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INSULIN-LIKE-GROWTH-FACTOR-BINDING-PROTEIN-3 (IGFBP-3) CONTRASTS MELANOMA PROGRESSION IN VITRO AND IN VIVO

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INTRODUCTION

Melanocytes

Our body is constantly under threat from external hazards. Skin, the main protection against many such environmental insults, is composed of 3 layers: the epidermis, dermis, and subcutaneous tissue. The main specialized cell types of the outermost layer, the epidermis, are the keratinocyte, the Langerhans cell, and the melanocyte. The melanocyte contains a unique organelle, the melanosome, that produces the pigment, melanin, to provide photoprotection against hazardous ultraviolet radiation (UVR).

Melanocytes are small and nucleated cells that derive embryologically from melanoblasts, cells residing in the neural crest, that from the third to the sixth month of embryonic life colonize various districts of the body (epidermis, hair follicles and ocular uvea). At the skin level, melanoblasts are established in the Basal Lamina, the layer between the dermis and epidermis. Only reaching the right place, melanoblasts can undergo maturation and differentiation in the form of melanocytes beginning the synthesis of melanin.

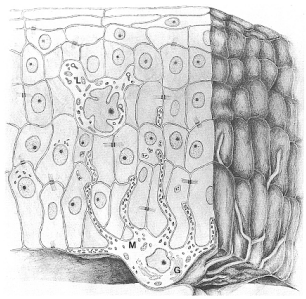


Figure 1. Dendritic extension permits to melanocytes to transfer melanin to the sorrounding cells.

The skin melanocytes (Fig. 1), are equipped with dendritic extensions, through which they can transfer the melanin via exocytosis of melanosomes to the surrounding keratinocytes, the most represented

cells in the epidermal layer.

The biochemical process leading to the production of melanin is called melanogenesis. This process is highly regulated by the interaction with keratinocytes. In response to UV radiation, keratinocytes secrete factors regulating the differentiation and homeostasis of pigmented cells, controlling also their survival, proliferation and motility.

One of the key enzymes of the melanogenesis is the Tyrosinase. This protein mediates the conversion from tyrosine to DOPA-quinone, the limiting step for the production of melanin. For this reason tyrosinase regulation takes place at different levels; transcriptionally, translationally, post-translationally, by its degradation and retention in the endoplasmic reticulum (Ebanks et al., 2009). The dysregulation of any of these processes may cause in humans a number of dysfunctions related to pigmentation, such as albinism. In such dysfunction, the absence of melanin makes the skin extremely sensitive to UV radiation, increasing the possibility of skin cancer.

In certain conditions, melanocytes can escape the adjustment made

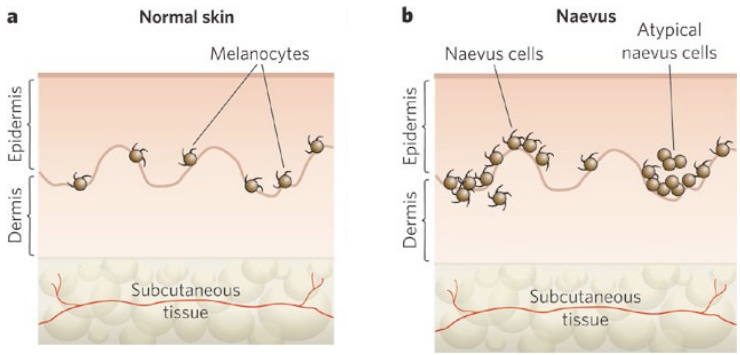


Figure 2. Agglomerates of melanocytes leads to the formation of nevi.

by keratinocytes, leading to the formation of agglomerates called nevi (Figure 2a/b).

These "islands" of melanocytes generally do not represent a pathological condition. However, benign nevi are very often characterized by a subset of mutations, rendering these cells more susceptible to evolve in melanoma malignancy, if crucial genes are affected by additional mutations.

Cutaneous melanoma

Melanocytes can undergo tumoral transformation giving rise to the malignancy known as Melanoma. Usually, but not always, melanoma is a cancer of the skin (cutaneous melanoma), since in this tissue is present the highest concentration of melanocytes in the body. However, Uveal and Mucosal melanoma represent two different categories of melanoma affecting the eyes and mucosal tissues such as the oral cavity. Cutaneous melanoma represents an aggressive and lethal form of skin cancer whose incidence is rising worldwide.

The growth of a melanoma follows several steps that determine its oncogenic potential. Initially, the expansion of the tumor is limited to the basal lamina (the so-called Radial Growth Phase, RGP: Figure 3). This condition may predispose these cells to evolve in an intra-epidermis lesion characterized by a local microinvasion into the dermis. In this stage a metastatic potential is acquired and iper-proliferating cells may progress to the Vertical Growth Phase (VGP: Vertical Growth Phase) culminating in the formation of proximal or distal metastasis (Meier F *et al*., 1998) (Figure 3). The production of

autocrine growth factors and the loss of adhesion receptors contribute to the destruction of the intracellular signaling of melanocytes.

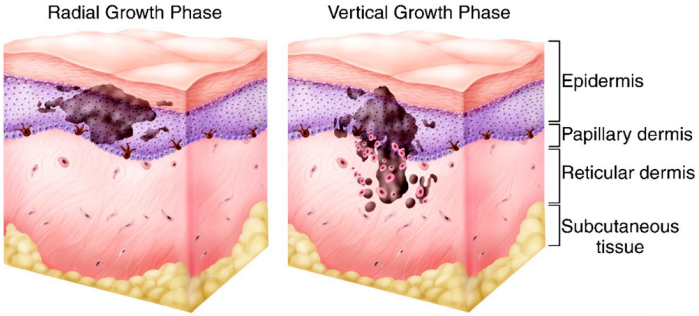


Figure 3. Radial growth phase, characterize primary melanoma growth while the vertical one permits the dermal invasion of metstatic cells.

Melanomas fall into four major histological subtypes :

1 . Superficial spreading melanoma (SSM): is the most common (approximately 70% of cases) and tends to occur on sun-exposed skin. It is characterized by a radial growth phase that lasts from 1 to 5 years, followed then by a vertical growth phase.

2 . Lentigo Malignant Melanoma (LMM): represents 5% of melanomas. LMM are generally located on the chronically sun damaged skin such as the face, head and neck and forearms. It originates from a melanoma in situ (lentigo maligna) that can persist for many years before moving to a vertical growth phase.

3 . Acral Lentiginous Melanoma (ALM) occurs on non-hair-bearing surfaces of the body, which may or may not be exposed to sunlight. It is also found on mucous membranes. Unlike other forms of

melanoma, acral lentiginous melanoma does not appear to be linked to sun exposure. It is the most common subtype in people with darker skin.

4 . Nodular Melanoma (NM) is the most aggressive form of melanoma. It tends to grow more rapidly in thickness (penetrate the skin) than in diameter. Instead of arising from a pre-existing lesion, it may appear in a spot where it did not previously exist. It represents 15-30% of melanomas.

The first three types start in situ and then sometimes become invasive. The NM is considered as a separate category because it is invasive from the outset and presents without a significant radial growth phase. These characteristics make NM difficult to detect.

Most melanomas occur as isolated lesions localized anywhere on the skin; invasive ones are much more serious, since they are able to penetrate deeper into the skin and may expand to other areas of the body: lungs, liver and brain are the favorite sites for metastases.

In 2002, the American Joint Committee on Cancer (AJCC) has proposed a clinical staging system for melanomas based on the classical TNM system, considering the thickness of the primary tumor (Breslow thickness) and the depth of invasion (Clark level). This new system assesses the degree of malignancy according to two different parameters:

A. Clark level of invasion: the skin breaks down into five levels, from I to V; the higher the level, the greater the malignancy.

B. The Breslow thickness: distinguishes four thicknesses measured with an ocular micrometer. The higher the thickness in millimeters, the greater the degree of malignancy.

The choice of a treatment for malignant melanoma depends greatly on its stage.

MOLECULAR PATHWAYS INVOLVED IN MELANOMA

Melanocytic transformation occurs by sequential accumulation of genetic and molecular alterations (Miller *et al*., 2006; Wolchok JD *et al*., 2007). Although the molecular mechanisms underlying melanoma development are still largely unknown, many genes and metabolic pathways have been described to be altered in melanoma.

The first step required for melanocytic transformation is a cellular or molecular change that is clonally inherited and contributes to the potential pathology. Genetic or epigenetic insults generally are the main target for an oncogenic activation. The result of such a change would be the generation of a melanocytic clone with a growth advantage over surrounding cells. Through many years of studies, numerous signaling pathways have been characterized to generate or contribute to this clonal selection, such as those inducing the cell proliferation (proliferative pathways) or bypassing the cell senescence (senescence pathway). Moreover, a secondary event, required for the progression to the advanced phase of melanoma development is to prevent the apoptotic responses (apoptotic pathways). More in detail, two of the most frequently dysregulated kinase cascades in human cancer are the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways (Figure 4)(Liu P et al., 2009; Santarpia et al., 2012.).

Such pathways represent a key mechanisms of signal transduction, regulating the proliferation and survival of cells, in response to the stimulation by growth factor receptors. The alteration of downstream components of these signaling cascades is frequently altered in cancer, thus contributing to tumorigenesis and resistance to anticancer therapies (McCubrey JA et al., 2011). Considering their

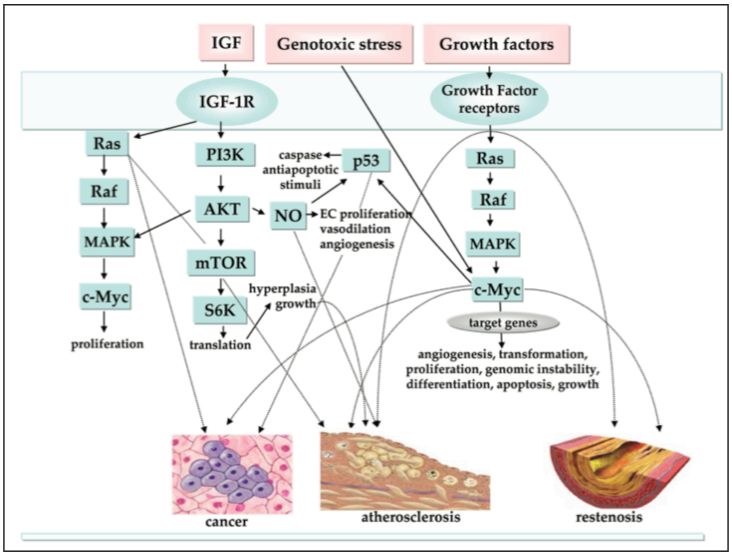


Figure4. MAPK and PI3K pathways represents one of the most important stimolous for sustaining cancer development.

importance, the future pharmacology is oriented in generating specific drugs, in order to specifically inhibit components of both pathways, counteracting the insurgence of mechanisms of resistance.

