been described until now: VMAT-1, considered as the “neuroendocrine form”, and VMAT-2, also known as the “neuronal form”, strictly expressed in the central and peripheral nervous systems. As for the molecular transporters previously described, also VMATs transport molecules against concentration gradient thereby exploiting the protonic gradient created by ionic H⁺ channels (Goldstein 2001).

In conclusion, fast enzymatic inactivation/reuptake of CA released into the synaptic cleft is a prerequisite for fine control over the effector system. Contrary to the usual depictions, vesicular stores of CA do not exist in a static state simply waiting for exocytotic release. Rather, they exist in a highly dynamic equilibrium with the surrounding cytoplasm, with passive outward leakage of CA, counterbalanced by inward active transport under the control of VMAT (Eisenhofer et al. 2004).

In spite of the fact that this process is highly activated by stress, the studies on stress-induced changes in activity and gene expression of CA transporters located in the peripheral neurons are quite rare. Very few reports deal with NET transporter protein and gene expression during oxidative stress in PC12 cells. These results support a functional role of oxidative stress in mediating the neuronal NE uptake associated with reductions in NE uptake binding sites and NET protein production, without changes in NET gene expression. The effect of oxidative stress on NET is a post-transcriptional event (Mao et al., 2004; Mao et al., 2005).

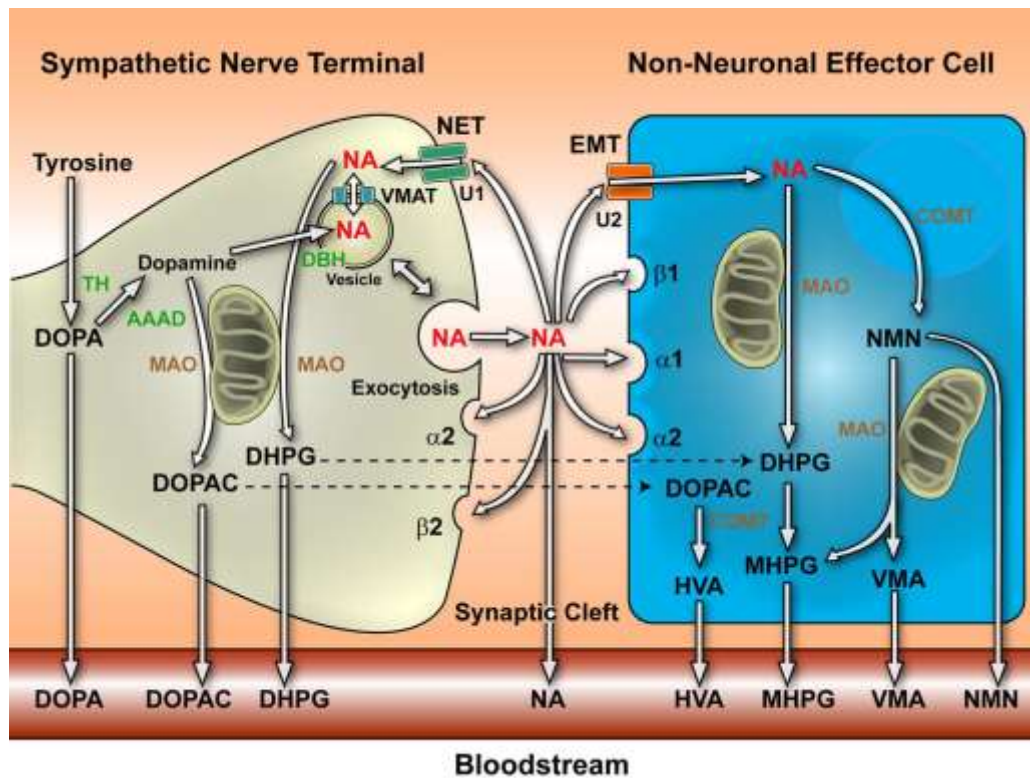


Fig. 12 Mechanisms of secretion, action and re-uptake of catecholamines in central and peripheral neuronal cells.

The adrenergic receptors

In order to mediate their biological effects, CA bind to specific receptors named adrenergic receptors, all included in the superfamily of transmembrane seven domains receptors coupled to G proteins. In 1948 Ahlquist first identified and classified these receptors in two main types, alpha (α) and beta (β), according to their responsiveness to *in vitro* stimulation with different classes of molecular agonists and antagonists (Ahlquist 1948). Almost fifteen years later α receptors have been further divided into two classes according to their anatomic location, thereby defining as α_1 post-synaptic receptors and as α_2 the pre-synaptic ones (Langer 1974). Further biological and pharmacological studies have led to the final classification of adrenergic receptors in distinct subtypes. In particular, α_1 receptors have been divided in α_{1a} , α_{1b} and α_{1d} , while α_2 receptors have been classified in α_{2a} , α_{2b} and α_{2c} adrenoceptors.

β receptors are also heterogenous and were initially subdivided into β_1 and β_2 adrenoceptors on the basis of functional and ligand affinity studies. Subsequently the β receptors have been classified using functional studies, receptor binding and genetic techniques. The β adrenoceptor family is now divided into three distinct subtypes, the β_1 , β_2 and the atypical β_3 receptor. There is an additional β -adrenoceptor subtype which has been recently identified in cardiac tissue and is a putative, atypical subtype named β_4 adrenoceptor (Lands et al. 1967; Strosberg e Pietri-Rouxel 1996; Kauman 1997) (fig. 13).

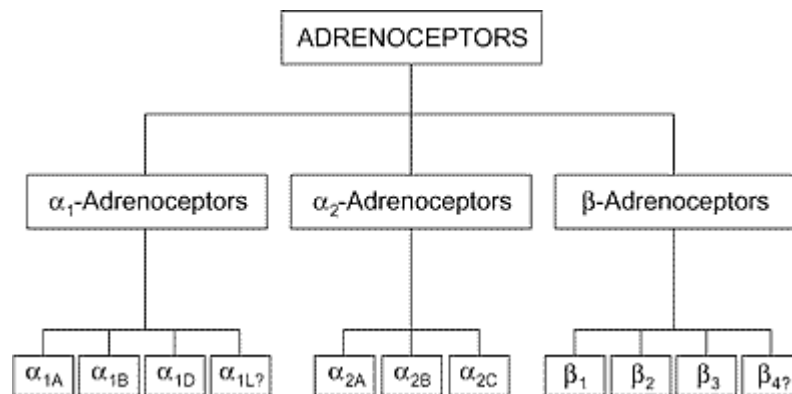


Fig. 13. α and β receptors classification.

- α_1 : these receptors are located in the central and periferic nervous system. In particular, in the central nervous system they are highly expressed post-synaptically where they mediate an excitatory role. On the other side, peripheral α_1 receptors are predominantly located intrasynaptically on both vascular and non-vascular smooth muscle where receptors activation results on muscle contraction and increase of blood pressure. They are also located on the cardiac muscle where they mediate a positive inotropic effect and on the liver, activating glycogen phosphorylation. (Bylund 1992; Aboud et al. 1993).

All these receptors are coupled to phospholipase C and their activation induces production of second messengers like inositole 3-phosphate (IP_3) and diacylglycerol (DAG). This signalling pathwat leads to activation of ionic channels voltage-dependent and stimulation of protein kinase C (PKC) and phospholipase A2, thereby regulating ciclic AMP (cAMP) and arachidonic acid (AA) production (Harrison et al. 1991; Berridge e Irvine 1989).

- **α_2** : the heterogeneous family of these receptors has been characterized studying their different binding affinity heterogenic, thereby allowing the identification of different receptors subtypes even within the same tissue. These receptors are now divided in α_{2a} , α_{2b} , α_{2c} e α_{2d} receptors (MacKinnon et al. 1994). In particular, the α_{2d} receptor shows a unique pharmacological profile, but it is still considered a splicing variant or receptor α_{2a} for its high sequence homology with this receptor (fig. 9) (Simonneaux et al. 1991).

α_2 receptors are expressed both at a pre and post-synaptic level where they mediate inhibitory effects on the central and peripheral nervous system (French 1991). In the central nervous system they regulate neurotransmitter release through autoreceptors located on noradrenergic nerve terminals and heteroreceptors located on other neurotransmitter signals. In particular, their sedative properties, mediated by somatodendritic autoreceptors on the locus coeruleus, have led to the development of α_2 -agonists as sedatives and anesthetics. Other central effects of α_2 adrenoceptors include the regulation of blood pressure, pupil diameter and body temperature. Peripheral activities include contraction of vascular smooth muscle and inhibition of lipolysis through activation of the receptors expressed by fat cells.

The activity of α_2 receptors is mediated by the activation of different G proteins including G_i/G_o proteins. All the receptor subtypes are negatively coupled to adenylyl cyclase, thereby mediating an inhibitory effect on cAMP production (fig. 14). In addition, it has been recently characterized a role of these receptors in activating several types of ionic channels like K^+ and Na^+/H^+ transporters (Bylund et al. 1995).

- **β_1** : β_1 receptors are located in high density in the striatum and a selective decrease in the expression of these receptors is related to Huntington's disease development (Waeber et al. 1991). β_1 receptors are also expressed in kidneys, cardiac muscle, airway muscle and fat cells and they show similar affinity for both NE and E binding. It is an excitatory receptor and its activation leads to positive cardiac inotropic and chronotropic effects, while in kidneys it stimulates renin secretion from juxtaglomerular cells.

β_1 , β_2 and β_3 receptors positively regulate adenylyl cyclase through activation of small G_s protein, thereby stimulating voltage-dependent Ca^{2+} ionic channels (fig. 14) (Bylund et al. 1994).

- **β_2** : it is an inhibitory receptor and shows higher affinity for NE binding in comparison with E. This receptor is mainly expressed on gastrointestinal and airway smooth muscle cells and its activation results in bronchodilation and general muscle relaxation. It is also located on blood vessels and coronaric arterie, increasing organs perfusion.
- **β_3** : is an excitatory receptor and it is located mainly in adipose tissue, both white and brown, where it is involved in the regulation of lipolysis and thermogenesis by NE through activation of the enzyme lipase. For this reason β_3 agonists are good candidates for obesity treatment (Kauman 1997). Some other β_3 agonists have also demonstrated antistress effects in animal studies, suggesting it also has a role in the central nervous system. β_3 receptors are found in the gallbladder and in urinary bladder. Their role in gallbladder physiology is unknown, but they are thought to play a role in lipolysis and thermogenesis in brown fat. In the urinary bladder it is thought to cause relaxation of the bladder and prevention of urination.

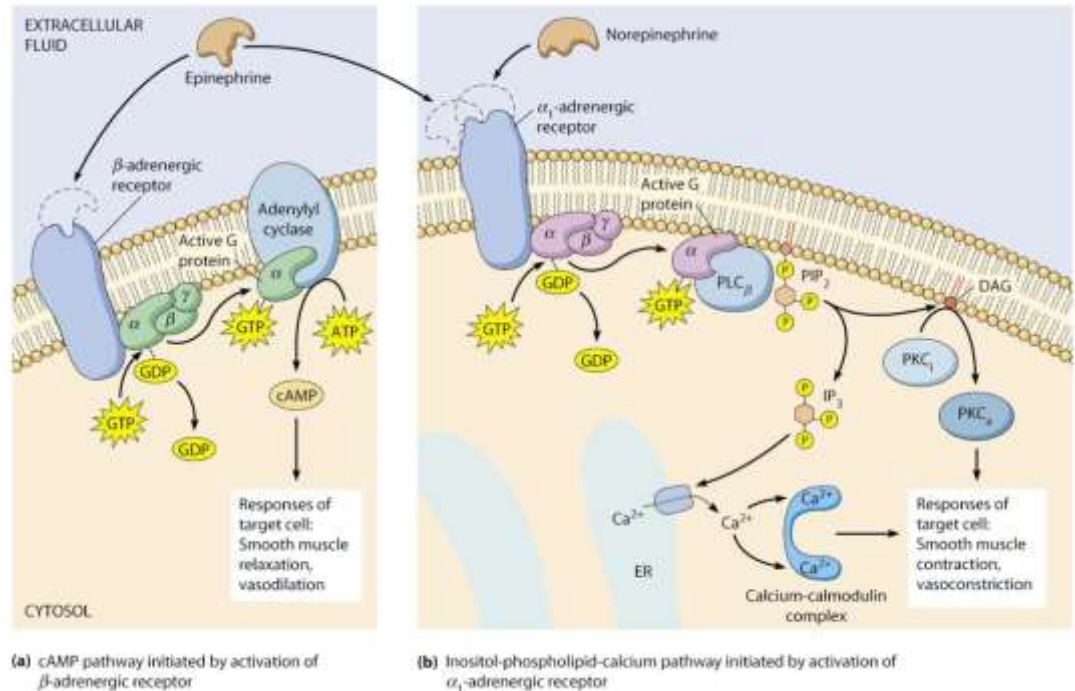


Fig. 14 α and β adrenergic receptors and their signalling pathways.

Catecholamines and cancer

Over the past 25 years, epidemiological and clinical studies have linked psychological factors such as stress, chronic depression, and lack of social support to the incidence and progression of cancer. In particular, several molecular and animal studies have begun to identify specific signaling pathways that could explain the impact of neuroendocrine effects on all stages of tumour growth and metastasis, from proliferation and growth of the primary tumour to metastatization of distant tissues (fig. 15) (Reiche et al., 2004; Sood and Thaker, 2008).

Adhesion to ECM

The tumour cell ability to adhere to ECM represents a key event leading to metastatization of distant organs. In particular, integrins receptors expressed on cells membrane are key mediators for ECM-cell interaction, and recent studies have started focusing on the role of CA in regulating cancer cells adhesion. Despite this mechanism have not been fully elucidated yet, it is well known that cAMP has the ability to regulate activity of the adhesion-mediating small GTPases RhoA and Rac through the activation of protein kinase A (Mercurio and Rabinovitz, 2001). Recent studies also showed that the exchange factor directly activated by cAMP (Epac) is also involved in integrin-mediated cell adhesion and cell-cell junction formation. According to these observations, recent data show that the β -agonist isoproterenol promotes ovarian cancer cell spreading and adhesion to laminin-5 in an Epac-dependent way and promotes adhesion to a fibronectin matrix in a cAMP mediated Epac-Rap1 pathway. Thus, stress hormones may promote cancer cell-matrix attachments and this mechanism represent a promising therapeutic target in order to avoid cancer cells migration from the primary tumour to distant peritoneal sites (Bos, 2006).

Proliferation and growth of primary tumour and metastasis

At both primary and metastatic sites, activation of autocrine, paracrine and/or endocrine pathways can promote tumour cell proliferation by disrupting the balance between positive, pro-proliferative and inhibitory signals (Langley and Fidler, 2007). Until now, data about the role of CA on cancer cells proliferation seem to be quite controversial and closely related to the studied cell type. In fact, some observation suggest that catecholamines repress normal cell proliferation, such as slowing keratinocyte growth, resulting in reduced wound healing in the context of stress (Flaxman and Harper, 1975).

On the other hand, other studies show that β -adrenergic inhibition with β_2 adrenergic receptor agonist pirbuterol reduces human tumour cell growth in xenograft models through inactivation of the Raf-1/Mek-1/Erk1/2 pathway (Carie and Sebti, 2007) and, in breast cancer, some studies have directly related β_2 -adrenergic receptor activation to increased tumour growth and progression. In other tumour models, CA inhibition on cancer cells proliferation have been related to dopamine and α -adrenoceptors signaling. For example, studies focused on a neuroblastoma model showed that NE inhibited cancer cell growth in cells expressing the dopamine transporter, where treatment with NE induced an increased ratio of cells undergoing G0/G1 phase (Pifl et al., 2001).

β_2 -adrenergic receptors may also act synergistically with other stimuli on cancer cells proliferation. A recent study showed that in a gastric cancer model these receptors contribute to nicotine-induced activation of the protein kinase C/Erk1/2/cyclooxygenase-2 (COX-2) pathway, leading to enhanced tumour cells proliferation (Shin et al., 2007). Another factor regulated by adrenergic signaling is represented by the transcriptional factor cAMP responsive element binding (CREB) protein, which can be activated in response to external stimuli such as stress hormones. It has been clearly demonstrated that CREB plays an important role in tumour cell proliferation, migration, angiogenesis, and apoptosis inhibition (Jean and Bar-Eli, 2000). Thus, β -adrenergic receptor signaling might interact with other CREB activators in order to modulate molecular processes involved in tumour progression, like viral infections. Infective events are key co-factors in the initiation of approximately 20% of human tumours, and all major human tumour-associated viruses have been found to be activated by either β -adrenergic or glucocorticoid-mediated signaling pathways (Antoni et al., 2006). For example, human herpesvirus 8, responsible for Kaposi's sarcoma, activates a cAMP response element in the promoter of a key viral transcription factor, thus β -adrenergic stimulation of the viral host cell induces CREB-mediated expression of viral oncogenes and growth factors that promote viral infection spreading and progressively lead to cancer initiation. Also Epstein-Barr virus and high-risk variants of the human papilloma virus are similarly activated by glucocorticoids action (Antoni et al., 2006).

Finally, in a prostate carcinoma model, treatment with cAMP agonists resulted in epithelial prostate cancer cells acquiring neuroendocrine characteristics (Cox et al., 1999). These characteristics were represented by dense core granules in the cytoplasm, the formation of neuron-like processes, loss of mitogenic activity, and new expression of neuroendocrine markers. The presence of these neuroendocrine-like cells has been

linked to poor prognosis in prostate cancer patients as they have minimal proliferative ability, but promote proliferation of surrounding cells through paracrine stimulation (Cohen et al., 1991).

Angiogenesis

Recently, it has been found that stress hormones may promote angiogenic mechanisms in human tumours, thereby leading to increased growth both of the primary tumour and metastasis. In adipose tissues, nasopharyngeal cancer cells and in two ovarian cancer cell lines, VEGF has been shown to be upregulated by NE through activation of β -adrenergic receptor signaling pathway cAMP/protein kinase A (PKA)-dependent, (Lutgendorf et al., 2003; Yang et al., 2006). The effect of NE on VEGF stimulation was completely reverted after treatment with non selective β -blocker propranolol and mimicked by agonists of β -adrenergic receptors (Thaker et al., 2006). Furthermore, NE modulates the expression of VEGF through activation of β_2 -adrenergic receptor in non-solid tumours, such as multiple myeloma (Yang et al., 2008). Observations about the effects of stress hormones on tumour angiogenesis have been confirmed both *in vivo* and *in vitro* cancer models. In an ovarian cancer orthotopic model, chronic stress induced by daily restraint resulted in higher levels of circulating and tissutal CA, increased tumour burden, increased number of microvessels, and a more invasive phenotype. Moreover, these same samples showed increased VEGF levels. According to that, clinical data have demonstrated that there is an association between higher levels of social support and lower serum VEGF levels in ovarian cancer patients (Lutgendorf et al., 2002). Continuous infusion of a non-selective β -blocker partially abrogated the effects of stress on tumour growth and progression, suggesting that β -adrenergic receptors play an important role in stress-mediated tumour growth (Thaker et al., 2006). Interleukin 6 (IL-6) is another key cytokine that plays a key role in tumour progression and angiogenesis and, according to this observation, it is related to increasing microvessel density and poorer cancer outcomes (Kiecolt-Glaser et al., 2003; Nilsson et al., 2005). Lowering stressful conditions exerts a protective effect and correlation between IL-6 with biobehavioral factors acts both at the primary tumour level and distant organism sites (Costanzo et al., 2005). These clinical data have been confirmed *in vitro* in an ovarian cancer model, showing that NE significantly increased IL-6 expression and that this increase was due to CA-mediated transcriptional regulation of IL-6. Additionally, NE induced IL-6 production in ovarian carcinoma cells was

regulated by a β -adrenergic receptor/Src tyrosine kinase pathway (Nilsson et al., 2007). The signal transducer and activator of transcription factor-3 (STAT-3) is activated by growth factors and cytokines, such as IL-6 and VEGF and has been found to promote angiogenesis and suppress apoptosis (Antoni et al., 2006). It is known that cytokines such as IL-6 can contribute to tumour growth and progression through the activation of STAT-3. Recent studies in an ovarian cancer model have determined that both NE and E activate STAT-3, promoting its translocation to the nucleus and binding to DNA. These effects on STAT-3 were mediated by β -adrenergic receptors and PKA signaling and were independent of IL-6, thereby suggesting that stress-mediated tumour progression may result, in part, through STAT-3 activation of downstream effector pathways (Landen et al., 2007).

Migration/invasion

There are now several lines of evidence suggesting that stress hormones can promote tumour cell movement and invasion in order to reach blood or lymphatic vessels that facilitate tumour spreading. For example, in a breast cancer model, NE induced not only a chemotactic response, but also promoted chemokinetic migration (Drell et al., 2003). NE can also promote migration in a phospholipase C γ and protein kinase C α dependent manner. In fact it has been demonstrated the role of CA and glucocorticoids on the invasive potential of ovarian cancer cells, and MMPs that are important for tumour cell penetration of extracellular matrix (Sood et al., 2006). NE concentrations similar to those found in the bloodstream in stress conditions significantly increased the *in vitro* invasiveness of ovarian cancer cells. E also promoted the invasive potential of ovarian cancer cells and these effects were blocked by a β -adrenergic receptor antagonist. Additionally, NE increased the *in vitro* production of MMP-2 and MMP-9 by tumour cells, and pharmacologic inhibition of MMPs blocked NE mediated increase in tumour cell invasion. Similar findings have been demonstrated in a nasopharyngeal carcinoma model where catecholamines increased the invasive potential by increasing MMP-2 and MMP-9 levels as well (Yang et al., 2006).

Resistance to apoptosis/anoikis

The continuation of the metastatic process depends on the ability of the tumour cell to avoid apoptosis and anoikis (Langley and Fidler, 2007). It has been shown that

dopamine and NE can promote cellular apoptosis through a G protein-mediated signaling in neuroblastoma cells (Chan et al., 2007). In addition, both acute and chronic stress reduced the sensitivity of prostate and breast cancer cells to apoptosis through a PKA-dependent BAD phosphorylation that was mediated by E interaction with β -adrenergic receptors (Sastry et al., 2007). These *in vitro* data have been confirmed by clinical trials showing a more positive outcome for patients suffering of prostate cancer under daily treatment with β -blockers (Perron et al., 2004).

Furthermore, CA may also act together with glucocorticoids to promote cancer growth. For example, cortisol increased the isoproterenol-induced cAMP and β -adrenergic receptor accumulation on the cellular membrane and substantially increased the effects of IL-1 α , IL-1 β , and TNF- α in lung carcinoma cells (Nakane et al., 1990). In addition, synergistic action of CA together with glucocorticoids can counteract chemotherapy-related cytotoxic effects in cervical and lung carcinoma cells through reduced expression of pro-apoptotic factors involved in intrinsic apoptotic pathway (Herr et al., 2003). According to these observations, breast cancer cell lines pre-treatment with dexametasone impaired chemotherapy effects through glucocorticoid receptors activation. In particular, glucocorticoids promote mitogen-activated protein kinase phosphatase-1 (MKP-1) and glucocorticoid-inducible protein kinase-1 (SGK-1)-mediated pathways. Glucocorticoids-mediated anti-apoptotic effects could be reversed through MKP-1 and SGK-1 gene silencing (Wu et al., 2004).

Finally, *anoikis* is a cell process by which normal cells enter apoptosis when separated from the extracellular matrix and neighboring cells. Recently, it has been demonstrated that CA can protect ovarian cancer cells from anoikis. These effects are mediated by focal adhesion kinase (FAK) phosphorylation through ADRB2-dependent activation of Src kinase. Parallel results were observed in ovarian carcinoma patients, linking increased levels of stress/depression to increased FAK activation and demonstrating accelerated cancer progression in patients with high levels of FAK activity (Sood et al., 2010).

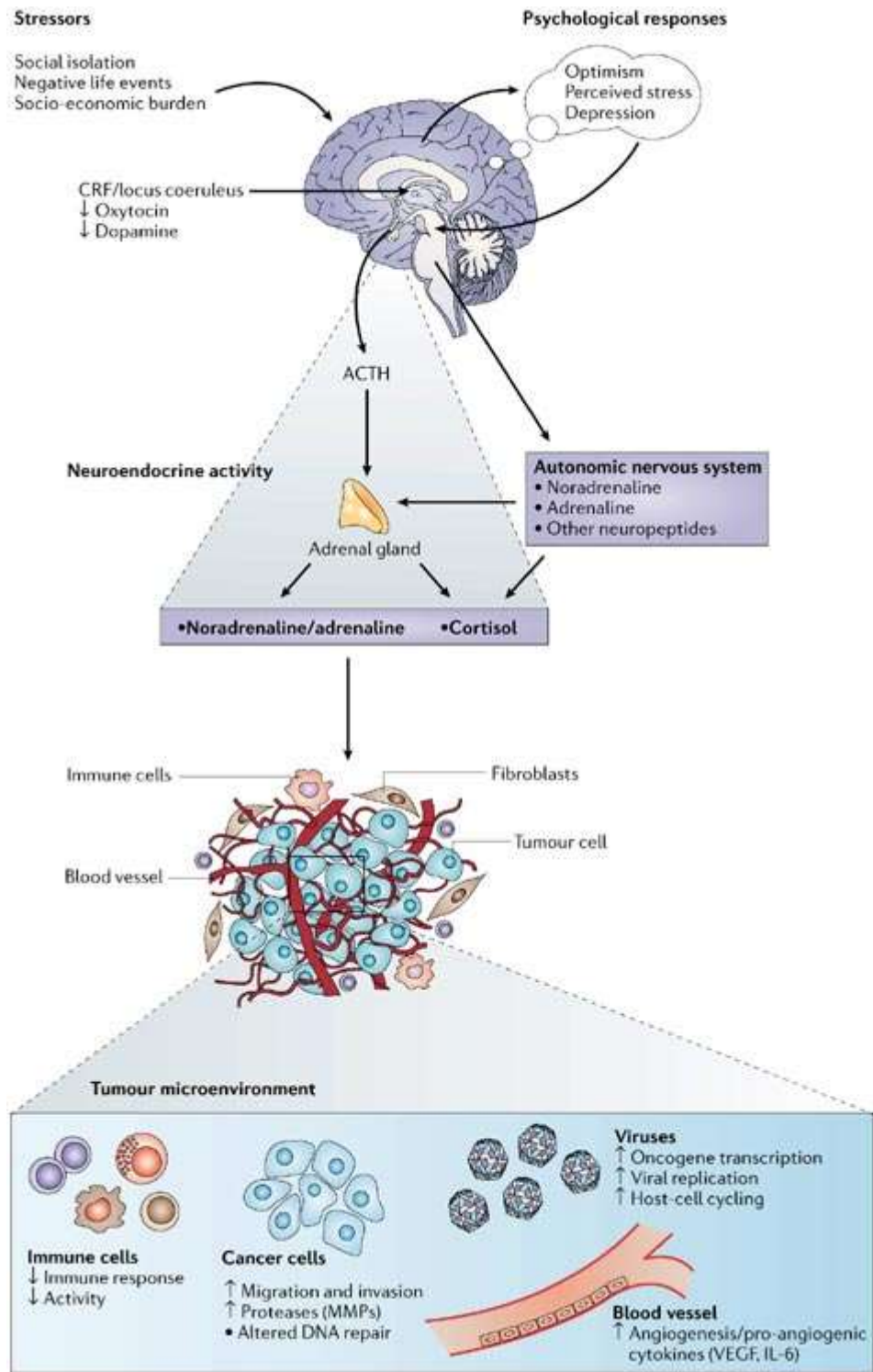


Fig. 15 Effect of stress and related factors on cancer cells and tumour microenvironment.

HYPOXIA AND HYPOXIA INDUCIBLE FACTORS (HIFs)

Tumour microenvironment alterations like hypoxia, resulting from lack of correct vascularization and/or excessive oxygen consumption (O_2), can directly influence cancer growth and progression through disruption of structures and functional tissue responses. All these events contribute to resistance to pharmacological therapy and bad prognosis. Major causes implicated in creation of hypoxic microenvironment are mostly structural/functional alteration of tumour vessels and a growing distance between blood vessels and cancer cells which strongly impairs O_2 diffusion and delivery (Fig. 16).

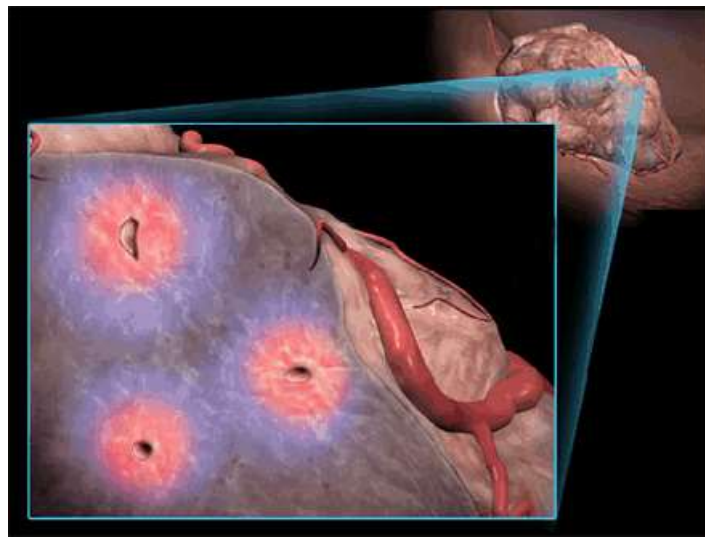


Fig. 16 Tumoural hypoxia. Violet areas represent hypoxic areas, while the grey ones represent the necrotic areas.

Solid malignant tumours are usually characterized by elevated number of hypoxic tissutal areas, with an O_2 pression ≤ 2.5 mmHg, eterogenously located inside the tumour burden. These regions may be located adjacent to regions with normal O_2 partial pressures (pO_2) but, in contrast to normal tissue, neoplastic tissue can no longer fulfill physiologic functions (Vaupel et al. 2001).

Hypoxia can be related to various factors dependent from perfusion, diffusion or anemic conditions (Vaupel et al., 2001; Vaupel et al., 2002). Perfusion-correlated hypoxia is caused by inadequate blood influx towards tissues. Tumoural microvessels often show both structural and functional abnormalities like disorganized microcirculation, perivascular detachment, vessels dilatation, irregular shape, loss of physiological/pharmacological receptor and complete absence of blood flux regulation. So new tumoural blood vessel formation is a relatively fragile process, subject to

disruptive interference at several levels and leads to frequent, but transient, ischemic events.

Diffusion-related hypoxia is characterized by a progressively growing distance between cancer cells and tumour microcirculation as a result of tumour mass growth. The final effect is a continuously reduced O₂ delivery towards cells >70µm distant from blood vessels, thereby leading to a condition of chronic hypoxia. Chronic hypoxia is also correlated to changes in the “geometry” of O₂ diffusion like modifications of blood flux paths inside cancer vessels.

Finally, reduced ability of red blood cells to deliver O₂ following anemic conditions related either to therapy or tumour itself causes anemic hypoxia.

In conclusion hypoxia acts as a continuative selective pressure by promoting growth and proliferation of genetically altered tumoural clones with diminished apoptotic potential, thereby promoting cancer aggressiveness. The increasing inability of tumour cells to activate apoptotic pathways can explain many of the clinical consequences of malignant progression, such as locoregional and distant tumour propagation and resistance to nonsurgical therapy. Survival and proliferation of occult peritumour cells with diminished apoptotic potential, located in hypoxic surgical scars, appear to be major pathogenetic events in the formation of local recurrences (Graeber et al. 1996; Soengas et al. 1999).

Hypoxia is also responsible for accumulation of genomic mutations by cancer cells, thereby increasing genomic instability favouring tumour progression and aggressiveness (Bindra e Glazer. 2005; Bindra e Glazer 2007; Huang et al. 2007; Koshiji et al. 2005). Hypoxic stress, both *in vivo* and *in vitro*, can generate DNA damage and mutations (Møller et al. 2001). Hypoxia-induced DNA damage has been detected throughout the bodies of individuals exercising at high altitude and has been attributed to ROS produced upon reoxygenation (Risom et al. 2007) or to stress-induced leakage of ROS from mitochondria (Møller et al. 2001). Also, severely hypoxic conditions and the subsequent reoxygenation progressively decrease the activity of DNA repair mechanisms (Bindra e Glazer. 2005). All these evidences have been confirmed through *in vitro* and *in vivo* studies where exposition of cancer cells to hypoxia have been demonstrated to actively promote their invasive and metastatic potential (Young et al. 1988; Young e Hill 1990; Cairns et al. 2001; Postovit et al. 2002; Rofstad et al. 2002).

Recent studies also suggest that sustained or intermittent hypoxic stress like cancer cells exposition for 6-8 hours at pO₂ ≤ 7 mmHg induces genetic alterations through both post-

transcriptional and post-translational modifications that modify cancer cells proteomic profile (Vaupel et al 2001; Vaupel et al 2002; Höckel e Vaupel 2001). These modifications include blockage of cell growth or damages to mechanisms of cell cycle arrest, differentiation, apoptosis and necrosis (Moudler e Rockwell S. 1987; Durand 1991; Giacca 1996; Riva et al. 1998; Haroon et al. 2000). Physiologically hypoxia-induced cell cycle arrest at G₁/S checkpoint is related to hypoxia inducible factor-1 α (HIF-1 α) which activates inhibitors of cycline-dependent kinases p21 e p27 (Goda et al. 2003). This mechanism of regulation seems to be p53-independent despite its accumulation during hypoxic conditions (Koumenis et al. 2001). In fact, hypoxia-induced apoptosis has been shown to be dependent on p53, Apaf 1, caspase 9, and caspase 3, indicating that the mitochondrial apoptosis pathway plays a significant role in this form of death, but hypoxia is also able to activate p53-independent apoptosis through a Bcl-2-dependent mechanism (Soengas et al. 1999).

Alternatively, hypoxia-induced proteomic changes induce cancer dissemination promoting cancer cells ability to adapt to nutrients deprivation or facilitating proliferation, local invasion and/or metastatization in order to escape from a hostile microenvironment.

As extensively debated in the next paragraph, cancer cells have developed elaborated mechanisms in order to detect pO₂ alteration and promote adaptation to hypoxia. The master regulator of this complex system is represented by transcription factor HIF-1, first identified by Semenza and colleagues as a protein directly binding EPO promoter DNA sequence (Wang et al. 1995). Its accumulation in response to low O₂ level induces activation of more than 100 genes involved in O₂ delivery like erythropoietin, angiogenesis (VEGF), anaerobic metabolism (glucose transporters and glycolitic enzymes) and other key mechanisms involving survival and cancer diffusion (Vaupel et al 2002; Höckel e Vaupel 2001).

HIF-1 α (Hypoxia-Inducible Factor-1 α)

HIF-1 is a heterodimeric complex characterized by an α subunit of 120 kDa O₂-dependent (HIF-1 α), and a β subunit of 91-94 kDa constitutively expressed (HIF-1 β) also known as aryl hydrocarbon nuclear translocator (ARNT), as it has been first identified inside the heterodimeric complex with the aryl hydrocarbon receptor (AHR) (Reyes et al., 1992). Both subunits belong to the protein superfamily of basic helix-loop-helix-Per/ARNT/Sim domains (bHLH-PAS) proteins (Wang et al., 1995) containing a N-terminal bHLH domain and two PAS domains (PAS-A e PAS-B) in order to specifically bind to DNA sequences called hypoxia responsive elements (HREs) located in target genes promoters and to form the heterodimeric complex HIF-1 α -HIF-1 β , respectively (Crews 1998) (Fig. 15). At the C-terminus HIF-1 α shows two transactivation domains named N-terminal transactivation domain (N-TAD), between aminoacids 531-575, and C-terminal transactivation domain (C-TAD), between aminoacids 786-826 (Ruas et al., 2002). In particular, the latter interacts with specific co-activators in order to activate genic transcription (Lando et al., 2002). In addition, stability and degradation of HIF-1 α subunit at different O₂ tensions is regulated through modifications of an O₂-dependent degradation domain (ODDD) (Fig. 15).

Finally, nuclear localization sequences (NLS) have been identified both at the N-terminal, between aminoacids 17-74, and at the C-terminal, between aminoacids 718-721. These sequences are crucial for HIF-1 α translocation into the nucleus independent from dimerization with β subunit (Kallio et al. 1998).

Other proteins of the HIF family are HIF-2 α , also known as endothelial PAS protein 1 (EPAS-1), HIF-like factor (HLF), HIF-related factor (HRF) and member of the PAS superfamily 2 (MOP2) alternatively, (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997) and the HIF-3 α factor (Fig. 17).

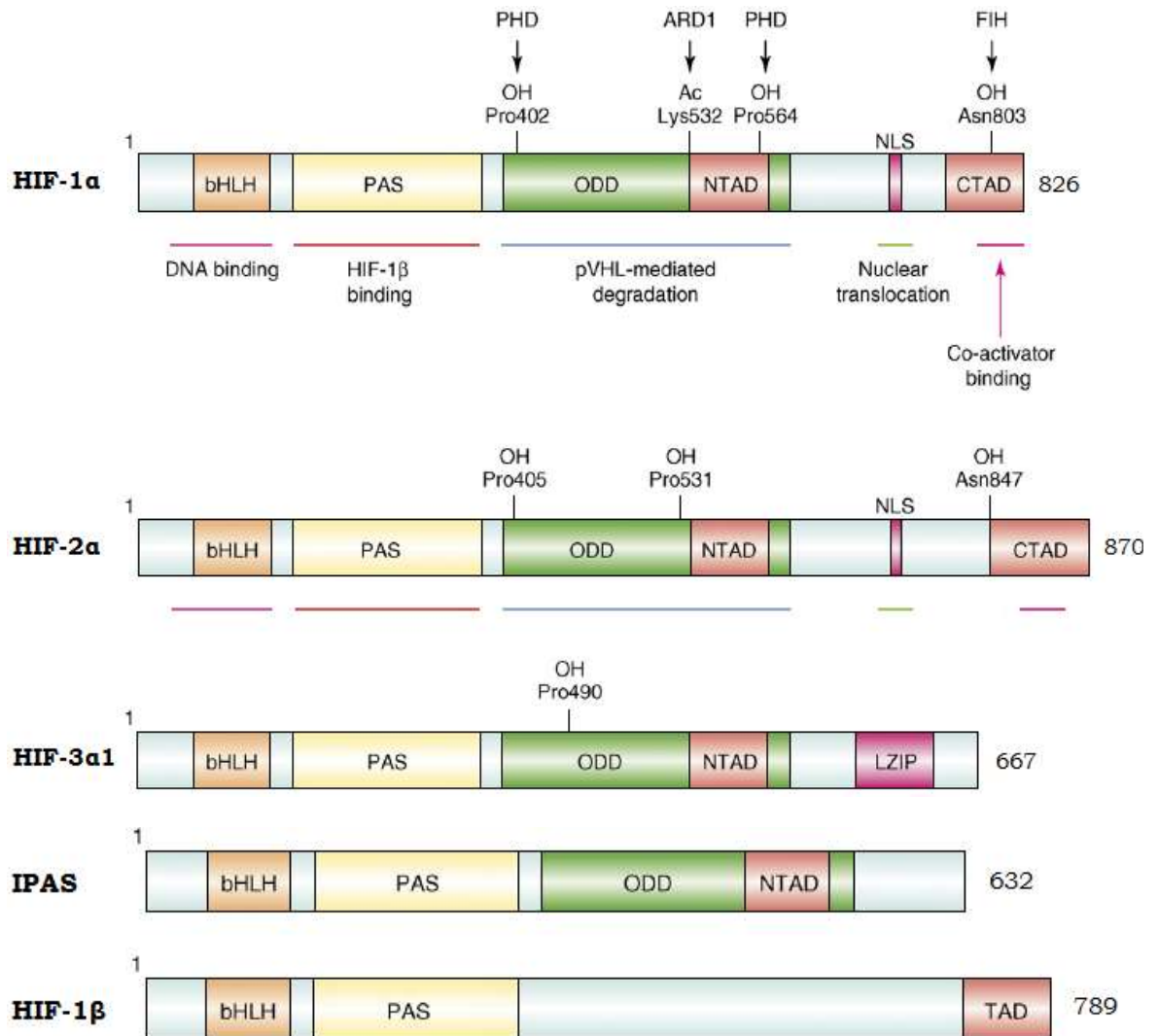


Fig. 17 HIF-1 α , HIF-2 α , HIF-3 α 1, IPAS e HIF-1 β structures and functional domains.

HIF-2 α (Hypoxia-Inducible Factor-2 α)

Subunit HIF-2 α , first named Endotelial PAS domain protein 1 (EPAS1) for its elevated expression in endothelial cells and highly vascularized tissues, has been first identified through cDNA library screening in ovarian carcinoma HeLa cells. HIF-2 α shows a 48% sequence homology with HIF-1 α and high conservation level of protein domains. As its isoform 1, this protein belongs to the family of bHLH-PAS proteins. In the light of these observations it was hypothesized that HIF-2 α had similar, redundant biological roles to HIF-1 α (Kim et al. 2007). Both proteins share similar transactivation mechanisms O₂-dependent that involve both C-TAD and ODD domains. In particular, as in the case of HIF-1 α , its O₂-dependent regulation is related to ODD domain hydroxylation on Pro405 and Pro 531, which in turn drives HIF-1 α to degradation via proteasome.

Anyway HIF-1 α and HIF-2 α exert different biological roles according to their tissutal expression and to N-TAD domain, which gives specificity for target genes. In fact while HIF-1 α is ubiquitously expressed, HIF-2 α expression is strictly related to lungs endothelium and pneumocytes type II, cardiomyocytes, duodenal epithelial cells and interstitial cells in the kidneys (Gordan et al. 2007).

HIF-2 α is complexed with subunit ARNT/HIF-2 β in order to form the functionally active heterodimeric complex HIF-2 α/β . In the nucleus HIF-2 α binds to the same DNA HRE sequence (5'-RCGTG-3') as HIF-1 α .

Biological functions

As previously stated, HIF-1 α and HIF-2 α show different genic activation patterns. For example, it has been observed that genes coding proteins related to cell metabolism are almost all activated by HIF-1 α , while other genes like EPO are preferential targets for HIF-2 α . In particular, HIF-2 α activates renal and hepatic production of EPO according to hypoxic conditions in order to promote erythropoiesis.

In addition HIF-2 α regulates iron homeostasis. An adjustment of iron metabolism is needed to satisfy increased iron demand in the bone marrow. Cytochrome *b* (*DcytB*) reduces ferric iron (Fe³⁺) to its ferrous form (Fe²⁺), which is then transported into the cytosol of enterocytes by divalent metal transporter-1 (*DMT1*). *DcytB* and *DMT1* are both hypoxia inducible and HIF-2 α regulated. Absorbed iron is released into the circulation by ferroportin (*FPN*) and is then transported in complex with transferrin to liver, reticuloendothelial cells, bone marrow, and other organs. Transferrin (*Tf*) is HIF regulated, and hypoxia increases its serum levels. When intracellular iron levels are low, iron regulatory protein (*IRP*) inhibits HIF-2 α translation and diminishes hypoxia-induced erythropoiesis (Haase 2010).

Several studies have also pointed out the role of HIF-2 α in vascular tissues adaptation to hypoxia for its higher transactivation ability on the promoter of the VEGF receptor (VEGF-R) gene (Tian et al., 1997). Other genes regulated only by HIF-2 α are for example genes coding for embryonal transcription factor Oct-4, cyclin D1, Twist1 and TGF- α (Patel e Simon 2008).

HIF-2 controls cellular proliferation is through modulation of c-Myc activity. C-Myc promotes cellular proliferation by regulating the expression of genes involved in cell cycle control including cyclins like cyclin D2 and cyclin kinase inhibitors like p21 and p27. Unlike HIF-1 α , HIF-2 α promotes C-Myc-dependent activation of cyclin D2 and

repression of p27 in renal carcinoma cells. In particular, HIF-1 α specifically disrupts c-Myc/Max and c-Myc/Sp1 complexes, allowing more Mad/Max interaction and DNA binding and causing cell cycle arrest in G1/S phase. On the other hand, HIF-2 α stabilizes c-Myc/Max complexes, in turn promoting c-Myc DNA binding at both E boxes and Inrs. HIF-2 may also drive cell cycle progression through the activation of cyclin D1. Cyclin D1 is a well-characterized cell cycle regulatory protein that is upregulated in many cancers. Recent studies have shown a correlation between HIF-2-mediated cyclin D1 expression and tumour growth in renal cancer cells (Loboda et al. 2010).

In addition HIF-2 α , but not HIF-1 α , is able to promote tumour growth in *in vivo* renal cancer models. Stable overexpression of HIF-2 α in renal cancer cells 786-O expressing pVHL promote xenografts growth similar to 786-O deleted for pVHL, while stable HIF-1 α overexpression has the opposite effect on cancer cells growth. Additional studies from the same research group also showed that only HIF-2 α positively correlates with the grade of dysplasia in pre-neoplastic lesions (Maranchie et al., 2002; Raval et al., 2005).

