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Hallmarks of tumor environment: Role of acidity in melanoma aggressiveness and metastatic dissemination

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ABSTRACT

Tumor microenvironment is a complex system consisting of several important components in constant, reciprocal interactions. Aspects of tumorigenicity such as tumor growth, migration, invasion and metastasis formation, as well as the secretion of soluble factors and the abnormal structure and function of tumor blood vessels depend on these interactions. In this way microenvironment acts as a coconspirator during carcinogenesis and neoplastic progression. Tumor microenvironment is often characterized by an acidic pH due to abnormal vascularisation, reduced lymphatic network, uncontrolled cell growth frequently associated with hypoxia, and extracellular accumulation of glucose metabolites even in the presence of an adequate oxygen level ("the Warburg effect"). Evidence is accumulating that acidity participates to tumor progression and is associated with poor prognosis. Since melanoma, as most solid tumors, often shows extracellular acidosis, in this study we investigate whether acidity is able to induce in melanoma cells different aspects of malignancy such as the epithelial-to-mesenchymal transition (EMT) or vascular endothelial growth factor (VEGF) C expression and whether this is associated with a change in the metabolic profile of acidic cells. In parallel, the effects of acidity on melanoma cell/host cell interactions have been investigated.

It is known that during melanoma progression, malignant melanocytes are reprogrammed into mesenchymal-like cells through to an EMT process associated with the acquisition of an invasive and pro-metastatic phenotype. A375M6 melanoma cells were exposed to an acidic extracellular environment (pH 6.6-6.8) and tested for markers and biological properties of EMT. We found that acidic cells express a significant up-regulation of mesenchymal markers (N-cadherin, Vimentin), typical transcription factors (Twist, NF-κB) and a reduction in E-cadherin expression. Acidic cells, also, express an increased MMP-9 activity and invasiveness through Matrigel filters, inhibited by Ilomastat, a MMP inhibitor. Invasiveness and N-cadherin up-regulation in acidic cells were NF-κB dependent, suggesting a pivotal role of NF-κB transcription factor in acidity-promoted EMT in melanoma cells. We also demonstrated that all these changes are transient and after seven days in standard medium, cells recover their original phenotype. When we

injected acidic EMT cells into blood stream of immunodeficient animals, they gave a number of lung micrometastases similar or even lower than non-acidic cells. Acidic cells were found more resistant to an apoptotic stimulus, such as H_2O_2, whereas they showed a reduction in cell proliferation rate and in cloning efficiency, changes that might interfere with the organ colonization ability. To understand the effective role exerted by acidic cells during melanoma dissemination, we investigated *in vitro* invasiveness and lung colonization of a mixed population of acidic and non-acidic tumor cells. We found that acidic cells promote *in vitro* invasiveness of non-acidic cells and this cooperation leads to a migration rate higher than that of acidic cells. Moreover, acidic cells cooperate for a better lung colonization of non-acidic cells that represent the greater part of cells participating to lung micrometastases.

A critical early step in the metastatic pathway is the invasion of cancer cells not only into surrounding stroma but also into peritumoral lymphatics. There is clear evidence that melanoma cells can induce lymphangiogenesis and this phenomenon is correlated with lymph node metastases. VEGF-C represents the most potent and well-recognized lymphangiogenic growth factor secreted in tumor milieu by melanoma cells and tumor-associated macrophages, however the mechanism underlying VEGF-C secretion is not completely understood. We demonstrated that an acidic extracellular pH promotes the expression of VEGF-C both in A375P melanoma cells and in melanoma cells isolated from a human spontaneous metastatic lesion, through the NF-κB transcription factor. We also demonstrated that esomeprazole, a proton pump inhibitor which requires acidosis to be activated, is able to prevent VEGF-C expression in acidic melanoma cells by interfering with NF-κB activation. Furthermore, we showed that esomeprazole abrogates the enhanced VEGF-C expression in tumor cells grown in acidic medium and stimulated by IL-1β. On the whole, our results reveal that acidity may be considered a strong promoter of VEGF-C expression in melanoma cells and provides a new pharmacological target to limit the development of tumor lymphangiogenesis.

We also investigated if acidity could modify the metabolic program of melanoma cells. It is known that acidity in tumor microenvironment is a direct consequence of the metabolism of cancer cells, which use glycolysis even in the areas of ample oxygen supply, but, on the contrary, the effect of the acidity on cell metabolism is less known. Our experiments, conducted using A375M6 melanoma cells, indicate that acidity reduces the

glycolytic metabolism, as evidenced by the decreased expression of glucose transporters, reduced lactate efflux and increased lactate uptake, all inverse features compared to those detected in A375M6 grown under hypoxic condition. We also found that the pyruvate kinase M2 (PKM2), a key player in the Warburg effect on cancer cells, is reduced in acidic cells. In order to inhibit the oxidative phosphorylation of acidic melanoma cells we used metformin, the most widely prescribed oral hypoglycemic agent which acts through inhibition of mitochondrial respiration and which has recently received increased attention for its potential antitumorigenic effects. The enhanced invasiveness acquired by melanoma cells exposed to an acidic culture medium was reduced by metformin treatment. Recently, lactate, the end-product of glycolysis, was shown to be associated with metastasis, tumor recurrence, and poor survival, and identified as the keystone of an exquisite symbiosis in which glycolytic and oxidative tumor cells mutually regulate their access to energy metabolites. We hypothesized that tumor microenvironment might facilitate a symbiosis between non-acidic/glycolytic cells that use glucose and produce lactate, and acidic cells that capture the lactate produced by non-acidic cells and oxidize it to produce energy to increase their aggressiveness. We shown that lactate, used by acidic cells for respiration, does not induce an increase of cell proliferation, but stimulates motility of acidic cells, maintaining their high invasiveness and the EMT profile. Lactate also acts on non-acidic cells increasing their motility, invasiveness, and inducing the EMT. We manipulated lactate uptake using the α-cyano-4-hydroxycinnamate (CHC), a drug known to reversibly inhibit MCT1. We found that 10 mM CHC kills acidic cells,0 while has only an antiproliferative effect on non-acid cells, suggesting that glycolytic cells can better tolerate the effects of MCT1 inhibition. The use of lower doses of CHC shows that MCT1 inhibition represses not only the lactate-dependent stimulation of motility and invasiveness, but also the invasiveness of untreated acidic cells. Medium conditioned by control (non-acidic) cells, probably containing lactate, is able to stimulate the motility of acidic cells and CHC blocks this effect, confirming the hypothesis that the lactate produced by non-acidic cells may stimulate acidic cells and that this cross-talk could be inhibited by MCT1 inhibitor.

In addition to observing the direct effect of the acidic environment on cancer cells, we also studied how acidity influences the interactions between mesenchymal stem cells (MSC) and tumor cells. It is well known that tumors are composed of malignant tumor cells and non-malignant benign cells, including endothelial cells, myofibroblasts,

macrophages, lymphocytes, dendritic cells, and MSC. Large number of MSC are recruited into the stroma of developing tumors and recent evidence suggests that these cells play a role in facilitating cancer progression influencing the behavior and aggressiveness of tumor cells. Our experiments, conducted using A375M6 human melanoma cells and human MSC derived from umbilical cord, demonstrated that condition medium collected from acidic MSC promotes the migratory and invasive abilities of melanoma cells as well as the expression of EMT related genes in a way much more evident compared to the effect of the media conditioned by non-acidic MSC. Whereas the medium conditioned by non-acidic MSC elicits a marked increase in tumor cell proliferation, the medium conditioned by acidic MSC has no significant effect on tumor cell growth; both types of conditioned media (from acidic or non-acidic MSC) induce a mesenchymal morphology of melanoma cells, but neither of them is able to induce resistance to apoptosis. A375M6 cells grown in medium conditioned by acidic MSC show also stimulated expression of TGF-β and TGF-β receptors. We evaluated the expression of several cytokines in MSC grown in acidic medium and we found that the only factor with enhanced expression in acidic MSC was the TGF-β, that we assumed to be the soluble factor secreted by acidic MSC and involved in the stimulated aggressive phenotype of melanoma cells. In effect, we demonstrated that TGF-β is able to increase the invasiveness of melanoma cells and to induce the expression of TGF-β and of EMT markers. In order to inhibit the effect of acidity on MSC and consequently on tumor cells grown in conditioned media, we used esomeprazole. Our experiments indicate that esomeprazole prevents the increase of TGF-β expression and of MMP2 activity in acidic MSC. Moreover, A375M6 cells grown in media conditioned by acidic MSC treated by esomeprazole shown invasiveness and motility comparable with that of cells grown in media conditioned by non-acidic MSC, much lower that invasiveness and motility induced by media conditioned by untreated acidic MSC. Esomeprazole was also able to block the stimulation of EMT in cancer cells grown in conditioned media. Probably, acidic microenvironment activates MSC that, through the TGF-β secretion, stimulates aggressiveness of the neighboring tumor cells. Esomeprazole, counteracting the effect of acidity on MSC, prevents the release of TGF-β and the consequent activation of tumor cells. This study also provides evidence that acidity-induced TGF-β secreted by MSC is able to promote tumor growth *in vivo*. Subcutaneous co-injection of melanoma cells and acidic/non-acidic MSC in

immunodeficient mice resulted in more rapid tumor growth compared with injection with tumor cells alone, but acidic MSC maximally promoted growth of melanoma tumors. When we treated mice with esomeprazole, tumor growth was attenuated, especially in mice injected with acidic MSC. Our findings are in agreement with a large number of results indicating that MSC promote tumor growth *in vivo*; furthermore, our study explores the relationship between MSC and tumor cells in an acidic microenvironment, proposing esomeprazole, which make acidic MSC incapable of communicating with tumor cells as therapeutic strategy against cancer.

In conclusion, acidosis represents a key factor contributing to a more aggressive phenotype of melanoma cells and strategies directed to target acidic environment and/or the intrinsic cell metabolism of low pH-adapted cells are a new and prominent issue in tumor therapy.

1. INTRODUCTION

Human carcinogenesis is a multistage process involving sequential alterations in protooncogenes (activation) and in tumor suppressor genes (inactivation) and in which epithelial cells progress through a series of premalignant phenotypes until an invasive cancer emerges. Experimental observations have demonstrated that this process can be divided into three general steps: initiation, promotion and progression (Hennings *et al.*, 1993). Initiation consists in the formation of the pre-malignant lesions caused either by genetic alterations or by environmental factors, such as viral infection or external genotoxic agents, which confer a permanent increase in susceptibility to cancer formation. Once a cell has been mutated by an initiator, it is susceptible to the effects of promoters; these compounds promote the proliferation of the cell, giving rise to a large number of daughter cells containing the mutation created by the initiator agent (Yamagiwa and Ichikawa, 1918). At this stage of primary tumor expansion, the cells are not invasive and metastatic. The progression stage of carcinogenesis is an extension of the tumor promotion stage: cell proliferation caused by promoting agents allows the cellular damage inflicted by initiation to be propagated, and the initiated cells are clonally expanded. This propagation of damage leads to the production of more genetic alterations in the genome of the neoplastic cell, resulting in an increased growth rate, invasiveness, and metastatic capability.

Metastasis, the spread of cells from the primary neoplasm to distant organs and their growth inside those, is the most fearsome aspect of cancer (Fidler, 1990). The process of cancer metastasis consists of a long series of sequential interrelated steps where cancer cells, after the activation of signaling pathways that control cytoskeletal dynamics and the turnover of cell-matrix and cell-cell junctions, detach from the primary tumor invading the surrounding tissue and its extracellular matrix (ECM) and penetrate blood and lymphatic vessels (intravasation) through the endothelial lining and the basement membrane. Subsequently, tumour cells survived in the circulation are transported to secondary organs, where extravasation occurs. If the microenvironment is permissive in these target organs, tumor cells can expand to form growing metastases (colonization) or remain quiescent for long periods (latency). Like cells in primary tumors, cells in metastases also proliferate,

invade, and enter blood vessels, leading to secondary metastasis. During each stage, tumor cells have to detach, migrate, invade, adapt and re-attach by involving matrix degrading enzymes and mechanical processes such as cell adhesion, changes of cell fate, cell movements and motility, and the generation of forces (Ungefroren *et al.*, 2011). Indeed, an understanding of the invasion process is only possible in the context of detailed insights into the cancer cell's interactions with the microenvironment.

Until recently, the study of cancer has been concentrated on the genetic and molecular description of cancer cells itself, or the laboratory techniques used to study these cells as they become abnormal during tumor progression. After years of research focused almost exclusively on oncogenes and tumor suppressors, researchers have begun to think about how the rest of our body influences the cancer cell and it is becoming clear that the microenvironment acts as a coconspirator during carcinogenesis and neoplastic progression. The tumor microenvironment is a complex system that consists of several important components including cancer cells, non-neoplastic stromal cells, signaling molecules, soluble factors and extracellular matrix; stromal elements include blood and lymphatic vessels, cancer-associated fibroblasts (CAF), mesenchymal stem cells (MSC), a variety of different immune cells such as lymphocytes and tumor-associated macrophages (TAMs), inflammatory cells and extracellular molecules as collagen, elastin, fibronectin, fibrin required for structural support. The relative amount of stroma and its composition vary considerably from tumor to tumor and the amount of stroma doesn’t correlate with the degree of tumor malignancy (Dvorak *et al.*, 1983). Each of these microenvironment components and the reciprocal dynamics interactions between them can regulate all aspects of tumorigenicity, promoting neoplastic transformation, influencing a metabolic reprogramming, supporting tumor growth, migration, invasion and metastasis formation, neovascularisation, apoptosis and chemotherapeutic drug resistance. It is through these mechanisms that tumor environment, in initially trying to suppress growth of early malignancy, exerts evolutionary pressure and facilitates the selection of tumor cell clones ideally suited to survive in different, more hostile, tissue environments and able to perform a metastatic cascade. The interaction between tumor cells and host cells is also recognized as crucial for the decision whether tumor cells progress toward metastatic dissemination or remain dormant (Heppner *et al.*, 1998; Fidler, 1978; Foulds, 1954; Nicolson, 1984). Indeed, tumor growth and metastasis is significantly reduced in fibroblast-deficient mice, while injection of wild-type fibroblasts into these mice can reverse this phenotype,

providing a clear evidence for the involvement of fibroblasts in the emergence of metastasis (Witz, 2008; Rose *et al.*, 1991; Rose *et al.*, 1994). Furthermore, while quiescent macrophages respond to immune or bacterial stimuli by expressing new functional activities capable to recognize and destroy transformed cells, macrophages isolated from experimental and spontaneous tumors show a reduced level of cytotoxic activities and it was proved to be relevant to tumor progression and metastases (Coussens *et al.,* 2002; Robinson *et al.,* 2005). A number of clinical studies have shown a correlation between increased TAMs and angiogenesis, metastasis and poor prognosis in patients with cancer (Wu *et al.,* 2012) and Skobe and Fusening (1998) demonstrated that activated stromal cells, not only support tumor growth, but may induce tumorigenic conversion of epithelial cells. These findings further support the hypothesis that stromal cells are not passive companion cells but can play a crucial role in epithelial tumor development. Evolving neoplastic cells, in their turn, respond to tumor microenvironment and modulate it by releasing a number of growth factors and cytokines able to activate the stroma, including colony stimulating factor (CSF), transforming growth factor-β (TGF-β), chemokines, basic fibroblast growth factor (bFGF), platelet–derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) A, C and D. These factors, that are chemoattractive for monocyte and macrophages, act as activators of fibroblasts, endothelial cells, adipocytes and stimulate stromal cells to release other mediators that affect tumor cell progression, remodel ECM, stimulate inflammation (Robinson and Coussens, 2005) and tumor angiogenesis (Bergers and Benjamin, 2003).

Remodeling of ECM is a necessary step in local invasion (Werb, 1997) and most of the enzymes and inhibitors needed at the invasion front are provided by host cells and not by the invading tumor cells (Liotta and Kohn, 2001). The principal enzymes that degrade the ECM are the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases able to modulate many physiological and pathological processes as apoptosis, angiogenesis and inflammation influencing the bioavailability or functionality of multiple important factors as TGF-β (Yu and Stamenkovic, 2000), EGFR (Cowden Dahl *et al.*, 2008) or VEGF (Bergers *et al*, 2000). Increased expression of MMP-1, MMP-2 and MMP-3 is linked with lymphatic invasion and lymphnode metastasis; inhibition of MMP-2, -9 and -14 attenuates angiogenesis and lymphangiogenesis, the processes whereby new blood and lymphatic vessels form. Both processes have an essential role in

the formation of a new vascular network to supply nutrients, oxygen and immune cells, and also to remove waste products (Folkman, 1971). The induction of tumor neovascularization results from the interrupted balance between pro-angiogenic factors as VEGF-A, FGF, heparinase, angiopoietin 2 (Ang2) and interleukin 8 (IL-8) and anti-angiogenic factors as interferons (IFN-α,β,γ), interleukins (IL-4) or tissue metalloproteinases inhibitors (TIMP). In addition, tumors can activate lymphangiogenesis by VEGF-C and VEGF-D. The new vessel formation is often disturbed in tumors leading to a vasculature characterized by dilated, hyperpermeable, tortuous and incomplete vessels. Many studies on different tumors have provided significant prognostic information with high vascularity associated with poor treatment outcome (Weidner *et al.*, 1992; Uzzan *et al.,* 2004). Tumor expansion after angiogenesis is dependent not only on delivery of oxygen and nutrients, but also on the release of anti- apoptotic and survival factors from the endothelial cells.

In summary, tumor cells recruit vasculature and stroma through production and secretion of stimulatory growth factor and cytokines, and the locally activated host cellular and extracellular elements in turn modify the proliferative and invasive behavior of tumor cells. The role of the tumor microenvironment in cancer progression has made it a target for anti-cancer therapies.

Another dimension in the interactions between tumor cells and host cells is represented by constant physical changes of the tumor and its surrounding associated with the transformation of a normal tissue into a high-grade malignancy. Most tumours develop an environment characterized by low oxygen tension (hypoxia), elevated interstitial fluid pressure, low glucose concentration and high lactate concentration. These changes are largely caused by a combination of poor tissue perfusion due to abnormal tumor vasculature, uncontrolled proliferation and altered energy metabolism (Hanahan and Weinberg, 2011). Both, the high permeability of tumor blood vessels and the absence of properly functioning lymphatics, are keys contributors to the decreased interstitial fluid drainage and the development of an interstitial hypertension in neoplastic tissues (Fukumura and Jain, 2007). Deviant vasculature and vessel density contribute to deficient perfusion that, together with elevate metabolic rates, is the cause of hypoxia. When hyperplastic cells grow and their blood supply is limited, hypoxia selects for a glycolytic phenotype and consequently extracellular spaces become acid. Thus, tumor environment is frequently hypoxic and acid (Hanahan and Weinberg, 2011). The relevance of these

changes in tumor progression is well ascertained and offers new targets to control tumor progression.

1.1 Hypoxia

Hypoxia is defined as a state of reduced oxygen availability (O_2 tension) or decreased O_2 partial pressure (pO_2 values) resulting from an imbalance between oxygen supply and consumption; it characterizes the microenvironment of many solid tumors and it has been shown to affect many biological properties of tumor cells implicated in tumor progression, response to therapy, including clinical outcome of patients (Vaupel and Mayer, 2007; Chan and Giacca, 2007; Bristow and Hill, 2008). Major causative factors of tumor hypoxia are abnormal structure and function of the microvessels supplying the tumor, increased diffusion distances between the nutritive blood vessels and the tumor cells, and reduced O_2 transport capacity of the blood due to the presence of anemia (Vaupel and Harrison, 2004). Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 μm of blood vessels. Without blood vessels, tumors cannot grow beyond a critical size or metastasize to another organ, thus tumor cells and host cells must recruit new blood vessels by vasculogenesis and angiogenesis. The newly formed vascular network differs greatly from that found in normal tissue, typically displaying a broad range of structural and functional abnormalities, including dilations, incomplete or absent endothelial linings and basement membranes, leakiness, irregular and tortuous architecture, arteriovenous shunts, blind ends, and a lack of contractile wall components and pharmacological/physiological receptors (Vaupel *et al.*, 1989). This abnormal architectural features of tumor vessels perpetuates regions of chronic hypoxia, which again stimulates angiogenesis in a "never end story". The oxygenation status of the tumor can be worsened further by increases in diffusion distances, which occur when tumor cells extend beyond the distance that allows adequate O_2 delivery from the blood vessels, or by a reduced ability of the blood to transport O_2 due to the presence of cancer-related or cancer treatment-induced anemia (Vaupel, 2004). Hypoxic areas of tumors are known to be decisive in inducing the development of a tumor phenotype more aggressive, resistant to therapeutic treatment and associated with adverse clinical outcomes (Vaupel

and Mayer, 2007). Hypoxia, more than the reduced levels of nutrients and the increased levels of waste products, drives genomic instability and alters DNA damage repair pathways (Bristow and Hill, 2008). A key regulator of the cellular response to hypoxia is the hypoxia-inducible factor-1 (HIF-1), a master-transcriptional activator for a group of genes that are responsible for cellular adaption to hypoxia (Simon, 2007). HIF-1 is a heterodimeric transcription factor composed of the basic helix-loop-helix-PAS-domain, containing the proteins HIF-1alpha (HIF-1α), regulated at the protein level in an oxygen-sensitive manner, and the arylhydrocarbon receptor nuclear translocator (HIF-1β), which is constitutively expressed (Ziello *et al.*, 2007). Under normal oxygen tension (normoxia), active prolyl hydroxylases hydroxylate HIF-1α making it a substrate for ubiquitylation by the von Hippel Lindau (VHL) E3 ubiquitin ligase complex followed by degradation by the proteasome; whereas under hypoxia prolyl hydroxylases are inactivated, HIF-1α protein is stabilized, translocates to the nucleus and heterodimerizes with HIF-1ß to form the complex HIF-1, which binds to hypoxic response elements (HRE, i.e., the DNA sequence where HIF-1 binds) and activates gene transcription that enhance cellular adaptation to hypoxia (Levy *et al.*, 1996) (Figure A).

HIFs act as a rheostat in increasing in tumor cells the expression of more than 100 genes involved in cell survival, angiogenesis, erythropoiesis, iron homeostasis, stimulation of invasion and migration, adaptive changes in energy metabolism and pH regulation (Pouysségur *et al.*, 2006; Denko, 2008). It activates the expression of these genes by binding to a 50-base pair *cis*-acting HRE located in their enhancer and promoter regions (Semenza *et al.*, 1991). HIF is involved in cell survival and proliferation by inducing growth factors, such as insulin-like growth factor-2 (IGF2) (Feldser *et al.*, 1999) and transforming growth factor-α (TGF-α) (Krishnamachary *et al.*, 2003). Experimental evidence suggested that hypoxia induces apoptosis in tumor cells and it provides a pressure in tumors selecting for cell with survival and growth advantage (Graeber *et al.*, 1996). Hypoxia is also able to stimulates the transcription of VEGF-A, a major regulator of angiogenesis, which promotes endothelial cell migration toward a hypoxic area, and regulates genes involved in governing the vascular tone such as nitric oxide synthase (NOS2) (Melillo *et al.*, 1995), heme oxygenease 1 (Lee *et al.*, 1997), endothelin 1 (ET1) (Hu *et al.*, 1998) and adrenomedulin (ADM) (Nguyen and Claycomb, 1999). Moreover, HIF induces genes involved in matrix metabolism and vessel maturation such as matrix

metalloproteinases (MMPs) (Ben-Yosef *et al.*, 2002), plasminogen activator receptors and inhibitors (PAIs) (Kietzmann *et al.*, 1999), and collagen prolyl hydroxylase (Takahashi *et al.*, 2000) and it promotes metastasis through regulation of a number of proteins involved in the metastatic process including vimentin, fibronectin, keratin 14, 18, 19, cathepsin D, and the urokinase plasminogen activator receptor (UPAR).

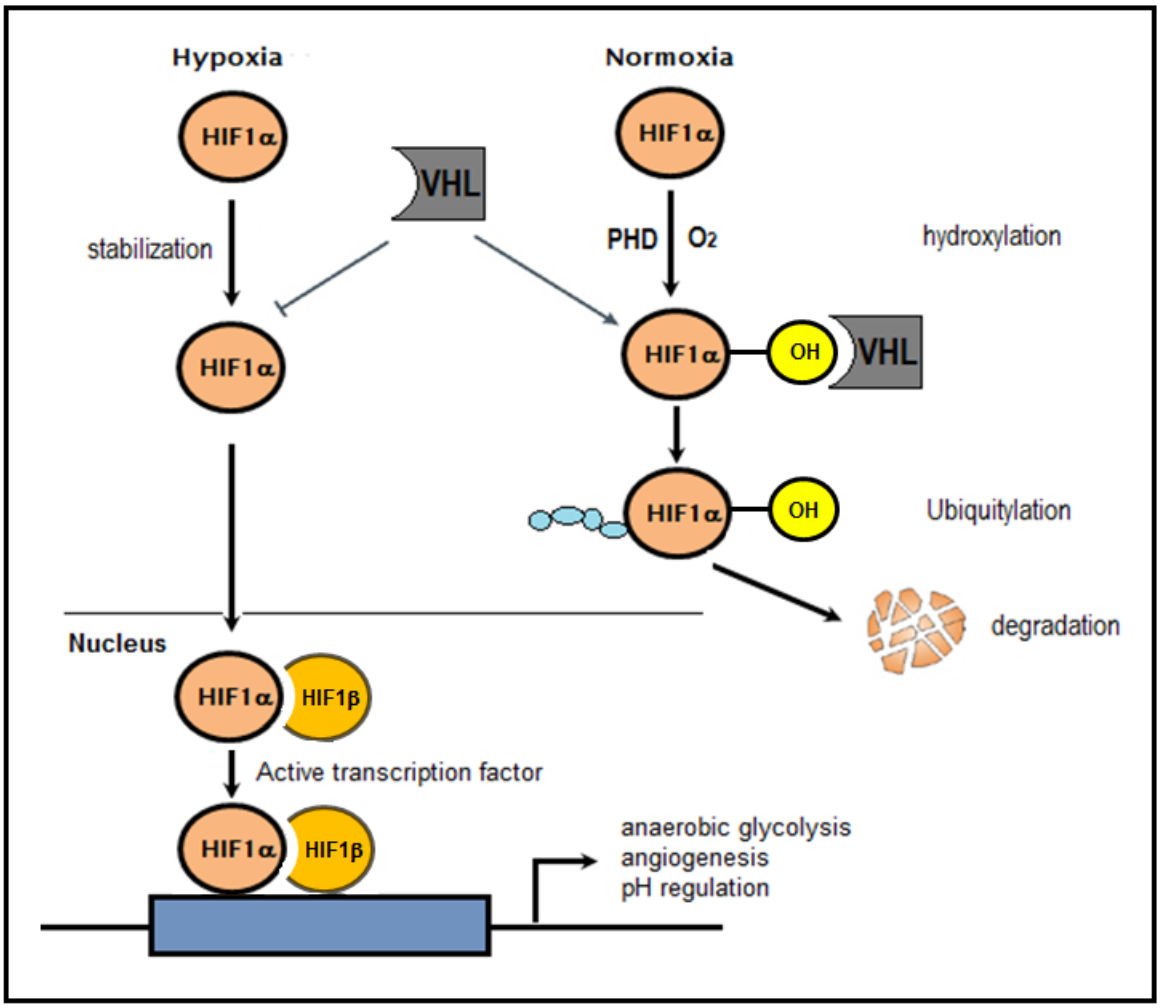


Figure A- Mechanism of hypoxia-induced gene expression mediated by the HIF-1α transcription factor. At normal oxygen levels (normoxia), prolyl-4-hydroxylases are active and hydroxylate HIF-1α making it a substrate for ubiquitylation by the von Hippel Lindau (VHL) E3 ubiquitin ligase complex followed by degradation by the proteasome; whereas under hypoxia prolyl hydroxylases are inactivated, HIF-1α protein is stabilized, translocates to the nucleus and heterodimerizes with HIF-1ß to form a complex which binds to hypoxic response elements and activates gene transcription that enhance cellular adaptation to hypoxia. Figure modified from Supuran, 2008. "Carbonic anhydrases: novel therapeutic applications for inhibitors and activators". Nat Rev Drug Discov 7:168-81.

Disruption of cell-cell and cell-extracellular matrix interactions are the first step to release epithelial cells from tissues, and tumor cells acquire a mesenchymal phenotype that confers the ability to move through extracellular matrix. Reduction of E-cadherin, a transmembrane protein functional to maintain intercellular adhesion of epithelial cells, is a fundamental event during the epithelial-mesenchymal transition (EMT) and is promoted by HIF-1α through activation of lysyl oxidase (LOX) (Krishnamachary *et al.*, 2006). EMT is fundamental for embryonic development and involves changes that include the loss of cell-cell adhesion and the acquisition of migratory and invasive properties (Thiery *et al.*, 2009). The acquisition of EMT features in cancer cells has been associated with metastasis and is involved in carcinogenesis, invasion, and tumor recurrence (Guarino, 2007; Azab *et al*., 2012). HIF-1α is an inducer of epithelial-to-mesenchymal transition (EMT) in cancer cells, either indirectly by TGF-β induction, or directly activating Snail, Twist and members of Zeb family (Pouysségur *et al*., 2006; Polyak *et al.*, 2009).

Low oxygen concentration also determines migration of tumor cells in specific district promoting a chemokine/chemokine receptor axis. Both, CXCR4 and its ligand, the stromal-derived factor-1 (SDF-1), have been demonstrated to be regulated by HIF-1α, and have a role in homing metastatic cells of different tumor histotypes to secondary sites (Staller *et al.*, 2003; Ceradini *et al.*, 2004). Vitexin, a HIF-1α inhibitor, was shown to significantly inhibit the migration of rat pheochromocytoma PC12 cells along with hypoxia-induced activation of c-jun N-terminal kinase (JNK) (Choi *et al*., 2006), suggesting that hypoxia or HIF-1α may play a role in cell migration. In another study it was found that hypoxia-induced increased motility and invasiveness of tumor cells require Notch signaling, and activated Notch mimics hypoxia in the induction of EMT (Sahlgren *et al.*, 2008).

The complex and redundant scenario organized by hypoxic tumor cells through HIF-1α signaling is amplified by the interception with the nuclear factor (NF)-kB. The synergistic HIF-1α-NF-κB pathway represents an elegant control mechanism for the specialized activities of inflammatory cells, such as β2-integrin expression, production of antimicrobial molecules and potent inflammatory cytokines (TNFα) and nitric oxide (Eltzschig and Carmeliet, 2011). This leads to a deregulated inflammation, with stimulatory signals higher than inhibitory, that contributes to tumor progression (Robinson and Coussens, 2005; Vakkila and Lotze, 2004). Inflammation is part of tumor progression,

and inflammatory cytokines, including TNFα, are the crucial mediators of fatigue, depression, cognitive impairment and cachexia of tumor-bearing patients (Seruga *et al.*, 2008).

Among the many genes induced by HIF are genes responsible for glucose transport and glucose metabolism (Brahimi-Horn *et al*., 2007). Under low oxygen supply, cells switch their glucose metabolism pathway away from the oxygen-dependent tricarboxylic acid (TCA) cycle to the oxygen-independent glycolysis (Dang and Semenza, 1999; Seagroves *et al*., 2001). HIF renders cancer cells more efficient in mobilizing glucose (glucose transporters, GLUT1 and GLUT3) and reduces the production of reactive oxygen species by repressing oxygen-consuming protein and lipid synthesis, increasing expression of proteins involved in anaerobic glucose metabolism (Aldolase A and C, enolase 1, esokinase 1 and 3, lactate dehydrogenase A, phosphofructokinase L and 1) (Semenza, 2002) and repressing mitochondrial oxidative respiration (Denko, 2008). Furthermore, the glycolysis metabolic products, such as lactate and pyruvate, have been reported to cause HIF-1α accumulation under normoxia and regulate hypoxia-inducible gene expression, hence establishing a potential positive feedback loop (Lu *et al.*, 2002). An important consequence of the switch to glycolytic metabolism is increased lactic acid production that needs to be removed in order to avoid a drop in intracellular pH that could result in cell demage. To contrast this effect HIF-1 induces the monocarboxylate transporter 4 (MCT4) and the carbonic anhydrase IX (CAIX) expression that mediate respectively the lactic acid efflux and the catalysis of reversible CO_2 hydration (Svastová *et al*., 2004). In this way, across the cell membrane in tumors there is a pH gradient with intracellular pH greater than extracellular pH (acidic outside, which is reverse of that found in normal tissue). Hypoxia, elevated interstitial fluid pressure, low glucose and high lactate concentration resulting from a predominant anaerobic metabolism, are responsible of low extracellular pH (pHe) in tumor tissues. As a consequence, the second metabolic hallmark of tumor environment is tumor acidosis.

1.2 Acidity

In normal differentiated adult cells, intracellular pH (pHi) is in most of the cases 7.2 and it is lower than the extracellular pH (pHe), which is approximately 7.4 (Figure B). In general, the extracellular pH of human tumors is lower than that in normal tissue and several studies revealed that cancers show a "reversed" pH gradient across tumor cells plasma membrane, with an increased intracellular pH (from 7.12 to 7.56), higher than the extracellular pH (from 6.2 to 6.9) (Gillies *et al.*, 2002). Thus, the range of pH values in tumors is much greater than that the normal tissues and it has been reported that the pH value in metastases is higher than those in primary tumors (Wike-Hooley *et al*., 1984).

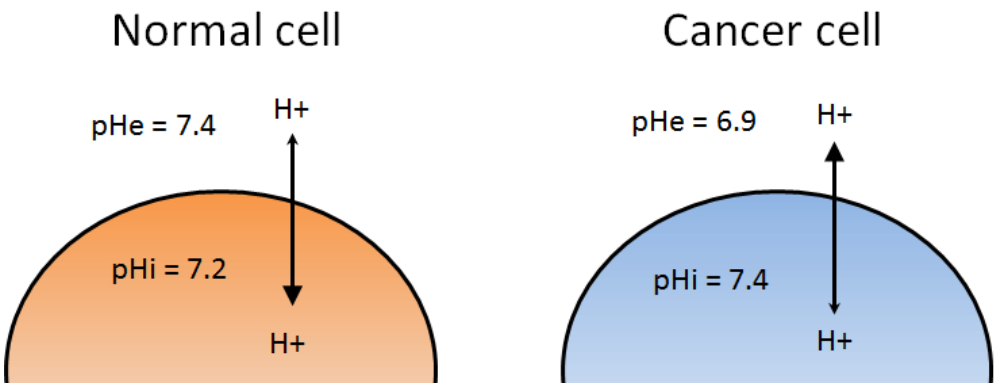


Figure B- The"reversed" pH gradient of cancer cells. In normal cells, intracellular pH (pHi) is in most of the cases 7.2 and it is lower than the extracellular pH (pHe), which is approximately 7.4. The intracellular tumour pH is slightly alkaline (pH 7.4), whereas the extracellular pH (pH_e) is slightly acidic (pH 6.9).

It is commonly accepted that the acidic extracellular pH of tumors is a result of a poor oxygenation due to heterogeneous blood supply (Gillies *et al*., 1999); Hemlinger and colleagues (1997) have shown a relationship between local blood flow, pO2 and pH. Both interstitial pH and pO_2 decrease gradually increasing the distance of the tissue to the vessel (Figure C).