RAS/RAF/MAPK/ERK

The mitogen activated protein kinase (MAPK) is an extensively studied signal transduction pathway in molecular oncology (Fig. 5). Being implicated in the control of physiologic processes such as cell

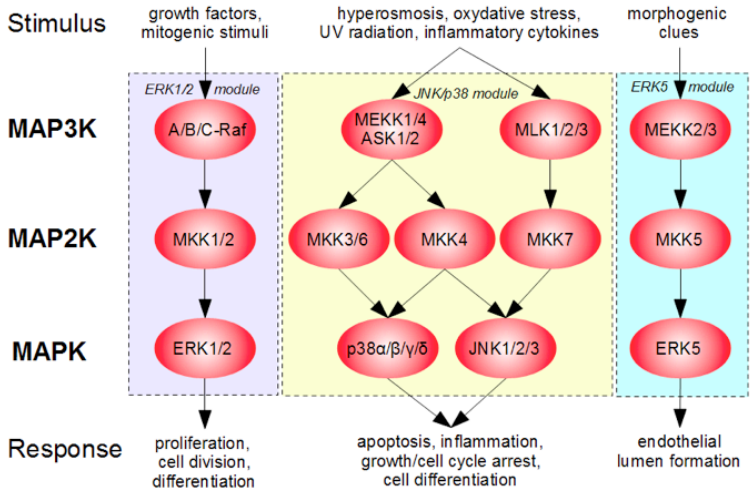


Figure 5. Simplified overview of mammalian MAPK cascades.

growth, survival, and invasion, its alteration is involved in the onset of a broad spectrum of cancers. MAPK plays a crucial role in melanoma development and is the primary subject of molecular studies since the discovery of frequent activating mutations of BRAF kinase in both melanomas and nevi (Pollock PM et al., 2003; Davies H et al., 2002). This signaling initiates at the cell membrane via activation of RAS GTPase through different receptor tyrosine kinases (RTKs), binding ligand or integrin adhesion to extracellular matrix. (Giehl K. et al., 2005; Campbell PM. et al., 2004). The active form of RAS (GTP-RAS), efficiently transduces the signal to

effector proteins or crosstalks with other signaling pathways, modulating cell proliferation, differentiation and survival. (Giehl K. et al., 2005). RAF and phosphatidylinositol 3-kinase (PI3K) represent the two most important effectors activated by RAS. In particular, RAF activation is the sufficient and necessary event to continue the MAPK phosphorylative cascade. (Marshall CJ et al., 1994). A-RAF, B-RAF, and C-RAF are translated from unique genes and represent the three isoforms of RAF, differentially expressed in cells and tissues, activating the MEK substrates. (Beeram M et al., 2005). In turn MEK, activates ERK, the downstream effector of the MAPK, which releases proliferative or survival signals through phosphorylation of a variety of targets: cytoplasmic proteins such as ribosomal S6 kinase (p90rsk) or proapoptotic bcl-2 interacting mediator of cell death (BIM) (Kohno M. et al., 2006; Panka DJ. et al., 2006); cytoskeletal proteins, such as microtubule-associated proteins 2 and 4 (MAP 2/4) (Panka DJ. et al., 2006); nuclear transcription factors, such as c-MYC, c-FOS, and hypoxia-inducible factor-1 alpha. (Panka DJ. et al., 2006; Sridhar SS. et al., 2005).

BRAF and Melanoma

RAF has been identified as a proto-oncogene many years ago (Rapp UR. et al., 1983). In recent years, the identification of activating RAF mutations in a large percentage of melanomas has led to considering this kinase as a crucial factor for melanoma development. (Davies H. et al., 2002). Strikingly, BRAF somatic missense mutations have been identified in 66% of malignant melanomas; 80% of these mutations consist in a single substitution (V600E) within the kinase domain (Davies H. et al., 2002; Brose MS et al., 2002; Pollock PM et

al., 2002). The kinase activity of this mutant is increased over 10-fold respect to wild-type BRAF. (Davies H et al., 2002). Interestingly, further studies revealed that a BRAF-activating mutations is also present in up to 82% of benign nevi, suggesting that activation of the MAPK pathway is a necessary but not sufficient event for melanomagenesis. (Pollock PM et al., 2003). This has also been confirmed by experiments in animal models. (Luo C et al., 2013).

Considering that BRAF mutation have been observed also in other malignancies as papillary thyroid carcinoma (69%) (Cohen Y et al., 2003; Nikiforova MN. et al., 2003), where they were associated with a worse prognosis (Xing M. et al., 2005), whether UV radiation has indeed a role in generating BRAF mutation is still a matter of debate (Davies H et al., 2002; Albino AP. Et al 1989).

Although BRAF mutations in nevi are considered a marker of melanoma susceptibility, their importance in nevi and melanomas remains unclear. Histopathologic analysis suggest that most melanomas evolve de novo, without a precursor melanocytic lesion. (Bevona C. et al., 2003).

Ras and Melanoma

RAS is an upstream factor of the MAPK pathway commonly deregulated in human cancer (Giehl K. et al., 2005). Activating mutations of RAS typically occur at codons 12, 13, or 61, maintaining it constitutively active (Barbacid M. et al., 1987). K-RAS represents the RAS isoform most frequently deregulated in many tumors, while N-RAS is the main isoform important in

melanoma development (van Elsas A et al., 1995; Ball NJ. Et al., 1994). RAS mutations contribute to MAPK activation in 15% of cutaneous melanomas (Albino AP. Et al., 1989; van't Veer LJ. Et al., 1989; Ball NJ. Et al., 1989). A number of in vivo experiments have demonstrated the importance of RAS in transforming murine and human cells. (Campbell PM. et al., 2004). In fact, constitutive RAS expression targeted to melanocytes in p16ink4a/p19arf-null mice leads to cutaneous melanomas with a 60% penetrance (Chin L. et al., 1997). Moreover, activated RAS is required to maintain these melanomas, since its loss leads to the tumor regression (Chin L. et al., 1999).

As it happens with BRAF, the isolated mutation of RAS is also not sufficient to initiate melanomas in mice (Powell MB. Et al., 1995; Chin L. et al., 1997). Interestingly, tumors with constitutively active RAS did not have a high metastatic potential, suggesting that additional oncogenes must be involved in generating a metastatic phenotype. As mentioned before, RAS mutations in human melanoma do not correlate with the degree of sun exposure.

Since BRAF and N-RAS act as activators of the same pathway, BRAF and N-RAS mutations are seldom found together in a same melanoma (Curtin JA. Et al., 2005; Davies H. et al., 2002). Rare coexistent BRAF (V600E) and N-RAS (Q61R) mutants have been described, but the functional significance of this remains unclear (Petti C. et al., 2006; Sense M. et al., 2006).

Pi3k / Akt

The phosphatidylinositol-3-kinase (PI3K) pathway is one of the most commonly altered pathways in human cancer. (Cully M. et al., 2006). Class I PI3Ks are heterodimers of a regulatory p85 subunit and a catalytic p100 subunit. (Hennessy BT. et al., 2005).

The main function of PI3K is to phosphorylate the PIP2 (phosphatidylinositol-4,5-biphosphate) substrates to PIP3 (phosphatidylinositol-3,4,5-triphosphate) (Robertson GP. Et al., 2005)(Fig. 3), to recruit target proteins to the plasma membrane (Cully M. et al., 2006) initiating a downstream signaling upon stimulation of RTK or G-protein coupled receptors. Akt (protein kinase B), is one of the main target of PIP3, with multiple functions and regulating a number of cellular processes, and is a commonly deregulated kinase in cancer. (Robertson GP. Et al., 2005) (Fig. 6).

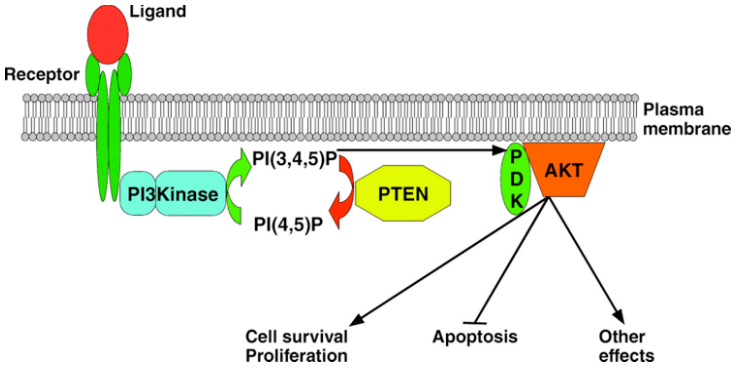


Figure 6. AKT represents one of the main target of the PI3K pathway

PIP3 is negatively regulated by PTEN (Phosphatase and tensin homolog deleted on chromosome 10) through dephosphorylation of PIP3 and is considered an important oncosuppressor. (Hennessy BT.

et al., 2005). The PI3K pathway interacts with many other pathways in the cell and controls diverse cellular processes such as proliferation, apoptosis, cytoskeletal rearrangement, and tumor cell chemoresistance. (Hennessy BT. et al., 2005; Cully M. et al., 2006).

Interestingly, RAS is able to crosstalk with, and to activate, the PI3K signalling.

Although PI3K represents the second main disregulated pathway in melanoma, its genetic alterations are not common. Amplification of components of the PI3K complex or PIK3CA mutation (p110 alpha subunit of PI3K) are detected in less than 1% of primary melanomas and 3% of melanoma metastases (Omholt K et al., 2006). However, PI3K inhibitors as Wortmannin and LY294002 exert antitumor activity in vitro, inhibiting proliferation and sensitizing cell lines to chemotherapy and radiation treatment (Hennessy BT. et al., 2005).

Akt is closely related to PKA and PKC, consisting of a kinase domain, a pleckstrin homology domain, and a regulatory tail (Hennessy BT. et al., 2005 Robertson GP et al., 2005). Once active, Akt phosphorylates a number of substrates, including MDM2, nuclear factor-B, mammalian target of rapamycin (mTOR), bcl-associated death promoter, human telomerase reverse transcriptase (hTERT), and p27, which promote cell survival, proliferation, and invasion (Cully M. et al., 2006; Robertson GP et al., 2005). Akt3 is the major isoform deregulated in melanoma (Stahl JM et al., 2004). Although no activating mutations of Akt3 have been reported in primary melanomas, overexpression has been seen (Stahl JM et al., 2004).

Targeted decrease of Akt3, either using small interfering RNA or via increased activation of PTEN, stimulated apoptosis of melanoma cell

lines, indicating a prosurvival function of Akt in melanoma (Stahl JM et al., 2004). There have been several studies of Akt expression in melanoma. Using IHC, phospho-Akt was detected in 54% of nevi, 71.3% of primary melanomas, and 71% of melanoma metastases, (Slipicevic A et al., 2005) and increased expression was seen in severe dysplastic nevi and metastatic melanomas versus benign nevi (Stahl JM et al., 2004). Increased phospho-Akt expression in melanoma is associated with tumor progression and lower survival rate (Meier F et al., 2005).