The role of HIF-2 α in promoting general tumorigenic mechanisms has also been confirmed by Covello et al., who demonstrated that subcutaneous teratomas generated from embryonic stem cells (ESCs) “knocked in” for HIF-2 α gene in the HIF-1 α locus show a fourfold higher xenograft growth in comparison to wild type cells (Covello et al., 2005). This event is not only related to loss of HIF-1 α expression as several studies have pointed out that teratomas generated from ESCs *HIF-1*^{-/-} do not show the same proliferative ability (Carmeliet et al., 1998). Finally, recent studies on human neuroblastoma models have demonstrated HIF-2 α stabilization in a condition of mild, but prolonged, hypoxia, thereby suggesting that HIF-2 α expression is able to promote tumorigenesis even in tumours exposed to hypoxic stress of lesser extent (Holmquist-Mengelbier et al., 2006).

HIF-3 α (Hypoxia-Inducible Factor-3 α)

HIF3 α gene codificates for protein HIF-3 α , formed by 662 aminoacids with a 73 kDa molecular weight. HIF-3 α mRNA can be detected in a variety of tissues, including the thymus, lung, brain, heart, kidney, liver, eye, and brain.

N-terminal bHLH-PAS domain shows homolgy sequence with HIF-1 α e HIF-2 α proteins of the 57% and the 53%, respectively, while HIF-3 α C-terminal ODD domain shows a sequence of 36 aminoacids with a 61% identity with HIF-1 α ODD. *In vitro* studies show that HIF-3 α dimerizes with protein ARNT/HIF-3 β similar to HIF-1 and HIF-2 β subunits. The resulting hheterodimeric complex HIF-3 α / β binds to HRE sequence 5'-RCGTG-3' thereby leading to activation of gene target expression.

In addition, there is a particular HIF-3 α isoform which generates from alternative splicing of the same mRNA as original HIF-3 α , first identified in murine models and named inhibitory PAS domain protein (IPAS).

Biological functions

Biological functions of HIF-3 α are still unclear, while there are evidences about IPAS protein inhibitory role. IPAS do not show a C-TAD domain and does directly regulates genic, but it semms to act as a direct negative dominant of HIF-1 α and HIF-2 α , as it binds to HIF-1 α and HIF-2 α N-terminal regions and prevent their binding to DNA (Makino et al. 2001). In addition, alternative splicing IPAS-specific is hypoxia-inducible in murine lung and cardiac tissues, thereby showing another O₂-dependent post-translational modification of HIF- α subunits. IPAS expression in normoxia has been observed just in Purkinje neural cells and in epithelial corneal cells. In the latter, IPAS seems to negatively regulate VEGF and angiogenic mechanisms (Makino et al. 2002).

Regulation of HIFs expression

Regulation of gene expression is similar for all proteins of the HIFs family. As for every protein, HIFs expression is related to a balance between expression and degradation mechanisms: in the case of HIF-1 α , transcriptional and synthesis seem to be constitutional mechanisms, while degradation is O₂-dependent.

In normoxic conditions HIF-1 α shows a very short half-life of about 1/2 ~ 5 minutes and is rapidly degraded via ubiquitin-proteasome system (Salceda et al., 1997). When cells are exposed to low O₂ tensions, HIF-1 α half-life is of about 30 minutes, the protein is stabilized and translocates to the nucleus, where it binds to subunit HIF-1 β forming the transcriptionally active HIF complex. The resulting heterodimer binds to HRE sequence of target genes and binds to transcriptional coactivators, thereby promoting gene expression (Lando et al., 2002).

HIF-1 α stability and transactivation ability are principally regulated by post-translational modifications on HIF-1 α domains, like hydroxylation, ubiquitination, acetylation and phosphorylation (Brahimi-Horn C et al., 2005).

This complex regulation mechanism allows cells to quickly adapt to variations of O₂ concentration according to its availability. HIF-1 α degradation in normoxic conditions is mainly mediated by ODD domain hydroxylation and the reaction is catalyzed by specific enzymes called prolyl hydroxylases-domain proteins (PHDs) (Huang et al., 1998).

The prolyl hydroxylase-domain proteins (PHDs)

In normoxic conditions, newly synthesized HIF-1 α is quickly hydroxylated on Pro 402 and 564 located in the ODD domain. This reaction is catalyzed by three enzymes known as prolyl hydroxylase-domain protein (PHDs) 1-3. Pro 402 and 564 are highly conserved in HIF-2 α (Pro405 e Pro530) and HIF-3 α proteins and they are located inside a highly conserved consensus sequence, LXXLAP. Only HIF-3 α shows a different aminoacidic sequence, LXXLHP (Bruick et al., 2001; Masson et al., 2001).

PHDs are dioxygenases 2-oxoglutarate-dependent which use O₂ and α -ketoglutarate as substrates and Fe²⁺ and ascorbate as cofactors. The hydroxylation mechanism is characterized by scission of molecular O₂ and one atom is transferred on a Pro residue, while the other one reacts with α -ketoglutarate, thereby creating succinate and CO₂ (Bruick RK et al., 2001). In physiologic conditions O₂ is a limiting substrate and this mediates HIF-1 α O₂-dependent regulation (Jiang et al., 1996).

All three PHD isoforms hydroxylate HIF- α *in vitro*, but PHD2 shows higher activity (attività relativa PHD2>>PHD3>PHD1) and it has been demonstrated as the master regulator of HIF-1 α turnover *in vivo* (Huang et al. 2002). According to these data, PHD2 gene silencing induces HIF-1 α stabilization in normoxia, while PHD1 e PHD3 inhibition does not show same effect (Berra et al. 2003). In addition, hypoxia induces both PHD2 and PHD3 expression, but it does not seem to have effects on PHD1 (Epstein et al. 2001). This could represent a mechanism of regulation of HIF-1 α activity induced by HIF-1 α itself. According to this hypothesis, PHD2 is mainly located in the cytoplasm, while PHD1 is mainly expressed in the nucleus and PHD3 in both compartments. Anyway, PHD2 is able to translocate to the nucleus, thereby contributing to HIF-1 α degradation in both nucleus and cytoplasm (Metzen et al. 2003). Pro-402 and Pro-564 hydroxylation is a key event for HIF-1 α degradation because it allows the interaction between oncosuppressor von-Hippen-Lindau (pVHL) protein and hydroxylated HIF-1 α ODD domain (Ivan et al. 2001). pVHL is a component of the E3 ubiquitin-ligase complex which drives protein digestion via proteasome (Cockman et al., 2000). In fact pharmacological inhibitors of proteasome activity and/or mutations of E1 enzyme stabilize HIF-1 α , showing the importance of ubiquitination and proteasome in normoxic HIF-1 α degradation (Huang et al. 1998; Salceda et al. 1997). After hydroxylation and binding to pVHL protein, the complex associates to proteins elongin C, elongin B cullin-2 and Rbx-1, forming the complex VCB-Cul2 E3 ligase. HIF-1 α interaction with this multiproteic complex induces HIF-1 α poliubiquitination, leading to HIF-1 α proteasomic digestion (Kamura et al. 2000).

pVHL is an oncosuppressor protein whose mutations are involved in development of several types of cancer (Iliopoulos et al., 1998; Schoenfeld et al., 1998). This protein was first characterized in the von Hippen-Lindau syndrome, hereditary disease with development of multiple tumours in several body tissues. Cells with mutated pVHL show more stable and active HIF-1 α e HIF-2 α in normoxic conditions, thereby causing an overexpression of hypoxia-induced genes and promoting cancer progression (Iliopoulos et al. 1996). The complex pVHL- E3 ligase is ubiquitarily expressed in different tissues and it is preferentially located in the cytoplasm, but like PHD2 is able to translocate to the nucleus, promoting HIF-1 α degradation in both compartments in normoxia (Berra et al. 2001). On the contrary, low O₂ tension directly inhibits PHDs activity because the consequent alteration of the mitochondrial chain transport leads to an excess of reactive oxygen species (ROS). ROS in turn change the oxidation state of

iron inside PHDs active site from Fe^{2+} to Fe^{3+} . Subsequently ferric iron is no longer active (Epstein et al. 2001; Simon 2006). In this condition, HIF-1 α is no longer hydroxylated and led to degradation, but stabilized and accumulated (Fig. 18). In addition, PHD activity can be regulated by intracellular Ca^{2+} concentration (Berchner-Pfannschmidt et al. 2004) and E3 ubiquitin-ligase Siah1 e Siah2 enzymes, whose trascription is hypoxia-induced (Nakayama e Ronai 2004).

Inside the HIF-1 α ODD domain, Lys 532 can be acetylated by an acetyl-transferase enzyme named arrest-defective 1 (ARD1) (Jeong et al. 2002). Lys532 acetylation promotes HIF-1 α -pVHL interaction, thereby destabilizing HIF-1 α . According to the importance of this post-translatinal modification, Lys 532 mutation in Arg 532 induces HIF-1 α stabilization (Tanimoto et al. 2000). In addition, when cells accumulate butirric acid, general inhibitor of deacetylases, there is an increase in HIF-1 α acetylated state leading to significative reduction of expression (Kim et al., 2001). As acetyl transferases is not influenced by O_2 tension, ARD1 can be active and acetylate HIF-1 α in an O_2 -independent manner, even if same studies have shown that ARD1 mRNA is less expressed in hypoxia, thereby leading to a reduction of HIF-1 α acetylation in comparison with normoxic condition (Jeong et al. 2002).

Factor inhibiting HIF (FIH)

Despite post-translational modifications, HIF-1 α stabilization alone is not enough to guarantee its activity as transcriptional activator.

Another key mechanism for HIF-1 α activity regulation is modulation of transactivation domains N-TAD and C-TAD. These two domains are separated from each other by an inhibition domain (ID) located between aminoacids 576-785, which downregulates their function in presence of high O_2 tension (Jiang et al., 1997-b).

Regulation of transcriptional activity is mediated by a molecular mechanism different, but correlated to the one that influences HIF- α stability in hypoxia. In normoxic condition, hydroxylation of Asn803 in HIF-1 α and Asn851 in HIF-2 α induces a steric inhibition of interaction between HIF-1 α and its coactivator CBP/p300, impairing its recruitment that is necessary for HIF-1 α activity (Sang et al., 2002). In hypoxic condition there is not Asn hydroxylation, so HIF-1 α can associate to CBP/p300 through C-TAD domain in order to activate target genes transcription (Lando D et al., 2002).

This molecular regulation is mediated by an asparagyl-hydroxylase enzyme named factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). As PHDs, also FIH-1 is a dioxygenase 2-oxoglutarate-dependent and has Fe^{2+} and ascorbate as cofactors and O_2 as a substrate, making this enzyme a proper oxygen levels sensor. FIH-1 is primarily located in the cytoplasm, but there is also a little nuclear fraction whose transcription is O_2 -dependent and that regulates HIF-1 α activity rather than its stability (Metzen et al., 2003). According to this, structural analysis have demonstrated that, when HIF-1 α is complexed to CBP/p300, Asn 803 is buried inside the proteic complex in order to prevent its hydroxylation. In fact, mutation of Asn 803 with Ala 803 allows interaction between HIF-1 α and its coactivators even in presence of high O_2 tension (Dames et al., 2002; Freedman et al., 2002; Lando et al., 2002). Although interaction of FIH-1 with pVHL is not required for its activity, it has been demonstrated that pVHL binds to FIH-1, forming a complex with HIF-1 α (Mahon et al., 2001). This allows histone deacetylases recruited by pVHL to interfere with the transcriptional process, thereby promoting FIH-1 inhibitory activity on HIF-1 α (Hewitson et al., 2002; Sang et al., 2002) (Fig. 18).

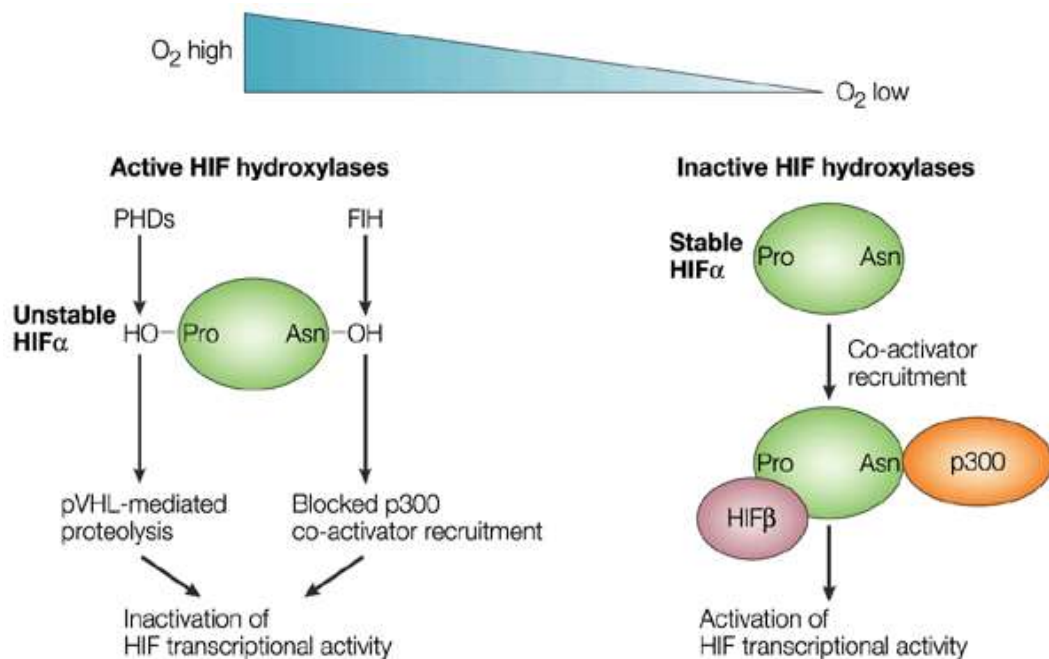


Fig. 18 Regulation of HIFs expression in normoxia and hypoxia.

In the same way as PHD, FIH-1 activity depends not only upon O₂ and 2-oxoglutarate availability, but also requires cofactors Fe²⁺ and ascorbate, while its transcription is O₂-independent (Lando et al., 2002; Metzzen et al., 2003). FIH-1 structural analysis have pointed out that FIH-1 requires formation of a homodimeric complex in order to bind its substrates and to perform its catalytic activity. As O₂ availability is a limiting factor for FIH-1 activity, it has been hypothesized that this enzyme has a role in sensing extracellular O₂.

In conclusion, O₂-dependent HIF-1 α functional regulation depends on “two steps” model: i) O₂-dependent prolyl-hydroxylation in the ODDD domain in order to prevent association with the multiproteic complex E3 ubiquitin ligase and subsequent proteasomal degradation; ii) O₂-dependent inhibition of Asn hydroxylation in the C-TAD domain to allow interaction with coactivator with CBP/p300 and functionally active complex formation (Fig. 18).

Sirtuins (SIRT5)

Sirtuins are a family of histone-deacetylase enzymes NAD⁺-dependent which have several roles in regulating gene transcription, DNA repair and cell metabolism. These enzymes respond to variations of the NAD⁺ oxidized/ NADH reduced ratio and represent important redox intracellular sensors (Denu, 2003). In mammals there are seven homologous of yeast Sir2 protein: SirT1, 6 e 7 proteins are located in the nucleus, SirT2 is expressed in the cytoplasm, while SirT3, 4 e 5 are strictly located in the mitochondria (Haigis e Guarente 2006).

SirT1 has been recently described as a negative regulator of HIF-1 α activity through its direct acetylation. This regulation is independent from intracellular levels of NAD⁺ e NADH, as when hypoxia increases NADH level, HIF-1 α deacetylation SirT1-mediated is downregulated (Lim et al. 2010).

SirT1 also regulates HIF-2 α transcriptional activity through deacetylation. In particular, it has been demonstrated that SirT1 directly binds to HIF2 α , but not to HIF1 α , and deacetylates specific lysin residues. For example, Sirt1 co-localizes with HIF2 α at the Epo gene promoter, thereby stimulating HIF2 α pro-transcriptional activity and promoting hepatic and renal EPO production both in *in vitro* and *in vivo* models. These data demonstrate that EPO production is dependent from both O₂ availability and cellular redox state (Dioum et al. 2009) (Fig. 19).

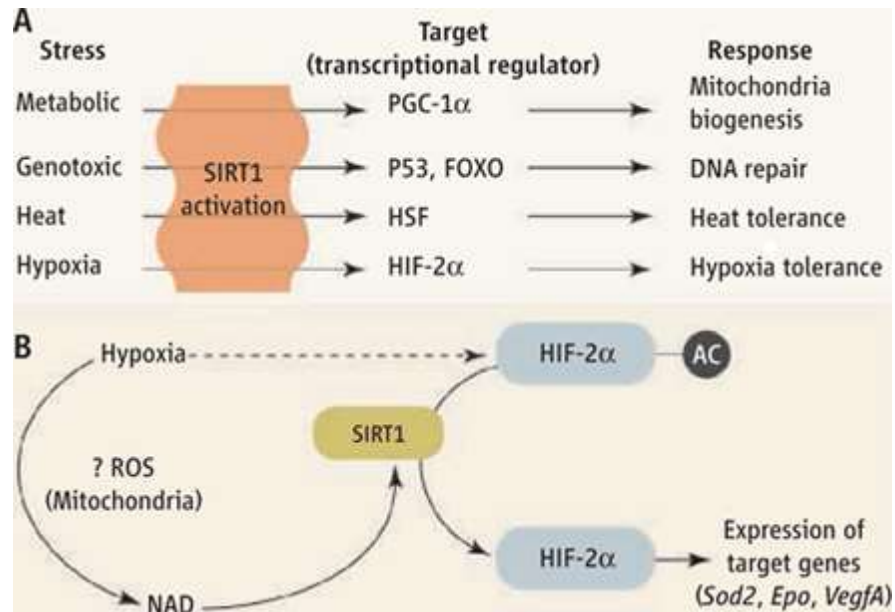


Fig. 19 HIF-2 α stabilization by SirT1 activity.

Recently it has been demonstrated that also SirT6 can inhibit HIF-1 α -mediated genic transcription through direct binding to HREs sequences and subsequent histone deacetylation in promoters of genes related to cell metabolism. According to that cells *Sirt6*^{-/-} show increased expression of glycolytic enzymes and increased glucose consumption, both *in vitro* and *in vivo* models (Zhong et al. 2010).

In the last years also SirT3 has been subject of numerous studies. In particular, Kim and colleagues have demonstrated that this protein acts as a mitochondrial oncosuppressor gene. In a model of embrional murine fibroblasts ingegnerized to express oncogenes RAS and Myc, total deletion of SirT3 is sufficient to promote neoplastic transformation and tumour development in murine models. In addition, transformed cells show a glycolitic metabolic behaviour, with increased glycolysis and oxidative stress and oxidative phosphorylation inhibition. According to that, excess ROS production due to loss of SirT3 expression promotes DNA damage process and thus accelerates cancer progression (Kim et al. 2010).

The role of SirT3 as oncosuppressor gene has been confirmed by Bell and colleagues colleghi who have pointed out that loss of SirT3 promotes murine embrional fibroblasts proliferation in hypoxic condition. Cells *Sirt3*^{-/-} show higher ROS levels compared to control cells, thereby favouring HIF-1 α stabilization throught inhibition of PHDs

activity. These data have been then confirmed reverting HIF-1 α stabilization following both antioxidants administration like N-acetyl-cysteyn (NAC) and SirT3 (Bell et al. 2011) (fig. 20).

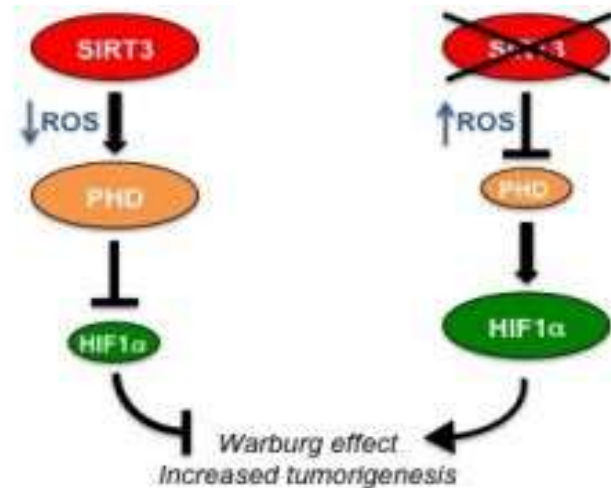


Fig. 20 Regulation of HIF-1 α stabilization by SirT3.

Phosphorilation, nitrosation and sumoilation of HIF- α

An other molecular mechanism that regulates HIF-1 stability in a PHDs independent manne ris represented by molecular chaperon Hsp90, which acts independently from pVHL expression and O₂ tension (Isaacs et al., 2002).

In addition, it has been described a HIF-1 α regulation through direct phosphorilation by mytogen-activated protein kinases (MAPK), where both Erk1/2 and p38 phosphorilates both HIF-1 α and HIF-2 α , as observed in *in vitro* and *in vivo* models (Richard et al., 1999). This post-translational modification does not affect HIF-1 α stability or its DNA binding ability, but positively regulates its transcriptional activity. The most accepted explanation for this event is that HIF-1 β subunit preferentially binds to phosphorilated HIF-1 α subunit, thereby leading to increased HIF-1 α activation and target genes transcription like VEGF and EPO (Richard et al., 1999).

Other described post-translational modifications regulating HIF-1 α are S-nitrosation on Cys800, which promotes direct interaction with coactivator CBP/p300, thus promoting HIF-1 α transcriptional ability, and SUMOilation, which affects both degradation and protein stability of HIF-1 α (Yasinska et al., 2003).

In particular, hypoxia promotes expression of the small ubiquitin-like modifier-1 (SUMO) protein, thereby favouring HIF-1 α sumoilation (Shao et al., 2004). At the beginning it was hypothesized that this modification could improve protein stability, while recent observations have demonstrated that this molecular mechanism promotes HIF-1 α degradation by promoting HIF-1 α binding to pVHL-E3 ligase complex. This regulatory mechanism is completely independent from PHDs hydroxylation state.

In hypoxic condition, HIF-1 α degradation SUMO-mediated is inhibited by expression of SUMO1/sentrin specific peptidase 1 (SEN1), a nuclear protease that removes the SUMO protein from HIF-1 α . As a demonstration of the importance of this mechanism for HIF-1 α stabilization, cell lines knock-out for SEN1 gene show reduced HIF-1 α expression. According to these observations, SEN1^{-/-} murine fetus showed a severe fetale anemia caused by failing regulation of EPO expression, thereby showing SEN1 physiological role in regulating HIF-1 α (Cheng et al., 2007).

Regulation of HIF activity and expression by growth factors

HIF-1 α expression induced by growth factors stimulation differ from HIF-1 α hypoxia-induced expression because it is not associated to a decreased degradation, but to an increased production through activation of phosphatidylinositol-3-kinase (PI3K) and MAPK pathways (Fukuda et al., 2002; Fukuda et al., 2003; Laughner et al., 2001; Zhong et al., 2000). According to that, cell stimulation with molecules like the epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), insulin and insulin-like growth factors 1 and 2 (IGF-1-2) and IL-1 β promote HIF-1 α expression, DNA binding and target genes activation even in normoxic condition (Feldser et al 1999; Hellwig-Burgell et al 1999; Laughner et al 2001; Zelzer et al 1998). Binding of these factors to their tyrosine-kinase receptors activates several molecular pathways like to MAPK and PI3K ones.

MAPK pathway leads to activation of both ERK1-2, also named p42/p44 MAPK, and p38 MAPK, following previous activation of Ras/Raf-1/MEK-1/ERK1-2. It has been demonstrated that HIF-1 α is phosphorylated by p42, p44, p38 α and p38 γ *in vitro*, but aminoacids involved in this modifications have not been discovered yet (Richard et al 1999; Sodhi et al 2000).

PI3K pathway directly activates Akt kinase (protein kinase B), which is related to several cellular mechanisms like apoptosis, cell cycle regulation and proteic translation (Vivanco e Sawyers 2002). One of Akt target proteins is FRAP, protein associated to

rapamycin FKBP12 (mTOR) which activates ribosomal protein p70^{S6} kinase (p70^{S6k}) and promotes mRNA translation with polypyrimidinic sequences at 5' end (Brown et al 1995; Zhong et al 2000). In addition, Akt also phosphorylates 4E-binding protein (4E-BP), known transcriptional regulator, its hyperphosphorylation leading to an increased proteic translation. This molecular pathway is negatively regulated by tumour suppressor phosphatase end tensin homologue (PTEN) coding for a phosphatase that, dephosphorylating PI3K substrates, downregulates all PI3K-AKT-FRAP pathway (Fig. 21). Differing from hypoxia which upregulates HIF-1 α expression in all cell types, growth factors stimulation induces HIF-1 α expression just in certain cell lines.

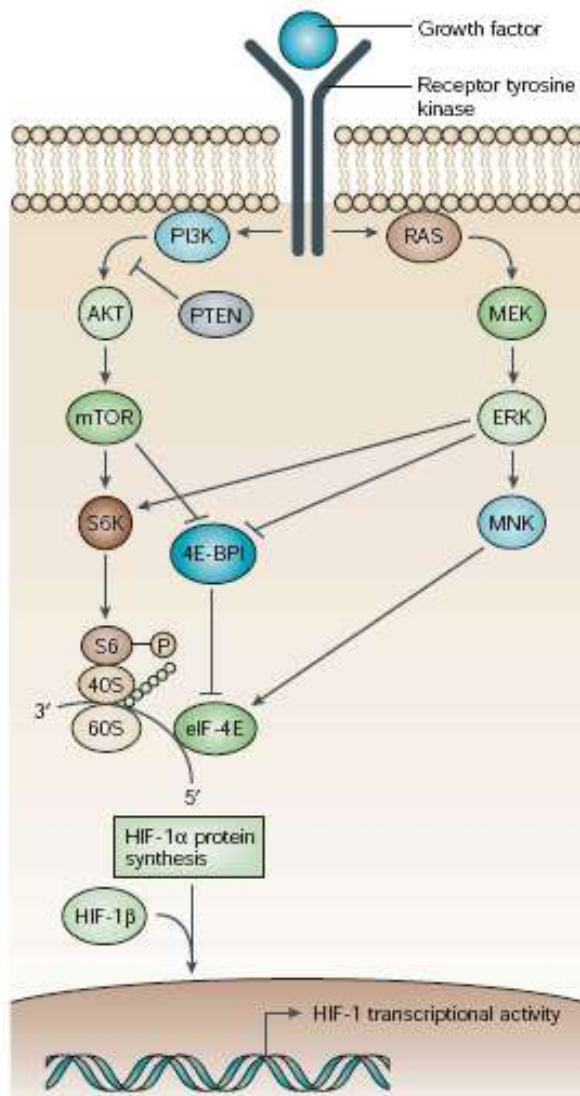


Fig. 21 Molecular pathway of HIF-1 α expression growth factors-induced.

It is now well understood how inflammation represents one of the key events of cancer development. Recent studies have thus suggested the role of HIF-1 α in regulating inflammatory mechanisms, showing that IL-1 β positively regulates normoxic HIF-1 α expression and promotes the HIF-1 α -dependent VEGF production through activation of the nuclear factor- κ B (NF- κ B) molecular pathway (Jung et al. 2003). Anyway, IL-1 β also mediates HIF-1 α expression through a post-translational mechanism that blocks the pVHL-dependent HIF-1 α degradation, thereby leading to HIF-1 α stabilization (Jung et al. 2003). According to that, HIF-1 α is activated in normoxia through genetic alterations of O₂-related signaling pathways and pVHL has a central role in regulating HIF-1 α transcriptional activity (Rankin and Giaccia 2008).

Cyclooxygenase-2 (COX-2)-dependent NF- κ B expression mediated by IL-1 β is one of the HIF-1 α positive effectors genes. In fact, it has been demonstrated that IL-1 β positively regulates HIF-1 α through activation of the classical inflammatory pathway NF- κ B and COX-2-mediated that leads to VEGF activation, tumour and metastasis growth (Yung et al. 2003). Despite reduction of IL-1-mediated HIF-1 α induction after administration of COX-2 inhibitors, prostaglandin E₂ (PGE₂), physiological product of COX-2 activity, promotes HIF-1 α expression in a dose-dependent manner.

In conclusion, in hypoxic condition, PHDs and FIH are inactive and HIF-1 α can positively modulate expression of its target genes.

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are O₂ partially reduced metabolites, showing higher reactivity than O₂ itself. Major ROS produced within the cell are superoxide anion (O₂^{-•}), hydroxyl radical (OH[•]), nitrogen monoxide (NO) and oxygen peroxide (H₂O₂). Thanks to their higher reactivity, excessive amounts of these molecules can severely impair cell functions. Physiologically, high ROS production is first related to cells involved in immunitary defense, as transient ROS production promotes expression of chemokines, cytokines and endothelial/leukocytic adhesion molecules, thereby activating anti-inflammatory response like elimination of bacterial/viral exogen pathogenic agents (Remick and Villarete 1996). As production of ROS can be highly toxic for cells and neighboring tissues, their production must be severely regulated through various molecular mechanisms.

Several studies have then showed that ROS can have an important role also in cells not related to immunitary system. In particular, it has been hypothesized that ROS can act

as second messengers in almost all cellular pathways like genic expression, celldifferentiation, apoptosis, proliferation and cell adhesion (Poli et al 2004; Aslan and Ozben 2003; Chiarugi 2005; Chiarugi 2001; Giannoni et al 2005). On the other hand, aberrant signaling ROS-dependent leads to pathologic events like cell cycle dysregulation (Boonstra and Post 2004), apoptosis and senescence (Gourlay and Ayscough 2005; Johan et al 2005), ischemic/reperfusion events (Otani 2004) and diabetes-related complications (Niedowicz and Daleke 2005).

Cellular energetic metabolism is based upon ATP production through the activity of the mitochondrial electron transport which leads to complete O_2 reduction into H_2O by accepting electrons and H^+ ions. During oxidative phosphorylation in the mitochondrial inner membrane, electrons are transferred from molecular carriers like nicotinamide adenine dinucleotide (NADH) or flavinadenine dinucleotide $FADH_2$ to the electron transport chain, composed by three enzymatic transmembrane complexes which contribute to produce a transmembrane proton electrochemical gradient. If protons flow back through the membrane, they enable mechanical work, such as rotating bacterial flagella. ATP synthase, an enzyme highly conserved among all domains of life, converts this mechanical work into chemical energy by producing ATP from ADP and P_i (inorganic phosphate) (fig. 22).

During these complex series of redox reactions, a single electron can be directly transferred to O_2 , thereby producing $O_2^{\cdot-}$ in the complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxidoreductase) (fig. 16). The electron chain transport is a highly efficient mechanism and usually consumes the majority of O_2 available. Anyway, it has been clearly demonstrated that almost 1-2% of all transported electrons are lost by transmembrane complexes, thereby generating $O_2^{\cdot-}$ through reactions catalyzed by Q coenzyme and ubiquinone and related complexes (Forman and Boveris 1982). According to their role in aerobic cellular respiration, mitochondria are major ROS producers *in vivo* (Boveris e Chance 1973).

Another electron chain transport is located on the endoplasmic reticulum, where $O_2^{\cdot-}$ is produced from nicotinamide adenine dinucleotide-3-phosphate (NADPH) cytochrome P450 oxidoreductase (Cross e James 1991). Anyway, $O_2^{\cdot-}$ can be generated also from ipoxantine/xanthine oxidase, NADPH oxidase, lipoxigenase (5-LOX) and COX enzymes. Also radiant energy absorption can generate ROS as the ionizing radiations like UV and γ rays are able to hydrolyze H_2O into OH^{\cdot} and radicalic hydrogen (H^{\cdot}) (fig. 22).

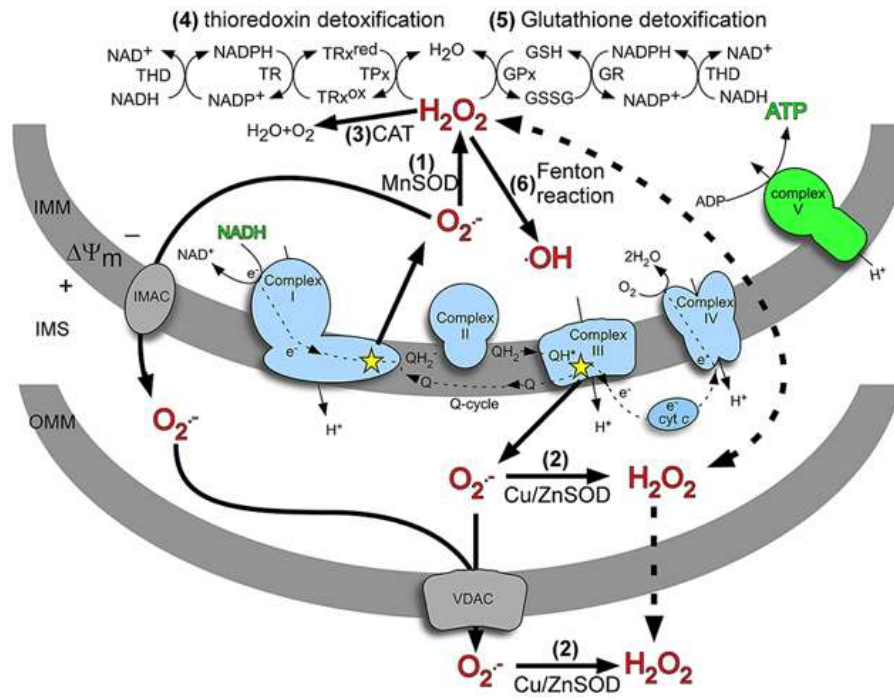


Fig. 22 Molecular pathways of ROS production.

HIF- α proteins can be stabilized in a ROS-dependent manner. Several evidences have showed that inhibition of mitochondrial ROS productions strongly impairs HIF- α stabilization in hypoxic condition (Chandel et al 1998; Weinberg and Chandel 2009). In agreement with these observations, molecules used in therapy can promote O_2 redistribution in the cytoplasm, outside the mitochondria, thereby leading to increased PHDs activity even in conditions of mild hypoxia (Hagen et al 2003; Vaux et al 2001). In addition, genetic studies demonstrate that complex III disruption, cytochrome C and Rieske Fe-Cu protein block HIF hypoxic stabilization (Mansfield et al 2005; Guzy et al 2005), while disruption of complex IV do not has the same result (Brunelle et al 2005). These results suggest that cellular respiration is not required for HIF stabilization, while ROS generated from the electron transport chain play a fundamental role in this event. ROS-dependent HIF-1 α stabilization is mainly related to modulation of PHDs activity, as they need oxygen, oxoglutarate and Fe^{2+} in order to fully sustain their activity. In fact, it has been demonstrated that PHDs regulation Fe-dependent is mediated by ROS in a model of *junD*^{-/-} cells, characterized by a condition of chronic oxidative stress (Gerald et al., 2004). According to this model, ROS production interferes with Fe^{2+} availability in the prolyl-hydroxylase catalytic site, thereby impairing PHDs activity in normoxic conditions. In fact, high H_2O_2 levels promote Fe^{2+} oxidation to Fe^{3+} , with a consequent increase of Fe^{3+} , inactive intracellular PHDs percentage. Subsequently,

exogenous addition of Fe^{2+} in *junD*^{-/-} cells strongly reduces HIF-1 α levels. The same effect is obtained after administration of reducing agents like cysteine or glutathione, or antioxidants molecules like ascorbate, whose physiological concentrations increase PHDs activity and thus HIF-1 α degradation, reverting the oxidation state of the atom of Fe in PHDs catalytic site (Knowles et al., 2003).

Like PHDs, also FIH activity is highly O_2 , oxoglutarate and Fe^{2+} -dependent, thus also FIH is highly sensitive to ROS-induced Fe^{3+} oxidation state, thereby promoting HIF-1 α accumulation. In addition it has been showed that some glucose metabolites like the 2-oxiacids can directly interact with PHDs oxoglutarate-binding domain, impairing their enzymatic activity. In addition, these molecules also change the catalytic Fe oxidation state, as this inhibitory effect 2-oxiacids-mediated is fully reversed after exogenous administration of Fe^{2+} , ascorbate and/or reducing agents (Lu et al., 2005) (Fig. 23).

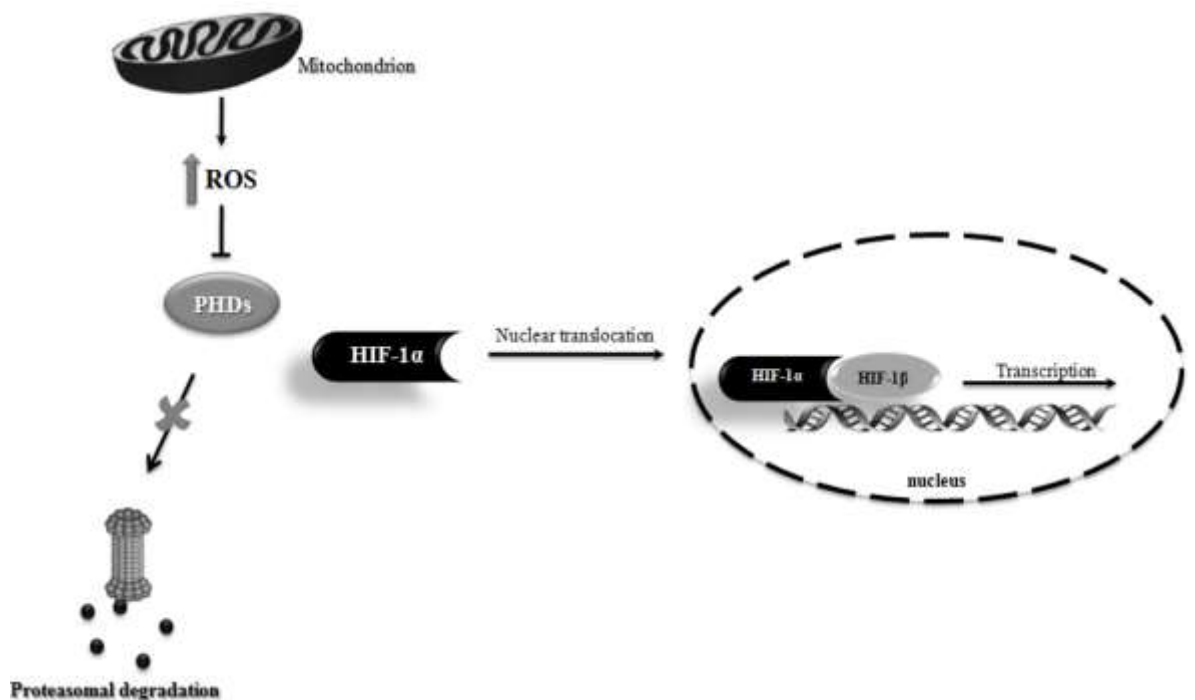


Fig. 23 HIF-1 α stabilization through ROS-mediated PHDs inactivation.

Anyway, molecular mechanism by which hypoxia causes an increase in ROS production has not been clearly understood yet. From a thermodynamic point of view it

seems unlikely reduced substrate concentration would cause an increase in the rate of the electron transport chain, thus it has been hypothesized that other factors may interact with these mechanisms.

According to the 'vectorial transport' hypothesis, ROS lacking from complex III can be released to either the matrix side of the membrane or towards the intermembrane space (Muller et al. 2004). Thus O₂ dissolved in the lipid bilayer might affect the ROS balance, thereby leading to higher oxidants release to the intermembrane space direction than to the matrix side. Although the specific mechanism responsible for this shift has not been elucidated yet, an oxygen-dependent change in the direction of ROS release could explain oxidant stress increases in the cytosol during hypoxic conditions. Interestingly, such a shift could theoretically rely on an increase in cytosolic ROS signalling even if overall ROS production were decreased.

The 'semiquinone lifetime' hypothesis proposes that O₂ interaction with protein or lipids at complex III could regulate the lifetime of ubisemiquinone at the Q_o or Q_i sites. Reduced electron removal from ubisemiquinone by the b cytochromes during hypoxia could subsequently promote superoxide production even under lower O₂ concentrations. Any small molecule or drug that alters the kinetics of electron removal from the semiquinone to the b cytochromes can potentially affect superoxide generation (Guzy e Schumacker 2006).

Finally, the 'oxygen access' hypothesis suggests that hypoxia might increase the access of O₂ to the semiquinone moiety at complex III. According to this hypothesis, if the molecular structure of one or more proteins in complex III were affected by the level of oxygen in the membrane, the ability of O₂ to attack the semiquinone were improved under low oxygen conditions and this could lead to an increase in ROS production, even under decreased O₂ availability.

Anyway, all these models require a modification of lipid-protein structure mediated by molecular oxygen, or a change in the concentration of oxygen, thereby increasing the transfer of an electron from ubisemiquinone to O₂ despite the lowered concentration of oxygen itself (Guzy e Schumacker 2006).

HIFs target genes

As stated in previous paragraphs, HIF-1 α is the master regulator of processes related to cellular hypoxic response like erythropoiesis, angiogenesis and glycolytic metabolism. In hypoxic conditions, complex HIF-1 α / β interacts in the nucleus with its coactivators like p300 or CBP (Ruas et al., 2005) and regulates genes transcription after binding to HREs DNA sequences (Kasper et al., 2005; Kasper et al., 2006).

Given the role of HIF-1 α in mediating also cancer-related pathological processes, the study of genes and molecular pathways activated by HIF is becoming more and more important in order to find always new therapeutic anti-cancer therapies (Fig. 24).

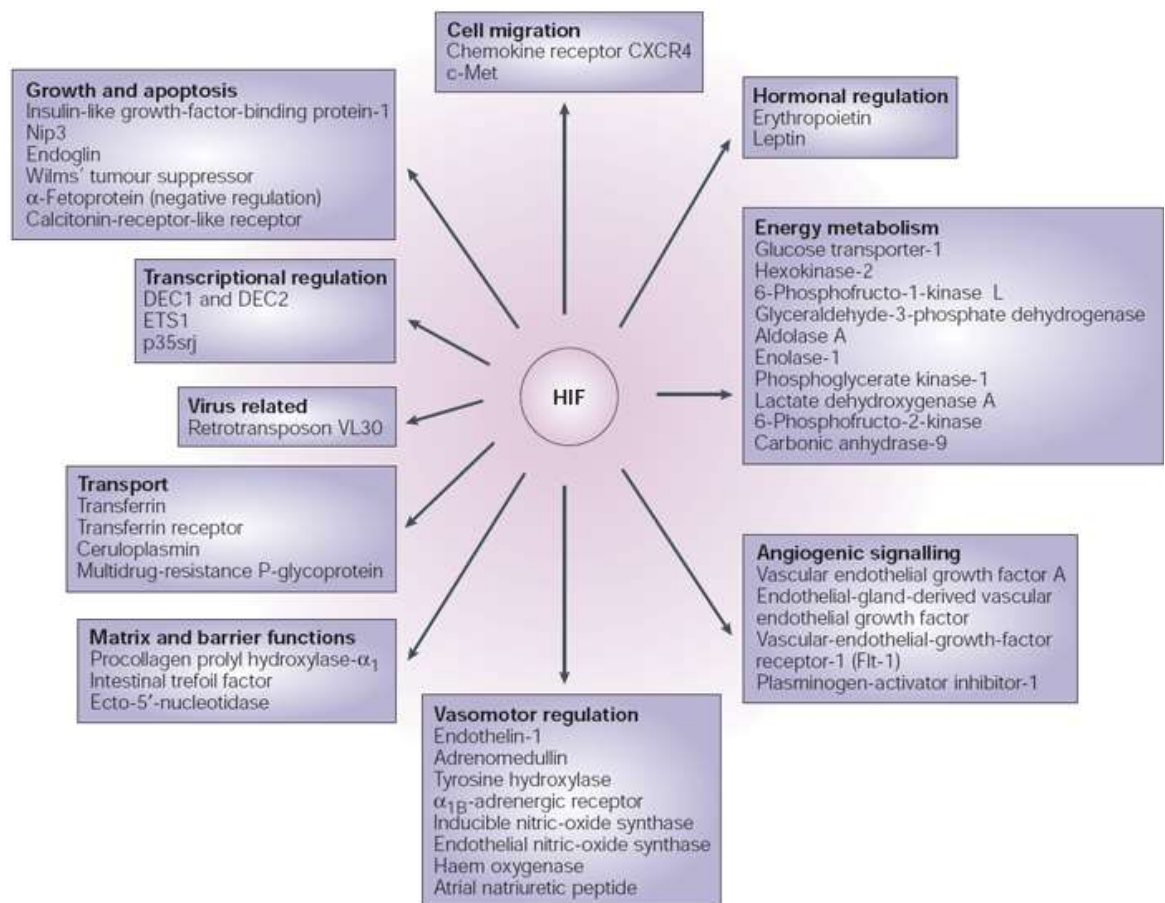


Fig. 24 HIF-1 target genes: a complex network.

Angiogenesis

Angiogenesis is a key process for tumour growth and dissemination and is characterized by recruitment and proliferation of endothelial cells from the stroma to the tumour microenvironment. The ability of cancer cells to metastasize distant tissues is positively correlated to tumour angiogenic potential, thereby leading to formation of new vessels in order to promote cancer cells dissemination in the bloodstream (Hill 1990; Weidner 1993; Weidner et al 1991). At the same time tumours also exploit lymphoangiogenesis to favour tumour growth also through lymphatic vessels. In addition, new vessels can originate from endothelial and tumour cells or from tumoural, non-endothelial cells through a process known as “vascular mimicry”.

