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GROWTH AND APOPTOSIS PATHWAYS IN HUMAN CUTANEOUS MELANOMA: *IN VITRO* AND *IN VIVO* STUDIES BY USING BIOLOGICAL AND PROTEOMICS APPROACHES

PhD Thesis
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ABSTRACT

Purpose. Melanoma is the most aggressive cutaneous cancer without effective treatment. Diagnosis is achieved very often too late and prognosis is poor. Aim of this work is to identify proteomic pathways potentially involved in melanoma aggressiveness.

Materials and Methods. We analyzed 5 human metastatic melanoma cell lines and human keratinocyte and melanocyte cell lines as a control. Their proliferation and apoptotic behaviors under serum stimulation and starvation were analyzed in order to identify the most aggressive one. Melanoma cell proteome from the most aggressive cell line (A375), compared to the less aggressive one (SK mel 28), was analyzed by means of two complementary approaches: 1) multiplexed assay to measure the levels of 27 cytokines both in cell extracts and in conditioned media; 2) proteomic study through LC-MS/MS analysis of cell extracts. Data obtained were analyzed using bioinformatic analysis.

Results. A375 cells were found to possess the highest growth rate both under serum stimulation and under serum starvation, while SK mel 28 cells under similar conditions were significantly less aggressive, confirmed also by invasion assays. The effect was markedly cell-density dependent, suggesting that cell-cell interaction and/or secretory signals dependent phenomena are important. Proteome analyses indicated that several proteins are differentially expressed and possibly related the aggressiveness. Some of these proteins have been identified as transport and proteasome components. The Bio-Plex analysis of the melanoma cell lines under study indicated that melanoma cells contain significantly ($p<0.001$) different levels of some inflammatory cytokines and angiogenic growth factors: the most significantly modified factors were IL-6, Il-7, RANTES and VEGF, suggesting a novel interesting viewpoint to explain melanoma cell aggressiveness.

Conclusions. The reported results show that transport, proteasome components and an altered cytokine balance may be responsible for human melanoma aggressiveness.

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1. INTRODUCTION

1.1 TRAITS OF CANCER

Scientific attention is focalized on an important disease: cancer.

This pathology is considered as by the aberrant cells unable to make functional structures, which duplicate unchecked and spread into the organism (Coldizt et al., 2005). Cancer cells show acquired traits like uncontrolled proliferation, morphologic alterations, transplant capability, resisting cell death and enabling replicative immortality (Hanahan and Weinberg, 2011). The differential study of *in vivo* growth identifies two different tumor groups: the first one is the metastatic cancer and the other one is the benign tumor (Pontieri, 2004). The uncontrolled proliferation is involved in processes like invasion and metastasis that require a sequence of different steps termed metastatic cascade. The multistep process begins with proliferation of the primary tumor and basement membranes (proliferation and angiogenesis), then tumor invades blood or lymph to a distant organ (invasion and circulation), so tumor cells arrest in a small vessels within the distant organ (arrest vessels). Next, cancer cells escape from the *lumina* of such vessels into the parenchyma of distant tissues (extravasation) and the formation of small nodules of cancer cells starts (micrometastases). Finally micrometastatic lesions (tumor) grow to macroscopic tumors (colonization) [Talmadge, Fidler 2010, Hunter et al, 2008,] (*Figure 1*)

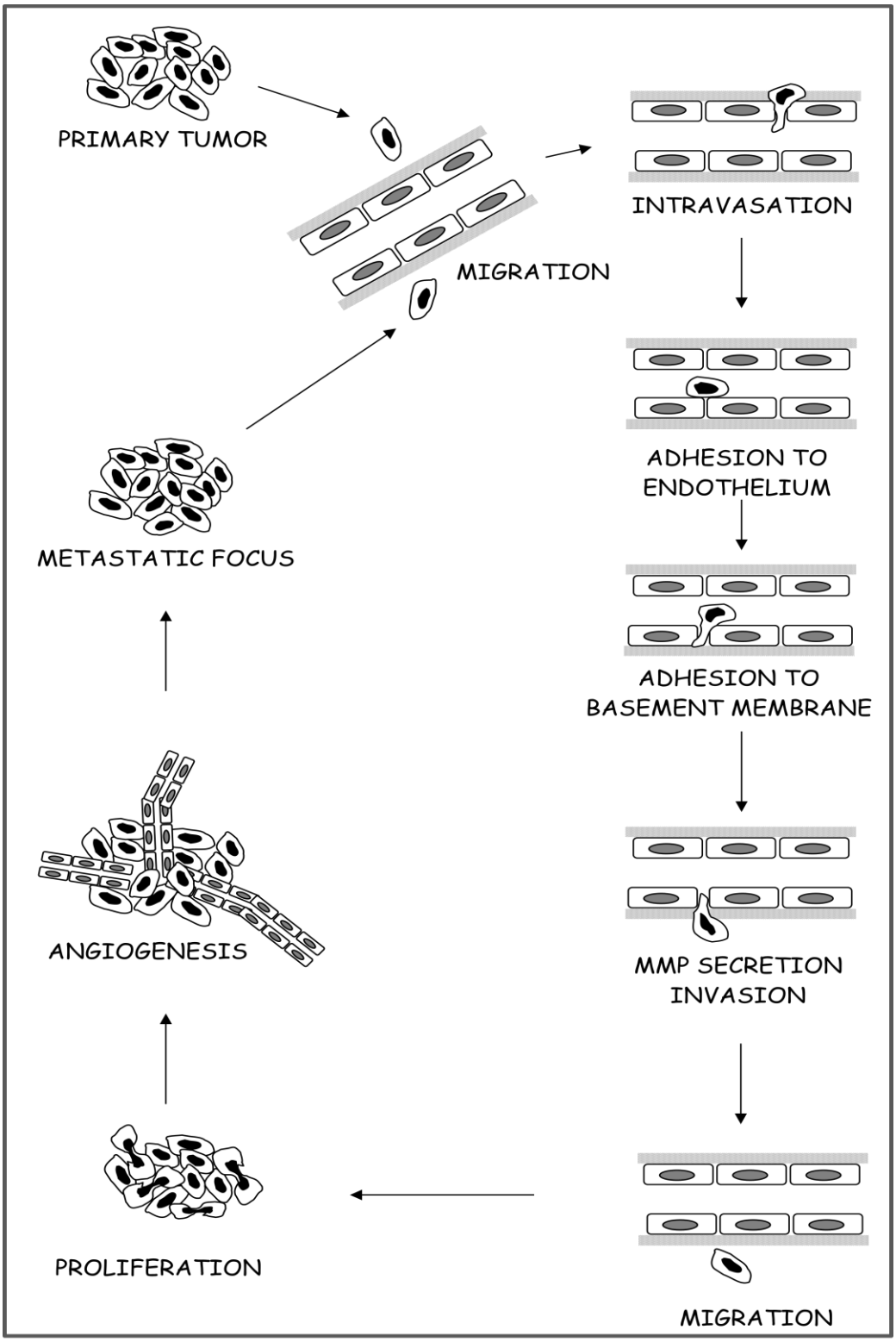


Figure 1. *Metastatic serial steps process (Lentini et.al., 2012 with modifications)*

1.2. MELANOMA

Melanoma is the most aggressive skin cancer and originates from the malignant transformation of melanocytes in the skin and mucous membranes or in eye, meninges and inner ear (Miller and Mihm MC Jr , 2006, Grichnik, 2008) *(Figure 2).*Cutaneous Melanoma (CM) is the most common form of melanoma.

The incidence and mortality of melanoma have steeply increased in the last century, but it continues to rise faster than that any other malignancy (Ricker et al., 2010).

Melanoma increased 15-fold in the last 50 years with a rate of increase of approximately 3-7% per year (De Giorgi et al., 2012, de Braud et al., 2003) due to many factors like environment, improvement of diagnosis and others. Therefore today melanoma is not yet considered a rare cancer, above all in the western countries. Many risk factors are involved in the tumor growth, like high number of nevi, alteration in an existing nevus, adult age, family history positive for melanoma, use of sunbeds and sunlamps and others constitutional host factors like phenotypic characteristics (Kari et al, 2011; Nielsen et al, 2011). Melanoma diagnosis often achieved too late (Psaty et al, 2010). Since prognosis in melanoma is directly proportionate to the depth of the neoplasm, early detection of melanoma is of crucial importance and accurate early diagnosis, leading to earlier treatment, is also extremely significant for a successful management. In fact, when melanoma is diagnosed early, it can be cured by surgical excision; nevertheless, metastatic melanoma remains generally incurable and is largely resistant to current therapies, with a median survival time of 6 to 9 months from the time of diagnosis and a 5-year survival rate of 5.5% (Walsh et al, 2010).

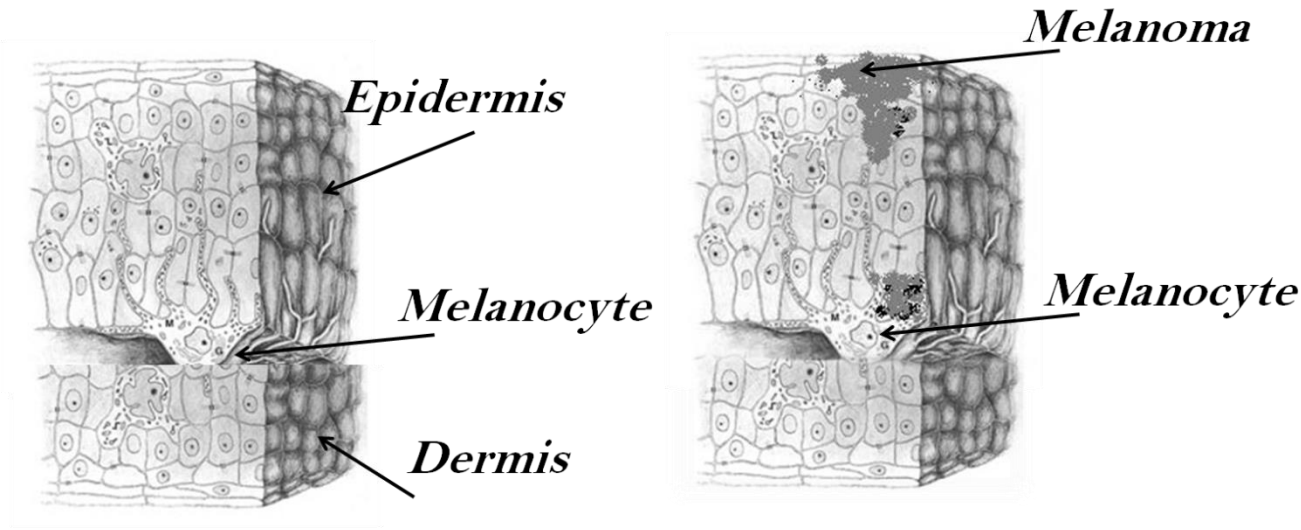


***Figure 2**. Melanoma origin.*

Specific visual features are used to diagnose a melanoma lesion; in particular the selection of traits was based on the ABCDE rule, which considers 5 visual criteria.

(A: lesion Asymmetry; B: Border irregularity; C: Color variegation; D: Diameter > 6mm; E: Evolution) *(Figure 3).*

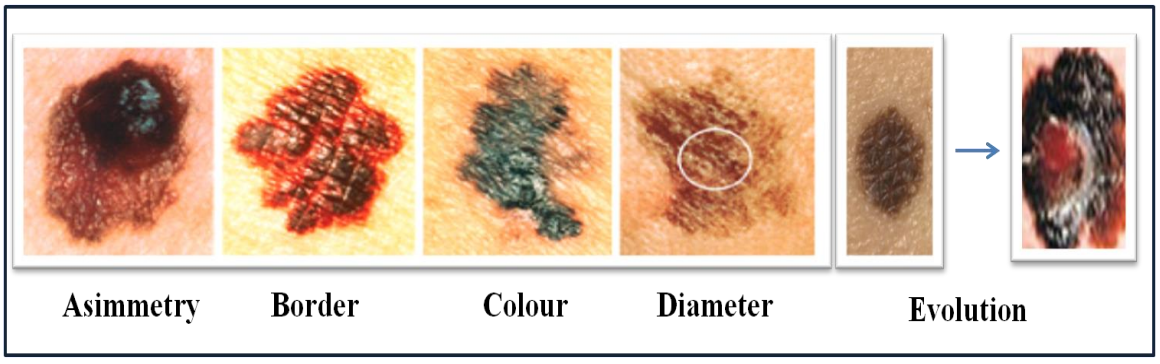


***Figure 3**. Melanoma features.*

The Clark and Breslow indexes are used to stage a melanoma lesion (Clark et al., 1984; Breslow, 1978) *(Figure 4)*. The evolution process of melanoma performs in two different layer of growth: a radial growth phase, which is characterized by intra-epidermal growth (Clark's index) and a vertical growth phase also called the dermal invasion phase (Breslow's index) (Meier et al., 1998). The vertical growth phase requires high angiogenic activity, which contributes to a very rapid development of melanoma metastatization.

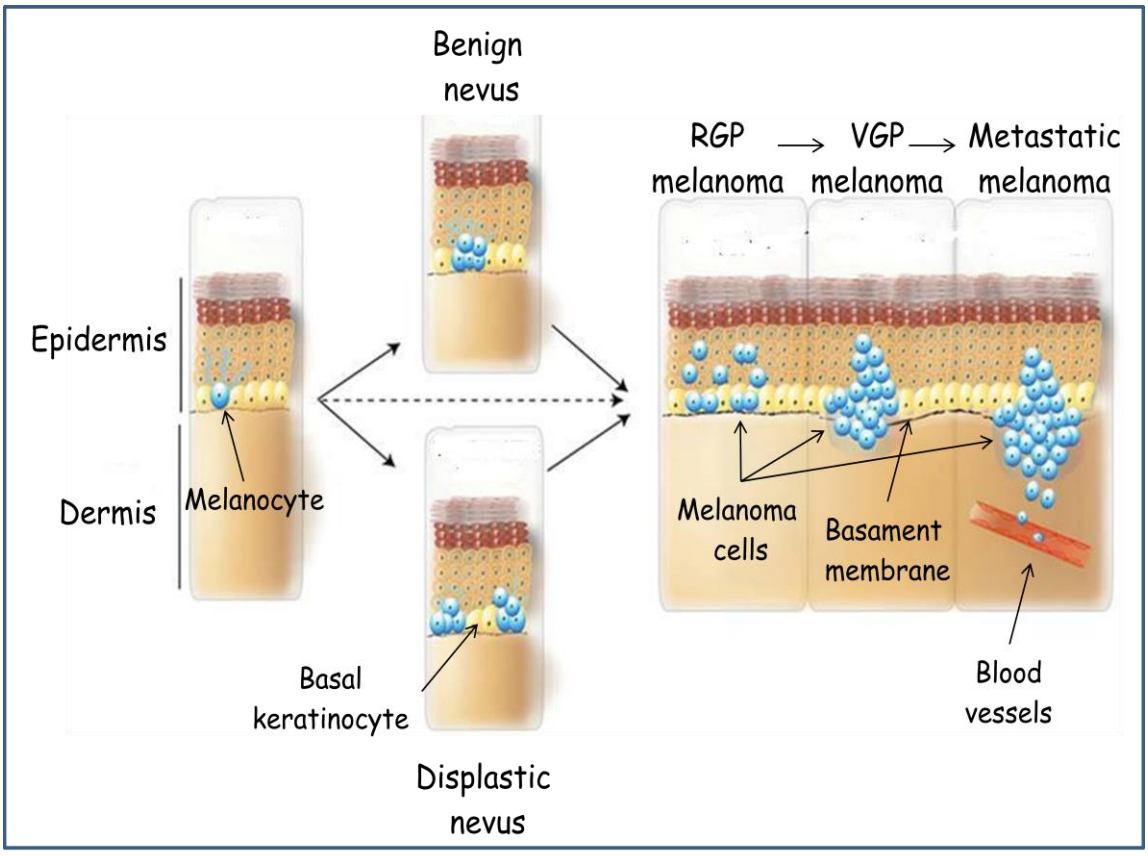


***Figure 4**. Melanoma Development in three different steps: radial (RGP), vertical (VGP) and metastatic growth.*

Although recent discoveries in the complex pathways involved in melanoma development created new opportunities for targeted drugs (Russo et al., 2009), the identification of novel molecular specific markers and pathogenic mechanisms is still an urgent need.

The elucidation of the molecular mechanisms likely related to the melanoma aggressiveness, may be therefore extremely important to develop novel molecular approaches correlating with different stages of the disease, potentially useful for diagnostic, prognostic and therapeutic options.