PTEN is a tumor suppressor gene that has many functions. It inhibits the MAPK pathway, (Slipicevic A et al., 2005) causes cell cycle arrest by upregulating p27, (Wu H et al., 2003) increases cell migration through dephosphorylation of focal adhesion kinase (FAK) and loss of focal adhesion formation, (Slipicevic A et al., 2005) up-regulates proapoptotic proteins including caspases and BH3 interacting domain death agonist, and downregulates antiapoptotic proteins such as bcl-2. (Wu H et al., 2003). Ectopic expression of PTEN in PTEN-deficient melanoma cells suppresses cell growth, inhibits colony formation, and reduces tumorigenicity and metastasis in mice. (Wu H et al., 2003) PTEN germline mutations result in Cowden disease, an autosomal dominant cancer predisposition syndrome (Slipicevic A et al., 2005) in which there is not an increased risk of melanomas (Goel VK et al., 2006).

Loss of tumor suppressor genes on chromosome 10 (including PTEN) is involved in 30% to 60% of noninherited melanomas, (Stahl JM et al., 2003) and loss of PTEN expression is seen in 30% to 50% of melanoma cell lines and 5% to 20% of primary melanomas (Wu H et al., 2003). PTEN somatic mutations are seen in melanomas (Wu H et al., 2003), where they occur in association with activating mutations in BRAF but not N-RAS (Tsao H et al., 2004). This is

consistent with the ability of N-RAS to activate both the PI3K and MAPK cascades, abrogating the need for specific inactivation of PI3K.

In the recent evaluation of genomic alterations in primary melanomas, tumors with BRAF mutations had fewer copies of PTEN than those with N-RAS mutations, suggesting that dual activation of the PI3K and MAPK pathways are important events in melanoma development. (Curtin JA et al., 2005). A recent study suggests that epigenetic silencing of PTEN through promoter methylation may also be an important means of inactivating this pathway in melanoma. (Mirmohammadsadegh A et al., 2006).

IGF System

The activation of two intracellular pathways, the Ras-Mek-Erk and the PI3K, are stimulated by the same receptor tyrosine Kinase located on the cell surface, which can bind several growth factors, among which the insulin-like growth factors (IGF-I And IGF-II) (Fig. 4) (Siddle K et al., 2001).
The IGF system plays critical roles in somatic growth in a endocrine fashion as well as in the proliferation and differentiation of normal and malignant cells in a paracrine-autocrine fashion. (Stewart CE et al., 1996).
The established components of the IGF system include GH, IGF-I-II peptides, type I and II IGF receptors, IGF binding protein (IGFBPs), and IGFBP proteases (Fig. 7).

GH is the major regulator of IGF synthesis in the liver. GH binding to the hepatic GH receptor stimulates IGF-I synthesis and release from the liver, and IGF-I is transported to the main target organs via circulation to act as an endocrine factor (Butler AA et al., 2001). Circulating IGF-I further exerts negative feedback on the somatotropic axis and suppresses the release of GH from the pituitary.

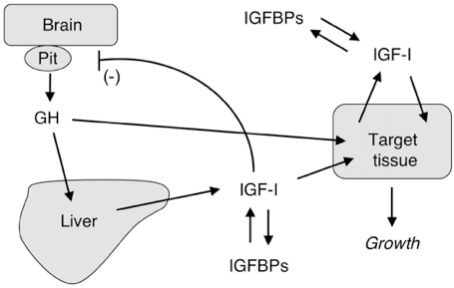


Figure 7. The GH/IGF system.

IGF-I/II peptides share approximately 50% homology to insulin. They are ubiquitously expressed, highly homologous small peptide hormones of approximately 7 kDa molecular mass with multiple endocrine and paracrine/autocrine activities (Stewart CE et al., 1996).

Most circulating IGF-I is produced by the liver and is responsible for growth and development. (Stewart CE et al., 1996). IGFs interact with specific cell surface receptors, designated type I and type II IGF receptors, and can also interact with the insulin receptor (IR). The type I IGF receptor (IGF-IR) is a transmembrane heterotetramer consisting of two α-subunits and two β-subunits. (Siddle K et al., 2001). There is approximately 60% sequence homology between IGF-IR and IR (Sjo¨ gren K et al., 1999). IGF-IR, like IR, possesses intrinsic tyrosine kinase activity. IGF-II and insulin also bind to IGF-IR but with 2- to 15-fold and 1000-fold lower affinity, respectively (Sjo¨ gren K et al., 1999).

The type II IGF receptor (IGF-IIR), which is identical to the cation-independent mannose-6-phosphate receptor, binds IGF-II with 500-fold increased affinity over IGF-I (Siddle K et al., 2001). IGF-IIR does not bind insulin. Most of the biological actions of IGF-II are thought to be mediated via IGF-IR (Siddle K et al., 2001). IGF-IIR is known to function primarily as a scavenger receptor, regulating the internalization and degradation of extracellular IGF-II, thus regulating the circulating IGF-II levels.
IR does not bind IGF-I and IGF-II with high affinity. However, the IR isoform and the IR-IGF-IR hybrid bind IGFs as well as insulin. IR-A, the IR fetal isoform, binds not only insulin but also IGF-II. These IR isoforms and hybrid receptors mediate certain actions of IGFs and insulin (Belfiore A et al., 2007).

Insulin-Like-Growth-Factor-Binding-Proteins.

A total of seven high-affinity IGF-binding proteins have been identified: IGFBP-1 through IGFBP-7 (Walker GE et al., 2004). Hepatic IGF-I circulates almost entirely (about 99%) bound to IGFBPs. IGFBP-3, a major IGFBP species in circulation, binds 75 to 90% of circulating IGF-I in a large ternary complex consisting of IGFBP-3, acid-labile subunit (ALS), and IGF. (Phillips LS et al., 1998). It has been postulated that circulating IGFBP-3 originates in the liver and is regulated by GH based upon the presence of a putative GH-response element in IGFBP-3 gene. (Phillips LS et al., 1998; Albiston AL et al., 1995). However, other findings demonstrated that GH administration had no effect on the expression of hepatic IGFBP-3 mRNA but increased circulating IGFBP-3 in human subjects, due to increased formation of the ternary complex (Olivecrona H et al., 1999). ALS is produced in the liver as a direct

effect of GH. The ALS stabilizes the IGF–IGFBP-3 complex, reduces the passage of IGF-I to the extravascular compartment, and extends its half-life (Domene´ HM et al., 2005).
It has become clear that IGFBPs 1–7 have intrinsic biological activity (IGF/IGFIR- independent actions) in addition to their actions to bind IGFs and sequester the active hormone, thereby reducing IGF biological activity (IGF/IGF-IR-dependent actions). (HwaV,OhY et al., 1999; Walker GE et al., 2004).

Structure of IGFBP-3

IGFBP-3 is a multifunctional protein that is found to play a variety of roles in circulation, in the extracellular environment, and inside the cell. Mature deglycosylated human IGFBP-3 has a molecular mass of 28.7 kDa and is comprised of 264 amino acids. The primary structures of mammalian IGFBPs contain three distinct domains of roughly equivalent sizes, with additional critical subdomains or functional motifs within each major domain that contribute to their diverse actions.

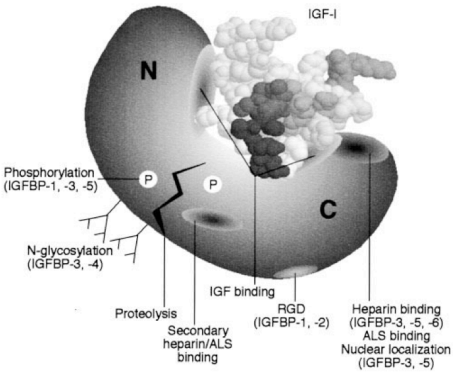


Figure 8. IGFBPs share a common structure and posses an high variable regulatory mid-region.

In addition to the structural and sequence homology among IGFBPs (Fig. 8), some IGFBPs

possess distinctive characteristics such as integrin recognition sequences in IGFBP-1 and IGFBP-2; phosphorylation on serine residues of IGFBP-1 and IGFBP-3; heparin binding motifs in IGFBP-3, -5, and -6; and nuclear localization sequences in IGFBP-3 and IGFBP-5. These distinctive characteristics could be important for the ability of IGFBPs to modulate IGF/IGFIR- dependent actions as well as IGF/IGF-IR-independent actions. (Walker GE et al., 2004).

The conserved N-terminal domain

In the mature IGFBP-3 peptide, the N-terminal third of the protein contains 87 amino acid residues after the signal peptide. IGFBP-3 along with all the other members of the IGFBP superfamily (both high affinity and low affinity IGF binders) share a common IGFBP motif —GCGCCXXC— a conserved N-terminal cysteine rich domain (Imai Y et al., 2000).

IGFBP-3 contains a total of 18 cysteines, 12 of which are located in this domain, which leads to the presence of six disulfide bonds within this domain. Important IGF-binding residues are known to be located within this domain (Buckway CK et al., 2001; Imai Y et al., 2000). Although no other major functional motifs have been identified in the amino-terminal domain, the observation that amino-terminal proteolytic fragments of IGFBP-3 cause IGF-independent inhibition of mitogenesis (Lalou C et al., 1996; Salahifar H er al., 2000) implies the presence of another active subdomain in this region. This subdomain remains to be identified.

The highly variable midregion

The midregion segment of IGFBP-3 contains 95 amino acids. This region separates the N-terminal domain from the C-terminal domain and shares less than 15% similarity with other IGFBPs. (Firth SM et al., 1999). Intriguingly, post-translational modifications (glycosylation, phosphorylation) of the IGFBPs have been found in the midregion, but not in the N- or C-terminal domains.

IGFBPs might be differentially targeted to tissues depending upon both their primary structure and their posttranslational modifications. Thus, glycosylation can affect cell interactions, phosphorylation can affect IGF-binding affinity and susceptibility to proteases, and proteolysis can affect both IGF/IGF-IR-dependent and IGF/IGF-IR-independent actions.(Firth SM et al., 2002).

Three sites of N-linked glycosylation exist in IGFBP-3. Carbohydrate increases the core protein size of IGFBP-3 from 29 kDa to forms estimated to be 40–43 kDa. Of the three potential glycosylation sites at Asn89, Asn109, and Asn172, the first two are always glycosylated, carrying an estimated 4 kDa and 4.5 kDa of carbohydrate, respectively, whereas the third site alternatively contains either undetectable or about 5 kDa of carbohydrate, accounting for the characteristic doublet form of the protein (Firth SM et al., 1999). Other sites of post-translational modification such as potential phosphoacceptor sites also reside in this central domain. Studies conducted by Yamanaka et al. suggest that IGFBP-3 binds to human breast cancer cell surface with typical receptor-ligand interaction and the midregion of the IGFBP-3 molecule is responsible for the interaction.

The conserved C-terminal domain

The C-terminal domain is cysteine rich (six cysteines), with three disulfide bonds within this domain. This IGFBP-3 region is also important in IGF binding (Devi GR et al., 2000; Spencer EM et al., 1995).

Because residues involved in IGF binding are present in both the N-terminal and C-terminal domains, the findings imply the likely existence of an IGF-binding pocket involving both domains. A functionally important 18-residue basic motif with heparin-binding activity has also been identified at residues 215-232. Apart from heparin binding, certain other glycosaminoglycans (Fowlkes JL et al., 1996) as well as cell surface proteoglycans (Smith EP et al., 1994) are bound by this 18-residue basic motif.