VEGF is the most powerful and selective endothelial mitogenic agent. It induces a quick and complete angiogenic response through interactions with its receptors, VEGFR-1/Flt-1 e VGFR-2/Flk-1/KDR, expressed on pre-existent endothelial cells, thereby leading to cells proliferation and migration (Leung et al 1989). High VEGF levels are present in several types of solid tumours and are positively correlated to increased cancer vascularization, metastatic ability, resistance to therapy and bad prognosis (Toi et al 1996; Takahashi et al 1995; Toi et al 1994).

As already stated, hypoxia induces VEGF production through several molecular mechanisms like increased protein and mRNA stability. According to that, HREs have been identified in both 3' and 5' regions flanking the VEGF human gene (Michenko et al 1994; Forsythe et al 1996; Levy et al 1996; Mizukami et al 2004), and its expression induced by hypoxia involves both HIF-dependent (Forsythe et al 1996; Shinojima et al 2007) and HIF-independent (Mizukami et al. 2004) molecular pathways. Anyway, VEGF expression and tumour vessels density *in vivo* are primarily correlated to a HIF-dependent mechanism which can be highly influenced by several factors like genetic mutations and the peculiar characteristics of tumour microenvironment (Carmeliet al 1998).

Several studies have pointed out how several genes correlated to different phases of angiogenesis are more expressed under low O₂ tension and thus are controlled by HIF-1 α expression (Levy et al., 1995; Bunn e Poyton, 1996; Forsythe et al., 1996; Berra et al., 2000; Giordano e Johnson, 2001; Semenza, 2002). In fact HIF-1 α can regulate expression of both pro-angiogenic cytokines/growth factors and of their respective receptors. Among them the most important are stromal-derived factor-1 (SDF-1) and its receptor CXCR4 (Staller et al., 2003), placental growth factor (PLGF), angiopoietin 1

and 2, platelet-driven growth factor-B (PDGF-B), VEGF and its receptor VEGF-R1 also known as Flt-1 (Fms-like tyrosine kinase-1), which binds both PLGF and VEGF (Gerber et al., 1997; Okuyama et al., 2006). Those ligands directly interact with their receptor expressed on the membrane of endothelial cells and endothelial progenitors, smooth muscle cells, mesenchymal stem cells and other cells bone marrow-derived (Bosch-Marce et al., 2007; Ceradini et al., 2004; Forsythe et al., 1996; Kelly et al., 2003; Simon MP et al., 2008), thereby promoting their recruitment and proliferation/vessels organization in the avascularized tumour areas (Neufeld et al., 1999; Joško et al., 2000; Conway et al., 2001).

Expression of VEGF and other pro-angiogenic factors induces increased vascular density, facilitating O₂ and nutrients diffusion in all cancer areas. In addition, HIF-1 α activates genes related to control of vessels muscular tone like nitric oxide synthase-2 (NOS-2) (Melillo et al., 1995), heme-oxygenase 1 (Lee et al., 1997), endothelin 1 (ET1) (Hu et al., 1998), adrenomedullin (ADM) (Nguyen e Claycomb, 1999) and α_{1B} adrenergic receptor (Eckhart et al., 1997). Finally, in hypoxic condition, there is also a general upregulation of all proteins involved in ECM degradation and vascular maturation like MMPs (Ben-Yosef et al., 2002), plasminogen activator, its receptors and its molecular inhibitors (PAIs) (Kietzmann et al., 1999).

Invasion and cell motility

After acquiring a metastatic phenotype, neoplastic cells are still physically separated from neighbouring tissues by basal membrane and ECM. In order to avoid this barrier, cells need to digest matrix proteins through the expression of proteolytic enzymes like the MMPs family proteins. These enzymes degrade all the main components of interstitial matrix and basal membrane, thereby allowing cancer cells penetration and diffusion in distant tissues (Brinckerhoff e Matrisian 2002).

MMPs are zinc-dependent endopeptidases ubiquitously expressed in human tissues and associated to cancer metastatic ability. They are divided into five groups according to their substrate: matrilysines, collagenases, stromalysins, gelatinases and membrane MMPs. These enzymes are also classified according to their structures in eight groups, five of them are secreted and three are bound to the cell membrane.

MMPs are synthesized as pre-pro-peptides containing both zinc and calcium ions. Their structures contain an amino-terminal pre-peptide, or signal peptide, of 17-29 aminoacids which directs the protein to the endoplasmic reticulum; a catalytic domain of 170

aminoacids with a zinc binding domain; a pro-peptide of 77-87 aminoacids containing a thiolic group (SH) which interacts with zinc and keeps the enzyme into an inactive form. In the majority of the MMPs, the cysteine residue is in the conserved sequence PRCGxPD. Some MMPs have a prohormone convertase cleavage site Furin-like as part of this domain, which, when cleaved, activates the enzyme.

Recent studies have demonstrated that the role of MMPs in cancer development is way more complex than the simple degradation of matrix proteins (Hojilla et al 2003). According to that MMPs act on several types of substrates including growth factors receptors, adhesion molecules, chemokines, cytokines, apoptotic ligands and angiogenic factors, thereby influencing most of the cancer progression phases.

MMP-2 and MMP-9, also named gelatinase A and B respectively, are the major MMPs implicated in tumour progression as key enzymes for collagen IV digestion, which is the main component of ECM (Stamenkovic 2003). Experimental evidences suggest that there is a strong correlation between MMPs increased expression and tumour cells invasive ability (Vihinen e Kahari 2002) and studies conducted on a pulmonar epithelial cells revealed that they are upregulated in hypoxia in a HIF-dependent manner (Leufgen et al 2005).

ECM digestion also requires activity of uPA/uPAR complex where uPA is activated after interaction with its receptor uPAR. Active uPA turns plasminogen in plasmin through proteolytic cut. Plasmin is directly involved in ECM proteins degradation and activation of several growth factors and MMPs which in turn contribute to further degrade ECM. In addition, uPAR can regulate other membrane proteins like integrins, growth factor receptors and receptors coupled with G proteins in order to promote cell migration (Yebra et al 1996). For example, uPAR acts as receptor for vitronectin to promote cell adhesion to ECM (Waltz e Chapman 1994), while interaction between uPAR and $\beta 1$ and $\beta 2$ integrins impairs cell adhesion (Wei et al 1996). In hypoxic condition increased uPAR mRNA expression results from an increased mRNA stability and higher activity of its gene promoter (Maity et al 2000). In addition, experimental evidences have pointed out a positive correlation between HIF and uPAR expression as HREs are located in the upper domain of uPAR promoter sequence (Graham et al 1998) and both uPAR expression and *in vitro* invasive ability of colon carcinoma cells are increased by HIF-1 α overexpression and thus impaired by its gene silencing (Krishnamachary et al 2003).

Anyway, there are also several pathways HIF-independent involving inhibition of NO production, activation of soluble guanylate cyclase and protein kinase G cGMP-dependent (PKG) (Postovit et al. 2002) or activation of MAPK like ERK1/2 and p38 (Yoon et al 2006). In order to escape from a hostile hypoxic microenvironment, cancer cells exploit migrating strategies in order to move toward higher availability of oxygen and nutrients. For example, HIF-1 α activation correlates with metastatization through direct regulation of key factors governing tumor cell metastatic potential, including Snail-dependent downregulation of E-cadherin expression and loss of cell-to-cell contact, lysyl oxidase (LOX) activation, and upregulation of both stromal-derived factor 1 (SDF-1) and its receptor CXCR4, thereby directly regulating metastatic cancer cells directional migration (Rankin and Giaccia, 2008). In addition, some factors like HGF play a mandatory role in mediating HIF-1 α -dependent cancer cells invasiveness.

HGF is a cytokine secreted from stromal cells which binds to its high specific receptor, coded by the oncogene MET, which expression is induced in hypoxia in several types of cancers. Several studies have demonstrated that hypoxia has a major role in promoting MET expression, leading to higher metastatization *in vivo* and higher invasivity *in vitro*. Hypoxic upregulation of MET promoter, its expression and phosphorylation have been observed in both cell lines derived from hypoxic and normal tissues, even if its expression and HGF production is increased by interaction between cancer cells and hypoxic tissues-derived fibroblasts (Ide et al 2006). In addition, it has been demonstrated that hypoxic MET expression is related to HIF-1 binding to two sites located at the 5' terminus of the non coding region of MET promoter Met (Pennacchietti et al 2006). According to that, the synergic effect between hypoxia and HGF production on cancer cells invasive ability is impaired after HIF-1 gene silencing (Hara et al 2006). A positive feedback regulatory mechanism contributes in sustaining and amplifying MET overexpression in solid tumours, as MET molecular pathways induce both HIF-1 α and MET itself activity (Boccaccio et al 1994; Tacchini et al 2001; Tacchini et al 2003).

Met is a tyrosine kinase receptor first expressed as a single chain precursor, which is then cleaved at a furine site thereby forming an extracellular, highly glycosilated α subunit, and a β subunit with an extracellular portion, that interacts with the ligand, a transmembrane domain and an intracellular portion, containing a catalytic site. The subunits are linked by a disulfure bond.

Met extracellular domain contains an homology region with the semaphorin, called Sema domain, that includes α subunit and the N-terminus of the β chain, an affine-Met

sequence rich in cysteines (MRS) followed by a motif made by repetition of prolynglycin (G-P) and four structures immunoglobulin-like (Birchmeier et al 2003).

The intracellular domain contains three regions:

- a juxtamembrane sequence with a Ser 985 that can be phosphorylated by PKC or Ca²⁺-calmodulin-dependent kinases in order to regulate receptor activity (Gandino et al 1994)
- Tyr 1003 that can be bound by ubiquitin ligase Cbl, leading to polyubiquitination, endocytosis and degradation of the receptor (Peschard et al 2003).
- Tyrosine kinasic activity domain which, following receptor activation, is transphosphorylated on Tyr 1234 and 1235 and a C-terminal region that contains Tyr 1349 e 1356 which are inserted into the multisubstrate docking site, capable of recruiting downstream adapter proteins with Src homology-2 (SH-2). These two Tyr are necessary and sufficient to activate signal transduction both *in vitro* (Ponzetto et al.,1994) and *in vivo* (Zanetti et al 1998).

Met ligand is HGF, or scatter factor (SF), secreted by fibroblasts *in vitro* or secreted by platelets, as a potent epathic mitogenic agent, of patients suffering from acute epathic insufficiency (Nakamura et al 1989; Zarnegar e Michalopoulos 1989). Met expression is usually related to epithelial cells, while HGF expression is restricted to mesenchymal cells. Following stimulation with HGF, Met activation mediates several biologic effects both *in vivo* and *in vitro* collectively known as “invasive growth”. *In vivo* invasive growth HGF-induced involves cellular spreading, loss of cell-to-cell contact, acquisition of a motile phenotype, also named *scatter*, that promotes cell migration towards a different microenvironment. During this process cells lose their adherent junctions that preserve their monolayer organization, change their polarization after cytoskeleton rearrangements, digest ECM through proteases and dynamically remodel their adhesion contacts to ECM. Finally, when cells reach the tissue, they proliferate and avoid apoptotic mechanisms, eventually forming new vascular structures (Comoglio 2002).

After its activation, MET receptor autophosphorylates and binds to signal transducers and adapting molecules involved in several molecular pathways that induce activation of MAPK, PI3K, and Jak/STAT (Trusolino e Comoglio 2002). MET molecular pathway alters expression and activity of cadherins, integrins and MMPs. These arrangements cause loss of cell-to-cell contact, basal membrane disruption and alterates interaction

among components of the ECM, promoting tumour invasion across the stroma (Trusolino e Comoglio 2002). Anyway HGF-dependent integrins regulation also allow cancer cells to avoid *anoikis* (Longati et al 1996; Amicone et al 1997).

HGF-induced cell migration is related to increased expression/activity of SNAIL, MAPK-EGR-1-mediated, with subsequent downregulation of E-cadherin expression, while SNAIL expression alone in absence of HGF is not sufficient to induce a migratory phenotype (Grotegut et al 2006). HGF-mediated signaling promotes integrin aggregation and recruitment of adhesive contacts and motile structures, thereby leading to an increase in the percentage of integrins bound to actin cytoskeleton in order to form actin-rich cells protrusions (Trusolino et al 2000).

Met promoter positively responds to several stimuli growth factors and HGF-induced. In addition, it has been demonstrated that several oncogenes like Ras and Ret induce its overexpression (Engelman et al 2007; Ivan et al 1997). Met promoter analysis reveals presence of four ETS sites, transcriptional factors that control genes involved in the invasive growth (Shirasaki et al 1999). For example, Ets1 promotes Met transcription *in vitro* and is activated by Met itself through the MAPK pathway, suggesting a positive feedback mechanisms that sustains invasive growth (Gambarotta et al 1996).

Another Met transcriptional regulator is tissutal O₂ tension. In fact hypoxia upregulates Met expression through HIF-1 binding to Met promoter, leading to amplification of HGF signaling and invasive spur induction (Pennacchietti et al 2003).

Metastatic ability

Metastatization is promoted by tumour hypoxia and is related to a complex network of events where chemokines direct cells migration, adhesion molecules mediate colonization of distant organs and proteases digest/alterates ECM. Several proteins involved in these processes include vimentin, fibronectin, keratins 14, 18, 19, MMPs, cathepsin D, uPA/uPA_r system are HIF-induced (Semenza 2003) (figura 24). Studies on models of breast and renal cancer show that expression of chemokine receptor CXCR4, major metastatic mediator, MMP-2 and 9 are all upregulated by HIF-1 (Leufgen et al 2005; Staller et al 2003).

Another key mediator of the metastatic process is the lysil oxidase enzyme, an HIF-1 target closely related to hypoxia and bad prognosis in several types of cancer. Lysil oxidase modifies ECM components like elastin and collagens and it has been observed that its inhibition impairs *in vitro* migration and metastatization *in vivo* following

subcutaneous transplants and intravenous injection (Erler et al 2006). In addition, it has been demonstrated that this family of enzymes promotes conformational changes in nuclear factor SNAIL structure, promoting its resistance to degradation and repression of E-cadherin expression, leading to EMT process (Pouyssegur et al 2006; Perinando et al 2005).

According to the role of hypoxia in cancer cells survival, several studies show that HIF-1 α has an anti-apoptotic. In fact, HIF-1 α downregulation promotes hypoxia-induced apoptosis through caspases activation and inactivation of Bcl-X_L, while HIF-1 α positively correlates with resistance to apoptosis in pancreatic cancer cells and hepatic cancer cells HepG2 (Akakura et al 2001). In addition, a direct action by HIF-1 α has been hypothesized in pro-apoptotic Bid downregulation, as its promoter sequence contains an HRE sequence (Earler et al 2004).

Genes transcription HIF-induced have a key role in tumour biology. For example Oct 4, HIF-2 α target, is a master regulator of both adult and embrional stem cells behavior (Tai et al 2005). The mechanism through which Oct4 could modulate tumour growth has not been elucidated yet, but it has been hypothesized that Oct-4 can promote cancer stem cells development and growth, thereby promoting cancer self-renewal and resistance to chemotherapy.

HIFs also directly regulates transcription factors like c-Myc and Notch. In fact, HIF-1 α interacts with Notch-1 intracellular domain, thereby increasing its half-life and transcriptional activity (Gustafsson et al 2005). The effect of this interaction *in vivo* has not been cleared yet but could be important for tumour biology given the role of Notch in mediating cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life (Wilson e Radtke 2006).

On the other hand, HIF-1 α exerts an inhibitory effect on c-Myc. This inhibition deregulates p21 and p27 (Koshiji M. et all 2004) and c-Myc target genes involved in DNA “mismatch repair”, thereby suggesting a role of HIF in promoting hypoxia-induced genetic instability (Koshiji et all 2005), while HIF-2 α promotes c-Myc transcriptional activity, promoting cancer progression (Gordan et al 2007).

miRNAs

It has been observed that HIF-1 not only regulates gene expression, but also activates trascription of microRNAs (Fig. 25), a family of small, non coding, mRNAs made of

19-24 nucleotides, that influence stability and translation of mRNAs (Kent e Mendell, 2006), thereby promoting cell survival in the tumour microenvironment (Kulshreshtha et al., 2007; Hebert et al., 2007).

In particular, HIF-1 induces expression of miRNA-210 (Kulshreshtha et al., 2007; Camps et al., 2008; Fasanaro P et al., 2008), an ubiquitarily hypoxia-responsive factor. In several tumours miRNA-210 expression is increased in hypoxic areas rather than in non-transformed tissues (Volinia et al., 2006; Iorio et al., 2005; Yanaihara et al., 2006; Camps et al., 2008). In humans it has been pointed out that miRNA-210 is able to regulate expression of genes involved in proliferation, DNA damage repairing systems, chromatin remodelling, metabolism and cell migration (Kulshreshtha et al., 2007-a; Camps et al., 2008; Fasanaro et al., 2008; Kulshreshtha et al., 2008; Kulshreshtha et al., 2007-b).

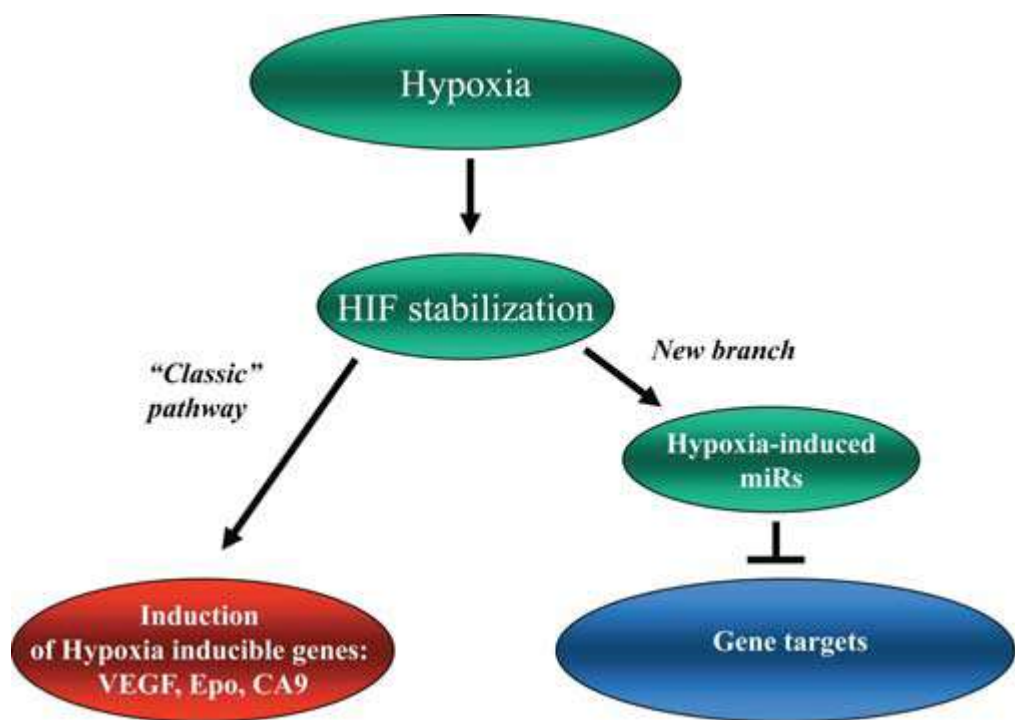


Fig. 25 Proposed model implicating select microRNAs in the hypoxia response.

Cellular metabolism

Aerobic energetic metabolism is dependent from oxidative phosphorylation and low O₂ level severely impairs ATP production and cell survival. When there is lower O₂

availability, cells need to adapt reprogramming their metabolic behaviour. As extensively described later, tumour cells undergo a “glycolitic switch” that allows them to change their metabolism into an anaerobic one, thereby keeping themselves metabolically active even under lower O₂ availability.

Physiologically non-neoplastic cells metabolize glucose into pyruvate, which is in turn moved in the mitochondria, thereby entering the Krebs cycle and undergoing oxidative phosphorylation. This metabolic pathway produces high ATP levels (38 molecules for each glucose molecule), but needs O₂ for mitochondrial respiration. On the other hand, hypoxic cancer cells maintain high glycolytic activity but, in absence of oxygen, they convert pyruvate into lactate, as high intracellular pyruvate concentration can severely impair cell survival itself. Anyway, this metabolic change alone is not sufficient in order to satisfy cells metabolic needs as glycolysis leads to production of only two ATP molecules, thus cancer cells need other adaptive mechanisms.

Glycolysis metabolic products, mainly secreted lactic acid, induce a strong acidification of the extracellular milieu, thereby lowering external pH level till 6.2-6.8 against physiological values of 7.2-7.4 in normal tissues. This microenvironmental acidification is direct consequence of increased glucose uptake and consumption, in order to sustain cell vitality (Gatenby RA., Gillies RJ. 2004). In 1920 Otto Warburg first demonstrated that cancer cells preferentially undergo glycolysis even in normoxic conditions. This mechanism, thus named “*Warburg effect*”, allows cancer cells to take advantage on the other not-transformed cell clones in hypoxic condition (see next chapter).

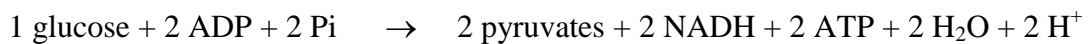
At the same time, cancer cells release pro-angiogenic factors that stimulates new vessels formation in order to promote O₂, glucose and other nutrients delivery to sustain metabolic needs of highly proliferative cells. Also in this case hypoxia exerts a selective pressure on cells unable to promote their angiogenic ability (Jiang et al 1997; Maxwell et al 1997). In addition, hypoxia favours O₂ delivery by erythrocytes promoting expression of genes involved in erythropoiesis and Fe metabolism. There is an increased EPO production, that stimulates red blood cells formation, thereby increasing the number of circulating red blood cells (Semenza et al., 1991). In addition, in order to ensure correct hemoglobin synthesis, as heme production is related to Fe availability, there is an upregulation of genes involved in Fe metabolism. Hypoxia upregulates expression of transferrin (Tf), that delivers Fe³⁺ in the bloodstream, of its receptor (Tfr), which promotes Fe uptake, and of ceruloplasmin, which oxidizes ferrous Fe²⁺ to ferric Fe³⁺ (Rolfs A et al., 1997; Bianchi L et al., 1999; Mukhopadhyay CK et al., 2000).

THE METABOLISM OF CANCER CELLS

Glycolysis

Glucose, a 6-carbons-aldehydic monosaccharide, is the primary source of energy for plants, animals and most microorganisms. Its total oxidation in CO_2 and H_2O leads to a free energy production of almost -2840 kJ/mole. Glycolysis is the key metabolic pathway for glucose catabolism in most cells of the organism and it does not require O_2 to carry on its reactions.

The overall reaction of glycolysis is: a molecule of glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) reacts with 2 NAD^+ molecules, 2 ADP molecules and 2 Pi molecules in order to produce 2 molecules of NADH, 2 molecules of pyruvate (CH_3COCOOH), 2 ATP molecules and 2 molecules of H_2O and 2 H^+ ions.

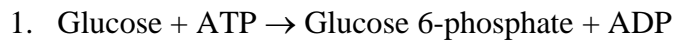


The overall molecular pathway is divided in ten reactions, The first five steps are regarded as the preparatory phase, since they consume energy to convert the glucose into two three-carbon sugar phosphates, while the second half of glycolysis is known as the pay-off phase, characterised by a net gain of the energy-rich molecules ATP and NADH (fig. 26).

During the preparatory phase, each glucose molecule undergoes two phosphorylations through consumption of two ATP molecules and is then divided into two molecules of glyceraldehyde 3-phosphate. For every reaction there is a variation of standard free energy ΔG° (Gibbs energy), defined as $\Delta G^{\circ} = -RT \ln K'_{\text{eq}}$ where R is the gas constant ($R = 8,315 \text{ J/mole} \cdot \text{K}$), T is the absolute temperature and K'_{eq} is the equilibrium constant. ΔG is the chemical potential that is minimized when a system reaches equilibrium at constant pressure and temperature. Its derivative with respect to the reaction coordinate of the system vanishes at the equilibrium point. As such, it is a convenient criterion of spontaneity for processes with constant pressure and temperature. Hence, out of this general natural tendency, a quantitative measure as to how near or far a potential reaction is from this minimum is when the calculated energetics of the process indicate that the change in Gibbs free energy ΔG is negative. In essence, this means that such a reaction will be favoured and will release energy. The energy released equals the maximum amount of work that can be performed as a result of the chemical reaction. In contrast, if conditions indicated a positive ΔG , then

energy—in the form of work—would have to be added to the reacting system to make the reaction go.

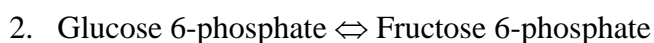
PREPARATORY PHASE REACTIONS :



$$\Delta G^{\circ} = -17,7 \text{ kJ/mole}$$

The first step in glycolysis is phosphorylation of glucose on the C-6 by a family of enzymes called hexokinases to form glucose 6-phosphate (G6P). This enzyme uses Mg^{2+} ions as cofactors and consumes ATP, but it acts in order to keep the glucose concentration low, promoting continuous transport of glucose into the cell through the plasma membrane transporters. In addition, it blocks the glucose from leaking out as the cell lacks transporters for G6P, and free diffusion out of the cell is prevented due to the charged nature of G6P. In addition, the disruption of the phosphoanhydridic bond between ATP phosphate groups generates high free energy variation which is partially conserved in the formation of the phosphoesteric bond of G6P. Finally, the interaction between a phosphoric group and an enzymatic catalytic site contributes in lowering activation energy, thereby promoting catalysis.

In animals, an isozyme of hexokinase called glucokinase is also used in the liver, which has a much lower affinity for glucose (K_m in the vicinity of normal glycemia), and differs in regulatory properties. The different substrate affinity and alternate regulation of this enzyme are a reflection of the role of the liver in maintaining blood sugar levels.

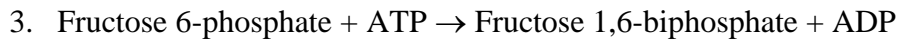


$$\Delta G^{\circ} = -1,7 \text{ kJ/mole}$$

G6P is then rearranged into fructose 6-phosphate (F6P) by glucose phosphate isomerase, which uses Mg^{2+} ions as cofactors. Fructose can also enter the glycolytic pathway by phosphorylation at this point.

The change in structure is an isomerization, in which the G6P has been converted to F6P. The reaction requires an enzyme, phosphohexose isomerase, to proceed. This reaction is freely reversible under normal cell conditions. However, it is often driven forward because of a low concentration of F6P, which is constantly consumed during

the next step of glycolysis. Under conditions of high F6P concentration, this reaction readily runs in reverse.

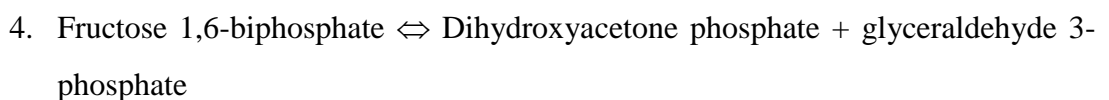


$$\Delta G^{\circ} = -14,2 \text{ kJ/mole}$$

Phosphofructokinase 1 (PFK-1) catalyzes the transfer of ATP phosphoric group to the oxydrilic group of F6P. The reaction catalyzed by PFK-1, which requires Mg^{2+} ions as cofactors, is coupled to the hydrolysis of ATP, an energetically favorable step, which makes the reaction irreversible, and a different pathway must be used to do the reverse conversion during gluconeogenesis. This makes the reaction a key regulatory point as PFK-1 is positively regulated by AMP, ADP and fructose 2,6 biphosphate, catalytic product of PFK-2 enzyme, concentrations, while it is negatively regulated by high concentrations of ATP and citrate, intermediate of the Krebs cycle.

Furthermore, the second phosphorylation event is necessary to allow the formation of two charged groups in the subsequent step of glycolysis, ensuring the prevention of free diffusion of substrates out of the cell.

The same reaction can also be catalyzed by pyrophosphate-dependent phosphofructokinase (PPi-PFK), which is found in most plants, some bacteria, archaea, and protists, but not in animals. This enzyme uses pyrophosphate (PPi) as a phosphate donor instead of ATP. It is a reversible reaction, increasing the flexibility of glycolytic metabolism. A rarer ADP-dependent PFK enzyme variant has been identified in archaean species.



$$\Delta G^{\circ} = 23,8 \text{ kJ/mole}$$

Destabilizing the molecule in the previous reaction allows the hexose ring to be split by aldolase into two triose sugars, dihydroxyacetone phosphate, a ketone, and glyceraldehyde 3-phosphate, an aldehyde. There are two classes of aldolases: class I aldolases, present in animals and plants, and class II aldolases, present in fungi and bacteria which use different mechanisms in cleaving the ketose ring.

Electrons delocalized in the carbon-carbon bond cleavage associate with the alcohol group. The resulting carbanion is stabilized by the structure of the carbanion itself via resonance charge distribution and by the presence of a charged ion prosthetic group.

The enzyme aldolase catalyzes an aldolic condensation that, despite the positive free energy variation, can be reversible given the high intracellular concentrations of the two reagents.

5. Dihydroxyacetone phosphate \leftrightarrow glyceraldehyde 3-phosphate

$$\Delta G^{\circ} = 7,5 \text{ kJ/mole}$$

Triosephosphate isomerase rapidly reversibly interconverts dihydroxyacetone phosphate with glyceraldehyde 3-phosphate that proceeds further into glycolysis. This is advantageous, as it directs dihydroxyacetone phosphate down the same pathway as glyceraldehyde 3-phosphate, simplifying regulation.

PAY-OFF PHASE REACTIONS:

Since glucose leads to two triose sugars in the preparatory phase, each reaction in the pay-off phase occurs twice per glucose molecule. This yields 2 NADH molecules and 4 ATP molecules, leading to a net gain of 2 NADH molecules and 2 ATP molecules from the glycolytic pathway per glucose.

6. glyceraldehyde 3-phosphate + Pi + NAD⁺ \leftrightarrow 1,3-bisphosphoglycerate + NADH + H⁺

$$\Delta G^{\circ} = 6,3 \text{ kJ/mole}$$

The triose sugars are dehydrogenated and inorganic phosphate is added to them, forming 1,3-bisphosphoglycerate.

The hydrogen is used to reduce two molecules of NAD⁺, a hydrogen carrier, to give NADH + H⁺ for each triose.

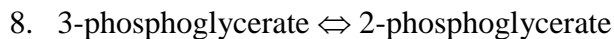
Hydrogen atom balance and charge balance are both maintained because the phosphate (P_i) group actually exists in the form of a hydrogen phosphate anion (HPO₄²⁻), which dissociates to contribute the extra H⁺ ion and gives a net charge of -3 on both sides.



$$\Delta G^{\circ} = -18,5 \text{ kJ/mole}$$

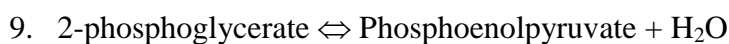
This step is the enzymatic transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP by phosphoglycerate kinase, forming ATP and 3-phosphoglycerate. At this step, glycolysis has reached the break-even point: 2 molecules of ATP were consumed, and 2 new molecules have now been synthesized. This step, one of the two substrate-level phosphorylation steps, requires ADP; thus, when the cell has plenty of ATP and little ADP, this reaction does not occur. Because ATP decays relatively quickly when it is not metabolized, this is an important regulatory point in the glycolytic pathway. Reactions 6 and 7 represent an energetic coupling where 1,3-BPG is the common intermediate. Total reaction is favoured as the ΔG° positive value of reaction 6 is balanced by ΔG° negative value of reaction 7.

ADP actually exists as ADPMg^{-} , and ATP as ATPMg^{2-} , balancing the charges at -5 both sides.



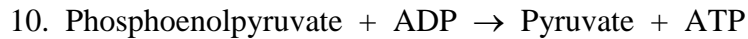
$$\Delta G^{\circ} = 4,4 \text{ kJ/mole}$$

Phosphoglycerate mutase, which uses Mg^{2+} ions as cofactors, now forms 2-phosphoglycerate through placement of the phosphoric group from C-3 to C-2 of glycerate. This is a two-phases reaction with generation of the intermediate of 2,3-bisphosphoglycerate.



$$\Delta G^{\circ} = 7,5 \text{ kJ/mole}$$

This reaction is catalyzed by enzyme enolase, which catalyzes the reversible removal of an H_2O molecule from 2-phosphoglycerate thereby forming phosphoenolpyruvate (PEP). Despite the little free energy variation, free energy derived from hydrolysis of the phosphoric group increases.



$$\Delta G^{\circ} = -31,4 \text{ kJ/mole}$$

This substrate-level phosphorylation is catalyzed by enzyme pyruvate kinase, which requires K^{+} or Mg^{2+} or Mn^{2+} ions as cofactors. The reaction product is first in its enolic form as enolpyruvate and then quickly tautomerizes in the chetonic form as pyruvate, which is the commonest form at pH 7.

In aerobic conditions the NADH molecules are completely reoxydized by mitochondrial complex I of the electron transport chain. The final electron acceptor is O_2 which is then reduced to H_2O . in presence of O_2 , pyruvate is converted into acetyl-CoA by pyruvate dehydrogenase complex, thereby entering the Krebs cycle.

Under low O_2 tension, the pyruvate is reduced by lactate dehydrogenase to L-lactate in a process called lactic acid fermentation, which is necessary for NAD^{+} regeneration, as in hypoxic conditions NADH cannot be reoxydized. Loss of generation of NAD^{+} can be harmful for cells because is the electrons acceptor required for oxydation of G3P and its absence can block the glycolisis.



$$\Delta G^{\circ} = -25,1 \text{ kJ/mole}$$

Lactate, produced for example in muscles undergoing high physical efforts, can be then delivered to the liver and converted into glucose through gluconeogenesis. After that glucose can be delivered again to muscles and catabolyzed through glycolisis. This shunt between liver and muscles is also named Cori Cycle.

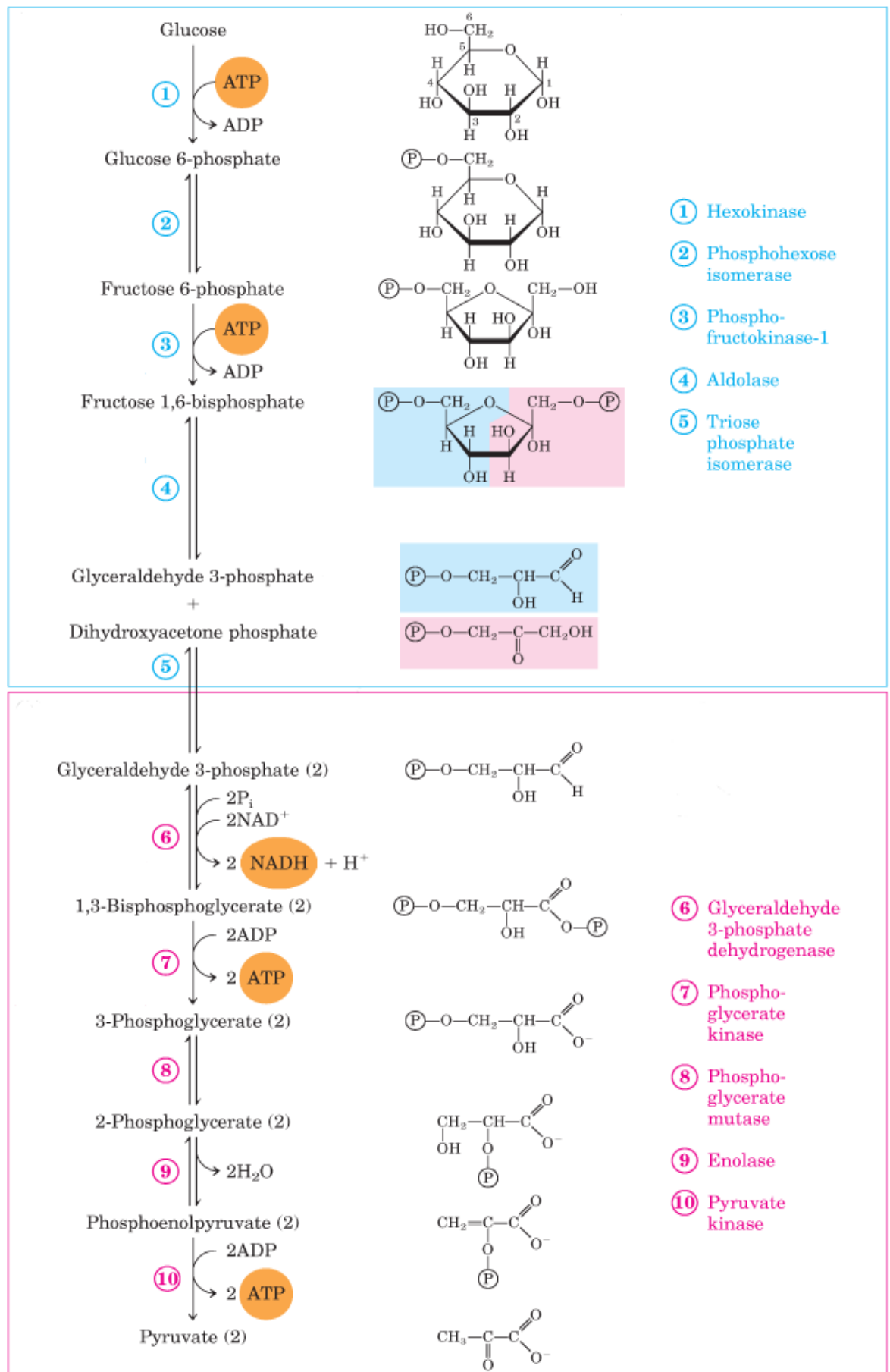


Fig. 26 Reactions of glycolysis.

The " Warburg Effect"

In 1924 Otto Warburg first observed that cancer cells metabolize higher amounts of glucose in comparison with cells from normal tissues, producing high levels of lactate. In addition, he showed that cancer cells preferentially have a glycolytic behaviour, even in presence of high O₂ tension (Warburg 1927). This peculiar characteristic was called "aerobic glycolysis" or "Warburg Effect". Further studies by Warburg himself hypothesized that the Warburg effect was mainly related to a mitochondrial deficiency developed by tumour cells, for example, through genetic mutations, thereby leading to a low energy delivery from mitochondrial respiration. Finally, aerobic glycolysis had to be the only possible metabolic mechanism that allowed cells to survive (Warburg 1956).

Anyway, subsequent studies demonstrated that mitochondrial deficiency was a rare condition even in cancer cells (Weinhouse 1956). Today it has been clarified that several factors, including tumour microenvironment and transcription factors, are needed in order to sustain cancer progression.

In the case of metabolism, the choice by cancer cells to prefer a glycolytic behaviour seems almost paradoxal because glycolysis is less efficient than mitochondrial respiration in providing ATP. In fact, fermentative metabolism of a molecule of glucose generates only 2 ATP molecules, while mitochondrial respiration leads to 36-38 ATP molecules/glucose molecule.

The advantages coming from an aerobic glycolytic metabolism are still under investigation. In multicellular organisms there are two main types of metabolism, the one characteristic for highly proliferating and tumoural cells, and the quiescent metabolism, typical of already differentiated cells. In order to divide quickly and efficiently, highly proliferating cells need to replicate their genome, produce proteins, lipids and all cellular components that are needed in order to form other cells. These activities require an efficient delivery of nutrients from the extracellular space and their conversion in biosynthetic products.

On the other hand, differentiated cells enter a quiescence phase (G₀ phase) characterized by high energetic demand in order to sustain cell vital. Thus cells convert glucose into ATP and CO₂ with the mitochondrial respiration in presence of high O₂ tension, while they catabolyze glucose into lactate just in hypoxic conditions.

An example of a proliferative cell energetic demand is the synthesis of palmytate, the major component of cell membranes. Its production requires 7 ATP molecules, 16 atoms of carbon from 8 acetyl-CoA molecules and 28 electrons from 14 NADPH

molecules. Similarly, aminoacids and nucleotides synthesis requires more carbon atoms and NADPH than ATP. A glucose molecule can generate 36 ATP molecules, or 30 ATP molecules and 2 NADPH molecules if metabolized through the pentose phosphate pathway. The same glucose molecule can also generate 6 atoms of carbon for the synthesis of macromolecules but, in order to form a 16-atoms carbonic chain, mitochondrial respiration needs 7 glucose molecules to produce the required amount of NADPH. This high intermediates demand is just partially compensated by catabolism of 3 glucose molecules. In conclusion, it is clear that a highly proliferative cell, like the tumoural one, can not use glucose just in order to produce ATP, otherwise the increased ATP/ADP ratio would lower the glycolytic intermediate flux, thereby reducing acetyl-CoA and NADPH production, required for macromolecules synthesis. A part of the uptaken glucose must be delivered to production of macromolecules precursors like acetyl-CoA for fatty acids, glycolytic intermediates for non essential aminoacids and ribose for nucleotides. According to these observations, Increased ROS was documented to modify a critical sulfhydryl group of pyruvate kinase M2 (PKM2), rendering it inactive and resulting in the shunting of glucose away from glycolysis toward the PPP (Anastasiou et al. 2011). The PPP generates NADPH, which reduces glutathione into an active antioxidant that protects the cell. In this manner, the shunting of glucose away from glycolysis toward the PPP is an essential element of redox homeostasis.

In addition to oxidation of PKM2, increased ROS can stabilize HIF-1. HIF-1, in turn, activates target genes such as PDK1, which diverts pyruvate away from mitochondrial oxidation, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 (PFKFB4), which degrades 2,6-fructose bisphosphate (2,6-FBP) (Keith et al. 2012; Semenza 2012). 2,6-FBP is a powerful allosteric activator of phosphofructose kinase 1 (PFK1), which converts fructose-1-phosphate to fructose-1,6-bisphosphate (1,6-FBP) at a rate-limiting step in glycolysis (Yalcin et al. 2009). Hence, increased PFKFB4, as observed in prostate cancer cell lines, would diminish PFK1 activity and divert glucose into the PPP shunt, elevating NADPH to titrate ROS (Ros et al. 2012). It is notable, however, that hypoxia also elevates PFKFB3, which drives glycolysis and can oppose PFKFB4; as such, the balance between PFKFB3 and PFKFB4 activities is critical for shunting glucose into glycolysis versus the PPP.

In addition, hypoxia adapting mechanisms involve coordinated expression of genes coding for glucose transporters (GLUT1, GLUT3), glycolytic enzymes

(phosphoglycerate kinase 1, pyruvate kinase M2, esokinase II), VEGF, EPO and heme-oxygenase-1 (HO-1) (Semenza 2003). In addition, there is induction of pyruvate dehydrogenase 1 (PDH-1) and lactate dehydrogenase (LDH) that reduce pyruvate availability, thus increasing its conversion to lactate and inhibiting Krebs cycle (Brahimi-Horn et al., 2007).

As a consequence, lowering of extracellular pH following lactic acid secretion acts as a selective agent upon those cells unable to survive under acidic conditions. On the other hand cells bearing low pH are able to produce ATP and alterate tumour microenvironment, thereby directly harming the cells they compete with (Gatenby e Gillies 2004). To this end HIF-1 α upregulates expression of the monocarboxylate transporter 4 (MCT4) that mediates lactic acid extrusion, and of the carbonic anhydrase IX (CA-IX), transmembrane enzyme that catalyzes conversion of extracellular CO₂ into carbonic acid (H₂CO₃). This mechanism further promotes extracellular acidification through H⁺ ions release, while HCO₃⁻ intake allows cancer cells to avoid intracellular acidification that could be highly disvital.

Finally, these data could at least partially explain the selective advantages of Warburg metabolism.

Molecular pathways involved in cell metabolic regulation

PI3K. PI3K enzyme catalyzes conversion of phosphoinositides into phosphoinositides-3,4,5-triphosphate (PIP₃) (Cantley 2002). PI3K activity is physiologically regulated by PTEN in non proliferative cells, but PTEN mutations can lead to a constitutive activation of the PI3K pathway. According to that, there is a deregulated cell growth and a continuous transduction of survival signals that also affect cell metabolism. One of the main PI3K effectors is Akt kinase and PIP₃ binding to Akt pleckstrin homology domain (PH domain) induces Akt translocation to the cell membrane, where it is phosphorylated by PI3K-dependent kinase 1 and thereby activated (Bahskar et al. 2007). Akt stimulates glycolysis and modulates ATP production through several mechanisms, thereby allowing cancer cells to receive adequate bioenergetic supply. Akt induces a glycolytic phenotype inducing GLUT expression on cell membrane.

In addition, Akt activates the glycolytic enzyme PFK-1. Akt phosphorylates and activates PFK2 that in turn produces fructose 2,6-bisphosphate, major PFK-1 allosteric activator (Deprez et al. 1997). Finally, Akt can promote activation of complex 1 of the rapamycin mammal target (mTOR). mTOR induces HIF-1 expression, leading to an overexpression of almost all genes involved in cell glycolytic metabolism (Brugarolas 2003).

HIFs and MYC. As already described, hypoxia is a common feature of solid tumours and complexes HIF-1 α , HIF-2 α and HIF-3 α are the master regulators of cell response to hypoxia. In particular, HIF-1 α regulates genes involved in cell metabolism (Hockel et al. 2001).

HIF-1 α can be activated in cancer cells in normoxic conditions. For example, mutations that lead to PI3K constitutive activation also activate in an aberrant manner mTOR pathway. In turn, mTOR phosphorylates ribosomal protein S6 kinase and binding protein eIF-4E 1. Both these proteins induce an increased HIF-1 α mRNA translation (Semenza et al. 2001).