The major genetic alterations in melanoma involve RAS-RAF- MEK and ERK pathways, referred to proliferative signals generated at the cell surface receptors and through cytoplasmic signaling into the nucleus (Davies et al., 2002, Russo et al., 2009).

In normal cells the signaling cascade is activated by the binding of a receptor tyrosine kinase on the cell membrane with its respective ligand. This binding activates RAS (a membrane-bound protein with GTPase activity) which then recruits RAF that in turn phosphorylates and activates MEK. Phosphorylated MEK activates ERK which induces several proliferative and survival processes (Inamdar et al, 2010). There are 3 isoforms of RAF in human cells: ARAF, BRAF and CRAF. Mutations in BRAF occur in 50-70% of melanomas; in particular a substitution of valine by glutamic acid codon 600 in exon 15 (V600E) accounts in 90% of BRAF mutations in melanomas (Corcoran et al., 2011). Mutant BRAF conveys survival signals through different cytoplasm and cytoskeletal targets; these signals initiate nuclear transcriptions turning out in expression of several cancer-associated genes. ERK activity is involved in immune evasion by melanoma cells by the decrease of the immunosuppressive soluble factor IL-10, vascular endothelial growth factor, or IL-6. Richmond et al reported that melanoma cells express a variety of

cytokines (small cell- signaling molecules), chemokines (chemotactic cytokines) and growth factors, and levels of expression are related to melanoma progression (*Figure 5*). A study on nevi, primary and metastatic melanoma lesions demonstrated as primary melanomas express low amounts of interleukin-8 (IL-8), tumor necrosis factor (TNF), TGF-β and c-kit (Moretti et al., 1999). On the contrary, metastases showed a up-regulation of IL-1α, IL-1 β, IL-8, TNF- α, TGF-β, granulocyte-macrophage colony stimulating factor (GM-CSF). So, it was concluded that IL-6 and IL-8 expression was associated with early malignancy, while TGF-β, IL-1α and GM-CSF were highly expressed in late metastasis. In particular, TGF- β is considered a marker of metastatic spreading (Moretti et al., 1999). Cytokines with known functions for inflammatory and immune cells might influence host immune response. Melanoma cells can activate and/or reshape their environment to secret metastatic progression factors. Further, melanoma cells can secrete inhibitory modulators and stop the recognition and maturation of effectors immune cells (Melnikova et al., 2009).

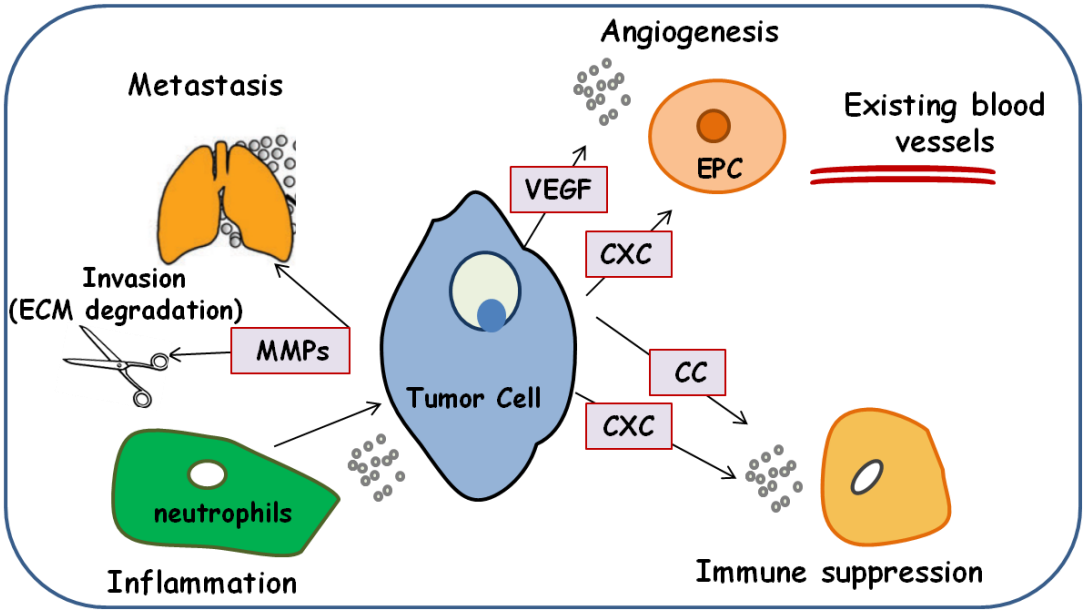


Figure 5*. Melanoma tumor cells develop CXC and CC chemokines that affect angiogenesis, inflammation, the tumor microenvironment, and immune cell response, recruitment of leukocytes, tumor growth and metastasis. In particular, melanoma cells also express chemokine receptors that guide metastasis to specific target organs. The chemokine gradient produced binds and activates these melanoma cell chemokine receptors. Chemokines also stimulate production of metalloproteinases that degrade the matrix and facilitate melanoma metastasis, so chemokine gradients, guide the metastasis progression (Richmond et al., 2009). ECM means Extra Cellular Matrix.*

In the last years a large number of biological data were generated and several new techniques enable the simultaneous detection of different modified elements. The *OMICS* sciences (Genomics, proteomics, transcriptomics, metabolomics and many others) use these high-throughput (HT) screening techniques to generate at the same time a large amount of data about genes, gene expression or proteins network. (Schneider MV et al., 2011).

1.2.1. THERAPEUTIC APPROACHES

The therapeutic approaches to melanoma depend on the stage of the tumor and they can be summarized as follows:

- Resection: surgery removal of the tumor and of the surrounding healthy tissue.
- Radiotherapy: the irradiation with high-energy ionizing electromagnetic rays blocks the growth and destroys cancer cells. This is a local therapy that attack a specific and detailed area, thus is not suitable to treat tumor too widespread.
- Chemotherapy: use of drugs that, through the blood circle, reach the tumor site and destroy cancer cells in a no-selective manner. Dacarbazine (5(-3, 3Dimehtyl-Triazenyl-Imidazole-4Carboxamide, DTIC) cisplatin (CDDP), methotrexate (MX) and others are used as chemotherapy drugs, each of them acting with different mechanism of action.
- Immunotherapy: treatment of cancer by inducing, enhancing, or suppressing an immune response; interferon- alpha (IFN-α) and interleuchin-2 (IL-2) are the most studied. In particular, IL-2 acts modulating effector cells, T-cells, NK (Natural Killer) and LAK (Lymphokine-Activated Killer). Regulatory T cells and myeloid-derived suppression cells seem to play an important role in inducing tolerance and in the down-regulation of immune response to tumor antigen, through the production of immune suppressive IL-10, transforming growth factor beta (TGF-beta), and the expression of inhibitory molecules such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). CTLA-4 is expressed on the activated T- lymphocytes and exerts a suppressive effect on the induction of immune responses after interaction between T-cell receptor (TCR) and human lymphocyte antigen (HLA) peptide molecules on the antigen presenting cell (APC). The

CTLA-4 block may enhance antitumor responses by allowing the immune system responsiveness against an antigen. Blocked CTLA-4 using anti-CTLA-4 monoclonal antibodies exert a tumor regression activity, so these antibodies are using as immunotherapy drug (Del Vecchio et al., 2011).

- Differentiation therapy: differentiation is the process by which totipotent cells (or pluripotent), initially identical and derived from a common progenitor element, acquire different characteristics that allow them to reach a structural and functional specialization. This phenomenon is essentially based on the transcription regulation, microRNA regulation and differential synthesis of specific proteins, in response to signals coming from the extracellular environment. Within a single organism, therefore, variously differentiated cells have the same genotype, but they may present phenotypes also very dissimilar, attributable to a different regulation of gene expression. So these cells are, distinguishable both for morphological characteristics and their molecular composition. Differentiation occurs, as a rule, during embryonic development and it is the basis of histogenesis and organogenesis; otherwise, differentiation plays an important role during post-natal life, existing in some tissues, such as bone marrow. The concept of differentiation therapy stems from the observation that some molecules, synthetic or natural, can stop the growth of cancer cells by inducing the differentiation and bring back cells to assume the same morphology and function of cells of the origin physiological tissue. This therapeutic approach does not set eradicating the cancer, but is able to block the growth and metastatic progression; also, unlike chemotherapy, it seems to be a more selective approach towards malignant cells and less toxic. One of the greatest achievements in this field was obtained with the administration of trans-retinoic acid in patients with

acute promyelocytic leukemia: in most cases, this molecule has led to partial clinical remission of the disease (Thiele et al., 2000). It is also important to emphasize that many molecules of natural origin show a significant differentiating power: genistein (a flavonoid) induces, in fact, a dendritic phenotype and an increase in melanin synthesis in murine melanoma cells (Hartmann et al., 1997); while, naringenin (a flavonoid) (Lentini et al, 2007), methylxanthines (caffeine, theophylline and theobromine) (Beninati, 1995) and antraquinones (aloin, aloe-emodin *etc*) (Tabolacci et al., 2011; Rossi et al., 2010;) are capable of inducing terminal differentiation of tumor cells likely through the activation of the enzyme transglutaminase as regulator of cancer metastasis (Lentini et al., 2012).

- Combination therapy: very often surgery, systemic medical therapy and radiotherapy are not-sufficient to eradicate the tumor. In particular, treatment of distant metastatic melanoma is unable to obtain a survival advantage. For this reason, a combination of different drugs with different mechanism of action and other therapeutic approaches, are used (Ascierto et al., 2012).

1.2.2. CYTOKINES, CHEMOKINES AND GROWTH FACTORS

Cytokines (Greek "cyto-, cell; and "-kinos", movement) are a family of signaling molecules that mediate and regulate immunity, inflammation, hematopoiesis, and many other cellular processes (Feldmann, 2008). Cytokines were at first identified as products of immune cells that act as mediators and regulators of immune process. Non-immune cells also produce cytokines which can have effects on non-immune cells as well. Cytokines alter the body's response to cellular damage or invasive pathogens. The first

cells to secrete cytokines in response to injury are epithelial and endothelial cells that initiate a potent immune response. The innate immune cells generate a range of cytokines and regulate macrophages/monocytes, dendritic cells (DCs), natural killer (NK) cells, mast cells, eosinophilis, and neutrophilis. Cytokines is the term used to refer to the immunomodulating agents, such as interleukin and interferons. Each cytokine bind cell-surface receptor; the interaction with the receptor, actives subsequent cascades of intracellular signaling then alter cell function. Cytokines can be characterized as autocrine (self modulating), paracrine (modulating cells in the immediate surroundings), juxtacrine (modulating through cell membrane signaling), retrocrine (modulating to stop host defense). The effects of cytokines are often pleiotropic, redundant, synergyc and antagonist and form a network (Grotzinger, 2002).

Since a unifying classification is still lacking, cytokines are identified by numeric order of discovery, by a given functional activity, by kinetic or functional role in inflammatory responses (early or late, innate or adaptive, pro-inflammatory or anti-inflammatory), by primary cell of origin (monokine = monocyte derivation; lymphokine = lymphocyte derivation), and, more recently, by structural homologies shared with related molecules (Kelso, 1998). It can be possible to identify cytokines as long and short chain structure, however these molecules can be classified also on their main function (Abbas et al., 2002). A variety of cells are capable of making cytokines, but the biggest producers are macrophages and T-helper lymphocytes. Cytokines are involved in hematopoiesis, adaptive immunity, innate immunity and inflammation. Cytokines have been classified as, interleukins (IL), interferon (INF), tumor necrosis factor (TNF), colony stimulating factor (CSF), chemokines and growth factors (*Table 1*). The term "interleukin" is used to identify cytokines that have the ability to act as signal molecules among different population of leukocytes (IL-1~IL-29). Interferons are a group of glycoproteins produced

by human or animal cells following the infection of virals and exposure to various inducing agents (INF-α; INF-β; INF-γ). TNFs (TNF- α; TNF-β) are known to have a multiplicity of actions. Colony stimulating factors (CSFs) [multi-CSF (IL-39); granulocyte macrophage-CSF (GM-CSF); monocyte-CSF (M-CSF); granulocyte-CSF (G-CSF); stem cell factor (SCF), erythropoietin (EPO)] stimulate proliferation or differentiation of pluripotent hematopoietic stem cells and different progenitors.

INTERLEUKINS	IL-1α/β, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 23, 27
INTERFERONS	IFN-α, β, γ, Ω
COLONY-STIMULATING FACTOR	G-CSF, GM-CSF, M-CSF, EPO, IL-3, MEG-CSF, SCF
IL6 FAMILY	IL-6, IL-11, CNTF, CT-1, OSM, LIF, NNT,-1/BSF-3
TNF FAMILY	TNF-α, TNF-β, LT-β, FasL, CD40L, CD27L, CD30L, NGF, TRAIL, TRANCE, APRIL, LIGHT, THANK, TALL-1
GROWTH FACTOR	FGFs, CTAP-3, EGF, ECGF, HGF, IGFs, PDGFs, PAF, TGFs, KGF, CTGF, GDF-15/MIC-1, Fibrosin
CHEMOKINES	**C**: lymphotactin **CC**: MPC-1,2,3,4, RANTES, MIP-1s, MIP-3,4,5, eotaxin **CXC**: IL-8, PF-4, NAP-2, GROs, ENA-78, IP-10, Mig **CX_3C**: fractalkine **Others**: IL-16, ecalectin

Table 1. *Cytokine classification (Xing and Wang, 2000).*

Cytokines are involved in a very elaborate network that is composed of two different immune mechanisms: innate and adaptative response (Abbas et al., 2002) (*Figure 6*).

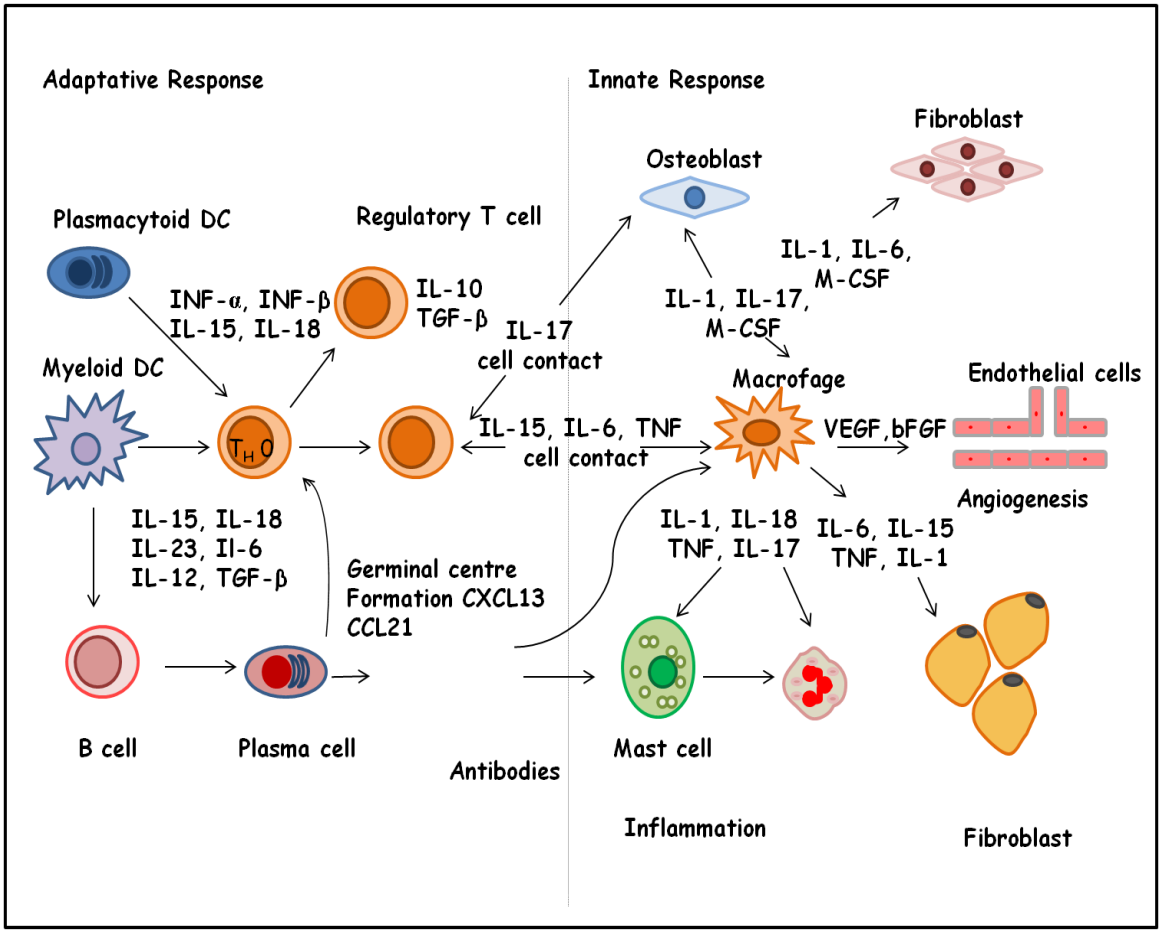


Figure 6. *Cytokines network*

Chemokines are a family of small cytokines or proteins secreted by cells. They have ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines. Proteins are classified as chemokines if they share structural hallmarks such as small size (8-10 kDa, with the mature peptide comprising between 70 and 120 amino acids), and the presence of four cystein residues inconserved locations that are important to form their 3-dimensional shape. Chemokines are classified into subfamilies denoted as CXC (one amino acid between the first two cysteine residues), CC (lacking the amino acid between the first two cysteine residues), CX3C (three amino acids between the first

two cysteines), and C (lacks one cysteine in the first pair of cysteine residues) (Richmond *et al*, 2009 ; McColl, 2002)(*Figure 7*).