Furthermore, IGFBP-3 as well as IGFBP-3-IGF-I complexes bind fibrinogen, fibrin (Campbell PG et al., 1999) and plasminogen (Campbell PG et al., 1998), via this binding domain. The basic region Lys 228-Arg 232 has also been shown to be essential for interactions with the ALS. Additional basic residues that reside within this domain interact with the cell surface and matrix, the nuclear transporter importin-β and other proteins. Singh et al. have also identified a short metal-binding domain in the C-terminal region of IGFBP-3 that binds metals and also has intrinsic effects. Although in vitro studies do not suggest a potential role for iron in IGF-independent biological actions of IGFBP-3, they reveal a dose-dependent effect of iron on IGFBP-3 binding to integrins αv and β1, caveolin-1, and transferrin receptor by unmasking of metal-binding domain epitopes in the IGFBP-3 molecule (Singh et al., 2004).

IGFBP-3 binding to transferrin has been shown to be dependent on a region in the C-terminal domain (Weinzimer SA et al., 2001).

Furthermore, a caveolin-scaffolding domain consensus sequence also resides in this region.

Recent studies have determined three-dimensional structures of IGFBPs including IGFBP-1, -2, -4, and -5 using nuclear magnetic resonance spectroscopy and x-ray crystallography (Kuang Z et al., 2007; Kalus W et al., 1998). These structural analyses not only confirm the previous findings indicating IGF binding sites in the N- and C-terminal domains of IGFBPs but also further reveal that a rigid disulfide bond ladder-like structure and the first five amino acids in the N-terminal domain are critical for IGF binding and masking IGF residues responsible for IGF-I receptor binding.

The C-terminal domain and the midregion of IGFBPs also contribute to inhibiting the interaction between IGF-I and IGF-IR either by directly blocking the IGF-IR-binding region of IGF-I or by steric hindrance. Thus, these structural data provide an understanding of the roles of each domain of IGFBP in enhancing/stabilizing IGF binding and modulating IGF binding to IGF-IR.

Although the structure of IGFBP-3 has not yet been determined, IGFBP-3 should have very similar structures in those critical domains responsible for cooperative binding to IGF and blocking of the IGF-IR binding region of IGF-I.

IGF/IGF-IR-Dependent and IGF/IGF-IR Independent Actions of IGFBP-3

IGF binding to and subsequent activation of the IGF-IR usually results in diverse biological effects in a wide range of cell types, including cellular proliferation and differentiation, an increase in metabolic activity, and cell survival via antiapoptotic pathways.

The IGFBPs are known to modulate the actions of IGFs in the circulation as well as the immediate extracellular environment (Rajaram S et al., 1997). Interestingly, apart from the ability of IGFBPs to inhibit or enhance IGF actions, IGFBPs also exhibit very clear, distinct biological effects independent of the IGF/IGF-IR axis. These IGF/IGF-IR-independent actions contribute to the diversity of biological outcomes due to IGFBPs. IGFBP-3 is a well-documented inhibitor of cell growth and/or promoter of apoptosis. Although its antiproliferative functions are mainly achieved through the attenuation of IGF/IGF-IR interaction (Martin JL et al., 1995), to date much light has been shed on the ability of these bioactivities to occur via IGF/IGF-IR axis-independent means (Schedlich LJ et al., 2000; Oh Y et al., 1993).

The protein inhibitory diffusible factor 45 (now recognized to be IGFBP-3) was originally isolated as a novel inhibitory factor, being able to inhibit the growth stimulation of chick embryo fibroblasts (CEF) in the absence as well as the presence of IGF-I. When bound to IGF-I, serum-induced growth stimulation of CEF was attainable, suggesting two opposing activities for inhibitory diffusible factor 45 (Blat C et al., 1989).

This study was quickly followed by another supporting this hypothesis, where fibroblast-growth-factor-stimulated DNA synthesis in CEF and mouse embryo fibroblasts was inhibited by mouse IGFBP-3 an action independent of IGFs yet attenuated by the presence of IGFs (Liu L et al., 1992). Similarly, the overexpression of human IGFBP-3 in Balb/c mouse fibroblasts resulted in the inhibition of cellular proliferation in the presence/absence of IGFs or insulin (Cohen P et al., 1993).

Although it is clear that IGFBP-3 achieves biological effects independent of the IGF/IGF-IR axis, the mechanisms by which these

effects are achieved are still not entirely understood. Evidence to date suggests the existence of multiple pathways by which IGFBP-3 elicits its proapoptotic and antiproliferative IGF/IGF-IR-independent effects in an array of different cell systems.

On the other hand, IGFBP-3 also has been shown to stimulate cell growth or other cell functions in an IGF independent manner in a variety of cell types. Martin et al. (Martin JL et al., 2003) demonstrated that IGFBP-3 stimulates growth in MCF-10A human breast epithelial cells via increased epidermal growth factor receptor phosphorylation and activation of p44/42 and p38MAPKsignaling pathways. Similar effect of IGFBP-3 was observed in breast cancer cells. Although expression of IGFBP-3 initially inhibited the growth of T47D human breast cancer cells, long-term culture of these cells resulted in growth stimulation due to an enhanced responsiveness of these cells to the proliferative effects of epidermal growth factor (Baciuchka M et al., 1998).

Mitsui Y et al, reported that IGFBP-3 exerts dual effects on human umbilical vein endothelial cells, potentiating doxorubicin-induced apoptosis but enhancing survival in serum-starved conditions. This study further demonstrated that IGFBP-3 antiapoptotic effects were mediated through activation of sphingosine kinase and increased expression of sphingosine kinase 1. These studies indicate that IGFBP-3 enhances cell growth or other cell functions depending on specific conditions. However, the underlying molecular mechanisms involved in these biological actions of IGFBP-3 are largely unknown.

RESULTS

Previous work from the laboratory in which I carried out my PhD project showed that serum levels of IGFBP-3 are lowered significantly in stage-IV melanoma patients (Panasiti V et al., 2011). These results are briefly summarized in Figure 9.

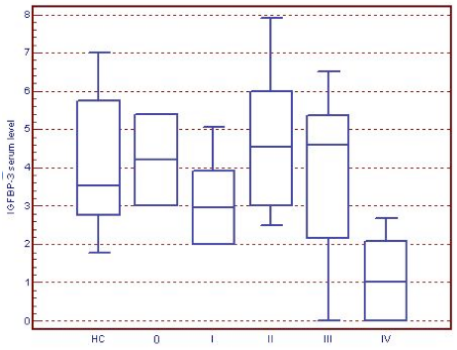


Figure 9. Serum concentration of full-size insulin-like growth factor binding protein (IGFBP)-3 in healthy control subjects (HC) and in patients with melanoma staged from 0 to IV.

Further measurements of the IGFBP-3 levels were done in sera taken from the same patients during the follow-up after the first diagnosis of melanoma. Surprisingly all the patients with progressive disease showed a significant reduction of IGFBP-3 concentrations in the second sample taken after some months, indicating a strong relation between melanoma progression and the drop of the protein.

Moreover in the same work, it was shown that the reduction of IGFBP-3 was due to its degradation, probably by a protease secreted by the tumour itself, whose amount increased with tumour progression.

In this work, the effect of IGFBP-3 on melanoma progression was investigated in vitro and in vivo. The cellular pathways mediating the action of the protein were also investigated.

IGFBP-3 in the tissutal microenvironment.

Considering the previous results, obtained at the systemic level, we firstly decided to determine the IGFBP-3 levels in the tissutal microenvironment. The aim of this analysis was to asses if the progression of primary melanoma to metastatic disease may be

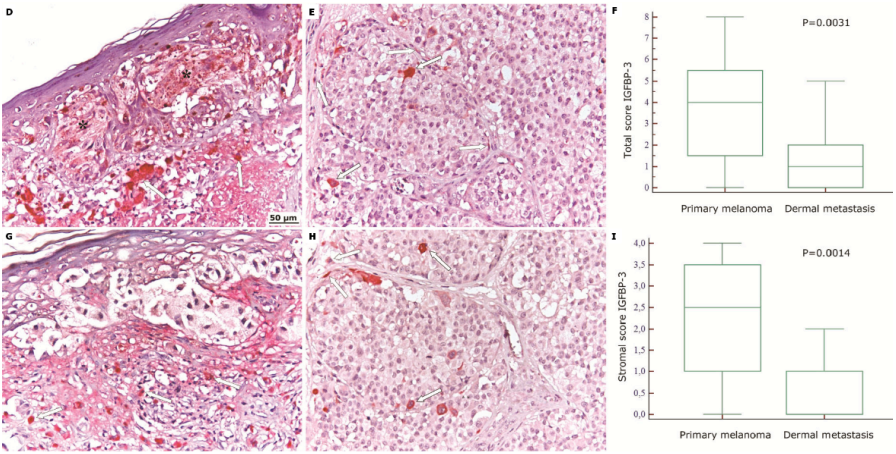


Figure 10. (D,G) IGFBP-3 immunostaining in tissue samples from primary melanomas. (E, H) IGFBP-3 immunostaining in tissue samples from metastatic melanomas. The asterisks indicate positive melanocytes nests in primary melanoma. The arrows indicate IGFBP-3 positive stromal cells. Original magnification, X100. (F,I) Graphic data processing for total (upper panel) and stromal (lower panel) IGFBP-3 immunostaining score.

related to, or influenced by, the presence of IGFBP-3 in the tissues.

Most of the circulating IGFBP-3 is produced by the liver; however, the protein is also secreted by several other inflammatory and mesenchymal cell types and by melanocytes themselves.

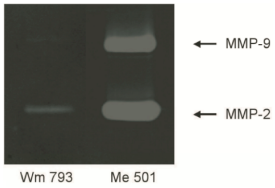


Figure 11. Detection of MMP-2 and MMP-9 activity in the culture media of WM793 and Me501 cells by zimographic assay.

Notably, an inverse correlation has been observed between the amount of IGFBP-3 produced by melanoma tissue and the metastatic capacity of the cells (Dar AA et al., 2010). To evaluate the presence of IGFBP-3 in the immediate environs of the tumour, immuno-histochemical analyses of primary and metastatic tumour samples taken from patients were performed. As shown in Fig.10 (D,G), a diffuse intracellular IGFBP-3 staining was observed in primary melanomas as well as in peri-tumoral stromal cells including monocytes/macrophages, lymphocytes, granulocytes and fibroblasts. In metastatic tumours IGFBP-3 staining was much weaker or absent (Fig.10 E,F,H,I). Previously, we hypothesized that IGFBP-3 loss in both blood and tissues could be accounted for degradation by tumour-produced proteases. This was further investigated. Firstly, secretion of proteases by cultured primary and metastatic melanoma cells lines was evaluated by zymography. As shown in Fig.11, the primary melanoma line WM793 secreted only small amounts of a collagenase (metalloprotease-2) (MMP-2). By contrast, the metastatic cells Me501 produced large amounts of proteases, especially MMP-2 and metalloprotease-9 (MMP-9). Accordingly, the culture media of metastatic, but not primary, cells caused extensive degradation of recombinant IGFBP-3 in vitro. (Fig.12).