Recent studies have demonstrated that in models of hereditary paraganglioma, glioblastomas and renal carcinoma several oncosuppressor genes code for enzymes of the Krebs cycle like succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase (IDH). SDH and FH loss of function leads to accumulation of succinate and fumarate, respectively. Both these metabolites inhibit

PHD2 activity competing with α -ketoglutarate for binding its catalytic site. Thus PHD2 inhibition reduces hydroxylation, ubiquitination and proteasomal degradation of HIF-1 α (Selak et al. 2005; Isaacs et al. 2005; Koivunen et al. 2007; Hewitson et al. 2007).

Once activated, HIF-1 α promotes gene transcription of SLC2A1 and SLC2A3, genes coding for GLUT1, mainly expressed in erythrocytes, and GLUT3, mainly located in neurons. Association of these proteins create a hydrophilic channel for glucose facilitated diffusion.

For what amplification of glycolytic pathway is concerned, HIF-1 induces expression of hexokinase 1 and 2 (HK1 e HK2), PFK1, aldolase A and C, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1, enolase 1, pyruvate kinase and lactate dehydrogenase A (LDHA).

In addition, recent studies on murine embryonal fibroblasts (MEFs) have demonstrated that gene coding for pyruvate dehydrogenase kinase 1 (PDK1) is a direct target of HIF-1. This enzyme phosphorylates and inhibits the pyruvate dehydrogenase (PDH) enzymatic complex, thereby blocking pyruvate entry into the Krebs cycle. These studies suggest that suppressing Krebs cycle and mitochondrial respiration, PDK1 could act as a survival factor for cells in condition of hypoxia (Kim JW et al. 2006).

c-Myc is a transcription factor overexpressed in almost the 40% of human cancers. This protein is a leucine-zipper HLH protein that dimerizes with the partner protein c-Myc-associated protein X (MAX). c-Myc/MAX complex binds to DNA specific sequences named E-boxes in order to activate or repress gene target transcription. The complex target genes seems to be dependent from the cellular type, but there is a common group of genes regulated by c-Myc like the ones involved in ribosomal biogenesis and glucose metabolism (Dang et al. 2008). It has been observed that c-Myc activates LDHA expression, thereby increasing lactate production in a model of lung carcinoma (Dang et al. 1997), while further studies suggest that also aldolase A, enolase, hexokinase 2, GAPDH, GPI, HK2, PFK1, PGK, PGM e TPI are upregulated by c-Myc activation (Osthus et al. 2000).

AMPK. Protein kinase activated by AMP (AMPK) is the main sensor of intracellular energetic state and has a central role in metabolic stress response. AMPK is activated through phosphorylation from protein kinase LKB1. In a condition of metabolic stress AMPK is activated by an increased AMP/ATP ratio. AMPK acts on glycolysis phosphorylating and activating the PFK2 inducible isoform (PFKFB3) (Marsin et al. 2000). PFK2 reaction product, fructose 2,6-biphosphate, is a PFK1 activator.

Observations from *in vivo* models lacking LKB1 and AMPK show a constitutive activation of the mTOR pathway. In the same conditions it has been observed an upregulation of HIF-1 α and its target genes in normoxic condition (Shackelford et al. 2009). These data show a strong dependence between LKB1/AMPK loss and HIF-1 α expression through an mTOR-dependent pathway.

p53 and OCT1. p53 is a transcription factor and oncosuppressor gene that has a major role in cell response to conditions of stress. It has been observed that p53 induces exokinase 2 expression through direct binding to the promoter, thereby stimulating glucose 6-phosphate production (Mathupala et al. 1997).

p53 regulates cellular glucose metabolism through activation of two effectors: TIGAR and SCO2. TIGAR is a p53-inducible gene and has a high functional affinity for PFK2 phosphatase domain (Bensaad et al. 2006). PFK2 is a bifunctional enzyme characterized by a kinase domain with high affinity for fructose 6-phosphate, and a phosphatase domain with high affinity for fructose 2, 6 biphosphate. Thus TIGAR expression induces fructose 6-phosphate production in the glycolytic pathway and its conversion to glucose 6-phosphate, according to the law of mass action. Finally, glucose 6 phosphate can enter the pentose phosphate pathway in order to produce NADPH and ribose-5-phosphate (R5P) for nucleic acids synthesis.

Second effector gene SCO2 is directly activated after binding to p53 as shown in models of colon cancer (Matoba et al. 2006). Together with SCO1, SCO2 encodes for two subunits of cytochrome c oxidase (COX), which catalyzes translocation of reducing equivalents from cytochrome c to O₂, pumping protons through the inner mitochondrial membrane.

Finally, it has been demonstrated that the promoter sequence of the gene coding PTEN contains a p53 binding region, through which p53 induces PTEN mRNA expression and thereby PI3K pathway inactivation (Stambolic et al. 2000).

OCT1 is a transcription factor from the POU domain family (Pit-1, Oct1/2, Unc-86). Studies on MEFs and adenocarcinoma epithelial cells have showed that OCT1 loss of function induces a metabolic change that suppresses tumour progression. In particular, OCT1^{-/-} cells show a decreased of glucose and lactate accumulation and an increased concentration of Krebs cycle intermediates like malate, succinate and isocitrate. These results demonstrate an increased mitochondrial activity and a decreased glycolytic flux due to OCT1 loss (Shakya et al. 2009).

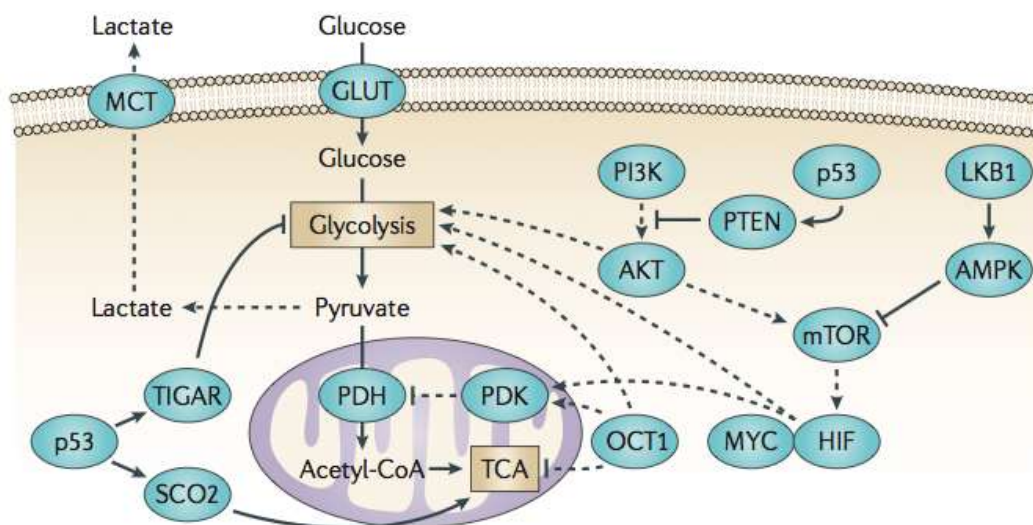


Fig. 27 Regulation of metabolic pathways by HIF-1, MYC, Oct-1 and p53.

Pyruvate Kinase M2 (PKM2). Pyruvate kinase (PK) catalyzes conversion of PEP to pyruvate with substrate-level phosphorylation of ADP to ATP.

There are several PK isoforms that are expressed according to specific metabolic needs of the tissue. PK L is expressed in tissues with high gluconeogenic activity like liver and kidneys, while isoform R is mainly expressed in erythrocytes and PK-M1 is located in tissue with high ATP demand like heart and brain. Isoform M2 (PKM2) is mainly expressed in lungs and, in general, by cells with high nucleic acids synthetic activity like embryonic cells, adult stem cells and cancer cells. PK L and R are coded from the same gene, PKLR, but are under control of two different promoters. Isoenzymes PKM1 and PKM2 are different splicing products from the same mRNA of gene *PKM2* and differ for 21 aminoacids (Reinacher et al 1981; Eigenbrodt et al. 1985; Eigenbrodt et al. 1992; Eigenbrodt et al. 1986; Steinberg et al. 1999). *PKM2* transcription is activated by transcription factor Sp1, while transcription factor Sp3 acts in hypoxic conditions repressing its transcription after physical dissociation from the promoter.

Immunohistochemistry studies of PKM2 have demonstrated that this protein can associate in a quaternary structure in a different manner in lung and cancer cells (fig. 28). In lungs and healthy proliferative cells PKM2 is expressed in a tetrameric form with four identical subunits, while in cancer cells, is always expressed in a dimeric form, and this particular PKM2 structure is also named “tumoural PKM2”. Tetrameric form PKM2 shows higher affinity for PEP, while dimeric form is characterized by

lower activity. In presence of PEP physiological concentrations, tetrameric PKM2 is highly active, while dimeric form is almost totally inactive. This catalytical difference is amplified when tetrameric form associates with glycolytic enzymes like HK, G3PDH, PGK, PGM, enolase and LDH-A and other enzymes like nucleotide diphosphate kinase, adenilate cyclase, glucose-6-phosphate dehydrogenase and proteins of the RAF/MEK/ERK pathway. On the other hand, dimeric PKM2 do not associates to glycolytic enzymes. This complex has two main functions: compartmentalization of metabolic reactions into the cytosol and increasing glycolysis efficiency through binding to enxyemes involved (Eigenbrodt et al. 2005). Transition from tetrameric to dimeric form is induced after direct interaction between PKM2 and several oncogenes (Eigenbrodt et al. 1988).

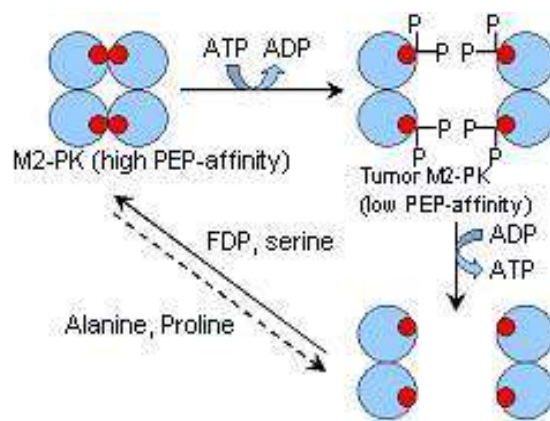


Fig. 28 PKM2 molecular structure.

Recent studies have clarified the molecular mechanism of M1- o M2- splicing of *PKM2* mRNA. In particular, it has been demonstrated that heterogenous ribonuclear proteins (hnRNP) I, A1 and A2 bind to RNA sequence on exon 9 inhibiting M1-specific splicing and c-Myc activates transcription of hnRNPI, hnRNPA1 and hnRNPA2. Thus it is clear that tumour cells with constitutive c-Myc expression have higher *PKM2* basal expression levels (Chen et al. 2010; Clower et al. 2010).

PK inactive isoform expression could represent an advantage for proliferating and tumour cells as, blocking glycolysis, carbonic flux stops at PEP formation. For the law of mass action, accumulation of this metabolite reverts the reaction flux, thereby leading glycolytic intermediates to biosintethic pathways, like the pentose phosphate pathway. These reactions are necessary to cancer cells as they provide both ribose-5-phosphate

for nucleotides synthesis and reducing power with NADPH, thereby avoiding excess of ROS and conditions of potentially harmful oxidative stress.

According to that, several mechanisms of PKM2 enzymatic activity inhibition have been described, like oxidation, phosphorylation and acetylation. Lv and colleagues have demonstrated in a prostatic carcinoma model that PKM2 acetylation on K305, induced by high glucose concentration, impairs its catalytic activity. In fact, acetylation favours PKM2 interaction with chaperon HSC70 and subsequent association with lysosomes, thereby leading to a mechanism of chaperone-mediated autophagy (CMA). Thus cancer cells accumulate glycolytic intermediates, thereby activating anabolic pathways and promoting proliferation and tumour growth in *in vivo* models (Lv et al. 2011). In addition, it has been recently showed in several cancer models, like breast and lung carcinomas, that PKM2 is phosphorylated on Tyr Y105 by FGFR1. This post-translational modification promotes dissociation of PKM2 tetrameric complex from its cofactor fructose 1, 6 biphosphate, impairing its activity (Hitosugi et al. 2010).

Finally, also ROS seem to have a key role in regulating PKM2. In a model of lung carcinoma it has been demonstrated that acute increase of intracellular ROS oxidizes the Cys358 of PKM2 catalytic site, modifying its structure and reducing catalysis. This event is strictly related to glucose catabolism through the pentose phosphate pathway, thereby leading NADPH synthesis in order to buffer excess of ROS. Cys358 oxidation is crucial because its mutation in a Ser358 blocks this event, thereby leading cells to higher sensitivity to oxidative stress and reducing their survival and tumour growth *in vivo* (Anastasiou et al. 2011). According to this data, Anastasiou and colleagues have recently demonstrated that both PKM1 overexpression and stimulation with pharmacological small-molecule PKM2 activators inhibits the growth of xenograft tumours. These small-molecule activators bind PKM2 and promote a constitutively active enzyme state that is resistant to inhibition by tyrosine-phosphorylated proteins, thereby leading to direct interference with anabolic metabolism (Anastasiou et al. 2012). In addition, PKM2 has a role as direct coactivator of HIF-1 α in target genes transcription (fig. 29).

As previously stated, PHD2 has a central role in hydroxylating and then promoting HIF-1 α degradation. For what concerns PHD3, recent studies have demonstrated that PHD3 hydroxylates prolines residues inside a highly conserved motif LRRLAP on the target protein (Xie et al. 2009). LRRLAP has been identified in an internal sequence of exon 10 of *PKM2* gene. In particular, Pro403 and Pro408 hydroxylation on PKM2 by PHD3

positively correlates with expression of HIF-1 α main target genes and probably promotes PKM2 transition from tetrameric to dimeric form (Chen et al. 2011). PKM2 dimeric form migrates in the nucleus, interacts with HIF-1 α through direct binding to HREs sequences on promoters of target genes and recruiting HIF coactivator p300. On the other hand, PKM1 do not associates to HIF-1 α (Semenza et al 2011). According to that, there is an increased transcription of HIF target genes like *PKM2* and *EGLN3*, encoding for PKM2 and PHD3, respectively (Pescador et al. 2005). These observations suggest a positive feedback mechanism of HIF-1 α activation in order to promote metabolic reprogramming of cancer cells (Semenza et al. 2011).

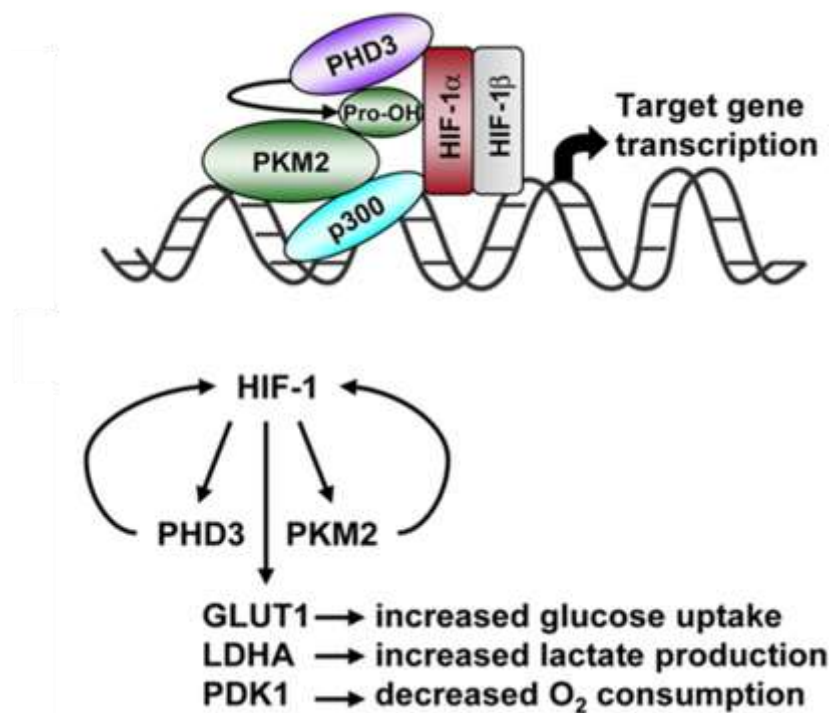


Fig. 29 Role of PKM2 as HIF-1 α direct coactivator.

Models of metabolic symbiosis

Nutrients and O₂ delivery to tumour mass is mediated by angiogenesis, but new vascularization is often made by arterious vessels not connected to venous circulation (Fukumura et al. 2010). In addition, tumour burden is characterized by impaired perfusion if compared to healthy tissues, thereby leading to formation of hypoxic areas inside the tumour, with subsequent HIF-1 α stabilization (Le et al. 2010).

This event leads cells to exploit different metabolic mechanisms inside the same tumour mass: normoxic regions, close to blood vessels, preferentially exploit mitochondrial respiration and phosphorilative oxidation, while hypoxic cells undergo glycolysis. According to that, it has been demonstrated that lactate, glycolysis end product, is the key mediator of a “metabolic symbiosis” model where both glycolitic and respiring cells reciprocally regulate nutrients availability through the transporter MCT-1. In fact, its inhibition shows antitunoral effects (Sonveaux et al. 2008).

According to this model, normoxic subpopulation of cancer cells exploit extracellular lactate to fuel oxidative phosphorylation, thereby avoiding extracellular glucose depletion, as glucose is metabolized through glycolysis by hypoxic cancer cells. Lactate used for mitochondrial metabolism gives several advantages to cancer cells:

- pyruvate oxidation to lactate by lactate dehydrogenase leads to production of reducing power that prevents harmful intracellular oxidative stress, thereby favouring cells survival (Lee et al. 2003; Pelicano et al. 2006);
- lactate oxidation do not requires ATP consumption;
- each molecule of lactate generates 18 ATP molecules, thereby allowing cells to save energy for glycolitic enzymes activity.

Lactate delivery between hypoxic and normoxic tumour areas is similar to the physiologic mechanism of muscles (Halestrap et al. 2004; Halestrap et al. 1999). During intense physical efforts, white muscle fibres produce and release lactic acid (Dubouchaud et al. 2000). Extracellular lactate is then uptaken by red muscle fibres in order to promote oxidative metabolism (Baker et al. 1998; Bonen et al. 1998; Dubouchaud et al. 2000) and this process is mediated by both MCT-1 and MCT-4 transporters (Halestrap et al. 2004; Halestrap et al. 1999). MCT-4 has low affinity for lactate (K_m=22 mM) and is consequently expressed by glycolitic cells in order to release the metabolite. MCT-4 expression is HIF-1 α -dependent (Dimmer et al. 2000; Ullah et al. 2006).

On the other hand, MCT-1 has high affinity for lactate ($K_m=3,5-10$ mM), thereby promoting its uptake in respiring cells (Halestrap et al. 1999; Dubouchaud et al. 2000; Ullah et al. 2006). It has been demonstrated that MCT-1 is the main canne for lactate intake by cancer cells (Sonveaux et al. 2008), while MCT-4 is responsible for protons extrusion from glycolitic cancer cells (Wahl et al. 2002). According to that, MCT-1 gene silencing is sufficient to impair oxidative phosphorylation lactate-dependent and cancer cells survival (Sonveaux et al. 2008). Despite being a pharmacological target to kill cancer cells close to blood vessels, MCT-1 inhibition undirectly promotes necrosis in hypoxic cancer cells usually resistant to conventional therapies and causing cancer recidival (Brown et al. 2004) (fig. 30). Finally, cancer cells death for lack of glucose is caused by metabolic modifications of respiring cells. In fact, oxidative cells modify their metabolic behavior to a glycolitic one, caused by inhibition of cellular respiration (Crabtree effect) (Crabtree 1929).

The lactate/MCT-1 pathway represents the key event of metabolic symbiosis in cancer and its discovery leads to new interpretations of data and relations previously observed (Sonveaux et al. 2008). For example, high energetic nature of lactate explains its autocrine activity as growth factor (Pike et al. 1991) and its bidirectional delivery among different cell types (Spencer et al. 1976; Cheeti et al. 2006; Wang et al. 2007). In addition, several studies relate to neoplastic cells a mechanism described just for stromal cells in order to exploit cancer cells metabolic products (Sonveaux et al. 2008).

Inhibition of MCT-1 impairs normoxic cancer cells ability to uptake lactate for their oxidative phosphorylation. Subsequently this cancer cells subpopulation exploits extracellular glucose for its metabolic needs, thereby depleting glucose from hypoxic cells that are unable to survive.

A second effect of MCT-1 inhibition, at least *in vitro*, is the lethal intracellular acidification (Wahl et al. 2002; Belt et al. 1979; Coss et al. 2003; Fang et al. 2006; Mathupala et al. 2004). In fact, it has been observed that MCT-1 expression is increased also in cancer cells that metabolize butyrate, as this transporter promotes its intake (Serpa et al. 2011). Butyrate is used by cancer cells to fuel β -oxidation, as demonstrated by their overexpression of acyl-dehydrogenase enzyme. Thus cancer cells able to metabolize butyrate are positively selected by tumour microenvironment (Serpa et al. 2010). These cells show a mesenchymal phenotype, with E-cadherin downregulation, increased expression of MMP-2 and 9 and expression of $\alpha 2$ and $\alpha 3$ integrins.

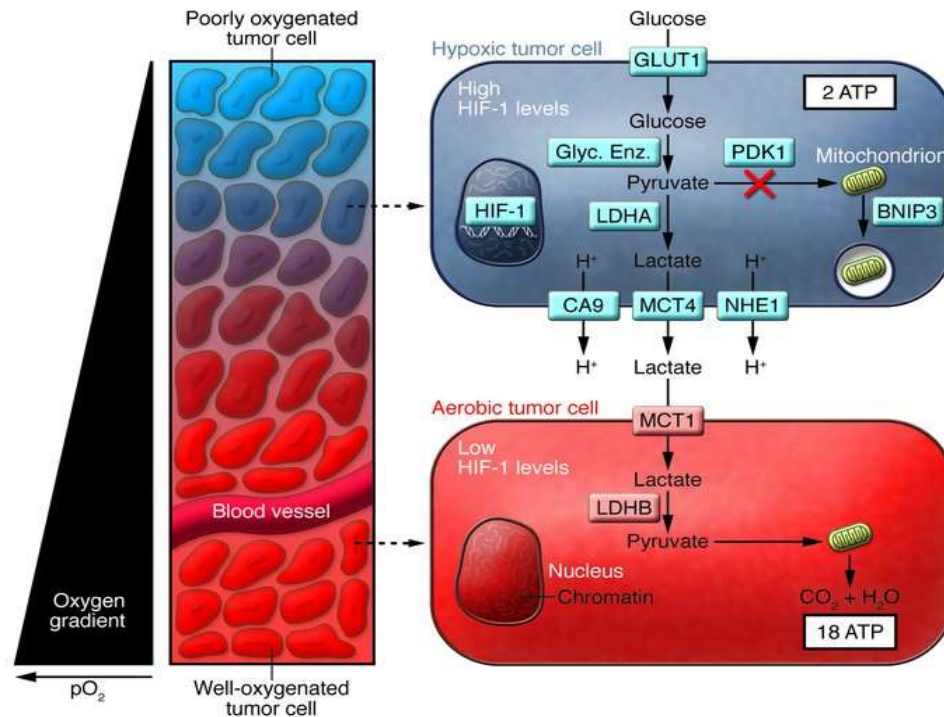


Fig. 30 Model of metabolic symbiosis between hypoxic and respiring cells.

In addition, recent studies suggest that tumour microenvironment, primarily cancer-associated fibroblast (CAFs), has a role in cancer metabolism. These observations lead to models according to which CAFs fuel neoplastic cells with aminoacids and nucleotides through autophagy, thereby promoting cancer progression and metastatization (Pavrides et al. 2010). In particular, several studies have focused on expression analysis of protein caveolin-1 1 (Cav-1) on CAFs. Cav-1 belongs to the caveolins transmembrane proteins family, main components of membrane microdomains called caveolae and involved in mechanisms of endocytosis receptor-independent. Cav-1 is mainly expressed on endothelial cells, adipose and stromal cells. In aprticular, Lisanti and colleagues have demonstrated that low Cav-1 expression on CAFs extracellular membrane is correlated to a highly proliferative and metastatic phenotype in a model of breast cancer (Migneco et al. 2010). Comparing this model to the relation between hypoxic and normoxic cancer cells, supported by an O_2 perfusion gradient inside the tumour, the metabolic relation between cancer cells and CAFs is mainly driven by a condition of high oxidative stress. According to that, correlation between oxidative stress and Cav-1 loss shows a biunivocal feature: Cav-1 degradation induces an increase in ROS accumulation and, in turn, oxidative stress induced by

neighbouring cancer cells further promotes Cav-1 degradation. Cav-1 has a role as negative regulator NO production through inhibition of NO syntase. Loss of Cav-1 thereby promotes increase in NO production, which can inhibit mitochondrial functions acting as an inhibitor of ferrous protein of the electron chain transport. This inhibition of oxidative phosphorylation NO-mediated induces higher mitochondrial ROS production in addition, studies on coc-culture models between fibroblasts and MCF-7 breast cancer cells showed that oxidative stress cancer cells-induced leads to Cav-1 digestion by fibroblasts and this effect is highly reduced after administration of antioxidant molecules like N-acetyl cystein (NAC) (Bonuccelli et al. 2010). Other studies hypothesized that ROS act on PHDs, inactivating them and promoting HIF-1 α stabilization.

It has been clearly demonstrated that phosphorylation or other post-traditional modifications ROS-dependent on PHDs induce modifications on their catalytic activity (Klimova et al. 2008). Subsequently fibroblasts exposed to oxidative stress undergo a metabolic shift towards glycolysis ROS and HIF-1 α dependent and produce and extrude high amounts of lactate through MCT-4. Excreted lactate is then taken up by cancer cells through MCT-1 in order to enter the Krebs cycle. This new metabolic model is also named “reverse Warburg effect” (Martinez-Outschoorn et al. 2010).

This metabolic model has been recently confirmed also in a prostatic carcinoma model. Studies conducted by our research group have demonstrated that, analyzing expression patterns of metabolic genes from both fibroblasts from benign hyperplasias and CAFs, the latter are characterized by a metabolic reprogramming to a Warburg phenotype and by a condition of mitochondrial oxidative stress. This event is caused by interaction with prostatic cancer cells that promotes fibroblasts activation, GLUT-1 expression, lactate production and extrusion after *de novo* expression of MCT-4. These metabolic modifications are HIF-1 α -dependent, expressed in normoxic condition following reduction of SIRT-3 expression. SIRT-3 is a NAD-dependent protein that, buffering excess of ROS, promotes PHDs activity and HIF1 α downregulation. Subsequently, lack of this protein expression leads to accumulation of ROS, impaired PHDs activity and HIF-1 α increased expression.

At the same time, cancer cells respond to CAFs contact modifying their metabolism to a respiring metabolism, reducing GLUT-1 expression and thereby increasing lactate intake through *de novo* expression of MCT-1. Thus cancer cells progressively become independent from glucose consumption, thereby developing high dependence from

extracellular lactate intake in order to promote anabolic reactions. According to these observations, MCT-1 pharmacological inhibition with 4-cyanohydroxycinnamide (CHC), blocking lactate influx, strongly reduces cancer cells survival and growth. In conclusion, taking advantage from modifying the surrounding microenvironment, cancer cells create a strong symbiotic interrelationship with CAFs, thereby surviving in presence of low glucose availability (Fiaschi et al. 2012) (fig. 31).

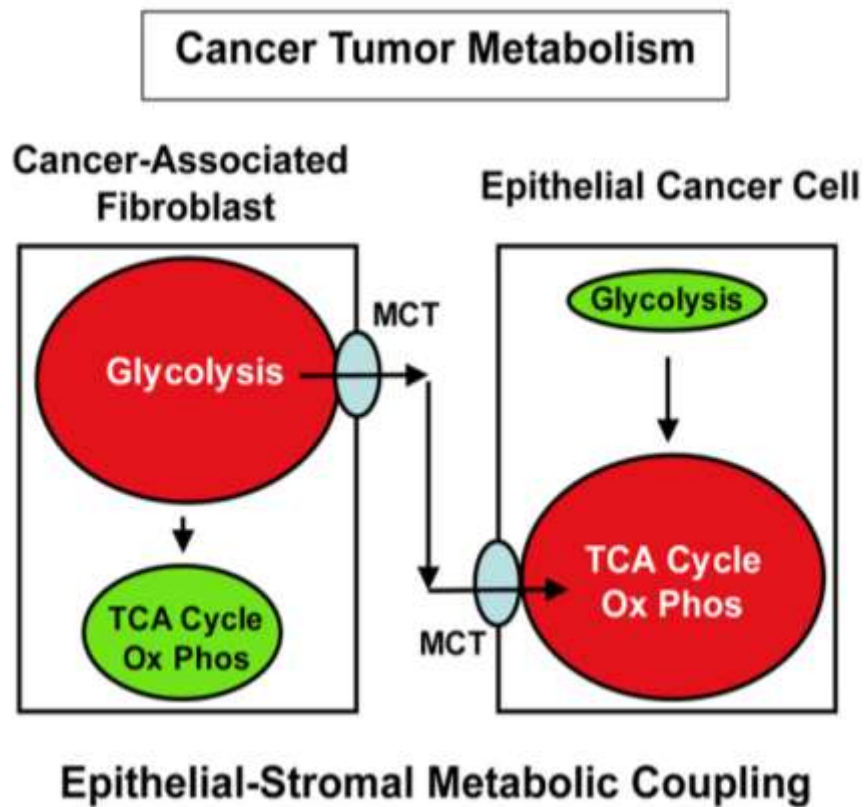


Fig. 31 Model of metabolic symbiosis between cancer cells and cancer-associated fibroblasts (CAFs).

MATERIALS AND METHODS

MATERIALS

- Unless specified, all reagents used for cell cultures were purchased from Euroclone Group, except DMEM 1x (no glucose) from Invitrogen, pyruvate and lactate from Sigma-Aldrich.
- Noradrenaline, adrenaline and propranolol were from Sigma-Aldrich.
- Cytokines used for cell stimulation were purchased from Peprotech.
- The metalloproteinase pharmacological inhibitor Ilomastat was purchased from Chemicon International.
- The HIF-1 α pharmacological inhibitor Topotecan was purchased from Sigma-Aldrich.
- Transwells for invasion assays were from Euroclone Group.
- The Diff-Quik staining kit was purchased from BIOMAP SNC.
- Matrigel was purchased from BD Biosciences.
- Proteases and phosphatases inhibitors were from Sigma-Aldrich.
- Bradford reagent for protein dosage and all materials for SDS-PAGE were from Biorad.
- PVDF membrane (Polyvinylidene fluoride) used for western blotting was from Millipore.
- All the antibodies used were purchased from Santa Cruz Biotechnology except antibodies against α -SMA (Sigma-Aldrich), HIF-1 α (BD Biosciences), p38/phospho-p38 and p42-p44 MAPK/phospho-p42-p44 MAPK (Cell Signaling).

- The secondary antibodies enzyme horseradish peroxidase –conjugated (HRP) were from GE Health Care.
- Chemiluminescence revelation kit is from GE Health Care.
- The photographic plates were from Kodak.
- The metalloproteinase catalytic activity evaluation kit Amplite™ Universal Fluorimetric MMP Activity Assay Kit - Red Fluorescence was purchased from AAT Bioquest.
- The cytofluorimetric apoptosis staining kit Annexin V-IP Fluos Staining Kit was from Roche.

Common use solutions

- SDS–PAGE 1X Sample Buffer. For 100 ml: 0.01 g of Bromophenol Blue, 1.52 g Tris Base, 20 ml glycerol, 2 g di SDS, 20 ml di 2-mercaptoethanol.
- SDS-PAGE 1X running buffer. For 1 litre: 25 mM Tris, 192 mM glycin, 0.1% (W/V) SDS, pH 8.3.
- SDS-PAGE 1X blotting buffer. For 1 litre: 25 mM Tris, 192 mM glycin, pH 8.3, 10% methanol.
- RIPA lysis buffer (50 mM Tris.HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1mM sodium ortovanadate, 100 mM NaF).
- Washing solution: tween 0.1 % in PBS.
- Blocking solution: non-fat dry milk 2 %, tween 0.05 % in PBS.
- Stripping solution: 62.5 mM Tris Hcl pH 6.8, 2 % SDS, 100 mM β-mercaptoethanol.
- PBS (Phosphate buffered saline). For 1 litre: 0.2 g di KH₂PO₄, 0.2 g KCl, 0.8 g NaCl , NaH₂PO₄ pH 7.4.

METHODS

Cell cultures and treatments

Human dermal fibroblasts (HDFs), human A375 cells isolated from amelanocytic melanoma and human Hs29-4T cells isolated from human melanoma lymphonodal metastasis were all cultivated in DMEM high glucose (4,5 g/L) (Dulbecco's Modified Essential Medium), supplemented with 10% bovine fetal serum (FCS), glutamine 2 mM, penicillin (100 U/ml) and streptomycin (100 µg/ml), at 37°C in a 5% CO₂ humidified atmosphere. Cells are usually stored in liquid nitrogen in a freezing solution containing 90% FCS and 10% dimethyl sulfoxide (DMSO) and then plated in Petri capsules.

All experiments were performed with 70%-80% confluent cultures, following 18-h incubation in serum-free culture medium. Cells were then stimulated with NE or E, at 1µM concentration. Where needed, cells were pre-treated with unselective β-AR antagonist propranolol (Sigma-Aldrich) (1 µM). After 1h, medium was removed and cells were stimulated with NE 1 µM with or without propranolol 1 µM.

For metabolism experiments, 70%-80% confluent A375 cells were incubated in normoxic condition for 18 h in serum-free and nutrient-free culture medium DMEM 1X w/o glucose/pyruvate/HEPES. Cells were then cultured for 24 h in presence of glucose (4,5 g/L), pyruvate (10 mM) and lactate (10 mM), in presence or absence of serum.

Isolation of human dermal fibroblasts

Human dermal fibroblasts (HDFs) were isolated from a surgical explantation taken from healthy patients. A small slice of the tissue piece was minced with sterile scalpels and pieces of <1 mm in size were plated in Petri plates and covered with covering glasses, favouring pieces compression and fragmentation. After having been digested overnight in 1 mg/mL collagenase I at 37 C°, fragments were spun down, re-suspended and plated in complete DMEM supplemented with 20% FCS, glutamine 2 mM, penicillin (100 U/ml) and streptomycin (100 µg/ml), Kanamicyn and AMFO (Amphotericyn B) 100 mg/lt.

After 3-4 days culture medium was removed and fresh medium was then added. Days required for epithelial and fibroblast cells exit from bioptic fragments is highly variable

and depends on the type of bioptic tissue and the number of fibroblasts composing it. Usually it takes 20-30 days and, after this period, tissutal fragments were removed with steril tweezers and trypsinyzed, thereby promoting fibroblasts isolation as epithelial cells are not able to re-adhere to plate surface. Obtained fibroblasts were then maintained in culture with complete DMEM supplemented with 10% FCS, glutamine 2 mM, penicillin (100 U/ml) and streptomycin (100 µg/ml). We also excluded contamination by skin stem cells of HDFs by evaluation of CD34 and cytokeratin-15, acknowledged as skin stem cell markers.

Preparation of conditioned media

For preparation of conditioned media, A375 cells were grown to 70% confluence in complete medium for 24 h. Then medium was removed and, after washing in PBS solution, cells were serum starved overnight in order to promote cells entry into a quiescent G0 phase, thereby better evaluating cells responsiveness to exogenous treatments. After this period cells were treated for 24 h with the indicated cytokines: TGF-β 10 ng/ml, NE 1 µM, VEGF (20 µg/ml) and IL-6 (50 µg/ml) added to serum-free culture medium.

Fresh serum-free medium was added for an additional 24 h before collection of conditioned medium (CM), in order to obtain CM free from cytokines (but conditioned by their earlier administration). CM were then harvested, clarified by centrifugation, and used freshly on HDFs cells that were then incubated with the obtained CM for 24 h and used for Western blot analyses.

Cell lysis and protein quantification

In order to identify proteins of interest, cell lysates were then separated through SDS-PAGE (polyacrylamide gel electrophoresis) and revealed with Western Blotting analysis.

After stimulations, cells were washed once with PBS solution and then lysated with RIPA lysis buffer. Obtained protein lysates were collected, always kept in ice and centrifugated at 13000 rpm for 15 minutes. After centrifugation, supernatant was collected and total proteins were quantified with Bradford assay.

Total protein quantification, expressed in $\mu\text{g/ml}$, is evaluated with Coomassie Brilliant Blue, which binds to basic and aromatic aminoacidic residues (specially arginins), leading to maximum absorption at 595 nm wavelength. Thus, Coomassie Brilliant Blue intensity is positively correlated to protein concentration.

For the standard curve bovine serum albumine was used (BSA), diluting BSA 2 mg/ml concentrated in deionized water and then obtaining rising BSA concentrations from 2 $\mu\text{g/ml}$ to 15 $\mu\text{g/ml}$.

Then Bradford reagent is prepared diluting 1/5 of starting solution with Coomassie Brilliant Blue in 4/5 of deionized water.

To run the assay, 50 μl of each sample, opportunely diluted in water in labelled eppendorfs, must be added to 950 μl of the working solution for each sample, resuspending well. After a 5 minutes incubation, the absorbance of each sample is evaluated at a wavelength of 595 nm, subtracting the blank value. From values obtained from the standard curve it is possible to create a curve of absorbance in function of its concentration, thus, interpolating absorbances values to the standard curve, it is possible to calculate final protein concentration.

Correlation between absorbance and concentration is expressed by Lambert-Beer law: $A = \epsilon dc$, where ϵ represents the extinction coefficient, d the path length and c represents sample concentration.

For each Western Blotting experiment usually 20-25 μg of total proteins are loaded on SDS-PAGE for each sample.

SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. This is achieved by adding SDS detergent to remove secondary and tertiary protein structures and to maintain the proteins as polypeptide chains. The anionic SDS coats the proteins (almost one SDS molecule binds every two aminoacidic residues of the polipeptidic chain), mostly proportional to their molecular weight, and confers the same negative electrical charge across all proteins in the sample.

SDS-PAGE samples are boiled for 5 minutes in a sample buffer containing SDS and β -mercaptoethanol, which leads to disulfuric bonds reduction and destabilization of

eventual protein tertiary structure. In addition, sample buffer is supplemented with bromophenol blue, ionizing coloured-tracking solution for the electrophoretic run, and glycerol, which increases sample density and promotes its stratification at the bottom of the loading well.

Once finished samples loading in the stacking gel, an electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive electrode (anode). Stacking gel, characterized by very low acrilamide concentration (4%), is required to better stratificate the samples before entering the separating gel. According to that, different ionic force and pH of running buffer and stacking gel are required: this technique is called isotachopheresis. In isotachopheresis the sample is introduced between a fast leading electrolyte and a slow terminating electrolyte. After application of an electric potential a low electrical field is created in the leading electrolyte and a high electrical field in the terminating electrolyte. The pH at sample level is determined by the counter-ion of the leading electrolyte that migrates in the opposite direction. In the first stage the sample constituents migrate at different speeds and start to separate from each other. The faster constituents will create a lower electrical field in the leading part of the sample zone and viceversa. Finally the constituents will completely separate from each other and concentrate at an equilibrium concentration, surrounded by sharp electrical field differences. Specific spacer or marker molecules are added to the sample to physically separate the sample constituents one is interested in.

Separation of SDS-proteins complexes is achieved according to separating gel acrylamide concentration. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins, while bromophenol blue is a very small molecule which is not affected by frictional forces, thereby representing the migration front. Proteins relative molecular mass is evaluated by comparison with protein ladder standard molecular weights, separated in the same gel. Running is carried on at 200 Volts for almost 1 h.

Western Blotting

After run, polyacrylamide gel is maintained for 5 minutes at room temperature in slow agitation in the transfer blot. In order to make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of polyvinylidene

difluoride (PVDF). The method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. Proteins transfer is carried out at 100 Volts for 1 h. PVDF membrane must be previously activated through treatment with methanol for 15 seconds and left at drying for at least 15 minutes at room temperature.

After electroblotting the PVDF membrane is incubated 1 h in slow agitation at room temperature with specific primary antibodies in a blocking solution containing non-fat dry milk 2% and Tween 0.05%. After incubation, the membrane is washed three times with a washing solution containing PBS 1X and Tween 0.1% and, in order to reveal the specific protein, the membrane is incubated with horseradish peroxidase conjugated secondary antibody for 30 minutes and then washed again for three times. In the chemiluminescence reaction horseradish peroxidase catalyzes the oxidation of luminol into a reagent which emits light when it decays. Since the oxidation of luminol is catalyzed by horseradish peroxidase, and the HRP is complexed with the protein of interest on the membrane, the amount and location of light that HRP catalyzes the emission of, is directly correlated with the location and amount of protein on the membrane. Chemiluminescent protein revelation is carried out with ECL-Amersham Pharmacia kit reagents and developing of blots is carried out in the developing room placing imaging films on top of the membrane. Exposure is repeated, varying the time as needed for optimal detection.

Crystal violet proliferation assay

Crystal violet (CV) is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) also known as Gentian violet (or hexamethyl pararosaniline chloride). The crystal violet assay is useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes thanks to its ability to bind to cells DNA.

For our experiments 2×10^4 cells have been plated in 24w multiwells and stimulated as previously described. Then cells have been washed with PBS and incubate with crystal violet solution for 5 minutes at 37 C°. The crystal violet solution contains 0,5% crystal violet in deionized water and methanol 20%. After incubation crystal violet was removed thorough aspiration and three washings in PBS solution.

Finally, crystal violet uptaken by cells was solubilized after incubation in slow agitation for 1 h at 37 C° with a solution containing sodium citrate 0,1 M, pH 4,2. After incubation, solution containing solubilized crystal violet was collected and its absorbance was evaluated at a 595 nm wavelenght. Each measurement was made in triplicate for each point of the curve of growth. Absorbance is positively correlated to crystal violet amount bound to cells DNA content.

Annexin V/Iodidium Propide cytofluorimetric staining

20×10^4 cells were plated in 60 mm Petri plates (p60) and treated with the different nutrients as previously described.

Cells were then washed in PBS solution and detached from plates with Accutase solution. The advantages of Accutase over the traditional Trypsin/EDTA treatment are that it is less damaging to cells. In this case cells treatment with Accutase carries lower risk to disrupt Annexin V antigens, expressed on the outer side of cell membranes during apoptotic events.

After cells removal from the plastes, they have been resuspended in PBS solution and centrifugated at 1000 rpm for 3 minutes. Finally, pelleted cells were resuspended in 100 µl containing 1 µl of annexin V, 1 µl of iodidium propide and 98 µl of buffer solution, all provided by the kit. After 15 minutes of incubation at room temperature, cells were evaluated by flow citometry for Annexin V/iodidium propide staining.

Intracellular ROS evaluation

For the evaluation of intracellular ROS amount, staining with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe was performed. H₂DCFDA is a chloromethyl derivative of H₂DCFDA. H₂DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. H₂DCF-DA is solubilized in DMSO at moment of usage and avoided from light exposure.

For our experiments, $1,5 \times 10^4$ cells were plated on 6 cm plates and stimulated as previously described, then stimulation culture medium was removed and fresh serum-free DMEM was added. Then H₂DCF-DA (5 µg/ml) probe was added and incubated for 3 minutes at 37°C. After the incubation time, cells are blocked through PBS washing,

quickly lysated in RIPA lysis buffer supplemented with proteases inhibitors and kept for 1 minute at 4°C. Lysates were then collected and centrifugated for 1 minute at 13000 rpm. For each sample 100 µl were transferred on a 96 wells multiwell and analyzed and fluorimetric quantification was performed at an excitation wavelength of 488 nm, while the emission wavelegth read was set at 510 nm. Obtained values were finally normalized on protein content of each sample.

Cell transfection with lipofectamine

Cells plated at a 90% confluence were transiently transfected using lipofectamine. Before transfection, DMEM cultur medium is removed and replaced with Optimem medium, lacking serum and antibiotics that could interfere with liposomes formation. Acoording to Petri plates diameter (60 or 100 mm), 4 µg or 12 µg of siRNA are diluted in 0.5 ml or 1.5 ml of Optimem medium and 10 µg or 30 µg of lipofectamine in 0.5 ml or in 1.5 ml of Optimem medium. SiRNAs are used at a final concentration of 200 nM for 10 µg di lipofectamine. After a 5 minutes incubation, solution containing siRNA is added to solution with lipofectamine and incubated at room temperature for 20 minutes, in order to promote liposomes formation, then equal amounts of final solution are added to each plate. Optimem medium was removed after 4-6 h from transfection, as lipofectamine could be slightly toxic for cells. Finally, cells were maintained in complete medium for 48 h and transfection efficiency was evaluated through immunoblotting assays using specific antibodies for the silenced protein.