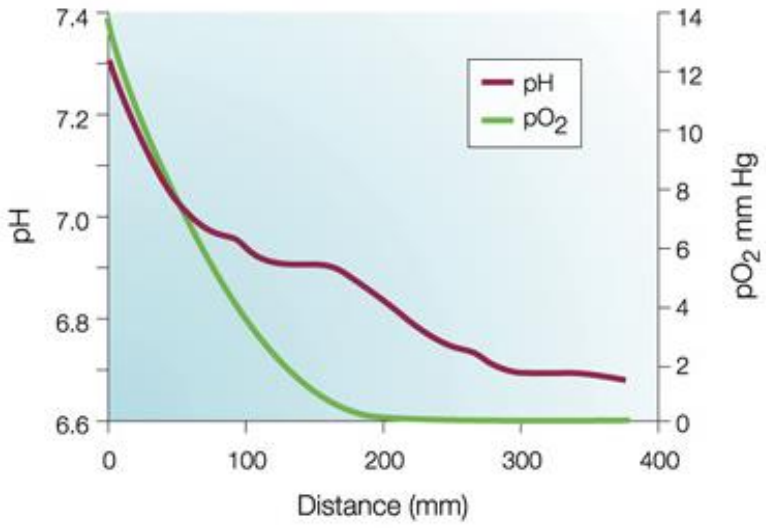


Figure C- Tumour interstitial pH and partial pressure of oxygen (pO_2) shown with distance from a vessel wall. Figure from Gatenby RA, Gillies RJ, 2004. "Why do cancers have high aerobic glycolysis?" Nat RevCancer 4:891-9.

In addition to poor oxygenation, the pH of cells and tissues is the result of the balance between metabolic processes, proton transport and buffering.

In most mammalian cells, which rely on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, glycolysis is inhibited by the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO_2 and H_2O. This inhibition is termed 'Pasteur effect', after Louis Pasteur, who first demonstrated that glucose flux was reduced by the presence of oxygen (Racker, 1974). In contrast to normal cells, cancer cells, even in the presence of sufficient oxygen to support mitochondrial respiration, use "aerobic glycolysis", a phenomenon termed "Warburg effect" (Gatenby and Gillies, 2004). Increased aerobic glycolysis is uniquely observed in cancers. This phenomenon was first reported by Warburg in the 1920s, leading to hypothesis that cancer results from impaired mitochondrial metabolism. Although the "Warburg hypothesis" has proven incorrect, an increased conversion of glucose to lactic acid in tumor cells has been continuously demonstrated (Semenza *et al*., 2001). The glucose analog 2-(^{18}F)-fluoro-2-deoxy-D-glucose (FDG) tracer used in PET (positron-emission tomography) imaging of thousands of oncology patients has unequivocally shown that most primary and metastatic

human cancers show significantly increased glucose uptake. The increased glucose uptake imaged with FdG PET was largely dependent on the rate of glycolysis.

Cells derived from tumors typically maintain their metabolic phenotypes in culture under normoxic conditions, indicating that aerobic glycolysis is constitutively upregulated through stable genetic or epigenetic changes (Gatenby and Gillies, 2004) and it could be a crucial component of the malignant phenotype. The metabolic products of glycolysis, such as hydrogen ions (H^+), cause a spatially heterogeneous but consistent acidification of the extracellular space, which might result in cellular toxicity. Some studies have shown that prolonged exposure of normal cells to an acidic microenvironment typically results in necrosis or apoptosis through p53- and caspase-3-dependent mechanisms (Park *et al*., 1999; Williams *et al*., 1999). So, constitutive upregulation of glycolysis requires additional adaptation to the negative effects of extracellular acidosis through resistance to apoptosis or upregulation of powerful mechanisms to prevent acidification of the intracellular environment. The maintenance of intracellular pH is attributed to the existence of short-term and long-term mechanisms (Roos and Boron, 1981). The short-term mechanisms consisting of rapid buffering responses such as physiochemical buffering of the acids, metabolic consumption of nonvolatile acid or transfer of acid from the cytosol to the organelles, act quickly but fail to maintain the intracellular environment at neutral pH for a long time. The mechanisms for long-term pHi regulation are represented by multiple and redundant families of H^+ transporters, which release protons and acidify tumor cell extracellular environment (Figure D): Na^+/H^+ exchanger 1 (NHE1), carbonic anhydrases (CAIX, CAXII and CAII), Cl^-/HCO_3^- transporters (Na^+/HCO_3^- co-transporters (NBCs) and anion exchange protein 1 (AE1)), proton-linked monocarboxylate transporters (MCT1 and MCT4) and vacuolar H^+-ATPases (V-ATPases) (Parks *et al*., 2013). The activity of Na^+/H^+ antiport depends on Na^+ and H^+ concentrations and it becomes inactive at alkaline pHi. The bicarbonate-dependent transporters are distinct from each others: the Na+ dependent HCO_3^-/Cl^- exchanger is quiescent at alkaline pHi and becomes activated as pHi falls below a certain threshold; the cation independent HCO_3^-/Cl^- is inhibited at acidic pHi in several cell types and appears to play at most a minor role in regulation of pHi under acidic conditions (Tannok and Rotin, 1989). In normal non-muscle cells MCTs have a limited role in pHi regulation but in cancer cells expression of MCTs is increased and it confers a selective advantage to cancer cells owing to the high affinity of these transporters for lactate (Halestrap and Price, 1999) and the

ability to promote the conversion of pyruvate to lactate (Harris *et al.*, 2009). Na^+–H^+ exchange (McLean *et al.*, 2000) and V-ATPases (Martinez-Zaguilan *et al.*, 1993) have both been observed to be upregulated in cancers, and V-ATPase might confer resistance to apoptosis.

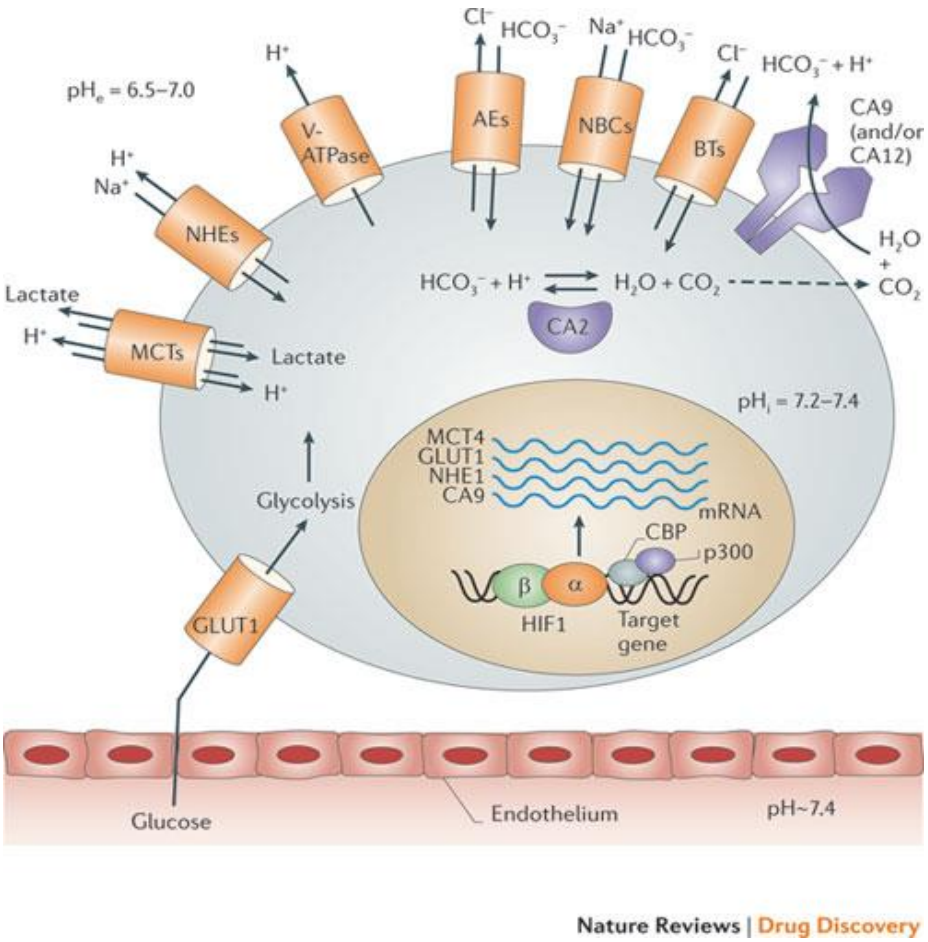


Figure D –Proteins involved in pH regulation within a tumour cell. The figure shows various proteins that are involved in regulating pH within tumours, including: monocarboxylate transporters (MCTs), which transport lactic acid and other monocarboxylates formed by the glycolytic degradation of glucose; Na^+/H^+ exchangers (NHEs); the plasma membrane proton pump vacuolar ATPase (V-ATPase); anion exchangers (AEs); Na^+/HCO_3^- co-transporters (NBCs); and carbonic anhydrases (CA2, CA9 and/or CA12). The glucose transporter GLUT1 transports glucose into tumour cells. The intracellular tumour pH (pH_i) is slightly alkaline (pH 7.2–7.4), whereas the extracellular pH (pH_e) is slightly acidic (pH 6.5–7.0). BT, HCO_3^- transporter; CBP, cyclic AMP-responsive element-binding (CREB) protein; p300, histone acetyltransferase p300. Figure from Neri & Supuran, 2011. "Interfering with pH regulation in tumours as a therapeutic strategy" Nature Reviews Drug Discovery 10, 767-777.

In reality, only a few proteins are differentially expressed in tumour cells compared with normal cells in the context of pH_i regulation. These include the hypoxia-regulated, extracellular-facing carbonic anhydrases, carbonic anhydrase IX (CAIX) and CAXII, which manage the acidic metabolic production of CO_2 by facilitating H^+ diffusion towards the vasculature and the hypoxia-induced monocarboxylate transporter 4 (MCT4), which removes H^+ in conjunction with lactate for the facilitation of continued glycolytic ATP production and thus contributes to pH_i regulation (Chiche *et al*., 2012; Le Floch *et al*., 2011; Ullah *et al.*, 2006). These proteins, coupled with other pH_i modifiers, such as H^+ pumps and cytosolic CAII, combine to result in a tumor cell that actively maintains a more alkaline pH_i than a normal cell, despite its acidic surroundings. The resulted high pHi of cancer cells confers the selective advantages of growth factors-independent proliferation and of evasion of apoptosis, for example through the negative regulation of the mitotic regulatory complex, cyclin-dependent kinase1 (CDK1)-ciclin B1 (Putney and Barber, 2003), or by modulating pro-apoptotic proteins as BAX (Khaled *et al.*, 1999) and SAPK (Stress-activated Protein Kinases, also known as JNK) (Zanke *et al.*, 1998).

Clinical investigations have shown that tumors with acidic environment are associated with high incidence of metastases, poorer prognosis (Walenta *et al*., 2000), resistance to chemotherapy (Raghunand *et al*., 2001), suppression of cytotoxic lymphocytes and natural killer cells tumoricidal activity (Fischer *et al.*, 1997). Tumor acidity also confers multiple advantages to the aggressiveness of tumor cells and affects several steps of metastatic cascade, stimulating invasiveness into host tissues, angiogenesis, lymphangiogenesis and secondary organ colonization (Webb *et al*., 2011; Calorini *et al*., 2012). Recent evidence reveals a molecular basis for pHi-dependent metastasis by showing that pH sensors control distinct stages of cell migration. One essential characteristic of migrating cells is their polarization along the direction of movement (Ridley *et al.*, 2003). Recent studies indicate that the intracellular pH may serve locally as a regulator of cell polarization and migration because it modulates cytoskeletal dynamics directly by affecting the actin-binding proteins cofilin (Pope *et al.*, 2004), profilin (McLachlan *et al*., 2007), twinfilin (Moseley *et al*., 2006), villin (Grey *et al*., 2006) and talin (Srivastava *et al*., 2008) and indirectly by enhancing the activity of the low-molecular-weight GTPase CDC42 (Frantz *et al.*, 2007). In addition to the *de novo* assembly of actin filaments, metastasis requires the remodelling of cell-substrate adhesions, a process that requires an increased pHi and a

decresed pHe (Stock *et al.*, 2008). Human melanoma cells migrate faster at a pHe = 7 compared with a pHe 7.4, because low pH regulates the dynamics of ECM-integrin attachments directly altering integrin conformation and affecting integrin-ligand binding (Paradise *et al.*, 2011). Through computational molecular dynamics simulations, Paradise *et al.* found that acidic extracellular pH promotes opening of the α(v)β(3) headpiece, indicating that acidic pH can thereby facilitate integrin activation. It has been reported that an acidic pHe is also necessary for the formation and maturation of invadopodia (Busco *et al*., 2010), proteolytic actin-rich protrusions involved in cancer cell invasion, and may enhance invasion of tumor cells facilitating the redistribution of active cathepsin B, a lysosomal aspartic proteinase with acidic pH optima, to the surface of malignant cells (Rozhin *et al.*, 1994; Webb *et al*., 1999). Acid-activated catepsins L also participate to amplify proteinase cascade through activation of urokinase-type plasminogen activator (uPA) (Goretzki *et al.*, 1992), that convert plasminogen to plasmin, which degrades various components of the ECM such as fibronectin, laminin, proteoglycan and collagen (Dano *et al.*, 1985) and also activates latent collagenase and growth factors (Lyons *et al*., 1988). Concomitant expression of uPA and uPA receptor (uPAR) by tumor cells results in a high invasive capability (Ellis *et al.*, 1992). Additionally acid-activated cathepsin B cleaves secreted, latent MMPs into active enzymes. MMPs, a family of structurally related zinc-dependent endopeptidases collectively capable of degrading all components of ECM, are classified into subgroups of collagenases, stromelysins and stromelysin-like MMPs, matrilysins, gelatinases and membrane-type MMPs (MT-MMPs) (Nagase and Woessner, 1999). MMPs have long been associated with invasiveness, dissemination of tumor cells and angiogenesis (Vihinen and Kähäri, 2002), due to their capacity to help tumor cells to cross structural barriers, including basement membranes, to migrate into the blood, extravasate and colonize in distant host tissues. Several studies have shown that acidity up-regulates in cancer cells expression and secretion of MMP-9 and MMP-2 (Kato *et al*., 1992; Rofstad *et al*., 2006), which have been demonstrated to be directly involved in tumor growth and invasion by studies with knockout mice: MMP-9-deficient mice showed reduced formation of melanoma metastases (Itoh *et al*., 1999) and MMP-2-deficient mice showed reduced melanoma tumor progression and angiogenesis (Itoh *et al*., 1998). Acidic pH*e* has also been shown to increase the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase (Griffiths *et al*., 1997), IL-8 (Xiong *et al*., 2001, Xu and Fidler, 2000), iNOS (inducible isoform of nitric oxide synthase) (Bellocq *et al.*,

1998) and VEGF (Fukumura *et al.*, 2001, Xu *et al*., 2002) in various types of cells through activation of the transcription factors NF-κB and/or AP-1 (Kato *et al*., 2005), induced under acidic pH (Xu *et al*., 2002). Other important components of basement membrane to be degraded by tumor cells to disseminate are the heparan sulphate chains. Toyoshima and Nakajima (1999) report that heparanase has an optimal pH of 4.2, but a significant heparanase activity persists at pH 6.0–6.5, suggesting that the acidic environment of tumors may activate the degrading properties of tumor heparanases.

A number of studies have suggested that the activity of two important H^+ transporters, NHE1 (Cardone *et al*., 2005) and the plasma membrane type of vacuolar H^+-ATPases (V-ATPases) (Hinton *et al.*, 2009) is important for cancer cell invasion. V-ATPases are overexpressed in many types of metastatic cancers and positively correlated to their invasion and metastasis (Sennoune *et al*., 2004, Chung *et al*., 2011). Blocking V-ATPase activity inhibits cancer cell invasion, reduces MMP-9 activity (Chung *et al.*, 2011) and abrogates metastatic diffusion of tumor cells (Lu *et al*., 2005). More recently it was found that the stimulation of breast cancer invasion (Reshkin *et al.*, 2000; Cardone *et al.*, 2005; Cardone *et al.*, 2007), melanoma migration (Stock *et al.*, 2005), and ECM degradation (Busco *et al.*, 2010) by acidic pH, is mediated by the activity of the Na^+/H^+ exchanger NHE1 (Vahle *et al*., 2014), which is able to regulate the expressions and activities of MT1-MMP (Vahle *et al*., 2014), MMP-2, MMP-9 and VEGF (Yang *et al*., 2010). NHE1 also regulates cortactin–cofilin interaction at invadopodia influencing the generation, maturation and function of invadopodia. Busco *et al*. proposed that the NHE1 acidifies the peri-invadopodial space and, in turn, this acidification up-regulates invadopodial morphology and proteolytic activity (Busco *et al*., 2010). NHE1 is able to regulate the activities of at least three mitogen-activated protein kinases (MAPK) subfamilies: extracellular signal regulated kinase (ERK)1/2, c-jun N-terminal kinase (JNK) and p38 MAPK (Lin *et al*., 2011). MAPKs are a family of serine/threonine kinases that are activated through a signaling pathway triggered by growth factors, stress, hormones, lymphokines, extracellular matrix components, and tumor promoters and their activity influences cell viability and proliferation; p38 MAPK, for example, inhibits cell cycle progression and promotes programmed cell death, while the converse is true for ERK (Xia *et al.*, 1995; Pedersen *et al.*, 2007). It was demonstrated that acid exposure activates MAPK signaling in a variety of cell type (Xue and Lucocq, 1997; Sarosi *et al*., 2005), which can induce proliferation, cycle progression and suppression of apoptosis. It is

known that extracellular acidity has a robust protective effect against apoptosis induced by multiple cytotoxic metabolic stresses but yet the enhanced survival associated with extracellular acidity has not been fully explained. Ryder *et al*., (2012) examined the role of the Bcl-2 family in this process showing the promotion of high anti-apoptotic Bcl-2 family member by acidosis, probably mediated by the acid-sensing G protein-coupled receptor, GPR65, via a MEK/ERK pathway. Previously, Kumar *et al.,* (2007) found that acidic pre-conditioning suppresses apoptosis increasing expression of Bcl-xl in coronary endothelial cells under stimulated ischemia. Another survival mechanism used by cells exposed to low pH is the autophagy (Wojtkowiak *et al.*, 2012) an evolutionarily conserved catabolic process through which cytoplasmic proteins and organelles are self-digested, maintaining cellular metabolism through recycling of the degraded components (Mizushima and Klionsky, 2007). Others researchers have shown that acidosis can induce the resistence to anticancer chemotherapies and a number of mechanisms have been postulated to be responsible for this effect, including reduction in cycling cell fraction (Newell *et al*., 1992), selection for apoptosis-resistant phenotypes (Ohtsubo *et al*., 2001), and direct effect of ion gradients on drug distribution or ion trapping (Raghunand and Gillies, 2002). The primary mechanism leading to a multidrug resistant (MDR) phenotypes assumed to be plasma-membrane localized overexpression of drug efflux transporters, such as P-glycoprotein (pGP), a member of the ABC (ATP-binding cassette) transporter family. The acidic extracellular pH doesn't modulate pGP expression but increase pGP pump activity, presumably as a result of lowered intracellular calcium levels and inhibition of PKC (Thews *et al*., 2006). In addition, intracellular pH may affect the molecular interaction between drugs and their targets such as various intracellular organelles, DNA, RNA, proteins involved in cell cycle progression and apoptosis. Acidity of tumors also reduces sensitivity of tumor cells to radiation therapy (Trowell, 1953; Haveman, 1980; Rottinger and Mendonca, 1982). Indeed, an acidic environment induces p53 expression (Ohtsubo *et al.*, 1997), prolongs radiation-induced G_2/M arrest (Park *et al.*, 2003), and markedly suppresses radiation-induced apoptosis in cancer cells (Lee *et al*., 1997, Choi *et al.*, 2004). It appears that the increase in radioresistence in acidic pH environment results also from an increased DNA damage repair during the prolonged G_2 arrest.

There is growing evidence that cancers can escape immune destruction by suppressing the anti-cancer immune response through maintaining a relatively low pH in their micro-environment. The acquired properties enable cancer cells to suppress the anti-cancer

activity of immune cells secreting chemokines to enhance recruitment of T-regulatory cells to the tumor site or producing immunosuppressive cytokines (Choi *et al.,* 2013). The acidic microenvironment has been shown to impair natural killer (NK) cells via restriction of IFN-gamma, IL-10 and TGF-beta (Lardner, 2001), to inhibit lymphocyte proliferation and dendritic cell maturation (Gottfriend *et al*., 2006), and to lead to loss of T-cell function of human and murine tumor-infiltrating lymphocytes; the T-cell function could be completely restored by buffering the pH at physiological values (Calcinotto *et al*., 2012). Extracellular lactic acid generated by cancer metabolism has been identified as primary cause responsible for the pH-dependent T-cell function-suppressive effect and new results suggest that tumor lactic acidosis suppresses CTL (cytotoxic T lymphocytes) function via inhibition of p38 and JNK/c-Jun activation (Mendler *et al.,* 2011). Ohashi *et al.* (2013) found that lactic acid secreted by tumor cells can play two independent roles in promoting tumor development: first, it inhibits the immune response by increasing arginase-1 (ARG1) expression in tumor associated macrophages; secondly, it induces inflammation within the tumor microenvironment by activating the IL-23/IL-17 pathway involved in tumor associated inflammation and lack of tumor immunosurveillance. Indeed, transient exposure of cells to acidic pH results in changes of Ca^{2+} dynamics stimulates pro-inflammatory cytokine release (Lee *et al.,* 2011) and suppresses the ability of macrophages to mount an adequate antibacterial proinflammatory response following TLR2 and TLR4 activation (Fernandez *et al.,* 2013). There are other studies on the pH dependence of antibody/antigen association showing that acidic pH decreases the synthesis of antibody (Zhuravskii *et al.,* 1980) and increases the avidity of human IgG binding to human neutrophils, monocytes and NK (natural killer) cells (Lopez *et al*., 1999) in addition to activating the complement system (Miyazawa and Inoue, 1990).

Although there are several studies regarding the role of acidic pHe in cancer, little is known about signal transduction pathway. Fukamachi *et al*. (2013) found that different signals transduction pathway function under acidic environment. Data suggest that MAPK including ERK1/2 and p38, AP-1, NF-κB and phospholipase D (PLD) mediate acidic pHe signaling thereby inducing expression of VEGF-A and IL-8, and MMP-9 production (Kato *et al*., 2013). The expression of HIF-1α induced by hypoxia is decreased by acidosis (Tang *et al.,* 2012) as well as mTORC1 activity that is inhibited rapidly and reversibly by acidic extracellular pH (Balgi *et al*., 2011), while mTORC2 is not affected by pH. Rafiee *et al.* (2006) demonstrated that acidic pH induces activation of Akt through PI3K activity

in human esophageal microvascular endothelial cells while Balgi *et al.* (2011) found that Pi3k-Akt pathway does not partecipate in pH sensing in human breast carcinoma and mouse embryo fibroblasts.

In order to respond to change of pH, cancerous cells require the ability to sense minute pH changes. Recently several hypotheses were made on the cellular sensors involved in the pH response. One mechanism by which pH sensing occurs is through post translational protonation of amino acid side chains, in particular the imidazole side chain of histidine residues located in critical regions of G-protein coupled receptors (GPCRs), intracellular molecules involved in actin assembly, membrane proton pumps and acid-sensing ion channels (ASICs) (Damaghi *et al.,* 2013). Some studies demonstrated the role of phosphatidic acid (PA) as a pH biosensor (Shin *et al*., 2011) while others support the importance of the focal adhesion kinase (FAK), a key regulator of focal adhesion remodeling (Choi *et al.,* 2013).

Acidity of tumor environment is not produced by the only accumulation of protons originated from different metabolic sources, but lactic acid participates to lowering pHe. Recent studies investigating lactate accumulation in human tumors have found that cervical tumors, head and neck cancers and rectal adenocarcinomas, with metastatic spread exhibit a wider range and significantly higher levels of lactate than non-metastatic tumors (Schwickert *et al.*, 1995; Walenta *et al*., 1997; Walenta *et al*., 2003). Elevated lactate levels correlate with poorer prognosis and poor disease-free survival in several epithelial cancers, such as cervical, head and neck, non-small cell lung and breast cancers (Dhup *et al*., 2012). In addition to the contribution to the immunologic escape already mentioned above, the lactate mediate cell mobility (Goetze *et al*., 2011), stimulates VEGF production by endothelial cells (Beckert *et al.,* 2006), NF-κB activity and IL-8 expression (Végran *et al*., 2011) and up-regulates genes associated with aerobic metabolism, including factors involved in the TCA cycle and electron transport (Chen *et al.,* 2008). These features make lactate metabolism of interest not only as a biological marker, but also as a potential therapeutic end point or target (Kennedy *et al.,* 2010).

1.3 Tumor metabolism

All the cells in the human body, whether benign or malignant, depend on metabolizing glucose both for proliferation and physiologic maintenance. Once ingested via nutrient transporters, glucose is broken down into pyruvate and two molecules of ATP. In the presence of oxygen, pyruvate enters the mitochondrion of the cell, where it is oxidized via the Krebs cycle, producing CO_2, H_2O, and 36 ATP molecules. Otherwise, pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH) and is then released from the cell.

The metabolic program of normal cells serves to meet the energetic requirements of maintaining homeostatic processes through ATP production, while proliferating cells need to satisfy the anabolic demands of macromolecular biosynthesis and to maintain cellular redox status. While energetically inefficient, glycolysis can provide cells with glycolytic intermediates necessary for biosynthesis of DNA and cell structural material. This is done via the pentose phosphate pathway and involves generating NADPH, which is then used for fatty acid synthesis, and ribose-5-phosphate, which is used for synthesis of nucleotides (Weinberg and Chandel, 2009). For this reason, a special property of cancer cells is their frequent persistence in using glycolysis even in the areas of ample oxygen supply capable of supporting oxidative phosphorylation, a phenomenon known as the Warburg effect (Vander Heiden *et al*., 2009). Although ATP production by glycolysis can be more rapid than by oxidative phosphorylation, it is far less efficient in terms of ATP generated per unit of glucose consumed; therefore tumor cells implement an abnormally high rate of glucose uptake. A number of oncogenes and tumor suppressor genes are involved in the metabolic switch from oxidative phosphorylation to glycolysis, such as myc, nfkb, Akt, EGF, PI3K, mTOR, KRAS (Kristen rat sarcoma viral oncogene homolog), AMPK (AMP-activated protein kinase) and HIF-1α (DeBerardinis, 2008; Hirschhaeuser *et al*., 2011). Particularly, HIF-1α is stabilized under normoxic conditions by the products of glycolysis, lactate and pyruvate (Lu *et al*., 2002). Consequently, expression of HIF1α-regulated genes as GLUT1, which encodes a glucose transporter that increases glucose uptake (Ebert *et al*., 1995), *LDHA*, encoding lactate dehydrogenase A, which converts pyruvate to lactate (Semenza *et al*., 1996), *PDK1*, encoding pyruvate dehydrogenase kinase 1, which inactivates the enzyme responsible for conversion of pyruvate to acetyl-CoA (Kim *et al*., 2006), leads to an enhanced flux of glycolysis resulting in lactate accumulation even in the

presence of oxygen. Lactate production in cancer cells is facilitate also by the increased expression of PK type M2 (PKM2) produced by alternative splicing of the *PKM* gene. PKM2 catalyses the rate-limiting step of glycolysis, controlling the conversion of phosphoenolpyruvate (PEP) to pyruvate, and thus ATP generation. By slowing the passage of metabolites through glycolysis, PKM2 promotes the shuttling of these substrates through the pentose phosphate pathway (PPP) and other alternative pathways, so that large quantities of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and other macromolecules needed to support the rapid cell division are produced (Cairns *et al.*, 2011). So, lactate, the end product of glycolysis, is produced in large excess in tumors and constitutes an alternative metabolic fuel for cancer cells (Sonveaux *et al*., 2008; Feron, 2009; Whitaker-Menezes *et al*., 2011), which is a phenomenon that has been well characterized in non-tumor tissues, including in skeletal muscle, brain and liver (Brooks, 2009; Gladden, 2004). Sonveaux *et al*. (2008) demonstrated the existence of a "metabolic symbiosis" between hypoxic and aerobic cancer cells, in which lactate produced by hypoxic cells is taken up by aerobic cells, which use it as their principal substrate for oxidative phosphorylation. The use of lactate as an energy source requires the conversion of lactate into pyruvate, regulated by LDH, as well as the transport of lactate into and out of tumor cells by way of specific transporters, the monocarboxylate transporters (MCTs). The MCT family includes 14 different members of whom only four are located at the cell membrane (MCT1-MCT4) (Halestrap *et al.*, 2012). Importantly for tumor cells, MCT1 and the hypoxia-induced MCT4 are specialized for the co-transport of lactate and H^+: exogenous lactate uptake by oxidative tumor cells occurs through the high-affinity lactate transporter MCT1, whereas glycolysis-derived lactate is released through the low-affinity lactate transporter MCT4 (Sonveaux *et al.*, 2008). High expression of MCT4 was found in glycolytic tissue, including several hypoxic and rapidly growing tumors (Pinheiro *et al*., 2012). Inhibition of MCT1 can shift the preference of oxidative tumor cells towards using glucose, thereby reducing the amount of glucose that can reach hypoxic tumor cells and altering their survival. Inhibition of MCT4 has instead the potential to directly target hypoxic tumor cells and promote their death by intracellular lactic acid accumulation (Draoui and Feron, 2011). Tumor-associated fibroblasts have also been documented to participate in lactate homeostasis in tumors. Koukourakis and colleagues reported that preferential expression of MCT1 and LDH-1 together with elevated PDH activity in tumor fibroblasts supports the metabolic use of lactate produced

by tumor cells, and thereby prevents the development of a hostile acidic environment (Koukourakis *et al.*, 2005; Koukourakis *et al*., 2006). Also Fiaschi *et al* (2012) demonstrated that fibroblasts in contact with epithelial cancer cells undergo myofibroblast differentiation and produce lactate through aerobic glycolysis which is used by cancer cells for respiration.

Cárdenas-Navia and colleagues have contributed to a large body of literature indicating that a cancer cell that is hypoxic at one moment may be aerobic an hour later and vice versa (Cárdenas-Navia *et al*., 2008). So tumor cells need to adapt not only to regional variation but also to temporal variation in oxygenation and it implies dynamic regulation of the metabolic symbiosis, such that cells may cycle between lactate-producing and lactate-consuming states.

1.4 Epithelial-to-mesenchymal-transition

Epithelial–to-mesenchymal transition (EMT) is an important contributor to the invasion and metastasis of epithelial-derived cancers. It is an orchestrated series of events in which cell-cell and cell-ECM interactions are altered. During EMT, epithelial cells are released from the surrounding tissue and alter their polarity, the cytoskeleton is reorganized to confer the ability to move through a three-dimensional ECM, and a new transcriptional program with multiple biochemical changes are induced to assume and maintain a mesenchymal cell phenotype (Radisky, 2005), which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components (Kalluri and Neilson, 2003). EMTs are classified into three subtypes based on the biological and biomarker context in which they occurs (Kalluri, 2009):

- Type I EMT is associated with implantation, embryo formation, and organ development; it neither causes fibrosis nor uncontrolled systemic invasion by high-grade epithelial cancer cells. This type 1 EMT generates mesenchymal cells able to create new tissues with diverse functions and that have the potential to subsequently undergo a MET to generate secondary epithelia.

- Type II EMT is associated with wound healing, tissue regeneration, and organ fibrosis. It is associated with inflammation and ceases once repair is achieved and inflammation is attenuated.
- Type III EMT occurs in epithelial cancer cells that differ genetically and epigenetically from untransformed epithelial cells. These changes, which mainly affect oncogenes and tumor suppressor genes, conspire with the EMT regulatory circuitry to produce cells that may invade and metastasize via the circulation and thereby generate systemic manifestations of malignant cancer progression.

In the type 3 EMT, several markers of EMT in cancer specimens have been associated with the presence of metastasis, the increased recurrence rate, and the decreased survival rate, supporting the concept that EMT represents aggressive behavioral changes of cancer cells (Krisanaprakornkit and Iamaroon, 2012). Recent studies have shown that transformation not only endows cancer cells with motility to detach from neighboring cells but also promotes anoikis resistance in cancer cells in anchorage-independent circumstances (Kumar *et al*., 2011; Jia *et al.,* 2012). TGF-β, which acts as a tumor suppressor at early stage of tumorigenesis and as a promoter of tumor progression and invasion in the late phase of tumorigenesis, was the first EMT inducer described in normal mammary epithelial cells by signaling through its receptor serine/ threonine kinase complex (Zavadil and Böttinger, 2005). A variety of others extracellular signals have been recently shown to induce transition of epithelial cells into mesenchymal or mesenchymal like cells during embryogenesis and in tumorigenesis, such as interleukin-6 (IL-6) (Yadav *et al*., 2011), epidermal growth factor (EGF) family members (Lo *et al.,* 2007), fibroblast growth factors (FGF) (Acevado *et al.,* 2007), hepatocyte growth factor (HGF) (Savagner *et al.,* 1997), and insulin-like growth factor (IGF) (Graham *et al.,* 2008). These factors, probably emanating from tumor-associated stromal cells, induce the loss of E-cadherin expression/function, a major constituent of the adherens junctions; when E-cadherin is lowered, *β*-catenin translocates to nucleus and participates to transcriptional regulation of EMT (Schmalhofer et al 2009). Furthermore, when E-cadherin is downloaded, cells express N-cadherin, a protein that is associated to migration invasion and metastasis. Indeed, the well-defined features of EMT include loss of epithelial markers as E-cadherin and a- and g-catenin and gain of mesenchymal cell markers as fibronectin, vimentin, and N-cadherin, changes that culminate in a front-to-back polarization of individual cells and an acquired ability to migrate and invade into the surrounding stroma as single cells

(Huber *et al.*, 2005). Acting in concert, these alterations are critical to completing the earlier steps of the metastatic cascade, which consists in local invasion, intravasation, survival through the circulation, and extravasation. Also Hedgehog, Notch and Wnt are capable of initiating EMT via up-regulation of a group of transcription factors (Takebe *et al*., 2011). The activation of EMT programs is mediated by intracellular signaling networks, such as signal transducing proteins ERK, MAPK, PI3K/Akt, Smads, RhoB, β-catenin, lymphoid enhancer binding factor (LEF), Ras, and c-Fos as well as cell surface proteins such as β4 integrins, α5β1 integrin, and αVβ6 integrin (Tse and Kalluri, 2007). PI3Kase/Akt pathway, for example, issues some properties of mesenchymal cells, such as resistance to apoptosis and proliferation effects (Cantrell, 2001). Studies show that EMT is controlled by a number of transcriptional factors, and many of them are transcriptional repressor as Zeb-1/2, Twist1, Snail, and Slug (Peinado *et al*., 2007) which directly repress mediators of epithelial adhesion (E-Cadherin) or the expression of claudins, necessary for the assembly of tight junctions between adjacent cells (Martinez-Estrada *et al*., 2006). Moreover activation of the NF-κB pathway is required for induction and maintenance of Ras- and TGF-β–dependent EMT (Huber *et al*., 2004). NF-kB binds to the promoter of the E-cadherin repressor ZEB-1/2 resulting in regulation of the EMT phenotype (Chua *et al.*, 2007). Many of the EMT-responsive genes activated by these transcription factors encode proteins involved in induction of EMT, and so create feedback loops that may help sustain the mesenchymal phenotype (Radisky, 2005). In other cases, activation of EMT involves more pleiotropic signals, as in the case of reactive oxygen species (ROS) produced in response to exposure to matrix metalloproteinases (MMPs) (Radisky *et al*., 2005). ROS can influence a number of signaling pathways (Finkel, 2003; Hussain *et al*., 2003), and can also directly induce EMT (Mori *et al*., 2004). HIF-1α, a potent transcriptional regulator of oxygen-dependent genes, is also an effective inducer of EMT in cancer cells, either indirectly by induction of TGF-β, or directly by activating Snail, Twist and members of Zeb family (Evans *et al.*, 2007). Of fundamental importance biologically, the activation of EMT programs has been associated with the acquisition of embryonic stem-cell like properties by normal and neoplastic cells (Mani *et al*., 2008). Zeb1 has been shown to negatively regulate expression of the microRNAs that function to suppress stemness, specifically members of the miR-200 family, able to enhance expression of Bmi-1 (Burk *et al.,* 2008). Many mouse studies and cell culture experiments have

demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers such as α-SMA, FSP1, vimentin, and desmin (Yang and Weinberg, 2008).