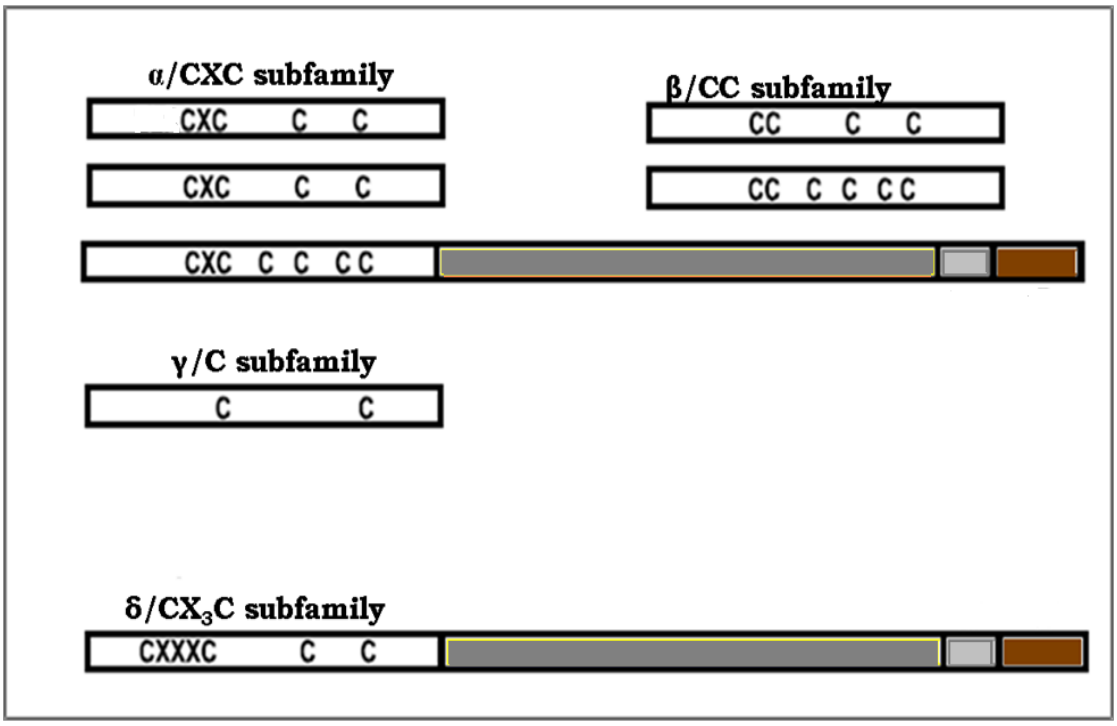


Figure 7. General structure of chemokine superfamily members (McColl, 2002).

Chemokines (L) bind to each specific receptors (R). In the *table 2* the chemokines nomenclature is reported.

Chemokine ligand nomenclature		
Systematic name	**Original names/s**	
	Human	***Mouse***
CXCL1	MGSA, gro-α	KC
CXCL2	MIP-2α, gro-β	MIP-2*
CXCL3	MIP-2β, gro-γ	
CXCL4	PF4	PF4
CXCL5	ENA-78	LIX
CXCL6	GCP-2	CKα-2
CXCL7	NAP-2	?
CXCL8	IL-8, NAF, NAP-1	?
CXCL9	Mig	Mig
CXCL10	IP-10	Crg2
CXCL11	I-TAC	I-TAC
CXCL12	SDF-1α, SDF-1β	SDF-1
CXCL13	BLC, BCA-1	BLC, BCA-1
CXCL14	BRAK, Bolekine	BRAK
CXCL15	?	Lungkine
CXCL16	CXCL16	CXCL16
CCL1	I-309	TCA-3
CCL2	MCP-1, MCAF	JE
CCL3	MIP-1α, LD78α	MIP-1α
CCL4	MIP-1β, LD78β	MIP-1β
CCL5	RANTES	RANTES
CCL6	?	C10, MRP-1
CCL7	MCP-3	MARC
CCL8	MCP-2	MCP-2
CCL9/10	?	MRP-2, CCF18, MIP-1γ
CCL11	Eotaxin	Eotaxin
CCL12	?	MCP-5
CCL13	MCP-4	?
CCL14	HCC-1	?
CCL15	HCC-2, Lkn1, MIP-1δ	?
CCL16	HCC-4, LEC	LCC-1
CCL17	TARC	TARC
CCL18	DC-CK1, MIP-4, PARK, AMAC-1	?
CCL19	MIP-3β, ELC, exodus-3	MIP-3β, ELC, exodus-3
CCL20	MIP-3α, LARC, exodus-1	MIP-3α, LARC, exodus-1
CCL21	6Ckine, SLC, TCA-4, exodus-2	6Ckine, SLC, TCA-4, exodus-2
CCL22	MDC, STCP-1	ABCD-1
CCL23	MPIF-1	?
CCL24	MPIF-2, eotaxin-2	?
CCL25	TECK	TECK
CCL26	Eotaxin-3	?

CCL27	CTACK, ILC	ALP, CTACK, ILC, Eskine
CCL28	CCL28	CCL28
CXCL1	Lymphoctactin, SCM-1, ATAC	Lymphotactin
***CX*3CL1**	Fractalkine	Neurotactin

Table 2*. Systemic nomenclature introduced at the 1998 Gordon Conference on chemokines (*McColl et al., 2002*).*

The role of Chemokines is to act as chemoattractant to guide the migration of cells through an increasing their gradient towards the source of the chemokine. Chemokines have an important role during both processes of immune surveillance and in development and inflammation. In particular, McCool suggests that chemokines can play an important role at several stages of DC (Dendritic Cell) development and function. Basal chemokines production and expression on the surface of endothelial cells can mediate DC precursor recruitment and differentiation of precursor to the immature DC.

Growth factors are subfamilies of cytokines capable of stimulating cellular growth, proliferation, differentiation. The growth factors belonging to cytokines family are: transforming growth-factors (cytokines which stimulate the growth of their target cells [transforming growth factor-β (TGF-β)]; epithelial growth factor (EGF); vascular endothelial cell growth factor (VEGF); fibroblastic growth factor (FGF).

2. AIM

Human cutaneous metastatic melanoma is the most aggressive skin cancer without effective therapies (Bandarchi et al., 2010). Presently systemic treatment for metastatic melanoma, including both cytotoxic and immunologic therapies, produces low rates of response (Sekulic et al. 2008). Human metastatic melanoma shows a very fast invasion and progression mechanism, and very often the actual therapeutic approaches are unable to contrast the evolution of cancer. So, there is an urgent need for effective novel therapies directed to attack the cause of the malignancy and molecular targets to arrest cancer progression. So, aim of this thesis is to understand the mechanisms related to melanoma aggressiveness that is related to a high proliferation rate, high invasion capability and high metastatic power. In particular, using different human melanoma cell lines as models of study, our first aim was to identify groups of melanoma cell lines highly different in aggressiveness and malignancy. After that, it is useful to study the differential protein expression of these cell lines using proteomic multi-approaches to identify potential molecules responsible of the malignancy. Recently the translational medicine, a new approach to the medicine based on the translational of novel approaches from bench of biomedical researchers to the bed of patients, is focalized on information derived from human experimentation and optimizes the interaction between the basic and clinical research (Wehling, 2008). For this reason, a specific goal of our study is to verify our results in specimens from patients and so act at different levels of study. Clarke and colleagues reported that cancer stem cells have a key role in cancer progression because they are a reservoir of self-sustaining cells with the unique ability to self-renew and maintain the tumor. These cancer stem cells can divide and expand the cancer stem cell

pool and to differentiate into the heterogeneous nontumorigenic cancer cell types. These cells in most cases seem to constitute the bulk of the cancer cells within the tumor (Clarke et al., 2006). Therefore, an additional target of this study is to investigate the cancer stem cells behaviors to find putative molecules and/or pathways related to the melanoma aggressiveness potentially useful to delay and revert this melanoma characteristic.

3. MATERIALS AND METHODS

a. Cell culture

Human metastatic cutaneous melanoma cell lines used in the present study were: SK mel 28, and A375 (purchased and authenticated from the American Type Colture Collection, ATCC, Manassas, VA), Mel-397 (kindly supplied by Dr. Stefania D'Atri, IDI-Roma), SK mel 110 (Faraone et al.,2009), human keratinocyte cell line HaCat (CLS, Eppelheim, Germany) and human Melanocytes (Cascade Biologics, Portland, OR, USA). In preliminary experiments, additional human melanoma cell lines (WM-115, SK 120, SK 147, and M14 from estabilished culture) have been used (not shown). HaCat and A375 were grown for the specified time in complete medium in Dulbecco's modified Eagle's medium (DMEM; Hyclone, South Logan, UT) and supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO) 2 mM L-glutamine, and 100 IU/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA) in humidified 5% CO_2 atmosphere, at 37 °C and, when required, under serum deprivation. Mel 397, SK mel 28 and SK mel 110 were grown in RPMI 1640 (Hyclone) 10% FCS, 2 mM L-glutamine, and 100 IU/ml penicillin/streptomycin, and, when required, under serum deprivation. Melanocytes were cultured in MGM4 medium according to supplier's instructions (Life Tecnologies, Painsley,UK). Cells were counted by hemocytometer and viability was measured by Trypan blue assay. Human metastatic stem cell clones, Mel 1 and Mel 3 (kindly supplied by Dr. Adriana Eramo, ISS-Roma) were also used. Cancer stem cells were cultured in serum-free medium containing 50 µg/ml insulin, 100 µg/ml apo-transferrin, 10 µg/ml

putrescine, 0.03 mM sodium selenite, 2 µM progesterone, 0.6% glucose, 5mM HEPES 0.1% sodium bicarbonate, 0.4% bovine serum albumin (BSA), glutamine and antibiotics, dissolved in DMEM-F12 medium (Gibco-Invitrogen, Carlsbad, CA) and supplemented with 20 µg/ml EGF and 10µg/ml bFGF. Flasks non-treated for tissue culture were used in order to reduce cell adherence and support growth as undifferentiated tumor-spheres. Medium was replaced or supplemented with fresh growth factors twice a week until cells started to grow forming floating aggregates. Cultures were expanded by mechanical partial dissociation of spheres, followed by re-plating of cells and residual small aggregates in complete fresh medium.

b. Proliferation assay

Semi-confluent cells were harvested, plated and propagated in medium containing 10% FBS in 6 well plates at 3 different densities: high ($9x10^4$ cells/well), intermediate ($6x10^4$ cells/well), low ($2x10^4$ cells/well) as previously reported (Faraone et al., 2009). The day after, two of the six wells were washed with PBS w/o calcium and magnesium and cells were harvested using Trypsin/EDTA (Euroclone) and counted with a Burker chamber (this number represents the T0). Media of other remaining wells were removed, cells were washed with PBS and medium was then replaced with serum-free medium. Cells were photographed, washed using PBS, harvested with trypsin/EDTA and counted with a Burker chamber after 24 and 48 hours of serum deprivation (*Figure 8)*. All experiments were carried out at least 3 times in duplicate.

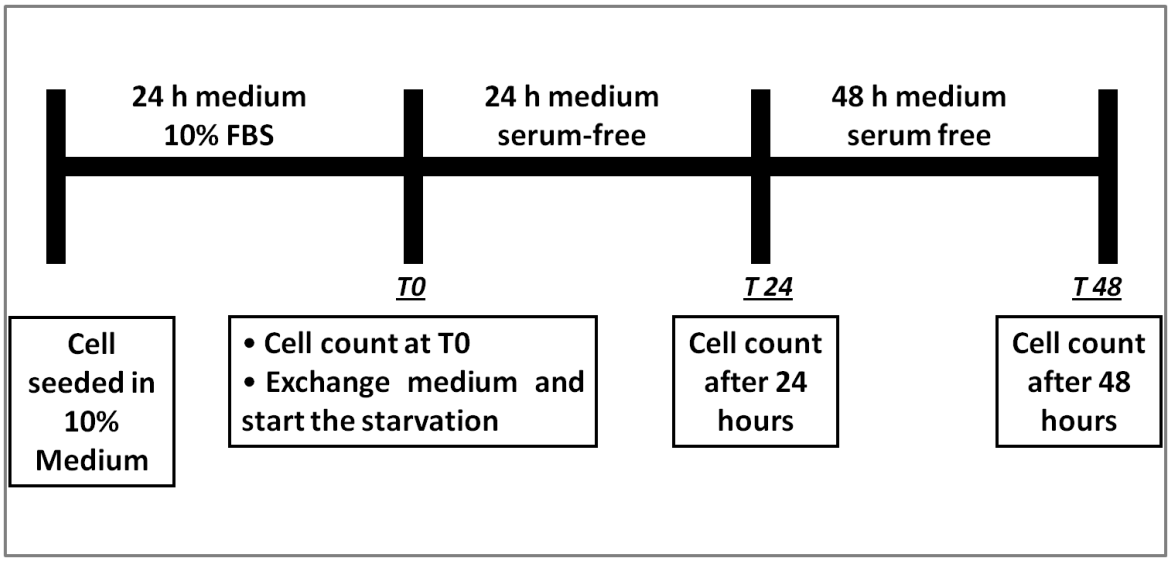


Figure 8. Schematic representation of the Proliferation Assay.

c. Invasion assays

Invasion assays were performed in Boyden Chamber as described (Aguzzi et al., 2004 ; Albini et al., 1987). Polycarbonate polyvinylpyrrolidone free membranes (8 µm pore size, Costar, Cambridge, MA), were coated with 0.5 mg/ml Basement Membrane Matrix Matrigel (BD Biosciences, Franklin Lakes, NJ). Cells were allowed to invade, using fetal bovine serum (FBS) as chemoattractant, for 5 h at 37 °C. The membrane was fixed in ethanol and stained with toluidine blue (2%). After the removal of non-migrating cells on the upper side of the membrane, the number of invasive cells was evaluated by means of the 4 field (x400).

d. Cell extract preparation

Postnuclear cell lysates were prepared using the following lysis buffer: NaCl 0.150 M, Igepal CA-630 (Sigma-Aldrich) 1%, Tris (hydroxymethyl) aminomethane 20mM, complete EDTA-free Protease Inhibitor Cocktail Tablets

(containing PMSF, Pefabloc Sc, Aprotinin, Leupetin, Pepstatin)(Roche, Milan, IT) and phophatase inhibitor (Sodium Floride 40mM and orthovanadate 1mM). Cells were plated in 100 mm Petri dishes (Corning, NY, USA) at an intermediate density ($6x10^5$ cells/well) in complete medium. The day after, cells were washed with PBS w/o Calcium and Magnesium and then replaced with a serum-free medium. After 48h of serum-deprivation, cells were washed 2 times with PBS (Euroclone), then 300 µl of lysis buffer were added and cells were harvested and cell lysates were collected using a scaper. Cell lysates were collected and incubated 20 minutes on ice. After that, the extracts were centrifuged at 13200 rpm x 10 minutes at 4 °C in an Eppendorf Centrifuge 5415R and the soluble fraction was collected. The protein concentration of each cell extract was then measured by Bradford's protocol (Bio-Rad kit, Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, using a cuvette spectrophotometer (Uvikon 860 Instrument, Kontron, Zurich, CH).

e. Programmed cell death analysis

Cells were grown in 6 wells plastic plates (Corning Inc.), trypsinized and at the specified time of growth, harvested (floating and attached cells) and fixed in 70% cold ethanol. Fixed cells were washed and incubated with 200 µg/ml ribonuclease A (RNase A, Life Tecnologies) for 30 min at 37 °C and 50 µg/ml Propidium Iodide (PI) and gated out electronically as previously described (Facchiano et al. 2001). The relative DNA content and cells distribution in various cell cycle compartments were determined with a FACScanto Becton Dickinson Instrument (Becton Dickinson, CA, USA) and the FACS Diva software (5.0.3 version).