Invasion assay

Transwell system, equipped with 8-µm pore polyvinylpirrolidone-free polycarbonate filters, was used. Cells (2×10^4 in 200 µl) stimulated as previously described were loaded into the upper compartment in serum-free growth medium, with or without 50 µmol/L ilomastat. The upper sides of the porous polycarbonate filters were coated with 50 µg/cm² of reconstituted Matrigel basement membrane in order to mimic an ECM and placed into six-well culture dishes containing 1 ml of complete growth medium. After 24 h of incubation at 37°C, noninvading cells and the Matrigel layer were mechanically removed using cotton swabs, and the microporous membrane was fixed in 96% methanol and stained with Diff-Quick solution. Chemotaxis was evaluated by counting

the cells that migrated to the lower surface of the polycarbonate filters (six randomly chosen fields, mean \pm SD).

Real-Time PCR

Total RNA was extracted from Hs29-4T and A375 derived from our experimental conditions using the RNeasy Minikit kit. Total RNA (1 μ g) was reverse-transcribed using the Quantitect Reverse Transcription Kit. Reverse transcription was performed in a final volume of 20 μ l containing reverse transcriptase, real-time buffer 1x and real-time primer mix. The amplification was carried out at 42°C for 2 minutes, then 42 C° for 15 minutes and 95°C for 3 minutes. Measurement of gene expression was performed by quantitative real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems), using the Qiagen Quantifast SYBR Green PCR kit. For each sample, 1 μ g of cDNA was added to 25 μ l of PCR mix. The samples were then subjected to 40 cycles of amplification at 95°C for 10 s and 60°C for 30 s. RNeasy Minikit, Quantitect Reverse Transcription Kit, all primer/probe mixes, and Qiagen Quantifast SYBR Green PCR Kit were from Qiagen, except the following primers:

ADRB1 FW: 5'-CAGGTGAACTCGAAGCCCAC-3'

ADRB1 REV: 5'-CTCCCATCCCTTCCCAAAC-3'

ADRB2 FW: 5'-ACGCAGTGCGCTCACCTGCCAGACT-3'

ADRB2 REV: 5'-GCTCGAACTTGGCAATGGCTGTGA-3'

VEGF FW: 5'-TACCTCCACCATGCCAAGTG-3'

VEGF REV: 5' ATGATTCTCCCTCCTCCTTC-3'

IL-8 FW: 5'-CTGGCCGTGGCTCTCTTG-3'

IL-8 REV: 5'-TTAGCACTCCTTGGCAAAC-3'

MMP-2 FW: 5'-ACGACCGCGACAAGAAGTTAT-3'

MMP-2 REV: 5'-ATTTGTTGCCAGGAAAGTG-5'

MMP-9 FW: 5'-GACAAGCTCTTCGGCTTCTG-3'

MMP-9 REV: 5'-TCGCTGGTACAGGTCGAGTA-5'

IL-6 FW: 5'-AGTTCCTGCAGTCCAGCC-3'

IL-6 REV: 5'-TCAAACCTGCATAGCCACTTTC-3'

Quantitative MMPs activity assay

Matrix metalloproteinases (MMPs) activity was measured with Amplite™ Universal Fluorimetric MMP Activity Assay Kit according to the manufacturer's instructions. Briefly, serum-free medium from confluent monolayer of cells was collected and 5 µl were added to 4-aminophenylmercuric acetate (AMPA; 1 nmol/L) at 37 °C for 1 hour to detect MMP-2 activity and at 37 °C for 2 hours to detect MMP-9 activity. A 50 µl portion of the mixture was then added to 50 µl of MMP Red substrate solution. After 60 minutes of incubation the signal was read by fluorescence microplate reader with excitation (Ex)/ emission (Em) = 540 nm/590 nm.

Statistical Analysis

In vitro data are presented as means ± SD from at least three experiments. Results were normalized versus control expression levels. Statistical analysis of the data were performed by Student's t test. $p \leq 0.05$ was considered statistically significant.

EXPERIMENTAL

PART I

AIM OF THE STUDY I

Stress is commonly defined as the general reaction of the organism in order to respond to endogenous and exogenous stimuli that can affect the homeostasis of the organism itself. The emotional arousal induced by the stimulus is processed in the CNS and occurs both at a biological/somatic level, with autonomic and endocrine changes, and at a psychological/behavioral level with sequences of motor behavior collectively known as the "flight or fight response". This response is usually time-limited and is progressively reduced when the stressful event is no longer present, but when stressors keep sustaining secretion of stress mediators, we have a condition of chronic stress-related disorder that constantly affects homeostatic mechanisms.

In the light of these observations, several epidemiological and clinical studies over the years have demonstrated the close interrelationship between chronic stress, depression and social isolation and cancer initiation and progression in several tumour models, pointing out the role exerted by CA through adrenergic receptors activation in almost all phases of cancer progression.

In particular, studies sight β -adrenergic receptor (AR) antagonists as novel therapeutic agents for melanoma, as they may reduce disease progression. Here within, preliminary studies have evaluated the expression of β -ARs in a series of human cutaneous melanocytic lesions, and we therefore studied the effect of their endogenous agonists, norepinephrine (NE) and epinephrine (E), on primary and metastatic human melanoma cell lines. Using immunohistochemistry, it has been demonstrated that both β_1 - and β_2 -ARs are expressed in tissues from benign melanocytic naevi, atypical naevi and malignant melanomas and that expression was significantly higher in malignant tumours.

Melanoma cell lines (human A375 primary melanoma cell line and human Hs29-4T metastatic melanoma cell lines) also expressed β_1 - and β_2 -ARs by measuring transcripts and proteins. NE or E increased metalloprotease-dependent motility, released interleukin-6 and 8 (IL-6, IL-8) and vascular endothelial growth factor (VEGF). These effects of catecholamines were inhibited by the unselective β -AR antagonist propranolol. The role of soluble factors elicited by catecholamines seemed pleiotropic as VEGF synergized with NE increased melanoma invasiveness through 3D barriers, while IL-6 participated in stromal fibroblast activation towards a myofibroblastic phenotype. Our results indicate that NE and E produce *in vitro via* β -ARs activation a

number of biological responses that may exert a pro-tumourigenic effect in melanoma cell lines. The observation that β -ARs are up-regulated in malignant melanoma tissues support the hypothesis that circulating catecholamines NE and E, by activating their receptors, favour melanoma progression *in vivo*.

RESULTS I

Preliminary results: expression of β -ARs in tissue sample

Melanoma represents the most aggressive type of skin cancer, with an increasing incidence found especially in young adults. Surgery continues to provide a cure for localized and regional disease. Despite increasing improvement in early diagnosis, a significant reduction in mortality has not been observed yet, as metastatic melanoma is still characterized by high resistance to drug therapies and radiation (MacKie et al., 2009; Rughani et al., 2013). Therefore, at present there is no therapeutic approach for the complete cure of metastatic melanoma and the only effective treatment for the eradication of the disease is represented by early phase surgery (Atallah et al., 2005). Hence, in the last decade several studies have contributed to a better understanding of the biological pathways underlying the process of melanoma dissemination and metastasis in order to identify new therapeutic targets.

There is growing evidence that some stress neurotransmitters, such as norepinephrine (NE) and epinephrine (E), directly contribute to promote tumour cell growth and invasion, at least in part through β -AR activation, thereby suggesting an important neuro-oncological link in tumour progression (Sood et al., 2006; Yang et al., 2006; Shang et al., 2009). This interrelationship among psychosocial stress, tumor growth and β -adrenergic activation has also been confirmed *in vivo* in mice under controlled stress conditions (Hasegawa and Saiki, 2002). Moreover, it has been shown that various human solid tumours, such as breast, colon, prostatic, ovary, nasopharyngeal and oral cancer, express β_2 -adrenoceptor (β_2 -AR), raising the possibility that such receptors may affect proliferation, invasion and dissemination processes (Sood et al., 2006; Palm et al., 2004; Drell et al., 2003). Interactions between tumour cells and soluble factors originated from the nervous system has recently been proposed to favour metastasis formation (Voss et al., 2010). Improved survival rates have been demonstrated in mice with metastatic tumour by combined administration of β -AR antagonists (Glasner et al., 2010). In addition, recent evidence suggests a dramatic role of β -AR blockers in reducing metastases, tumour recurrence and specific mortality in breast cancer patients (Powe et al., 2011). More recently, the use of β -blockers for concomitant disease was associated with a reduced risk of progression of thick melanoma and with an increased survival time of melanoma patients, suggesting that the interaction of catecholamines with β -ARs could be a useful target in this disease (De Giorgi et al., 2011; Lemeshow et

al., 2011). However, no detailed information regarding β -ARs expression in human cutaneous benign and malignant melanocytic lesions or catecholamine influence on melanoma cell migration has been provided so far.

According to these observation, studies conducted in collaboration between the Section of Clinical, Preventive and Oncologic Dermatology and the Division of Pathological Anatomy in the Department of Critical Care Medicine and Surgery of Florence by prof. Silvia Moretti and prof. Daniela Massi evaluated the expression levels of both β_1 and β_2 -AR in order to point out a possible correlation between adrenergic receptors expression and melanoma progression. To this aim, the study series included 5 common melanocytic naevi (CN) (2 females, 3 males, age 28-54 yrs, mean 35.8 yrs); 5 atypical (so-called dysplastic) melanocytic naevi (AN) (2 females, 3 males, age 30-47 yrs, mean 40.4 yrs); 5 *in situ* primary melanoma (PM) (2 females, 3 males, age 37-55 yrs, mean 44.2 yrs; site: 3 trunk, 1 lower extremity); 9 superficial spreading (SS) PM (7 females, 2 males, age 41-82 yrs, mean 58.4 yrs; site: 4 trunk, 3 leg, 2 arm; thickness 0.30-1.90 mm, mean 0.82 mm; 5 level II, 3 level III); 6 nodular (N) PM (3 females, 3 males, age 53-76 yrs, mean 61.5 yrs; site: 3 trunk, 1 leg, 2 arm; thickness 1.40-17 mm, mean 5.2 mm; 2 level III, 3 level IV, 1 level V), 10 metastatic melanoma (MM), 5 cutaneous and 5 lymph-nodal (1 female, 9 males, age 59-87 yrs, mean 77.1 yrs).

The presence of β -ARs was demonstrated in all melanocytic lesions examined. The specimens were obtained by surgical resection in all cases and fixed in 10% formalin before being processed in paraffin. Haematoxylin-eosin stained sections from each histological specimen were reviewed to confirm the histological diagnosis. The percentage of positive cells per lesion was scored according to semi-quantitative criteria. Since the percentage of positive naevus melanocytes/melanoma cells was always higher than 50%, semi-quantitative results were expressed as score 1 (50-80% positive naevus melanocytes/ melanoma cells), score 2 (81-90% naevus melanocytes/melanoma cell staining), and score 3 (91-100% melanoma cell staining). The cell staining intensity was scored on a scale as weak, moderate, strong, very strong. In particular, the staining for β_1 -AR was confined to the cell cytoplasm in naevus melanocytes and melanoma cells; the reaction for β_2 -AR was also confined to the cell cytoplasm in all cases, with an additional peripheral membrane pattern in some AN melanocytes and malignant cells. The cell staining intensity was always weak with regard to the reaction for β_1 -AR, whereas the immunostaining for β_2 -AR appeared to be weak in CN, moderate (except one very strong reaction), in AN; moderate or strong in

in situ PM; from weak to moderate or strong in SSPM, and from strong to very strong in NPM and MM, with no difference between cutaneous and nodal metastasis.

In regards to β_1 -AR expression, score 1 was evaluated in both CN and AN, score 2 was found in a minority (3 *in situ* and 2 SS) of PM, whereas in the other PM and MM score 3 was detected. β_1 -AR expression was significantly higher in malignant than in benign lesions ($p \leq 0.0001$) and in PM or MM than in naevi ($p \leq 0.0001$ and $p \leq 0.0001$). No difference was observed between CN and AN, or between *in situ*/SSPM compared to NPM/MM.

With regards to β_2 -AR expression, score 1 was observed in CN, score 2 in AN and score 3 was detected in all PM and MM but one (SSPM), which exhibited score 2. β_2 -AR reactivity was significantly higher in malignant lesions than in naevi ($p \leq 0.0001$), and in PM or MM respectively, than in naevi ($p \leq 0.0001$ and $p \leq 0.0001$). AN exhibited a significantly higher reactivity compared to CN ($p \leq 0.003$), and no difference was observed between *in situ*/SSPM and NPM/MM. In addition, no significant difference was detected between PM and MM for both receptors.

Epidermal keratinocytes were lightly coloured for β_2 -AR. In fact, it has been previously described that human keratinocytes primarily express β_2 -AR (Sivamani et al., 2007). Endothelial and stromal cells exhibited heavy reactivity for β_2 -AR in malignant lesions, and to some extent, in AN.

Taken together, these preliminary data showed that β_1 - and β_2 -ARs were variably expressed in human melanocytic lesions with a significant up-regulation in PM and MM, and, at least for β_2 -AR, a significant up-regulation was also observed in AN versus CN.

β -AR expression analysis and responsiveness of melanoma cell lines after catecholamines stimulation

In order to confirm the correlation between sensitivity to catecholamines and progression towards a malignant phenotype of melanoma cells hypothesized after the *in vivo* preliminary analysis, we performed our *in vitro* experiments on two different melanoma cell lines, namely Hs29-4T cells, selected from a lymph nodal metastatic lesion, and A375 cells, derived from human primary melanoma.

First we evaluated expression levels of both β_1 and β_2 -AR on both cell lines, performing Western Blot analysis and evaluation of mRNAs through Real Time PCR analysis. We observed that both cell lines express low and comparable levels of β_1 -AR, as shown in Figure 1, while they both express higher amounts of β_2 -AR (Figure 2A and 2B), with the primary A375 melanoma cell line exhibiting a significantly higher expression of β_2 -AR compared to the metastatic Hs29-4T cell line.

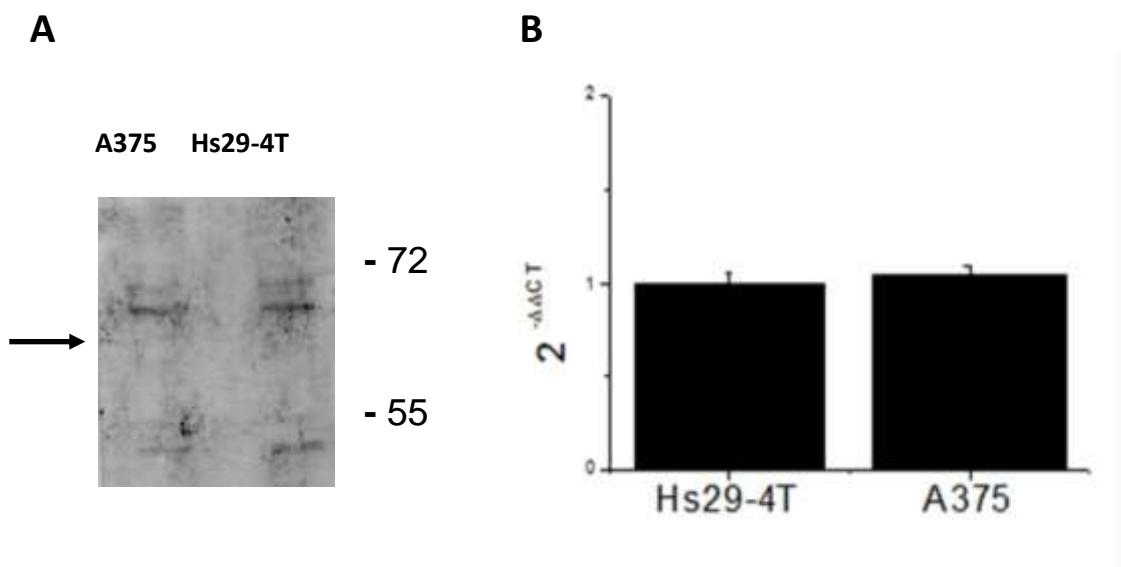


Fig. 1. Expression of β_1 -AR in melanoma cell lines. (A) Analysis of β_1 -AR expression by immunoblot in primary (A375) and metastatic (Hs29-4T) melanoma cell lines. (B) Amount of ADRB1 mRNA by real-time PCR. The amount of target, normalized to the endogenous reference (18S RNA), was given by the $2^{-\Delta\Delta ct}$ calculation and was reported as $2^{-\Delta\Delta ct}$. Both immunoblots and real-time PCR are the mean of three independent assays.

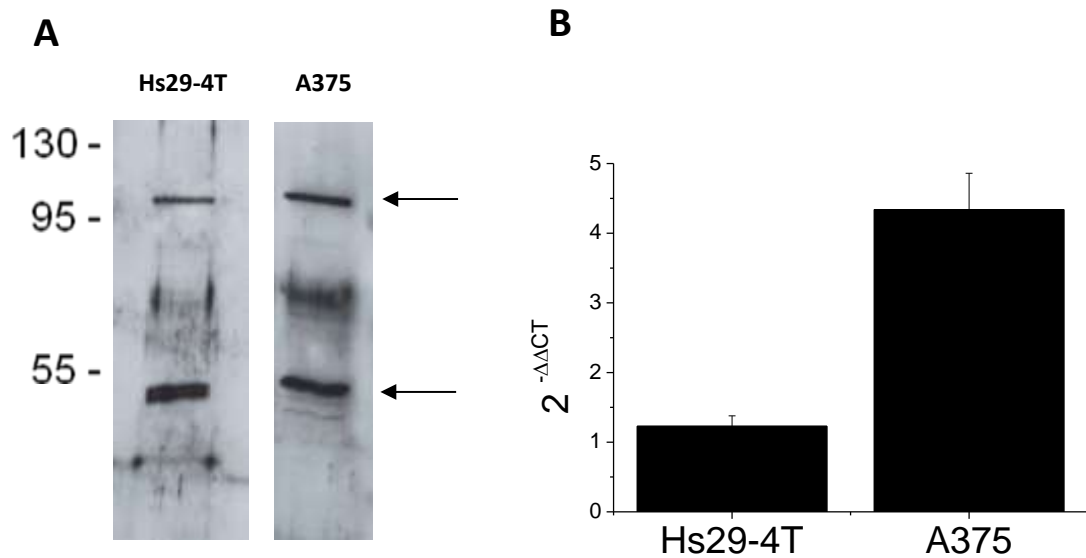


Fig. 2. Expression of β_2 -AR in melanoma cell lines. (A) Analysis of β_2 -AR expression by immunoblot in primary (A375) and metastatic (Hs29-4T) melanoma cell lines. (B) Amount of β_2 -AR mRNA by real-time PCR. The amount of target was given by the $2^{-\Delta\Delta Ct}$ calculation and was reported as $2^{-\Delta\Delta Ct}$. Immunoblot and Real-Time PCR are the mean of three different experiments.

The second messenger cyclic AMP (cAMP) mediates several cellular responses to external signals such as proliferation, ion transport, regulation of metabolism and gene transcription by activation of the cAMP-dependent protein kinase (cAPK or PKA). Activation of PKA occurs when two molecules of cAMP binds to the two regulatory subunits of the tetrameric PKA holoenzyme, leading to release of active catalytic subunits. Activation of transcription upon elevation of cAMP levels results from translocation of PKA to the nucleus where it phosphorylates serine or threonine residues on target proteins that bear PKA responsive motif R-R-X-(S/T)-Y like the transcription factor CREB, which in turn leads to TFIIB binding to TATA-box-binding protein TBP1, thus linking phosphorylated CREB to the Pol II transcription initiation complex (Taylor et al., 1990).

As already known, NE secretion leads β adrenergic receptors stimulation, resulting in $G_{\alpha s}$ -mediated activation of adenylyl cyclase and subsequent conversion of ATP into cAMP. Transient flux of intracellular cAMP activates PKA to phosphorylate multiple target proteins, including transcription factors of the CREB/ATF and GATA families, as well as β adrenergic receptor kinase (BARK). BARK recruitment of β -arrestin inhibits β adrenergic receptor signaling and activates Src kinase, resulting in activation of transcription factors such as STAT3 and metastasis-associated genes involved in

inflammation, angiogenesis, tissue invasion, and epithelial–mesenchymal transition (EMT), and in downregulation of genes promoting antitumour immune responses (Cole and Sood, 2012).

According to these observation, we performed a Western Blot analysis of activated PKA level in order to assess melanoma cell lines responsiveness to NE stimulation. The doses of NE and E used for our study were selected to reflect the physiological conditions of this hormone at the level of the tumour. Although circulating plasma levels of NE are only 10 to 1000 pM in a normal individual, they may reach as high as 100 nM in conditions of stress (Schmidt et al., 1996). Studies suggest that within the tumour microenvironment, concentrations may reach as high as 10 μ M (Lara et al., 2002). In our case, pilot experiment show best responsiveness of both cell lines at a catecholamines concentration of 1 μ M. As shown in figure 3A and 3B, both cell lines are able to respond to catecholamine stimulation with PKA activation in a range of time between 5 and 20 minutes of stimulation, while at 30 minutes the signal starts decreasing, according to a progressive receptor signaling downregulation.

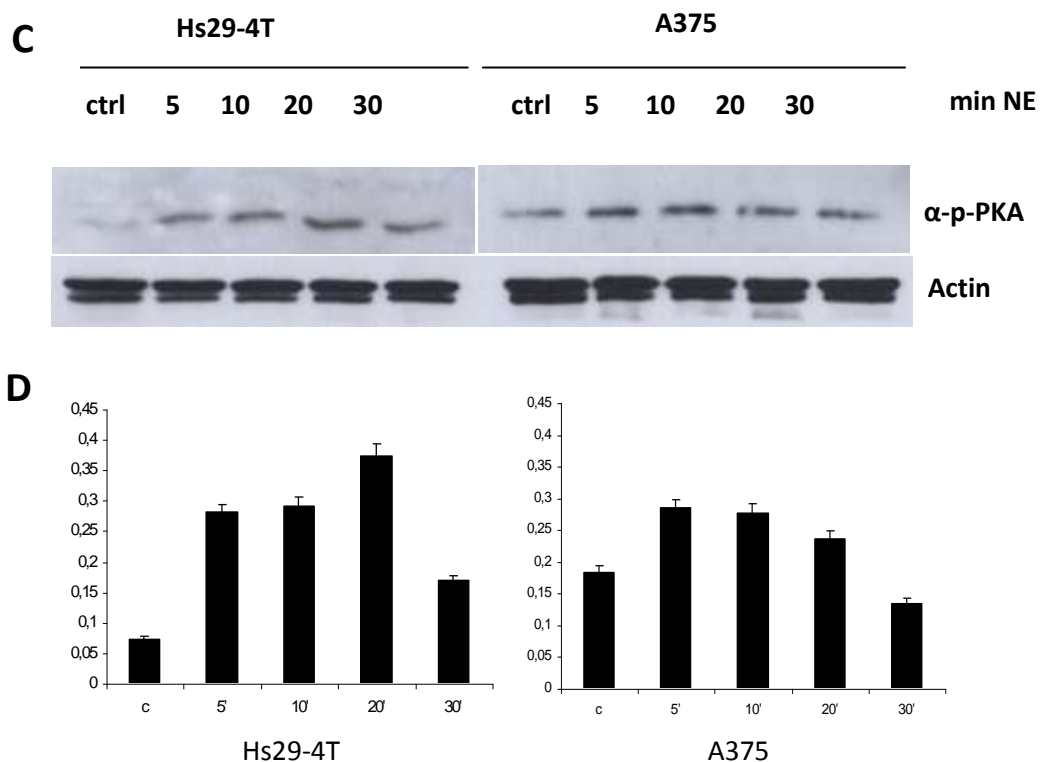
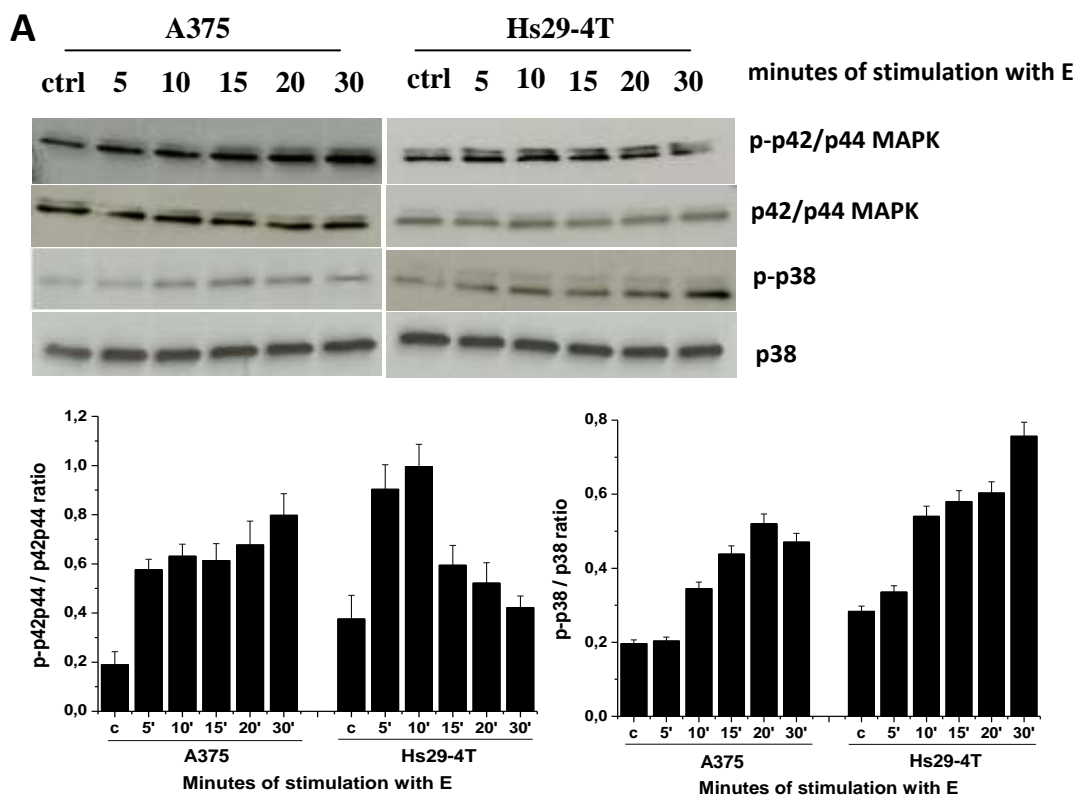


Fig. 4 (A, B). Activation of PKA. Melanoma cell lines were serum-deprived overnight and then stimulated with NE (1 μ M) for the indicated period and an immunoblot analysis for the detection of the phosphorylation level of p-PKA was shown. Actin immunoblot was used for normalization. The bar graph below represents the phosphorylation level of PKA in four different experiments. * $p < 0.005$.

To further elucidate the role of catecholamines in melanoma signaling, we evaluated the activation of the mitogen-activated protein kinase (MAPK) pathways. Several studies have in fact related β adrenergic-dependent MAPK signaling to increased growth and aggressiveness of breast (Cakir et al., 2002), pulmonary (Schuller and Cekanova, 2005) and pancreatic carcinoma (Weddle et al., 2001; Askari et al., 2005), but few studies until now have investigated the effects of β -adrenergic signaling on these molecular pathways in melanoma models. Thus we performed a Western Blot analysis in order to evaluate phosphorylated levels of both p42-p44 and p38 MAPK. In particular, we analysed phosphorylation of Thr202 and Tyr404, key aminoacidic residues for MAPK activation.

Our data demonstrate that A375 cells show a progressive activation of p42/p44 MAPK that reaches its maximal level at 30 minutes of stimulation with both E and NE (figure 4A and 4B). On the other hand, Hs29-4T cells respond to E stimulation within 15 minutes of stimulation, while after 20 minutes p42/p44 phosphorylated levels start decreasing. On the contrary, they show a similar activation pattern to A375 cells after NE stimulation, where p42/p44 reach maximal activation after 30 minutes (figure 4A and 4B).

For what concerns p38, also in this case both NE and E are able to elicit its activation in both cell lines, although with different kinetics (fig. 4A and 4B).



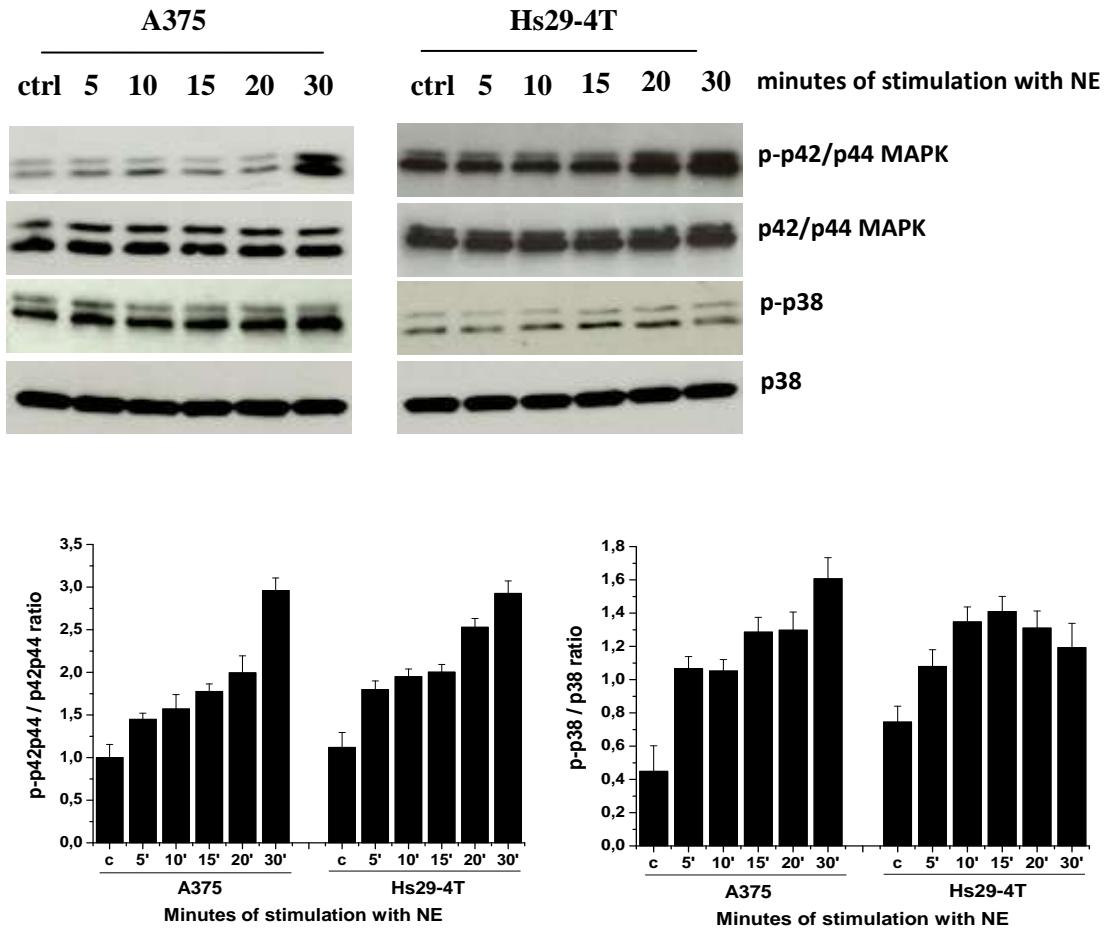
B

Figure 4. Analysis of activation of p42/p44 and p38 MAPK. Melanoma cell lines were serum-deprived overnight and then stimulated with E (A) or NE (1 μ M) (B) for the indicated period and an immunoblot analysis for the detection of the phosphorylation level of MAPKs were shown. Total p42/p44 and p38 MAPK immunoblot were used for normalization. The bar graphs below represent the phosphorylation level of MAPKs in four different experiments. * $p < 0.005$.

Taken together, our data further confirm that both primary and metastatic cell lines respond to catecholamines promoting activation of both PKA and p42/p44 and p38 MAPK pathway, acknowledged to play mandatory roles in regulating cancer cells growth, survival and invasive ability.

Catecholamines increase melanoma cells invasive ability

Activation of autocrine, paracrine and/or endocrine pathways At both primary and metastatic tumour sites can promote tumour cell proliferation by disrupting the balance between positive, pro-proliferative and pro-invasive, and inhibitory signals (Langley and Fidler, 2007). As already stated, several studies have demonstrated the close interrelationship between psychosocial factors and cancer progression. In particular, chronic stress and related continuative production of stress hormones are able to affect key tumour cells mechanisms like invasive/metastatic ability and angiogenesis in order to promote tumour spreading in distant sites. For example, it has been demonstrated in models of ovarian and nasopharyngeal carcinomas that NE increases cells motility and invasive ability through upregulation of MMPs secretion (Yang et al., 2006; Sood et al., 2006). In addition, the β blocker propranolol showed to have effect in impairing cancer cells invasion through inhibition of MMPs and VEGF secretion in an *in vitro* model of pancreatic carcinoma (Guo et al., 2009).

According to these observation, we therefore analysed the motility of melanoma cells upon catecholamine stimulation. The invasion assay was carried out by Boyden chambers placed in a well of a 24 multiwell. Cells were seeded on the upper part of the filter, while a chemoattractant stimulus, in this case culture medium supplemented with serum, was placed in the bottom of the well. In order to mimick the basal layer structure, the Boyden filter was covered with Matrigel, syntethic mixture of different structural proteins like laminin, entactin, proteoglycans and collagen, thereby creating a three-dimensional layer. After 24 h of stimulation with catecholamines, filters were subjected to haematoxylin-eosin staining in order to evaluate the number of cells migrated to the lower side of the Boyden filter. We selected adequate time point by means of pilot experiments showing 24 h as the best to obtain motility increase.

As showed in bar graphs in figure 5A and 5B, both NE and E are able to elicit invasive behaviour in both metastatic and primary melanoma cells. In particular, both NE and E appear to be more effective in primary melanoma cells with respect to metastatic melanoma cells, thereby according to an increase in cancer aggressive behaviour. In addition, E is the most efficient catecholamine in eliciting invasiveness of the metastatic Hs29-4T cell line. In order to further confirm the involvement of β subtypes of adrenergic receptors activation in modulating melanoma cells invasion, A375 and Hs29-4T cells were pre-treated with β blocker propranolol before performing stimulation with catecholamines, and seeded in the upper side of the Boyden chamber. As shown in

figure 5A and 5B, propranolol impairs invasive ability of both primary and metastatic melanoma cells, thereby reducing the number of invaded cells to control levels.

Given their ability to degrade the ECM, MMPs act directly on the motility and invasiveness of cancer cells, allowing them to overcome the boundaries of the tissues and therefore to exit from the tumour primary site and reach blood and lymphatic vessels in order to metastatize in distant tissues (Sternlicht et al. 1999; Boire et al. 2005). To further confirm the role of MMPs in mediating catecholamines-dependent cells motility, we performed Boyden assays incubating both cells lines with ilomastat, a broad range pharmacological inhibitor of MMPs activity. As demonstrated by both pictures and bar graphs in figure 5A and 5B, the pro-invasive effect of both NE and E is strongly sensitive to ilomastat, that reduces the number of invaded cells to control levels.

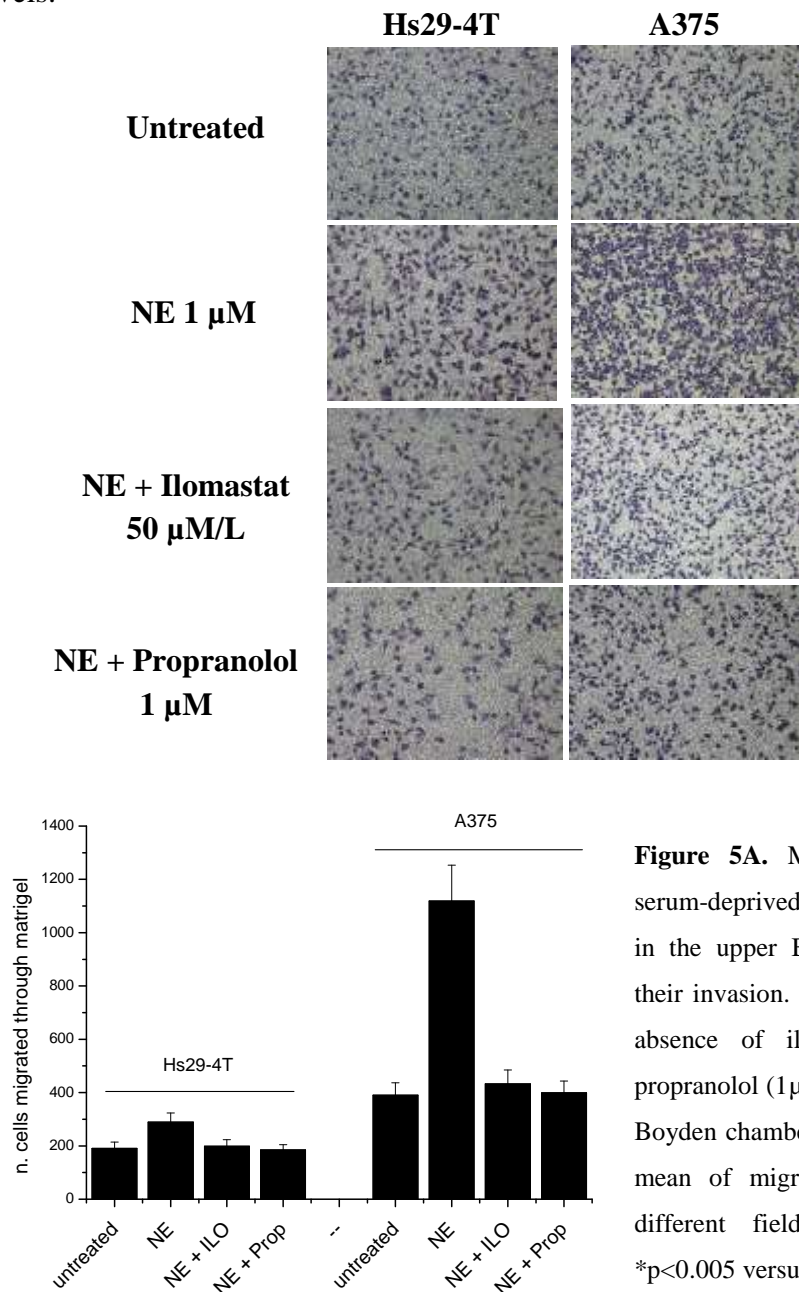


Figure 5A. Melanoma cell lines were serum-deprived overnight and then seeded in the upper Boyden chamber for assay their invasion. NE (1 μ M), in presence or absence of ilomastat (50 μ mol/L) or propranolol (1 μ M), was added in the upper Boyden chamber. Bar graphs represent the mean of migrated cells counted in six different fields for each experiment. * $p < 0.005$ versus untreated.

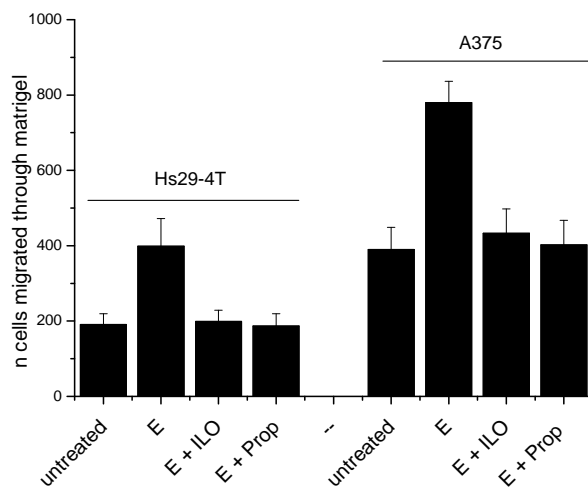
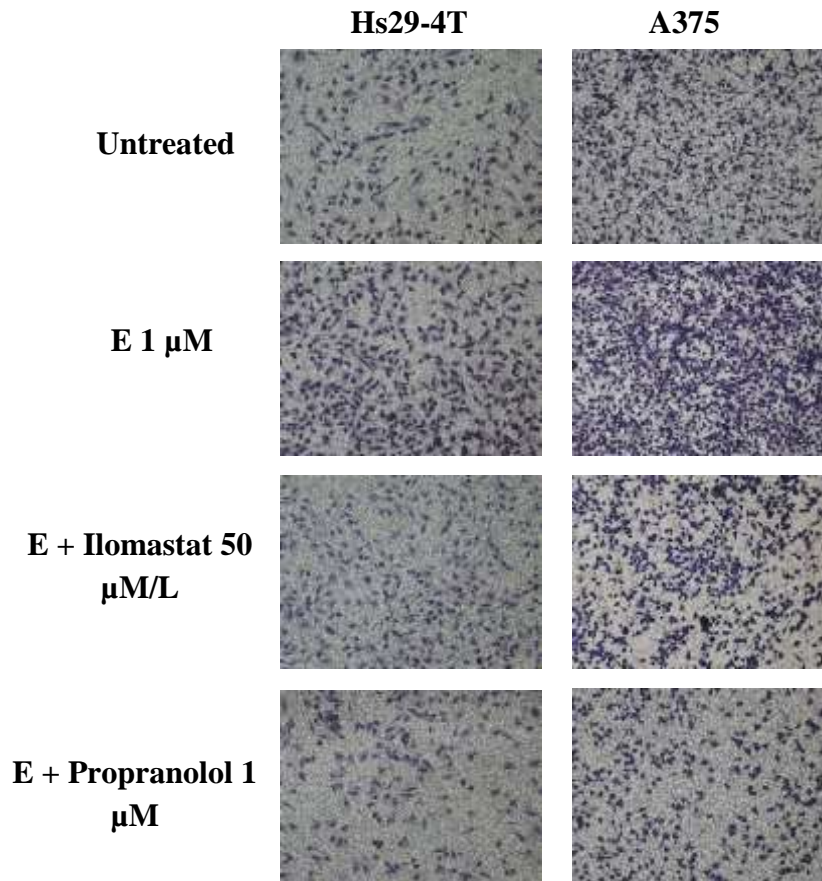


Figure 5B. Melanoma cell lines were serum-deprived overnight and then seeded in the upper Boyden chamber for assay their invasion. E (1 μM), in presence or absence of ilomastat (50 μmol/L) or propranolol (1μM), were added in the upper Boyden chamber. Bar graphs represent the mean of migrated cells counted in six different fields for each experiment. *p<0.005 versus untreated.

Taken together, our data demonstrate that catecholamines stimulation promotes melanoma cells invasion through activation of a β-adrenergic-dependent pathway that involves MMPs secretion, thereby confirming the involvement of a proteolytic degradation of the matrigel barrier during invasion.

We therefore analysed by Real Time PCR the expression of MMP-2 and MMP-9, the main proteolytic enzymes expressed by Hs29-4T and A375 cell lines. Melanoma cell

lines were serum-deprived overnight and then stimulated with E or NE 1 μ M for 24 h. At the end of the stimulation, RNA was extracted and analysed in order to maintain the same analysis time point as the one used for motility assays. Figure 5C and 5D reveal that, while NE and E do not influence MMP-9 production, the expression of MMP-2 is increased by NE in A375 primary melanoma cells and by E in metastatic Hs29-4T.

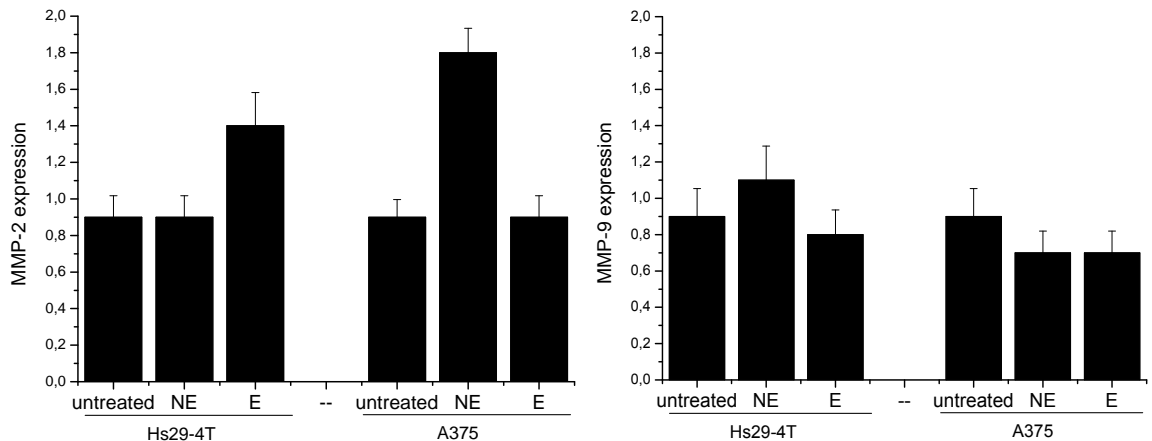


Figure 5C and 5D. Expression of MMP-2 (C) and MMP-9 (D) mRNA by real-time PCR. Melanoma cell lines were serum-deprived overnight and then stimulated with E or NE (1 μ M) for 24 h. The amount of target, normalized to the GAPDH mRNA amounts, was given by the $2^{-\Delta\Delta CT}$ calculation and was reported as arbitrary units (a.u.). The graphs reports data as the mean of three independent assays.

In order to further elucidate this data apparently in contrast with several studies in literature, we analysed enzymatic activity of MMP-2 and MMP-9 with a fluorimetric kit. This MMP activity assay kit uses a Tide FluorTM 3 (TF3)/Tide QuencherTM 3 (TQ3) fluorescence resonance energy transfer (FRET) peptide as a MMP substrate. In the intact FRET peptide, the fluorescence of TF3 is quenched by TQ3. Upon cleavage into two separate fragments by activated MMPs, the fluorescence of TF3 is recovered and its signal can be easily read by a fluorescence microplate reader at Ex/Em = 540/590 nm. Our data demonstrate that both NE and E are able to maintain a high activation state of secreted MMP-2 and MMP-9 following catecholamines stimulation for 24h on both cell lines (figure 5E and 5F).