An apparent paradox comes from the observation that the EMT-derived migratory cancer cells establish secondary colonies at distant sites that histopathologically resemble the primary tumor from which they derive; they no longer show the mesenchymal phenotypes attributed to metastasizing carcinoma cells. Having migrated to their target destinations, the invasive cells must revert the mesenchymal phenotype to their original epithelial phenotype, able to proliferate, through a process known as mesenchymal-epithelial transition (MET) to ensure the secondary tumor formation (Yang and Weinberg, 2008). This is supported by the fact that EMT inducing transcription factors can directly inhibit proliferation (Thiery *et al.,* 2009). The tendency of disseminated cancer cells to undergo MET likely reflects the local microenvironments that they encounter after extravasation into the parenchyma of a distant organ, quite possibly the absence of the heterotypic signals they experienced in the primary tumor that were responsible for inducing the EMT in the first place (Bissel *et al.,* 2002). Tumor cells that undergo MET lead to distal re-epithelialization metastases and re-expression of the E-cadherin/catenin complex is the only accepted criterion for defining MET (Ding *et al*., 2013). These considerations indicate that EMT is probably a transient and reversible event during tumor progression; it is a centrally important mechanism for the progression of carcinomas to a metastatic stage and implicates MET during the subsequent colonization process (Kalluri and Weinberg, 2009). Although many clinical reports fostered the concept of transient EMT-MET switches in metastasis, there are only a few experimental proofs (e.g., Chaffer *et al*., 2006; Korpal *et al.,* 2011). Particularly, two papers supported the role of an EMT in dissemination and the need of a MET for efficient metastasis (Tsai *et al*., 2012; Ocaña *et al.,* 2012). As argued by Brabletz (2012), these concepts have clinical impact for future therapeutic strategies against metastasis: targeting EMT alone might be counterproductive because it actives proliferation of disseminated cells; it should be combined with therapy against cycling cells (standard chemotherapy) and inhibiting MET, thereby maintaining dormancy or directly targeting the stem cell phenotype. Since there are few direct experimental data supporting MET in cancer metastasis, Tsuji *et al.* (2008) proposed a new model to understand the role of EMT during metastatic process. He hypothesized that EMT is necessary but not sufficient for metastasis. Indeed, to complete the entire complex

metastasis process, the EMT cells would need an incredible plasticity. On the other hand, cancers consist of a highly heterogeneous population of cells with a remarkable range of phenotypes. When subcutaneously injected, both EMT and non-EMT cells established primary tumors, but only cells with an EMT phenotype invaded into the adjacent connective tissues and blood vessels; neither cells formed lung metastatic nodules. Moreover, subcutaneous inoculation of a mixture of the two cell types resulted in intravasation of both cell types and formation of lung metastasis from non-EMT cells. Tsuji and colleagues showed that EMT and non-EMT cells cooperate to complete the spontaneous metastasis process: they hypothesized that EMT cells, with enhanced migratory and invasive phenotype are responsible for degrading the surrounding matrix to invade and intravasate; non-EMT cells then migrate together with EMT cells, enter the blood stream, survive in circulation and reestablish colonies in the secondary sites (Tsuji *et al.,* 2008 and 2009).

1.5 Therapeutic implications

Since acidity of tumor environment appears to contribute to cancer aggressiveness, chemo- and radiation-resistance and evasion of immune reactions, measures to interferate with H+ dynamics (both pHi and pHe) coupled with metabolic disruption could provide a new strategy for anticancer therapeutics. The strategies proposed consist of: the increase of the pH of the extracellular space; proton pump inhibition that decreases the intracellular pH; acute intracellular acidification that kills cancer cells directly or potentiates their sensitivity to adjuvant measures; acute extracellular acidification that enables tumor selective release of cytotoxic drugs encased in pH-sensitive nanoparticles (McCarty and Whitaker, 2010).

Previous studies have shown that buffering tumor acidity through systemic administration of an alkaline agent raises the pHe of tumors. The principal physiologic buffer used to control pH is bicarbonate; Robey *et al.,* (2009) have shown that chronic use of oral bicarbonate as a cancer intervention selectively increased the pH of tumors and reduced the formation of spontaneous metastases in mouse models of metastatic breast cancer and the rate of lymph node involvement. Computer simulation used to verify the ability of

sodium bicarbonate to increase pHe of tumors in vivo also indicates that the normalization of tumor acidity reduces invasiveness of tumor cells without altering the pH of blood or normal tissues (Silva *et al*., 2009). Other studies tested the efficacy of a non-bicarbonate/non volatile buffer as 2-imidazole-1-yl-3-ethoxycarbonylpropionic acid (IEPA) (Ibrahim Hashim *et al*., 2011) that can reduce spontaneous and experimental metastases as well as bicarbonate. As an alternative, several researchers have explored inhibition of the membrane ion pumps involved in the maintainance of an alkaline pHi by extruding protons or importing bicarbonate ions. Proton pump inhibition tents to decrease intracellular pH and the consequent intracellular acidification can have an impact on cancer cell behavior, for example suppressing the efficiency of glycolysis, sustaining the raise of pHe (Fais *et al*., 2007), and exerting anti-proliferative and pro-apoptotic effects on tumor cells themselves (De Milito *et al.,* 2007; Yeo *et al*., 2008; Supino *et al*., 2009). NHE is crucial in pH regulation and is expressed in every cell type. Of the nine members of the NHE family, NHE1 has received the most attention in the context of tumor cell-targeted therapy (Parks *et al*., 2013). Both the acidic pH_e and the constitutively active NHE1 play a key role in driving protease-mediated digestion and remodelling of the ECM and the turning on of invasive phenotypes of the cell, scavenging normal tissue and increasing motility through the formation of invasive structures such as leading-edge pseudopodia and invadopodia (Lauritzen *et al.,* 2012). Most recently it has also been advanced that deregulation of NHE1 activity is a major factor leading to metastasis in human breast cancer (Amith *et al*., 2013). Amiloride was the first NHE inhibitor developed and it was shown to decrease vascular endothelial growth factor (VEGF) production and the activity of urokinase-type plasminogen activator (μPA), metalloproteinases (MMP) and other proteases, all of which aid in the activation of the metastatic process (He *et al.,* 2007, Provost *et al.,* 2012). There are several molecules derived from amiloride investigated as NHE inhibitors. Double substitution of the nitrogen of the 5-amino group gave DMA, EIPA, MIBA and HMA, weak selective inhibitors of NHE1 that are much more effective than amiloride (Masereel *et al*., 2003). Treatment with EIPA was recently demonstrated to compromise the growth of KRAS-transformed tumour xenografts, an effect that was attributed to inhibition of nutrient uptake by macropinocytosis (Commisso *et al*., 2013). Cariporide, another NHE1 inhibitor, was found able to lower the intracellular pH of cancer cells (Wong *et al.,* 2002), to affect tumor angiogenesis so as to inhibit tumor growth (Gao *et al.,* 2011, Harguindey *et al*., 2013). Proton extrusion is also achieved by the V-ATPase

that plays various functions in tumors, including proliferation, tumorigenesis, drug resistance and tumor progression (Sennoune *et al.*, 2004). Several studies have shown that proton pump inhibitors (PPIs) such as esomeprazole, omeprazole and pantoprazole, activated by acidic pHe of tumors and highly effective at inhibiting V-ATPases, have an antineoplastic activity towards human hematopoietic and solid tumors (De Milito *et al*., 2009). Furthermore, PPIs revert chemoresistance and induce tumor cell killing (Luciani *et al*., 2004; De Milito *et al*., 2007). On the other hand, proton pump inhibitors are normally adopted in the treatment of gastritis, Zollinger-Ellison syndrome and, limitedly to veterinary oncology, gastric hyperacidity secondary to mast cell tumors in dogs and cats (Spugnini *et al*., 2010). PPIs induce a clear inhibition of B cell tumor growth that passes through an early massive reactive oxygen species (ROS) activation and lysosomal membranes perturbation (De Milito *et al.*, 2007). PPIs cause also alkalinization of acidic vesicles and acidification of the cytosol. In vivo experiments confirmed that PPIs induce tumour growth inhibition, consistent with changes in pH gradients, thus abolishing the typical tumour-dependent reversal of pH gradients (Harguindey *et al.*, 2009). These evidences render PPIs suitable to target critical mechanisms involved in tumor progression. Many biological and pharmacological data point to the possible use of the inhibition of tumor associated CA IX, with selective sulfonamide inhibitors, in the management of hypoxic tumors, which do not respond to the classical chemo- and radiotherapy. Sulphonamide inhibitors were found effective to block the growth of primary tumor and metastases in a mouse model of breast cancer (Chiche *et al.*, 2009) and reduction in CAIX expression has been shown to improve the effect of radiotherapy both in vitro and in vivo in xenograft experiments (Dubois *et al.*, 2011). MCTs are overexpressed in many tumors and contribute to the maintenance of cellular alkalinity through the active export of lactic acid. MCT inhibitors have been shown to decrease pHi in neuroblastoma (Fang *et al.*, 2006) and melanoma cells (Wahl *et al.*, 2002), resulting in cell death (Sonveaux *et al.*, 2008). By inhibiting MCT1, lactate is no available to the aerobic cells, so it induces a switch from lactate-fuelled respiration to glycolisis, accompanied by a retardation of tumor growth in a mouse model of lung carcinoma and in transplanted human colorectal carcinoma (Sonveaux *et al.*, 2008). Several MCT inhibitors have been described but none of them is specific, as cyanocinnamate derivatives (e.g. α-cyano-4-hydroxycinnamate or CHC), bioflavonoids (e.g. phloretin and quercetin), anion transport inhibitors (e.g. niflumic acid and 5-nitro-2-(3-phenylpropylamino) benzoate or

NPPB), or stilbenedilsulfonates (e.g. 4,4′-dibenzamidostilbene-2,2′-disulfonate or DIDS). A recent MCT1-specific inhibitor, AR-C117977, has been found to have immunosuppressive properties that prolong skin graft and heart allograft survival in mice (Bueno *et al.,* 2007). The organomercurial reagent p-chloromercuribenzene sulfonate (pCMBS) inhibits both MCT1 and MCT4 and its activity is probably mediated through its binding to CD147 (Basigin), required for the translocation of monocarboxylate transporters to the plasma membrane (Wilson *et al.,* 2005; Kennedy *et al*., 2010). In the context of therapies against tumor acidity, a novel class of non-invasive pH-selective PET imaging agents, able to target acidic tumors, opens new directions to enhance the effectiveness of diagnosis and therapy (Vāvere *et al*., 2009)**.** pHLIP (pH Low Insertion Peptide), a water-soluble membrane peptide that inserts across cell membranes as an α - helix when the extracellular pH is acidic, was used to detect tissue acidity and to diagnose primary tumors, metastatic lesions and lipid bodies in necrotic tissues (Reshetnyak *et al*., 2011; Macholl *et al.*, 2012).

Another class of anticancer agent for new therapeutic strategies could be the inhibitors of glycolytic pathway, which is important for ATP generation, essential for cancer cells to survive and growth. Metabolic adaptation renders cancer cells highly addictive to and dependent on the glycolytic pathway, so inhibition of glycolysis is expected to have a severe impact on these cells. The glycolytic inhibitors known include: the 2-deoxyglucose, an analogue of glucose, able to suppress hexokinase II (Geschwind *et al.*, 2004) and cause a depletion of cellular ATP, leading to blockage of cell cycle progression and cell death *in vitro,* and exhibit antitumor activity *in vivo* (Maher *et al*., 2004; Maschek *et al*., 2004); arsenate compounds that abolish ATP generation by causing arsenolysis in the glyceraldehyde-3-phosphate dehydrogenase reaction; 3-bromopyruvate (3-BrPA), another potent inhibitor of hexokinase II (Ko *et al.,* 2001), effective in killing liver cancer cells in in animal models when given by local infusion (Geschwind *et al.*, 2002). The ability of 3-BrPA to preferentially kill cancer cells with mitochondrial defects and that live in a hypoxic environment provides a biochemical basis to further develop this class of compounds as novel anticancer agents with potentially promising therapeutic activity and selectivity (Xu *et al*., 2005).

Metformin (N′,N′-dimethylbiguanide), one of most widely prescribed oral hypoglycemic agents used in type 2 diabetes and also in polycystic ovarian syndrome (PCOS), where insulin resistance is a key factor for the development of the metabolic disturbances, has

recently received increased attention because it may reduce cancer risk (Evans *et al.*, 2005; Lee *et al*., 2011; Zhu *et al.*, 2011) or improve cancer prognosis, independently of its hypoglycaemic effects (Kourelis and Siegel, 2011). Metformin acts through inhibition of mitochondrial respiration by inhibiting complex I of the electron transport chain and hence is an example of mitochondrial metabolic inhibitor blocking oxidative respiration (El-Mir *et al.*, 2000). This results in increased cellular AMP-to-ATP ratios and activates the AMP-activated protein kinase (AMPK) which increases GLUT1 expression to promote glucose uptake (Fryer *et al.*, 2002) and phosphorylates phosphofructokinase-2 to promote energy generation through increased glycolysis (Marsin *et al*., 2000). Metformin is also a potent blocker of cell proliferation by inhibiting the mTORC1 complex (Zakikani *et al*., 2006; Cantrell *et al.*, 2010) and inductor of cell apoptosis through activating JNK/p38 MAPK pathway (Xiong *et al*., 2012). Moreover, metformin and phenformin (both mitochondrial complex 1 disruptors) sensitize tumour cells to MCT inhibition or knockout, indicating the possibility that synthetic lethality could be achieved through combination therapy (Le Floch *et al*., 2012). Combined treatment with metformin and chemotherapeutics has been studied in in vivo models of breast, prostate and lung cancer. One example is the combination of metformin and doxorubicin, that kills both cancer stem cells and non–stem cancer cells in vitro and in vivo (Hirsch *et al.*, 2009), or the combination of metformin and 2-deoxyglucose that has a much stronger deleterious effect than either drug (Sahra *et al.*, 2010).

2. AIM

Tumor micro-environment is often characterized by an acidic pH due to abnormal vascularisation, reduced lymphatic network and uncontrolled cell growth frequently associated with hypoxia, and extracellular accumulation of glucose metabolites even in the presence of an adequate oxygen level ("the Warburg effect"). Evidence is accumulating that acidity participates to tumor progression, stimulating tumor cells aggressiveness and facilitating their invasiveness into host tissues. Acidic micro-environment is associated with poor prognosis, inducing resistance to chemotherapy, suppression of cytotoxic lymphocytes and tumoricidal activity of natural killer cells.

The aim of this thesis is to determine whether extracellular acidity induces different aspects of malignancy in tumor cells, either directly or through tumor cell/host cell interactions. For this study we used human melanoma cell lines. Melanoma represents one of the most aggressive tumor of human being, able to survive at very low pH (lower than 5), suggesting that at least melanomas are acidic and armed to tolerate extreme acidic conditions.

In the complex scenario of genetic, epigenetic and environmental cues that deal with malignancy, the proposal of the study are:

- To determine whether acidity induces in tumor cells an EMT program and its reversion*;* EMT is a complex series of events in which cell-cell and cell-extracellular matrix interactions are altered to release epithelial cells from tissues and confer them the ability to move through extracellular matrix and disseminate.
- To study if acidity induces VEGF-C expression in melanoma cells and to investigate the transcription factor involved; VEGF-C expression by tumor cells correlates with early metastatic dissemination to regional lymph nodes.
- To determine if phenotypic changes of acidic cells are associated with a change in their metabolic profile, and drugs targeting the metabolic programme acquired by acidic melanoma cells (metformin and CHC) may abrogate malignancy of acidic cells.
- To study the effects of acidity on melanoma cell/host cell interactions, in particular if acidity is able to activate mesenchymal stem cells (MSC) and if acidic MSC are

able to elicit a more aggressive phenotype in melanoma cells. MSC represent a population of stromal cells recruited in tumor environment, able to express several pro-tumoral activities.

- To verify if proton pump inhibitors (esomeprazole) activated by acidity, repress aggressiveness of acidic melanoma cells and the pro-tumoral effects of acidic MSC.

3. MATERIALS AND METHODS

Cell lines and culture conditions

In this study we used different human melanoma cell lines: a) A375P human melanoma cell line obtained from American Type Culture Collection (ATCC, Rockville, MD); b) A375M6 isolated in our laboratory from lung metastasis of SCID bg/bg mice i.v. injected with A375P cells human melanoma cell lines; c) the primary cell culture line SSM2c obtained from the Laboratory of Tumor Cell Biology, Core Research Laboratory-Istituto Toscano Tumori (CRL-ITT, Florence, Italy) (Santini *et al.,* 2012). We also used human mesenchymal stem cells (MSC) isolated from bone marrow aspirates of donors by Prof. Saccardi's group (AOUC, Florence). Human melanoma cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM 4500 mg/l glucose, GIBCO) supplemented with 10% foetal calf serum (FCS, Boerhinger Mannheim, Germany) and 1% glutamine; MSC were cultivated in Dulbecco's Modified Eagle Medium (DMEM 1000 mg/l glucose) supplemented with 20% foetal calf serum and 1% glutamine. All cells were mantained at 37°C in a 10% CO_2 humidified atmosphere. Cells were harvested from subconfluent cultures by incubation with a trypsin-EDTA solution (GIBCO), and propagated every three days. Cells were counted using Bürker chamber. Viability of the cells was determined by trypan blue exclusion test.

In some experiments A375M6 cells were treated with TGFβ 10 ng/ml, IL1β 1000 U/ml (from PeproTech), parthenolide 1,5-5 μM (from Calbiochem), esomeprazole 100 μM (donated from Dr. Stefano Fais, National Institute of Health, Rome), H_2O_2 200 μM (Merck), lactate 10 mM (from Sigma-Aldrich), CHC (alpha-cyano-4-hydirxycinnamate) 1-10 mM and metformin 1-10 mM (from Sigma-Aldrich).

Detection of mycoplasma contamination

Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test (Chen, 1977). Cells were grown on a coverslip (Bio-Optica), fixed with 4% formaldehyde, air-dried, stained with DNA-specific fluorescent Hoechst 33258, and examined microscopically. All cultures that were infected with mycoplasmas had readily

discernible, small, morphologically uniform, bright fluorescent bodies in the extranuclear and intercellular space in contrast to the non-contaminated control cultures in which the extra-nuclear background appeared uniformly dark.

Cell cryopreservation

Cells recovered from subconfluent cultures were washed in PBS, centrifuged and resuspended at concentration of 1-2x10^6 cells/ml, in colture medium containing:

- For human melanoma cell lines 45% FCS and 10% dimethyl sulfoxide (DMSO, Sigma Aldrich);
- For MSC 90% FCS and 10% DMSO.

Cell suspensions were distributed in sterile vials (Sarstedt), frozen at -80° and subsequently transferred in containers for cryopreservation by exposure to liquid nitrogen (-150°).

Hypoxic condition

Cells were changed to DMEM4500 containing 1% FCS and incubated for 2h in normal oxygen tension before exposure to hypoxia. For hypoxic conditions (0.3% O_2), cells were incubated for 24h in a humidified hypoxic workstation (Ruskin Concept 400 hypoxic chamber, UK), and in some experiments, cells were also re-exposed to normoxic conditions for 24h.

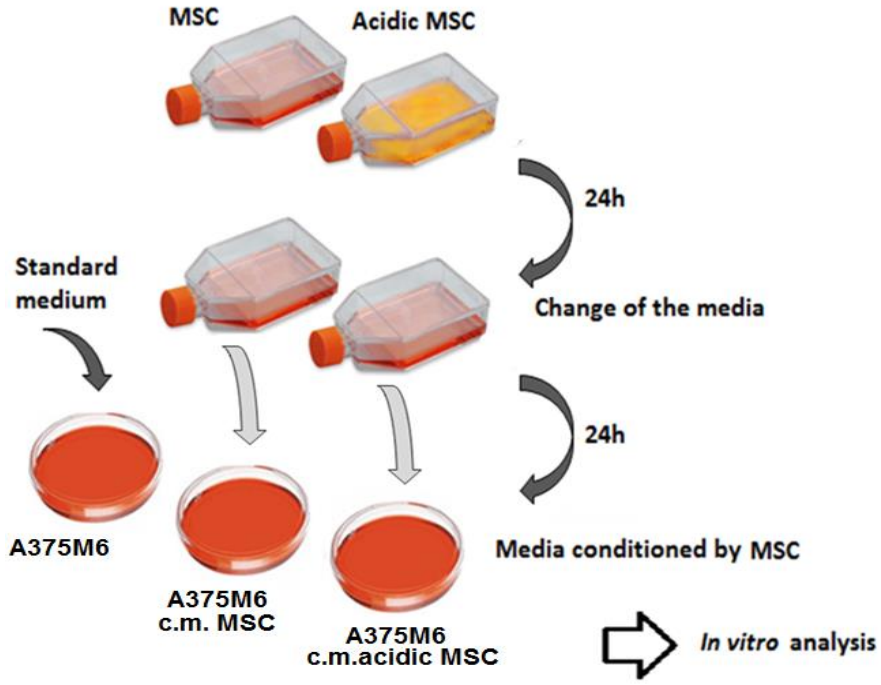
Acidic treatment

Low pH medium was obtained by addition of HCl 1N in DMEM 4500 (for melanoma cells) or 1000 (for MSC) containing respectively 10% or 20% FCS and pH value was monitored by a pHmeter (Orion PH Meter 520A-1). As pH value was stable (6.6-6.7), acidified medium was added to cultures and the seal caps of flasks were tightly closed. pH of acidified media were verified 2 hours later and at the end of the experiment. Cultures were exposed to the acidified medium for 4-24 hours. In some experiments esomeprazole or metformin were added to standard or acidified media. Treatments with parthenolide and

IL1-β were performed 2 hours before media acidification, whereas lactate and/or CHC were added to cells grown in acidic medium after changing acidic medium with standard medium.

Treatment with media conditioned by MSC

Mesenchymal stem cells were grown to sub-confluence in standard or acidic medium (DMEM 1000) and after 24 hours the medium was replaced by standard medium (DMEM 4500). After 24 hours this conditioned medium was collected and added to melanoma cells colture for additional 24 hours before melanoma cells analysis.



Flow cytometric analysis of cell cycle distribution

Cell cycle distribution was analyzed by the DNA content using PI staining method. Cells, harvested at 24 hours after treatment, were washed 2 times in PBS by centrifugation and stained with a mixture of 100 µg/mL propidium iodide (PI), 20 µg/mL RNase A, 1 mg/mL trisodium citrate and 0.3% (v/v) Triton X-100 in the dark at room temperature for 30 min. The stained cells were analyzed by flow cytometry (BD-FACS Canto) using red propidium-DNA fluorescence.

RNA extraction

Total RNA was extracted and purified from cells collected in TRI Reagent ® RNA Isolation Reagent (Sigma). One ml of the reagent was sufficient to lyse 5-10x10^6 cells. Samples were allowed to stand for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes and 0,2 ml of chloroform per ml of TRI Reagent used were added. Samples were shaked vigorously for 15 seconds, allowed to stand for 15 minutes at room temperature and centrifuged at 12000xg for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and 0.5 ml of iso-propanole per ml of TRI reagent used were added. Samples were vortexed, incubated for at least 30 minutes at -20°C and centrifuged at 12000xg for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed by adding 1 ml of 75% ethanol per 1 ml of TRI Reagent used. Samples were vortexed and centrifuged at 7500xg for 5 minutes at 4°C; when the RNA pellet was dry an appropriate volume of water was added. The concentration of RNA was determined by measuring the absorbance at 260 nm (*A*260) in a spectrophotometer; the ratio between the absorbance values at 260 and 280 nm gave an estimate of RNA purity (pure RNA has an *A*260/*A*280 ratio of 1.8–2.0). Purified RNA was stored at –80°C.

Retrotranscription of RNA to cDNA

RNA was retrotranscribed using ImProm-II™ (Promega). A suspension containing 1μg RNA, 1 μl random primers (Promega, 500 μg/ml) and nuclease free-water to reach the final volume of 5 μl was prepared for each sample. The tubes were heated to 70°C for 5 minutes to melt secondary structure within the template and incubated at 4°C for 5 minutes to prevent secondary structure from reforming. A solution containing the following components was added to each sample: $MgCl_2$ (final concentration 3 mM), deoxyribonucleotides (dNTP, final concentration 0.5 mM), 20 U RNAsin (Recombinant RNAsin® Ribonuclease Transcriptase), 1μl reverse transcriptase (ImProm-IIReverse Transcriptase), 5x Reaction Buffer (ImProm-II Reaction Buffer) and nuclease-free water to a final volume of 15 μl. Following an initial annealing at 25° for 5 minutes, the reaction was incubated at 42° for 1h and at 70° for 15 minutes. At the end of the reaction , cDNA were stored at -20°C. The reaction was performed using a Perkin-Elmer Thermal cycler.

End point PCR (Polymerase Chain Reaction)

Aliquots of 2-3 μl of the cDNA mixture were used for PCR amplification. The end-point PCR reactions were carried out in 20 μl of a solution containing a reaction buffer (GoTaq reaction buffer 5x), deoxyribonucleotides (dNTP, final concentration 0.2 mM), specific primers (final concentration 0.1 μM), 1 U/sample of GoTaq Polymerase. The specific primers sequences used for the identification of human VEGF-C, VEGF-A, GAPDH, IL-1β, IL-6, TNFα, TGF-β, β2-m were: **VEGF-C** forward: 5'-CCCCACATCTATACACACCTCC- 3', reverse: 5'-TCCGGACTCGACCTCTCGGAC-3' (60°C, 34 cycles); **VEGF-A** forward 5'-TTCTGCTGTCTTGGGTGCAT-3', reverse 5'-TGTCCACCAGGGTCTCGATT-3' (60°C, 34 cycles); **GAPDH** forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3' (58°C, 27 cycles); **IL1-β** forward 5'-TGAACTGCACGCTCCGGGACTC-3', reverse 5'-TTCTGCTTGAGAGGTGCTGATG-3' (58 °C, 34 cycles); **TGF-β** forward 5'-ATGACTCATTCAGTCACCATAGC-3', reverse 5'-CTATCCCCCACTAAAGCAGG-3' (58 °C, 34 cycles); **IL-6** forward 5'-AGTTCCTGCAGTCCAGCCTGAG-3', reverse 5'-TCAAACTGCATAGCCACTTTCC-3' (58 °C, 34 cycles); **TNF-α** forward 5'-ACCAGGGAGCCTTTGGTTCTGG-3', reverse 5'-AAGGCAGCTCCTACATTGGGTC-3' (58 °C, 34 cycles); **β2-m** forward 5'-TGCTATCCAGAAAACCCCTC-3', reverse 5'-GGACGTCTCAATTCGTACTG-3' (55 °C, 28 cycles).

Aliquots of 6-10 μl of each PCR mixture were applied to a 2% agarose gel (containing 0.5 μg/ ml of ethidium bromide), electrophoresed and visualized. cDNA products were evaluated on the basis of standard PCR markers (Promega). A digital camera (Kodak) was used to visualise the PCR products and aquire the images. The bands of genes of interest were compared with the bands of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) or β2-m (β2 microglobulin). In some experiments the intensities of the electrophoretic bands (OD) were determined with a specific program (Scion® Image) and the following calculations allowed to evaluate the changes of the RNA contents:

Final OD sample = (OD sample/ OD GAPDH) x100

Normalization compared to the control =

(% final OD sample / % final OD control) x100

Real-Time quantitative RT-PCR (qPCR)

Quantitative real time PCR (qPCR) was performed using the GoTaq® Probe Systems (Promega) and was carried out in triplicate using an Applied Biosystems 7500 Sequence Detector. The primers were designed according to published human cDNA sequences in Genbank database, using the FastPCR software: **VEGF-C** forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'; **VEGFR2** forward 5'-CCAGTCAGAGACCCACGTTT-3' and reverse 5'-AGTCTTTGCCA TCCTGCTGA-3'; **VEGFR3** forward 5'-TGGTACCGGCTC AACCTCTC-3' and reverse 5'-CACGTTTTTGCAGTCCAGCA-3'; **TGF-β** forward 5'-GACTACTACGCCAAGGAGGTC-3' and reverse 5'-TGCTGTGTGTACTCTGCTTGA-3'; **TGFβR1** forward 5'-TCC TTCAAACGTG CTGACATC-3' and reverse 5'-TGGAACATCGTCGAGCAATTT-3'; **TGFβR2** forward 5'-CAACCACCAGG GCATCCA-3' and reverse 5'-TCGAGGTC CAGCACTCA-3'; **TGFβR3** forward 5'-TTGCTGCGGGTGGATCTC-3' and reverse 5' TTAAAGTCCCTCCTTGCAAT TT-3'; **GLUT-1** forward 5'-CGGGCCAAGAGTGTG CTAAA-3' and reverse 5'-TGACGATACCGGAGCCAATG-3'; **GLUT-3** forward 5'-CGAACTTCCTAGTCG GATTG-3'and reverse 5'-AGGAGGCACGACTTAGACAT-3'; **N-Cadherin** forward 5'-CACTGCTCAGGACCCAGAT-3' and reverse 5'-TAAGCCGAGTGATGGTCC-3'; **E-Cadherin** forward 5'-CGGGAATGCAGTTGAG GATC-3' and reverse 5'-AGGATGGTGTAAGCGATGGC-3'; **Snail** forward 5'-CCCAGTGCCTCGA CCACTAT-3' and reverse 5'-CCAGATGAGCATTGGCAGC-3' and **18S** forward 5'-CGCCGCTAGAGGTGAAATTCT-3' and reverse 5'-CGAACCTCCGA CTTTCGTTCT-3'. mRNA was quantified with the ΔΔCt method as described (Livak and Schmittgen, 2001). mRNA levels were normalized to 18S as an endogenous control.

siRNA transfection

For tumor cell transfections, cells were allowed to reach 80% confluence. The siRNA for NF-κB or HIF-1α was diluted in Opti-Mem to a final concentration of 20 nM and it was incubated with Lipofectamine 2000 (Invitrogen) for 20 minutes at room temperature to allow complex formation to occur. DNA-Lipofectamine complex was then added to the

cells and after tranfection cells were incubated in standard medium for 24 hours before they were exposed to low pH.

Western blotting analysis

Cells, washed twice with ice cold PBS containing 1 mM Na_3VO_4, were lysed in 100 µl of RIPA buffer containing: 20mM Tris–HCl (pH 7.4), 150 mM NaCl, 100 mM HEPES, 1% Triton X-100, 10 mM glycerophosphate, 100 µM PMSF, 100 mM AEBSF, 5 mM bestatin, 2 mM leupeptin, 1 mM pepstatin A and 80 µM of aprotinin. Cells were centrifuged at 14000 rpm for 20 minutes and the supernatants were collected and stored at -20°C. Protein quantification was performed spectrophotometrically using Quick Start Bradford (BioRad).

Samples were prepared to have: 65% of protein extract, 25% Laemmli Buffer 4x, 10% Beta-mecaptoethanol 1M. Supernatants containing equal amounts of protein (45 µg) were denaturated at 95°C for 5 minutes and separated on 10% (v/v) SDS–PAGE gel for 1 hour at 180V. Fractionated proteins were transferred from the gel to a PVDF nitrocellulose membrane for 1 hour at 100V using BioRad system. Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins and then incubated for 1 hour at room temperature in a blocking solution consisting in Odyssey Blocking Buffer (Licor Bioscience)/ PBS (1:1). Subsequently, the membrane was probed with the appropriate antibody diluted 1:1000 with Odyssey Blocking Buffer /T-PBS buffer (PBS-Tween 0,1% solution) at 4°C overnight. After washing in T-PBS buffer, the membrane was incubated with an anti-rabbit (1:10000), anti-mouse (1:8000) secondary fluorescent antibody (Invitrogen), diluted with Odyssey Blocking Buffer /T-PBS buffer (1:1), for 1 hour at room temperature. After consecutive washing with T-PBS the immunoreactive bands were visualized using the Odyssey Infrared Imaging System (Lycor®Bioscience). Antibodies used for Western Blot analysis were: rabbit anti-human VEGF-C (ab9546) was from abcam; rabbit anti-NFκB (sc-372), rabbit anti-IkB (sc-371), rabbit anti-human Twist (sc-15393), rabbit anti-MCT1 (sc-50324**)** and rabbit anti-MCT4 **(**sc-50329) from Santa Cruz Biotechnology (Heidelberg, Germany); rabbit anti-PARP (#9542) from Cell Signaling (Danvers, MA, USA); rabbit anti-human N-Cadherin (orb11100) from Biorbyt (Cambridge, UK); rat anti-human E-Cadherin (GTX11512) from Gene Tex (Irvine, CA,

USA); mouse anti-human Vimentin was from Sigma (v6630); mouse anti-β-tubulin (05-661) was from Millipore (Billerica, MA, USA).

Immunofluorescence

Tumor cells were cultured on 25-mm coverslips, treated for 24 hours and washed 3 times in PBS. Cells were fixed for 20 minutes in 3,7% formaldehyde at room temperature, washed and then permeabilized with 0.1% Triton X-100 solution prepared in PBS. After three washes, permeabilized cells were incubated in blocking solution (PBS supplemented with 10% horse serum) to saturate nonspecific sites and then incubated overnight at 4°C with anti-NF-κB antibody (Santa Cruz Biotechnology) diluited 1:1000 in blocking solution. Cells were washed and then incubated for 1 hour using 1:100 goat anti-rabbit IgG-FITC. Cell nuclei were counterstained with DAPI (1 µg/mL for 30 minutes). Following two washes in PBS, coverslips were mounted with glycerol on glass slides and the cells were observed with an inverted confocal Nikon Eclipse TE2000 microscope equipped with a x60 S-Fluor oil immersion lens.

Zymography

Cells were cultured in medium containing 10% FBS for at least 2 days before being serum-deprived for 24 h. Cells were incubated in fresh serum-free medium and treated with acidic medium for 24h. The gelatinolytic activity released by tumor cells into their growth medium was tested by means of electrophoresis on a 8% SDS-PAGE gels co-polymerized with 0.1% (w/v) type A gelatin. After running, gels were washed for 30 min at room temperature in 2.5% (v/v) Triton X-100 to remove SDS, and then incubated overnight at 37°C in buffer containing 50 mM Tris–HCl, pH 7.4, 200 mM NaCl and 5 mM $CaCl_2$. In order to visualize the zone of lysis, the gels were stained for 60 minutes at room temperature with 0.1% Coomassie brilliant blue (Merck) in methano/water/acetic acid in a ratio 5:5:1, and destained for 2 hours in a destaining solution of methanol/acetic acid/water (3:1:6; v/v). The gels were visualized using the Odyssey Infrared Imaging System. The gelatinolytic activity was demonstrated as clear bands on a blue background.

Moreover, the gelatinolytic activities of HT1080 human fibrosarcoma cells were used as markers of molecular weight.

Invasion assay

Invasiveness of A375M6 melanoma cells grown for 24 hours in normal or low pH medium was determined *in vitro* by using BD Matrigel™ Basement Membrane Matrix and Millicell cell culture Insert (24-well PCF 8.0 μm, Millipore). The coated membranes were prepared by using Matrigel suspensions at 250 μg/ml. $1{,}5x10^5$ cells, resuspended in 200 μL of their own conditioned medium, were seeded in the upper compartment and incubated for 6 hours at 37°C in 10% CO_2 in air. The lower chamber contained complete culture medium, which include 10% FBS to trap invading cells.