f. Differentiation and culture of stem cells

Differentiation of melanosphere forming cells was obtained by replacing stem cell medium with Melanocyte Growth Medium (MGM4, Lonza, East Rutherford, NJ) and they were cultured in tissue culture-treated flasks, to allow cell attachment and differentiation toward the melanocytic lineage. Cells were harvested and conditioned media were taken after four days of differentiation (PC cells). PC cells were washed with PBS and fresh medium was replaced; after four days conditioned media were taken (PC^+ cells).

g. Cytokines and growth factor analysis: the Multiplex approach

Cytokines and growth factors were measured by using a Bio-Plex Pro human cytokine 27-plex panel (Bio-Rad Laboratories, Hercules, CA) as described (Tabolacci et al., 2011) for the following analytes: IL-1Ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, TNF-α, IFN-γ, MIP-1α, MIP-1β, Eotaxin, MCP-1, G-CSF, GM-CSF, basic FGF, VEGF, IP-10, RANTES, and PDGFbb. Conditioned media were collected, centrifuged and concentrated (from 10X to 100X) using a centrifugal filter units with microporous membrane (YM-3). The protein concentration was then measured as above described. Twenty five micrograms of proteins from total cell lysates were analyzed, according to the manufacturer's instructions. The analysis was carried out using 50 µl of the sample. After the incubation with antibodies-activated magnetic beads, samples were washed using a Bio-Plex Pro ^{TM} Station (Bio-Rad). The quantification was carried out on a Bio-Plex Array Analyzer (Bio-Rad) by a Bio-Plex Manager Software version 4.1.1. Results expressed as pg/ml were analyzed by a Bio-Plex Manager Software version 6. Normalization of samples

was achieved by correcting for the protein concentration and for number of harvested cells.

h. Electrophoresis

The protein electrophoretic profile of the melanoma cell lines and HaCat lysates was analyzed using gels (2.4 -15% continuous acrylamide-bisacrylamide gradient vertical gels, manually poured into 16×18 cm, thickness 1.5 mm, 15 wells) generated with a gradient maker (Model 385, Bio-Rad, Hercules California, US) and run with the SE 600 Ruby Apparatus (Hoefer, Inc. Holliston,MA), using fresh solutions as described (Facchiano et al 2010). The gel stacking region was hold. Protein extracts were also separated on 4–15% Mini-PROTEAN® TGX™ pre-cast polyacrylamide gel (8.6 x 6.7 x 0.1, Bio-Rad) as pubblished (Verdoliva et.al 2013).

i. Immunological analysis

Whole cell lysate (40 μg per well/120 μg per well), separated on 4–15% Mini-PROTEAN® TGX™ pre-cast polyacrylamide gel Bio-Rad), blotted onto nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, UK), was probed with rabbit anti-caspase 3 (1:1000, Cell Signaling, MA, USA) or anti-β actin (1:5000, Abcam, MA) in milk 1.25% TPBS 0.1% for 1 h at RT. After the membrane was washed three times with PBS-T(0.1% Tween 20 in PBS, pH 7.4) for 10 min, incubated in the appropriate HRP-conjugated secondary antibody at room temperature for 1 h. Horseradish peroxidase-conjugated secondary antibodies (Pierce) were used, followed by chemiluminescence according to manufactory's instracture (ECL; Euroclone Milan,IT).

j. Mass spectrometry analysis

For protein identification, the total lane of the gel was cut, proteins were reduced, alkylated and digested overnight with bovine trypsin sequencing grade (Roche Applied Science, Monza, IT) according to a standard protocol. Peptide mixtures were analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) using an HPLC Ultimate 3000 (DIONEX, Sunnyvale, CA) connected on line with a linear Ion Trap (LTQ, ThermoElectron, San Jose, CA). Peptides were desalted in a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) and then separated in a reverse phase column, a 10 cm long fused silica capillary (Silica Tips FS 360-75-8, New Objective, Woburn, MA), slurry-packed in-house with 5 μm, 200 Å pore size C18 resin (Michrom BioResources, CA) as described (Facchiano et al. 2012). Peptides were eluted using a linear gradient from 96% A (H_2O with 5% acetonitrile and 0.1% formic acid) to 60%B (ACN with 5% H_2O and 0.1% formic acid) in 40 min, at 300 nl/min flow rate. Analyses were performed in positive ion mode and the HV Potential was set up around 1.7-1.8kV. Full MS spectra ranging from m/z 400 to 2000 Da were acquired in LTQ mass spectrometer operating in a data-dependent mode in which each full MS scan was followed by five MS/MS scans, where the five most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) with normalized collision energy of 35%. Target ions already fragmented were dynamically excluded for 30 s. Tandem mass

spectra were matched against Swissprot database and through SEQUEST algorithm (Yates et al., 1995) incorporated in Bioworks software (version 3.3, Thermo Electron) using fully tryptic cleavage constraints with the possibility to have one miss cleavage permitted, static carbamidomethylation on cysteine residues and methionine oxidation as variable modification. Data were searched with 1.5 Da and 1 Da tolerance respectively for precursor and fragment ions. A peptide has been considered legitimately identified when it achieved cross correlation scores of 1.8 for [M+H]1+, 2.5 for [M+2H]2+, 3 for [M+3H]3+, and a peptide probability cut-off for randomized identification of $p<0.001$. Protein and peptide false discovery rate (FDR) was calculated dividing the number of false hits by the number of positive hits where the false hits are evaluated using a decoy database directly constructed by Bioworks software on the same human database used for the target search and adopting the same scoring criteria.

k. Bioinformatic analysis

The bioinformatic analysis of data was performed through DAVID portal (http://david.abcc.ncifcrf.gov/)(Huang et al., 2009a, 2009b; Facchiano et al., 2003). In particular proteins, identified by the UNIPROT accession number, were classified using several parameters (e.g. functional annotation chart, KEGG analysis of pathways). Moreover, to confirm the proteomic and Bio-Plex results, data from microarray dataset were analyzed through Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/gds).

l. Statistical analysis

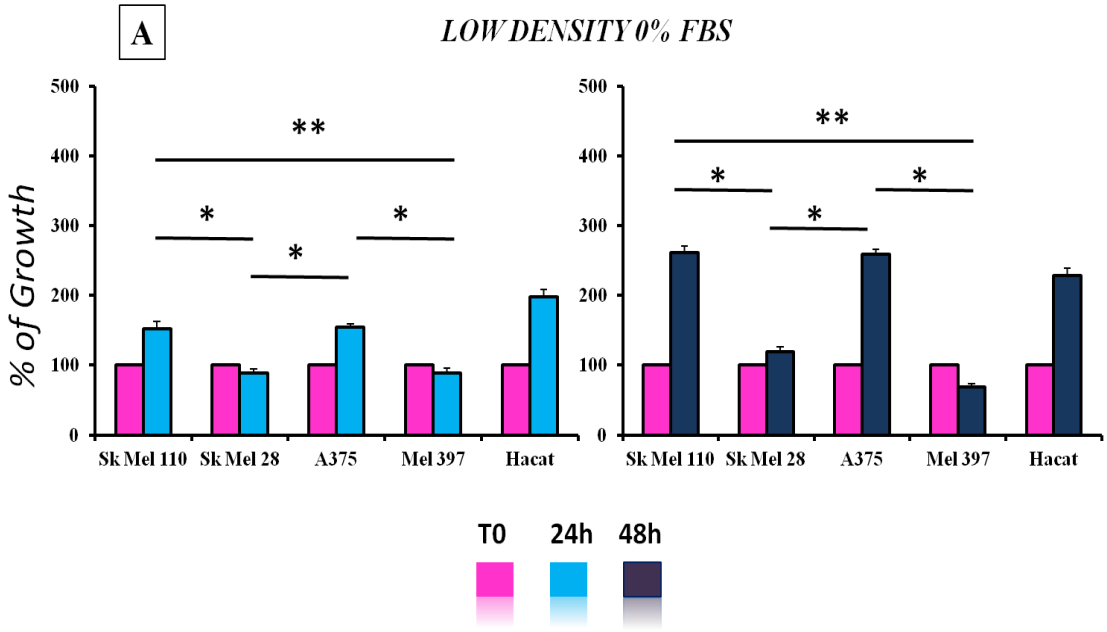
All experiments were repeated three times, and the results are expressed as the mean ± SD (standard deviation) of three different determinations. Data were analyzed by the *t*-Student test, differences were considered highly significant when $p<0.05$.

4. EXPERIMENTAL RESULTS

I. Cell proliferation studies

Human cutaneous metastatic melanoma cell lines, melanocyte cell line and a keratinocyte cell line were used to investigate and compare the aggressiveness of several cancer cell lines. Tumor aggressiveness includes migration, invasion, cell proliferation, angiogenesis capabilities of metastatic cells (White et al., 2011). This study allowed identifying the most and the less aggressive melanoma cell lines to be used for further analyses. Cell lines were seeded at three different densities identified as low (20,000 cells/well), intermediate (60,000 cells/well) and high (90,000 cells/well). Cells were grown in serum-free medium (*Figure 9A,9B,9C*) and in complete medium supplemented with 10% FCS (*Figure 9D*) and cell number was measured at 24 and 48 hours of serum deprivation by counting with and hemocytometer. Cell viability was checked by the Trypan blue assay (*Rossi et al 2010*). Different growth properties were observed in the different cell lines. SK mel 110 and A375 cells showed a higher growth rate in both conditions (*Fig.9A, B, C, and D*). On the contrary, SK mel 28, Mel 397 and HaCat cells, showed a lower growth rate under starvation conditions (*Fig. 9A, B, C*). Moreover, SK mel 110 and A375 in

serum-free medium and seeded in a low density (30,000/well), showed after 24 hours a 252.26% growth rate (time 0), and a 260 % growth rate after 48 hours, compared to cells at time 0 (T0), reported as 100%. A375 under the same conditions showed after 24 hours a 154.67% growth rate and 259% growth rate after 48 hours, in respect of cells at T0. Unlike SK mel 28 and Mel 397 cells under the same conditions showed a 88.35% growth rate after 24 hours and 119.90% and 68.75% after 48 hours, respectively. HaCat cells showed after 24 hours a 198% growth rate and 228.78% after 48 hours (Fig. 1A). Cells seeded at 90,000/well (high density) showed a different trend compared to the low density condition. SK mel 110 showed a 279.05% growth rate after 24 hours and a 343.2% growth rate after 48 hours. A375 cell line revealed after 24 hours a 248.16% growth rate and after 48 hours a 309.76% growth rate. SK mel 28 and Mel 397, showed after 24 hours a 136.74% and 92.2% growth rate respectively, and showed after 48 hours a 256.34% and 100.61% growth rate. HaCat cells after 24 hours and 48 hours showed a 238.8% and 301.34% growth rate respectively. Cells seeded at 60,000/well (i.e. intermediate density) showed intermediate behaviors, indicating that the observed effects are cell-density dependent indeed. All these results are summarized in the following figure (*Figure 9*).


A
LOW DENSITY 0% FBS
% of Growth
500
400
300
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0
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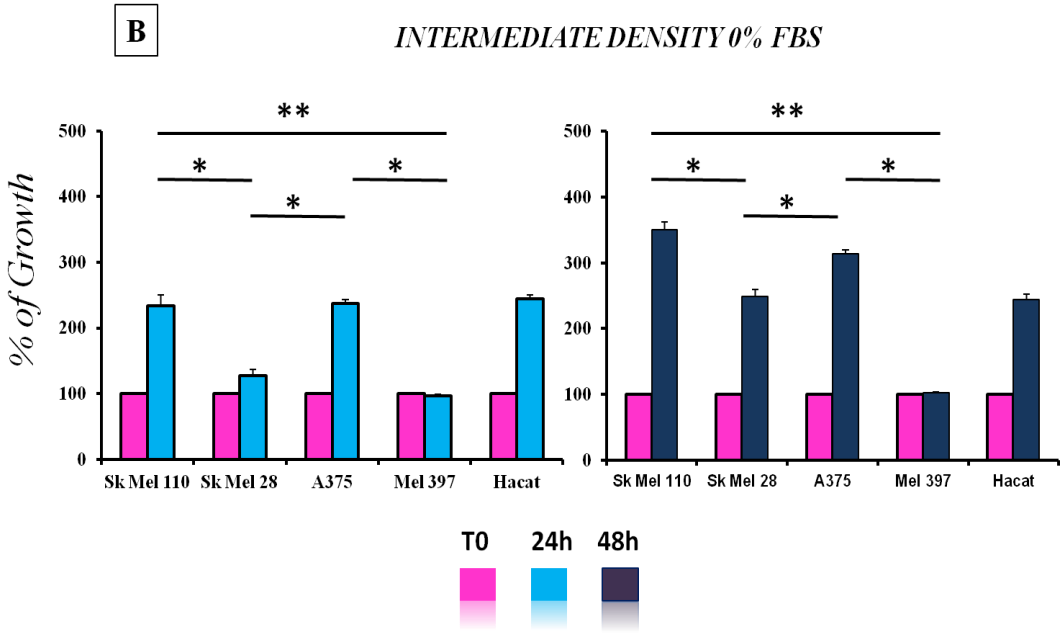
*
*
*
Sk Mel 110
Sk Mel 28
A375
Mel 397
Hacat
T0
24h
48h


B
INTERMEDIATE DENSITY 0% FBS
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Sk Mel 110
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A375
Mel 397
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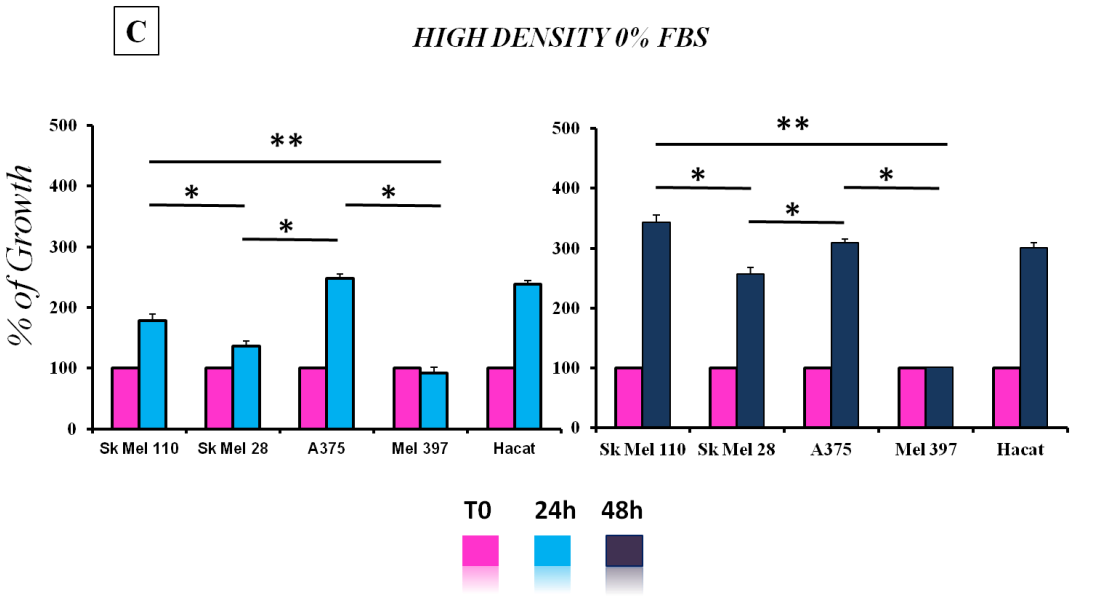
C
HIGH DENSITY 0% FBS
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Sk Mel 110
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Mel 397
Hacat
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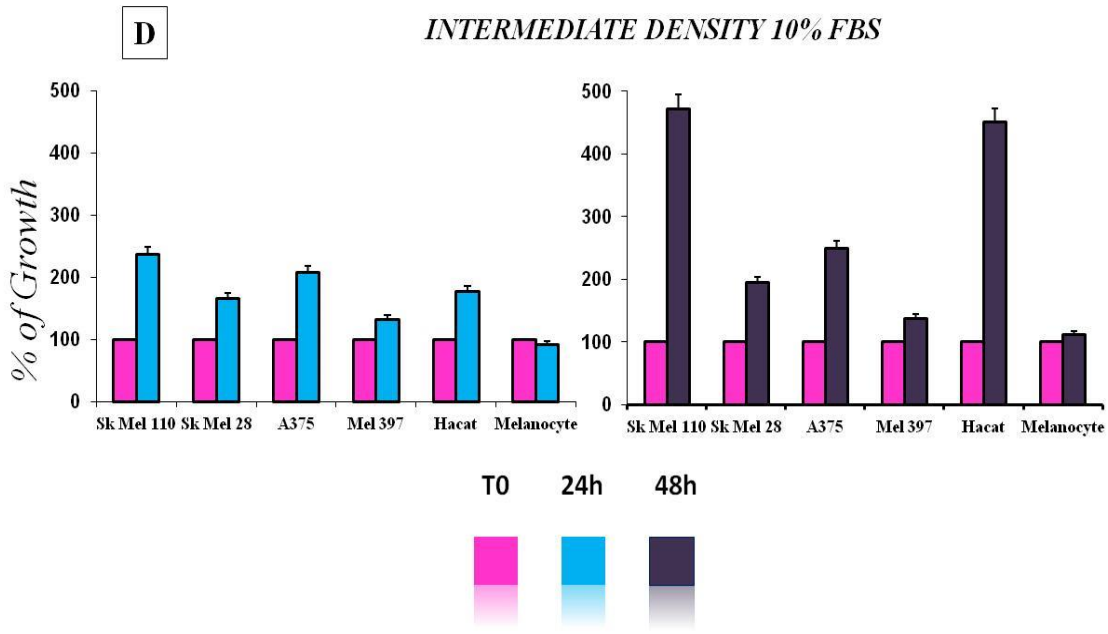

D
INTERMEDIATE DENSITY 10% FBS
% of Growth
500
400
300
200
100
0
Sk Mel 110
Sk Mel 28
A375
Mel 397
Hacat
Melanocyte
T0
24h
48h

Figure 9. *Growth Rate of Human Metastatic Melanoma Cell Lines. Panel (A)- (F) show growth rate of SK mel 110, SK mel 28, A375 and Mel 397 metastatic melanoma cells and human HaCat keratinocytes, in serum free-medium and at three different densities; (A) low density, (B) Intermediate density, (C) high density. Panels (D) show the growth rate of the same 5 cell lines in 10% FCS supplemented medium. Each data point represents the mean of three different determinations. Asterisks indicate statistical significance ($p<0.05$), comparing most to the less aggressive at the same time point.*

This studies allow to identify two different group of cell lines that differ significantly for growth rate; SK mel 110 and A375 (high growth rate) and SK mel 28 and Mel 397 (low growth rate). Starting from this observation, for our study we choose A375 cell line and SK mel 28 as models to study the melanoma aggressiveness (*Figure 10*).