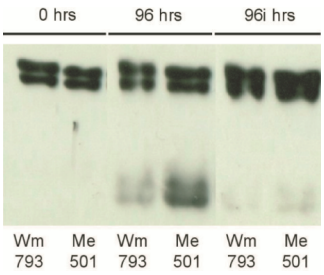


Figure 12. Western blot analysis showing degradation over time of IGFBP-3 by the culture media of WM793 and Me501 cells.

Secondly, tumour samples taken from patients were immuno-stained for both IGFBP-3 and MMP- 9.

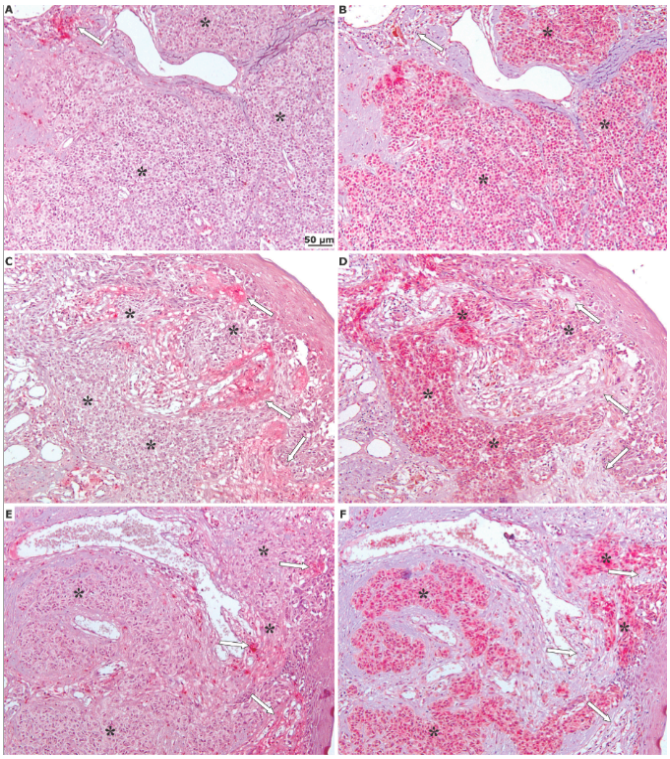


Figure 13. Comparison of the immunohistochemistry for IGFBP-3 (A, C, E) and MMP-9 (B, D, F) in sequential sections obtained from primary melanomas and dermal metastases. The asterisks mark the melanocytes nests while the arrows indicate the stromal tissue. Original magnification X100.

Strikingly, there was an almost perfect inverse correlation between the areas of low or no IGFBP-3 staining and those of high MMP-9

staining (Fig.13), suggesting that the enzyme could indeed have a role in the degradation of IGFBP-3.

IGFBP-3 inhibits motility and invasion in cell cultures of metastatic melanomas.

To investigate the ability of IGFBP-3 to influence the migratory and invasive behavior of metastatic melanoma cells, the effect of the protein was tested in vitro on human (Me501) and murine (B16) metastatic melanoma cell lines. To this end, scratch-repair and trans-well migration/invasion tests were performed in the absence and in the presence of IGFBP-3.

For scratch-repair analysis, the metastatic melanoma cell lines were grown to confluence in 10% serum. After introduction of the scratch, the cells were supplemented with serum-free medium without or with added IGFBP-3 (2 ug/ml) and followed for up to 24 hours. As the control, the same test was performed on the melanoma primary line WM793, which, as expected, had very scarce, if any, migratory capacity.

Strikingly, treatment with IGFPB-3 markedly retarded migration of both human and murine metastatic melanoma cells (Fig.14A, middle rows). Also, addition of anti-IGFBP-3 antibodies to the culture media, to sequester any cell-made IGFBP-3, accelerated scratch repair (Fig. 4A, last two rows). Trans-well-migration/invasion assays revealed that the invasive capacity of metastatic melanoma cells was also strongly impaired by treatment with IGFBP-3, while being enhanced by treatment with anti-IGFBP-3 antibodies (Fig.4B). As

expected, IGFBP-3 had no effect on the primary melanoma cells WM793.

Importantly, the effect of IGFBP-3 on both migration and invasion was independent of cell proliferation. FACS analyses performed on semi-confluent cells did not reveal any difference in cell-cycle distribution between the IGFBP-3 treated and untreated cells. As expected in dearth of serum, both groups underwent cell-cycle arrest after a while, but the timing of the arrest was unaffected by IGFBP-3 treatment (results not shown).

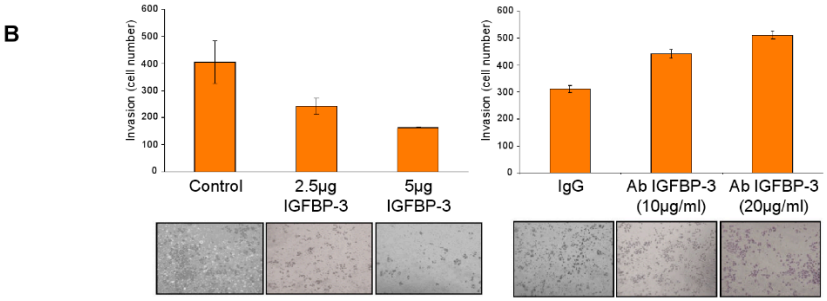


Figure 14B. Trans-well invasion assay with Me501 cell before and after treatment for 48h with increasing doses of IGFBP-3 and anti-IGFBP-3 antibodies.

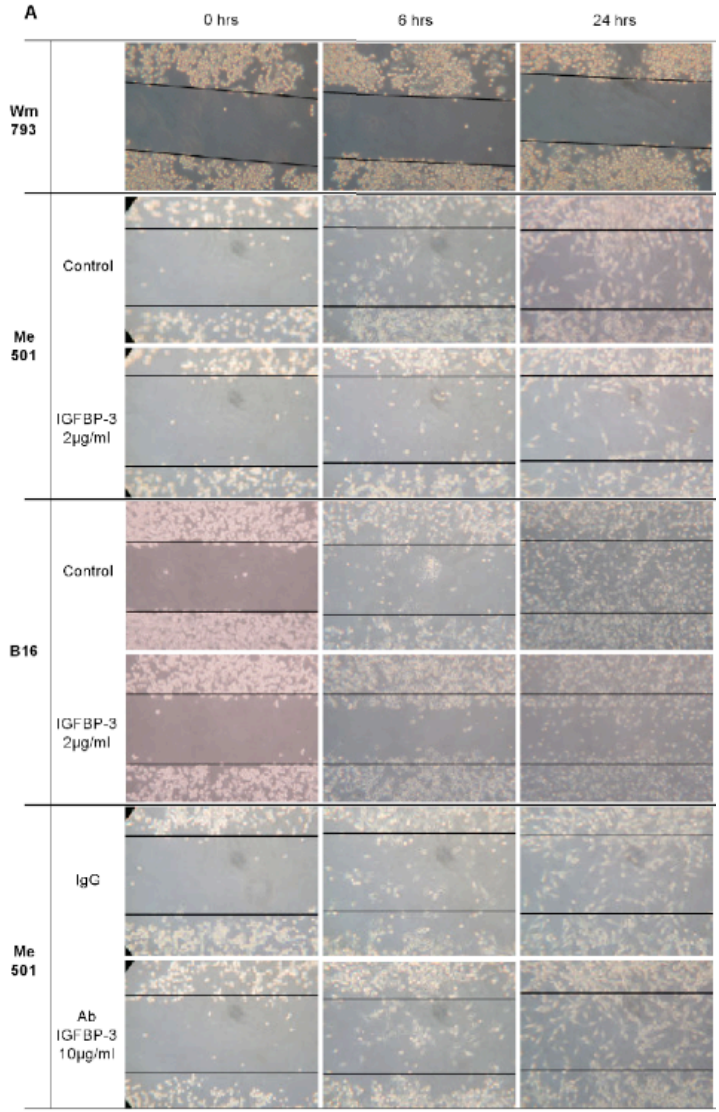
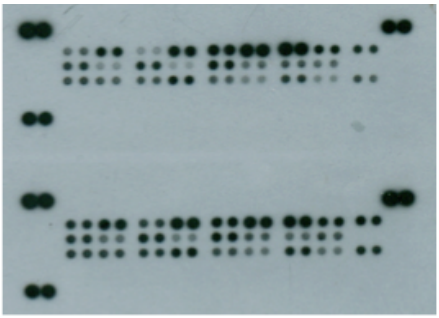


Figure 14A. Scratch-test analysis of primary human (WM793) and metastatic murine (B16) and human (Me501) melanoma cells, untreated, treated with 2µg/ml of IGFBP-3, and treated with 10 µg/ml of anti-IGFBP-3 antibody. The images were captured at 0, 6, 24h after incubation.

IGFBP-3 signals through the Akt pathway.

In order to understand the signaling pathways whereby cells respond to treatment with IGFBP-3, we analyzed the phosphorylation status of relevant kinases belonging to the MAPK and PI3K pathways, using pre-cast phosphor-proteome profiler arrays. The human metastatic melanoma cell lines were grown to semi-confluence, transferred to a serum-free medium and treated with IGFBP-3 for 24 hours. Fig.15 shows the phosphor-proteome profiles of the Me501 metastatic lines, treated or not with IGFBP-3.



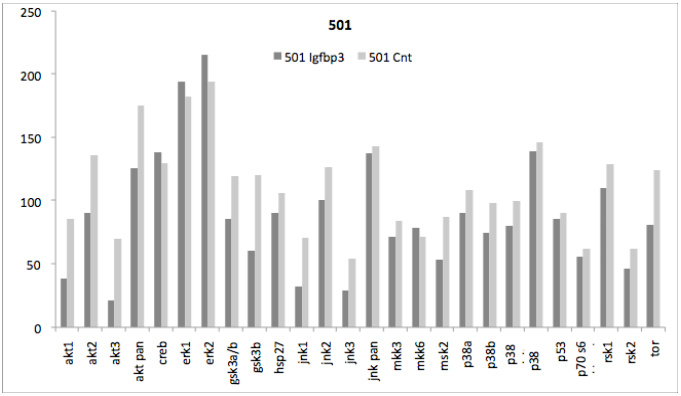


Figure 15. Analysis by phosphor-proteomic arrays of signal-transduction pathways involving in mediating IGFBP-3 action. Me501 cells before (light grey) and after (dark grey) treatment with IGFBP3.