After incubation, cells on the upper side of the filters were wiped off mechanically using cotton swabs and the membranes were fixed overnight in icecold methanol. Cells on the lower side of the membranes were stained with Diff Quick solutions and counted.

Motility assay

Motility of A375M6 melanoma cells grown for 24 h in normal or low pH medium was determined *in vitro* on polycarbonate filters, with 8 μm pore size, 6.5 mm diameter, mounted in Boyden's chambers. $1{,}5x10^5$ cells, resuspended in 200 μL of DMEM 2% FBS, were seeded in the upper compartment and incubated for 6 hours at 37°C in 10% CO_2 in air. 400 μl of DMEM supplemented with 10% FBS or of media conditioned by mesenchymal stem cells were added in the lower compartment as attractant. After incubation, cells on the upper side of the filters were wiped off mechanically using cotton swabs and cells on the lower side of the insert membrane were fixed overnight in icecold methanol. Cells were stained with Diff Quick solutions, washed and counted.

Wound Healing Assay

Cell migration was also evaluated by an in vitro wound healing assay. Cells were grown at 80-90% confluence in 35 mm dishes; the cell layer was wounded with a sterile 200 ml

pipette tip and incubated in 1% FBS colture medium for 24 hours. The wound was observed and photographed using phase contrast microscopy.

Lactate assay

Lactate was measured in the cultured media with Lactate Assay kit (Source Bioscience Life Sciences). Samples were prepared in 50 µl/well with Lactate Assay Buffer in a 96-well plate. 50 µl Reaction Mix containing 46 µl Lactate assay buffer, 2 µl Probe, 2 µl Enzyme Mix were added to each well and the reaction was incubated for 30 minutes at room temperature, protect from light. Lactate reacted with the enzyme mix to generate a product, which interacted with lactate probe to produce fluorescence.

Incorporation of lactate into proteins

Cells were grown in acidic medium for 24 hours and then [U-^{14}C] lactate was added for other 24 hours (2 µCi/ml, final concentration). Cells were then resuspended in 20% trichloroacetic acid, placed on ice for 30 minutes and centrifuged at 12,000 *x g* for 15 minutes at room temperature. The supernatant was removed and the pellet resuspended with 200 µl of water. The resuspended pellet was assayed for [14C] labelled proteins by scintillator.

Detection of released CO2 by radioactive lactate

Cells were grown in acidic medium for 24 hours and then 0.2 µCi/ml D-[U-^{14}C] lactate was added for 15 minutes in incubator at 37 °C, 5% CO_2. Each dish had a taped piece of Whatman paper facing the inside of the dish wetted with 100 µl of phenyl-ethylamine-methanol (1:1) to trap the CO_2. Then 200 µl of 4 M H_2SO_4 was added to cells. Finally, Whatman paper was removed, transferred to scintillation vials and radioactive CO_2 released was counted by scintillator for counting.

Soft-agar Colony Formation Assay

A375M6 cell suspensions (1000 cells sample^{-1}) were plated in a semisolid medium of 0.3% agar over a bottom layer of 0.9% agar (Agar Bacteriological, Acumedia). Dishes were incubated at 37°C in a humidified atmosphere containing 10% CO_2 and colonies were counted after 2–3 weeks.

Limiting dilution assay

Detached and washed A375M6 cells grown in acidic or standard medium were counted and diluted. 100 cells were taken and suspended in 10 ml of culture medium. Cells were plated by transferring 100 μl of cell dilution in each well of a of 96-well culture plate. The cells were monitored during two-three weeks, to count the obtained clones.

Lung colonization assay

Experiments were conducted in accordance with national guidelines and were approved by the ethics committee of the Animal Welfare Office of the ItalianWork Ministry and conformedto the legal mandates and Italian guidelines for the care and maintenance of laboratory animals. Subconfluent cultures of melanoma cells were harvested by EDTA/trypsin solution, centrifuged at 200 g, and then resuspended in DMEM medium at 5x 10^6 cells/ml. 0.2 ml of this suspension were injected into the lateral tail veins of eight-week-old female SCID-bg/bg mice (Charles River Laboratories). Mice (five animals per group) were monitored every 3 days and sacrificed after 40 days. Lungs were inspected for metastatic nodules and fixed overnight at 4 °C in formalin (5% in PBS) for histological analyses.

In vivo protumoral activity of acidic MSC

100 μl of PBS containing 1 x 10^6 A375M6 melanoma cells alone or in the presence of 0.5 x 10^6 acidic or non-acidic mesenchymal stem cells (MSC) were injected subcutaneously in the shoulder of eighth-week-old female SCID bg/bg mice (Charles River Laboratories). In some experiments, esomeprazole sodium salts was resuspended in PBS and injected into

the lateral tail veins at the 12, 14 and 16th day after tumor cell injection, at a dose of 25 mg/Kg. The animals were monitored daily and tumour size was measured every 2–3 days by a caliper. Tumour volumes were determined by the formula: (L x W ^{2})/2; where L and W are the length and width.

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue blocks were cut into 5μm consecutive sections and mounted on positively charged slides. Tissue sections were deparaffinazed and rehydrated before staining with H&E or HRP stain. Some sections were used for antigen retrieval, performed for 20 minutes with citrate buffer (pH 6.0), before incubation in a solution containing 0.1% Triton X-100. These sections were then incubated overnight at 4°C with the anti-GFP primary antibody and then incubated with biotinylated secondary antibodies streptavidin-horseradish-peroxidase-conjugated-HRP. Slides were then counterstained with hematoxylin. Positive and negative controls were used throughout all immunostaining protocols.

Statistical Analysis

Densitometric data are expressed as means ± standard errors of the mean (SEM) depicted by vertical bars from representative experiment of at least three independent experiments. Statistical analysis of the data was performed by Student's t-test, and $p \leq 0.05$ was considered statistically significant.

4. RESULTS

4.1. Profile of acidic human melanoma cells

4.1.1. Acidic melanoma cells and Epithelial-to-Mesenchymal Transition

EMT-like features of human melanoma cells grown in an acidified medium.

The chaotic and incomplete vasculature of tumor is frequently responsible for a transient or persistent oxygen deficiency and tumor cells respond converting to an anaerobic respiration, driving the environment of most solid tumor to be acidic. The acidic microenvironment contributes to tumor cells aggressiveness by increasing their mutation rate (Morita *et al*., 1992), invasiveness into host tissues and secondary organ colonization (Rofstad *et al.*, 2006; Moellering *et al*., 2008). *In vitro* and *in vivo* studies revealed that in several tumors, including melanoma, extracellular pH is 6.2-6.9 (pHe of normal extracellular space: 7.3-7.4) and that acidic environment of tumors is associated with a poorer prognosis. To analyze the influence of a reduced pH on human melanoma phenotype, we exposed A375P cells for 24 hours to an acidified medium (pH 6.5). Cultures were cultivated in a normoxic condition (21% O_2) and standard glucose content in order to evaluate the net effect of pH on tumor phenotype. First, we examined cellular morphology and we observed that cells grown in acidic medium lose their round cobblestone-like appearance, that characterizes epithelial cells, and adopt an elongated spindle-shaped morphology, similar to mesenchymal cells (Fig.1).

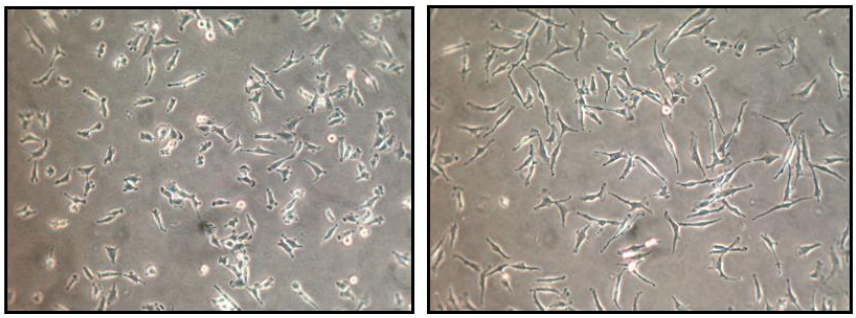


Figure 1- Cell morphology of A375P human melanoma cells grown in standard medium (pH 7.4) or acidic medium (pH 6.5) for 24 hours.

EMT, the epithelial-mesenchymal transition, is an essential developmental process by which cells of epithelial origin lose epithelial characteristics and polarity, and acquire a mesenchymal phenotype and markers such as N-cadherin, vimentin, Twist and Snail (Thiery *et al.*, 2006). It has been proposed that EMT-like processes might occur during tumor progression in carcinomas, particularly at specific stages (i.e., invasion and intravasation) where tumor cells disassemble and migrate to tissue/organ sites distant from the primary tumors. To test the effect of low pH on EMT induction in A375P melanoma cells, we analyzed by western blot analysis the expression of some EMT markers after 6 (Fig.2, panel A) or 24 (Fig.2, panel B) hours of acidic treatment. It is possible to observe the increase of mesenchymal markers as the mesenchymal cell–cell adhesion protein N-cadherin, the intermediate filament protein Vimentin, the transcription factors Twist and Snail already after 6 hours of acidic treatment, while the reduction of the epithelial cell–cell adhesion protein E-cadherin was observable only after 24 hours.

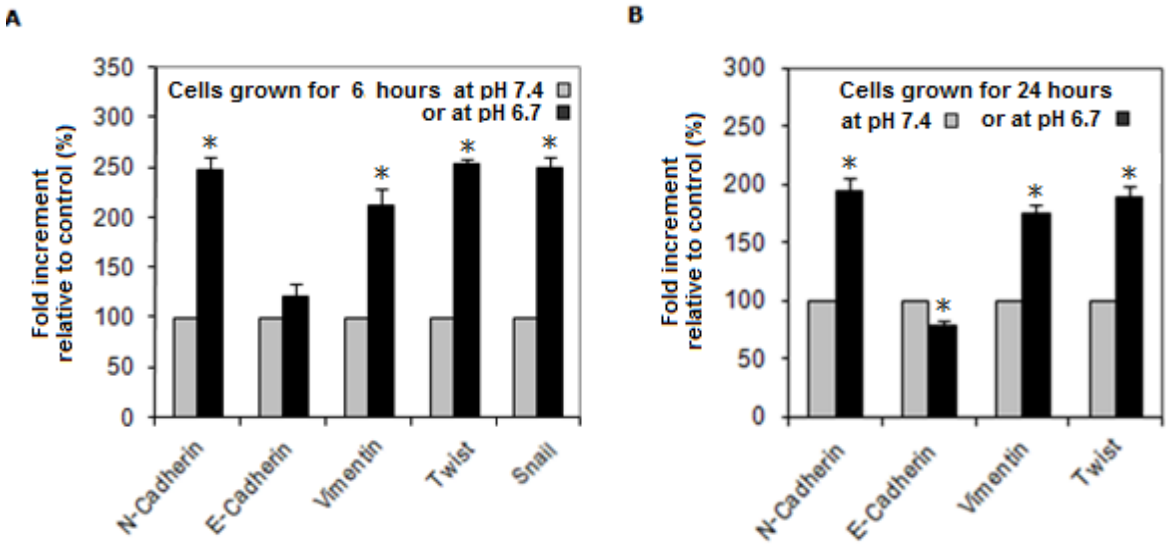


Figure 2- EMT markers' expression in acidic A375P melanoma cells. **A)** N-cadherin, E-Cadherin, Vimentin, Twist and Snail proteins detected by Western blot analysis (mean ± SEM), in melanoma cells grown in standard pH medium (7.4) or acidic medium (6.7) for 6 hours. **B)** N-cadherin, E-Cadherin, Vimentin and Twist proteins detected by Western blot analysis in melanoma cells grown in standard pH medium or acidic medium for 24 hours. Data are expressed as percentage of values compared to the control; densitometry data presented are normalized to intensity of β-tubulin bands. *$P < 0.05$ compared with control cells.

After these preliminary results using A375P cells, suggesting that acidity can induce cell modifications aligned with EMT program, we analyzed the influence of a reduced pH on A375M6 cell line, isolated in our laboratory from lung metastasis of SCID bg/bg mice i.v. injected with A375P human melanoma cells. We found that acidity did not modify viability of tumor cells, but it affects their grown (Fig.3) probably reducing their proliferative capacity.

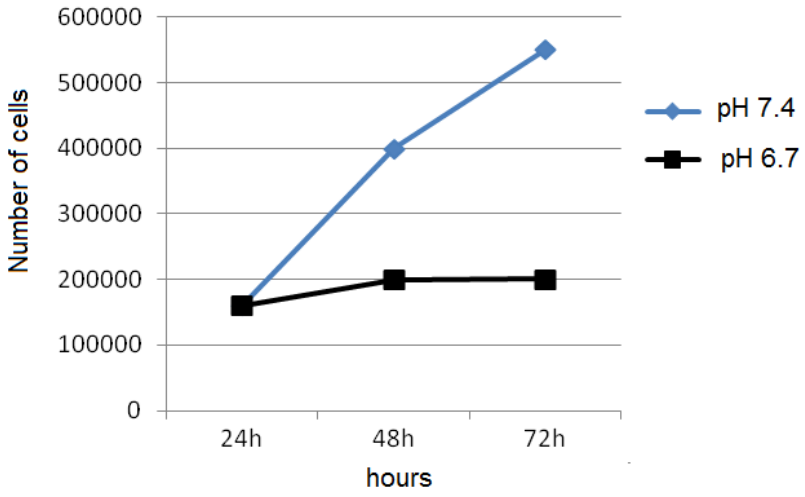


Figure 3- Growth curve of A375M6 melanoma cells in standard (pH 7.4) or acidic (pH 6.7) medium.

Low extracellular pH also promotes in these cells a spindle and dendritic shape which was associated with a higher resistance to the pro-apoptotic agent H_2O_2, as indicates the absence of a cleaved PARP in treated acidic cells (Fig.4, panel A and B). Acidic A375M6 cells also express a clear enhancement of characteristic mesenchymal markers of EMT, such as N-cadherin and vimentin, and an increase amount of Twist, one of the major transcription factors governing EMT in various tumor cells. Reduction of E-cadherin, although significant, was not as evident as expected (Fig. 4, panel C). It has been postulated that both EMT and the reverse process, mesenchymal–epithelial transition (MET), to be part of the process of metastatic tumor formation. EMT is critical for the initial transformation from benign to invasive carcinoma, whereas MET is critical for the later stages of metastasis. To test if the epithelial-mesenchymal transition promoted by acidity in human melanoma cells was a reversible phenomenon, the medium of A375M6

cells grown at low pH (6.7) for 24 hours was replaced by standard pH medium (7.4) and cell morphology was observed every day.

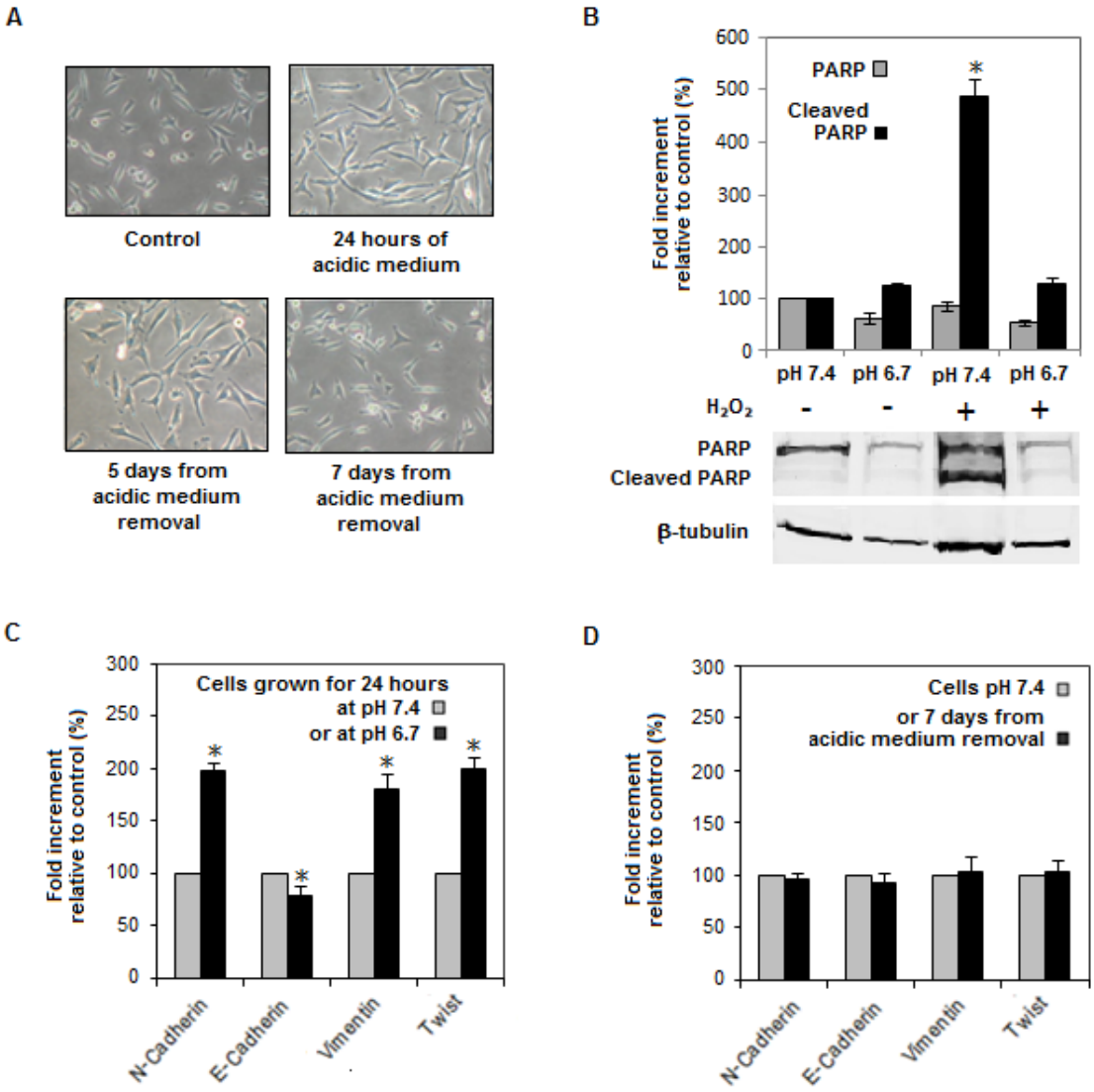


Figure 4- Acidity regulation tumor cell phenotype. A) Cell morphology of A375M6 human melanoma cells grown in acidic medium for 24 hours and then reconstituted in standard pH medium for 5 or 7 days. **B)** Western blot analysis of PARP expression in acidic melanoma cells treated with H_2O_2. Densitometry data presented are normalized to the intensity of β-tubulin bands. **C)** Densitometric analysis of N-cadherin, E-Cadherin, Vimentin and Twist proteins detected by Western blot analysis (mean ± SEM), in melanoma cells grown in standard pH medium or acidic medium; and **D)** densitometric analysis of N-cadherin, E-Cadherin, Vimentin and Twist proteins in melanoma cells grown for 24 hours in acidic medium and then grown for 7 days in standard pH medium. Data are expressed as percentage of values compared to the control; densitometry data presented are normalized to intensity of β-tubulin bands. *$P < 0.05$ compared with control cells.

Acidic melanoma cells grown in standard pH media recovered a more epithelial morphology (Fig.4, panel A) and molecular markers (Fig.4, panel D) like control cells after 7 days, signifying that acquisition of EMT phenotype by acidic melanoma cells is a transient phenomenon.

Acidic melanoma cells and invasiveness

EMT phenotype has been associated with increased production of MMPs, which can facilitate invasive and metastatic characteristics of tumor cells. Early investigations revealed that breast cancer cell lines expressing mesenchymal markers often expressed MMPs, and that suppression of these MMPs blocked their invasive and migratory characteristics (Martorana *et al.*, 1998). To assess if melanoma cells, that had undergone EMT driven by acidity, were associated with an increased invasiveness, we tested the ability of acidic A375M6 cells to migrate through filters coated with a reconstituted basement membrane of Matrigel.

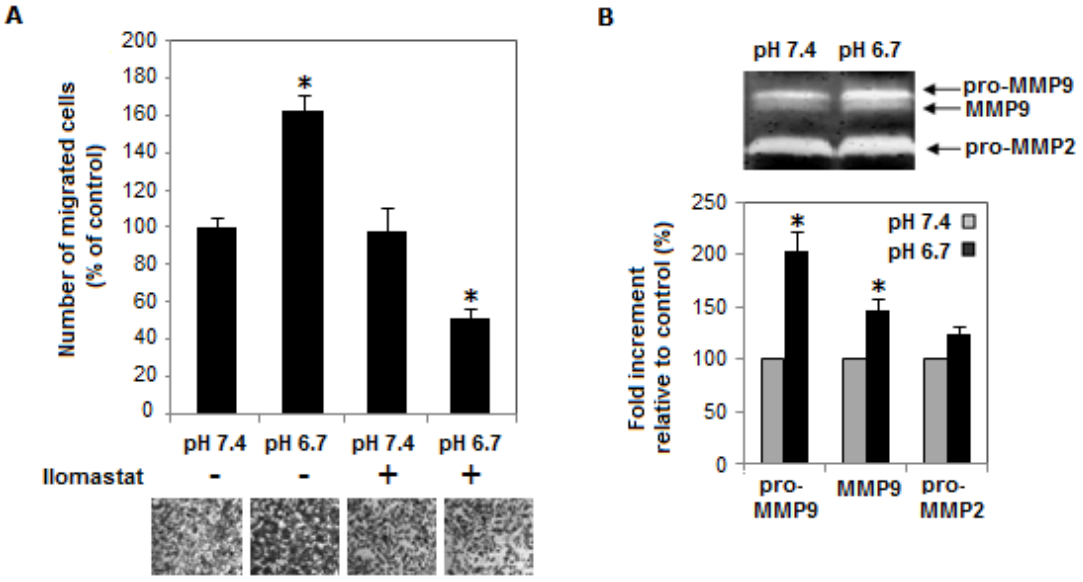


Figure 5- ***In vitro*** **invasive capacity of acidic A375M6 melanoma cells**. **A**) Invasiveness of melanoma cells grown in standard pH or acidic medium and in the presence or absence of Ilomastat was performed using Matrigel-coated filters. **B)** Gelatin zymography of media conditioned by melanoma cells grown in standard pH or acidic medium. Bar graph derived from densitometric analysis of pro-MMP9, MMP9 and pro-MMP2. Data represent the mean ± SEM of at least three independent experiments. *$P < 0.05$ compared with control cells.

We found that acidic melanoma cells, re-suspended in their conditioned medium, express a higher ability to invade Matrigel filters (Fig.5, panel A) that was inhibited when the migration occurred in the presence of Ilomastat, a broad MMP inhibitor. Medium conditioned by acidic cells was analyzed by gelatin zymography for gelatinase activity that revealed an increase of MMP-9 (Fig.5, panel B). All these data suggest that acidity is able to induct a mesenchymal type migration of melanoma cells.

To test if these phenomena were reversible, we analyzed the invasiveness of acidic cells and the MMP activity after 7 days of cell incubation in medium at standard pH. When acidic cells were grown in standard pH medium for 7 days, the increased invasiveness through Matrigel and MMP-9 activity turn to the level of control cells, and the only mesenchymal characteristic maintained by these cells was represented by a lasting responsiveness to Ilomastast (Fig.6, panel A and B).

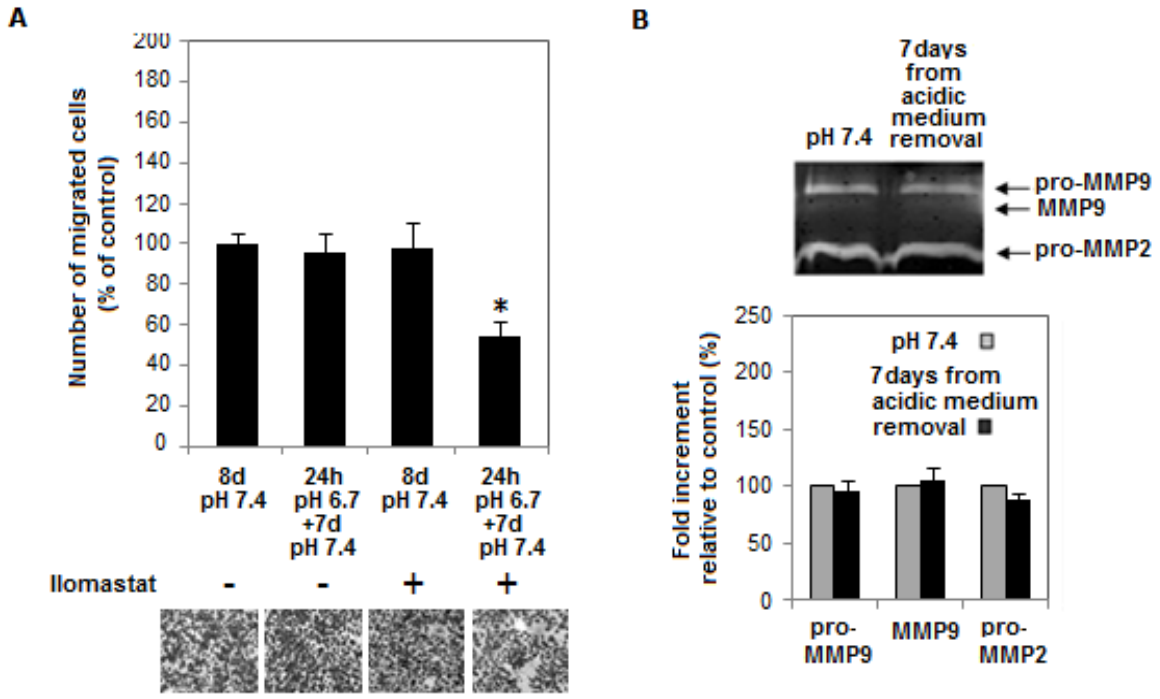


Figure 6- In ***vitro*** **invasive capacity of acidic A375M6 melanoma cells grown in standard pH medium for 7 days. A)** Invasiveness of melanoma cells grown in acidic medium for 24 hours and then grown in standard pH medium for 7 days, in the presence or absence of Ilomastat. **B)** Gelatin zymography of media conditioned by melanoma cells grown in standard pH or acidic medium and then grown in standard pH medium for 7 days. Bar graph derived from densitometric analysis of pro-MMP9, MMP9 and pro-MMP2. *$P < 0.05$ compared with control cells.

HIF-1α is not involved in the acidity-induced EMT

It is known that HIF-1α, a potent transcriptional regulator of oxygen-dependent genes, promotes cancer cell survival and invasion and correlates with metastasis in many kinds of tumors (Krishnamachary *et al*., 2003). Emerging evidences have demonstrated the direct effect of HIF-1α in EMT induction in cancer cells, either indirectly by induction of TGF-β, or directly by activating Snail, Twist and members of Zeb family (Evans *et al.*, 2007). We hypothesized that acidity could induce EMT and invasiveness through the transcription factor HIF-1α and we examined the expression of HIF1α in melanoma cells under acidic pH condition at different times (1, 3, 6, 24 hours). Figure 7 shows that HIF-1α, expressed in cells treated by cobalt chloride 100 μM (used as positive controls), is not expressed by cells grown at acidic pH and therefore it isn't the transcription factor involved in EMT induction by low pH.

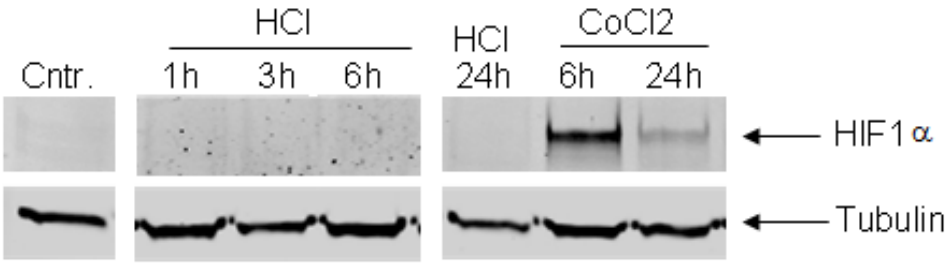


Figure 7- HIF-1α protein expression in A375P melanoma cells grown in acidic medium or treated by cobalt chloride ($CoCl_2$) for different times.

NF-κB is involved in the acidity-induced EMT

It is known that tumor microenvironment, consisting of infiltrated immune cells and their secretory cytokines and/or chemokines, facilitates cancer cell motility, invasiveness, and metastatic potential. Initially discovered and studied as a major activator of immune and inflammatory functions via its ability to induce expression of genes encoding cytokines, cytokine receptors, and cell-adhesion molecule and to be a critical mediator in the response to invading pathogens (Vallabhapurapu *et al.*, 2009), the nuclear factor kappa B

(NF-κB) has been implicated in the control of oncogenesis. Recent studies have demonstrated that the activation of the transcription factor NF-κB is linked to various signal transduction pathways and to transcription activation events that mediate cell proliferation, cell migration, and angiogenesis (Gilmore, 2006; Haffner *et al.*, 2006). Moreover, NF-κB is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, apoptosis and immune reaction (Baldwin, 1996) and it has been shown capable to bind to the promoter of the E-cadherin repressor ZEB-1/2 resulting in regulation of the EMT phenotype (Chua *et al*., 2007).

We demonstrated that acidity activates NF-κB by analyzing the protein expression of IκB, the inhibitor of NF-κB. When IκB interacts with the NF-κB, the complex is retained in the cytoplasm and NF-κB can't translocate to the nucleus to regulate target gene expression. The phosphorylation and the consequent degradation of IκB result in a rapid translocation and activation of NF-κB. We used cells treated with TGF-β, a known inducer of EMT through NF-κB (Huber *et al*., 2004; Li *et al*., 2012), and cells treated with parthenolide, a major sesquiterpene lactone from the plant *Tanacetum parthenium*, able to inhibit NF-κB both indirectly, by inhibiting the IκB kinase (IκK), and directly, by modifying p65 in its activation loop (Kwok *et al*., 2001, Hehner *et al*., 1999). Level of IκB in acidic cells decreased as in TGFβ-stimulated cells and parthenolide abolished this effect (Fig.8, panel A). These results mean that acidity, as well as TGF-β, activates NF-kB, which is inhibited by parthenolide. siRNA against NF-κB abrogated NF-κB and N-cadherin expression in acidic melanoma cells (Fig.8, panel B) suggesting that changes in EMT markers (Fig.4, panel C) are regulated through NF-κB pathway. In addition, the enhanced invasiveness of acidic melanoma cells was significantly inhibited by both parthenolide and siRNA for NF-κB (Fig.8, panel C, D). Altogether, these findings suggest a decisive role of NF-kB in acidity-promoted mesenchymal phenotype, also considering that melanoma cells grown in low pH medium do not show any changes in HIF-1α expression (Fig.7), a critical EMT promoter.

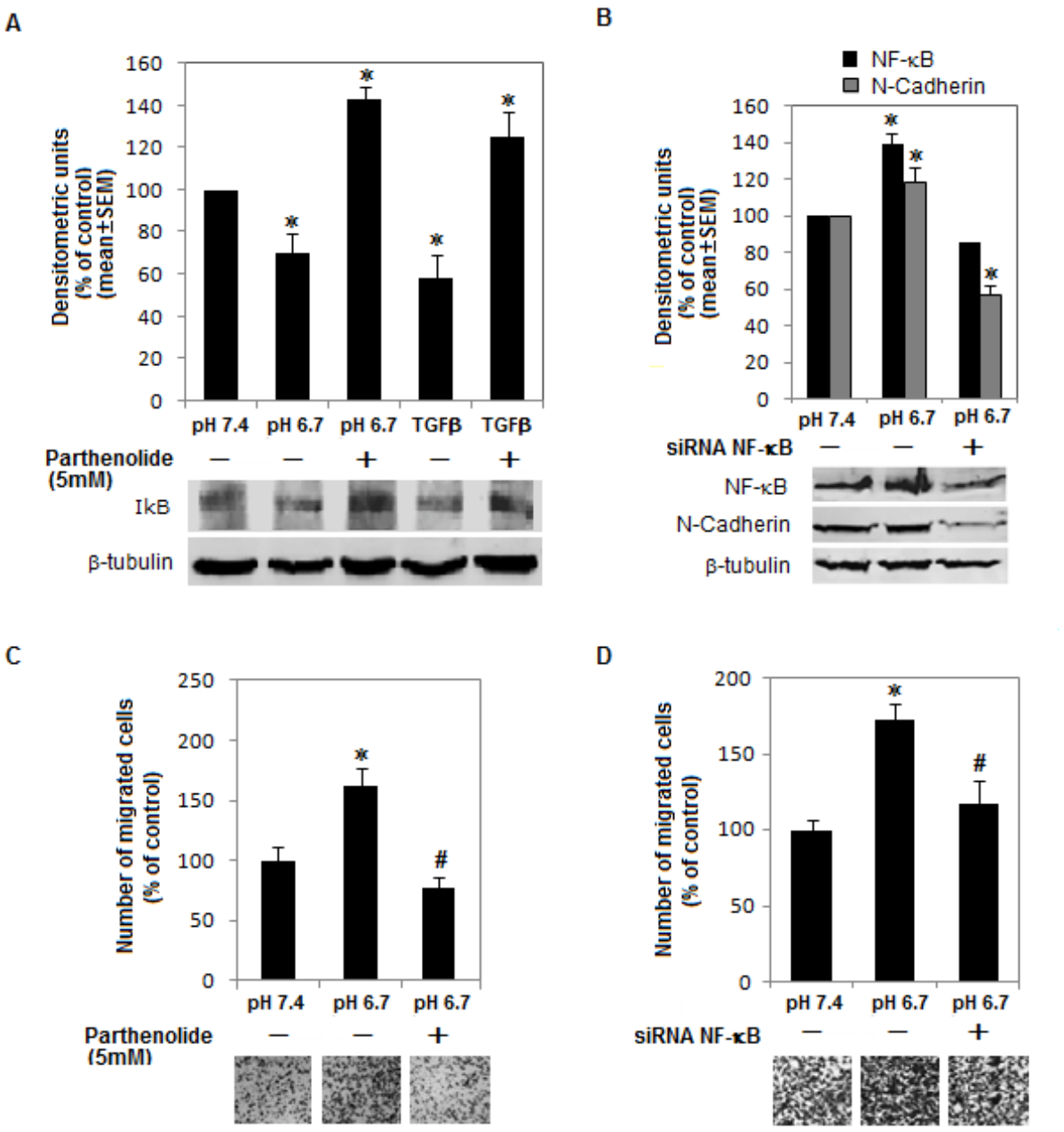


Figure 8- NF-κB role in invasiveness of acidic A375-M6 melanoma cells. **A)** IκB protein expression in cells grown in acidic medium or treated by TGFβ, in the presence or absence of Parthenolide. Data are expressed as percentage of values compared to the control; densitometry data presented are normalized to the intensity of β-tubulin bands. **B)** NF-κB and N-Cadherin expression in acidic cells after NF-κB siRNA treatment. Densitometry data presented are normalized to the intensity of β-tubulin bands. **C)** Invasiveness of melanoma cells grown in acidic medium and treated by Parthenolide. **D)** Invasiveness of melanoma cells grown in acidic medium and treated with NF-κB siRNA. *$P < 0.05$ compared with control cells; $^{\#}P < 0.05$ compared with acidified cells.