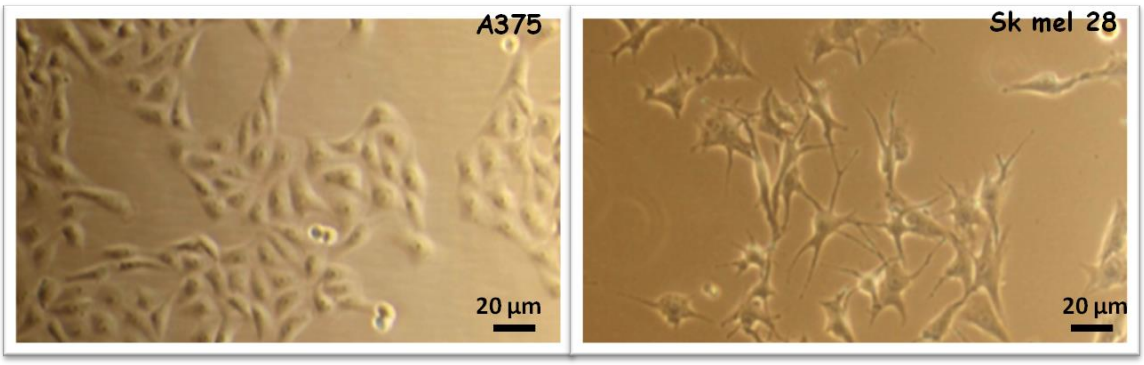


Figure 10. *A375 and SK mel 28 cell line under serum deprivation.*

II. *Invasion assay*

Invasion capability of SK mel 28 compared to A375 cell line, estimated by a Boyden Chamber assay, was impaired. The analysis performed on MG-coated porous filter, indeed, showed a high invasive power of the most aggressive (A375) compared to the less

aggressive (SK mel 28) cells; in particular a mean of 42 and 5cell per field were counted respectively (*Figure 11*).

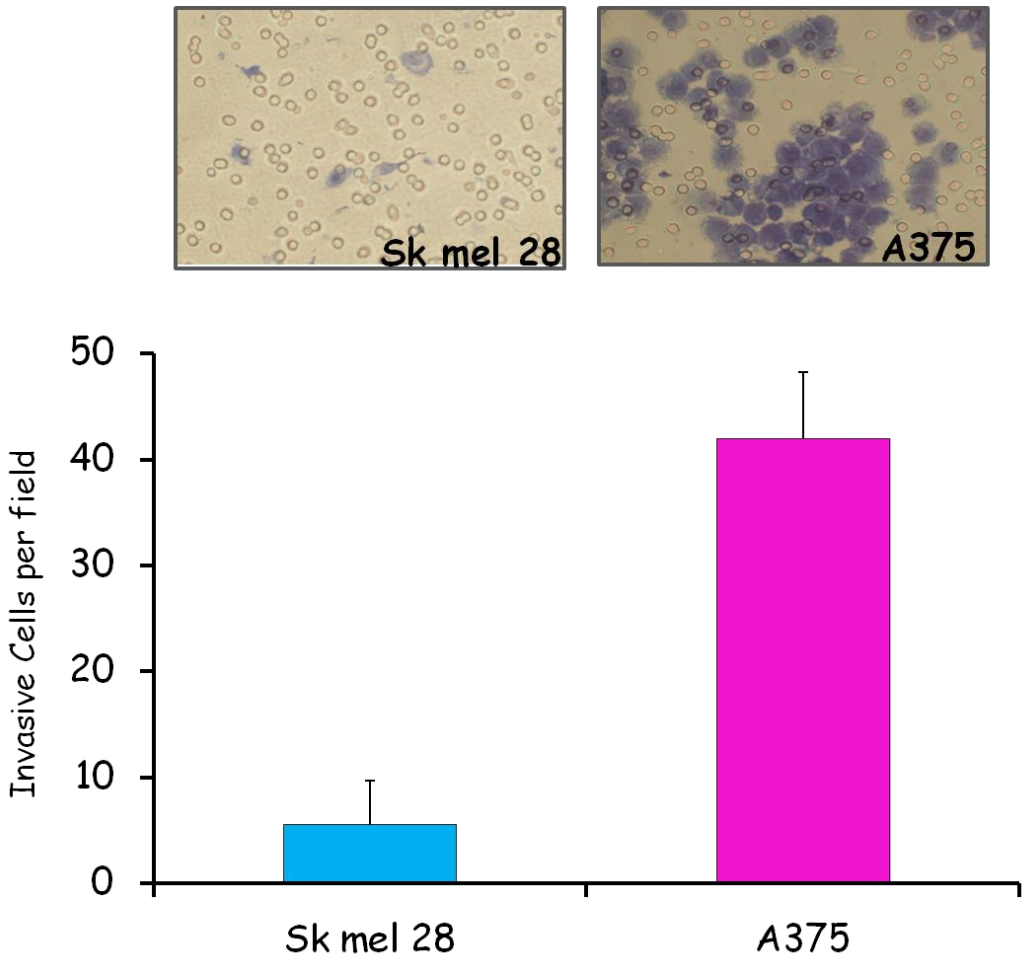


Figure 11 *Assessment of cell invasion SK mel 28 and A375 cell lines by Boyden Chambers assay. A375 resulted more invasive compared to SK mel 28 cells Quantification of the invasion is expressed as the number of invasive cells per field. Top of the panel, representative micrographs of Boyden Chambers invasion assay (400× magnification).*

III. *Cell death analysis*

To elucidate the consequence of the serum deprivation on the A375 and SK mel 28 proliferation, we evaluated the DNA content using flow cytometry after PI staining of nuclei. As shown in *figure 12*, the percentage of apoptotic cells under serum deprivation conditions (measured as sub-G1 population) was significantly higher in SK mel 28 cells than that measured in A375 cells (15.8% versus 3.3%, $p<0.05$).

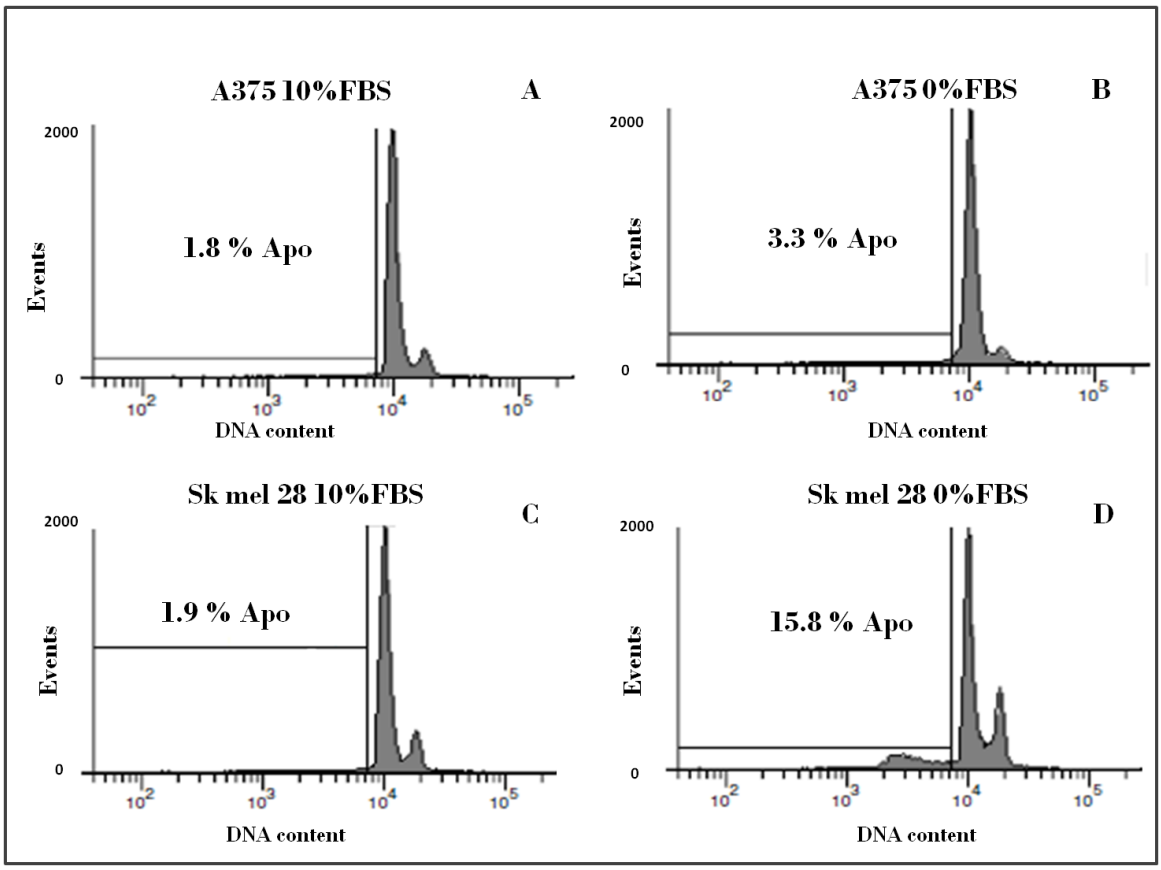


Figure 12. *Apoptosis evaluation of A375 and SK mel 28 cell grown in a complete medium (A and C) and in a serum-free medium (B and D) . Flow cytometric analysis of the cellular DNA content was performed after propidium iodide staining, and the*

subG0/G1 region represents the apoptotic cell population. Data were expressed as the percentage of total cells. Each point represented the mean ± SD of three different determinations.

IV. *Cytokines and Growth Factors Analysis*

Multiplex assay using the Bio-Plex technology (see methods) measured the content of 27 human cytokines and growth factors in protein extracts and in conditioned media from melanoma cell lines and stem cells under study. Data obtained, show in cell extracts (*Table 3a*) cytokines levels that result not significantly modified and they are following reported: PDGF-bb; IL-2; IL-4; IL-5; IL-10; IL-15; IL-17; G-CSF; GM-CSF; MIP-1b; and TNF-α. Otherwise, comparing A375 and SK mel 28 extracts, the most and the less aggressive cell lines respectively, we identified significantly different levels of some inflammatory cytokines and angiogenic growth factors, the modified analytes were IL-6, IL-1ra, IL- 1 β, IL-7, IL-8, IL-9, IL-12, Eotaxin, FGF-basic, INF-γ, RANTES, and VEGF . In particular, data show (*Table 3b*) that PDGF-bb, IL-1β, IL-1ra, IL-8 resulted higher in SK mel 28 than A375, while IL-6, IL-12 and VEGF were lower in SK mel 28 than A375 extracts.

In A375 conditioned media IL-1ra, IL-6, IL-7, IL-9, IL-12, Eotaxin, Rantes and VEGF are increased ($p<0.001$) while IL- 8 is reduced in A375 conditioned media when compared to SK mel 28 ($p<0.001$).

CYTOKINES LEVELS INCELL EXTRACT		
	SK mel 28	**A375**
PDGF-bb	2.75 ± 1.54	0.44 ± 0.39 *
IL-1b	9.07 ± 8.21	9.07 ± 8.21*
IL-1ra	3.20 ± 0.78	1.49 ± 0.71 *
IL-2	OOR	OOR
IL-4	0.02±0.01	0.03±0.02
IL-5	OOR	0.02±0.02
IL-6	0.07 ± 0.08	127.42 ± 0.08 **
IL-7	0.40 ± 0.06	0.03± 0.05
IL-8	81.97± 22.80	4.09 ± 0.08 *
IL-9	0.16 ± 0.05	0.25± 0.18
IL-10	OOR	OOR
IL-12	2.3 ± 0. 54	31.97 ± 1.08 **
IL-13	0.09 ± 0.08	0.03 ± 0.02
IL-15	OOR	OOR
IL-17	OOR	0.03 ± 0.01
Eotaxin	0.68 ± 0.30	0.41 ± 0.36
FGF basic	4.09 ± 0.98	11.36 ± 10.87
G-CSF	2.11±2.06	18.66±16.03
GM-CSF	0.28±0.24	0.28±0.17
IFN-g	0.61±0.10	0.78±0.32
IP-10	34.37±19.22	0.46±0.27
MCP-1	OOR	8.56±8.10
MIP-1a	OOR	2.66±1.76

MIP-1b	0.78±0.28	2.16±1.66
RANTES	0.54 ± 0.04	1.50 ± 1.20
TNF-a	OOR	0.07± 0.6
VEGF	4.56 ± 1.54	257.38 ± 59.25 **

Table 3a. Twenty seven human cytokines and growth factors levels have been measured (data are reported as pg/ml). The most significantly modified are shown *= $p<0.005$ or **= $p<0.001$. ORR (Out Of Range) identifies analytes that are not detectable by the assay.

CYTOKINES LEVELS IN CONDITIONED MEDIA		
	SK mel 28	**A375**
PDGF-bb	OOR	OOR
IL-1b	OOR	0.03± 0.04
IL-1ra	28.17 ± 3.30	48.61 ± 3.38 *
IL-2	1.67 ±1.89	2.36 ±2.34
IL-4	0.06±0.10	1.33±1.66
IL-5	OOR	0.10±0.14
IL-6	1 ± 0.04	6.75 ± 1.06 **
IL-7	0.40 ± 0.06	6.52 ± 4.89 **
IL-8	116.96 ± 5.25	2.75 ± 1.06 **
IL-9	9.50 ± 3.67	1686.88 ± 5.25 **
IL-10	OOR	1.19±1.69
IL-12	23.97 ± 3.11	66.72 ± 11 *
IL-13	OOR	19.24±2.46
IL-15	OOR	OOR
IL-17	OOR	OOR
Eotaxin	0.09 ± 0.01	88 ± 24.45 **
FGF basic	11.30±10.71	OOR
G-CSF	2.11±166	18.66±16.03
GM-CSF	10.22±9.28	39.93±24.54
IFN-g	47.26±31.76	382.04±237.34
IP-10	OOR	2.48±1.51
MCP-1	OOR	8.56±7.10
MIP-1°	4.11±3.12	1.75±2.47
MIP-1b	0.33±0.28	OOR
RANTES	15.24 ± 1.45	245.67 ± 24.25 **
TNF-a	OOR	0.19± 0.27
VEGF	117.29± 20.19	10134.32 ± 3785.37 **

Table 3b. Twenty seven human cytokines and growth factors levels have been measured (data are reported as pg/ml). The most significantly modified are shown *= $p<0.005$ or **= $p<0.001$. ORR (Out Of Range) identifies analytes that are not detectable by the assay. (Lower or higher than the limit of detection).