In these cells, treatment with IGFBP-3 produced a drastic inactivation of all three Akt isoforms, especially Akt3, with a concomitant dephosphorylation of GSK3β and mTOR. The

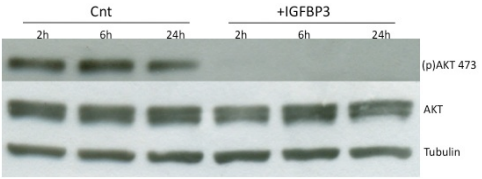


Figure 16. Western blot kinetic analysis of the phosphorylation of Akt (ser 473) in untreated and IGFBP-3 treated Me501 cells.

activation state of Akt was further analysed at different times of treatment with IGFBP-3 (Fig.16). Dephosphorylation was essentially complete after 2 hours and remained so for the ensuing 24 hours. The overall amount of Akt remained unchanged throughout the experiment, indicating that IGFBP-3 had no effect on either the expression or the stability of the protein. Overall these results are a strong indication that IGFBP-3 significantly affects the AKT-mTOR-GSK3B axis.

IGFBP-3 acts independently from the IGF1 sequestration.

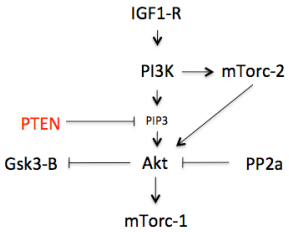


Figure 17. Brief description of the PI3K pathway.

Figure 17 briefly summarizes the PI3K pathway, an important regulator of AKT functions. As shown, the activity of AKT is regulated by multiple stimuli and its complete network of interactors is far more complex (Yong Liao et al., 2010; Y Lu et al., 2011). However, an important event, involved in the regulation of AKT is the association

between IGF1 and its receptor (Hernández-Sánchez C et al., 1995). As explained in the Introduction, it is known that one of the biological functions of IGFBP-3 is to modulate the access of IGF-1 to its receptor, thereby modulating the activation state of the PI3K-AKT pathway. Therefore, it was important to assess whether the capacity of IGFBP-3 to dephosphorylate AKT in melanoma cells was dependent of IGF-1.

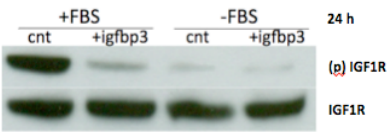


Figure 18. Analysis of the phoshorylation of IGF1-R (Tyr 1135), regulated by the IG1 interaction with the receptor.

A number of evidences suggest that this is not the case. First, the migration/invasion experiments were performed in the absence of serum, therefore in absence of IGF-1. The cells themselves did not produce IGF-1, as verified by PCR and western blot analysis (not shown). Finally, we determined the activation state of the IGF1-receptor, by analyzing its phosphorylation state. As shown in Fig. 18, in the serum starvation conditions employed in the migration/invasion assays, the basal activity of the receptor is rather low and remains unaffected by treatment with IGFBP-3. Control experiments in the presence of serum showed that the inhibition of IGF1-R mediated by IGFBP3 is in this case

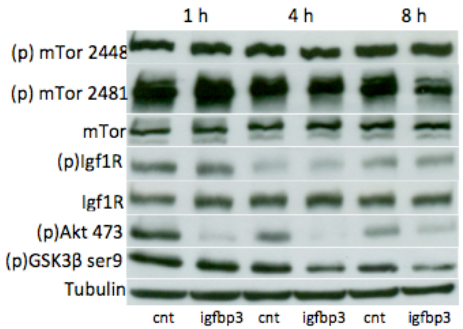


Figure 19. Western Blot analysis detecting the phosphorylation of kinases involved in the regulation of AKT (ser 473) or regulated by AKT.

very strong, as expected (Figure 18).

A kinetic analysis was also performed, whereby the phosphorylation state of the IGF-1R was assessed at different times of IGFBP-3 treatment. As shown in Fig. 19, activation of IGF1-R was poorly affected by IGFBP-3 during a treatment of 8 h. Importantly, AKT dephosphorylation, which was almost complete already after 1 h, was independent of IGF1-R state. In the same experiment, the phosphorylation status of several kinases upstream or downstream of AKT was also determined. As stated previously, GSK3-beta was dephosphorylated upon IGFBP-3 treatment, albeit following a slower kinetics than AKT. Dephosphorylation was also observed for mTOR 2481, a site diagnostic of the activity of mTORC2, an important kinase regulating AKT (Copp J et al., 2009; Xiaoqing Gan et al., 2011). The fact that mTOR 2481 dephosphorylation follows a slower kinetic than AKT suggests that mTORC2 is not involved in AKT inhibition.

IGFBP3 acts through the activation of cellular phosphatases.

Figure 20. IGFBP3 acts on AKT at only 15 m of treatment.

By further analyzing the kinetics of IGFBP-3 action, it was observed that dephosphorylation of AKT was appreciable after only 15 minutes of treatment.

Since we were not able to observe any correlation among IGFBP3 treatment and the inhibition of upstream activators of AKT (IGF1, mTORC2 and PI3K) and considering the speed of the AKT's inhibition, we wondered if this effect could be mediated by an active mechanism instead than a passive one. For this reason we

hypothesized that this phenomenon could be mediated by the activation of a phosphatase driving AKT dephosphorylation.

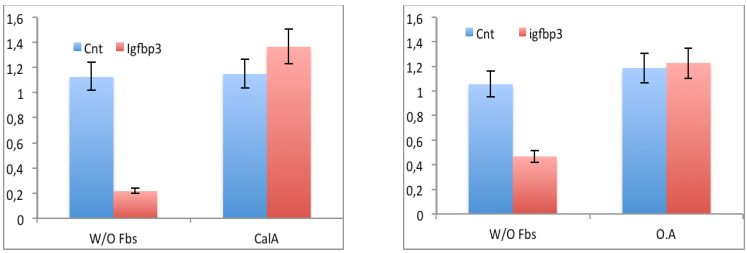
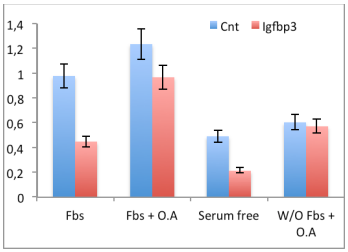


Figure 21. Levels of (p) AKT 473 assayed by W.B., after the treatment with Calyculin A (left panel) or Okadaic Acid (right panel) added to the medium, 1 h before IGFBP3.

As shown in Figure 17, PTEN, one of the most important tumor suppressors of the cell, is a phosphatase that negatively regulates AKT (Li L et al., 2007; Song MS et al., 2012). PTEN gene is frequently deleted or mutated in many kind of cancers; however, the PTEN protein was absent from our cell lines (not shown), indicating that it can not be involved in the IGFBP-3 signaling.

Besides PTEN, PP1 and PP2A represent the most relevant phosphatases of AKT involved in a number of cellular process such as the insulin signaling, apoptosis and cellular motility (Garcia A et al., 2003; Galbo T et al., 2013; Basu S, 2011).



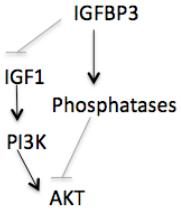


Figure 22. In the presence of IGF1, the blockage of IGFBP3 signaling mediated by Okadaic Acid is only partial. The scheme summarizes the ipotized mechanisms of IGFBP3's action

In order to

understand if these phosphatases could be involved in the regulation of AKT we inhibited their activity by using two different compounds. Okadaic Acid and Calyculin A have been widely employed for their specificity and strength in inhibiting PP1 and PP2A activity (Jamie L et al., 2009). Figure 21 shows the results obtained by treating Me501 cells (in the absence of serum) for 1 h with these compounds, plus IGFBP3. The graphs clearly show that both inhibitors are able to completely block the action of IGFBP3 on AKT. This result strongly suggests that the inhibitory action of IGFBP-3 on AKT is mediated by the activation of PP1 and PP2A phosphatases.

The experiments with Okadaic Acid were also performed in the presence of serum. Figure 22 shows that under these conditions IGFBP3 only partially inhibits AKT. This suggests that IGFBP3 indeed regulates AKT phosphorylation by two different mechanisms; an IGF-1 dependent one, which is not affected by phosphatase inhibition, and an IGF-1 independent one, which relies on the activation of either (or both) PP1 and PP2A phosphatases.

MAPK pathway inhibition in the presence of IGFBP-3

In the mutational landscape characterizing the oncogenic stimuli that sustain a tumor, the hyperactivation of AKT is an important event to bypass apoptotic triggers and increase the proliferation rate and metastatic potential of cancer cells (Bellacosa A et al., 2005). Particularly in melanoma, MAPK activation represents one of the most important stimuli in inhibiting apoptosis and triggering cellular growth.

Accordingly, Me501 cells have an hyperactivated MAPK pathway (see Fig.15), whose main components (ERK) are insensitive to IGFBP-3 inhibition, in agreement with the fact that IGFBP-3 does not affect the cell cycle. However,

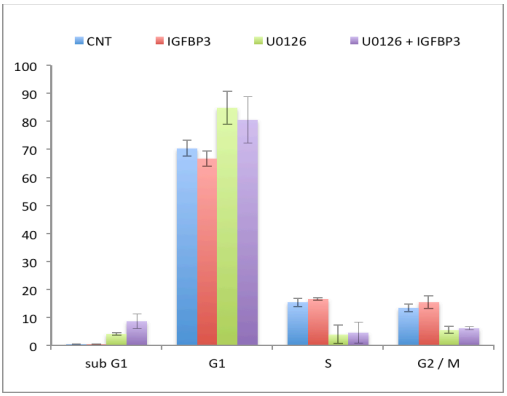


Figure 23. Cell cycle analysis with FACS sorter of Me501 cells treated with IGFBP3 and/or U0126 MEK Inhibitor.

since an hyper-active MAPK pathway seems to be an important hallmark of melanomagenesis, we tested whether inhibition thereof, in addition with IGFBP-3 treatment, could produce a stronger anti-tumoral effect, for instance inducing apoptosis of the malignant cells.

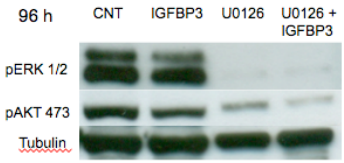


Figure 24. W.B of Me501 cells treated with IGFBP3 and/or U0126 confirmed the inhibition of the MAPK signaling.

For this purpose we utilized the drug U0126, a potent inhibitor of MEK, an important component of the MAPK pathway (Wu P et al.,

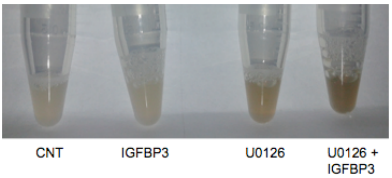


Figure 25. Me501 cells, treated with U0126 and U0126+IGFBP3, showed a deep darkening of the cell lysates, suggesting the stimulation of the melanogenesis process upon the inhibition of the relative pathways.

2013).

Firstly, we analyzed by FACS sorter the cell cycle of the MEK and/or IGFBP-3 treated cells. As mentioned before, IGFBP-3 does not have any effect on the cell cycle, while the MEK inhibitor is able to block a percentage of cells in the G1 phase, indicating a cell cycle arrest (Figure 23). The co-treatment with IGFBP3 and U0126, however, did not show any significant additive action on any stage of the cell cycle. Moreover, annexin V analysis that allows to detect apoptotic cells, also yielded negative results (data not shown).

Although co-treatment with IGFBP-3 and MEK inhibitor failed to induce apoptosis, it had the singular effect of apparently increasing the melanine content of the Me501 cells. A darkening of the cell lysates was already visually observable upon treatment with U0126, and was further increased with the addition of IGFBP-3 (Fig.26).