Cooperation between acidic and non-acidic cells

After observing that acidic pH makes tumor cells more invasive, we evaluated whether the medium conditioned by acidic cells was able to enhance the invasive potential of non-acidic cells. When tumor cells grown in standard pH medium were re-suspended in a medium conditioned by acidic cells, these cells express an increased capacity to invade Matrigel filters (*g*) that was comparable to the capacity of acidic cells (*d*) (Fig.9, panel A). To disclose the participation of amoeboid or mesenchymal migration, invasivity of tumor cells was also determined in the presence of Rho Kinase (ROCK) inhibitor or Ilomastast that inhibit amoeboid or mesenchymal migration, respectively. Indeed, amoeboid migration is dependent on actomyosin contractility induced by Rho/Rho-associated protein kinase (ROCK) signaling, whereas mesenchymal migration is dependent on secretion of matrix metalloproteases. Melanoma cells grown in standard pH media are significantly inhibited by ROCK inhibitor (*b*), but not by Ilomastat (*c*), indicating that the amoeboid-like migration is the predominant type of migration of these cells. In contrast, migration of acidic cells are inhibited by Ilomastat (*f*) (as already shown), but not by ROCK inhibitor (*e*). The enhanced migration of non-acidic cells re-suspended in a medium conditioned by acidic cells are inhibited by ROCK inhibitor (*h*) as well as Ilomastat (*i*), indicating that migration of these cells results from the promotion of MMP activities of media conditioned by acidic cells on the amoeboid-like style of migration of non-acidic cells. Thus, we tested whether the enhanced migration acquired by non-acidic cells, when they were exposed to a medium conditioned by acidic cells, might confer to a mixed population of non-acidic and acidic tumor cells a migration rate even higher than cells grown in a low pH medium. In fact, a mixture of acidic and non-acidic melanoma cells ($1.0x10^5$ cells, 1:1 ratio) gave a number of invading cells that was higher than we may expect considering the sum of individual ability of acidic and non-acidic cells, with no cooperation (the arrow indicates the invasiveness calculated by adding migration level of $5x10^5$ acidic cells plus migration level of $5x10^5$ non-acidic cells). Fig.9, panel B, also shows that a mixed population of acidic and non-acidic cells migrates more than acidic cells. Thus, cooperation between acidic and non-acidic tumor cells leads to maximized tumor aggressiveness.

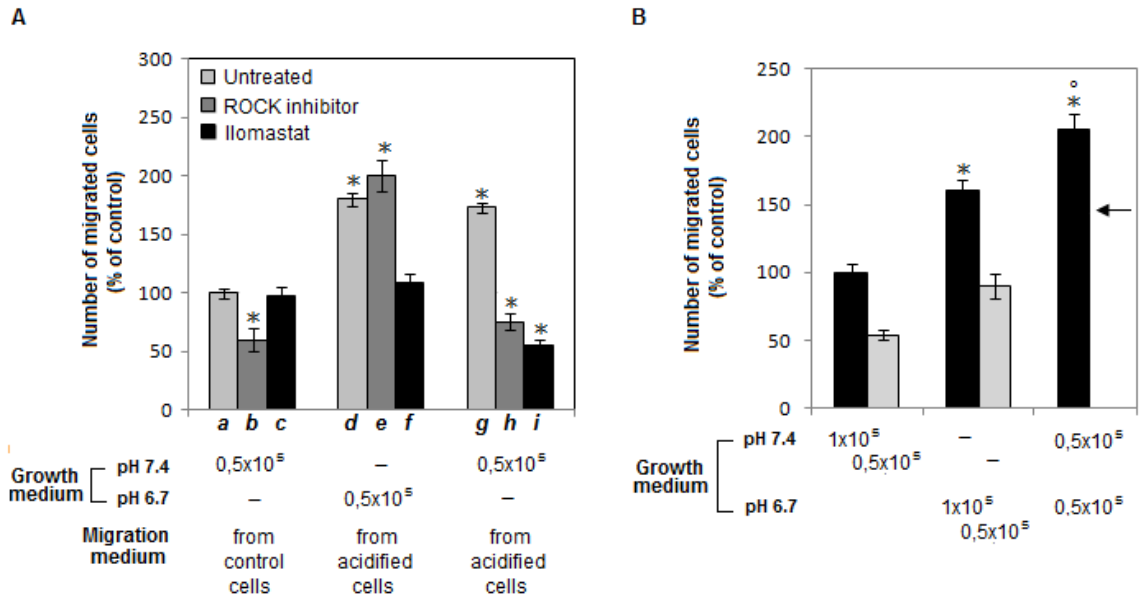
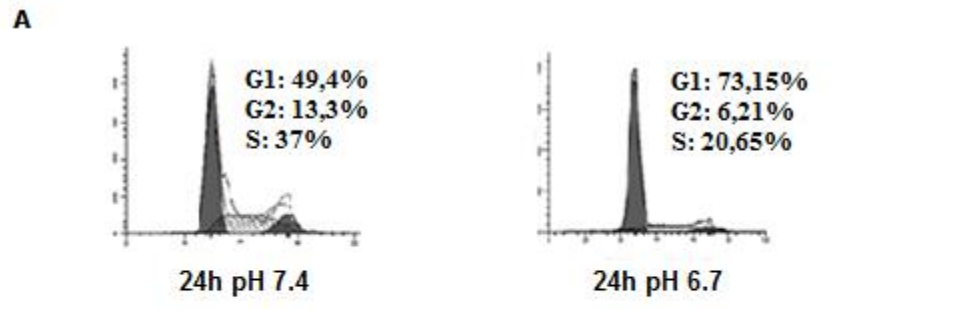


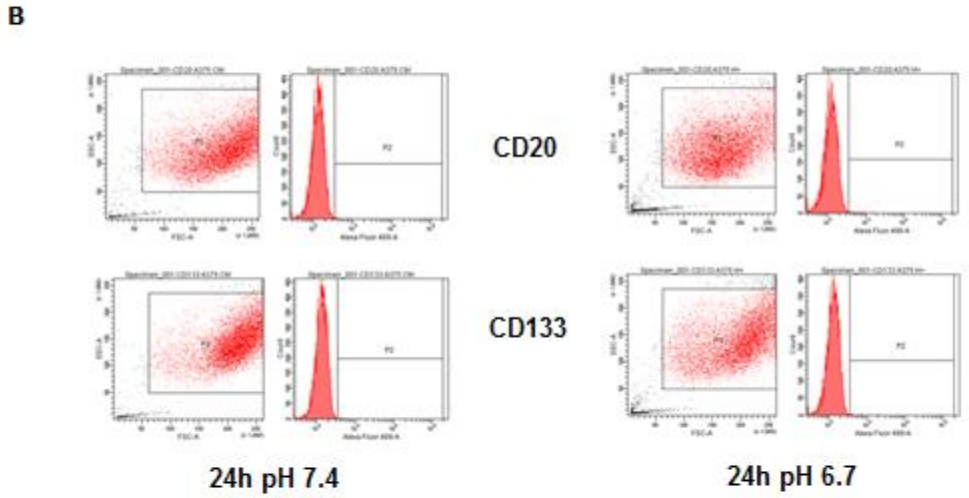
Figure 9- Cooperation between acidic and non-acidic A375-M6 melanoma cells in invasiveness. A) Change in invasiveness of melanoma cells grown in standard pH medium and then migrated in a medium conditioned by acidic cells. Migration of tumor cells is determined in the presence of Rho Kinase (ROCK) inhibitor or Ilomastast. B) Change in invasiveness of a mixed population of acidic and non-acidic melanoma cells (1:1, ratio), re-suspended in their own conditioned medium. **P* < 0.05 compared with control cells. °*P* < 0.05 compared with acidic cells.

Acidic melanoma cells and lung colonization

To investigate whether acidic melanoma cells undergoing EMT were also able to colonize host organs, final and crucial step of metastatic diffusion of tumor cells, we decided to inject acidic melanoma cells intravenously into immunodeficient animals. "Experimental metastases" assay is a complex process composed of sequential steps involving survival in blood stream, arrest in capillary bed of secondary organ, invasion into parenchyma and proliferation that tumor cells must carry out in order to colonize distant organs. Moreover, the "experimental metastasis" assay allows evaluation of final steps of metastatic cascade without biological influences of primary tumor growth. Before testing the cloning efficiency of acidic melanoma cells, we performed a cell cycle analysis revealing that acidic cells express a partial G1 arrest (73% vs 49%) (Fig.10, panel A), unchanged stem cell markers (CD133 and CD20) (Fig.10, panel B) and unchanged or even reduced cloning

efficiency by limiting dilution or in soft agar, compared to control cells (Fig.10, panel C and D).





C

Control	*18/1000*
6h HCl	*9/1000*
24h HCl	*0*

D

Control	*8%*
24h HCl	*0*

Figure 10- A) Cell cycle of acidic and non-acidic cells before injection into mice**; B)** Cytometric analyses of stem cell markers CD133 and CD20 expression in A375M6 grown in standard or acidic medium for 24 hours. **C)** Number colony formation in soft agar or **D)** using limiting dilution; cloning efficiencies were evaluated after 12 to 14 days of culture.

We injected non-acidic or acidic melanoma cells intravenously into SCID bg/bg immunodeficient animals, and we found that the number of lung micrometastases was comparable (Fig.11, panel A). This finding means that although acidic cells are more invasive than non acidic cells, the reduced number of proliferating and cloning cells

encumbers formation of lung colonies. As the mixed population of acidic and non-acidic cells shown the highest invasive capacity *in vitro*, we decided to use the mixed population also for in vivo experiments.

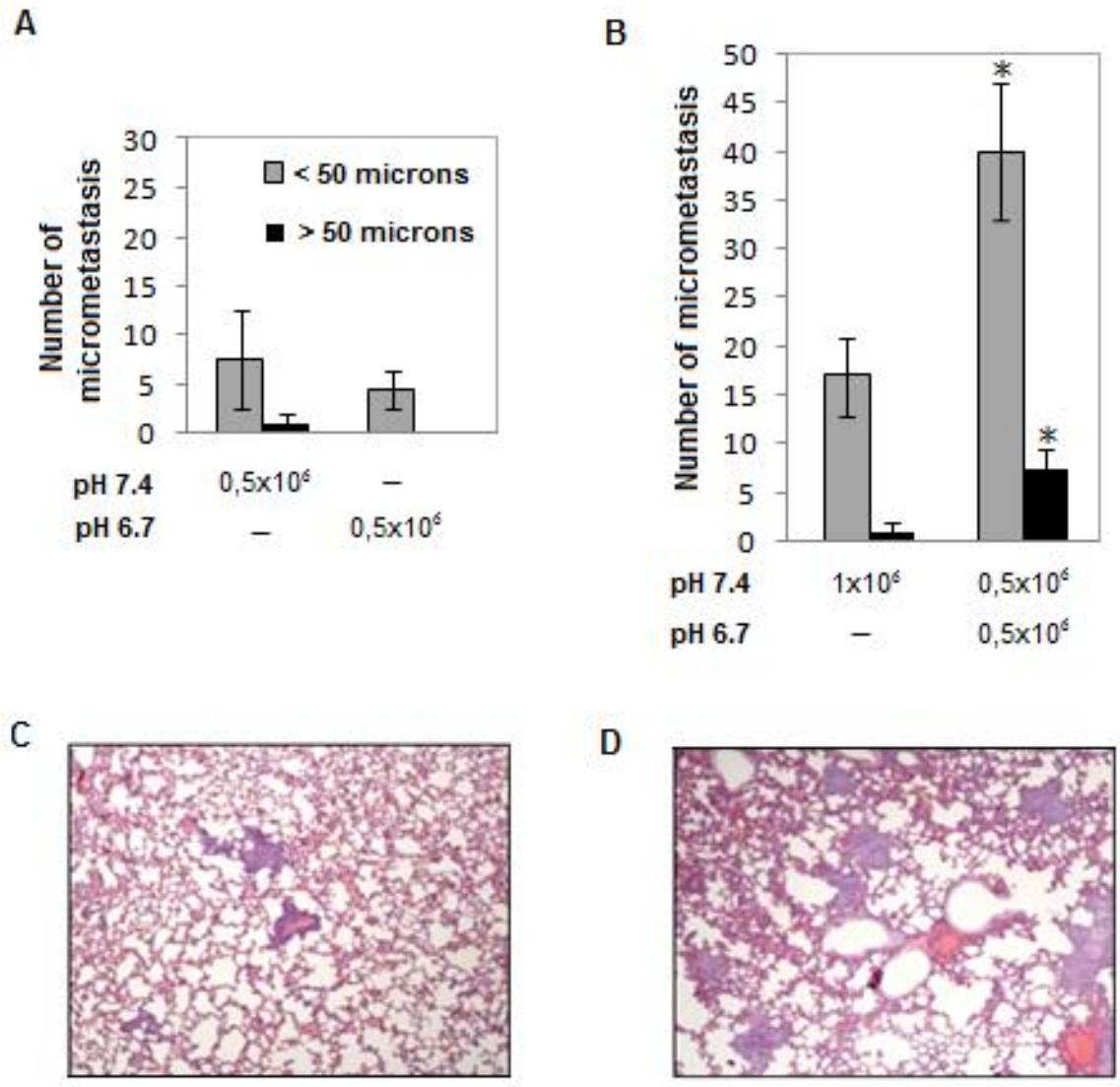


Figure 11- Cooperation between acidic and non-acidic A375M6 melanoma cells in lung colonization. **A)** Micrometastases identified by histological examination in lungs of mice injected with melanoma cells grown in standard pH or acidic medium. Data represent the mean ± SEM. **B)** Change in micrometastases identified by histological examination in lungs of mice injected with a mixed population of acidic and non-acidic melanoma cells (1:1, ratio). Data represent the mean ± SEM. **C)** Histological representative images of lung micrometastasis obtained injecting melanoma cells or **(D)** a mixed population of acidic and non-acidic melanoma cells, stained with H&E. **P* < 0.05 compared with control cells.

In order to distinguish the two cell types, we transfected non-acidic cells with GFP, while acidic cells were not transfected. When we injected into animals a mixed population of $0{,}5x10^6$ acidic cells (non-GFP-expressing) and $0{,}5x10^6$ non-acidic tumor cells (GFP-expressing), this mixed population gave a number of micrometastases significantly higher than that obtained injecting a similar amount ($1\ x10^6$) of non-acidic tumor cells (Fig.11, panel B).This effect is also evident comparing numbers of micrometastases greater than 50 μm in diameter (Fig.11, panel B) and the histological exams of lungs derived from the injection of non-acidic tumor cells (Fig.11, panel C) or of the mixed population (Fig.11, panel D). We tested GFP expression by tumor cells of lung micrometastases and we found that the greater fraction of tumor cells participating to lung colonies are non-acidic-GFP-expressing tumor cells (Fig.12, panel A and B), revealing that colony development depends on the cooperation between acidic and non acidic tumor cells, but non acidic tumor cells participate greater to the growth of colonies.

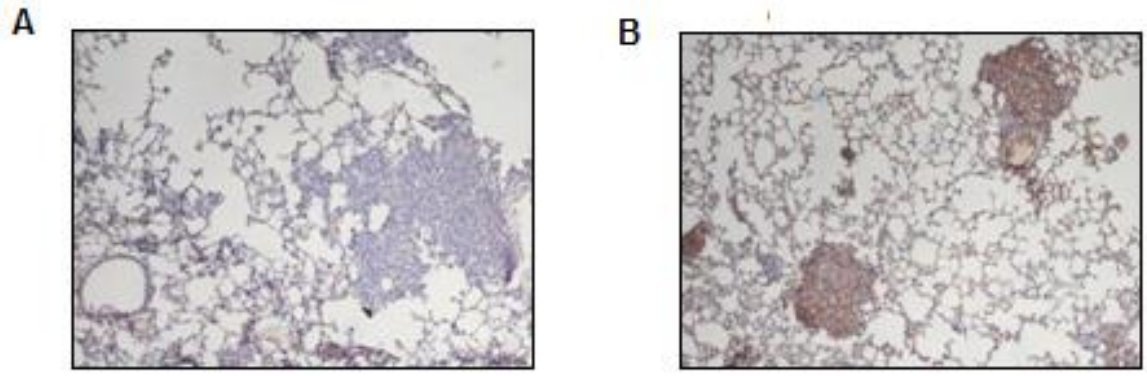


Figure 12- Immunohistochemistry of micrometastasis from a mixed tumor cell population to detect GFP-expressing cells, analysis in the absence (**A**) or in the presence (**B**) of anti-GFP primary antibody (original magnification x20).

The final publication is available at link.springer.com

http://link.springer.com/article/10.1007%2Fs10585-014-9637-6

(Peppicelli *et al*., 2014)

4.1.2. Acidic pH and VEGF-C expression in human melanoma cells

VEGF-C expression in Hypoxia

The contribution of lymphatic system to tumor metastasis is being increasingly demonstrated in experimental and human studies. The regional lymph nodes are the first and most common site of metastasis for various solid tumors (Rinderknecht and Detmar, 2008) and extent of lymph node involvement anticipates staging and prognosis of tumors (Dadras *et al.,* 2003). A member of the vascular endothelial growth factor (VEGF) family, the VEGF-C, was found the most potent lymphangiogenic growth factor, required for embryonic and postnatal lymphatic development (Karkkainen *et al.,* 2004; Su *et al.,* 2007). VEGF receptor-3 (VEGFR-3) is the determinant receptor of VEGF-C and it represents a marker for lymphatic endothelial cells (Karkkainen *et al.,* 2004; Su *et al.,* 2007). Stimulation of VEGF-C/VEGFR-3 axis increases lymph node metastasis (Su *et al.,* 2007; He *et al.,* 2002), while VEGFR-3 antagonists are able to inhibit lymphangiogenesis of primary tumor and lymphatic dissemination of tumor cells (He *et al.,* 2005). Moreover a lymphangiogenic therapy was found to be crucial in the control of regional lymph node and systemic metastasis, whereas angiogenic blockade reduces blood vessel density, systemic metastasis, but not nodal metastasis (Burton *et al*., 2008). Human tumor cells, including human melanoma cells, release VEGF-C that promotes colonization of regional lymph nodes and metastases outgrowth (Skobe *et al*., 2001).

Large regions of a tumor are often at a great distance from blood vessels and lymphatic vessels, and this distance causes low levels of O_2 tension (hypoxia) with the induction of HIF1α activity, a key regulator of the cellular response to hypoxia. Hypoxic areas of tumors are known to be decisive in inducing the development of a more aggressive tumor phenotype, resistant to therapeutic treatment and associated with adverse clinical outcomes (Vaupel and Mayer, 2008). Hypoxia, more than the reduced levels of nutrients and the increased levels of waste products, drives genomic instability and alters DNA damage repair pathways (Bristow and Hill, 2008). Here, we investigated if hypoxia, that it is well known for stimulating VEGF-A expression, a major regulator of angiogenesis (Gray *et al.,* 2005), modifies also VEGF-C expression.

A375P melanoma cells were grown for 24 hours in normoxic (20% O_2) or hypoxic conditions (0.3% O_2), or in hypoxic condition and then reoxygenated for other 24 hours. As revealed by RT-PCR and Western blot analyses, expression of VEGF-C in A375P melanoma cells was reduced by hypoxia, in contrast with the striking stimulation of VEGF-A expression, both at mRNA (Fig.13, panel A) and protein levels (Fig.13, panel B). The reoxygenation after hypoxia condition brings both the levels of VEGF-A and VEGF-C mRNA back to their control states (Fig.13, panel A), while VEGF-A protein remains high (Fig.13, panel B).

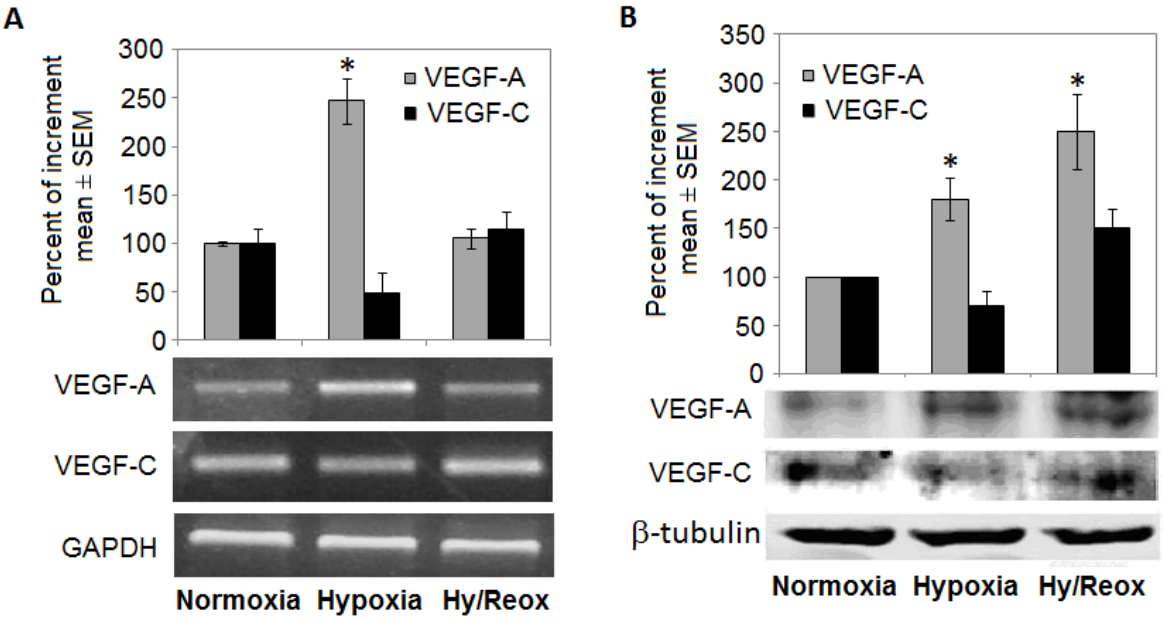


Figure 13- Change in VEGF-A and VEGF-C expression in A375P human melanoma cells grown under an hypoxic condition. A) VEGF-A and VEGF-C mRNA expression in melanoma cells grown for 24 hours in normoxic (20% O_2) or hypoxic conditions (0.3% O_2), or in hypoxic condition and then reoxygenated for other 24 hours (Hy/Reox). Each band was quantified by densitometric analysis and the corresponding histogram was constructed as relative to GAPDH. B) VEGF-A and VEGF-C protein levels in melanoma cells grown for 24 hours in normoxic or hypoxic conditions or in hypoxic condition and then reoxygenated. Each band was quantified by densitometric analysis and the corresponding histogram was constructed as relative to β-tubulin. * indicates $p<0.05$.

To explore in-depth the molecular mechanism by which hypoxia failed to induce VEGF-C in melanoma cells, A375P cells were transfected with siRNA for HIF-1α and monitored the levels of VEGF-C and VEGF-A in response to hypoxia. Selective inactivation of HIF-1α resulted in a clear inhibition of hypoxia-stimulated VEGF-A expression, whereas the reduced VEGF-C expression in tumor cells grown under hypoxic condition did not change (Fig 14). This finding suggests that HIF-1α does not have a role in the reduction of VEGF-C expression in melanoma cells grown under hypoxia.

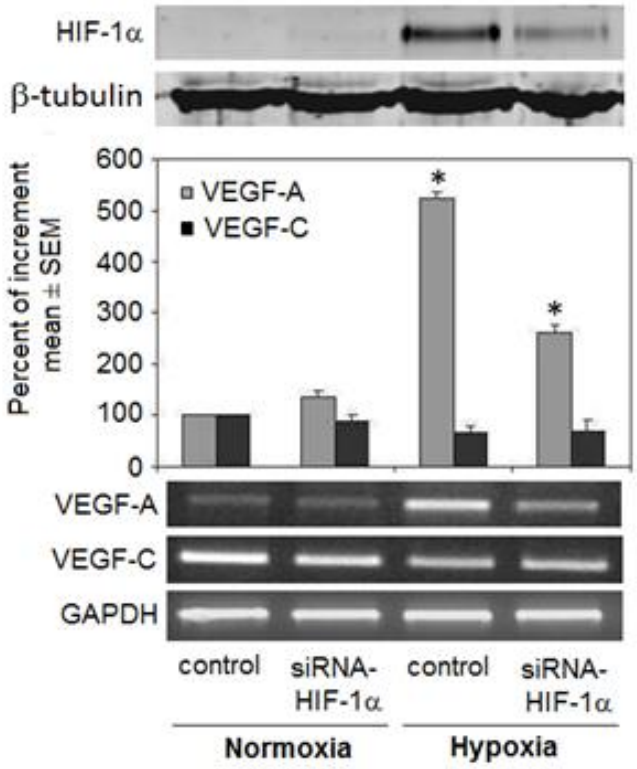


Figure 14- Levels of VEGF-C and VEGF-A in cells transfected with siRNA for HIF-1α. Upper panel: HIF-1α protein expression in A375P melanoma cells transfected with siRNA for HIF-1α and grown in hypoxia. Lower panel: VEGF-A and VEGF-C mRNA levels. Each band was quantified by densitometric analysis and the corresponding histogram was constructed as relative to GAPDH. * indicates $p<0.05$.

Expression of VEGF-C in human melanoma cells grown in media at different pH

The chaotic and incomplete vasculature of tumor is frequently responsible for a transient or persistent oxygen deficiency and tumor cells respond converting to an anaerobic respiration, driving the environment of most solid tumor, including melanoma, to be acidic. Tumors with acidic environment are associated with a poorer prognosis (Walenta *et al.*, 2000), resistance to chemotherapy (Raghunand *et al.*, 2001), suppression of cytotoxic lymphocytes and natural killer cells tumoricidal activity (Fischer *et al.*, 2007). Tumor acidity also contributes to tumor cells aggressiveness by increasing their mutation rate (Morita *et al.*, 1992), invasiveness into host tissues and secondary organ colonization (Rofstad *et al.*, 2006; Moellering *et al.*, 2008). Thereafter we examined the ability of an acidic medium to promote VEGF-A and VEGF-C expression in melanoma cells. A375P melanoma cells grown in standard culture conditions reach the confluence at the fourth day of growth and pH of media changes from 7.4 to 6.8 (Fig.15).

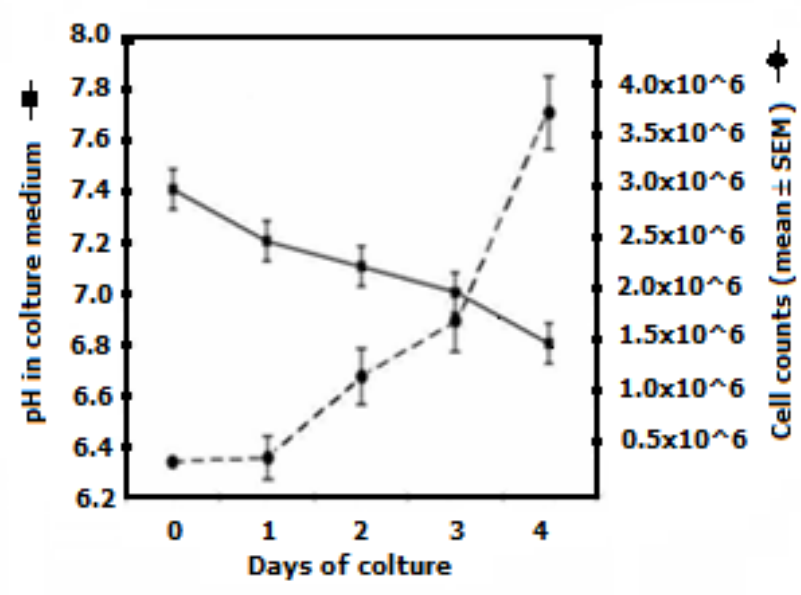


Figure 15- Growth curve and pH of media in melanoma cell cultures.

We found that mRNA level of VEGF-A of confluent cells (pH 6.8) was unchanged respect to VEGF-A level of sparse cultures (pH 7.2) (Fig. 16, panel A), while mRNA and protein levels of VEGF-C of confluent cells (pH 6.8 medium) were significantly higher than those of cells recovered from sparse cultures (pH 7.2 medium) (Fig.16, panel B).

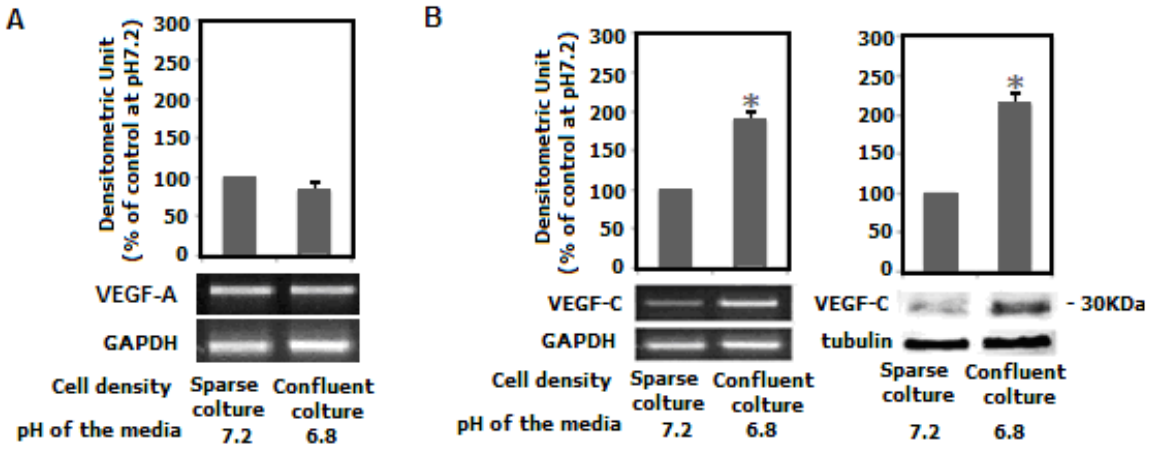


Figure 16- A) VEGF-A mRNA expression in sparse (pH 7.2) and confluent (pH 6.8) cell cultures. **B)** VEGF-C mRNA and protein expression in sparse (pH 7.2) and confluent (pH 6.7) cell cultures, each band was quantified by densitometric analysis and the corresponding histogram was constructed as relative to GAPDH and β-tubulin, respectively. Values presented are means ± SEM of three independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

We also found that VEGF-C expression was enhanced in tumor cells of sparse cultures when they were exposed to media collected from confluent melanoma cell cultures at pH 6.8, (Fig. 17, panel A). To ascertain whether induction of VEGF-C in A375P melanoma cells was related to a low pH, and not to agents released by tumor cells themselves in confluent cultures, we examined whether media collected from sparse cultures acidified to pH 6.6 (panel B) and media that have not been exposed to cells acidified to pH 6.7 (Fig. 17, panel C) may stimulate VEGF-C expression in melanoma cells. We found that both media stimulate VEFG-C expression in melanoma cells. However, VEGF-A expression is not modified by acidity (panel D). These results clearly indicate that acidity *per se* may promote VEGF-C in A375P melanoma cells. Finally, the pH response curve (Fig. 17, panel E) shows that a change of pH medium from 7.4 to 6.5 promotes VEGF-C expression in a progressive manner and the maximum effect on VEGF-C expression occurs at pH 6.8.

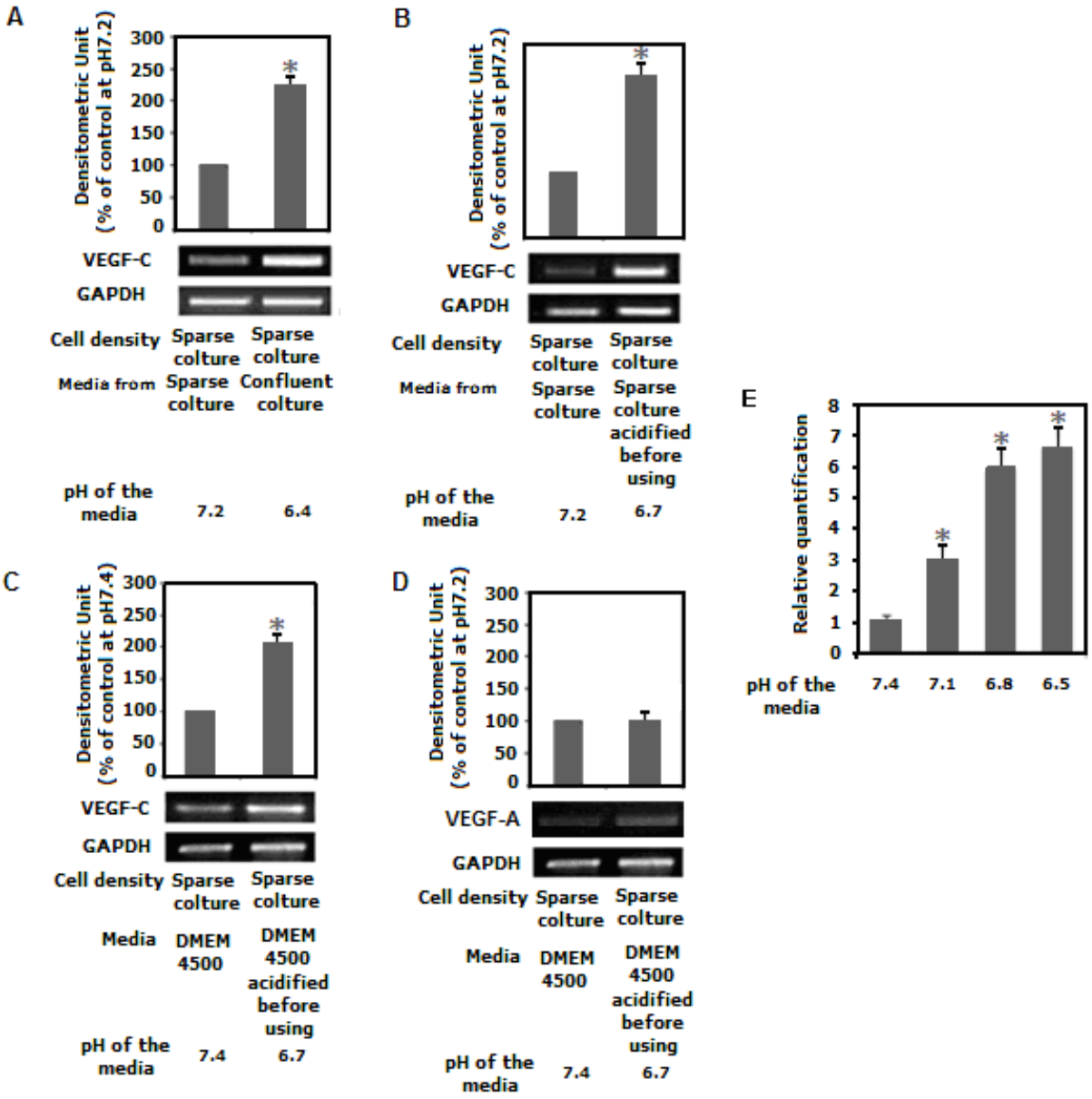


Figure 17- VEGF-C mRNA expression in melanoma cells of: **A)** sparse cultures grown in an acidic medium collected from confluent cultures, **B)** sparse cultures grown in acidified sparse culture medium, and **C)** sparse cultures grown in acidified medium that have not been exposed to cells. **D)** VEGF-A mRNA expression in melanoma cells of sparse cultures grown in acidified medium that have not been exposed to cells. Each band was quantified by densitometric analysis and the corresponding histogram was constructed as relative to GAPDH. **E)** Quantitative real-time PCR of VEGF-C expression in melanoma cells incubated for 24 hours in unexposed media at different pH. Values presented are means ± SEM of three independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

It is known that VEGF-C binds VEGF-C receptor 3 (VEGFR3) and induces tyrosine auto-phosphorylation of VEGF-C receptor 2 (VEGFR2) and 3 leading to angiogenesis and lymphangiogenesis (Joukov *et al.*, 1996). However, we found very low level of expression of VEGFR2 and VEGFR3 and pH response curve experiments indicate that acidity does not stimulate in melanoma cells the expression of VEGF-C receptors 2 (Figure 18, panel A) and 3 (Figure 18, panel B).