In the *table 4* the results of different melanoma stem cell clones at three different differentiation stages are reported. Mel SC (melanoma stem cells), Mel PC(differentiating cells) and Mel PC^+ (differentiated cells) show different cytokine levels related to the differentiation stage. Data indicate that PDGF-bb, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-15, Il-17, Eotaxin, FGF basic, GM-CSF, IFN-g, IP-10, MCP-1, TNF-α were not modified in both cell extracts and conditioned media, in the three conditions. On the contrary, MIP-1 α, MIP-1 β, RANTES, VEGF, IL-7, IL-12, IL-13 are high in SC, rise in PC and decrease in PC^+. IL-9 and IL-1 β levels are increase in PC and in PC^+ compared to SC cells and therefore, these levels are increased in PC^+ compared to PC cells.

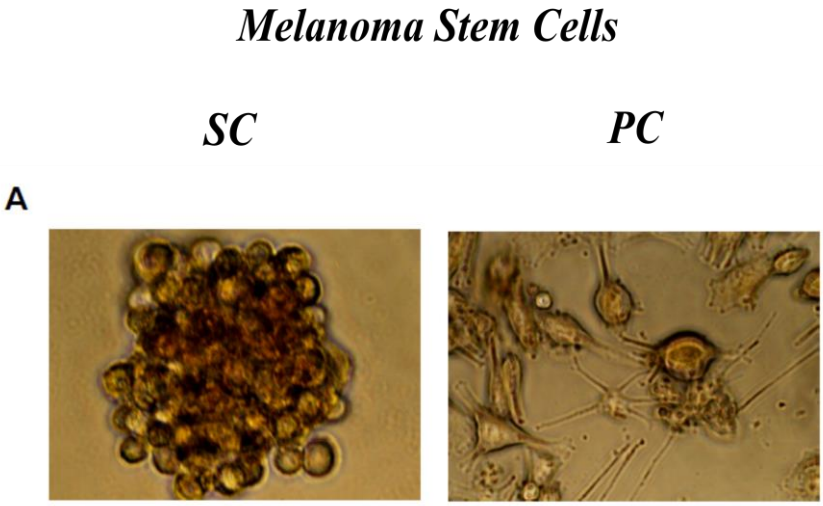


Figure 12. *SC and PC human melanoma stem cells (kindly purchased from Dr. Adriana Eramo, ISS). (Sette et al., 2013)*

CYTOKINES LEVELS IN CELL EXTRACTS			
	Mel 1 SC	**Mel 1 PC**	**Mel 1 PC^{+}**
PDGF-bb	12.45±1.25	7.55±0.76	9.32±0.93
IL-1b	8.20±0.82	432.74±287.07	453.78±326.59
IL-1ra	3.22±0.32	5.52±4.04	7.06±0.71
IL-2	14.22±20.08	40.48±44.55	40.58±48.10
IL-4	2.70±0.27	0.80±0.08	1.35±0.92
IL-5	1.63±0.16	1.69±1.23	1.70±2.09
IL-6	OOR	OOR	OOR
IL-7	2.17±0.22	453.55±599.67	44.00±21.41
IL-8	OOR	OOR	OOR
IL-9	256.86±286.86	20933.61±15561.24	4807.36±3176.37
IL-10	14.56±1.46	7.92±7.12	16.40±1.64
IL-12	0.88±0.09	5.76±6.51	5.74±0.57
IL-13	3.66±0.37	14.03±14.07	10.60±12.61
IL-15	1.33±0.13	1.69±0.17	3.83±0.38
IL-17	1.56±0.16	OOR	OOR
Eotaxin	OOR	OOR	OOR
FGF basic	OOR	OOR	OOR
G-CSF	67.31±34.64	98.41±106.64	115.10±29.20
GM-CSF	17.98±1.80	42.63±33.82	28.30±34.38
IFN-g	79.47±7.95	22.66±14.30	41.20±50.18
IP-10	27.98±2.80	37.13±26.52	67.74±6.77
MCP-1	OOR	72.85±11.78	39.22±32.70
MIP-1a	151.36±177.48	678.92±931.06	334.83±184.25
MIP-1b	44.91±12.43	402.13±352.84	136.51±24.67
RANTES	41.44±4.14	462.27±448.56	227.98±245.61
TNF-a	3.77±0.38	14.55±1.46	13.15±1.31
VEGF	147.45±14.75	1856.48±1676.98	759.71±958.24

Table 4a. Twenty seven human cytokines and growth factors levels have been measured (data are reported as pg/ml).

CYTOKINES LEVELS IN CONDITIONED MEDIA

	Mel 1 SC	Mel 1 PC	Mel 1 PC^{+}
PDGF-bb	0.15±0.01	9.56±0.96	0.30±0.03
IL-1b	1.63±0.77	26.65±15.53	27.16±28.00
IL-1ra	250.19±215.60	48.15±17.55	158.19±218.43
IL-2	15.44±5.79	22.35±10.52	23.94±11.66
IL-4	0.90±0.14	2.03±0.49	2.22±0.22
IL-5	0.43±0.07	1.20±0.62	1.79±0.18
IL-6	ORR	ORR	ORR
IL-7	14.99±10.72	415.59±140.28	236.67±314.39
IL-8	ORR	ORR	1.10±0.11
IL-9	4514.39±3236.60	ORR	8123.97±638.02
IL-10	6.75±1.87	12.46±7.76	11.82±6.82
IL-12	3.73±1.07	18.70±7.90	22.57±14.92
IL-13	27.82±14.38	46.07±11.86	43.46±15.83
IL-15	0.26±0.03	1.11±0.11	ORR
IL-17	1.92±1.89	3.27±2.84	1.44±0.14
Eotaxin	ORR	ORR	ORR
FGF basic	6.39±0.64	14.84±1.48	9.20±0.92
G-CSF	1909.68±728.94	68.16±48.62	1353.21±1355.41
GM-CSF	3.84±3.30	683.35±657.20	723.79±1014.64
IFN-g	8.98±1.92	24.10±10.65	17.11±14.47
IP-10	7.61±4.54	32.82±6.27	16.26±20.59
MCP-1	844.79±737.90	1258.29±520.14	981.96±56.81
MIP-1a	524.35±230.26	818.89±320.58	831.53±511.17
MIP-1b	604.11±370.62	539.63±163.21	1012.14±500.24
RANTES	410.88±91.97	1128.06±513.12	1750.77±279.28
TNF-a	1.97±0.20	8.27±2.06	7.51±2.08
VEGF	3384.90±947.46	11785.19±14787.10	6035.58±3102.81

Table 4b. Twenty seven human cytokines and growth factors levels have been measured (data are reported as pg/ml).

V. Proteomic studies - Mass Spectrometry Analysis

Cell extracts were treated and then separated as above reported. The protein bands were detected in the gels by silver staining procedure. Bands were cut and digested as reported. The cell proteome was analyzed and the total number of identified proteins was calculated. The UNIQUE proteins, i.e. those uniquely expressed in just one of the two cell lines, A375 or SK mel 28 melanoma cells, represent the most interesting candidates, directly or indirectly playing a role as molecules/pathways responsible for their different aggressiveness.

We identified in A375 cells 326 unique proteins out of 836 total proteins and in SK mel 28 186 unique proteins out of 696 total proteins. Therefore 510 common proteins were identified in both cell lines (*Figure 11*).

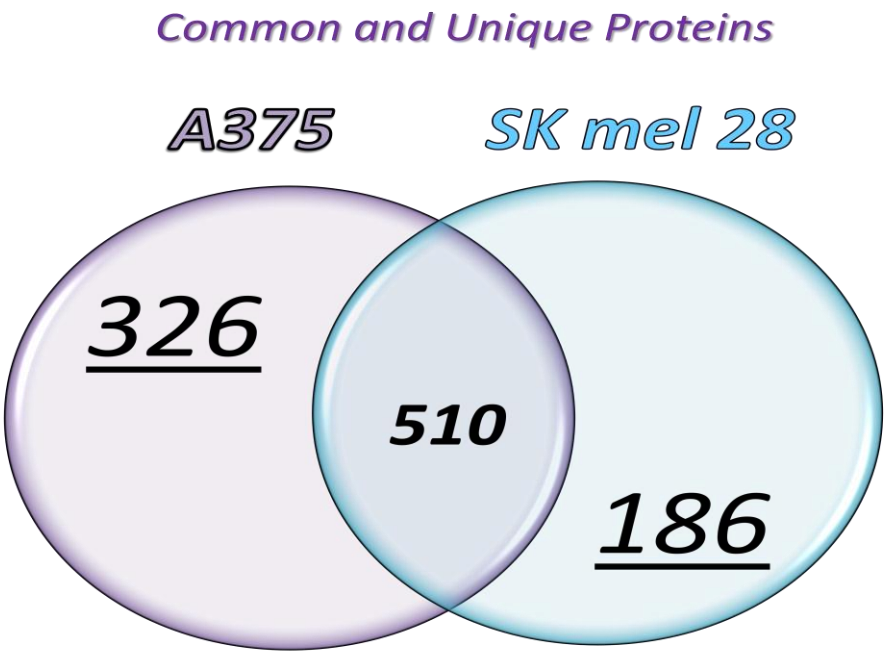


Figure 13. *Schematic representation of common and unique proteins in A375 and SK mel 28 cell lines.*

VI. Proteomic studies - Data Analysis

The obtained proteomic results indicated a large number of unique proteins that were analyzed by a Gene Ontology and clustering screening through DAVID software 6.7 version (http://david.abcc.ncifcrf.gov/). In the *table 4* the functional data of A375 cells obtained from GO analysis are reported, were proteins expressed only in SK mel 28 (less aggressive cells) were compared and classified by means of DAVID features to those expressed in A375 (more aggressive cells) only. Results were compared and functional pathways present in A375 but absent in SK mel 28 were identified. In particular, by the clustering analysis (*Figure 14*), the following pathways were recognized in SK mel 28 cell line: aminoacyl-tRNA biosynthesis, citrate cycle (TCA cycle), pentose phosphate pathway, selenoamino acid metabolism, fructose and mannose metabolism pathways. These pathways were identified in A375: butanoate metabolism, amino sugar and nucleotide sugar metabolism, endocytosis, spliceosome, insulin signaling pathway and others. Different common pathways were also identified like valine, leucine and isoleucine degradation, ribosome, proteasome, fatty acid metabolism, glycolysis / gluconeogenesis, pyruvate metabolism (*Table 5).* To make easier the interpretation of results, presently only pathways identified with more than 3% of the unique proteins have been reported, although a careful analysis of the whole body of results is ongoing by taking into account also categories/pathways represented by groups of protein corresponding to less than 3% of total.

Pathways	A375		SK mel 28	
	% Value	n° of proteins	% Value	n° of proteins
Valine, leucine and isoleucine degradation	7,69	10	8,22	6
Ribosome	9,23	12	19,18	14
Proteasome	6,93	9	9,59	7
Fatty acid metabolism	6,16	8	5,48	7
Glycolysis / Gluconeogenesis	4,62	6	9,59	4
Pyruvate metabolism	3,85	5	10,96	8
Butanoate metabolism	4,62	6	*< 3 or absent*	*< 3 or absent*
Amino sugar and nucleotide sugar metabolism	4,62	6	*< 3 or absent*	*< 3 or absent*
Endocytosis	10,00	13	*< 3 or absent*	*< 3 or absent*
Spliceosome	6,93	9	*< 3 or absent*	*< 3 or absent*
Insulin signaling pathway	6,93	9	*< 3 or absent*	*< 3 or absent*
Aminoacyl-tRNA biosynthesis	*< 3 or absent*	*< 3 or absent*	13,70	10
Citrate cycle (TCA cycle)	*< 3 or absent*	*< 3 or absent*	6,85	5
Pentose phosphate pathway	*< 3 or absent*	*< 3 or absent*	5,48	4
Selenoamino acid metabolism	*< 3 or absent*	*< 3 or absent*	5,48	4
Fructose and mannose metabolism	*< 3 or absent*	*< 3 or absent*	5,48	4
Others…	32,29	26	*< 3 or absent*	*< 3 or absent*

Table 4*. Schematic report of the David classification analysis. To make easer data evaluation* only pathways identified with more than 3% of the unique proteins have been reported.

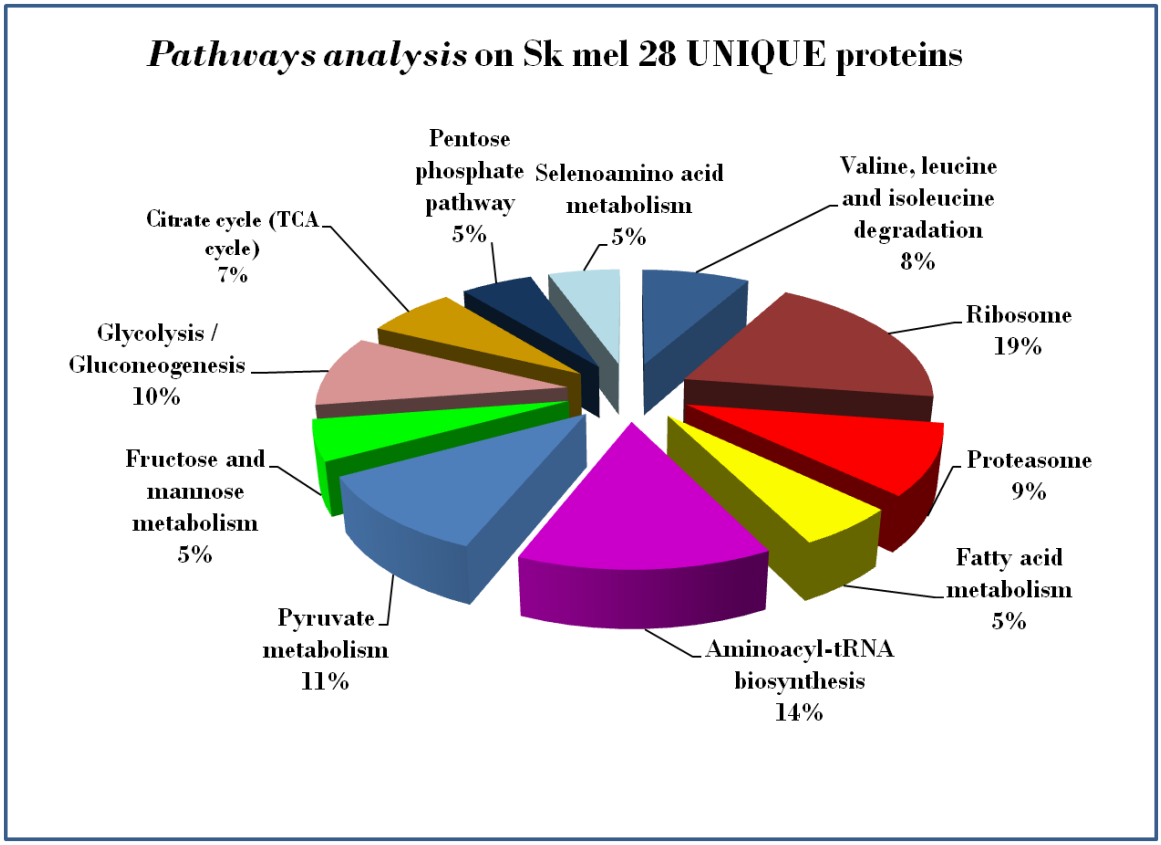


Figure 14a. *Schematic representation of the David classification analysis. Unique proteins, i.e. expressed in SK mel 28 only and absent in A375 cell line, were considered.*