Melanin synthesis by both normal and tumoral cells is regulated by multiple molecular pathways, including the MAPK and the PI3K ones (Mehdi Khaled et al., 2002; H.Y. Park et al., 2009; Shen T et al., 2012). Specifically, the ERK protein negatively regulates tyrosinase, the enzyme initiating melanine synthesis. For this reason melanoma cells harboring a BRAF mutation, very often show an hypopigmentation phenotype caused by reduced levels of tyrosinase (Rotolo S et al., 2005). Moreover, the kinase GSK3-beta, which is regulated by AKT, also plays an important role in melanogenesis by regulating the activity and the levels of tyrosinase. Also in this case the hyperactivation of AKT contrasts melanine production by repressing GSK3-beta (Mehdi Khaled et al., 2002; Mehdi Khaled et al., 2003).

IGFBP-3 induces melanocytic differentiation of metastatic melanoma cells

To investigate in greater depth the effect of IGFBP-3 on melanogenesis, we determined whether treatment of the Me501 cells with this protein indeed resulted in activation of the melanine synthesis pathway.

Firstly, we measured the melanine content in treated and untreated Me501 cells, detecting a significant increase in the production of the pigment (Fig. 26).

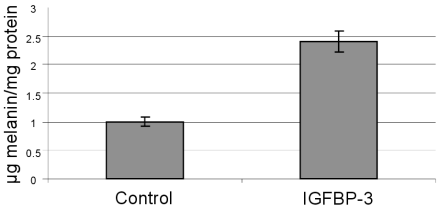


Figure 26. Me501 cells treated with IGFBP3, showed higher levels of melanine content. Analysis conducted by measuring the absorbance of melanine to the spectrophotometer.

Secondly, we assessed whether this depended on the activation of tyrosinase, the main enzyme controlling melanogenesis (H.Y. Park et al., 2009). This was indeed the case; enzymatic assays an western blot experiment revealed an increase of both enzymatic activity (Fig. 27) and protein amounts (Fig. 28).

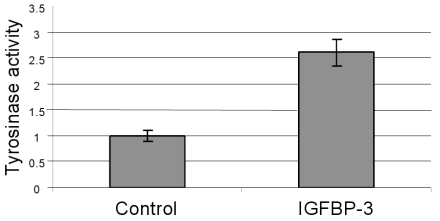


Figure 27. Me501 cells treated with IGFBP3, showed higher Tyrosinase's activity. Tyrosinase's activity measured by L-DOPA oxidation, by measuring the absorbance of DOPA-Quinone to the spectrophotometer.

This behaviour is in agreement with the fact that one the downstream targets of Akt most affected by IGFBP-3 treatment is GSK3β, a

factor whose activation by dephosphorylation triggers melanine synthesis and differentiation in melanocytes (Fig. 28) (Mehdi Khaled et al., 2002; Kazuhisa Takeda et al., 2000).

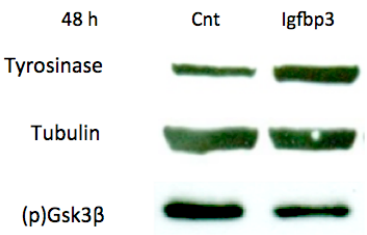


Figure 28. The levels of Tyrosinase are increased upon the 48h treatment with IGFBP3. Gsk3b activation by dephosphorilation is an upstream event involved in this process.

Besides losing the ability of migrate and invade, and enhancing melanogenesis, IGFBP-3 treated cells kept in culture for a few days acquired also a more dendritic appearance (Fig.29), further suggesting that they were steered towards melanocytic differentiation.

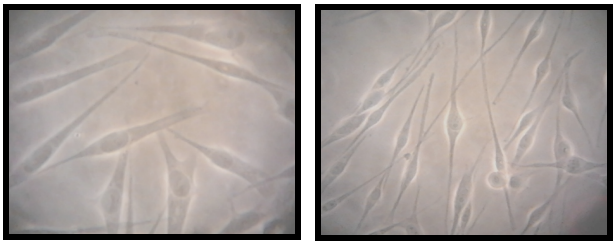


Figure 29. Me501 cells treated with IGFBP3 (right image) appear more dendritic than Cnt cells (left image).

In vivo anti-tumoral effects of IGFBP3 treatment.

To assess the potential clinical relevance of "in vitro" results, we evaluated the anti-tumoral activity of IGFBP-3 in SCID mice xenografted s.c. with Me501 human melanoma cell line.

Xenografts model	Compound	Dose (mg/Kg) and route	Schedule	TWVI %	s.e.m.	CR
Me-501	Saline	0	TTWx9 from day 3	0	18.53	0/8
	IGFBP3	0.37 i.p.	TTWx9 from day 3	40.33	9.51	2/8
		1.87 i.p.	TTWx9 from day 3	92.50	6.02	3/8

Table 1. Percentages of tumor weight inhibition (TWI) and percentages of mice tumor free in each group number of in Me501 bearing SCID mice treated with IGFBP3 and three times a week (TTW) with one day interval for three weeks. For each group standard error of the mean (s.e.m.) and complete response ratio (CR) were also reported. The values in this table are referred to the day of sacrifice (day 28 after tumor cell injection). Mean results are representative of two different experiments (each experiment on at least 8 mice). Tumor size was measured three times per week with calipers, and volume was calculated as described in the "Methods" section.

Three days after tumor injection, mice received subcutaneous injections of saline, or 0.37 mg/Kg or 1.87 mg/kg IGFBP-3 three times a week (TTW) for three weeks and tumor growth was estimated during the follow-up. As illustrated in Table 1 and Fig.30, the growth of Me501 tumors in mice treated with IGFBP-3 was significantly reduced as compared with control animals, with a dose-dependent efficacy, and the size of IGFBP-3-treated tumors was significantly smaller than control tumors at follow-up (P=0.0005), indicating that IGFBP-3 treatment may exert an antineoplastic activity in vivo. More in detail, Tumor Volume Inhibition percent

(TVI%) in IGFBP-3-treated mice was respectively of 40,33 and 94,61% at 28 days post-tumor implant. At sacrifice, tumor diameter was significantly lower (t-Test $p<0.001$) in IGFBP-3 (3± 1,5 and 4.5 ± 2 mm) in comparison to saline (9.0 ± 3 mm) treated mice. Moreover, complete response (CR) was obtained in 2/8 mice treated with the lower IGFBP-3 concentration, in 3/8 mice treated with the higher IGFBP-3 concentration but in none of the saline group (Table 1).

Moreover, tumors from IGFBP3 treated mice were consistently darker than those from untreated mice, suggesting upregulation of melanine synthesis. Indeed, as shown in Fig. 31, tyrosinase activity increased in tumor tissues from IGFBP-3 treated mice, confirming the observations made on cultured

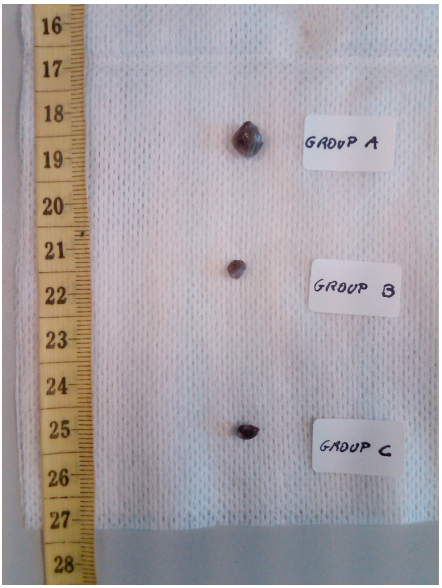


Figure 30. Tumour sample derived from each group.

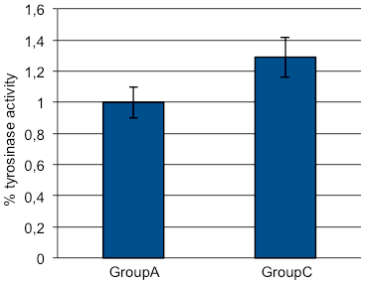


Figure 31. Tyrosinase activity I of melanomas excised from untreated and IGFBP-3 (high-dose) treated mice was measured on tissue homogenates as described in Methods for the cultured cells

melanoma cells.

Melanoma-bearing SCID mice treated with IGFBP3 did not show any signs of systemic toxicity such as weight loss, diarrhea, or hair ruffling during the dosing period until sacrifice. Considering the role of IGFBP3 in regulating glycaemia, glycemic values were also assessed at 4 and 24 hours after the injection of High dose IGFBP3. No alteration in glycemic level was observed in IGFBP3 treated mice.

EXPERIMENTAL PROCEDURES

Immunohistochemistry and immunofluorescence

The samples tissues was taken from patients affected by cutaneous melanoma in the Department of Dermatology of the University "Sapienza" of Rome.

Primary melanoma (20) and dermal metastases (20) were analyzed.

Immunohistochemistry was performed on 3–5µm thick sections obtained from formalin-fixed tissue embedded in paraffin. Antigen retrieval was performed using a PT module (pH 6; Thermo Fisher Scientific, Fremont, CA, USA).

The immunohistochemical procedure was performed according to alkaline phosphatase method.

Briefly, rabbit polyclonal anti-MMP-9 antibody (Thermo Scientific, RB-9234-P) diluted 1:200 and rabbit polyclonal antibody anti-IGFBP-3 antibody (AbD, 5345-5109) diluted 1:100 were used as primary antibody. After Tris-phosphate buffered saline (TBS) rinse, sections were further incubated with labelled polymer in accordance with the standard UltraVision AP detection system (Thermo Fisher Scientific). After a final TBS rinse, all immunohistochemical reactions were visualized by using Liquid Fast-Red Substrate System (Thermo Scientific, Runcorn, UK) as the chromogen and hematoxylin as the counterstain.

The immunohistochemical evaluation was performed independently by 2 researchers without knowledge of the patient's data, using a

double-headed microscope. Intra-observer agreement was higher than 90%. The number of positive cells was separately counted for IGFBP-3 and MMP-9 under a light microscope at 200X magnification. For each slide, at least 7-10 microscopic fields were randomly chosen.

A five grade-semi-quantitative scoring system (score 0-4) was adopted for the evaluation of IGFBP3 and MMP-9 immunohistochemical expression. The score was graded according to the percentage of stained cells: score 0 was defined as the presence of stained positive cells ≤ 5%, score 1, 2 and 3 were defined as the presence respectively of 6% to 25%, 26 to 50% and 51%- to 75% positive cells and score 4 as the presence of > 75% .

The immunohistochemical score was evaluated separately for melanocytic and stromal cells and expressed as tumour and stromal score respectively. A total score was derived for each sample as the sum of tumour and stromal score.