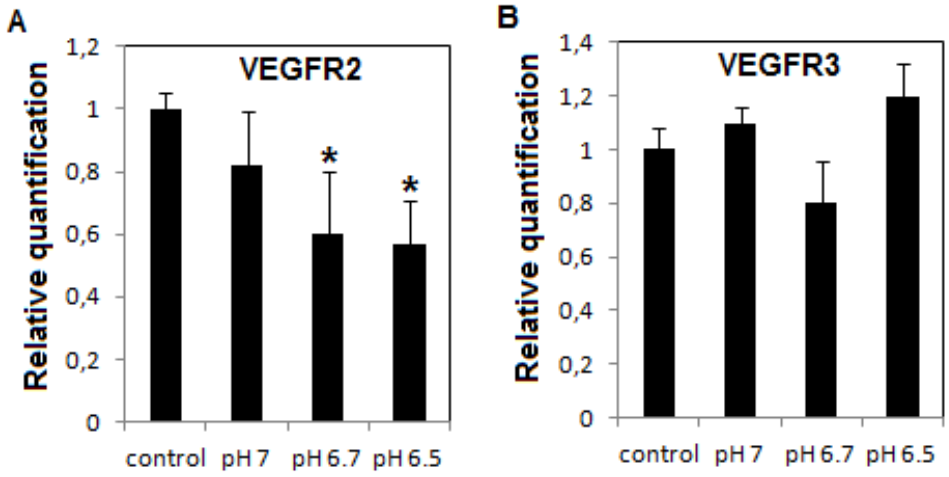


Figure 18- Quantitative real-time PCR of VEGFR2 and VEGFR3 expression in melanoma cells incubated for 24 hours in unexposed media at different pH. Asterisk indicates $p<0.05$.

Low pH stimulates expression of VEGF-C in melanoma cells through NF-κB activity

As we showed in Figure 7, HIF-1α is not expressed by cells grown in acidic medium and therefore it can't be the transcription factor involved in VEGF-C stimulation by low pH; whereas, we demonstrated in Figure 8 that acidity stimulates NF-κB activity in A375M6 cells. We confirmed this effect of acidity also in A375P melanoma cells, studing NF-κB location within the cells by using immunofluorescence assay. Analysis of intracellular location of NF-κB in A375P melanoma cells grown under a standard or acidic medium, revealed that these cells exposed to a standard pH medium (pH 7.4) express an exclusive labeling in cytoplasm, while cells exposed to a standard medium acidified to pH 6.7, immediately before its use, show nuclear localization of NF-κB /p65 subunit with a peak

at 6 hours after treatment (Figure 19). Thus, acidity promotes nuclear localization of NF-κB and stimulates its activity.

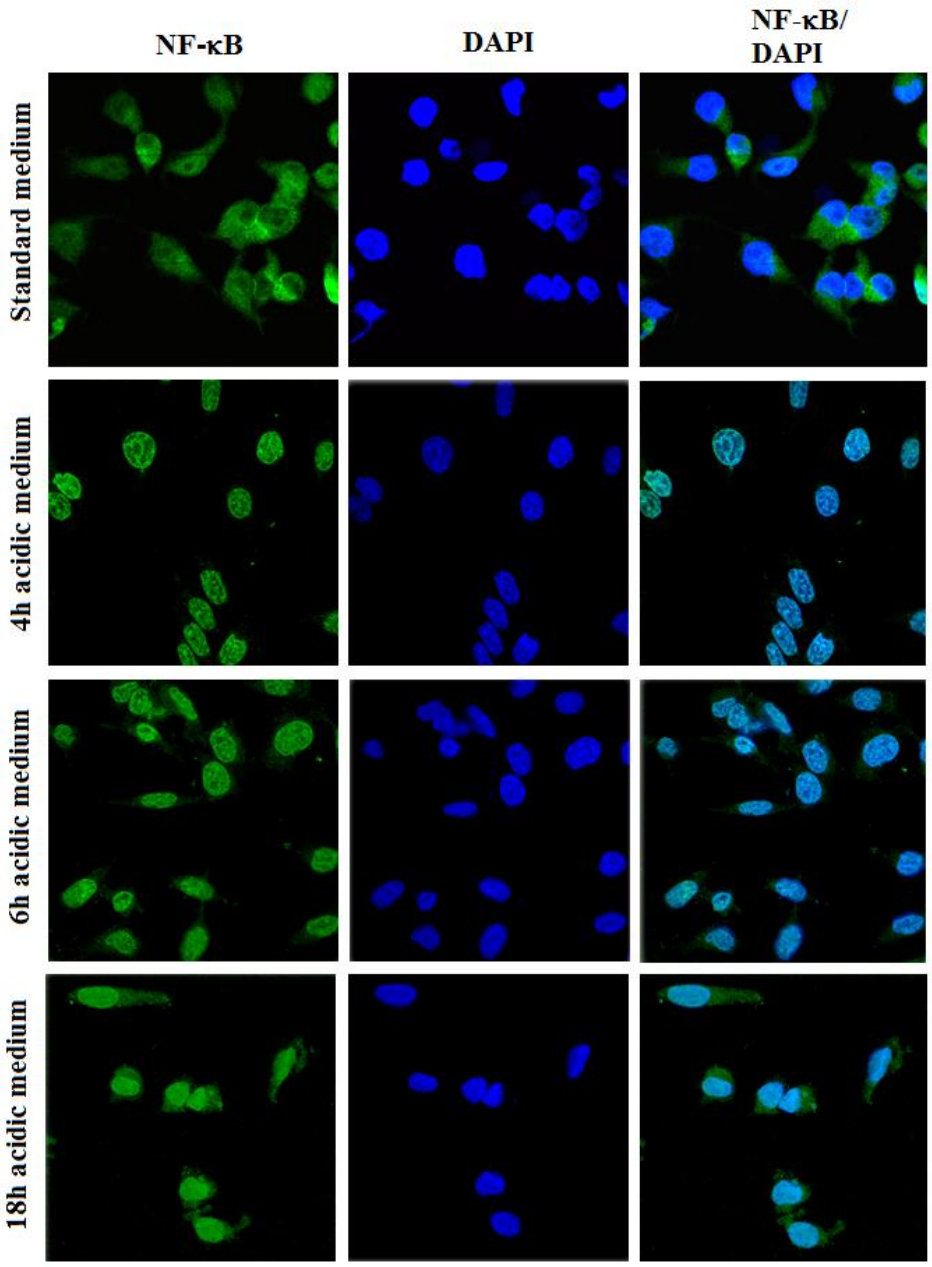


Figure 19- Acidic medium activates NF-κB /p65. Double immunofluorescence analysis of NF-κB /p65 nuclear translocation in melanoma cells exposed to an acidic medium for 4, 6 and18 hours (NF-κB/p65 *green*, DAPI *blue*).

The up-regulation of VEGF-C expression induced in A375P melanoma cells by an acidic medium was inhibited by the treatment with parthenolide (NF-κB inhibitor) either at 1,5 or 5μM (Fig. 20, panel A). The inhibitory effect of parthenolide was also demonstrated in melanoma cells incubated under normal pH (Fig. 20, panel A, column 1, 2, 3). Moreover, we found that VEGF-C up-regulation was inhibited in A375P melanoma cells transfected with p65 NF-κB siRNA and exposed to an acidic medium (Fig. 20, panel B). These findings suggest that VEGF-C expression of melanoma cells grown under normal and, more effectively, low pH is dependent from NF-κB signalling pathway.

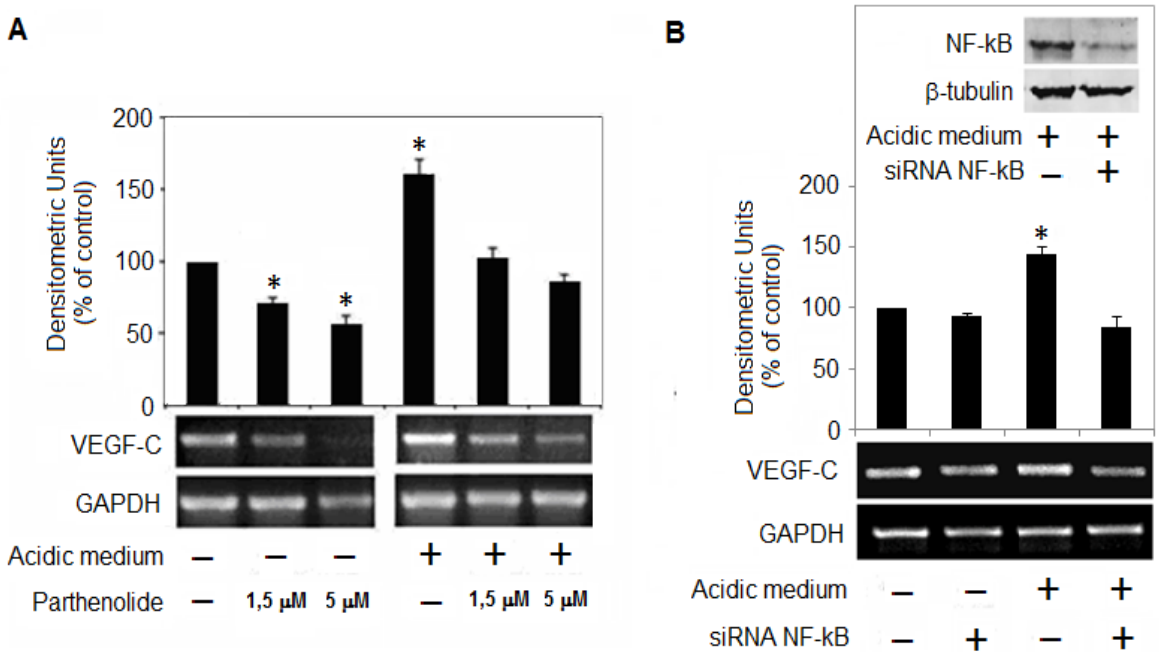


Figure 20- Change in VEGF-C expression in A375P melanoma cells exposed to an acidic medium and with NF-kB inhibition. A) Inhibition by parthenolide (1.5 or 5μM) of VEGF-C mRNA expression in acidic melanoma cells; **B)** Inhibition by siRNA for NF-κB of VEGF-C mRNA expression in acidic melanoma cells. In box of Figure, NF-κB protein expression in A375P melanoma cells grown in acidic medium transfected with siRNA for NF-κB or scramble. Densitometric assessment is expressed in arbitrary units. Values presented are means ± SEM of three independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

Esomeprazole inhibits VEGF-C expression in acidic human melanoma cells

A number of researchers have explored inhibition of the membrane ion pumps involved in the maintenance of an alkaline intracellular pH by extruding protons or importing bicarbonate ions. Proton extrusion is achieved by multiple and redundant families of H^+ transporters, including the V-ATPase that plays various functions in tumors, as proliferation, tumorigenesis, drug resistance and tumor progression (Sennoune *et al.*, 2004). Several studies have shown that proton pump inhibitors (PPIs) such as esomeprazole, omeprazole and pantoprazole, activated by acidic extracellular pH of tumors and highly effective at inhibiting V-ATPases, have an antineoplastic activity towards human hematopoietic and solid tumors (De Milito *et al*., 2009). In order to establish whether a proton pump inhibitor (PPI) might be effective to abrogate VEGF-C up-regulation induced by acidity, we chose esomeprazole. Esomeprazole is a common PPI which is protonated and activated in an acidic medium to express its pharmacological effects, among those, the recently described anti-inflammatory special property (Hashioka *et al*., 2009).

For this study, 100 μM esomeprazole was selected in accordance with PPI plasma level found in human beings after intravenous administration of these drugs. Incubation of human A375P melanoma cells for 24 hours in a acidic medium containing 100 μM esomeprazole does not significantly modify their viability (viable cells >95%) (Figure 21) and pH of the medium.

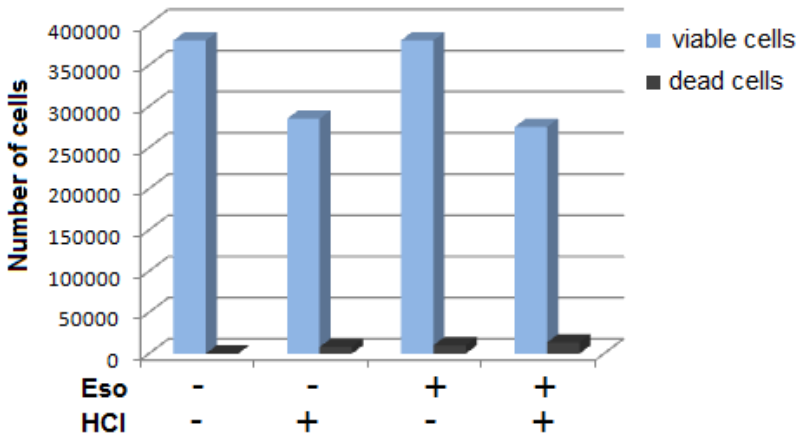


Figure 21- Viability of A375P cells grown in standard or acidic medium and treated with esomeprazole 100 μM.

Interestingly, a 100 μM esomeprazole concentration strongly inhibits NF-κB activation in A375P melanoma cells exposed for 6 hours to a reduced pH (pH 6.7); indeed nuclear localization of NF-kB after acidic treatment is inhibited when melanoma cells are exposed to acidic pH in the presence of esomeprazole (Fig.22, panel A). Importantly, we demonstrated that esomeprazole effectively inhibits VEGF-C expression in acidic melanoma cells (Fig. 22, panel B).

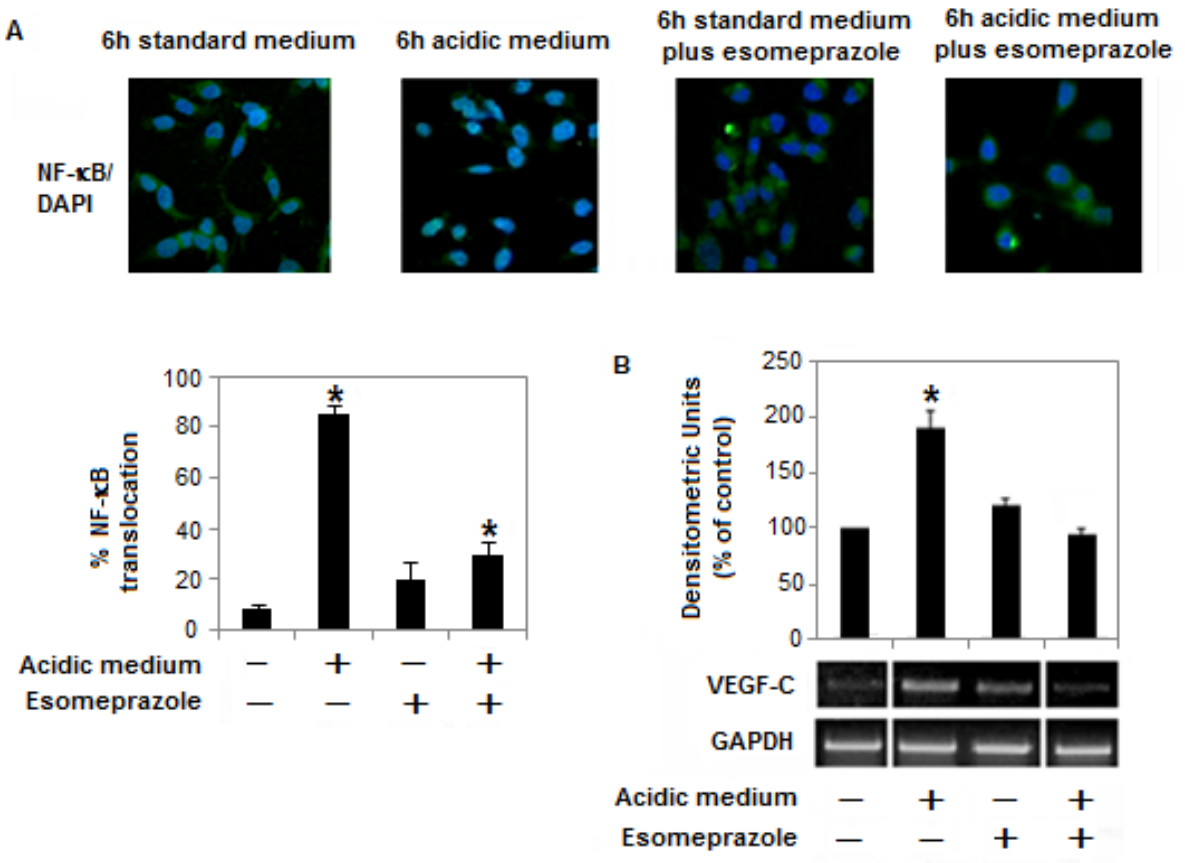


Figure 22- Inhibition by esomeprazole (100μM) of NF-κB-dependent up-regulation of VEGF-C mRNA expression in acidic A375P melanoma cells. A) Double immunofluorescence analysis of melanoma cells exposed for 6 hours to an acidic medium and treated with esomeprazole (NF-κB/p65 *green*, DAPI *blue*); the columns represent the percentage of cells expressing nuclear NF-kB. **B)** Inhibition by esomeprazole of VEGF-C mRNA expression in acidic melanoma cells. Densitometric assessment in arbitrary units and representative PCR blots. Values presented are means ± SEM of at least three independent experiments. Asterisk indicates $p<0.05$.

Inflammatory cytokines, such as TNFα and IL-1β stimulate VEGF-C expression in human vascular endothelial cells mainly through NF-κB activation (Ristimäki *et al*., 1998; Chilov e*t al*., 1997; Cha *et al*., 2007), and represent suitable promoters of VEGF-C also in melanoma cells. We found that TNFα and, in a more evident way, IL-1β stimulate VEGF-C mRNA in A375P melanoma cells, and this promotion was inhibited by the NF-κB inhibitor Parthenolide (Fig.23, panel A).

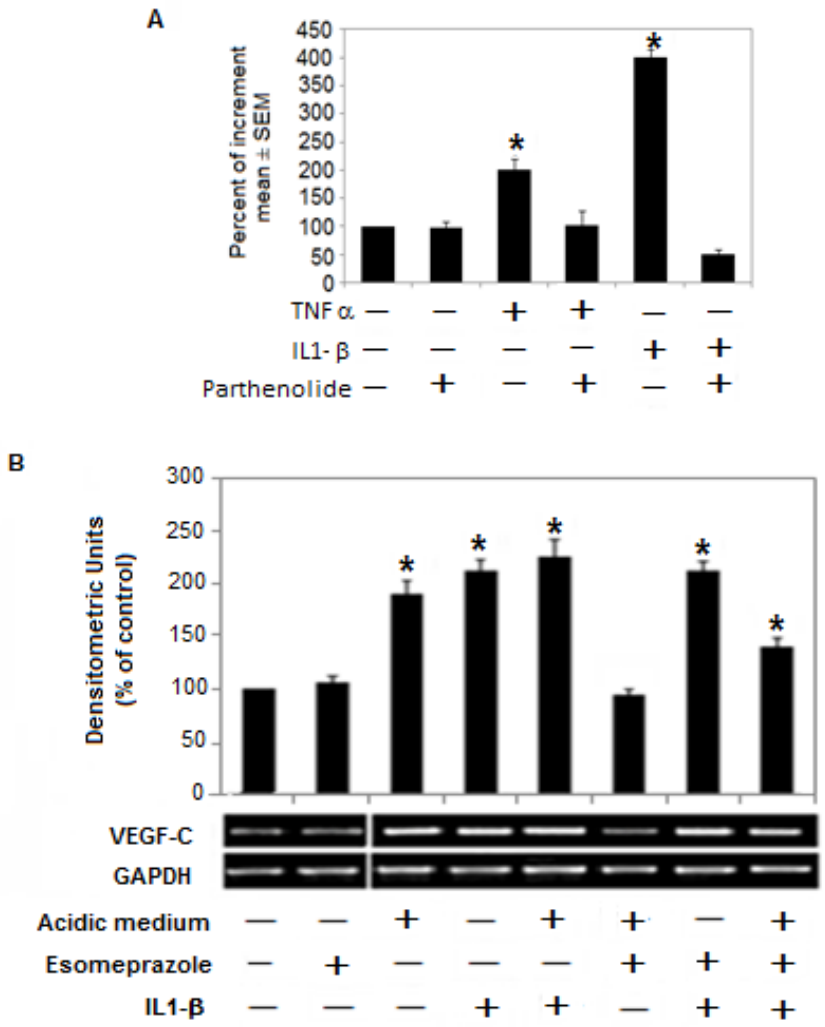


Figure 23- Inhibition of NF-κB-dependent up-regulation of VEGF-C mRNA in melanoma cells. A) VEGF-C expression in melanoma cells stimulated with TNFα or IL-1β, in the presence or in the absence of Parthenolide, an NF-kB inhibitor. **B)** Inhibition by esomeprazole of NF-κB-dependent up-regulation of VEGF-C mRNA in melanoma cells treated with IL1β and/or an acidic media for 24 hours. Densitometric assessment in arbitrary units and representative PCR blots. Values presented are means ± SEM of at least three independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

Fig.23, panel B indicates that the IL-1β-stimulated expression of VEGF-C was inhibited in melanoma cells that were incubated within an acidic medium containing esomeprazole. Esomeprazole, on the other hand, does not abrogate VEGF-C up-regulation induced by IL-1β when tumor cells were incubated in a standard medium (pH 7.4), because esomeprazole activation necessitates an acidic environment. These results indicate that esomeprazole may inhibit VEGF-C expression in A375P melanoma cells stimulated by inflammatory cytokines, only in case acidity takes part to the environmental changes.

Esomeprazole reduces VEGF-C expression in human melanoma cells freshly isolated from a metastatic human melanoma lesion.

We demonstrated that acidity stimulates VEGF-C expression in human melanoma cells collected from a spontaneous metastatic lesion and esomeprazole blocked it. Also in primary human melanoma, Parthenolide 5μM inhibits VEGF-C expression promoted by low pH (Fig. 24, panel A), indicating that, also in these cells, VEGF-C stimulation under acidic pH condition is dependent from NF-κB signalling pathway. In unstimulated cells, NF-κB dimmers are retained in an inactive form in the cytosol through their interaction with IκB proteins. Degradation of these inhibitors upon their phosphorylation by the IκB kinase (IKK) complex leads to nuclear translocation of NFκB and induction of transcription of target genes. As shown in fig. 24, panel B, esomeprazole stimulates, in these primary tumor cells, the expression of the well known inhibitor IκB, and consequently it inhibits NF-κB. As we found in A375P melanoma line, we then demonstrated that esomeprazole abolishes VEGF-C expression induced by acidity through NF-κB signaling (Fig.24, panel C). In addition, esomeprazole abolishes the up-regulation of VEGF-C expression in tumor cells grown in an acidic medium containing IL-1β (Fig.24, panel D). On the whole, esomeprazole exerts an effective anti-VEGF-C activity also in melanoma cells of a primary culture derived from a spontaneous metastatic lesion.

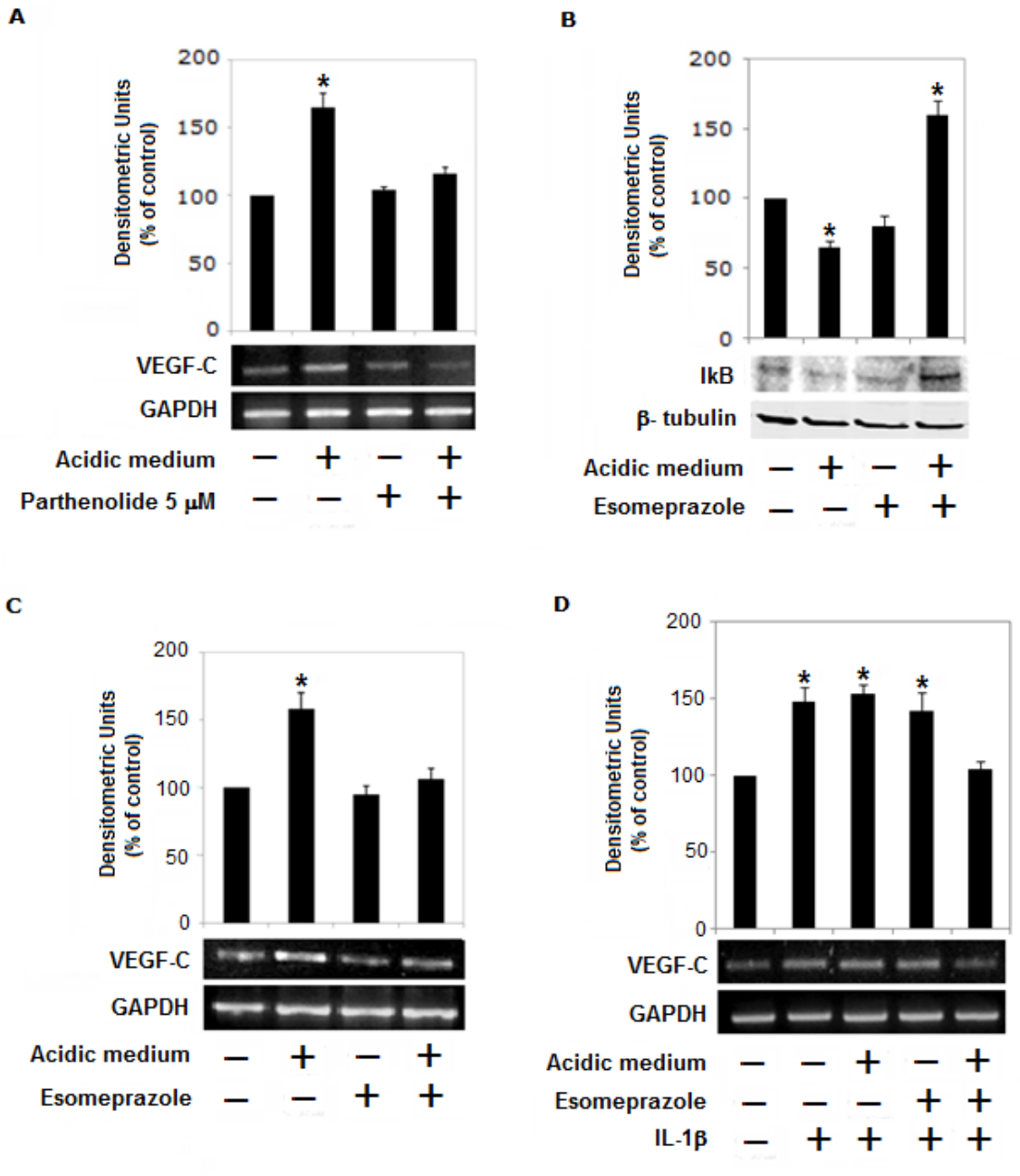


Figure 24- Inhibition of NF-κB-dependent up-regulation of VEGF-C mRNA in acidic primary culture of melanoma. **A)** Inhibition by parthenolide 5µM of VEGF-C mRNA in melanoma cells grown in an acidic medium. **B)** IκB protein expression in acidic melanoma cells treated with esomeprazole (100µM) for 24 hours; **C)** Inhibition by esomeprazole of VEGF-C mRNA of acidic melanoma cells. **D)** Inhibition by esomeprazole of NF-κB-dependent up-regulation of VEGF-C mRNA in melanoma cells treated with IL1β and/or an acidic media for 24 hours. Densitometric assessment in arbitrary units and representative PCR blots or western blots. Values presented are means ± SEM of at least three independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

Acidity and VEGF-C expression in breast and prostate carcinoma cells

In order to establish whether the effect of low pH on VEGF-C expression was limited to melanoma cells or a generalized effect, we used also others carcinoma cell lines for example DU145 prostate carcinoma cells and MCF-7 breast carcinoma cells. Fig.25 indicates that the stimulation of VEGF-C expression by acidity is not limited to melanoma cells, indeed mRNA levels of VEGF-C of breast (Panel A) and prostate carcinoma cells (Panel B) grown at acidic pH is significantly higher than levels of cells grown at standard pH.

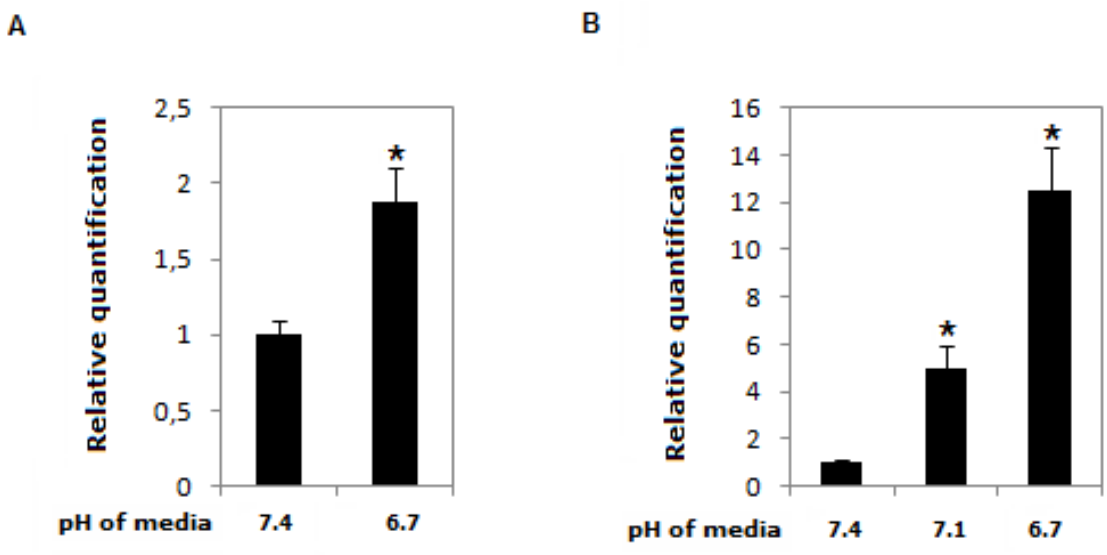


Figure 25- VEGF-C expression in breast and prostate carcinoma cells grown in an acidic medium. **A)** Quantitative real time PCR of VEGF-C mRNA of MCF-7 breast carcinoma cells incubated for 24 hours at pH 7.4 and 6.7; **B)** quantitative real time PCR of VEGF-C mRNA of DU145 prostate carcinoma cells incubated for 24 hours at pH 7.4, 7.1 and 6.7. Values presented are means ± SEM of at least two independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

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4.1.3. Acidity and the metabolic reprogramming

Metabolic profile of acidic cells

Ninety years ago, Otto Warburg published a body of work linking metabolism and cancer through enhanced aerobic glycolysis that distinguishes cancer from normal tissues (Warburg 1956; Hsu and Sabatini, 2008; Vander Heiden *et al.*, 2009; Koppenol *et al.*, 2011). The conversion of glucose to lactate, which can occur in hypoxic normal cells, persists in cancer tissues despite the presence of oxygen that would normally inhibit glycolysis through a process termed the Pasteur Effect. Therefore, most cancer cells exhibit increased glycolysis and use this metabolic pathway for generation of ATP as a main source of their energy supply. This phenomenon, known as the Warburg effect, is considered as one of the most fundamental metabolic alterations during malignant transformation. The phenomenon is characterized by increased glucose uptake and reliance on glycolysis for ATP production despite available oxygen source. After observing various differences in behavior of acidic cells compared with non-acidic cells, we decided to investigate whether these changes were also accompanied by a different metabolism. Cells can adapt to extracellular changes by regulating their transport across the plasma membrane and, subsequently, their compartmentalization and metabolism through alterations in the expression of genes involved in anaerobic glycolysis, oxidative phosphorylation and oxygen delivery (Mobasheri *et al*., 2005). Transport of glucose across the plasma membrane is the first rate-limiting step for glucose metabolism and is mediated by facilitative glucose transporter proteins (GLUTs). The up-regulation of these transporters may represent a key mechanism by which malignant cells may achieve increased glucose uptake to support the high rate of glycolysis. Elevated levels of GLUT1 have been shown in almost all human cancers (Macheda *et al.*, 2005). Also GLUT3 is expressed in human malignant tissue, but not so frequently as GLUT1. However, GLUT3 protein expression is indicator of poor prognosis outcome in non-small lung carcinoma (Younes *et al*., 1997), oral squamous cell carcinoma (Ayala *et al*., 2010) and laryngeal carcinoma (Baer *et al*., 2002). Both GLUT1 and GLUT3 glucose transporters are HIF-1α target genes. In our study we analyzed the expression of glucose transporters GLUT 1 and

GLUT 3 by Real Time PCR in A375M6 melanoma cells grown in acidic medium for 24 hours. We used also cells grown for 24 hours under hypoxia condition to compare the expression levels of these transporters. Figure 26, panel A, shows that, while the expression of GLUT1 slightly increases under hypoxia, its expression significantly decreases when melanoma cells are grown in acidic pH medium. GLUT1 mRNA is reduced to comparable levels even when the cells are treated with TGF-β, a multifunctional cytokine that induces epithelial-mesenchymal transition (Fig. 26, panel A). Also GLUT3 mRNA, which significantly increases in hypoxia, shows a clear reduction both when the cells are grown at acidic pH and when cells are treated with TGF-β (Fig. 26, panel B). Therefore acidic cells, as in general the cells that have undergone the EMT, appear to reduce the uptake of glucose.

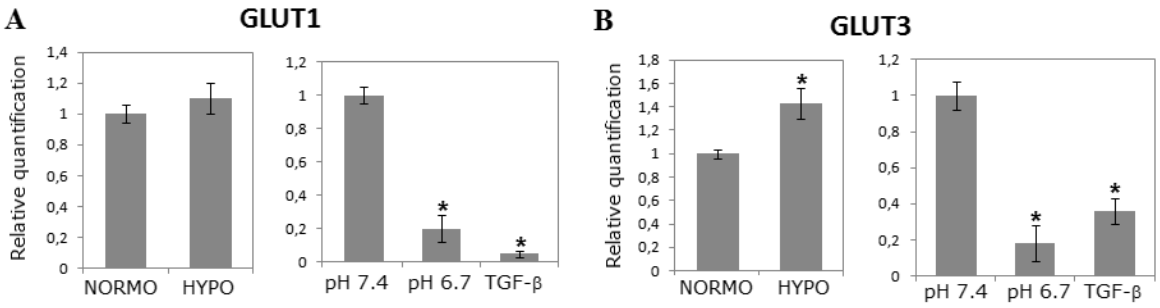

Figure 26- Expression of glucose transporters. Expression of GLUT1 **(A)** and GLUT3 **(B)** mRNA in A375M6 melanoma cells grown in normoxia, hypoxia or acidity for 24 hours, or treated by TGFβ 10 ng/ml. *$P < 0.05$ compared with control cells.

The use of lactate as an energy source requires the transport of lactate into and out of tumor cells by way of specific transporters, the monocarboxylate transporters (MCTs). Importantly for tumor cells, MCT1 and the hypoxia-induced MCT4 are specialized for the co-transport of lactate and H^+: exogenous lactate uptake by oxidative tumor cells occurs through the high-affinity lactate transporter MCT1, whereas glycolysis-derived lactate is released through the low-affinity lactate transporter MCT4 (Sonveaux *et al.*, 2008). High expression of MCT4 was found in glycolytic tissue, including several hypoxic and rapidly growing tumors (Pinheiro *et al*., 2012). We compared the protein expression analyzed by Western blot of MCT1 and MCT4 under hypoxia and acidic conditions. While MCT1 protein expression remained comparable to control levels in cells grown in hypoxia and

increased in cells grown at acidic pH (Fig. 27, panel A), MCT4 showed the opposite trend, increased in hypoxic cells and reduced in acid cells (Fig. 27, panel B).