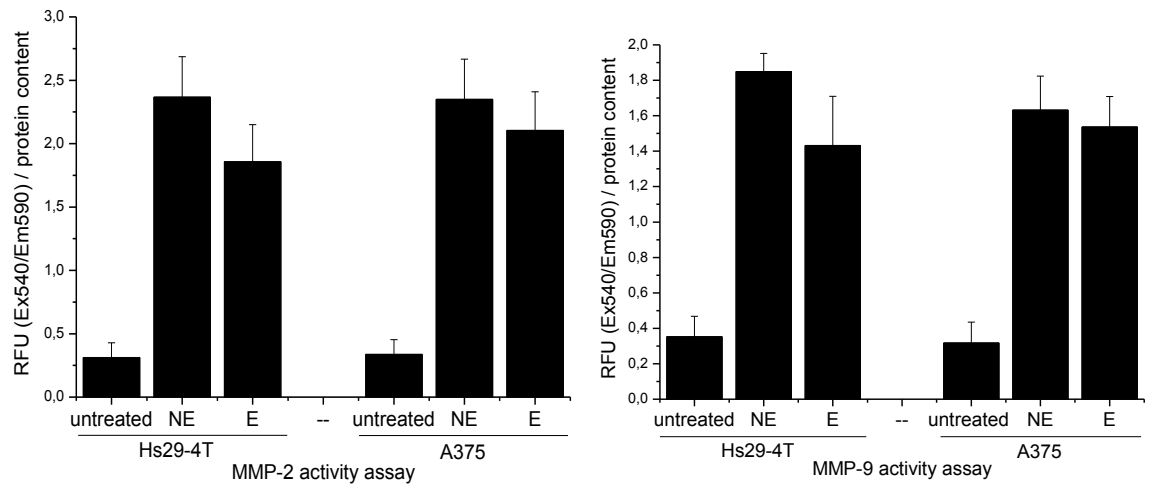


Figure 5E and 5F. MMP-2 and MMP-9 activity assay. Melanoma cell lines were serum-deprived overnight and then stimulated with NE (A) or E (1 μ M) (B) for 24h. The media obtained were then tested for MMPs activity with a fluorimetric kit, following the manufacturer's instructions. Data are presented as RFU versus concentration of test compounds. The graphs reports data as the mean of four independent experiments. * $p < 0.005$.

In conclusion, data obtained so far give new insights about the role of catecholamines in influencing MMPs activity both at a transcriptional and post-translational level. Indeed, we observed a prolonged and sustained activation of both MMP-2 and MMP-9 by catecholamines in melanoma cells, while analysis of RNA revealed a control of expression, mainly focused on MMP-2.

Effects of catecholamines on cytokine production and tumour microenvironment

Cytokines are key molecules secreted by the immune system and play important roles in the cross-talk between different immunologically active cells and tissues, but cytokines are secreted also by non immune-related cells like endothelial and epithelial cells, thus mediating systemic responses to physiological or pathological stimuli.

In the light of these observations, it has been demonstrated that cancer cells secrete many cytokines, chemokines and growth factors that can affect their own aggressiveness, in terms of proliferation, invasion or survival, as well the reactivity of the surrounding stroma. For example, cancer cells production of IL-8 promotes proliferation and survival of cancer cells through autocrine signaling pathways. In addition, tumour-derived IL-8 activates endothelial cells in the tumour vasculature to promote angiogenesis and induce a chemotactic infiltration of neutrophils into the tumour site (Wilson and Waugh, 2008). Also IL-6 plays an important role both in regulating proliferation, invasiveness/scattering and resistance to apoptosis of tumor cells. IL-6 has also been shown to enhance endothelial cell migration, a key step in angiogenesis, and dissemination of solid tumors, also through a synergistic action with VEGF, main promoter of cancer angiogenic sprouting. Thus increased expression of these cytokines, either in the circulation or in tumour tissue, is correlated with worse prognosis in several cancers (Dankbar et al., 2000; Ara et al., 2010; Otrrock et al., 2011).

To address the role of catecholamine stimulation in these features we first analysed the expression of a panel of cytokines/growth factors by quantifying mRNA levels through real time PCR analysis after stimulating cells for 24 hours with NE or E. We found that catecholamine stimulation leads to an increase in the expression of VEGF, IL-6 and IL-8 in both cell lines (Figure 6A, 6B, 6C). Interestingly, the two catecholamines used show differential effects for VEGF, IL-6 and IL-8 in Hs29-4T and A375 cells. As shown in the bar graphs, A375 primary melanoma cells respond to both NE or E eliciting an almost twenty-fold higher expression of IL-6 and an almost five-fold greater expression of VEGF and IL-8. Conversely, the Hs29-4T metastatic cell line senses NE to increase expression of IL-6 and IL-8, while E seems to play a more important role in promoting VEGF expression.

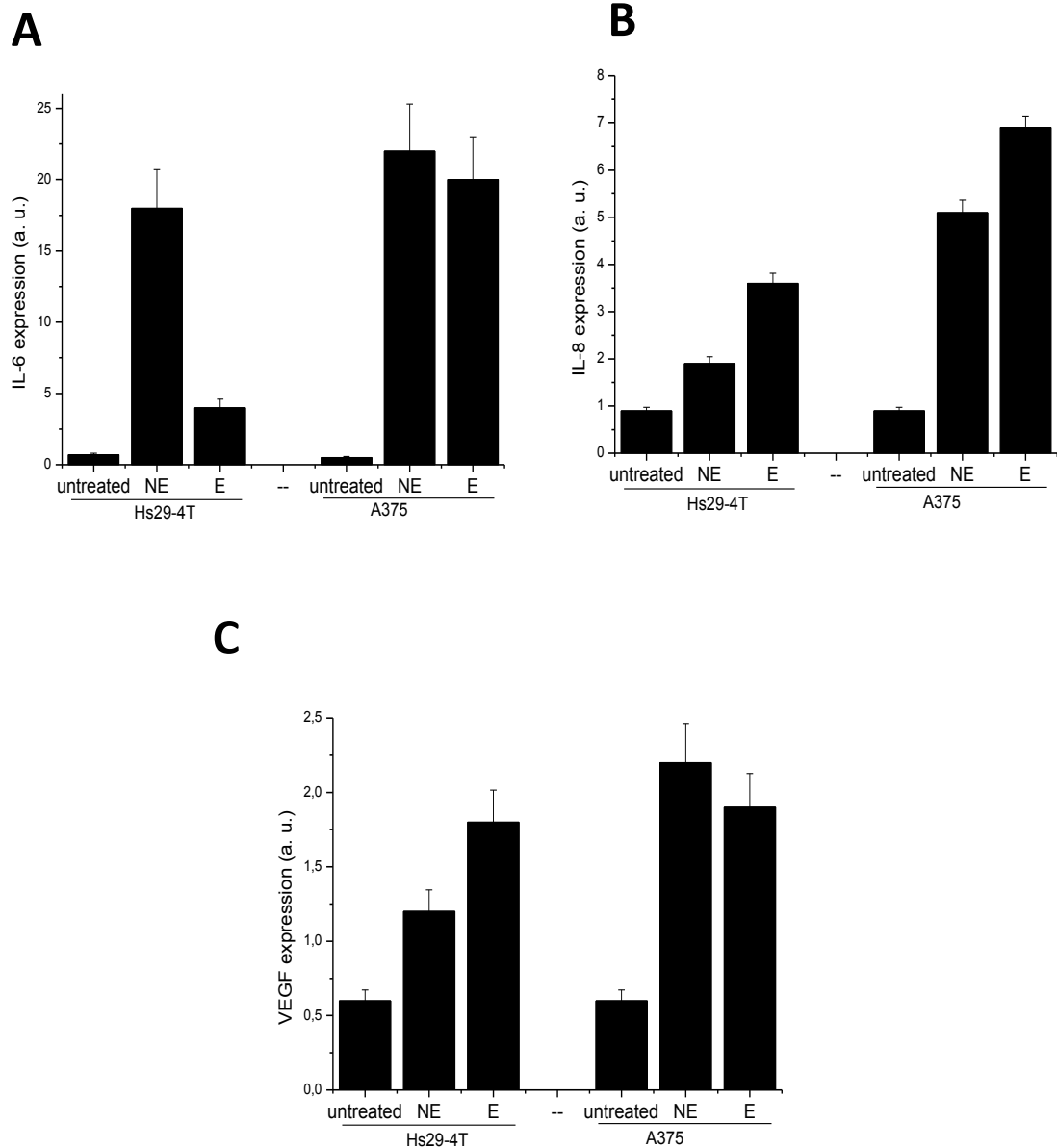


Figure 6. Expression of VEGF, IL-6 and IL-8 in E- and NE-treated melanoma cell lines. Melanoma cell lines were serum-deprived overnight and then stimulated with E or NE (1 μ M). Total RNA was used for the amplification of mRNA of IL-6 (A), VEGF (B) and IL-8 (C), using as housekeeping gene GAPDH mRNA. The amount of target, normalized to the GAPDH mRNA amounts, was given by the $2^{-\Delta\Delta CT}$ calculation and was reported as arbitrary units (a.u.). The graphs reports data as mean of three independent assays. * $p < 0.005$.

As already stated, cancer cells keep creating a complex and continuative “cross-talk” with surrounding, non-malignant cells and/or with the extracellular architecture through direct cell-to-cell contacts and paracrine/exocrine signals. These interactions are not static, but they evolve along with tumour progression. For example, CAFs express various growth factors and cytokines like IGF-1 and HGF that promote survival,

migration and invasion of tumour cells (Li et al. 2003; De Wever et al. 2004; Lewis et al. 2004).

In order to evaluate a possible synergy between catecholamines stimulation and cytokines in promoting primary melanoma aggressiveness, we performed invasion assays where A375 primary melanoma cells were seeded in the upper Boyden chamber and stimulated with NE (1 μ M) alone or in combination with IL-6 (50 ng/ μ l) or VEGF (20 ng/ μ l). We observed that, while VEGF and IL-6 alone do not affect cancer cells invasion, stimulation with VEGF increases the invasive spur induced by NE in A375 primary melanoma cells. On the contrary, combined A375 cells stimulation with NE and IL-6 does not seem to play a significant role (Figure 7A).

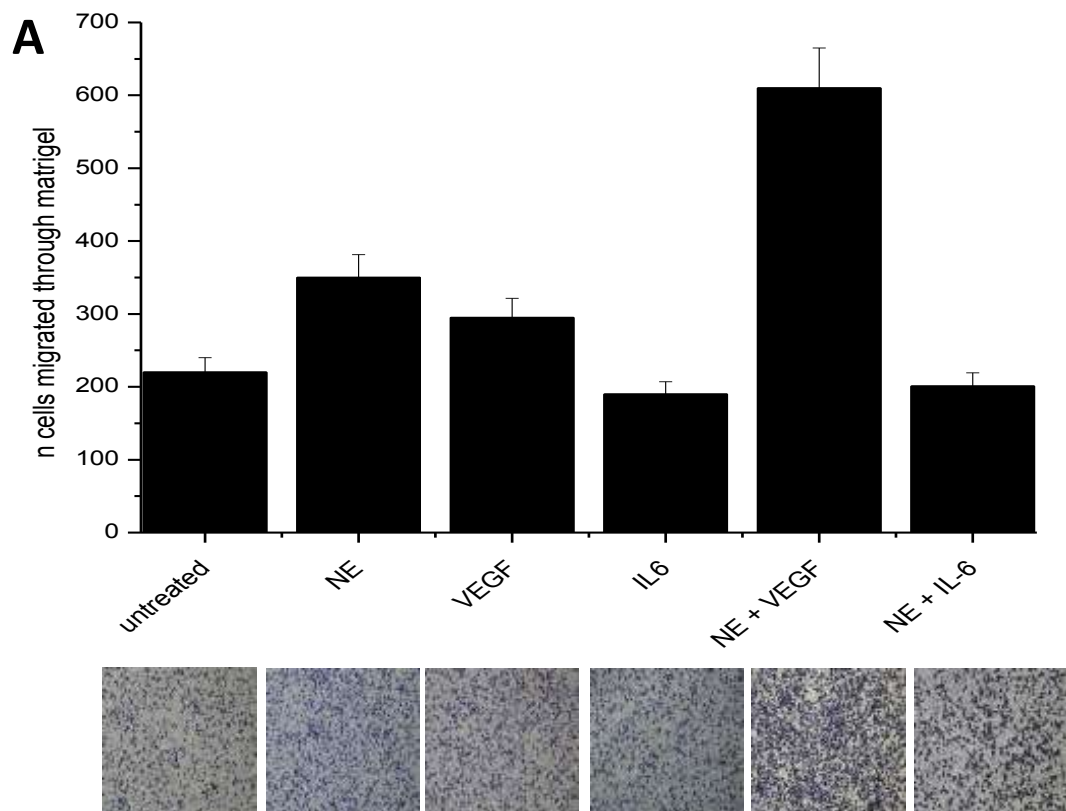


Figure 7A. Synergy among NE, cytokines and tumour microenvironment. (A) A375 primary melanoma cells were serum-deprived overnight and then seeded in the upper Boyden chamber for assaying their invasion. NE (1 μ M), IL-6 (50 ng/ μ l), VEGF (20 ng/ μ l), in combination with NE or alone, were added in the upper Boyden chamber. Bar graph represents the mean of migrated cells counted in six different fields for each experiment. * $p < 0.005$ versus untreated.

As a result of specific environmental stimuli produced by neighboring cancer cells, healthy fibroblasts may undergo an activated state, named “myofibroblastic”, which is characterized by the *de novo* expression of α -smooth muscle actin (α -SMA) protein, the actin isoform typical of smooth muscle cells. Among molecules produced by tumour cells, IL-6 has been described as a key molecule in activating surrounding stromal and/or inflammatory cells infiltrating the tumour burden (Ara et al., 2010).

We therefore analyzed the ability of IL-6, in association with NE, to induce an activated state in healthy dermal fibroblasts. To this aim we performed a Western Blot analysis in order to evaluate the protein level of α -SMA in human dermal fibroblasts (HDFs) treated with conditioned media (CM) A375 cells. To obtain CM from melanoma cells, A375 cells were stimulated in starvation medium with NE, IL-6 or VEGF. After 24 hours of stimulation, the media was removed and replaced with fresh serum-free culture medium in order to obtain CM free from NE, IL-6 or VEGF, but conditioned by their previous administration. HDFs were then incubated with the obtained CM for 24 h and then used for Western blot analysis. We observed that IL-6 is able to activate dermal fibroblasts, as demonstrated by their ability to elicit α -SMA expression in comparison with the untreated condition. In addition, in dermal fibroblasts, the conditioned medium of NE-treated A375 cells induce an activation state very similar with respect to treatment with IL-6 alone. Conversely, VEGF treatment is almost ineffective in eliciting a reactivity of fibroblasts (Figure 7B).

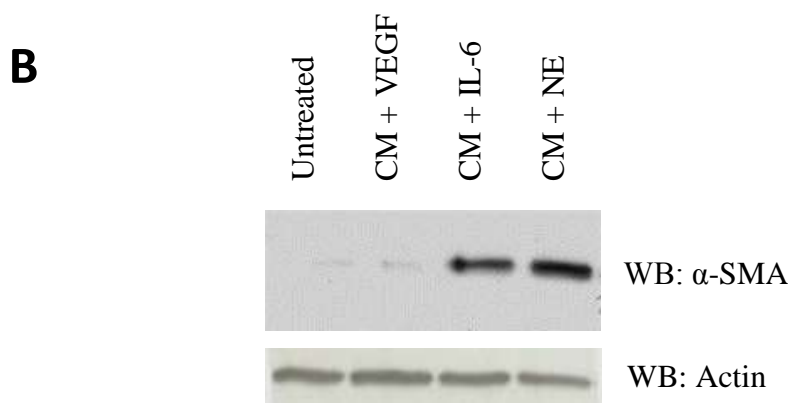


Figure 7B. Analysis of human dermal fibroblasts (HDFs) activation state through evaluation of α -SMA expression. A375 cells were grown to sub-confluence and treated for 24 h with 1 μ M NE, IL-6 (50 ng/ μ l) or VEGF (20 ng/ μ l). Fresh serum-free medium was added for additional 24 h before collection of conditioned medium (CM) free from NE, IL-6 or VEGF, but conditioned by their earlier administration. HDFs were then incubated with the obtained CM for 24 h and then used for Western blot analysis of α -SMA expression. The blot is representative of three different experiments.

Taken together, these data suggest that *in vitro* the treatment of human melanoma cells with catecholamines dramatically affects their aggressiveness, inducing expression of MMP-2, VEGF, IL-6 and IL-8. These factors orchestrate a feed-forward loop leading to increase of proteolytic invasiveness of tumour cells, as well as activating surrounding fibroblasts.

DISCUSSION I

The mediators of stress norepinephrine (NE) and epinephrine (E) exert stimulant effects on the invasion process of a number of neoplastic cells, and this effect seems to be due, at least in part, to the interaction with β -adrenoceptors (AR), which are expressed by various malignant tumours. In particular, the expression of β -AR has been studied in various human solid tumours, such as breast, colon, prostatic, ovary, nasopharyngeal and oral cancer, raising the possibility that such receptors could affect invasion and dissemination processes.

My study therefore aimed to evaluate the expression of β -AR and the influence of NE and E on the malignant behaviour of melanoma cell lines. Preliminary results assessed by immunohistochemistry on a series of human benign and malignant melanocytic lesions showed that both β_1 and β_2 -AR were expressed in melanocytic tumours, according to the fact that both benign and malignant lesions can theoretically be affected by catecholamines *in vivo*. Anyway, both β_1 and β_2 -AR showed significantly higher expression in malignant than benign lesions, and, for what β_2 -AR is concerned, its expression is primarily related to atypical than common naevi. These findings suggest that malignant lesions can be more deeply influenced by catecholamines than benign counterparts, as the staining intensity for β_2 -AR progressively increased from CN toward AN and to PM and MM, whereas the reaction intensity for β_1 -AR was weaker in all groups of lesions. Such a difference can rely on a lower expression of β_1 -AR than β_2 -AR on naevus melanocytes and melanoma cells. This hypothesis was thereby supported by *in vitro* data, as both real time PCR and western blot analysis of primary and metastatic melanoma cell lines demonstrated a lower expression of β_1 -AR versus β_2 -AR.

We also demonstrated that A375 primary and Hs29-4T metastatic melanoma cell lines respond to catecholamine stimulation enhancing motility and invasion and producing molecules closely related to neoplastic progression. In keeping, we show that NE and E are able to elicit activation of p42/p44 and p38 MAPKs, acknowledged to play mandatory roles for cell growth, survival and invasive ability, in both primary and metastatic cell lines. These data could be of striking interest in order to find new strategies for melanoma treatment. In fact, Meier et al. showed that combined targeting of the p42/p44 and Akt signaling pathways significantly inhibited growth and enhanced apoptosis in melanoma cell cultures (Meier et al., 2007).

Moreover, the two cell lines tested show differential responsiveness to NE and E. Concerning the invasion assay, the primary cell line responded to NE and, to a lesser degree, to E, while the metastatic cell line showed a clear reaction only to E. The inhibition induced by ilomastat strongly suggests a proteolytic degradation of matrigel and the probable intervention of MMPs in both cell line invasive process. In fact, real time PCR analysis demonstrates that MMP-2, rather than MMP-9, is produced at a significantly higher level compared to baseline, by A375 cells under NE stimulus and by Hs29-4T cells under E stimulation. Furthermore, our data show that both NE and E are able to elicit sustained activation of MMP-2 and MMP-9 both in primary and metastatic cell lines. Thus, increased motility of melanoma cells seems to be due to a proteolytic invasive capacity, typical of a mesenchymal phenotype (Hoffmann et al., 2000; Parri et al., 2009), and catecholamines seem able to influence MMPs activity both at a transcriptional and at a post-translational level. This disparity could be explained by the fact that activation of pro-MMP-2 and pro-MMP-9 is closely related, as it has been demonstrated that active MMP-2 can convert pro-MMP-9 to its active form through direct proteolytic action. In the light of these findings, MMP-9 activation could be a downstream effect of MMP-2 direct regulation by catecholamines (Bauvois, 2012). The migration of neoplastic cells appears to be increased through activation of β -ARs, because it is completely abolished by β -ARs antagonist propanolol. As stimulation of both cell lines with NE and E induces activation of the protein kinase PKA, we can speculate that catecholamines exert their functions on melanoma cell lines primarily through a PKA-dependent pathway.

Also for what the production of cytokines is concerned, NE and E show differential effects on melanoma cell lines. In fact, primary A375 cells significantly increase levels of IL-6 and VEGF under NE and E challenge, whereas metastatic Hs29-4T cells increase IL-6 expression under NE stimulus, and produce significant amounts of VEGF, especially under E activation. Concerning the expression of IL-8, the primary cell line responded to both NE and E at a significantly higher degree compared to the metastatic one, and both cell lines reacted more intensely to NE. This result is in agreement with the angiogenic role of IL-8, since *de novo* angiogenesis is particularly useful for primary tumours to escape the hostile microenvironment and disseminate metastasis (Singh and Varney, 2000). In addition, the catecholamine-induced IL-8 enhancement is in agreement with the described IL-8 stimulation produced by NE in prostate cancer (Voss et al., 2010).

We do not know exactly why such a discrepancy exists between primary and metastatic cell line response, but it is possible that at least in part this difference is due to a higher expression of β_2 -ARs, assessed as protein and transcript, on the primary melanoma cell line. Another possibility is that IL-6 and IL-8, whose expression was associated with early malignancy of melanoma *in vivo* are actually secreted more efficiently by a cell line derived from a primary melanoma, thereby leading to enhanced aggressiveness towards a more metastatic phenotype (Moretti et al., 1999).

In vitro experiments clearly show that catecholamines can increase the malignant behaviour of melanoma cells affecting both invasion capacity and cytokine production. Preliminary data in melanoma tissue sections also showed a strong reactivity for β_2 -AR in most endothelial and stromal cells, including macrophages, suggesting the possible influence of catecholamines on cells of the tumour microenvironment. In keeping, recent studies showed that phagocytic cells like neutrophils and macrophages are direct sources of catecholamines and that stimulation with both NE and E can enhance macrophagic release of proinflammatory cytokines as TNF- α , IL-1 β and IL-6 through a NF- κ B-dependent pathway (Flierl et al., 2009). These data could support the hypothesis of potential feed-forward biologic loops capable of affecting metastatic behaviour of neoplastic cells.

Our *in vitro* experiments could further confirm that some pro-metastatic loops could work in *in vivo* melanoma model too. In fact, we demonstrate that IL-6 and NE in melanoma cells can activate dermal fibroblasts toward a myofibroblastic phenotype, as demonstrated by upregulated α -SMA expression (Kalluri et al., 2006). It is well known that stromal fibroblasts within tumours undergo a process, commonly called mesenchymal-mesenchymal transition (MMT) to myofibroblasts, leading them to a more contractile phenotype and allowing a cross talk with tumour cells dramatically affecting their aggressiveness (De Wever and Mareel, 2003; Dvorak et al., 2011). In turn, activated fibroblasts can secrete other pro-metastatic cytokines, such as VEGF, capable of inducing further tumour angiogenesis. In keeping with these observations, in our experiments, VEGF can increase melanoma cell migration and invasion ability, particularly when associated with NE (Dong et al., 2004).

Previous data support the hypothesis that various types of stress, such as surgical procedure or neuroendocrine stress due to psycho-social factors, can stimulate tumour progression both in animals and humans (Voss et al., 2010). This seems to be true also for melanoma at least in *in vivo* models (Azpiroz et al., 2008; Vegas et al., 2006).

Our work provides evidence that stress hormones like NE and E can significantly stimulate the malignancy of melanoma cells at various levels and that β -ARs, likely involved in this response, are largely expressed in melanoma cell lines and cutaneous melanomas. It is the first time, to our knowledge, that β -ARs are demonstrated in a large series of human cutaneous melanocytic lesions, and even in melanocytic naevi, suggesting a potential influence of catecholamines also in benign counterparts. Moreover, the detection of β_2 -AR also in the stromal cells of melanoma microenvironment implies further possible effects of catecholamines on melanoma progression. Consequently, it is possible that the interaction catecholamines- β -ARs could play a dramatic role during the clinical course of melanoma patients. The efforts to understand molecular events underlying such an interaction could therefore be very useful for indicating new targets in therapy.

CONCLUDING REMARKS I

On the basis of the results obtained we can speculate that in aggressive melanoma, the action exerted by catecholamines both on the tumour cells and their microenvironment could be related at least in part to the preservation of a tumour stem niche, which may in turn contribute to resistance to conventional pharmacological therapies. At this purpose, it has been recently reported that melanoma stem cells isolated from patients showed higher expression of multiple ABC transporters and IL-8, suggested to be related with increased chemoresistance of melanoma cells (Luo et al., 2012). Also VEGF directly acts on the cancer stem niche through blood vessel formation *via* its primary function on endothelial cells, and thus increasing the area of the niche required for preservation of stem cell-like properties. In addition, VEGF can act on melanoma stem cells in an autocrine way, promoting their self-renewal, through its receptor VEGFR-2 (Takakura, 2012). We can therefore hypothesize that catecholamines could promote melanoma aggressiveness both enhancing angiogenesis and motile ability of cancer cells and also providing a possible protective mechanism for melanoma stem cells from drugs delivery, likely in an IL-8 and VEGF-dependent way. This fascinating hypothesis could be strengthened by our recent observation that melanoma cells treated with NE and β 3-AR selective antagonist SR59230A show diminished percentage of cells positive to stem marker CD133, thereby suggesting a role of β 3-AR in controlling stem-like properties of a small population of melanoma cells (Calvani M., unpublished data). In the light of this hypothesis, also the action exerted by catecholamines on the tumour microenvironment could be related to protection and prevention of premature exhaustion of the stem cell niche with cancer cells characterized by stem cell-like properties. To this purpose, it has been reported that myofibroblasts and endothelial cells are key components of the tumour stem niche and support *in vivo* tumour initiation from a small number of cancer stem cells (Vermeulen et al., 2010; Calabrese et al., 2007). Our experiments showed that IL-6 and NA in melanoma cells can activate dermal fibroblasts toward a myofibroblast phenotype, allowing a cross-talk with tumour cells, thereby dramatically increasing their aggressiveness. In turn, activated fibroblasts can secrete other pro-metastatic cytokines, such as VEGF and SDF-1, capable of further inducing tumour angiogenesis and recruitment of distant cells, thus activating further pro-metastatic circuits (De Wever 2003; Dvorak 2011; Dong 2004). This could be confirmed in our model by recent observations that melanoma cells stimulated with NE

recruit CAFs, macrophages, adult endothelial cells and endothelial precursor cells (EPCs) (Calvani M., unpublished data). Of note, both macrophages and endothelial cells have been recently described as sources of catecholamines (Flierl et al., 2009; Sorriento et al., 2012). Hence, both systemic and tumour-neighbouring sources of catecholamines could promote recruitment and differentiation of CAFs and EPCs, inducing a triple cross-talk among populations, maybe creating a specific microenvironment to control self-renewal and undifferentiated state of melanoma stem cells and thereby fostering melanoma aggressive potential and treatment failure. Future study combining β -blockers with common therapies should therefore have important implications for treatment of malignant melanoma and its total eradication, counteracting at multiple stages this complex and continuative, catecholamines-mediated interplay between cancer cells and their microenvironment.

EXPERIMENTAL

PART II

AIM OF THE STUDY II

In 1926 Otto Warburg demonstrated that cancer cells undergo a glycolytic metabolism even in presence of oxygen, thereby metabolizing high amounts of glucose into lactate (Warburg effect), but just recently metabolic reprogramming towards a Warburg metabolism has been considered as a real hallmark of cancer. Indeed, several studies have now established that this metabolic shift is crucial for proliferating cells that require, in addition to ATP, macromolecules in order to sustain cancer progression. In addition, it has been recently proposed a new metabolic model where cancer cells induce the Warburg effect in stromal fibroblasts, which undergo a myofibroblastic differentiation, and secrete lactate and pyruvate in the extracellular space through the monocarboxylate transporter 4 (MCT-4). Cancer cells then take up these metabolites through the monocarboxylate transporter-1 (MCT-1) and use them in the mitochondrial TCA cycle, promoting energy generation via oxidative phosphorylation.

In particular, the aim of this study is to start focusing on the ability of nutrients to regulate transcription factors involved in cancer cells metabolism. One of these factors is represented by hypoxia inducible factor-1 α (HIF-1 α). It is well established that, under hypoxic condition, HIF-1 α activates target genes whose protein products mediate a switch from oxidative to glycolytic metabolism (ex. GLUT-1, LDH-A), while it is not well established yet the role of HIF-1 α in regulating these complex pathways under normoxic condition. To this purpose, A375 primary melanoma cell line was cultured in normoxic condition in presence/absence of nutrients like glucose, pyruvate and lactate in order to understand how different nutrients can activate key pathways for cancer progression. Cytofluorimetric analysis demonstrate that A375 show strong dependence on glucose content of media in order to sustain survival, thereby according to a Warburg metabolic phenotype, and that nutrients alone are able to induce the expression of HIF-1 α and of its target gene carbonic anhydrase-IX (CA-IX), probably through an increased reactive oxygen species (ROS) production. In order to further understand the role of nutrients-induced HIF-1 α , we therefore performed migration and invasion assays with Boyden chambers, respectively in absence or presence of Matrigel mimicking the extracellular matrix. Nutrients alone are also able to elicit a pro-invasive response of A375 and this effect is reverted after both treatment with Topotecan, a HIF-1 α pharmacological inhibitor, and HIF-1 α gene silencing. Finally, data obtained so far allow

us to hypothesize a novel important role of nutrients-induced HIF-1 α normoxic stabilization in order to sustain cancer progression.

RESULTS II

Role of nutrients in regulating melanoma cells proliferation and survival

Today, metabolism reprogram is universally accepted as one of the hallmarks of cancer. In fact, cancer cells are able to completely modify their metabolic behaviour in order to sustain cancer progression. Rising evidence have now established that cancer cells undergo a specific metabolic reprogramming, leading cancer cells to gain several advantages that are not only related to mere ATP production.

Non-neoplastic cells usually metabolize glucose into pyruvate, which is in turn moved in the mitochondria, thereby entering the Krebs cycle and undergoing oxidative phosphorylation. This metabolic pathway produces high ATP levels, quantified in 38 molecules for each glucose molecule, but needs O₂ to support mitochondrial respiration. On the other hand, Otto Warburg demonstrated that cancer cells undergo a glycolytic metabolism, named “Warburg Effect”, even in presence of oxygen, thereby metabolizing higher amounts of glucose into lactate, which is then extruded in the extracellular space through monocarboxylate transporter-1 (MCT-1) (Vander Heiden et al., 2009). Glucose fermentation does not require oxygen, but it is far less efficient than the TCA cycle coupled to oxidative phosphorylation in generating ATP. Despite decreased efficiency in ATP production, fast-growing cells rely primarily on glucose fermentation during proliferation regardless of oxygen availability. Indeed, several studies have now established that the Warburg effect, coupled with increased glucose uptake due to incomplete glucose oxidation, promotes in cancer, high-proliferating cells the efficient anabolism of macromolecules from glycolytic intermediates, providing the key carbon precursors needed for the synthesis of nucleic acids, phospholipids, fatty acids, cholesterol and porphyrins, needed to sustain uncontrolled and continuative cellular mitosis (Lunt and Vander Heiden, 2011; Pedersen, 2007).

Since the metastatic ability of cancer cells is strongly related to microenvironmental conditions like nutrients availability, stromal cells and vascularization, their cellular metabolic fluxes and nutrient demand may show considerable differences. Moreover, their stage-dependent metastatic ability may further create metabolic alterations depending on its microenvironment. Although recent studies have aimed to elucidate cancer cell metabolism in different cancer models, in most of the cases the nutrient demand and metabolic activity of tumour cells still remains poorly understood due to their high heterogeneity.

For what the melanoma is concerned, the glycolytic phenotype of some melanoma cell lines has been only recently identified by metabolic profiling using isotopically labeled nutrients (Scott et al., 2011).

In order to further elucidate the metabolic behavior of melanoma cells, we started performing crystal violet assay on A375 primary melanoma cell line cultured in normoxic condition for 24, 48 and 72 hours in total absence (black line) or presence of nutrients like glucose (red line), pyruvate (green line) and lactate (light blue line). As culture control condition we cultured melanoma cells in presence of both serum and glucose (violet line). This assay is useful in order to obtain quantitative information about the number of cells adhering to multi-well cluster dishes. The dye in this assay, crystal violet, stains DNA. Upon solubilization, the amount of dye taken up by cultured cells can be quantitated in a spectrophotometer at 595 nm wavelength. The graph in Figure 1 shows normalized data as ratio between crystal violet absorbance (nm) and protein content, thereby allowing us to correlate with the nuclear DNA content and thus with cell number.

Data obtained demonstrate that nutrients alone are not able to sustain cancer cells proliferative ability. In fact, A375 cells proliferation rate in presence of glucose, pyruvate or lactate alone does not show significant modifications if compared with cells maintained for 24 hours in absence of nutrients while, as expected, the optimal culture condition represented by both serum and glucose induced a four-fold increase in cells proliferation rate (Figure 1).

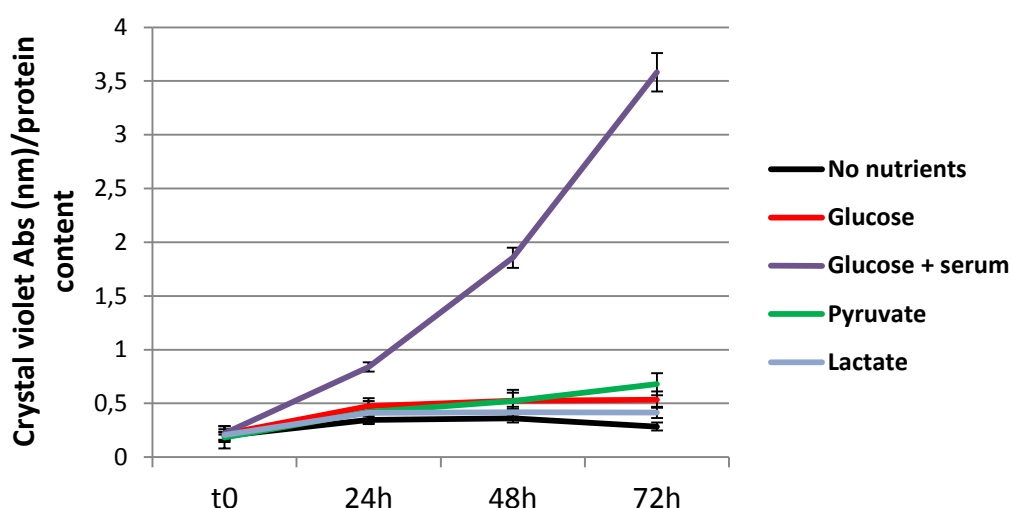


Figure 1. Crystal violet assay on A375 melanoma cells treated for 24, 48 and 72 h with glucose (4,5 g/L), pyruvate (10 mM) or lactate (10 mM). Culture condition in presence of glucose and serum was considered as standard control condition. Data are represented as crystal violet Abs (nm)/protein content ratio and each measurement was made in triplicate for each point of the curve of growth. Experiment was repeated at least 3 times. p value < 0,005.

To further elucidate the effect of the different nutrients in cancer cells survival, we therefore performed an Annexin V-iodidium propide staining assay in order to evaluate apoptotic cells percentage. Annexin-V is a Ca^{2+} dependent phospholipid-binding protein that has high affinity for phosphatidylserine, which itself is translocated from the inner to the outer leaflet of the plasma membrane during the early phase of the apoptotic process. Apoptotic cells were identified by flow cytometry using Annexin-V conjugated to fluorescein isothiocyanate (FITC), in conjunction with propidium iodide (PI) to distinguish apoptotic cells (Annexin-V-FITC positive, PI negative) from necrotic cells (Annexin-V-FITC positive, PI positive).

Using this method as a marker of apoptosis, exposure of melanoma cells to 4,5 g/L glucose, alone or in combination with serum, under serum-free conditions for 24 h led to a significant decrease (17.4% compared to 6.4%) in the number of cells in early and late apoptosis as compared to cells cultured in total nutrients absence for the same time. In contrast, addition of pyruvate or lactate alone induced melanoma cells apoptosis to levels comparable to that related to nutrients withdrawal, while only combination of both nutrients with serum was able to significantly reduce apoptotic cells percentage (Figure 2).

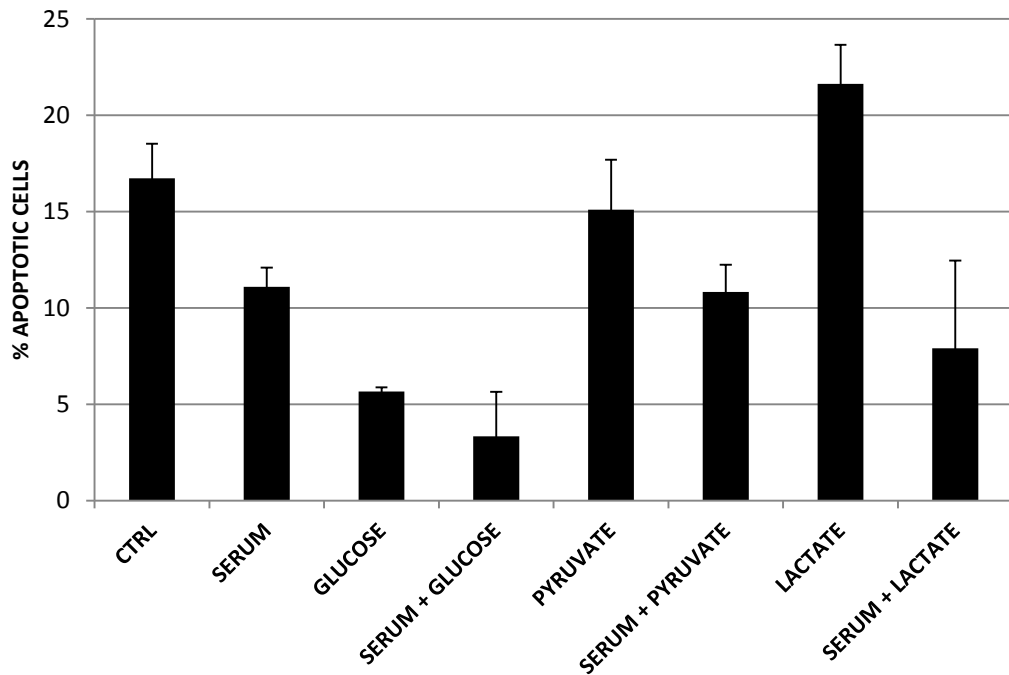


Figure 2. Annexin V/Iodidium Propide cytofluorimetric staining on A375 melanoma cells treated for 24h with glucose (4,5 g/L), pyruvate (10 mM) or lactate (10 mM), alone or in combination with serum. Cells were then pelleted and labeled with annexin V and iodidium propide. Finally cells were evaluated by flow citometry. Data are represented as total percentage of cells positive to annexin V staining alone (early apoptosis) and together with PI (late apoptosis). Experiment was repeated at least 3 times. p value < 0,005.

To further elucidate the molecular mechanisms underlying nutrients-based cell survival, we therefore evaluated the possible role of nutrients in directly influencing Akt activation. Akt is a central player in processes downstream of activated growth factor receptor signaling like the insulin receptor, EGF-R and HGF-R regulating cells proliferation and survival. After ligand binding and subsequent receptor activation, Akt recruitment to the plasma membrane is mediated mainly through phosphatidylinositol 3-kinase (PI3K), which phosphorylates phosphoinositides to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Akt amino terminal pleckstrin homology (PH) domain binds PIP3, thereby promoting the translocation of Akt to the plasma membrane where it is phosphorylated and activated. The tumor suppressor PTEN acts as a regulator of Akt activity by dephosphorylating PIP3, although it is frequently downregulated or lost during tumor progression, contributing to deregulation of the pathway in cancer cells. After activation, Akt is able to translocate to the nucleus where it affects both directly or indirectly cellular proliferation and survival. Among Akt substrates there are transcriptional regulators like CREB, E2F, NF- κ B, the FOXO

transcription factors and murine double minute 2 protein (MDM2), master regulator of p53 activity. In addition, Akt is able to target a number of other molecules to affect the survival state of the cell including the pro-apoptotic molecule Bcl-2-associated death promoter (BAD) and caspase 9 (Altomare and Khaled, 2012).

In order to evaluate Akt activation, melanoma A375 cells were serum-deprived overnight and then cultured for 24 h in presence of glucose (4,5 g/L) or pyruvate (10 mM), alone or in combination with serum. As demonstrated by the western blot represented in figure 3, melanoma cells cultured for 24h in presence of nutrients alone do not show activation of the Akt-mediated pathway, while the combination of nutrients and serum in the culture medium induced a strong increase in the level of phosphorylated protein (Figure 3).

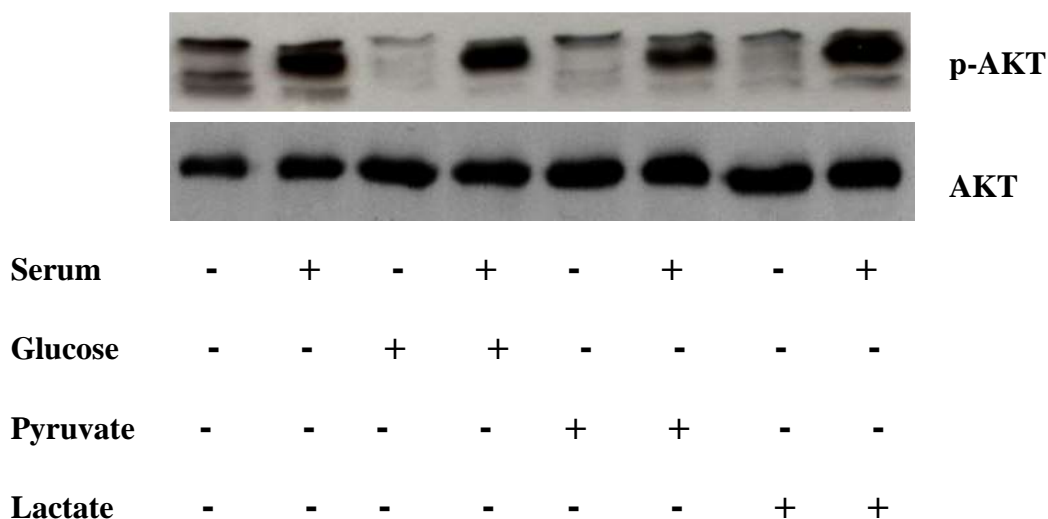


Figure 3. Activation of AKT. Melanoma A375 cells were serum-deprived overnight and then cultured for 24 h in presence of glucose (4,5 g/L) or pyruvate (10 mM), alone or in combination with serum. An immunoblot analysis for the detection of the phosphorylation level of p-AKT was shown. Total AKT immunoblot was used for normalization. The western blot is representative of three independent experiments.

Taken together, these data demonstrate that nutrients alone do not affect significantly A375 melanoma cells proliferative ability, as confirmed by lacking of Akt activation in conditions of serum withdrawal. On the other hand, according to a Warburg-like metabolic phenotype, Annexin V-IP staining assay showed a high dependence of

melanoma cells from glucose in order to sustain their survival, likely through an Akt-independent pathway.

Nutrients promote normoxic HIF-1 α and CA-IX expression and affect ROS production

Hypoxia is a common feature of solid tumours and complexes HIF-1 α , HIF-2 α and HIF-3 α are the master regulators of cell response to hypoxia. In particular, HIF-1 is an heterodimeric complex characterized by an α subunit of 120 kDa O₂-dependent (HIF-1 α), and a β subunit of 91-94 kDa constitutively expressed (HIF-1 β) also known as aryl hydrocarbon nuclear translocator (ARNT), as it has been first identified inside the heterodimeric complex with the aryl hydrocarbon receptor (AHR) (Reyes et al., 1992). In normoxic conditions HIF-1 α shows a very short half-life of about 1/2 ~ 5 minutes and is rapidly degraded via ubiquitin-proteasome system (Salceda et al., 1997). When cells are exposed to low O₂ tensions, HIF-1 α half-life is of about 30 minutes, the protein is stabilized and translocates to the nucleus, where it binds to subunit HIF-1 β forming the transcriptionally active HIF complex. The resulting heterodimer binds to HRE sequence of target genes and binds to transcriptional coactivators, thereby promoting gene expression (Lando et al., 2002). As already stated, HIF-1 α directly regulates several genes coding for enzymes involved in cells metabolism, in particular glycolytic metabolism, including glucose transporters and glycolytic enzymes like hexokinase 1 and 2 (HK1 e HK2), PFK1, aldolase A and C, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1, enolase 1, pyruvate kinase and lactate dehydrogenase A (LDHA).

We therefore analyzed if melanoma cells exposure to different nutrients can regulate transcription factors involved in cancer cells metabolism. We focused on the expression of HIF-1 α as it is well established that, under hypoxic condition, HIF-1 α is a master regulator of anaerobic metabolism, while it is not well established yet the role of HIF-1 α in regulating these complex pathways under normoxic conditions.