Cell lines and culture conditions

The Wistar melanoma (WM) cell lines were kindly provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA. The cell lines Me501 were established from metastases obtained from melanoma patients surgically resected at the Istituto Nazionale dei Tumori, Milan, Italy. All melanoma cell lines were seeded in 3-cm Petri dishes (2 × 105 or 4 × 105 per dish) in RPMI 1640 supplemented with 100IU/mL penicillin, 100μg/mL streptomycin (Life Technologies, Gaithersburg, MD), and 2mmol/L glutamine (Life Technologies) with 10% FCS in a 5% CO2 environment at 37°C.

Phospho-Proteome Profiler

The phospho-proteome profiler array was performed by utilizing the RD System "Human Phospho-Kinase Array Kit".

Western blot analysis

Cells were grown to 80% confluence and then harvested on ice using cell lysis buffer (20mM tris, pH 7.5, 150mM NaCl, 1% Triton X, 1X protease and phosphatase inhibitor mixture (Roche Applied Science). Cells were dounce-homogenized and centrifuged at 10,000 Xg for 10min. The supernatant was quantitated using Bradford assay; 30μg of each lysate was run out on 12.5% SDS-polyacrylamide on under reducing conditions and transferred onto 0.2-μm nitrocellulose. The membranes were probed with antibodies: (p)Akt Ser 473, total Akt (p)Gsk3β Ser 9, (p) Tyr 1135 IGF1-R, total IGF1R, (p) Thr202/204 ERK1/2, total mTOR, (Cell Signaling), β-Tubulin (Sigma-Aldrich), (p) Ser 2448 mTOR, (p) Ser 2481 mTOR (Millipore).

Visualization of signal was performed by using the ECL system (Pierce).

Pharmacological Inhibitors

Okadaic Acid (at 60nM - 100nM) and Calyculin A (at 15 nM) phosphatase inhibitor, was taken from Santa Cruz Biotechnology. Both inhibitors were added to the medium 1 hour before adding 2μg/ml IGFBP3.

U0126 was taken from Calbiochem and added at 10 μM, 1 hour before adding 2μg/ml IGFBP3.

Gelatin substrate zymography

For gelatin zymography, 20 μl of serum-free medium were separated on 10% SDS-polyacrylamide gels containing 1mg/ml bovine gelatin (Sigma, Deisenhofen, Germany) under non-reducing condition. Following electrophoresis, the gels were washed twice for 30 minutes in 2.5% Triton X-100 to remove SDS. After equilibration in enzyme substrate buffer (50mM Tris-HCl, pH 7.5; 150mM NaCl; 5mM CaCl2, the gels were incubated in the same buffer overnight at 37°C. They were then stained with Coomassie Blue R 250 and destained in water.

IGFBP-3 protease assays

To assay for protease activity in the culture media of melanoma cells, 20pmol of full-length, glycosylated IGFBP-3 was mixed with 20μl of serum-free medium from Me501 cells and the samples were incubated at 37°C for 96h and then subjected to SDS-PAGE under reducing conditions. IGFBP-3 proteolysis was analyzed by Western blot.

Scratch-Wound Assay

To evaluate the effect of IGFBP-3 on cell motility and migration, a scratch-wound assay was done on WM793, Me501 and B16 cells. Monolayer cells were scraped with a pipette tip to generate a scratch wound after cells reached confluence. The wounded surface was washed with 1×PBS and incubated in RPMI without fetal bovine serum in the presence or in the absence of recombinant IGFBP3 (ABd serotec). Cell migration into the wound was monitored by

phase microscopy using an Axiovert 200M microscope with digital camera (Carl Zeiss, Thornwood, NY), taking pictures at 2, 4, 6, 24, and 48 h. The images were captured by AxioVision 4.0 software (Carl Zeiss). The closure of the initial gap area was assessed by calculating the difference between the initial and the remaining wound area at each time point.

BD biocoat matrigel invasion assay

BD matrigel chambers, stored at -20°C, were kept at room temperature for 1h, after which 500µl of DMEM was added to the wells. The inserts were then transferred to the medium-containing wells. DMEM (500µl) was added to the inserts and kept at 37°C in an incubator with 5% CO2 for equilibration. After 2h, the medium in the inserts was aspirated and inserts were placed into the wells containing DMEM and 5% FBS which acts as a chemo-attractant for the cells to migrate. Overall, 50000 cells in 500µl of DMEM were added to the inserts. The plates were incubated for 22h in a CO2 incubator at 37°C. The chamber inserts were then stained using the Diff-Quick staining kit (Dade-Behring) according to the manufacturer's instructions. Finally, the membranes were separated with a sterile scalpel and observed using a light microscope. The number of cells that had passed through the membranes were counted as a measure of their migration potential.

In Vivo Tumor Growth Analyses

Female CB.17 SCID/SCID mice aged 4–5 weeks (Harlan; Correzzana, Milan, Italy) were kept under specific pathogen-free

conditions and fed ad libitum. Mice were housed in micro-isolator cages, and all food, water, and bedding were autoclaved prior to use. Each mouse was injected subcutaneously in the right flank with 0.5 × 106 human melanoma Me501 cells derived from metastatic lesions that had been resuspended in 0.2 mL of RPMI-1640 containing 10% FCS. Three days after tumor injection IGFBP3 resuspended in normal saline was administered subcutaneously three times a week with one day interval, at the doses of either 0.37 or 1.87 mg/kg. Tumor dimensions were measured three times per week with calipers. Tumor weight was estimated according to Geran et al. using the following formula: tumor weight (mg) = length (mm) × width2(mm)/2.

At least 8 mice were used for each treatment group. Data are expressed as the mean value of tumor weight with 95% confidence intervals. Mice were monitored for the duration of the in vivo experiments for body weight, hair ruffling, and the presence of diarrhea. All mice were killed at the end of the experiments, 28 days after the injection of the human tumor cells.

Ethic Statement

Animal experiment were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Istituto Superiore di Sanità, Italy . All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Statistical analysis

In order to establish which variables were statistically related to high IGFBP-3 serum level in melanoma patients, data were entered into a Microsoft Excel spreadsheet and analyzed by the ANOVA test; a P value <0.05 was considered statistically significant (Table 1).

A receiver operating characteristic (ROC) curve analysis was used to find an average value of metastatic volume, IGFBP-3 serum level and IGF1/IGFBP-3 molar ratio, in order to dichotomize the patients into two subgroups, according to the cut off obtained.

Overall survival (OS) was calculated as the time lasting from the first sample withdrawal to the date of death or last follow-up; the OS curves were compared using the Kaplan-Meier method in order to correlate the survival time with IGFBP-3 serum level and IGF1/IGFBP-3 ratio.

Statistical analysis for immunohystochemical results was conducted with the Mann Whitney U test. To assess significant correlations, the Spearman correlation coefficient was calculated.

A t test analysis was performed to compare the mean tumor volumes in xenografted SCID mice.

P values <0.05 were regarded as statistically significant.

Statistical analyses was performed through MedCalc® version 12.1.4.0 (Schoonjans F et al., 1995).

DISCUSSION

In this study we show that IGFBP-3 has a remarkable anti-tumoral activity on malignant melanoma, both in vitro and in vivo.

Previous clinical observations revealed that IGFBP-3 serum levels of melanoma patients were highly correlated with disease stage and progression. Indeed, stage IV patients underwent a significant loss of circulating IGFBP-3, whose extent was a strong predictor of survival time (Panasiti V et al., 2011). In this work it is shown that IGFBP-3 loss is also appreciable in the tissutal microenvironment, where the progression to the metastatic phase of melanoma was accompanied by a reduction in IGFBP-3 immunostaining both in the stromal cells surrounding the tumour and in tumour cells themselves.

This may suggest that melanomas have an active role in destroying IGFBP-3, and indeed many tumors are known to produce proteases. Accordingly, metastatic, but not primary, melanoma cells secreted large amounts of a MMPs, mostly MMP-9. These results agree with a former study reporting that blood serum from IV-stage melanoma patients was uniquely able to degrade IGFBP-3 in vitro (Panasiti V et al., 2011). It is possible, therefore, that melanomas ingrain a self-sustaining loop, destroying IGFBP-3 as they grow and becoming as a consequence ever more aggressive. However, the alternative hypothesis that melanomas inhibit tissutal, or even hepatic, production of IGFBP-3 cannot yet be discounted and is currently being tested.

In vitro assays on cell cultures revealed that IGFBP-3 affects the ability of cells to migrate and invade rather than that to proliferate. In fact, the highly motile and invasive behavior of the two cell lines of metastatic tumors analyzed (Me501 and the murine line B16) was

strongly inhibited by treatment with human recombinant IGFBP-3. IGFBP-3-induced proliferation arrest and apoptosis induction were described by other investigators (Gribben L et al., 2012; Mehta HH et al., 2011), but to the best of our knowledge inhibition of cell motility and invasiveness has not been reported previously.

Phosphor-proteome profiling of certain signal transduction pathways involved in tumoral transformation revealed that the metastatic cells treated with IGFBP-3 turned off AKT. AKT inactivation caused the concomitant dephosphorylation of its primary target GSK3β. That GSK-3β in particular may be a mediator of the anti-metastatic effect of IGFBP-3 is suggested by the fact that IGFBP-3-treated cells showed a tendency to revert to a more differentiated melanocytic phenotype, as indicated by the increase of both tyrosinase activity and melanine content. In fact, GSK3β is known to promote melanine synthesis and melanocyte differentiation (Mehdi Khaled et al., 2002; Kazuhisa Takeda et al., 2000).

The molecular mechanism whereby IGFBP-3 promotes AKT dephosphorylation remains to be deciphered; however, the data presented here point to an important involvement of cellular phosphatises PP1 and PP2A. In fact, treatment of melanoma cells with a specific inhibitor of these phosphatases was able to abolish IGFBP-3-triggered AKT dephosphorylation.

This surmise is in agreement with the observation that the inhibitory effect of IGFBP-3 on AKT appears to be independent of IGF-1. This is borne out by the facts that the motility/invasiveness assays were performed in the absence of IGF-1, that the cells under study did not produce IGF-1 and had very low levels of (p)IGF-1 receptor that IGFBP3 treatment is not able to further deregulate. Therefore,

IGFBP-3 apparently exerts its anti-migratory/invasive effect independently of the IGF1R/PI3K pathway.

The anti-tumoral effect of IGFBP-3 was also investigated in vivo using a murine model, namely SCID mice which were inoculated with human melanoma (Me501) cells. Tumor growth was strongly inhibited already upon administering low doses of IGFBP-3, and completely arrested in 2 out of 8 cases already at lower doses, and in 3 out of 8 cases at higher doses. In agreement with in vitro observations on cultured cells, tumours from IGFBP-3 treated mice appeared darker and had higher melanine content and tyrosinase activity, suggesting that also in vivo IGFBP-3 directed tumoral cells to revert towards a more melanocytic phenotype.

Importantly, IGFBP-3 did not produce evident toxic effect even at the higher doses, nor had detectable negative impact on glucose metabolism.

Altogether, the results presented here suggest that IGFBP-3 is a potentially interesting anti-tumoral agent, all the more because it is a physiological factor that is not expected to have major adverse effects when given therapeutically. At the least, it may qualify for a valid adjuvant therapy in melanoma (and perhaps other cancers) during treatment with conventional anti-tumoral drugs.

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