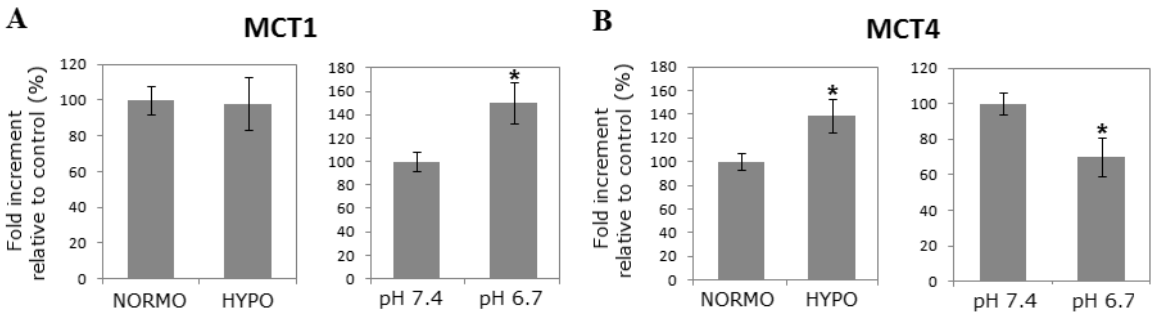

Figure 27- Expression of lactate transporters. MCT1 **(A)** and MCT4 **(B)** protein expression in A375M6 melanoma cells grown in normoxia, hypoxia or acidity for 24 hours. Data are expressed as percentage of values compared to the control; densitometry data presented are normalized to intensity of β-tubulin bands. **P* < 0.05 compared with control cells.

These data suggest that acidic cells, with a low capacity to capture glucose and release lactate and with high lactate uptake transporters expression, could be characterized by a lower glycolytic capacity compared to control cells and by an oxidative metabolism. A result that confirms this hypothesis is the reduced expression of the pyruvate kinase M2 isoform (PKM2) (Fig. 28), which plays a critical role in regulating anabolic metabolism and aerobic glycolysis and facilitates the lactate production in cancer cells (Mazurek *et al.*, 2011).

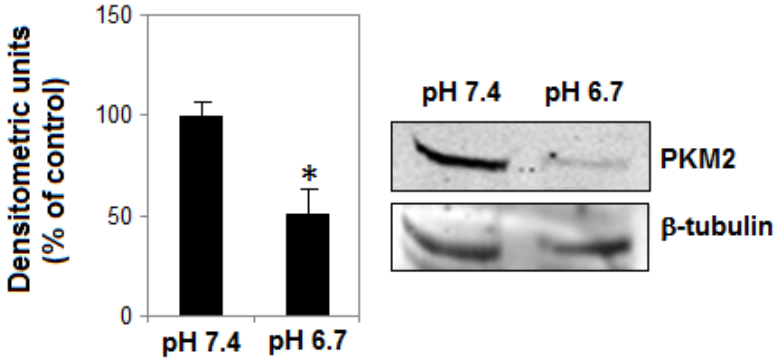

Figure 28- Western blot analysis of PKM2 expression in melanoma cells grown in acidic o non-acidic medium. Densitometry data presented are normalized to the intensity of β-tubulin bands. **P* < 0.05 compared with control cells.

We measured the concentration of lactate released in media by cells grown at low pH after 24 or 48 hours. Lactate concentration was normalized to the number of cells (Fig. 29, panel A) or to the protein concentration (Fig. 29, panel B) and in both cases the lactate released by acid cells was lower compared to the lactate released by control cells.

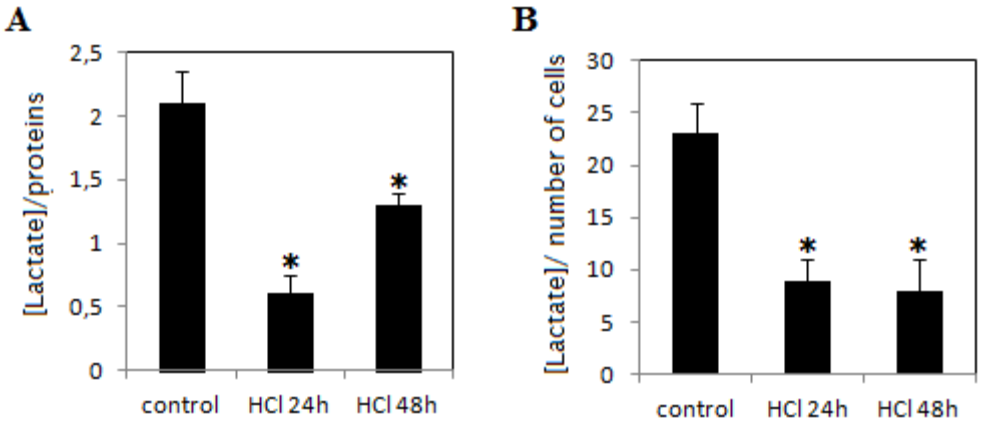

Figure 29- Assay of lactate extruded by acidic melanoma cells. A) Lactate extruded normalized on protein concentration. **B)** Lactate extruded normalized on number of cells. **P* < 0.05 compared with control cells.

We also evaluated lactate reconversion towards anabolic pathways (Fig. 30, panel A), through analysis of [14C]-proteins and respiration of lactate (Fig. 30, panel B) through analysis of released [14C]-CO2. The results clearly indicate that acidic medium induces a metabolic change in melanoma cells reducing the extrusion of lactate, the lactate incorporation into proteins and increasing respiration.

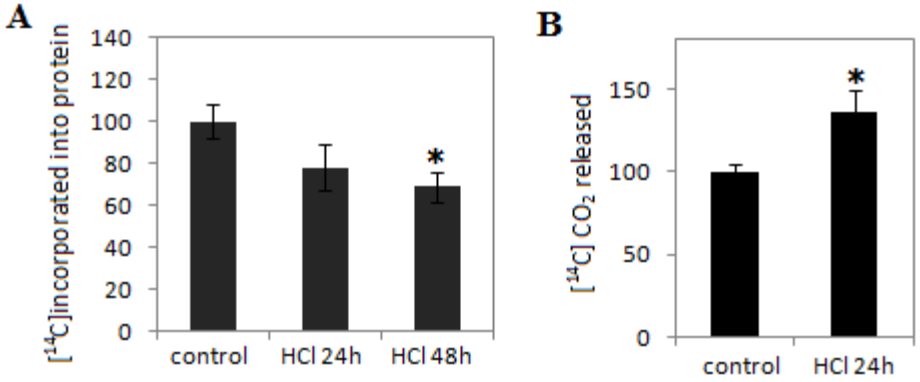

Figure 30- A) Incorporation of [14C] into proteins and **B)** respiration of [14C]-lactate, evaluated as [14C]-CO2 released. **P* < 0.05 compared with control cells.

Metformin inhibition

Acidity acts on melanoma cells not only promoting EMT and resistance to apoptosis but also induces a metabolic reprogramming shifting cell metabolism to oxidative phosphorylation. As cell metabolism of acidic cells may represent a potential therapeutic end point, we decided to test drugs interfering with the metabolism acquired by acidic melanoma cells, e.g. the preferential mitochondrial oxidative phosphorylation. In our experiments we used metformin to target oxidative phosphorylation of acidic melanoma cells. Metformin (N0,N0-dimethylbiguanide) is the most widely prescribed oral hypoglycemic agent used not only in type 2 diabetes, but also used in polycystic ovarian syndrome (PCOS), where insulin resistance is a key factor for the development of the metabolic disturbances. Several reports suggest that metformin acts through inhibition of mitochondrial respiration by inhibiting complex I of the electron transport chain and hence is an example of mitochondrial metabolic inhibitor blocking oxidative respiration (El-Mir *et al.,* 2000). This results in increased cellular AMP-to-ATP ratios and activates the AMP-activated protein kinase (AMPK) which increases GLUT1 expression to promote glucose uptake (Fryer *et al*., 2002) and phosphorylates phosphofructokinase-2 to promote energy generation through increased glycolysis (Marsin *et al*., 2000). Multiple epidemiological studies show that metformin is associated with decreased cancer incidence and cancer-related mortality and it has recently received increased attention for its potential antitumorigenic effects. For example, metformin inhibits the growth of a subpopulation of breast cancer cells and reduces their ability to form tumors in mice (Hirsch *et al.,* 2009). Interestingly, metformin may also be involved in regulating breast cancer-initiating cell ontogeny by transcriptionally repressing the process of epithelial to mesenchymal transition (EMT) (Vazquez-Martin *et al.,* 2010) and reduced the growth of a variety of tumor xenografts in mice including those established from breast and prostate cancer cells (Liu *et al.,* 2009), and suppressed the development of breast, colon and other tumors in transgenic mice (Anisimov *et al*., 2005; Tomimoto *et al*., 2008).

We treated acidic or non-acidic A375M6 melanoma cells with metformin (1-10 mM) for 24 hours and we evaluated cell vitality using the trypan blue exclusion test. We found that acidic cells are more sensitive to metformin treatment compared to control cells that do not die but show a proliferative arrest (Figure 31). For the following experiments we used

metformin 1 mM and we evaluated the ability of metformin to block the increase of invasiveness that melanoma cells show when are grown in an acidic medium.

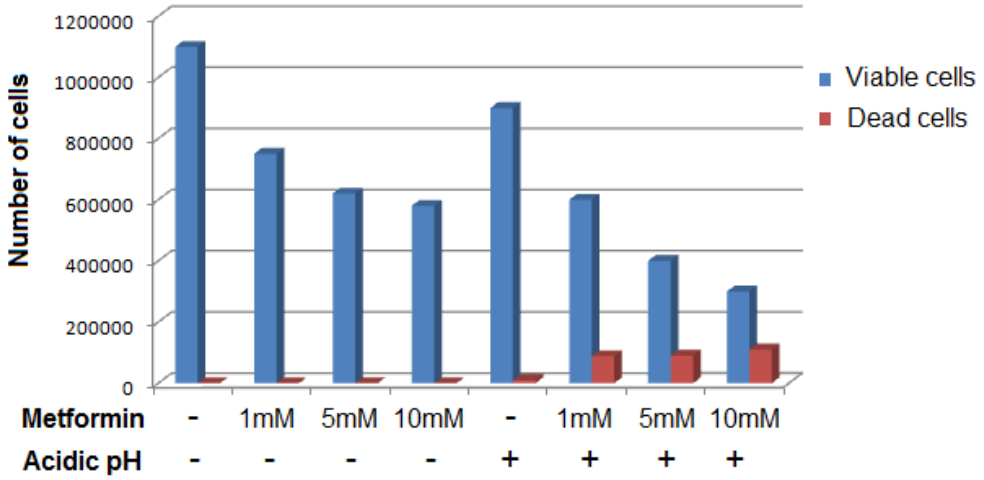

Figure 31- Viability of A375M6 cells grown in standard or acidic medium and treated with metformin at different concentrations (1mM, 5mM, 10mM).

As shown in figure 32, metformin 1mM blocks the enhanced invasiveness acquired by melanoma cells exposed to an acidic culture medium, but not the invasiveness of control cells.

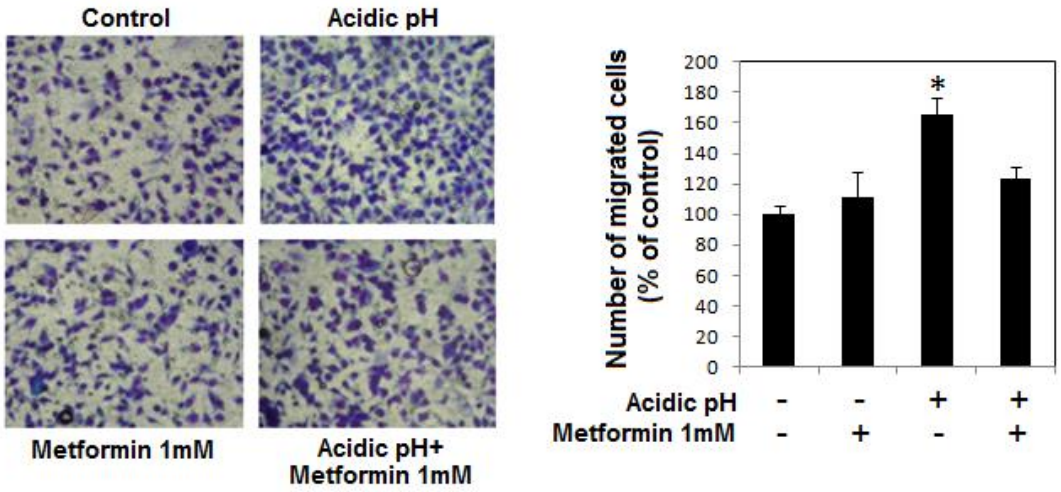

Figure 32- Metformin inhibits the invasiveness of acidic cells. Invasiveness of A375M6 cells grown in standard or in acidic medium, in the presence or absence of metformin 1mM, performed using Matrigel-coated filters. Migration is measured as a percentage of the control. *P < 0.05 compared with control cells.

Phenotypic features of cells treated with lactate

Since the cells grown at acidic pH showed an increase in uptake of lactate (Figure 27) we investigated what are acidic cells using lactate for, what phenotipic features are they changing. A375M6 cells were grown in acidic or non-acidic medium; after 24 hours, media were changed and standard media with or without lactate were added to the flask for other 24 hours. Sodium lactate was used at a concentration of 10 mM which corresponds to the range of lactate detected in tumors (Walenta *et al*., 2001). We first observed the morphology of cells treated with lactate that was unchanged compared to control cells or to acidic cells not treated with lactate (Figure 33).

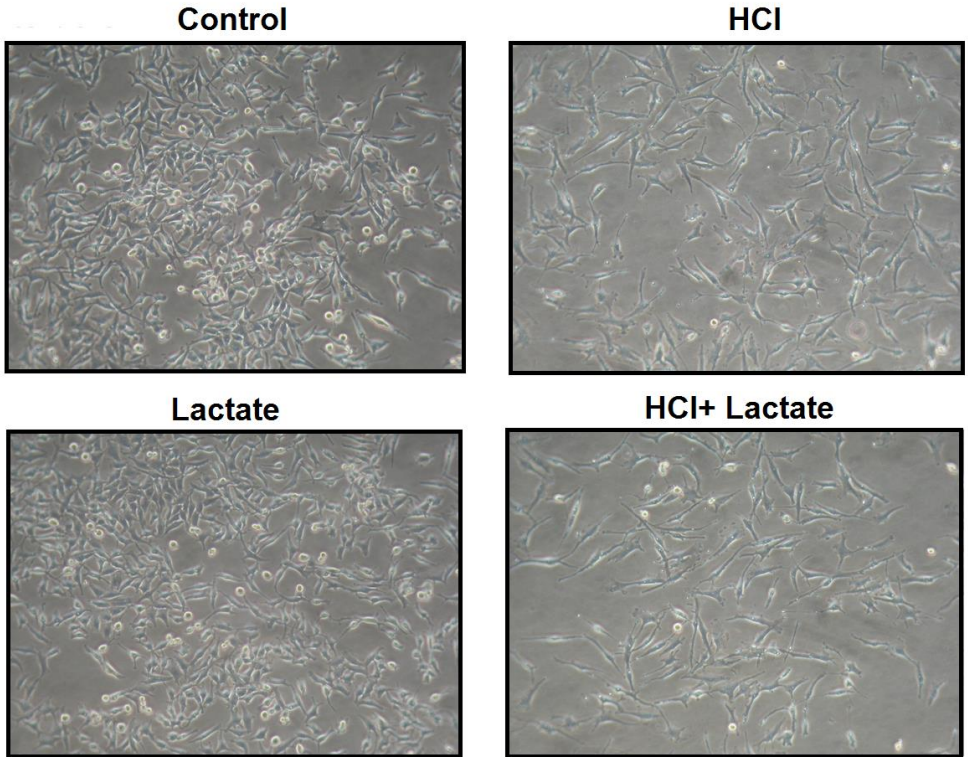

Figure 33- Effect of lactate on cell morphology. Cell morphology of A375M6 human melanoma cells grown in standard or acidic medium for 24 hours and then treated with lactate 10 mM for other 24 hours.

Cell growth analysis showed that lactate is not used by the cells to increase their proliferative capacity, but on the contrary, cell growth decreases when acidic or non-acidic cells are treated with lactate (Figure 34).

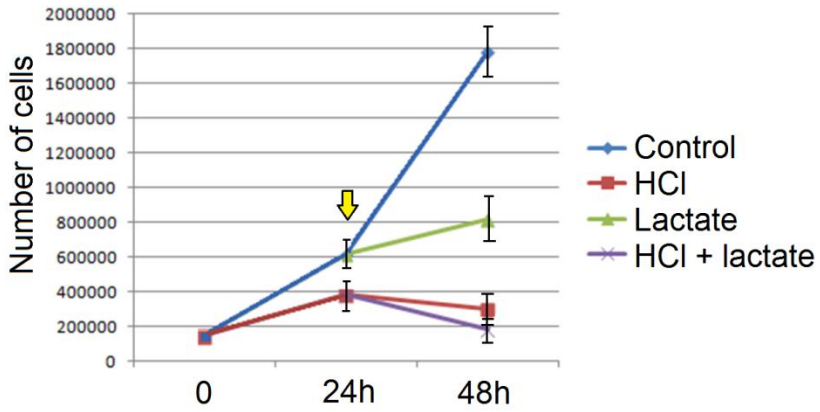

Figure 34- Effect of lactate on cell growth. Growth curve of A375M6 melanoma cells grown in standard (control) or acidic (HCl) medium for 24 hours (0-24h) and then grown in standard medium in the presence or absence of lactate (24-48h). The yellow arrow indicates the time at which the medium was changed and the lactate was added to the cultures.

Other characteristics that we examined in cells treated with lactate were the invasive capacity through Matrigel-coated filters and the motility, using the wound healing assay. Figure 35 shows that non-acidic melanoma cells treated with lactate and re-suspended in their conditioned medium express a higher ability to invade Matrigel filters that was inhibited when the migration occurred in the presence of Ilomastat. Also acidic cells treated with lactate show invasiveness stimulated compared to control, but not significantly higher than invasiveness of acidic cells.

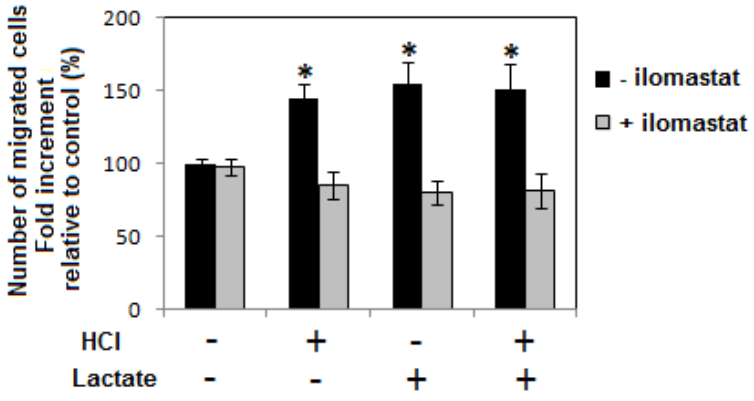

Figure 35- Effect of lactate on cell invasiveness. Invasiveness of A375M6 cells grown in standard or in acidic medium for 24 hours and then grown in standard medium in the presence or absence of lactate for other 24 hours. Migration was performed in the presence or absence of ilomastat and was measured as a percentage of the control. *P < 0.05 compared with control cells.

Acidic or non-acidic cells treated with lactate were grown to subconfluence and scraped with a sharp edge to make a cell-free area that was observed for 24 hours. Lactate appears to be very efficient in stimulating cell motility of both acidic and non-acidic cells (Fig.36). Cells grown in acidic medium are less able to move (percent closure = 27%) compared to cells grown in medium at standard pH (percent closure = 45%), but when acidic cells are treated with lactate 10 mM reach a percent wound closure (42%) comparable to the control. Non-acidic cells treated with lactate appear to be the cells with the greatest ability to move (percent closure = 70%).

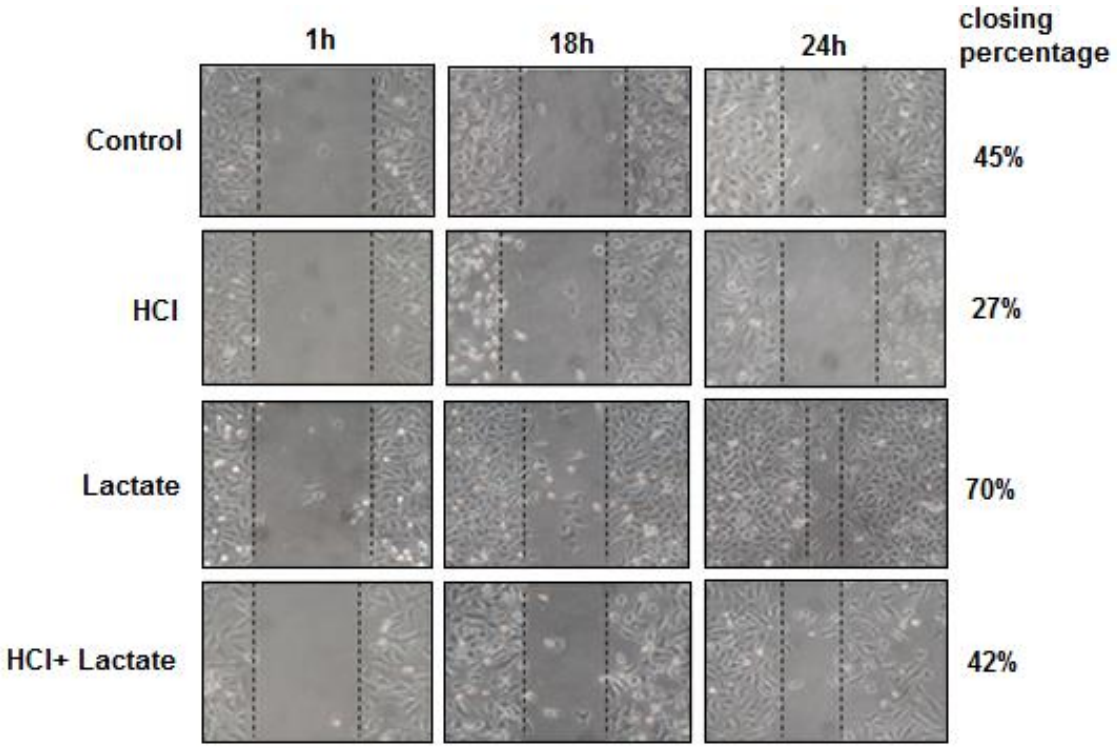

Figure 36- Effect of lactate on cell motility. Wound healing assay of A375M6 melanoma cells grown in standard or in acidic medium for 24 hours and then grown in standard medium in the presence or absence of lactate for other 24 hours. The wound closure was evaluated 18 hours and 24 hours since its creation.

Finally, we tested the effect of lactate on EMT induction in A375M6 melanoma cells. We analyzed by Real Time PCR the mRNA expression of N- Cadherin, a mesenchymal marker, and E-Cadherin, an epithelial marker. An increase of N-cadherin and a reduction of E-cadherin expression are observable in cells grown in acidic medium (as already showed by western blot in figure 4, panel C) and in cells treated with lactate; these effects

are even more evident when cells are grown in acidic medium and then treated with lactate (Fig.37).

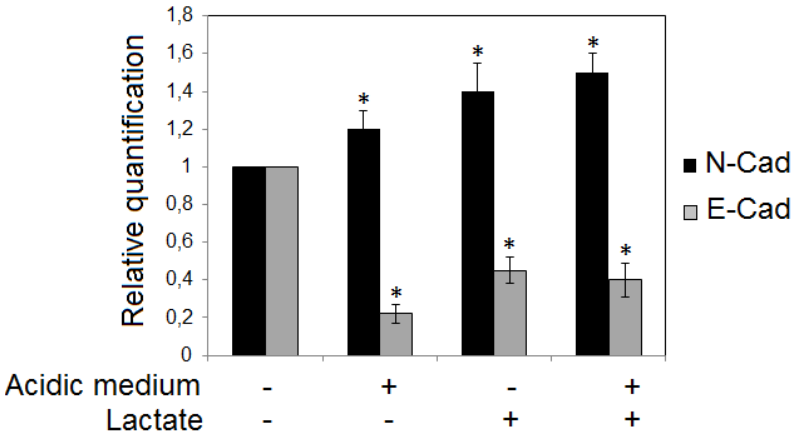

Figure 37- Effect of lactate on EMT induction. Quantitative real time PCR of N-Cadherin and E-Cadherin mRNA of A375M6 human melanoma cells grown in standard or in acidic medium for 24 hours and then grown in standard medium in the presence or absence of lactate for other 24 hours. *P < 0.05 compared with control cells.

Therefore, treatment with lactate reduces cell growth but induces in acidic cells a more marked EMT and an increased motility, while the invasive capacity remains comparable to the levels of acidic cells not treated with lactate. These effects could contribute to increase the aggressiveness of acidic cancer cells.

Targeting MCT1 inhibits lactate-induced motility and invasiveness

It was found that lactate, the end-product of glycolysis, is the keystone of an exquisite symbiosis in which glycolytic and oxidative tumor cells mutually regulate their access to energy metabolites. For example, lactate released as the end-product of glycolysis in the hypoxic tumor cell compartment, prominently fuels the oxidative metabolism of the oxygenated tumor cell subpopulation, thereby sparing glucose for glycolytic cells (Sonveaux *et al*., 2008). Another example of symbiosis in which glycolytic and oxidative tumor cells mutually regulate their access to energy metabolites was demonstrated by

Fiaschi and collegues: fibroblasts in contact with epithelial cancer cells undergo myofibroblast differentiation and produce lactate through aerobic glycolysis and Warburg metabolism which is used by cancer cells for respiration (Fiaschi *et al.*, 2012). We therefore thought that tumor microenvironment might facilitate a symbiosis between non-acidic/glycolytic cells that use glucose and produce lactate, and acidic cells that capture the lactate produced by non-acidic cells (a process mediated by MCT1) and oxidize it to produce energy increase their aggressiveness. This metabolic symbiosis could be disrupted by MCT1 inhibition using α-cyano-4-hydroxycinnamate (CHC), a drug known to reversibly inhibit MCT1 with approximately 10-fold selectivity compared with other MCTs (Manning *et al.*, 2000).

First, we tested different concentrations (10mM, in figure 38, panel A; 5mM, 1mM and 500 μM, in figure 38, panel B) of CHC on acidic and non-acidic cells to observe the cell viability and choose the best concentration which was the 5 mM (the 10 mM slightly reduced cell viability of non-acidic cells but killed most of cells grown in acidic medium, Figure 38 A).

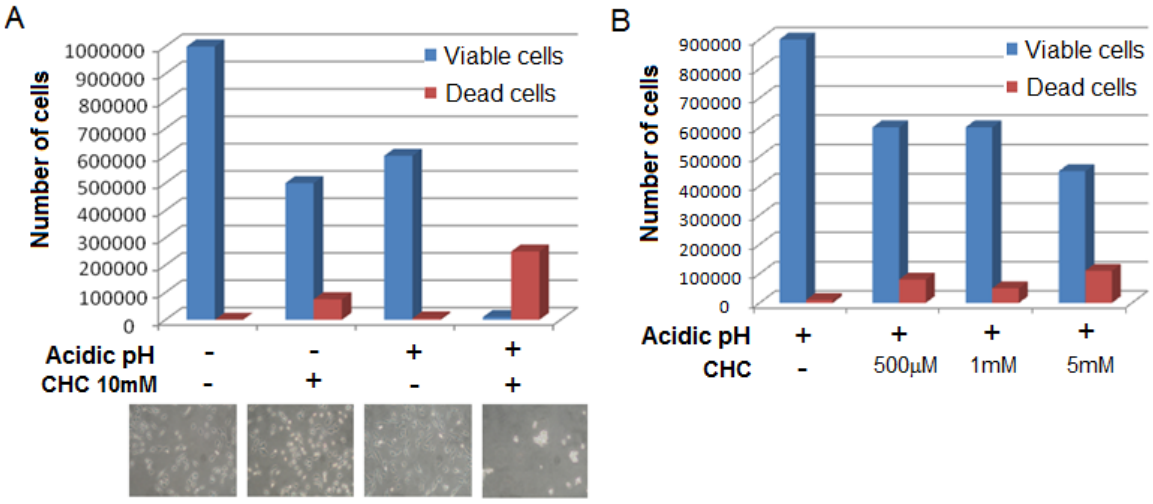

Figure 38- Viability of A375M6 cells grown in standard or acidic medium and treated with CHC at different concentrations: A) 10 mM**, B)** 500 mM, 1 mM, 5 mM.

We next investigated whether treatment with CHC 5mM was able to block lactate-induced motility and invasiveness. Lactate-induced motility was quantified in vitro using the wound healing assay. Figure 39 , panel A, shows that lactate added to the culture medium

stimulates highly the wound closure (closure = 100%) and treatment with CHC blocks this effect (closure = 30%) reducing the wound closure to the level of control cells (closure = 34%). The treatment with CHC does not appear to affect the motility of control cells (closure = 35%). Also the analysis of the invasive capacity showed that CHC has no effect on the control cells, but is able to block not only the increased invasiveness of cells treated with lactate, but also the invasiveness of cells grown in acidic medium and of cells treated with both acidic medium and lactate (Figure 39, panel B).

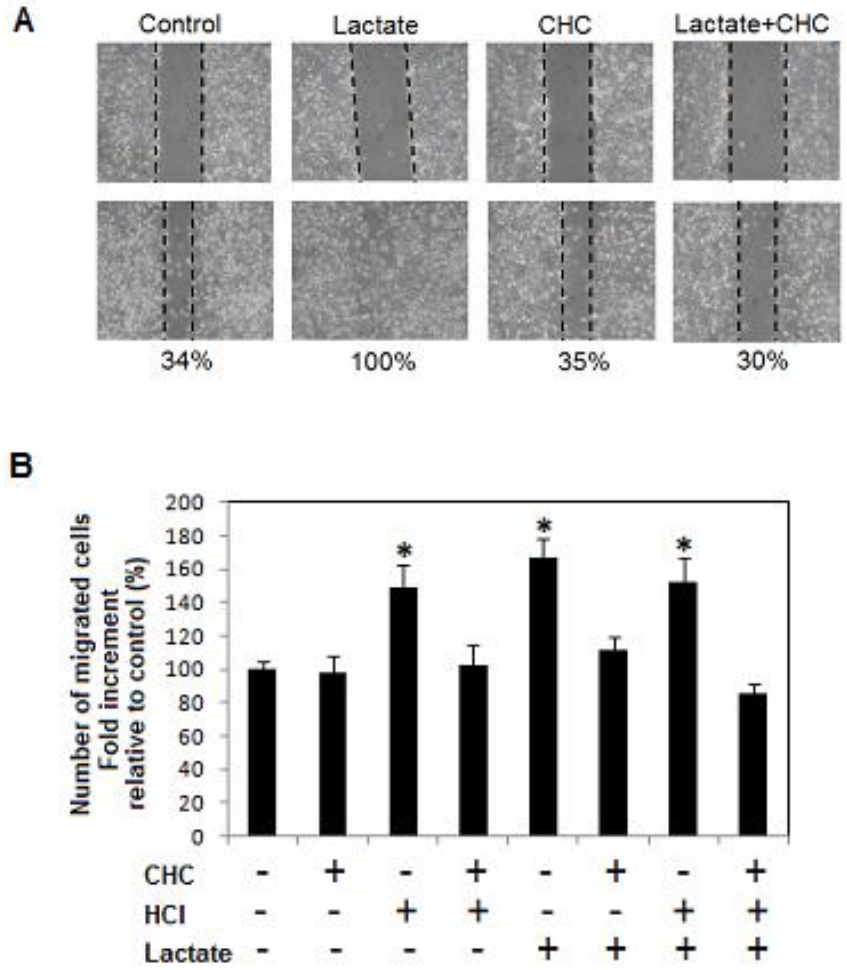

Figure 39- CHC inhibits the lactate-dependent stimulation of motility and invasiveness of melanoma cells. A) Wound healing assay of A375M6 melanoma cells grown in the presence of lactate 10mM and treated with CHC 5mM. The wound closure was evaluated 24 hours since its creation. **B)** Invasiveness of A375M6 cells grown in standard or in acidic medium for 24 hours and then grown in standard medium in the presence or absence of lactate (10 mM) and/or CHC (5mM) for other 24 hours. Migration is measured as a percentage of the control. *P < 0.05 compared with control cells.

Finally, we added to acidic or non-acidic cells medium conditioned by control cells, that we think contains lactate, and we evaluated cell motility using the wound healing assay. Figure 40 confirms the low ability of acidic cells to close the wound (12% of closure), compared to control cells (39% of closure). Medium conditioned for 24 hours by control cells does not affect the motility of control cells (from 39% to 41%) but is able to stimulate the motility of acidic cells (from 12% to 38% of closure). This stimulation is blocked when CHC, the inhibitor of MCT1, is added to the medium (from 38% to 10% of closure), confirming the hypothesis that the lactate produced by non-acidic cells may stimulate acidic cells and that this cross talk could be inhibited by CHC.

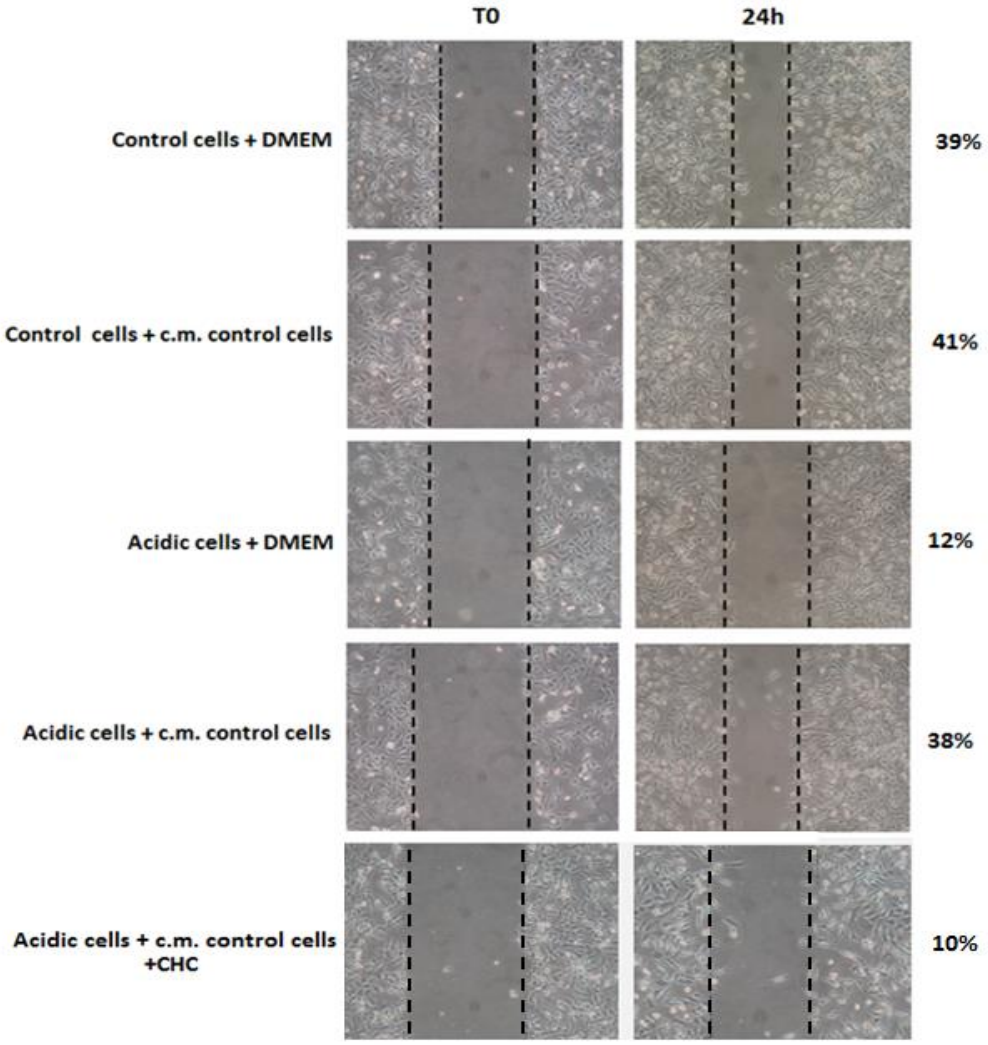

Figure 40- CHC inhibits the stimulation of motility of acidic melanoma cells caused by non-acidic cells medium. Wound healing assay of acidic or non-acidic A375M6 melanoma cells grown medium conditioned by non-acidic cells and treated with CHC 5mM. The wound closure was evaluated 24 hours since its creation.