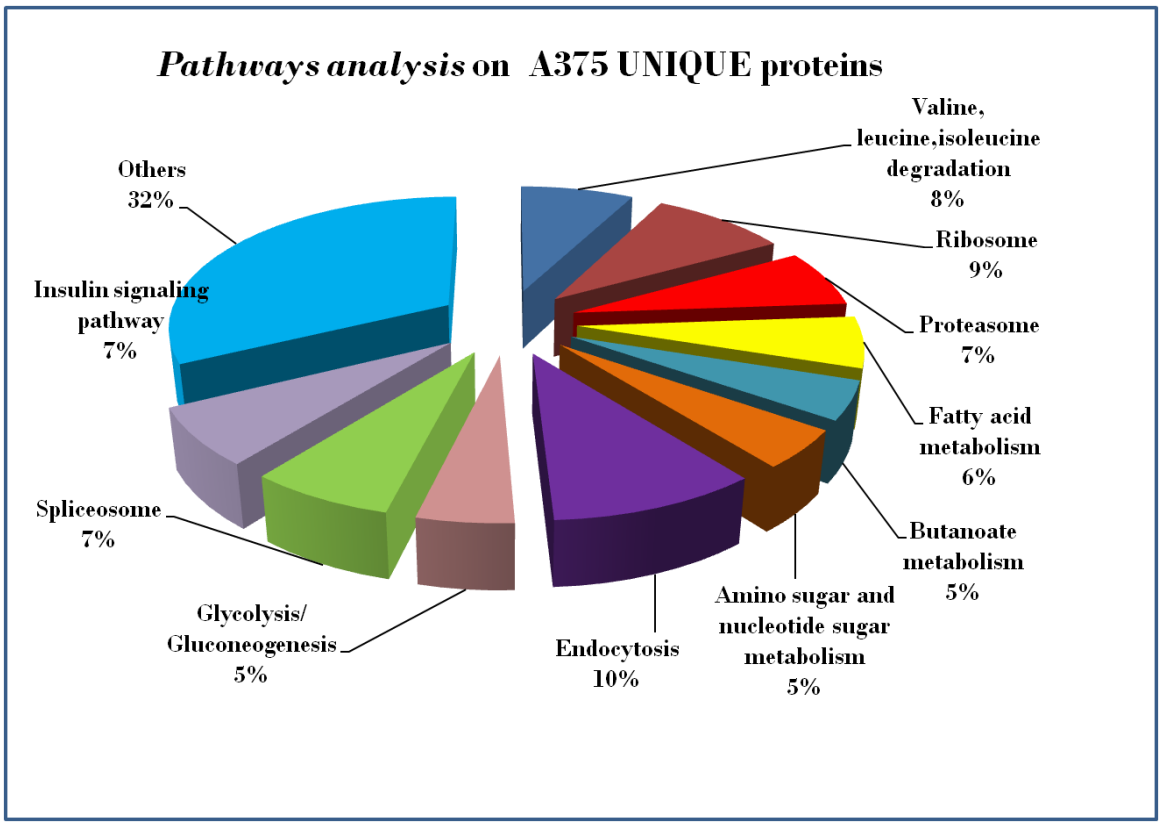


Figure 14b. *Schematic representation of the David classification analysis. Unique proteins, i.e. expressed in A375 only and absent in SK mel 28 cell line, were considered.*

Functional Classification	A375		SK mel 28	
	% Value	n° of proteins	% Value	n° of proteins
acetylation	19.34	182	18.36	108
cytoplasm	13.50	127	10.2	60
mitochondrion	5.42	51	*< 3 or absent*	*< 3 or absent*
transit peptide	3.83	36	*< 3 or absent*	*< 3 or absent*
phosphoprotein	19.77	186	20.92	123
protein transport	3.40	32	*< 3 or absent*	*13*
oxidoreductase	3.40	32	*< 3 or absent*	*10*
disease mutation	6.16	58	*< 3 or absent*	*26*
nucleotide-binding	6.06	57	7.48	44
endoplasmic reticulum	2.98	28	*< 3 or absent*	*16*
transport	5.10	48	*< 3 or absent*	*< 3 or absent*
RNA-binding	*<3 or absent*	*16*	4.25	25
ribonucleoprotein	*1,59*	*15*	3.06	18
ATP-binding	*< 3 or absent*	*38*	4.76	28
nucleus	*< 3 or absent*	*< 3 or absent*	10.71	63
Others	11.06	104	20.24	119

Table 5*. Schematic report of the David Gene Ontology analysis.*

A further Gene Ontology analysis using DAVID software 6.7 version (http://david.abcc.ncifcrf.gov/) (*Figure 15*) was performed also on the proteins obtained from the MS analysis and results are reported in the *table 5*. A375 proteins were pooled in

different functional categories as mitochondrion, transit peptide, protein transport, oxidoreductase, disease mutation, endoplasmic reticulum, transport. SK mel 28 proteins were pooled in RNA-binding, ribonucleoprotein, ATP-binding, nucleuos functional categories. Both A375 and SK mel 28 unique proteins were clustered in cytoplasm, phosphoprotein, nucleotide-binding and acetylation cathegories.

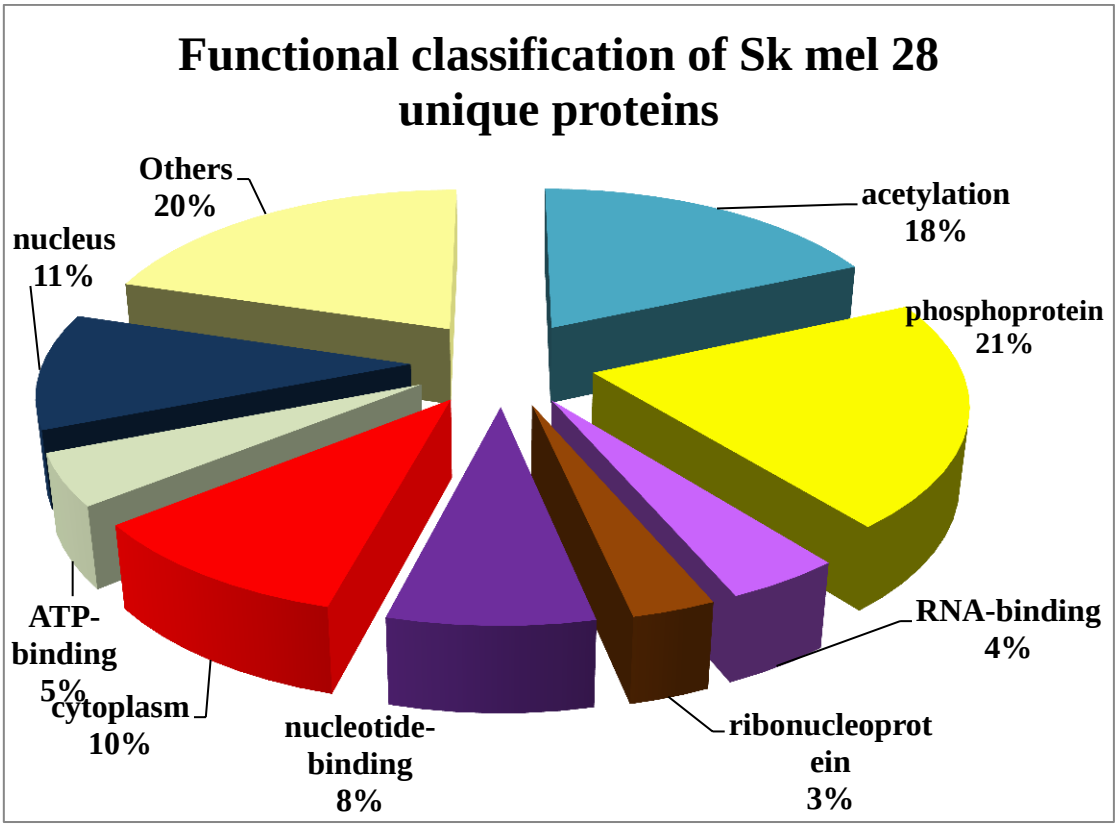


***Figure 15a**. Schematic representation of David analysis for functional classification.*

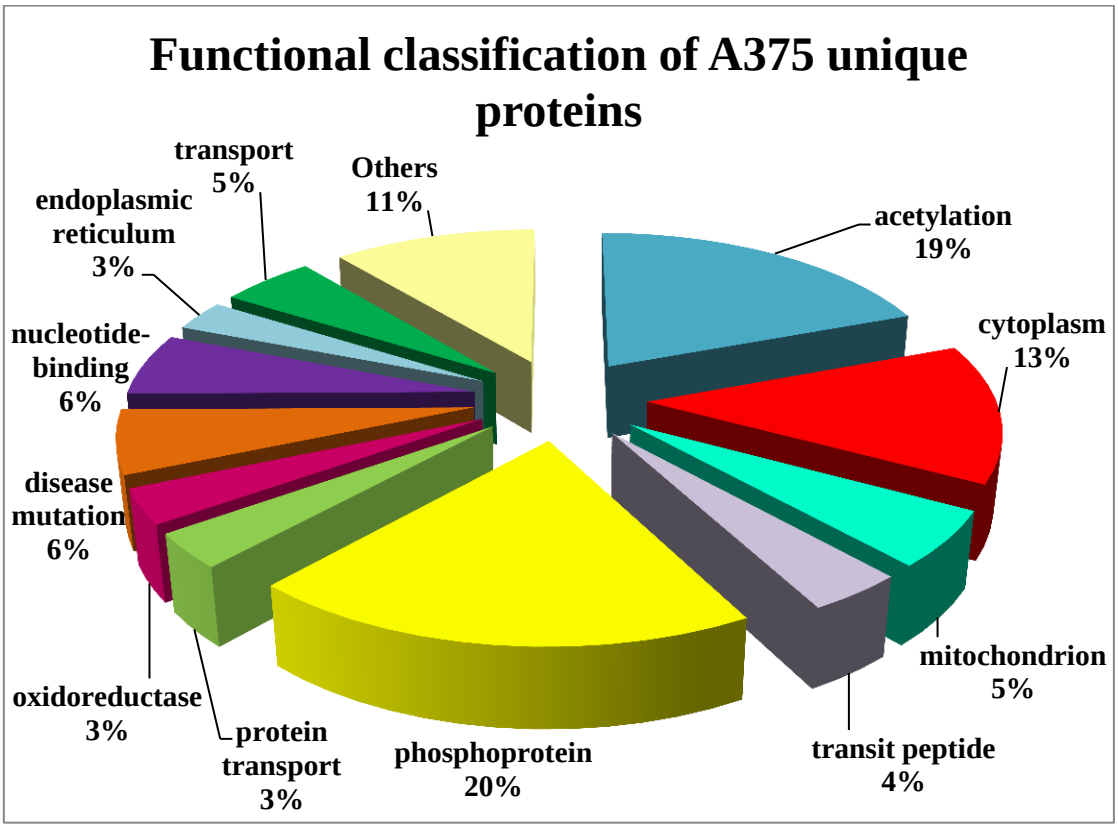


***Figure 15b**. Schematic representation of David analysis for functional classification.*

VII. Validation of proteomic data by correlation to GEO dataset

To confirm this preliminary analysis, we compared our results obtained through proteomic and Bio-Plex analyses to a gene expression dataset concerning human metastatic melanoma, at the NCBI's Gene Expression Omnibus (GEO) database (Neveol et al., 2012). In particular the dataset GDS1314, (http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1314) was examinated. This dataset analyzed contains expression profiling of Mel JUSO, A375, 607B, 518A2, and SK mel 28 malignant melanoma cell lines. Results correlated with regions of chromosomal breakpoints, and indicate an association between chromosomal breakpoints and altered gene expression. By the dataset analysis, we obtained gene expression of IL-1ra, IL-6, IL-7 and Rantes. We compared the GEO results to the proteomic analysis (Bio-Plex assays

above reported) and data showed that, choosing SK mel 28 as control (100%), Rantes, IL-6 and IL-7 are increased in A375 in both transcriptomic and proteomic analysis, while IL-1ra decreased in A375 in the same analysis (*Figure 16*).

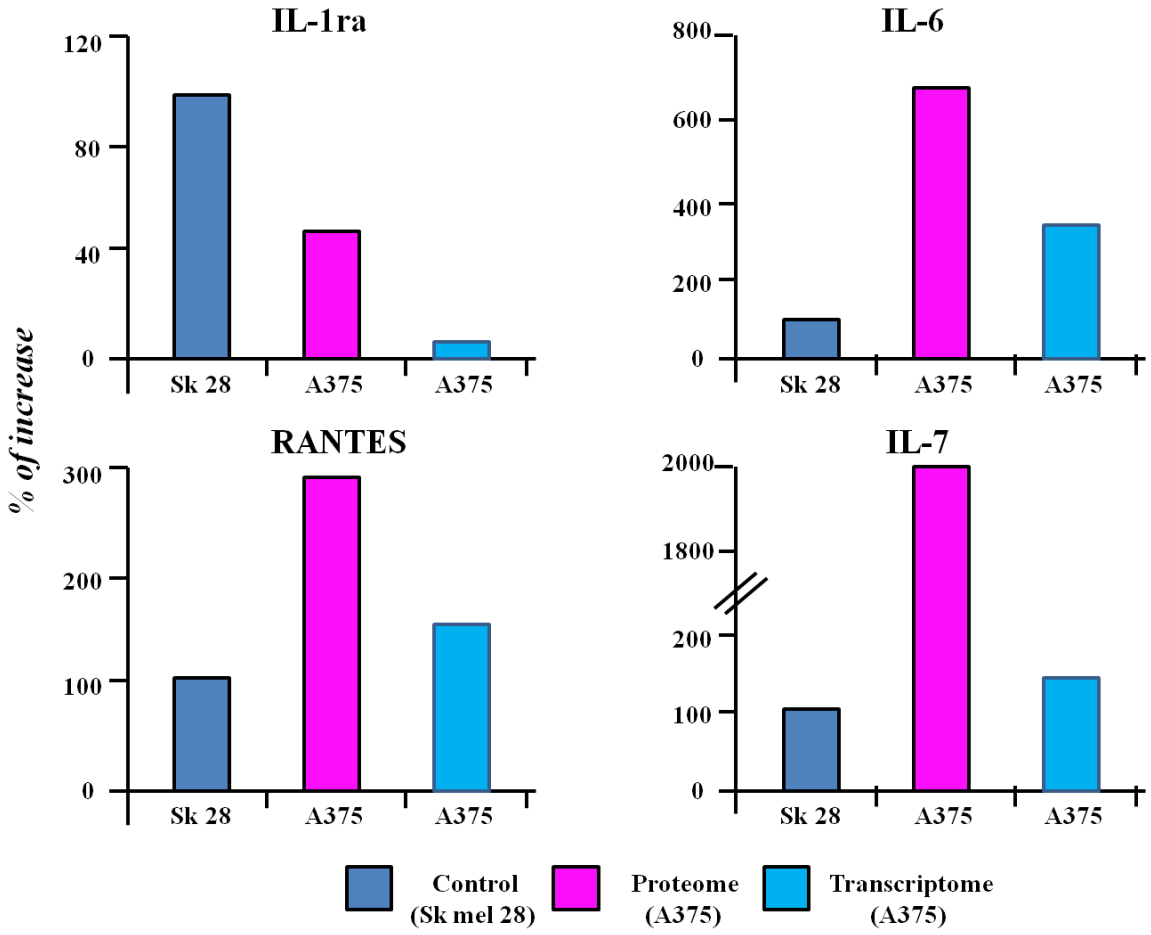


Figura 16. *Bio-Plex and GEO analysis comparison. A375 cytokines increase by trascriptomic and proteomic analysis compared to SK mel 28 as control.*

Another study available through GEO Series accession number GDS1375 from a cutaneous malignant melanoma study (http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1375) was analyzed. This dataset contains expression profiling of primary malignant melanoma (n= 45), benign skin nevi (n=18), and normal skin samples (n=7) (Talantov et al., 2005).

Results identify potential molecular markers for lymph node staging assays, and provide insight into melanoma tumorigenesis. Searching for cytokines expression into the study dataset, we found several findings confirming the data obtained in cell lines and cancer stem cell clones. In particular, we found that 3 out of 5 present in the dataset, namely IL-1ra, IL-7 and VEGF were significantly modified and in the same way modulated (*Figure 17*).

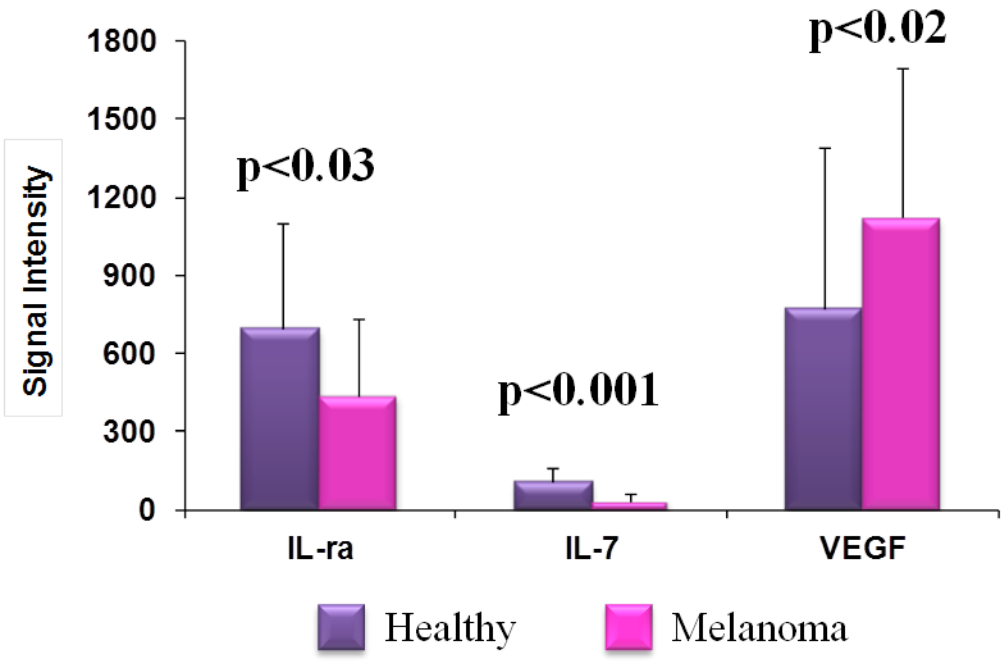


Figura 17. *GDS1314 GEO analysis. Cytokines significantly modified in human metastatic melanoma patients compared to healthy individuals.*

In Figure 18 a schematic representation of cytokines level expressed in three different models is reported.