To this purpose, A375 primary melanoma cell line was nutrients-starved overnight and then cultured in normoxic condition for 24h in presence/absence of nutrients like glucose, pyruvate and lactate. Cells were then lysed and a western blot analysis of HIF-1 α expression was performed in order to understand how different nutrients can activate key pathways for cancer progression. As a control condition cells were cultured for 24h also in presence of glucose, pyruvate and lactate in combination with serum. In fact, HIF-1 α expression induced by growth factors stimulation is associated to an increased production through activation of phosphatidylinositol-3-kinase (PI3K) and MAPK pathways (Fukuda et al., 2002; Fukuda et al., 2003; Laughner et al., 2001; Zhong et al.,

2000). According to that, it has been demonstrated that MAPK pathway leads to activation of both p42/p44 MAPK and p38 MAPK, following previous activation of proteins Ras/Raf-1/MEK-1. It has been demonstrated that HIF-1 α is phosphorylated by p42, p44, p38 α and p38 γ *in vitro* (Richard et al 1999; Sodhi et al 2000).

As shown in figure 4, all nutrients alone are able to promote normoxic HIF-1 α stabilization at levels comparable to HIF-1 α expression in melanoma cells cultured in presence of nutrients in combination with serum. In particular, glucose shows higher effectiveness in inducing HIF-1 α expression.

We therefore evaluated the expression of HIF-1 α main target gene, carbonic anidrase IX (CA-IX). Again, western blot analysis shows that nutrients alone, and in particular glucose, are able to promote also normoxic CA-IX expression (figure 4).

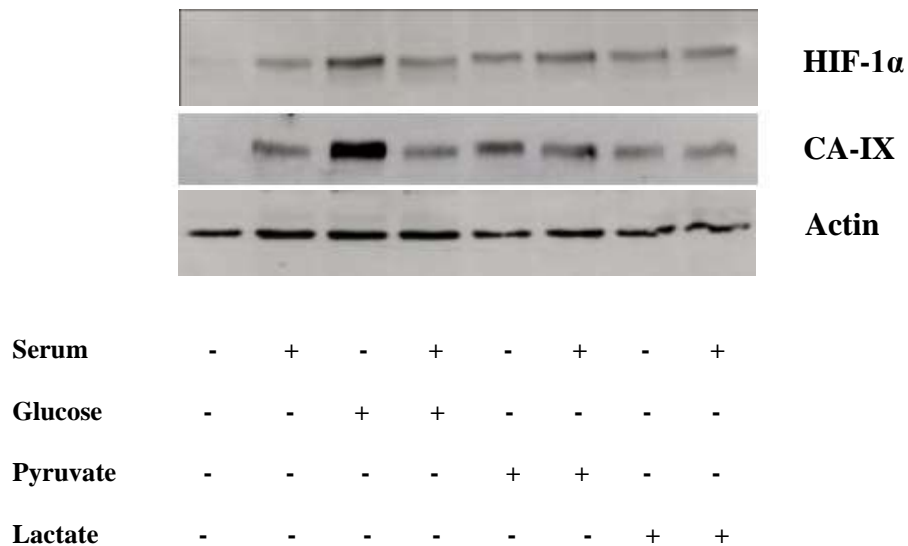


Figure 4. Evaluation of HIF-1 α and CA-IX expression. Melanoma A375 cells were serum-deprived overnight and then cultured for 24 h in presence of glucose (4,5 g/L), pyruvate (10 mM) or lactate (10 mM), alone or in combination with serum. An immunoblot analysis for the detection of HIF-1 α and CA-IX was shown. Actin immunoblot was used for normalization.

In the light of these data, we next analyzed the level of ROS produced by melanoma cells in presence of different metabolites in order to rely their role to normoxic nutrients-mediated HIF-1 α expression. In fact, it has been already demonstrated that ROS can act as second messengers in several signaling pathways regulating cancer progression, like epithelial-mesenchymal transition (EMT), migration/invasion and angiogenesis. In particular, hypoxia is directly related to higher mitochondrial ROS

production, thereby promoting HIF-1 α stabilization through inhibition of PHDs activity (Klimova 2008; Wheng-sheng Wu 2006).

To evaluate the amount of ROS produced, staining with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe was performed. H₂DCFDA is a chloromethyl derivative of H₂DCFDA. H₂DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. The assay was performed on A375 cells serum-starved overnight and then cultured in normoxic conditions for additional 24 hours in presence of glucose, pyruvate and lactate, alone or in combination with serum. Analysis of H₂DCFDA absorbance revealed that melanoma cells show significantly higher production of ROS after culture in presence of metabolites alone, which is in agreement with the nutrients-induced HIF-1 α stabilization. Again, culture of melanoma cells with glucose alone induced the highest spike of ROS production, according to its role in promoting highest HIF-1 α protein expression (figure 5).

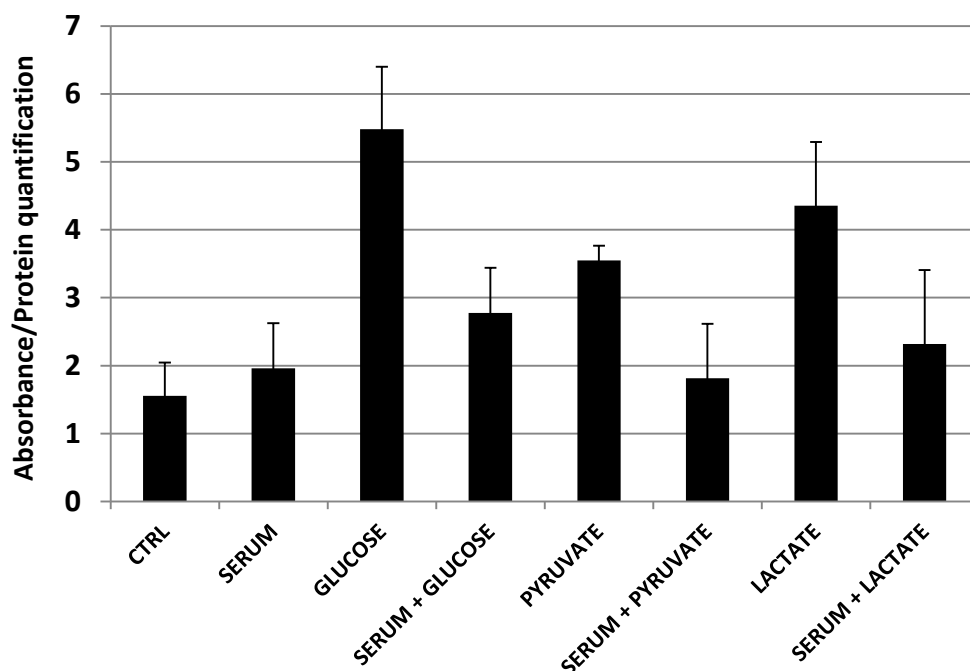


Figure 5. Intracellular ROS amount evaluation using fluorimetric probe H₂DCFDA. A375 melanoma cells were serum-deprived overnight and then treated for 24 h with glucose (4,5 g/L), pyruvate (10 mM) or lactate (10 mM), alone or in combination with serum. Then H₂DCF-DA (5 μ g/ml) probe was added and incubated for 3 minutes at 37°C. After the incubation time, cells were quickly lysated in RIPA lysis buffer and lysates were then collected, centrifugated, transferred on a 96 wells multiwell and analyzed. Data are represented as absorbance / protein quantification ratio. Experiment was repeated at least 3 times. p value < 0,005.

Taken together, these data demonstrate that nutrients alone are able to directly regulate expression of HIF-1 α and CA-IX in the human melanoma model. In addition, glucose, pyruvate and lactate induce high ROS production, thereby allowing us to speculate that nutrients promote normoxic HIF-1 α stabilization in a redox-dependent manner.

Nutrients promote CA-IX expression in a HIF-1 α -dependent manner

Carbonic anhydrase IX (CA-IX) is a highly active, tumor-associated transmembrane carbonic anhydrase isoform composed by an extracellular catalytic domain, whose activity is hypoxia-dependent. In fact, CA-IX expression pattern is strictly related to HIF-1 α -mediated transcriptional activation of *CA9* gene, as *CA9* promoter region contains several HRE elements that induce CA-IX expression in response to hypoxia (Kaluz et al., 2002).

The most important CA-IX role is represented by hydration of pericellular CO₂. This reaction generates an extracellular proton, which contributes to generating an increasingly acidic extracellular pH, facilitating tumor cell invasiveness, and bicarbonate ion, which is delivered to cytoplasm through the bicarbonate exchangers directly interacting with CA-IX in order to maintain an alkyline intracellular pH favorable for tumor growth (Svastova et al., 2004). In addition to its role in the regulation of tumoral pH and tumor cell survival, there is evidence that CA-IX contributes to cell processes such as adhesion and migration, both of which are crucial for metastatic progression in human cancer (McDonald et al., 2012).

To further confirm the involvement of HIF-1 α in the normoxic, nutrients-based induction of CA-IX expression, we performed specific experiments of inhibition of HIF-1 α expression. We first examined HIF-1 α expression levels in melanoma cells stimulated with glucose, pyruvate and lactate for 24 h, alone or in combination with the camptothecin analogue Topotecan, a topoisomerase I pharmacological poison which has been already demonstrated as an HIF-1 α protein accumulation blocking agent (Rapisarda et al., 2004).

Western blot analysis shown in figure 6 clearly demonstrates that melanoma cells treatment for 24 h with Topotecan impairs normoxic nutrients-induced HIF-1 α expression. Consistent with this finding, Topotecan significantly reduced CA-IX accumulation as well (figure 6).

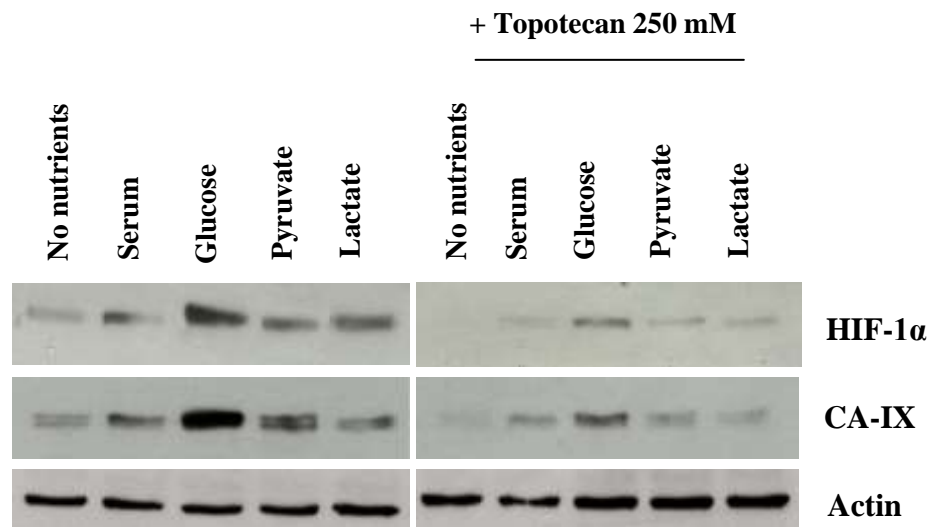


Figure 6. Evaluation of HIF-1 α and CA-IX expression in absence or presence of Topotecan 250 nM. Melanoma A375 cells were serum-deprived overnight and then cultured in presence of glucose (4,5 g/L), pyruvate (10 mM) or lactate (10 mM) and stimulated or not with Topotecan for 24 h. An immunoblot analysis for the detection of HIF-1 α and CA-IX was shown. Actin immunoblot was used for normalization.

To further confirm the results obtained so far we then used a specific siRNA targeting HIF-1 α mRNA for knockdown of gene expression. Again, HIF-1 α knockdown cells resulted in almost total abrogation of HIF-1 α and CA-IX protein accumulation, according to siRNA highest efficiency in downregulating protein expression if compared to pharmacological treatment (figure 7).

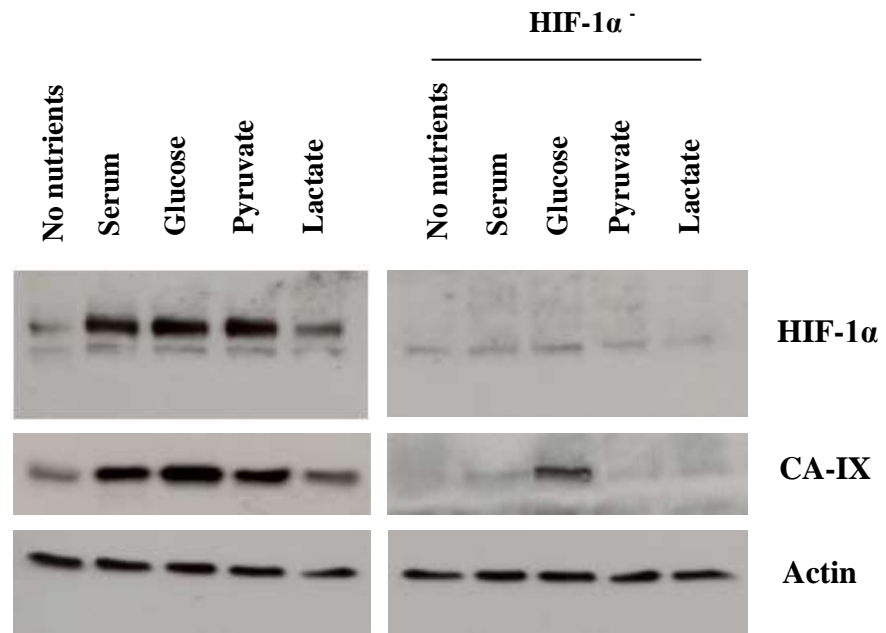


Figure 7. Evaluation of HIF-1 α and CA-IX expression after HIF-1 α gene silencing. Melanoma A375 cells were silenced for HIF-1 α as previously described and then cultured in presence of glucose (4,5 g/L), pyruvate (10 mM) or lactate (10 mM) for 24 h. An immunoblot analysis for the detection of HIF-1 α and CA-IX was shown. Actin immunoblot was used for normalization. $p < 0,05$.

Nutrients-induced HIF-1 α expression promotes melanoma cells invasiveness

We therefore evaluated the role of nutrients-induced HIF-1 α expression in regulating cancer cells invasive ability. In fact HIF-1 α activation correlates with metastasis in multiple tumors and can promote metastasis through the regulation of key factors regulating tumor cell metastatic potential, including E-cadherin, lysyl oxidase (LOX), CXCR4, and stromal-derived factor 1 (SDF-1) (Rankin and Giaccia, 2008).

To this aim we performed *in vitro* Boyden assays. The Boyden chamber is composed by an upper and a lower compartment separated by a microporous membrane with pores of 8 μ m of diameter. In order to evaluate cancer cells ability to degrade and invade through an organic matrix, the Boyden chamber microporous membranes were covered with Matrigel, a synthetic preparation rich in laminin, collagen IV and entactin and heparan sulfate proteoglycan (perlecan). This matrix acts as a reconstituted basement membrane *in vitro*, occluding the pores of the membrane and blocking non-invasive cells from migrating through the membrane, while invasive cells can secrete proteases that enzymatically degrade the Matrigel matrix and enable invasion through the membrane pores.

For our experiments, A375 were plated in the upper chamber in 200 μ l of serum-free medium in absence or presence of serum, glucose, pyruvate or lactate, while the lower compartment was filled with 500 culture μ l of medium with 10% FBS as a standard chemotactic agent. Cells were then allowed to migrate through the pores to the other side of the membrane and, after a 24h incubation time in normoxic conditions, they were stained with haematoxylin-eosin staining. Finally, the number of cells that had migrated to the lower side of the membrane was determined by image capture of cells attached through a microscope and then counting the number of cells invaded to the lower membrane surface.

As shown in both figures 8 and 9, all nutrients increase the invasive spur of melanoma cells, with glucose greatly increasing A375 cells invasive ability. In addition, the pro-invasive effect of all nutrients is strongly sensitive to both Topotecan treatment (figure 8) and HIF-1 α gene silencing (figure 9). Again, melanoma cells treatment with Topotecan resulted in high impairment of A375 motility, while HIF-1 α knockdown cells showed almost total abrogation of invasive ability, in keeping with the results obtained from western blot analysis (figures 6 and 7).

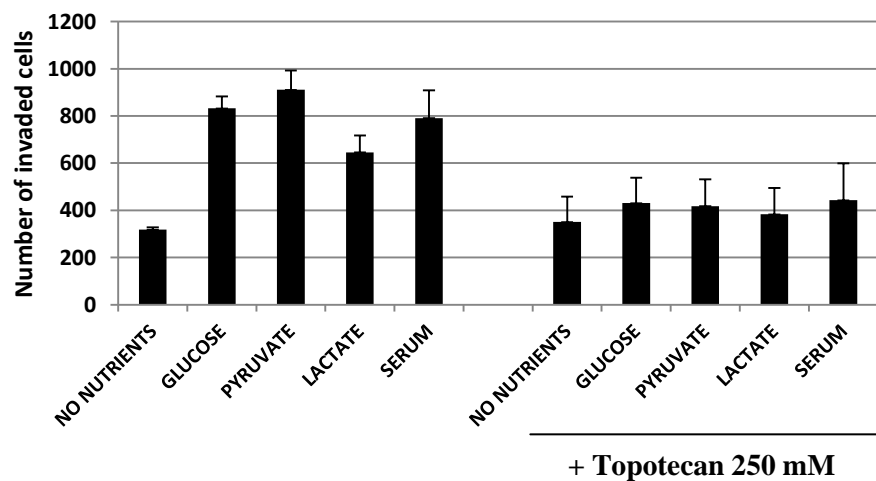
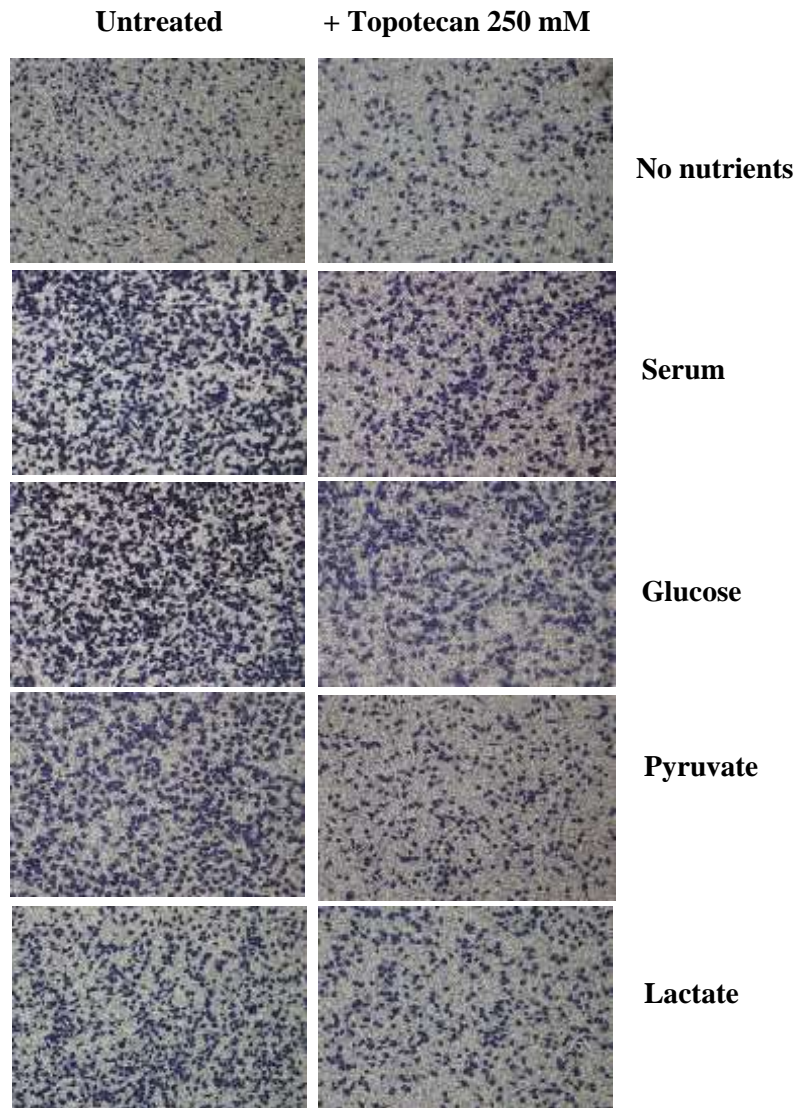


Figure 8. Evaluation of A375 invasive ability through Boyden chamber assay. Melanoma A375 cells were treated as previously described and then loaded into the upper compartment of the Boyden chamber in serum-free growth medium, with or without 250 mM Topotecan. After 24 h of incubation at 37°C, the microporous membrane was fixed in 96% methanol and stained with Diff-Quick solution. The bar graph shows the number of migrated cells to the lower surface of the polycarbonate filters from three independent experiments. P value < 0,05.

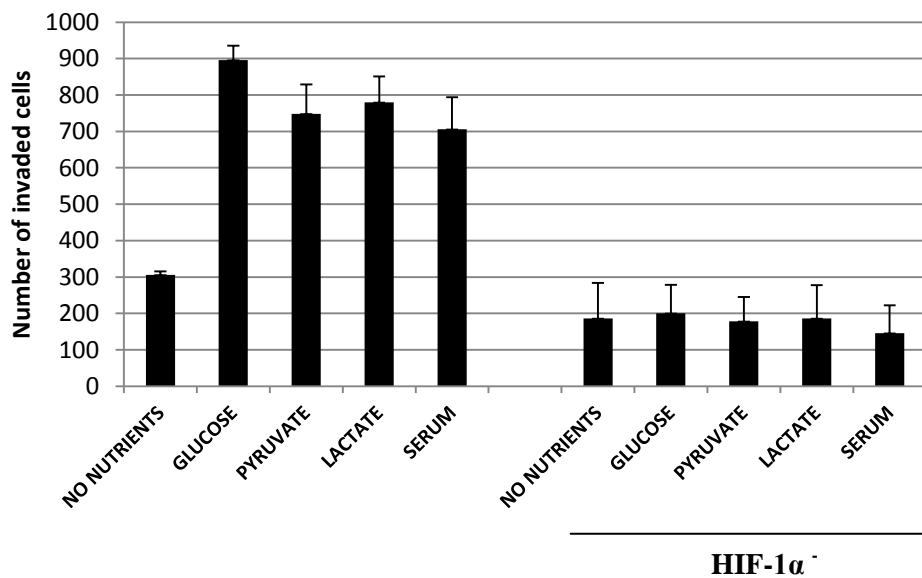
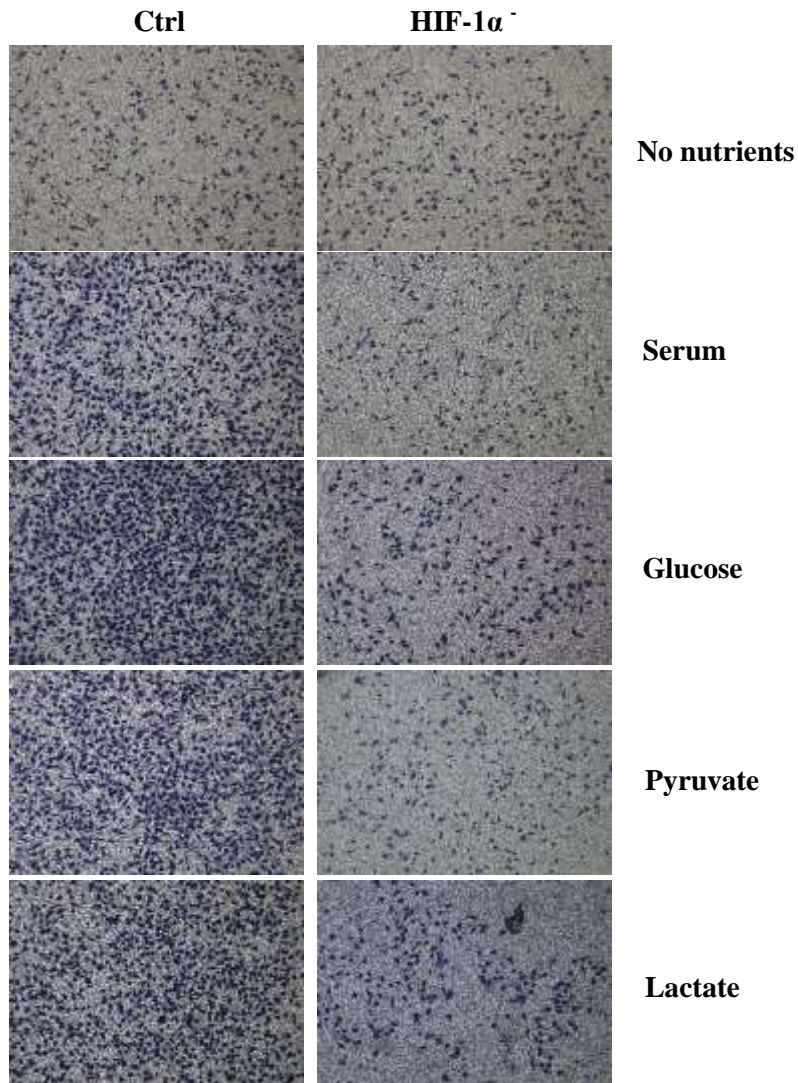


Figure 9. Evaluation of A375 invasive ability through Boyden chamber assay. Melanoma A375 cells were silenced for HIF-1 α as previously described and then loaded into the upper compartment of the Boyden chamber. After 24 h of incubation at 37°C, the microporous membrane was fixed in 96% methanol and stained with Diff-Quick solution. The bar graph shows the number of invaded cells to the lower surface of the polycarbonate filters from three independent experiments. P value < 0,05.

In conclusion, data obtained so far indicate that nutrients are able to directly regulate HIF-1 α and CA-IX expression in normoxic conditions, and it is likely that this stabilization is redox-dependent. In addition, experiments performed using siRNA targeting HIF-1 α and Topotecan treatment demonstrate that melanoma cells exploit nutrients by increasing invasiveness through a HIF-1 α -dependent mechanism.

DISCUSSION II

The urgent need of always new antineoplastic drugs is at present a real challenge for molecular oncologists. In particular, the recently renewed interest for the metabolic deregulation of tumors has led to new pharmacological strategies targeting metabolic pathways to impair cancer cells survival. In fact, approximately 60% to 90% of cancers display a Warburg-like metabolic profile that is, the capacity of non-hypoxic tumor cells to entirely rely on glycolysis without exploiting oxidative phosphorylation in order to support the high biosynthetic and energy demands of actively proliferating cells. Therefore, tumours characterized by aerobic glycolysis and/or high glucose dependence could be more sensitive than other tumours to agents targeting metabolic-related key cancer pathways.

Metastatic melanoma represents a very aggressive tumour, which relays to more than the 80% of total deaths caused by skin cancers, thanks to its intrinsic resistance to common pharmacological therapies (Helmbach et al., 2001). Just recently a report by Scott and colleagues characterized the metabolic profile of several melanoma cell lines, showing that cancer cells all exhibited a Warburg metabolism with higher glucose consumption rate and lactate production compared to healthy melanocytes (Scott et al., 2011).

According to these observations, our data indicate that metastatic melanoma cells A375 display a high dependence from glucose, as the Annexin V/IP staining assay demonstrated that only glucose administration was able to significantly reduce the percentage of apoptotic cells. As a further confirmation, pyruvate alone was not able to sustain melanoma cells survival, thereby allowing us to hypothesize reduced TCA cycle and oxidative phosphorylation activity in favor of a Warburg metabolism. On the other hand, we observed that glucose, pyruvate and lactate are not able to elicit melanoma cells proliferation, as confirmed also by the western blot analysis on Akt phosphorylation levels.

Additionally, we report that increased aggressiveness of human metastatic melanoma A375 cells is tightly correlated to a normoxic, nutrients-induced HIF-1 α stabilization. Our finding of HIF-1 α acting as a nutrients-responsive transcription factor unravels a pathway independent from the O₂ extracellular concentration which may connect tumor cell metabolism and cancer cells motility. These data are proposed to further support the importance of Warburg metabolism for aggressive tumour cells. According to that, as

many genes coding for glycolytic enzymes, glucose transporters, and glucose regulatory hormones are induced by hypoxia (Dang and Semenza, 1999; Feldser et al., 1999), the Warburg effect may represent a feed-forward mechanism to maintain a high intracellular concentration of metabolites and intermediates in order to foster the expression of HIF-1 α -related genes even in normoxic condition. In addition, in our experimental model all nutrients promoted high normoxic ROS accumulation. Previous studies by Lu and colleagues reported that several TCA cycle intermediates can stabilize HIF-1 α through inhibition of PHD enzymes (Lu et al., 2002; Lu et al., 2005). Therefore, it is highly conceivable that in our experimental model nutrients-based ROS delivery during normoxia could play an important role in promoting HIF-1 α expression through inactivation of PHDs activity.

In addition, our data also indicate HIF-1 α as the driver of a nutrient-dependent pathway mediating melanoma motility. This conclusion was supported by experiments performed using both Topotecan, a known HIF-1 α pharmacological inhibitor, and a specific siRNA targeting HIF-1 α , which almost completely blocked nutrients-induced cancer cells invasiveness. Our data are in agreement with a recent report by Goetze and colleagues who observed that both pyruvate and lactate have been shown to increase the migration of head and neck cancer cells (Goetze et al., 2011). However, to our knowledge, our study is the first to show that nutrients have the potential to increase the invasive ability and metastatic potential of aggressive melanoma cells through an HIF-1 α -dependent pathway also in normoxic condition. HIF-1 α has already been proposed as a key player of cancer cells invasiveness, and mechanisms proposed are mainly correlated to its ability to regulate key factors governing tumor cell metastatic potential, including E-cadherin, lysyl oxidase (LOX), CXCR4, and stromal-derived factor 1 (SDF-1) (Rankin and Giaccia, 2008). We also recently reported that aggressive melanoma cells respond to hypoxia upregulating their motile/invasive abilities, based on redox stabilization of HIF-1 α and activation of the Met protooncogene, allowing a proteolytic motility enhancing metastatic dissemination to lungs (Comito et al., 2011). It is then conceivable that lack of proliferation in presence of nutrients alone could be seen as a strategy leading to protection against a condition of metabolic stress, thereby favouring cancer cells motogen escaping strategy. Furthermore, also HIF-1 α has been recently described as a negative regulator of cancer cells proliferation through activation of different mechanisms. For example, it has been reported that HIF-1 α promotes cell cycle arrest through induction of p27 overexpression regardless of hypoxic conditions

(Hackenbeck et al., 2009), and Kaidi and colleagues demonstrated that HIF-1 α induces overexpression of the main cyclin-dependent kinase (CDK) inhibitors p21 and p27 by antagonizing c-Myc transcriptional activity, thereby leading to cell cycle arrest into G1 phase (Kaidi et al., 2007).

In the light of these observations, we can speculate that also nutrients-dependent carbonic anhydrase IX (CA-IX) expression could contribute to enhanced melanoma aggressiveness. CA-IX overexpression has been demonstrated in several cancer models as a master regulator of pH homeostasis, which is crucial for cancer cells in order to avoid potentially harmful effects of an highly glycolytic and therefore pro-acidic metabolism (Robertson et al., 2004; Neri and Supuran 2011). For this reason, several studies have started clarifying the role of CA-IX in eliciting cells motility. Initially Parkila and colleagues demonstrated that acetazolamide, a potent specific CA inhibitor, reduced renal cancer cells invasive ability *in vitro* (Parkkila et al., 2000). More recently, in colorectal cancer cells it has been proved that COX-2-dependent expression of CA-IX correlates with tumour stage and increases cancer cells invasiveness (Sansone et al., 2008). Additionally, Svastova et colleagues pointed out the ability of CA-IX to modulate E-cadherin-dependent cell adhesion through direct colocalization with β -catenin and to promote loss of cell-cell contact, the key initial step of cancer invasion (Svastova et al., 2003). Therefore, gene silencing experiments will be crucial to completely elucidate the ability of nutrients to possibly regulate melanoma cells invasiveness through a CA-IX-dependent mechanism.

In conclusion, our data propose the normoxic, nutrients-and redox-induced HIF-1 α as a mandatory player in melanoma aggressiveness, acting as a promoter of melanoma cells invasiveness. In this light, future studies aimed on targeting specific metabolic pathways could therefore have important implications for treatment of malignant melanoma.

CONCLUDING REMARKS II

On the basis of the results obtained we can speculate that also protein kinase M2 (PKM2) expression could be directly affected by nutrients, thereby suggesting a positive feedback mechanism of HIF-1 α activation in order to further promote a Warburg-like metabolic behavior in aggressive melanoma cells. In particular, Pro403 and Pro408 hydroxylation on PKM2 by prolyl hydroxylase-3 (PHD-3) promotes PKM2 transition from tetrameric to dimeric form, which migrates in the nucleus and acts as HIF-1 α direct coactivator. In turn, there is an increased transcription of HIF target genes like *PKM2* and *EGLN3*, encoding for PKM2 and PHD3, respectively, thereby engaging a feed-forward loop leading to amplified HIF-1 α activity (Semenza et al., 2011).

Another fascinating hypothesis is that metabolites in the diffusion-limited tumor microenvironment could be acting as paracrine signaling molecules. For example, it has been recently demonstrated that tumour-secreted lactate can act as a paracrine molecule able to favour motility *in vitro* of human mesenchymal stem cells (hMSC) (Rattigan et al., 2011). We can therefore speculate that nutrients could have a dramatic effect on tumour progression acting both on cancer cells, promoting HIF-1 α -dependent invasiveness, and on the surrounding stroma, maybe favouring recruitment of neighbouring stromal cells like cancer-associated fibroblasts (CAFs), thus activating further pro-metastatic cross-talks. In particular, as we report that glucose, pyruvate and lactate induce normoxic HIF-1 α stabilization, likely through a redox-dependent mechanism, it is conceivable that nutrients delivered to microenvironment by growing tumour vasculature could directly modulate stromal cells metabolism, promoting Warburg metabolism through normoxic stabilization of HIF-1 α and favouring metabolic reprogramming of melanoma cells towards a reverse Warburg phenotype in conditions of low glucose concentration. At this purpose, we recently demonstrated a metabolic cross-talk in which PCa cells elicit an HIF-1 α and redox-dependent Warburg metabolism in CAFs, which produce and extrude lactate in turn uploaded by PCa cells in order to foster their own anabolic pathways and ensure cancer cell proliferation (Fiaschi et al., 2012). This adaptative mechanism engaged by cancer cells demonstrates their high metabolic plasticity, as these cells can either use Warburg metabolism in high glucose environment, but are able to quickly change their metabolic behaviour to a reverse Warburg programme after CAFs contact during glucose starvation. Finally, according to this observation, we can speculate that this hypothetical nutrients-driven

metabolic liaison between cancer cells and CAFs could involve recruitment and activation of other key components of tumour reactive stroma, like macrophages and endothelial cells. Accordingly, lactate has been described to directly modulate cell signaling networks in endothelial cells upon uptake through the monocarboxylate transporter-1 (MCT-1), stimulating an autocrine NF- κ B/IL-8 pathway driving cell migration and new vessels formation (Vegran et al., 2011). Moreover, lactate has also a role in directly promoting macrophage activation and production of proinflammatory molecules such as IL-6 (Samuvel et al., 2009). It is therefore conceivable that nutrients can directly modulate metabolic-related intracellular signaling pathways of different stromal cells in order to foster this complex metabolites-mediated interplay, offering a both structural and metabolic support essential for tumour survival/growth and resistance to common therapeutic agents.

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APPENDIX: PUBLICATIONS

Q1 β -adrenoceptors are upregulated in human melanoma and their activation releases pro-tumorigenic cytokines and metalloproteases in melanoma cell lines

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Recent studies sight β -adrenergic receptor (AR) antagonists as novel therapeutic agents for melanoma, as they may reduce disease progression. Here within, we evaluated the expression of β -ARs in a series of human cutaneous melanocytic lesions, and studied the effect of their endogenous agonists, norepinephrine (NE) and epinephrine (E), on primary and metastatic human melanoma cell lines. Using immunohistochemistry, we found that both β 1- and β 2-ARs are expressed in tissues from benign melanocytic naevi, atypical naevi and malignant melanomas and that expression was significantly higher in malignant tumours. Melanoma cell lines (human A375 primary melanoma cell line and human Hs29-4T metastatic melanoma cell lines) also expressed β 1- and β 2-ARs by measuring transcripts and proteins. NE or E increased metalloprotease-dependent motility, released interleukin-6 and 8 (IL-6, IL-8) and vascular endothelial growth factor (VEGF). These effects of catecholamines were inhibited by the unselective β -AR antagonist propranolol. The role of soluble factors elicited by catecholamines seemed pleiotropic as VEGF synergized with NE increased melanoma invasiveness through 3D barriers, while IL-6 participated in stromal fibroblast activation towards a myofibroblastic phenotype. Our results indicate that NE and E produce *in vitro* via β -ARs activation a number of biological responses that may exert a pro-tumorigenic effect in melanoma cell lines. The observation that β -ARs are upregulated in malignant melanoma tissues support the hypothesis that circulating catecholamines NE and E, by activating their receptors, favour melanoma progression *in vivo*.

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KEYWORDS: β -adrenoceptors; immunohistochemistry; melanoma; melanoma progression; norepinephrine/epinephrine

Melanoma represents the most aggressive type of skin cancer, with an increasing incidence found especially in young adults. A significant reduction in mortality has been not observed, despite a noteworthy improvement in early diagnosis achieved in recent years.¹ At present, no medical option can cure metastatic melanoma (MM) and the only effective treatment for the eradication of the disease is early-phase surgery.² Hence, increased knowledge of the biological pathways underlying the process of melanoma dissemination and metastasis is crucial in order to identify new therapeutic targets.

Previous studies have shown that various human solid tumours, such as breast, colon, prostatic, ovary, nasopharyngeal

and oral cancer, express β 2-adrenoceptor (β 2-AR), raising the possibility that such receptors may affect invasion and dissemination processes.^{3–8} Moreover, some stress neurotransmitters, such as norepinephrine (NE) and epinephrine (E), have been demonstrated to contribute to the regulation of tumour cell invasion, at least in part through β -AR activation.^{6,7,9} Interactions between tumour cells and soluble factors originated from the nervous system has recently been proposed to favour metastasis formation.¹⁰ Improved survival rates have been demonstrated in mice with metastatic tumour by combined administration of β -AR antagonists.¹¹ In addition, recent evidence suggests a dramatic role of β -AR blockers in reducing metastases, tumour

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recurrence and specific mortality in breast cancer patients.¹² More recently, the use of β -blockers for concomitant disease was associated with a reduced risk of progression of thick melanoma¹³ and with an increased survival time of melanoma patients,¹⁴ suggesting that the interaction of catecholamines with β -ARs could be a useful target in this disease.

Expression of β -ARs has been found in melanoma cell lines and in human melanoma biopsies and NE was demonstrated to enhance cytokine production from melanoma cells.¹⁵ However, no detailed information regarding β -ARs expression in human cutaneous benign and malignant melanocytic lesions or catecholamine influence on melanoma cell migration has been provided so far. Our aim was to evaluate the expression of β -ARs on a series of human cutaneous melanocytic naevi and malignant melanoma, and assess the potential influence of NE and E on the malignant behaviour of human melanoma cell lines. We could demonstrate a significant upregulation of β -ARs expression in melanoma *in vivo* and the activation of pro-tumorigenic biological responses induced by NE and E *in vitro*.

MATERIALS AND METHODS

Histologic Samples

Forty human cutaneous melanocytic lesions from 40 different patients were evaluated. Tissue samples were retrieved from the archives of the Division of Pathological Anatomy, Department of Critical Care Medicine and Surgery, University of Florence, Florence, and from the Division of Pathology, Pistoia Hospital, Pistoia, Italy.

The study series included five common melanocytic naevi (CN) (two females, three males, age 28–54 years, mean 35.8 years); five atypical (so-called dysplastic) melanocytic naevi (AN) (two females, three males, age 30–47 years, mean 40.4 years); five *in situ* primary melanoma (PM) (two females, three males, age 37–55 years, mean 44.2 years; site: three trunk, one lower extremity); nine superficial spreading (SS) PM (seven females, two males, age 41–82 years, mean 58.4 years; site: four trunk, three leg, two arm; thickness 0.30–1.90 mm, mean 0.82 mm; five level II, three level III); six nodular (N) PM (three females, three males, age 53–76 years, mean 61.5 years; site: three trunk, one leg, two arm; thickness 1.40–17 mm, mean 5.2 mm; two level III, three level IV, one level V), ten MM, five cutaneous and five lymph-nodal (one female, nine males, age 59–87 years, mean 77.1 years).

Materials

Rabbit polyclonal anti- β 1- or anti- β 2-AR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Chemicon (Temecula, CA, USA), streptavidin-biotin peroxidase complex from Ultravision (LabVision, Fremont, CA, USA), DAKO EnVision System HRP from Dako (Carpenteria, CA, USA). For *in vitro* experiments, unless specified all reagents were obtained from Sigma (St Louis, MO) except PVDF membrane (Millipore, Bedford, MA); Matrigel

(BD Biosciences, Bedford, MA); Diff-Quik staining kit (Medion Diagnostics, Miami, FL); Transwell (Corning Incorporated, Corning, NY); ilomastat (Chemicon International, Bedford, MA); p38 MAPK (mitogen-activated protein kinase), phospho-p38 MAPK (Thr180/Tyr182), p44/p42 MAPK and phospho-p44/p42 (T202/Y204) monoclonal antibodies (Cell Signalling, Danvers, MA). The Amplite™ Universal Fluorimetric matrix metalloproteinase (MMP) Activity Assay Kit-Red Fluorescence was supplied by AAT Bioquest, Sunnyvale, CA.

Immunohistochemistry

The specimens were obtained by surgical resection in all cases and fixed in 10% formalin before being processed in paraffin. Haematoxylin-eosin stained sections from each histological specimen were reviewed to confirm the histological diagnosis. The protocol was approved by the Institutional Review Board for use of human tissues.

For immunohistochemical analyses, a representative section of 3 μ m for each lesion was selected. All sections were deparaffined in Bio-Clear (Bio-Optica, Mi, Italy) and hydrated with grade ethanol concentrations until distilled water. Antigen retrieval was performed by calibrated water bath capable of maintaining the Epitope Retrieval Solution EDTA (pH 9.0) at 97 °C for 15 min. The sections were then allowed to cool down to room temperature (RT) for 20 min. To block endogenous peroxidase activity, slides were treated with 3.0% hydrogen peroxidase in distilled water for 10 min and subsequently washed two or three times with PBS. Then polyclonal antibodies anti- β 1- or anti- β 2-AR were incubated for 1 h at RT at 1:100 dilution or at 1:30 dilution, respectively. Immunohistochemical analysis was performed using the streptavidin-biotin peroxidase complex for β 2-AR, or DAKO EnVision System HRP for β 1-AR. Finally, aminoethylcarbazole (LabVision) was applied for 5 min as chromogen.

Normal eccrine sweat glands intensely express β 2-AR, and this parameter was used as a positive internal control.¹⁶ Negative control was performed by substituting the primary antibody with a non-immune serum at the same concentration. The control sections were treated in parallel with the samples. The sections were lightly counterstained with Mayer's haematoxylin.

Immunostaining was independently assessed by two observers (DM, SM). Discrepancies in the reading were resolved by a second parallel reading of the slides. The percentage of positive cells per lesion was scored according to semi-quantitative criteria. As the percentage of positive naevus melanocytes/melanoma cells was always higher than 50%, semi-quantitative results were expressed as score 1 (50–80% positive naevus melanocytes/melanoma cells), score 2 (81–90% naevus melanocytes/melanoma cell staining) and score 3 (91–100% melanoma cell staining). The cell staining intensity was scored on a scale as weak, moderate, strong, very strong.

For statistical analysis, non-parametric tests were used to determine significant differences between groups. The distribution of the scored values of positive lesions after immunological staining in each group was the unit of analysis. Groups were: naevi, PM, MM. Statistical evaluation was performed also comparing CN vs AN, naevi vs malignant lesions, and *in situ* PM plus SSPM vs NPM and MM. Differences were assessed using the non-parametric Mann–Whitney *U*-test for independent samples and were considered significant at $P \leq 0.05$.

Cell Lines and Culture Conditions

Human dermal fibroblasts (HDFs) isolated from a surgical explantation taken from healthy patients, human A375 PM cell line and human Hs29-4T MM cell line were cultivated in DMEM supplemented with 10% FCS at 37 °C in a 5% CO₂ humidified atmosphere. All experiments were performed with 70–80% confluent cultures, following 18-h incubation in serum-free culture medium. Cells were then stimulated with NE or E, at 1 μ M concentration. Where needed, cells were pre-treated with unselective β -AR antagonist propranolol (Sigma-Aldrich) (1 μ M). After 1 h, medium was removed and cells were stimulated with NE 1 μ M with or without propranolol 1 μ M. For fibroblast activation, cells were grown to sub-confluence and treated for 24 h with the indicated cytokines. Fresh serum-free medium was added for an additional 24 h before collection of conditioned medium (CM), in order to obtain CM free from cytokines (but conditioned by their earlier administration). HDFs cells were then incubated with the obtained CM for 24 h and then used for western blot analyses.