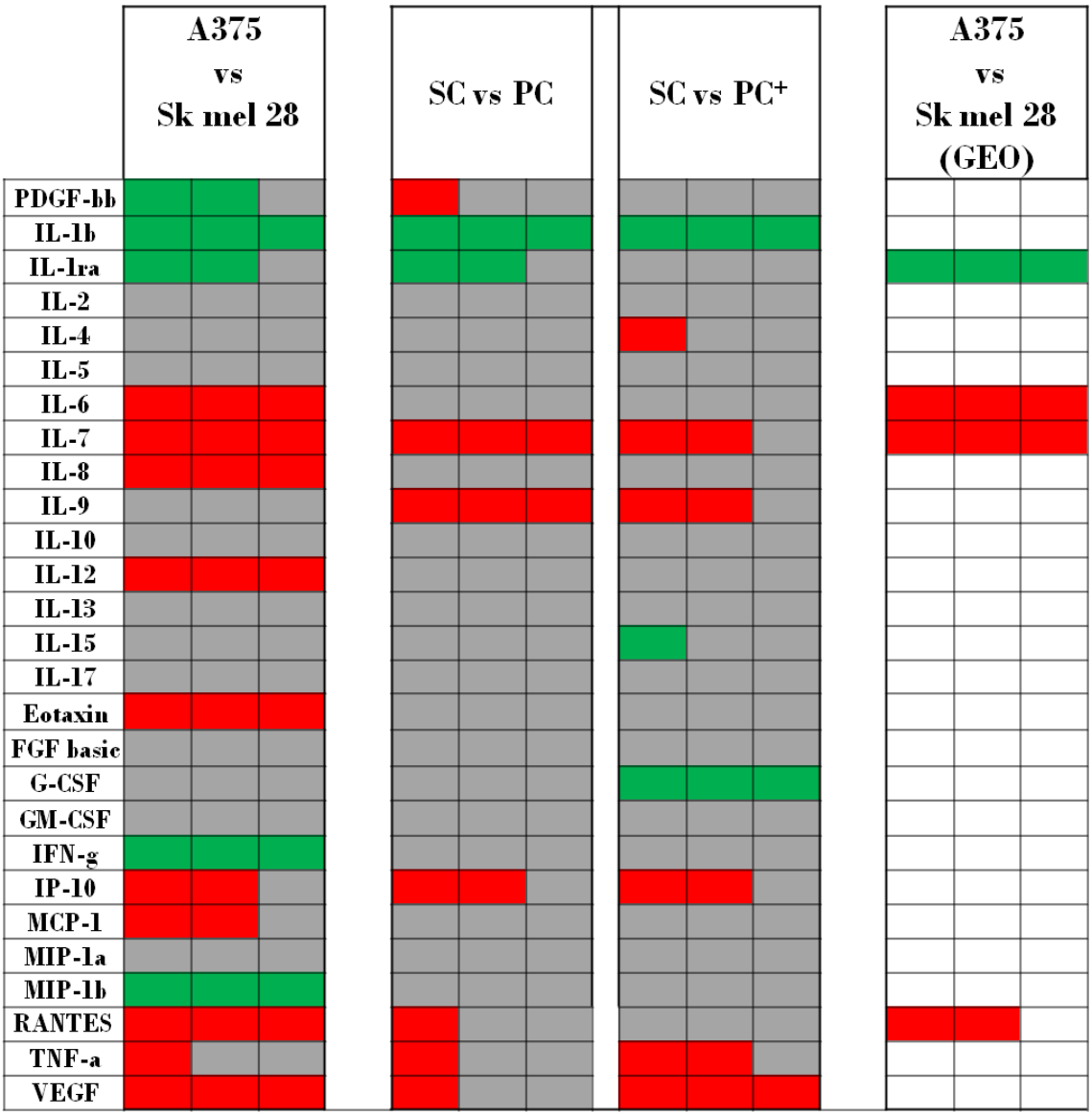
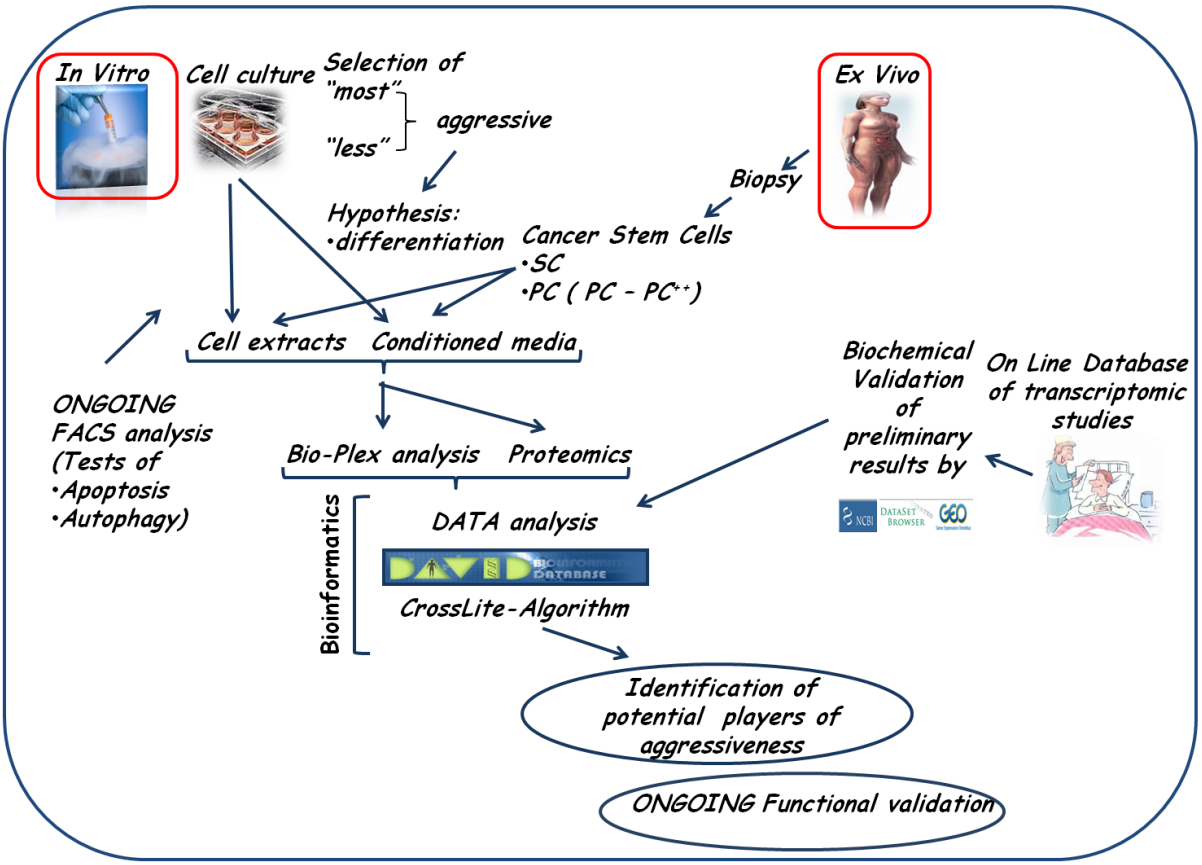


Figure 18. *Schematic representation of cytokines level expressed in three different models such as the most aggressive (A375) versus the less aggressive (SK mel 28) melanoma cell lines; the most aggressive (SC) versus the less differentiated (PC)and the most aggressive (SC) versus the most differentiated (PC^{+}) melanoma stem cells the most aggressive (A375) versus the less aggressive (SK mel 28)according to the GEO analysis. Green box means downregulation; red box means upregulation; grey box means not modified expression; empty box indicates un-matched condition. 1 box means 1-3 fold modulation; 2 boxes means 3-8 fold modulation; 3boxes means 8 to higher fold modulation. Statistical significance was always very high ($p<0.001$).*

5. DISCUSSION & CONCLUSION

Concept Flow



Human metastatic melanoma is a devastating skin cancer characterized by a poor prognosis (Mathieu et al., 2012). Due to the lack of therapeutic strategies against advanced stages, there is an urgent need of novel therapeutic approaches to contrast the molecular mechanism underlying the fast progression and metastatic process. In the mechanisms related to melanoma aggressiveness to find novel melanoma modulators was analyzed. At first we selected as model of study several melanoma cell lines and we performed a growth assay at three different densities of seeding and in serum deprivation

conditions. This analysis led to identify cells that, under extreme stimuli conditions, show adaptive and reactive traits. As above reported we identified two groups of cell lines highly different for proliferation rate, confirmed by *in vitro* invasion assays, and we selected as model the less (SK mel 28) and the most (A375) aggressive cell lines. In fact the proliferation assay showed that A375 cells behave very high growth rate compared to SK mel 28 cells at three different cell density. Moreover, results suggested a key role played by cell signaling mechanisms like secretory stimuli and/or cell-cell interaction. As shown in figure 8, a high proliferation rate was revealed in A375 cells after 24 and 48 hours compared to SK mel 28, that presented a low proliferation at the same time. A375 cells in the time between 24 and 48 hours of growth showed the same trend that may be explained by a high expression of adhesion and secretory molecules that stimulate the surrounding cells to proliferate.

These observations were confirmed carrying out an invasion assay demonstrating that A375 are more invasive and therefore, more aggressive than SK mel 28. Therefore the reason of this higher aggressiveness was investigated. It is known that differentiation is closely related to aggressiveness (Mohlin et al., 2011), many literature data, in fact, reported important anticancer effects of phytochemicals linked to the induction of tumor cell differentiation, through the activation of intracellular transglutaminase (TGase; EC 2.3.2.13) (Facchiano et al. 2006; Lentini et al. 2010; Lentini et al., 2000), an enzyme involved in melanoma progression. Previously reported, using proteomic approaches we identified a number of possible players in melanoma aggressiveness, to be further investigated. In fact, by a comparative analysis between several melanoma models with different aggressivity, IL-6, IL-12, VEGF, IL-7, IL-9, Eotaxin, RANTES, PDGF-bb, IL-17, INF-γ and G-CSF were identified as positive modulators of aggressiveness.

To verify these data, a biochemical validation using GEO dataset was performed and IL-6, IL-7 and RANTES were confirmed to be related to a high aggressiveness.

In previous studies an increasing level of RANTES was related to metastatic dissemination (Mrowietz et al., 1999) and promotes the IL-1b expression which in turn, in other studies, was demonstrated to stimulate the IL-6 increase (Li et al., 2009). To further investigate these preliminary results, the differential protein-expression was studied and it is noteworthy that one of identified interesting pathways was the selenoamino acid metabolism. Selenocystine (SeC), a naturally occurring selenoamino acid, has an anticancer activity triggering the apoptosis process in human metastatic melanoma cell

lines through the activation of the mitochondria-mediated and death receptor-mediated apoptosis pathways (Chen and Wong, 2008). The matching of literature reports with our proteomic results confirms that our approach is useful and reliable and allows to hypothesize that this pathways play a key role in melanoma aggressiveness. To identify additional putative players responsible of the aggressiveness, through a DAVID analysis the functional classifications was studied and the attention was focused on proteins transport and proteins related to proteasome which we found more expressed in A375 than in SK mel 28 cells. E3 ubiquitine-protein ligase NEDD4 (E3 Ub ligase NEDD4, also known as cell proliferation-inducing gene 53) is an ubiquitylation mediator. Melan-A/MART-1 (also called Melan-A) is a protein expressed by melanocytic cells (Romero et al., 2002). Melan-A interacts with E3 Ub and so it can be degraded in the lysosomes; the resulted peptides are exposed on the surface of the melanoma cells. So ubiquitylation of a melanosomal protein concerns its sorting and degradation and could therefore impact on pigmented cells (Levy et al., 2005).

Aberrant cellular proliferation and damaged apoptotic pathways are traits of cancer aggressiveness; proteasome is involved in both processes. A high proteasome activity is related to an absent apoptosis activity as seen in cancer progression and some inhibitories were used to block the proteasome activity and therefore, to induce apoptotic process (Cecarini et al., 2011). So, the inhibition of proteasome is a strategy to contrast cancer progression. On the basis of these observations, and above all based on our reported proteomic results we can hypothesize that in our aggressive cells, proteasome activity can be related to the high malignancy.

In conclusion we identified cell lines that differ for malignancy, in particular A375 are more aggressive than SK mel 28. Using an immunometric analysis we identified several cytokines probably involved in cutaneous melanoma aggressiveness such as IL-6, IL-7, RANTES and VEGF and others (SEE *Table 6*). *In vivo* these molecules are involved in inflammation, immune response, apoptosis and angiogenesis processes, so it can be possible that modulating their expression could act on the melanoma metastatic dissemination process. The following molecular pathways likely very important to explain the higher cell aggressiveness were identified: selenoamino acid metabolism involved in apoptosis process and proteasome activity. *In vitro* and *in vivo* validation studies are presently ongoing to demonstrate these interesting findings, with the aim to develop novel strategies against cutaneous melanoma.

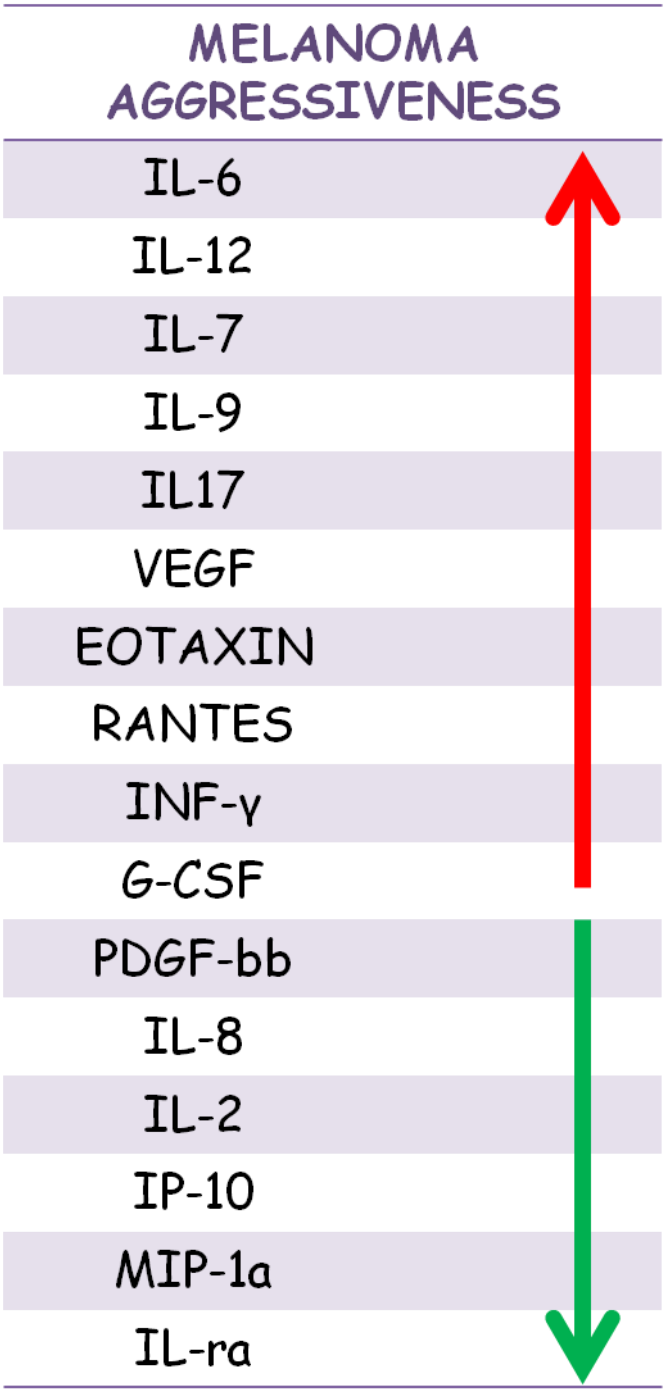


Table 6. Cytokines involved as positive (green arrows) or negative (red arrows) modulators of human melanoma cell aggressiveness.

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