Western Blot Analysis

Cells were lysed for 20 min on ice in 500 μ l of complete RIPA lysis buffer (50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% NP40, 2 mmol/l EGTA, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin). Lysates were clarified by centrifuging, separated by SDS-PAGE and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA and 0.1% Tween 20 for 1 h at RT and were probed first with specific antibodies and then with secondary antibodies. For chemiluminescence detection, we used Gel Logic 2002 Kodak Imaging System, equipped with a charge-coupled device camera, which guarantees high linearity. Quantity One software (Bio-Rad) was used to obtain quantitative analyses.

Invasion Assay

Transwell system, equipped with 8- μ m pore polyvinylpyrrolidone-free polycarbonate filters, was used. Cells (5×10^4 in 300 μ l) were loaded into the upper compartment in serum-free growth medium with or without 50 μ mol/l ilomastat. The upper sides of the porous polycarbonate filters were coated with 50 μ g/cm² of reconstituted Matrigel

basement membrane and placed into six-well culture dishes containing 1 ml of complete growth medium. After 24 h of incubation at 37 °C, noninvasive cells and the Matrigel layer were mechanically removed using cotton swabs, and the microporous membrane was fixed in 96% methanol and stained with Diff-Quick solution. Chemotaxis was evaluated by counting the cells that migrated to the lower surface of the polycarbonate filters (six randomly chosen fields, mean \pm s.d.).

Real-Time PCR

Total RNA was extracted from Hs29-4T and A375 derived from our experimental conditions using the RNeasy Minikit kit. Total RNA (1 μ g) was reverse-transcribed using the Quantitect Reverse Transcription Kit. Reverse transcription was performed in a final volume of 20 μ l containing reverse transcriptase, real-time buffer 1 \times and real-time primer mix. The amplification was carried out at 42 °C for 2 min, then 42 °C for 15 min and 95 °C for 3 min. Measurement of gene expression was performed by quantitative real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems), using the Qiagen Quantifast SYBR Green PCR kit. For each sample, 1 μ g of cDNA was added to 25 μ l of PCR mix. The samples were then subjected to 40 cycles of amplification at 95 °C for 10 s and 60 °C for 30 s. RNeasy Minikit, Quantitect Reverse Transcription Kit, all primer/probe mixes and Qiagen Quantifast SYBR Green PCR Kit were from Qiagen, except the following primers:

ADRB1 FW: 5'-CAGGTGAACTCGAAGCCCAC-3'
ADRB1 REV: 5'-CTCCCATCCCTTCCCAAAC-3'
ADRB2 FW: 5'-ACGCAGTGCGCTCACCTGCCAGACT-3'
ADRB2 REV: 5'-GCTCGAACTTGGCAATGGCTGTGA-3'
VEGF FW: 5'-TACCTCCACCATGCCAAGTG-3'
VEGF REV: 5'-ATGATTCTCCCTCCTCCTTC-3'
IL-8 FW: 5'-CTGGCCGTGGCTCTCTTG-3'
IL-8 REV: 5'-TTAGCACTCCTTGGCAAAACTG-3'
MMP-2 FW: 5'-ACGACCGCGACAAGAAGTTAT-3'
MMP-2 REV: 5'-ATTTGTTGCCAGGAAAGTG-5'
(NM_00453; Digestive and liver disease 37 (2005) 584–592)
MMP-9 FW: 5'-GACAAGCTCTTCGGCTTCTG-3'
MMP-9 REV: 5'-TCGCTGGTACAGGTCGAGTA-5'
IL-6 FW: 5'-AGTTCCTGCAGTCCAGCC-3'
IL-6 REV: 5'-TCAAACGTCATAGCCACTTTC-3'

Quantitative MMP Activity Assay

MMPs activity was measured with Amplitude™ Universal Fluorimetric MMP Activity Assay Kit according to the manufacturer's instructions. Briefly, serum-free medium from confluent monolayer of cells was collected and 5 μ l were added to 4-aminophenylmercuric acetate (AMPA; 1 nmol/l) at 37 °C for 1 h to detect MMP-2 activity and at 37 °C for 2 h to detect MMP-9 activity. A 50 μ l portion of the mixture was then added to 50 μ l of MMP Red substrate solution. After 60 min of incubation the signal was read by fluorescence

microplate reader with excitation (Ex)/emission (Em) = 540 nm/590 nm.

Statistical Analysis

In vitro data are presented as means ± s.d. from at least three experiments. Results were normalized vs control expression levels. Statistical analysis of the data were performed by Student’s *t*-test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Expression of β-ARs in Tissue Samples

The presence of β-ARs was demonstrated in all melanocytic lesions examined. The staining for β1-AR was confined to the cell cytoplasm in naevus melanocytes and melanoma cells; the reaction for β2-AR was also confined to the cell cytoplasm in all cases, with an additional peripheral membrane pattern in some AN melanocytes and malignant cells. The cell staining intensity was always weak with regard to the reaction for β1-AR, whereas the immunostaining for β2-AR appeared to be weak in CN, moderate (except one very-strong reaction), in AN; moderate or strong in *in situ* PM; from weak to moderate or strong in SSPM, and from strong to very strong in NPM and MM, with no difference between cutaneous and nodal metastasis.

The immunostaining of each lesion taking into account both reaction intensity and percentage of positive cells is shown in Figure 1. In regards to β1-AR expression (Figure 1a), score 1 was evaluated in both CN and AN, score 2 was found in a minority (3 *in situ* and 2 SS) of PM, whereas

in the other PM and MM score 3 was detected. β1-AR expression was significantly higher in malignant than in benign lesions ($P \leq 0.0001$) and in PM or MM than in naevi ($P \leq 0.0001$ and $P \leq 0.0001$). No difference was observed between CN and AN, or between *in situ*/SSPM compared with NPM/MM.

With regards to β2-AR expression (Figure 1b), score 1 was observed in CN, score 2 in AN and score 3 was detected in all PM and MM but one (SSPM), which exhibited score 2. β2-AR reactivity was significantly higher in malignant lesions than in naevi ($P \leq 0.0001$), and in PM or MM, respectively, than in naevi ($P \leq 0.0001$ and $P \leq 0.0001$). AN exhibited a significantly higher reactivity compared with CN ($P \leq 0.003$), and no difference was observed between *in situ*/SSPM and NPM/MM.

In addition, no significant difference was detected between PM and MM for both receptors.

Examples of reactions of melanocytic lesions for β1- and β2-AR are shown in Figure 2. Epidermal keratinocytes were lightly coloured for β2-AR, as previously described.¹⁷ Endothelial and stromal cells exhibited heavy reactivity for β2-AR in malignant lesions, and to some extent, in AN.

Taken together, our data showed that β1- and β2-ARs were variably expressed in human melanocytic lesions with a significant upregulation in PM and MM, and, at least for β2-AR, a significant upregulation was also observed in AN vs CN.

Effects of Catecholamines on Cancer Cell Motility

In order to confirm the correlation between sensitivity to catecholamines and progression towards a malignant phe-

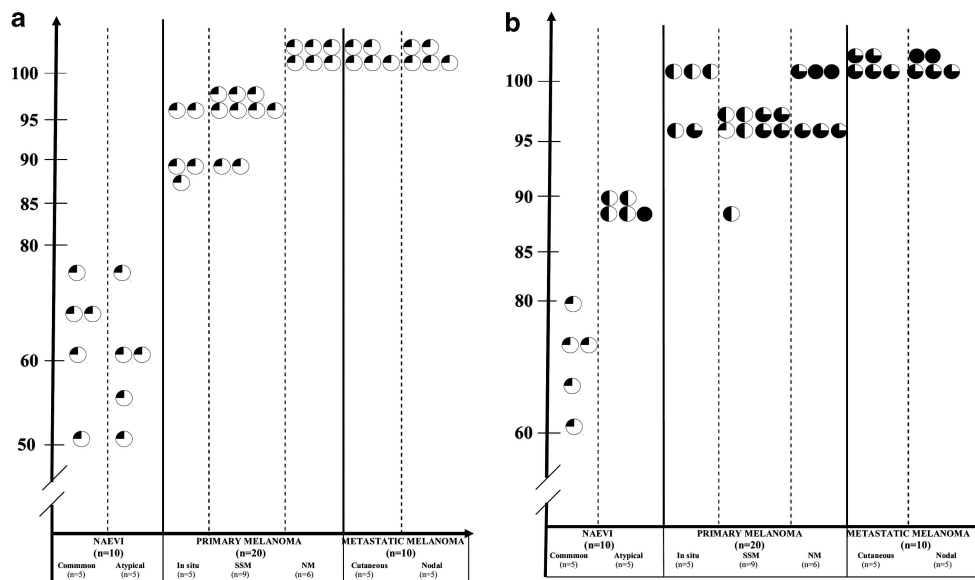


Figure 1 Immunohistochemical expression of β1-AR (a) and β2-AR (b) in cutaneous human melanocytic lesions: percentage of positivity and staining intensity in each lesion. Each circle represents the percentage of stained cells for one lesion. A quarter-black circle indicates positive weak staining; a half-black circle indicates positive moderate staining; a three-quarter-black circle indicates positive strong staining; a solid-black circle indicates very-strong staining.

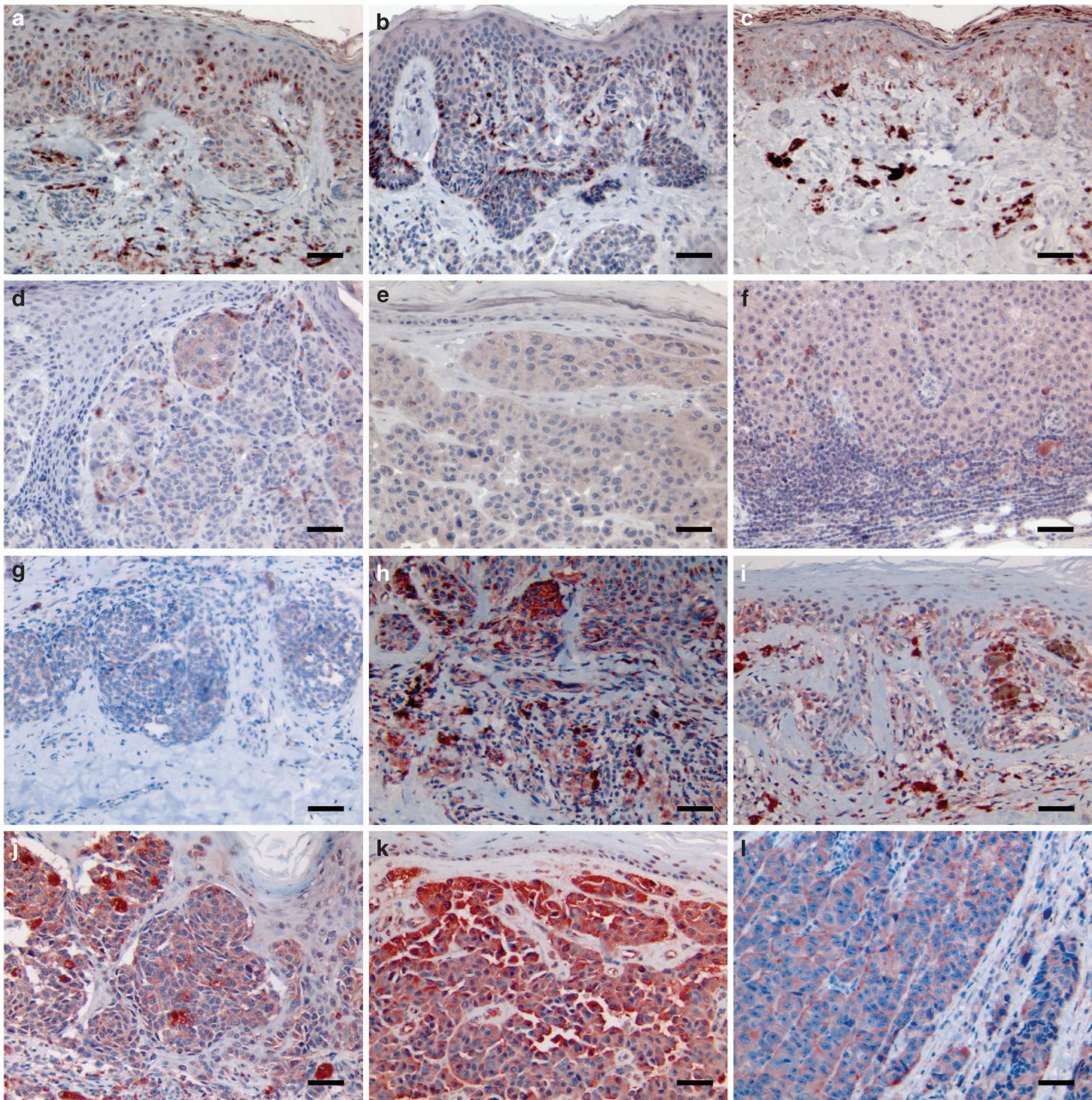


Figure 2 Expression of β 1-AR and β 2-AR in human cutaneous melanocytic lesions. β 1-AR immunostaining in CN (a), AN (b), *in situ* PM (c), SSPM (d), NPM (e), nodal MM (f): all lesions show a low reaction intensity, confined to the cell cytoplasm. β 2-AR immunoreactivity in CN (g), AN (h), *in situ* PM (i), SSPM (j), NPM (k), nodal MM (l): except the weak staining intensity observed in CN, a moderate to strong or very-strong reactivity intensity is detected in all the other lesions. While CN cells show only cytoplasmic staining for β 2-AR, AN and melanoma cells show cytoplasmic and membranous positivity; stromal cells show some reactivity in malignant lesions and AN (Scale bar, 50 μ m).

notype of melanoma cells, we treated with NE or E two human melanoma cell lines, namely Hs29-4T cells, selected from a metastatic lesion, and A375 cells, derived from PM. We observed that both cell lines express low and comparable levels of β 1-AR, as shown in Figure 3, while they both express higher amounts of β 2-AR (Figures 4a and b), with the primary A375 melanoma cell line exhibiting a significantly higher expression of β 2-AR compared with the metastatic

Hs29-4T cell line. Both cell lines are able to respond to catecholamine stimulation with protein kinase A (PKA) phosphorylation, a known trait of β -AR stimulation (Figures 4c and d). In addition, we evaluated the effects of both catecholamines on the MAPK pathways, as few studies until now have investigated the effects of β -adrenergic signalling on these molecular pathways in melanoma models. As shown in Figure 5, both NE and E are able to induce activation,

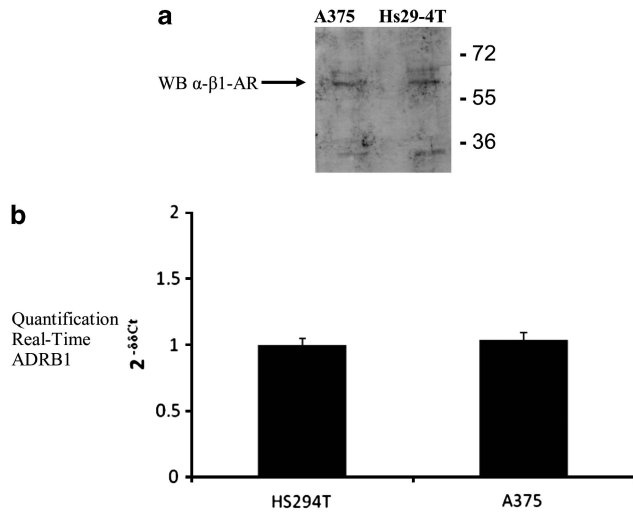


Figure 3 Expression of β 1-AR in melanoma cell lines. (a) Analysis of β 1-AR expression by immunoblot in primary (A375) and metastatic (Hs29-4T) melanoma cell lines. (b) Amount of ADRB1 mRNA by real-time PCR. The amount of target, normalized to the endogenous reference (18S RNA), was given by the $2^{-\Delta\Delta ct}$ calculation and was reported as $2^{-\Delta\Delta ct}$. Both immunoblots and real-time PCR are the mean of three independent assays.

although with different kinetics, of p42-p44 and p38 MAPK in both cell lines (Figures 5a and b).

We then analysed the 3D motility of these cells upon catecholamine stimulation. The invasion assay, carried out by Boyden chambers covered with Matrigel to mimic a 3D barrier, revealed that both NE and E are able to elicit invasive behaviour in both metastatic or PM cells. Both NE and E appear to be more effective in PM cells with respect to MM cells. In addition, E is the most efficient catecholamine in eliciting invasiveness of the metastatic Hs29-4T cell line (Figures 6a and b). In both cell lines the increase in 3D invasiveness is sensible to treatment with ilomastat, a broad range inhibitor of MMPs (Figures 6a and b). The pro-invasive effect of both NE and E is strongly sensitive to propranolol, thereby confirming the involvement of β subtypes of AR. The last finding suggests the involvement of a proteolytic degradation of the Matrigel barrier during invasion. We, therefore, analysed by Real-Time PCR the expression of MMP-2 and MMP-9, the main proteolytic enzymes expressed by Hs29-4T and A375 cell lines, during stimulation with catecholamines. Figures 6c and d reveal that, while NE and E do not influence MMP-9 production, the expression of MMP-2 is increased by NE in A375 PM cells and by E in metastatic Hs29-4T. More importantly, both NE and E are able to maintain a high activation state of secreted MMP-2 and MMP-9 following catecholamines stimulation for 24 h on both cell lines (Figures 6e and f).

Effects of Catecholamines on Cytokine Production and Tumour Microenvironment

Cancer cells secrete many cytokines, chemokines and growth factors that can affect their own aggressiveness, in terms of

proliferation, invasion or survival, as well the reactivity of the surrounding stroma. To address the role of catecholamine stimulation in these features we first analysed the expression of a panel of cytokines/growth factors by real-time PCR. We found that catecholamine stimulation leads to an increase in the expression of VEGF, IL-6 and IL-8 (Figures 7a–c). Interestingly the two catecholamines used show differential effects for VEGF, IL-6 and IL-8 in Hs29-4T and A375 cells. In A375 PM cells both NE or E are able to elicit expression of VEGF, IL-6 and IL-8. Conversely, the Hs29-4T metastatic cell line senses NE to increase expression of IL-6 and IL-8, and E to express VEGF.

The role of VEGF, IL-6 and IL-8 in tumour progression varies from increase in invasiveness/scattering and growth of angiogenic sprouting for VEGF and IL-8, to activation of stromal and/or inflammatory cells for IL-6.^{18–20} We observed that VEGF stimulation increases the invasive spur induced by NE in A375 PM cells, while IL-6 does not have a role (Figure 8a). On the other side, we analysed the ability of IL-6, in association with NE, to activate dermal fibroblasts. We observed that IL-6 is able to activate dermal fibroblasts, as demonstrated by their ability to express α -smooth muscle actin (α -SMA), an acknowledged marker of myofibroblasts.^{21,22} In addition, in dermal fibroblasts, the CM of NE-treated A375 cells elicits an activation state very similar with respect to treatment with IL-6 alone. Conversely, VEGF treatment is almost ineffective in eliciting a reactivity of fibroblasts (Figure 8b).

Taken together, these data suggest that *in vitro* the treatment of human melanoma cells with catecholamines dramatically affects their aggressiveness, inducing expression of MMP-2, VEGF, IL-6 and IL-8. These factors orchestrate a feed-forward loop leading to increase of proteolytic invasiveness of tumour cells, as well as activating surrounding fibroblasts.

Discussion

The present study shows that the immunohistochemical expression of β -1 and β -2 ARs is significantly upregulated in melanoma tissues. Interestingly, all tested melanocytic lesions exhibited some immunoreactivity, suggesting that both benign and malignant lesions can theoretically be affected by catecholamines *in vivo*. However, since β -ARs staining significantly increases in PM and MM compared with melanocytic naevi, it appears that malignant lesions can be more deeply influenced by catecholamines than benign counterparts. The staining intensity for β 2-AR progressively increased from CN, towards AN, to PM and MM, whereas the reaction intensity for β 1-AR was weaker, in all groups of lesions. Such a difference can rely on a different reactivity of the used antibodies, but it is likely that β 1-AR is expressed on naevus melanocytes and melanoma cells of sections at a lower level than β 2-AR. This hypothesis was supported by PCR and western blot analysis of primary and MM cell lines,

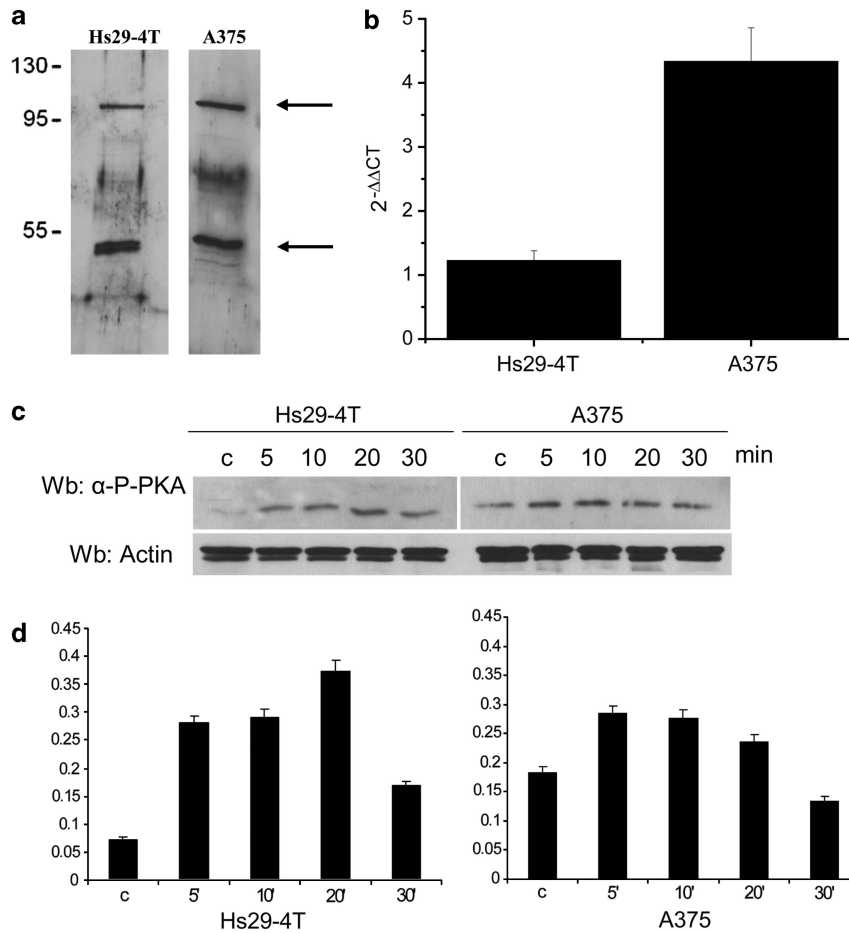


Figure 4 Expression of β 2-AR in melanoma cell lines and analysis of the signalling pathway activated by NE stimulation. (a) Analysis of β 2-AR expression by immunoblot in primary (A375) and metastatic (Hs29-4T) melanoma cell lines. (b) Amount of ADRB2 mRNA by real-time PCR. The amount of target was given by the $2^{-\Delta\Delta Ct}$ calculation and was reported as $2^{-\Delta\Delta Ct}$. Immunoblot and real-time PCR are the mean of three different experiments. (c, d) Analysis of activation of PKA. Melanoma cell lines were serum-deprived overnight and then stimulated with NE (1 μ M) for the indicated period and an immunoblot analysis for the detection of the phosphorylation level of p-PKA was shown. Actin immunoblot was used for normalization. The bar graph below represents the phosphorylation level of PKA in four different experiments. * $P < 0.005$.

both of which exhibited a lower expression of β 1-AR vs β 2-AR.

In melanoma tissue sections a strong reactivity for β 2-AR was also observed in most endothelial and stromal cells, including macrophages, suggesting the possible influence of catecholamines on cells of the tumour microenvironment and the chance of further potential biologic loops capable of affecting metastatic behaviour of neoplastic cells.

We also demonstrated that A375 primary and Hs29-4T MM cell lines respond to catecholamine stimulation enhancing motility and invasion, and producing molecules closely related to neoplastic progression. In keeping, we show that NE and E are able to elicit activation of p42/p44 and p38 MAPKs, acknowledged to have mandatory roles for cell growth, survival and invasive ability, in both primary and metastatic cell lines. This observation is in agreement with the findings of Pak *et al.*,²³ also indicating the Ras-MAPKs

pathway as a target of β -adrenoceptors. These data could be of striking interest in order to find new strategies for melanoma treatment. In fact, Meier *et al.*²⁴ showed that combined targeting of the p42/p44 and Akt signalling pathways significantly inhibited growth and enhanced apoptosis in melanoma cell cultures.

Moreover, NE and E exhibited a diverse stimulation capacity on the two cell lines. Concerning the invasion assay, the primary cell line responded to NE (and, at a lesser degree, to E), while the metastatic cell line showed a clear reaction only to E. The inhibition induced by ilomostat strongly suggests a proteolytic degradation of Matrigel and the probable intervention of MMPs. In fact, we demonstrate that MMP-2, rather than MMP-9, is produced at a significantly higher level compared with baseline, by A375 cells under NE stimulus and by Hs29-4T cells under E stimulation. Furthermore, our data show that both NE and E are able to elicit

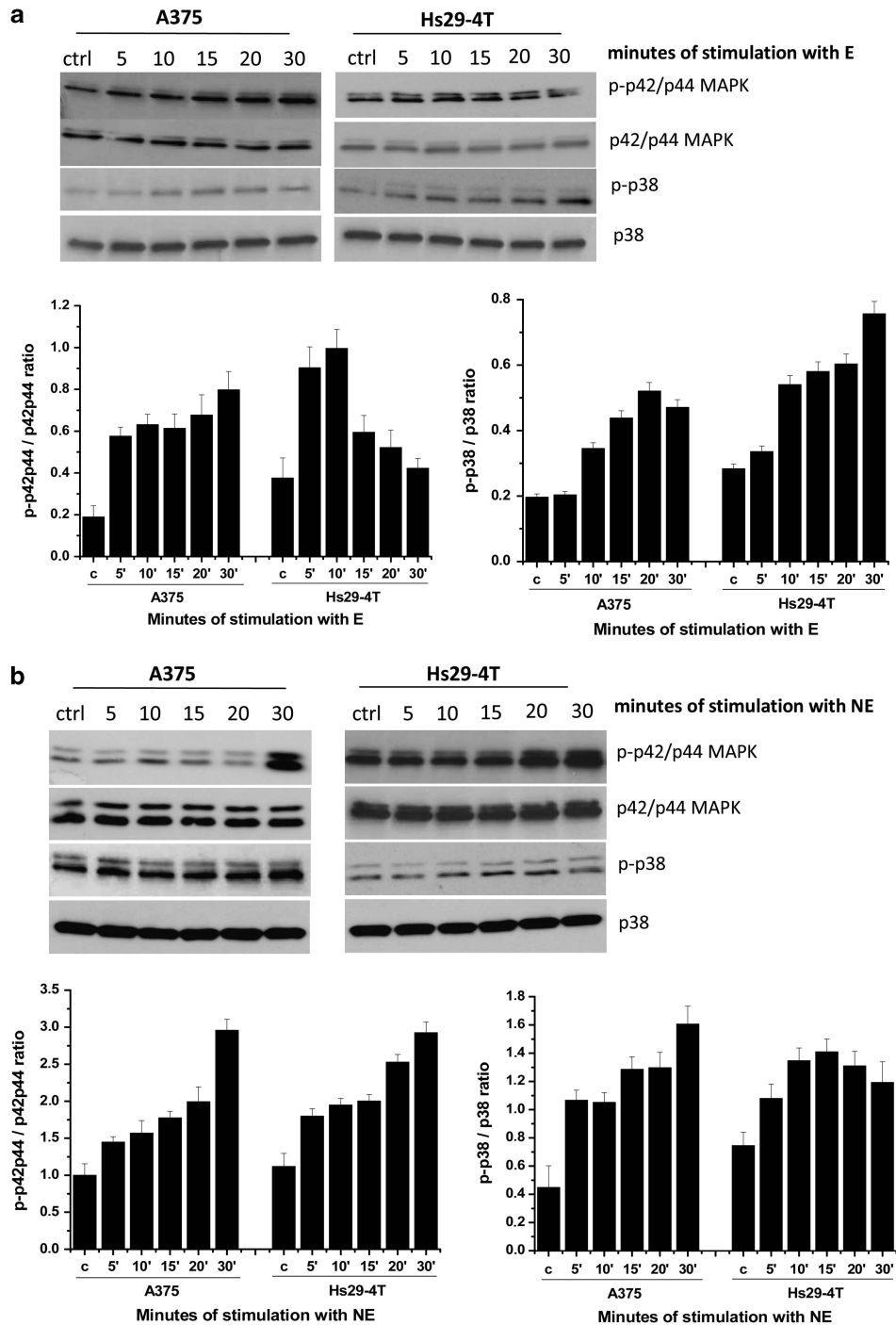


Figure 5 Analysis of activation of p42/p44 and p38 MAPK. Melanoma cell lines were serum-deprived overnight and then stimulated with E (a) or NE (1 μ M) (b) for the indicated period and an immunoblot analysis for the detection of the phosphorylation level of MAPKs were shown. Total p42/p44 and p38 MAPK immunoblot were used for normalization. The bar graphs below represent the phosphorylation level of MAPKs in four different experiments. * $P < 0.005$.

sustained activation of MMP-2 and MMP-9 both in primary and metastatic cell lines. Thus, increased motility of melanoma cells seems to be due to a proteolytic invasive capacity, typical of a mesenchymal phenotype,^{25,26} and catecholamines

seem able to influence MMPs activity both at a transcriptional and at a post-translational level. The migration of neoplastic cells appears to be increased through activation of β -ARs, because it is completely abolished by propranolol.

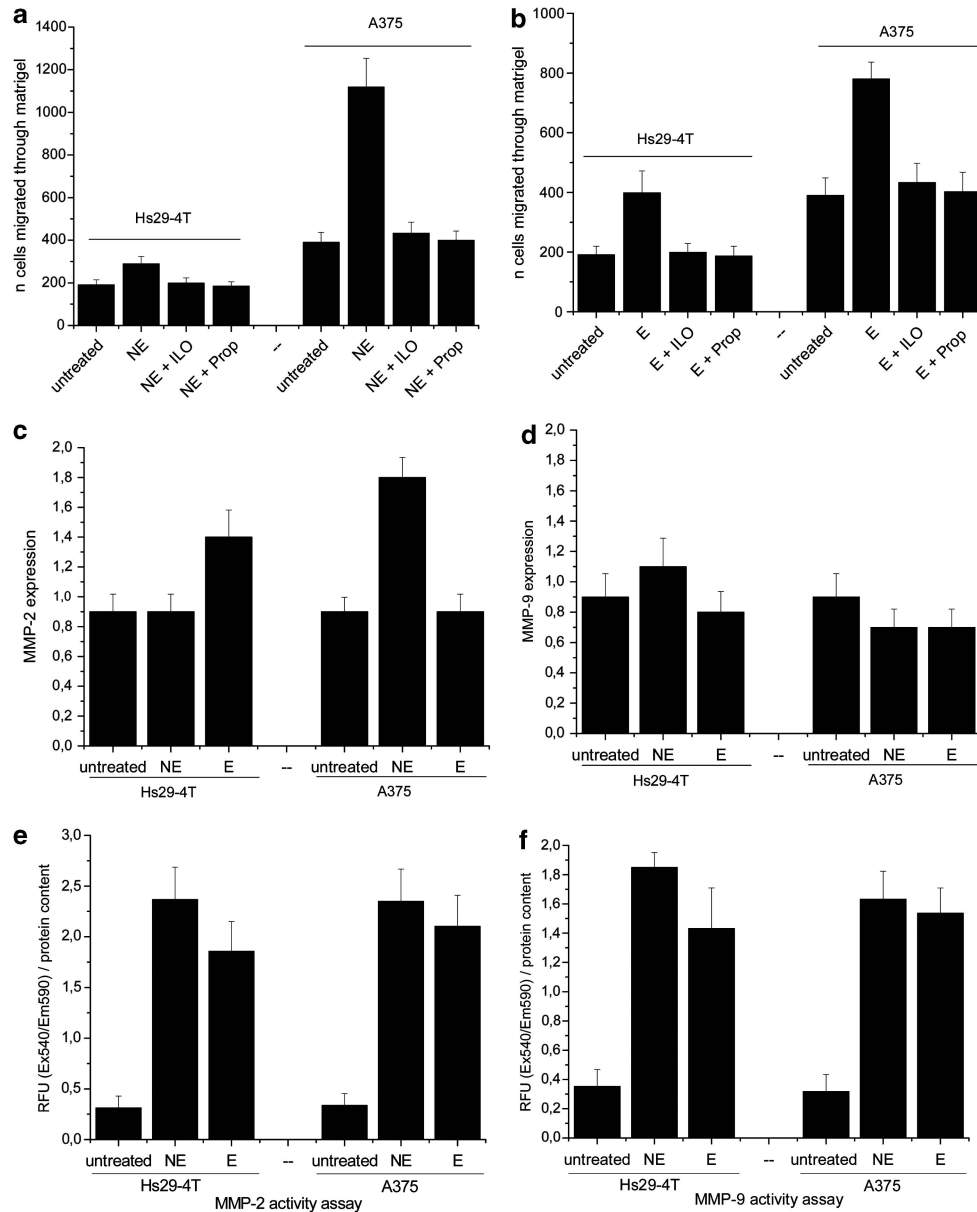


Figure 6 Effects of E and NE stimulation on melanoma cell lines invasion. Melanoma cell lines were serum-deprived overnight and then seeded in the upper Boyden chamber for assay their invasion. (a, b) NE (a) or E (b) ($1 \mu\text{M}$), in the presence or absence of ilomastat ($50 \mu\text{mol/l}$) or propranolol ($1 \mu\text{M}$), were added in the upper Boyden chamber. Bar graphs represent the mean of migrated cells counted in six different fields for each experiment. $*P < 0.005$ vs untreated. (c, d). Expression of MMP-2 (c) and MMP-9 (d) mRNA by real-time PCR. Melanoma cell lines were serum-deprived overnight and then stimulated with E or NE ($1 \mu\text{M}$) for 24 h. The amount of target, normalized to the GAPDH mRNA amounts, was given by the $2^{-\Delta\Delta\text{CT}}$ calculation and was reported as arbitrary units (a.u.). The graphs report data as the mean of three independent assays. (e, f) MMP-2 and MMP-9 activity assay. Melanoma cell lines were serum-deprived overnight and then stimulated with NE or E ($1 \mu\text{M}$) for 24 h. The media obtained were then tested for MMPs activity with a fluorimetric kit, following the manufacturer's instructions (see Materials and Methods). Data are presented as RFU vs concentration of test compounds. The graphs report data as the mean of four independent experiments. $*P < 0.005$.

With regard to the production of cytokine transcripts, A375 cells significantly increase levels of IL-6 and VEGF under NE and E challenge, whereas Hs29-4T cells increase IL-6 expression under NE stimulus, and produce significant amounts of VEGF, especially under E activation. Concerning the expression of IL-8, the primary cell line responded to

both NE and E at a significantly higher degree compared with the metastatic one, and both cell lines reacted more intensely to NE. This result is in agreement with the angiogenic role of IL-8, as *de novo* angiogenesis is particularly useful for primary tumours to escape the hostile microenvironment and disseminate metastasis.²⁷ In addition, the catecholamine-induced

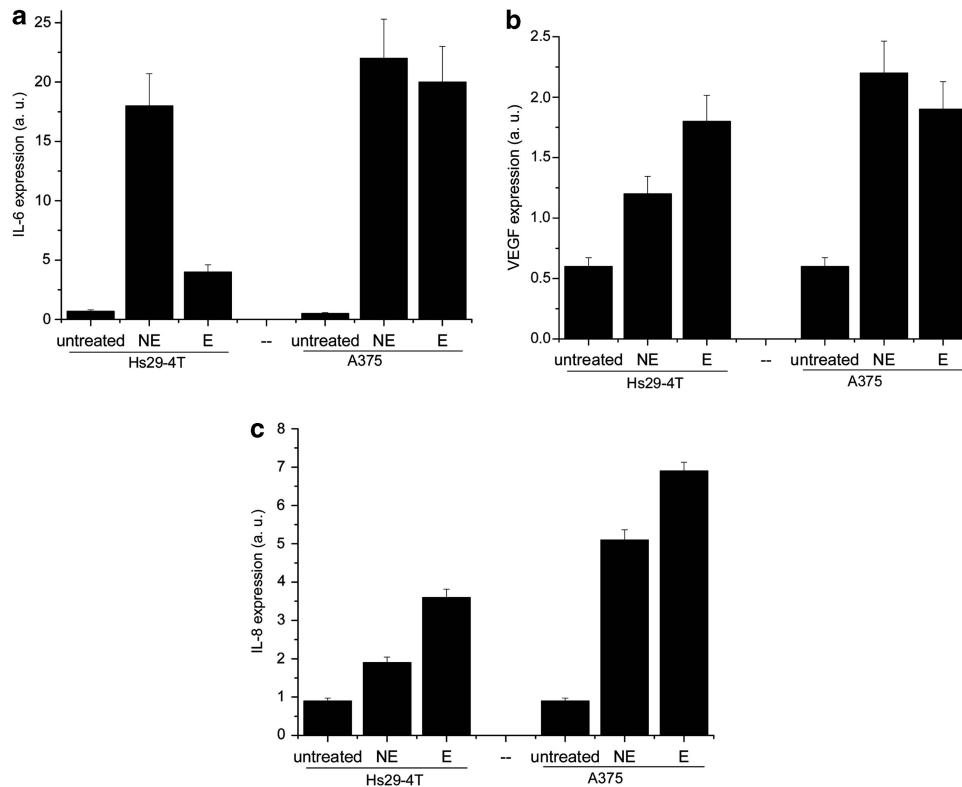


Figure 7 Expression of VEGF, IL-6 and IL-8 in E- and NE-treated melanoma cell lines. Melanoma cell lines were serum-deprived overnight and then stimulated with E or NE (1 μ M). Total RNA was used for the amplification of mRNA of IL-6 (a), VEGF (b) and IL-8 (c), using as housekeeping gene *GAPDH* mRNA. The amount of target, normalized to the *GAPDH* mRNA amounts, was given by the $2^{-\Delta\Delta CT}$ calculation and was reported as arbitrary units (a.u.). The graphs report data as mean of three independent assays. * $P < 0.005$.

IL-8 enhancement is in agreement with the described IL-8 stimulation produced by NE in prostate cancer.¹⁰

We do not know exactly why such a discrepancy exists between primary and metastatic cell line response, but it is possible that at least in part this difference is due to a higher expression of β 2-ARs, assessed as protein and transcript, on the PM cell line. Another possibility is that IL-6 and IL-8, whose expression was associated with early malignancy of melanoma *in vivo*²⁸ are actually secreted more efficiently by a cell line derived from a PM.

In vitro experiments clearly show that catecholamines can augment the malignant behaviour of melanoma cells affecting both invasion capacity and cytokine production.

Our *in vitro* experiments suggest that some pro-metastatic loops could work in melanoma *in vivo* too. We demonstrated that IL-6 and NE in melanoma cells can activate dermal fibroblasts towards a myofibroblastic phenotype, identified by α -SMA expression.²¹ It is well known that stromal fibroblasts within tumours undergo a process, commonly called mesenchymal–mesenchymal transition to myofibroblasts, leading them to achieve a more contractile phenotype and allowing a cross talk with tumour cells dramatically increasing their aggressiveness.^{29,30} In turn, activated fibroblasts can secrete other pro-metastatic cytokines, such as VEGF,³¹

capable of inducing further tumour angiogenesis. In addition, in our experiments, VEGF can increase, particularly when associated with NE, melanoma cell migration and hence invasion capacity.

Previous data support the hypothesis that various types of stress, such as surgical procedure and the immediate post operative period, or neuroendocrine stress due to psychosocial factors, can stimulate tumour progression both in animals and humans.¹⁰ This seems to be true also for melanoma at least in *in vivo* models.^{32,33}

Our work provides evidence that stress hormones like NE and E can significantly stimulate the malignancy of melanoma cells at various levels and that β -ARs, likely involved in this response, are largely expressed in melanoma cell lines and cutaneous melanomas. It is the first time, to our knowledge, that β -ARs are demonstrated in a large series of human cutaneous melanocytic lesions, and even in melanocytic naevi, suggesting a potential influence of catecholamines also in benign counterparts. Moreover, the detection of β 2-AR also in the stromal cells of melanoma microenvironment implies further possible effects of catecholamines on melanoma progression. Consequently, it is possible that the interaction catecholamines- β -ARs could have a dramatic role during the clinical course of melanoma patients. The efforts

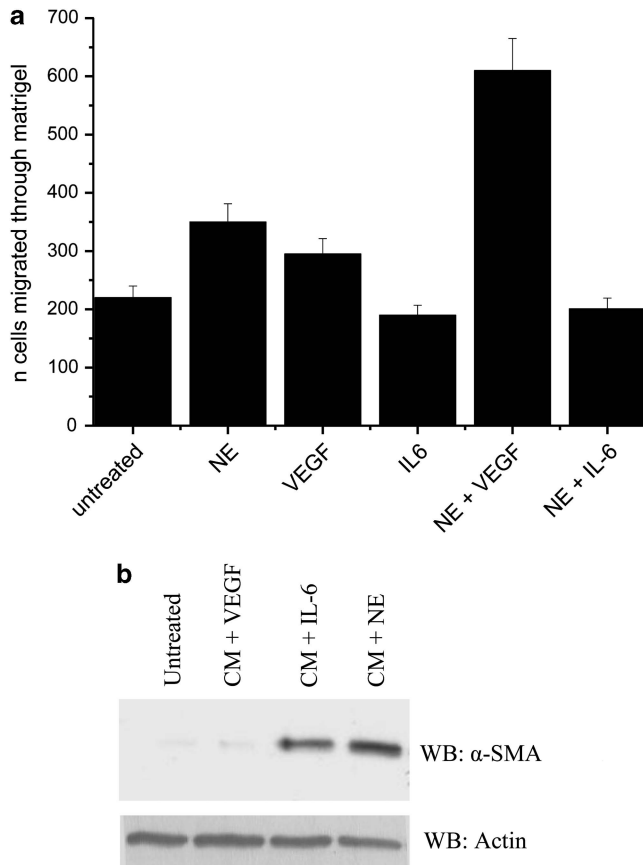


Figure 8 Synergy among NE, cytokines and tumour microenvironment. (a) A375 primary melanoma cells were serum-deprived overnight and then seeded in the upper Boyden chamber for assaying their invasion. NE (1 μ M), IL-6 (50 ng/ μ l), VEGF (20 ng/ μ l), in combination with NE or alone, were added in the upper Boyden chamber. Bar graph represents the mean of migrated cells counted in six different fields for each experiment. * $P < 0.005$ vs untreated. (b) Analysis of human dermal fibroblasts (HDFs) activation state, through evaluation of α -SMA expression, after treatment with conditioned medium (CM) from NE/IL-6/VEGF-treated A375 cells. A375 cells were grown to sub-confluence and treated for 24 h with 1 μ M NE, IL-6 (50 ng/ μ l) or VEGF (20 ng/ μ l). Fresh serum-free medium was added for an additional 24 h before collection of CM, in order to obtain CM free from NE, IL-6 or VEGF (but conditioned by their earlier administration). HDFs were then incubated with the obtained CM for 24 h and then used for western blot analysis of α -SMA expression. Actin immunoblot was used for normalization. The blot is representative of three different experiments.

to understand molecular events underlying such an interaction can be very useful for indicating new targets in therapy.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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*Quando ti metterai in viaggio per Itaca
devi augurarti che la strada sia lunga,
fertile in avventure e in esperienze.*

I Lestrigoni e i Ciclopi

*o la furia di Nettuno non temere,
non sarà questo il genere di incontri
se il pensiero resta alto e un sentimento
fermo guida il tuo spirito e il tuo corpo.*

*In Ciclopi e Lestrigoni, no certo,
nè nell'irato Nettuno incapperai
se non li porti dentro
se l'anima non te li mette contro.*

Devi augurarti che la strada sia lunga.

*Che i mattini d'estate siano tanti
quando nei porti - finalmente e con che gioia -
toccherai terra tu per la prima volta:
negli empori fenici indugia e acquista
madreperle coralli ebano e ambre
tutta merce fina, anche profumi
penetranti d'ogni sorta; più profumi inebrianti che puoi,
va in molte città egizie
impara una quantità di cose dai dotti.*

Sempre devi avere in mente Itaca -

*raggiungerla sia il pensiero costante.
Soprattutto, non affrettare il viaggio;
fa che duri a lungo, per anni, e che da vecchio
metta piede sull'isola, tu, ricco
dei tesori accumulati per strada
senza aspettarti ricchezze da Itaca.*

*Itaca ti ha dato il bel viaggio,
senza di lei mai ti saresti messo
sulla strada: che cos'altro ti aspetti?*

*E se la trovi povera, non per questo Itaca ti avrà deluso.
Fatto ormai savio, con tutta la tua esperienza addosso
già tu avrai capito ciò che Itaca vuole significare.*

(Itaca – Konstantinos Kavafis)