4.2. Role of acidic mesenchymal stem cells in human melanoma malignancy

Acidic MSC drive an EMT program in A375M6 human melanoma cells

It is well known that tumors are composed of malignant tumor cells and nonmalignant benign cells. The "benign" tumor compartment includes blood vessels, infiltrating inflammatory cells, and stromal cells such as fibroblasts and mesenchymal stem cells (MSC). MSC are multipotent precursors endowed with the ability to differentiate into a variety of mesenchymal cells, including osteoblasts, chondrocytes, adipocytes, muscle cells, pericytes, reticular fibroblasts, and even neural cells (Chamberlain *et al.*, 2007). Large number of MSC is recruited into the stroma of developing tumors and recent evidences suggest that these cells play a role in facilitating cancer progression, influencing the behavior and aggressiveness of tumor cells (Karnoub *et al.*, 2007; Jing *et al*., 2012). Indeed, the relationship between MSC and tumor cells appears dual: primary and metastatic tumors attract MSC in their microenvironment where they affect tumor cell survival, angiogenesis, motility and invasiveness (Feng and Chen, 2009); vice versa in the bone marrow MSC attracts tumor cells and contribute to a microenvironment that promotes osteolysis, tumor growth, survival, and drug resistance (Bergfeld and DeClerck, 2010). Moreover, MSC are an important source of inflammatory cytokines that affect tumor and immune cells, thus have an immunomodulatory function (Sotiropoulou and Papamichail, 2007).

Our past studies revealed that an acidic pH medium promotes in human melanoma cells clear markers of EMT, such as a mesenchymal morphology, N-cadherin, vimentin and Twist expression, higher resistance to apoptosis and cell invasiveness. Thus, after showing that the acidity induces a more aggressive phenotype of melanoma cells, we proposed to investigate its role in the interaction between tumor cells and mesenchymal stem cells. We exposed MSC for 24 hours to a pH 6.7-6.8 acidified medium or to a pH 7.4 standard medium. Media conditioned for 24 hours by MSC grown in standard or acidic medium were used to grow A375M6 human melanoma cells for other 24 hours. First, we examined A375M6 morphology and we observed that both cells grown in medium conditioned by

acidic MSC and cells grown in medium conditioned by non-acidic MSC showed a longer morphology, similar to mesenchymal cells, compared with cells grown in standard medium (Fig.41, panel A). It was evaluated also the proliferative capability of these cells and Figure 41, panel B shows that cells grown in medium conditioned by acidic MSC proliferate more than control cells but lower than cells grown in medium conditioned by non acidic MSC.

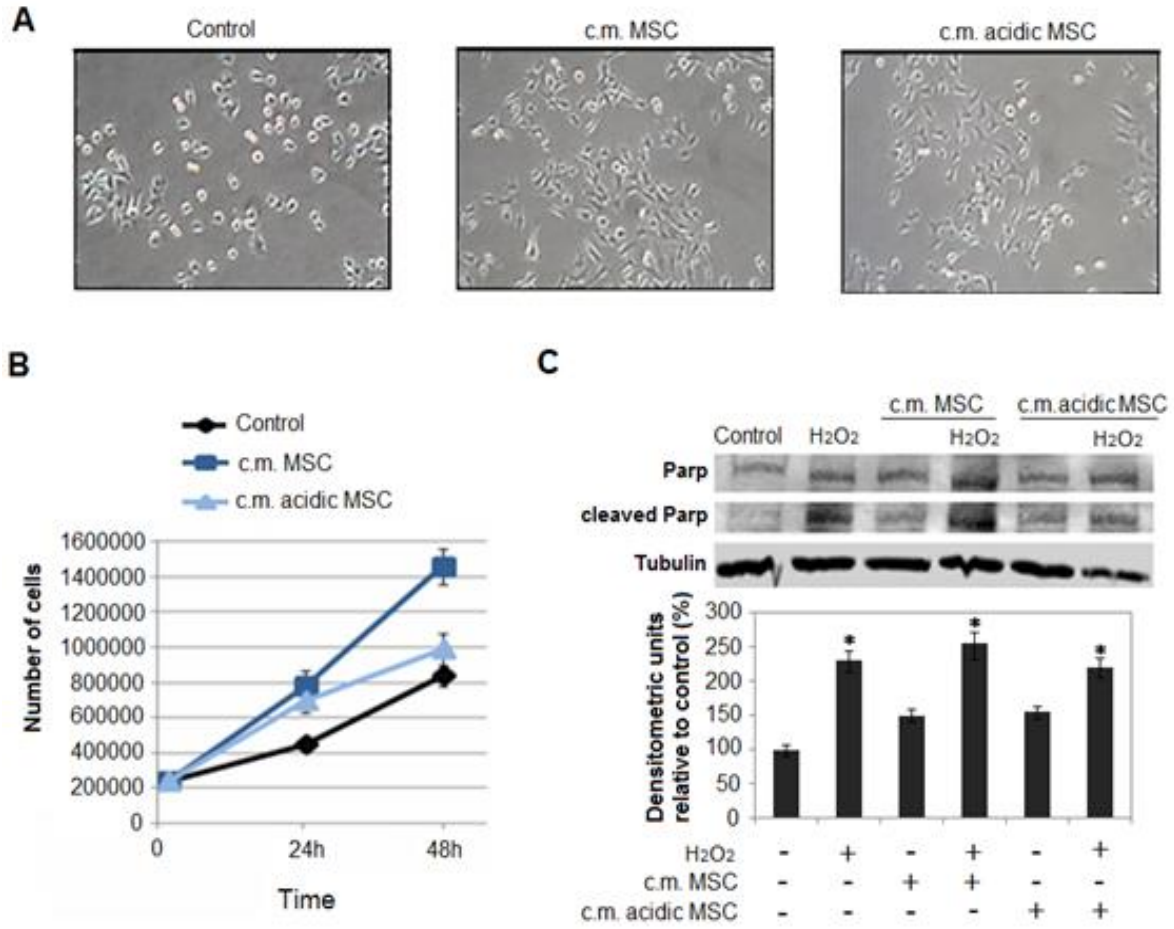

Figure 41: **Characteristics of A375M6 melanoma cells grown in media conditioned by acidic or non-acidic MSC**. **A)** Cell morphology of A375M6 human melanoma cells grown in standard medium (control), in a medium conditioned by MSC (c.m. MSC) or by acidic MSC (c.m. acidic MSC) and their respective growth curves (**B**). **C**) Western blot analysis of PARP expression in melanoma cells grown in medium conditioned by acidic or non-acidic MSC and treated with H_2O_2. Densitometry data presented are normalized to the intensity of β-tubulin bands. *$P < 0.05$ compared with control cells.

As we found that acidic pH induced in A375M6 cells resistance to the pro-apoptotic agent H_2O_2, we tested whether medium conditioned by acidic MSC would induce the same protection. We treated cells grown in conditioned media with H_2O_2 200 µM for 6 hours and we evaluated the apoptotic activity through cleaved parp fragment expression. Data show that media conditioned by acidic or non-acidic MSC, do not confer to A375M6 cells increased resistance to apoptosis (Fig.41, Panel C). To test the effect of medium conditioned by acidic MSC on EMT induction in A375M6 melanoma cells, we analyzed by Real Time PCR analysis the mRNA expression of some EMT markers as N- Cadherin, a mesenchymal marker, and E-Cadherin, an epithelial marker. An increase of N-cadherin and a reduction of E-Cadherin expression are already observable in cells grown in medium conditioned by non-acidic MSC; these changes are more evident and significant when A375M6 are grown in medium conditioned by acidic MSC (Fig.42, panel A).

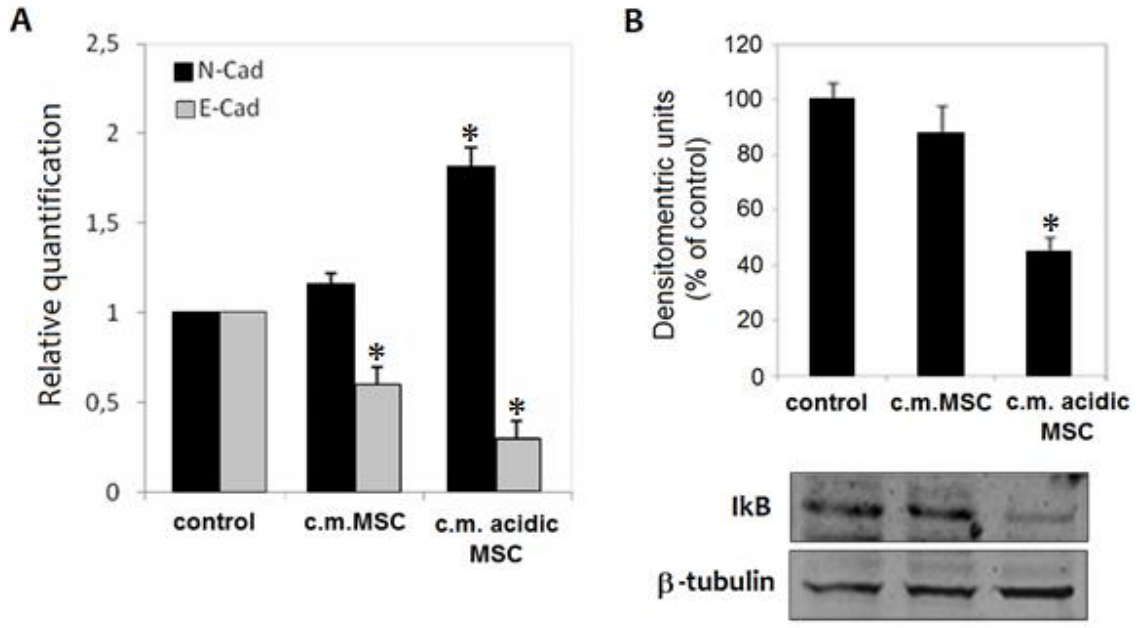

Figure 42: **Acidic MSC drive an EMT program in A375M6 human melanoma cells. A**) Quantitative real time PCR of N-Cadherin and E-Cadherin mRNA of A375M6 human melanoma cells grown in standard medium or a medium conditioned by MSC or by acidic MSC. **B**) **)** IκB protein expression in A375M6 human melanoma cells grown in standard medium or a medium conditioned by MSC or by acidic MSC. Values presented are means ± SEM of at least two independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

As in past experiments we observed the involvement of NF-κB in the acid-induced EMT, we analyzed the expression of IkB, the NF-κB inhibitor, in A375M6 cells grown in media conditioned by acidic or non-acidic MSC. Level of IκB was significantly decreased in cells grown for 24 hours in medium conditioned by acidic MSC (Fig.42, panel B). Thus, it is probable that medium conditioned by acidic MSC is capable of inducing in melanoma cells an EMT program via NF-κB pathway.

Another feature that we evaluated was the invasive ability, often associated with the epithelial-mesenchymal-transition. We tested the ability of A375M6 cells grown in media conditioned by acidic or non-acidic MSC, to migrate through filters coated with a reconstituted basement membrane of Matrigel and we found that melanoma cells grown in medium conditioned by acidic MSC express a higher ability to invade Matrigel filters (Fig.43, panel A) that was inhibited when the migration occurred in the presence of Ilomastat, a broad MMP inhibitor.

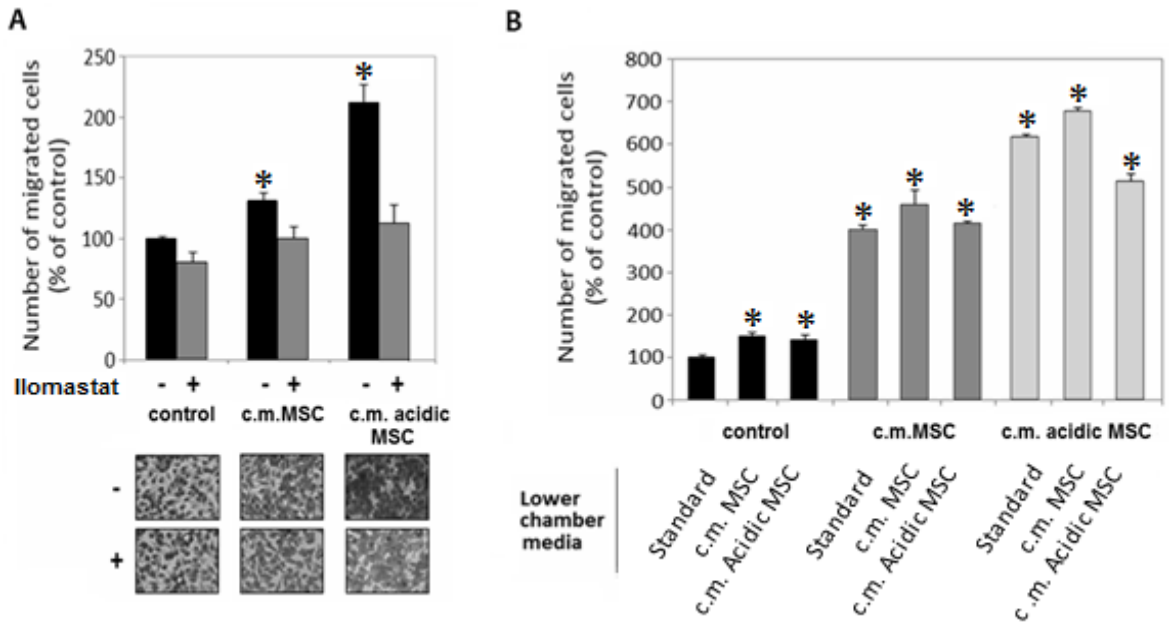

Figure 43- Invasiveness and migration of melanoma cells grown in media conditioned by acidic or non-acidic MSC. A) Invasiveness of A375M6 cells grown in media conditioned by acidic or non-acidic MSC performed using Matrigel-coated filters. **B)** Boyden chamber migration assay of A375M6 melanoma cells grown in standard medium or in medium conditioned by acidic or non-acidic MSC. Lower compartment contained standard medium (with 10% FCS) or medium collected from acidic or non-acidic MSC, used as chemoattractant. Data represent the mean ± SEM of at least two independent experiments. Asterisk indicates $p<0.05$.

The motility was tested using Boyden chambers and conditioned media used to grow A375M6 cells were also used as chemoattractant. This test demonstrated that medium conditioned by acidic MSC was able to stimulate A375M6 motility, but not their chemotaxis (Fig.43, panel B). In fact, changing media in lower chambers (used as chemoattractant), the motility of cells grown in media conditioned by acidic MSC remained unchanged. The only cells that proved to be sensible to the change of medium on the lower chamber were the control cells. Panels A and B in figure 43 show that also medium conditioned by non-acidic MSC can stimulates both invasiveness and motility, but the effect of medium conditioned by acidic MSC is much higher.

Finally, we investigated the expression of mRNA for the TGF-β and his receptors and we observed that the mesenchymal phenotype of A375M6 cells grown in medium conditioned by acidic MSC is also characterized by an increased mRNA for TGF-β (Fig.44, panel A) and TGFβR1 and R2 while no significant difference was observed for the TGFβR3 expression levels (Fig.44, panel B).

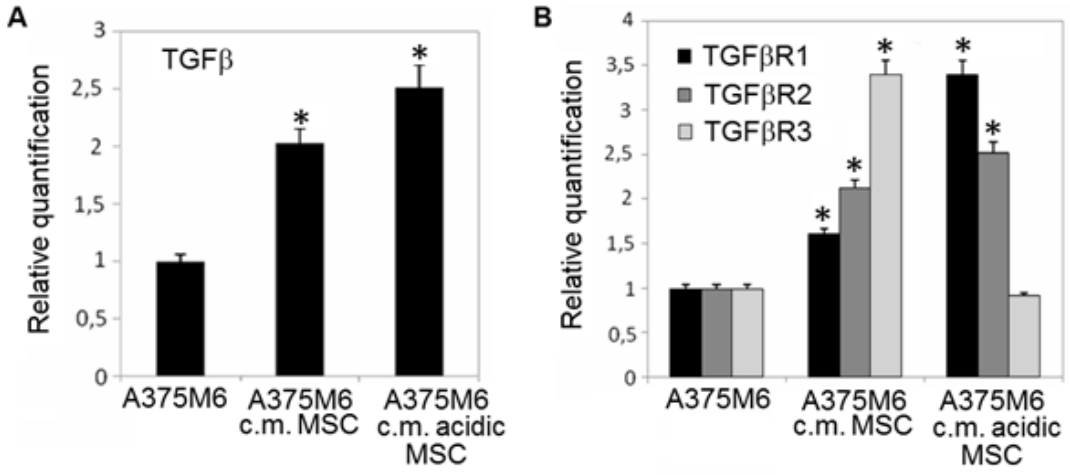

Figure 44- Quantitative real-time PCR of TGF-β (A) and TGFβ receptors (B) in melanoma cells grown in medium conditioned by acidic or non-acidic MSC. Values presented are means ± SEM of two independent experiments. Asterisk indicates $p<0.05$ compared with control cells.

TGF-β as a possible pro-EMT factor secreted by acidic MSC

After the observation that medium conditioned by acidic MSC is able to induce different phenotypic changes similar to those that occur during the EMT, we hypothesized that MSC grown at acidic pH produce something which can then act on tumor cells inducing more aggressiveness. Studies conducted in rodents, primates and humans have provided evidence that proinflammatory cytokines may play an important role in the regulation of aggression behavior and cytokines and that growth factors produced by MSCs are able to affect angiogenesis, cellular migration and apoptosis (Boomsma *et al.*, 2012). We evaluated the expression of several cytokines (such as IL-6, IL1β, TGF-β, TNF-α) in MSC treated with acid for 24 hours. It resulted that IL-6 was not expressed (data not shown), IL-1β expression decreased in acid cells (Fig.45, panel A) and TNF-α was highly expressed but its expression didn't change in acidic MSC compared to the control (Fig.45, panel B). The only one factor that increased his expression in acidic MSC was TGF-β (Fig.45, panel C). Therefore, we hypothesized TGF-β could be the factor secreted by acidic MSC and involved in the changes of melanoma cell behaviors.

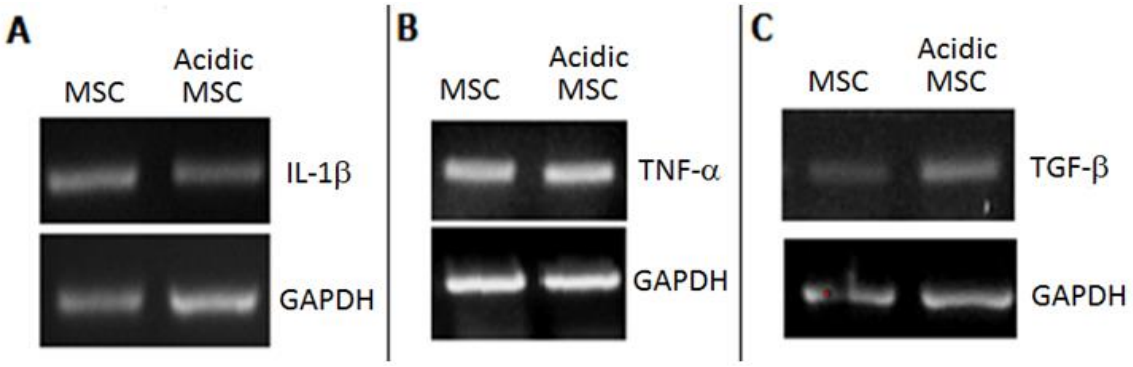

Figure 45- Cytokines mRNA expression in MSC grown in acidic medium. A) IL-1β, **B)** TNF-α, and **C)** TGF-β.

To confirm our hypothesis we treated tumor cells with 10 ng/ml exogenous TGF-β and we evaluated some phenotypic traits previously analyzed in cells grown in media conditioned by acidic or non-acidic MSC. A375M6 cells treated with TGF-β expressed higher level of

TGF-β mRNA expression (Fig.46, panel A) and higher levels of EMT markers as N-Cadherin and Snail, while mRNA level of E-Cadherin was significantly reduced (Fig.46, panel B). To examine the effect of TGF-β on cell invasion, Matrigel-coated transwells were used. As shown in figure 46, panel C, treatment with TGF-β significantly induced A375M6 cell invasion, which was inhibited by Ilomastat. Thus, TGF-β is probably the factor that participates to the elicited EMT program promoted in melanoma cells by medium conditioned by acidic MSC.

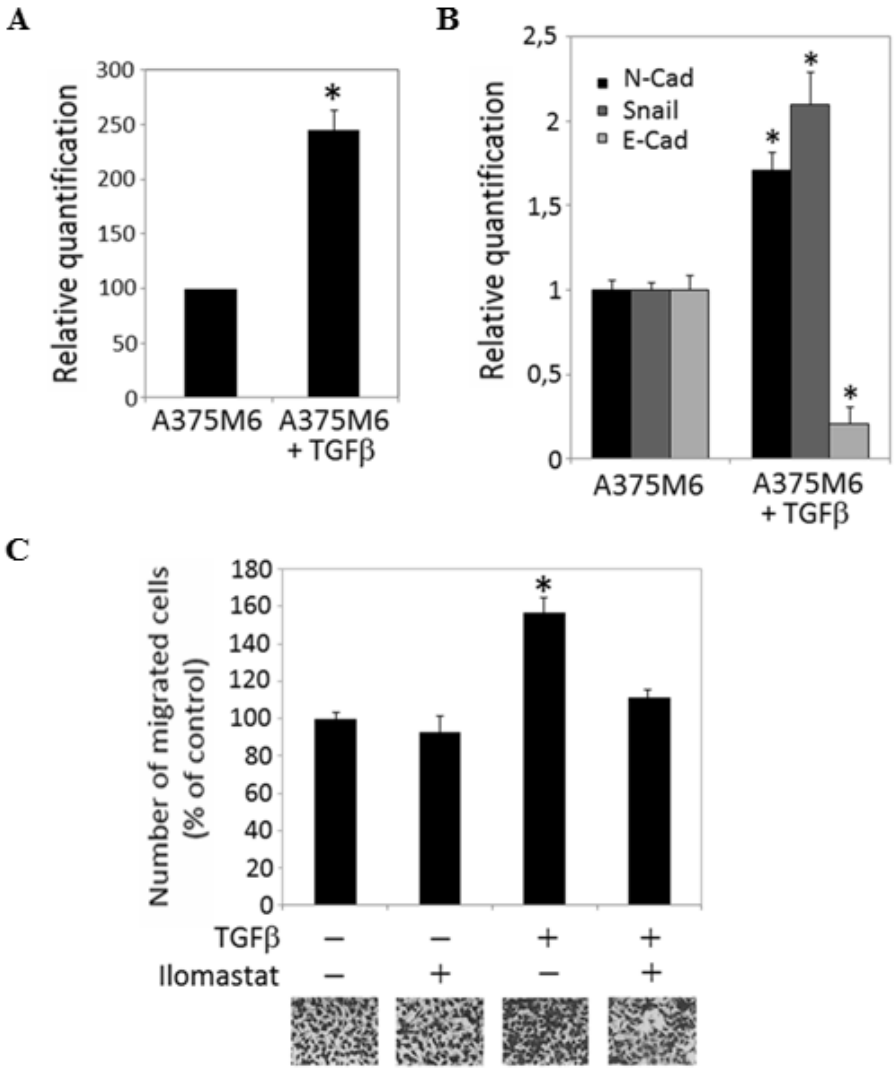

Figure 46- TGF-β is a pro-EMT factor. A) Quantitative real-time PCR of TGF-β in melanoma cells treated with TGFβ 10 ng/ml for 24 hours. **B)** Quantitative real-time PCR of N-Cadherin, Snail and E-Cadherin in melanoma cells treated with TGFβ 10 ng/ml for 24 hours. **C**) Invasiveness of A375M6 cells treated with TGFβ 10 ng/ml, in the presence or absence of Ilomastat and performed using Matrigel-coated filters. **P* < 0.05 compared with control cells.

Esomeprazole inhibition of in vitro aggressiveness of melanoma cells conditioned by acidic MSC

Since acidity of tumor environment appears to contribute to cancer aggressiveness, chemo- and radiation-resistance and evasion of immune reactions, strategies to interfere with H^+ dynamics could provide a new strategy for anticancer therapeutics. An option is the use of agents that inhibit the function of pumps generating the pH gradient, such as vacuolar H(+)-ATPases (V-ATPases). A class of V-ATPase inhibitors, called proton pump inhibitors (PPIs), has emerged as the drug class of choice for treating patients with peptic diseases. These drugs (including omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole) inhibit gastric acid secretion by targeting the gastric acid pump, but they also directly inhibit V-ATPases. For our experiments we used esomeprazole 100 μM. MSC were grown for 24 hours in standard medium or in acidic medium in the presence or absence of esomeprazole.

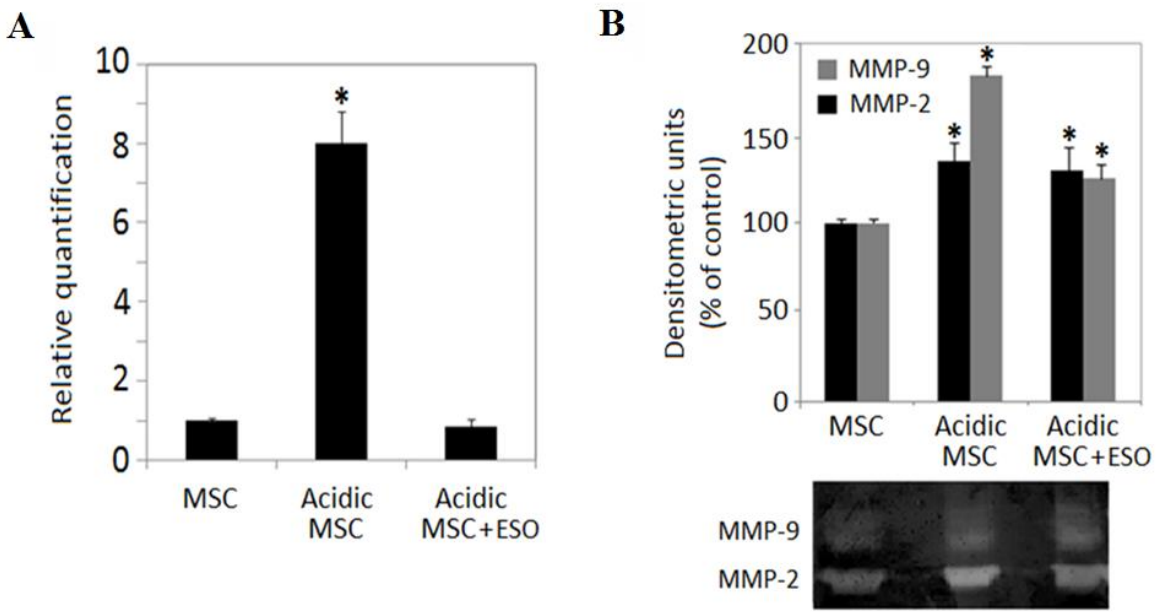

Figure 47- Esomeprazole inhibits activation of acidic MSC. A) Quantitative real-time PCR of TGF-β in MSC grown in acidic medium for 24 hours in the presence or absence of esomeprazole (ESO) 100 μM. **B)** Gelatin zymography of media conditioned by MSC grown in standard medium or in acidic medium in the presence or absence of esomeprazole 100 μM. Bar graph derived from densitometric analysis of MMP-9 and MMP-2. Data represent the mean ± SEM of at least two independent experiments. *$P < 0.05$ compared with control cells.

When we analyzed mRNA level of TGF-β using real time PCR, we found that the increased level of TGF-β in acidic MSC was reduced when acidic MSC were treated with esomeprazole (Fig.47, panel A). Moreover, gelatin zymography of media conditioned by acidic MSC revealed an increased MMP2 activity that was inhibited when acidic cells were treated with esomeprazole (Fig.47, panel B).

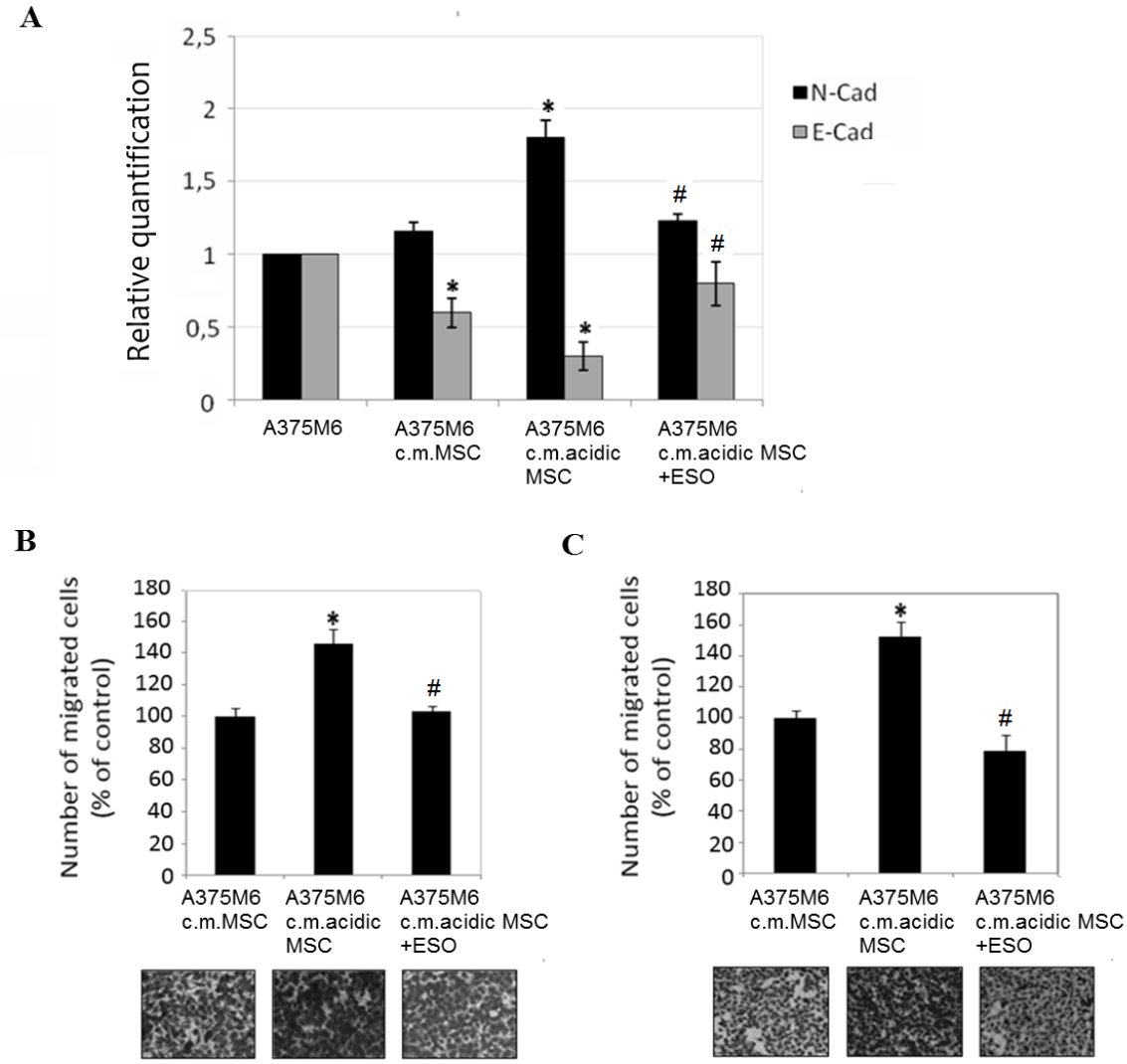

Figure 48- Esomeprazole inhibition of in vitro aggressiveness of melanoma cells conditioned by acidic MSC A) Quantitative real time PCR of N-Cadherin and E-Cadherin mRNA of A375M6 human melanoma cells grown in standard medium or in a medium conditioned by MSC grown in their turn in standard medium, in acidic medium or in acidic medium in the presence of esomeprazole 100 μM. **B)** Invasiveness and **(C)** migration of human melanoma cells grown in medium conditioned by non-acidic or acidic MSC treated with esomeprazole 100 mM. *$P < 0.05$ compared with control cells. #< 0.05 compared with cells grown in medium conditioned by acidic MSC.

We therefore used esomeprazole to treat MSC, and their conditioned media were used to grow tumor cells. We investigated whether esomeprazole, with which acidic mesenchymal cells were treated, was able to block the stimulatory effect of medium conditioned by the same acidic MSC on cancer cells. The increased of N-Cadherin and the reduction of E-Cadherin due to the treatment of A375M6 cells with medium conditioned by acidic MSC were lower when acidic MSC conditioning media were treated by esomeprazole (Fig.48, panel A). In addition, also the enhanced invasiveness (Fig.48, panel B) and motility (Fig.48, panel C) of acidic melanoma cells grown in medium conditioned by acidic MSC were significantly inhibited when A375M6 were grown in medium conditioned by acidic MSC treated by esomeprazole.

Esomeprazole inhibition of in vivo aggressiveness of melanoma cells/acidic MSC

To investigate *in vivo* effect of acidic MSC on tumor growth, human melanoma cells and human MSC were used in mouse models. A375M6 cells ($1x10^6$) and acidic or non-acidic MSC ($0,5x10^6$) were mixed together and subcutaneously implanted into immunodeficient mice. Tumors were measured every two-three days after their appearance. Starting from the twelfth day from the subcutaneous inoculations, Esomeprazole was administered by intravenous injection for three times (at the 12, 14 and 16th day) and we observed tumor growth.

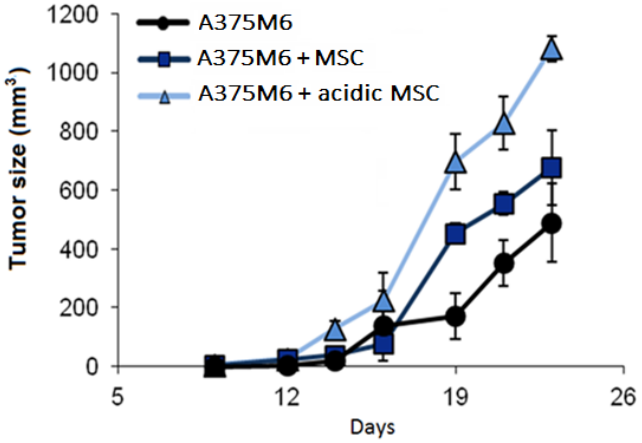

Figure 49- Growth kinetic of subcutaneous tumors obtained by injecting A375M6 cells alone, A375M6 with MSC or A375M6 with acidic MSC into immunodeficient mice.

Figure 49 shows that the growth kinetic of subcutaneous tumors obtained by injecting A375M6 cells is lower than that obtained by injecting A375M6 cells together with MSC or acidic MSC. In particular, acidic MSC maximally promote growth of melanoma tumors. Esomeprazole had no effect on the growth of tumors obtained by injecting A375M6 cells alone (Fig.50, panel A); it had a slight effect on the growth of tumors obtained by injecting A375M6 cells together with MSC (Fig.50, panel B) and it was able to significantly reduce the growth of tumors obtained by injecting A375M6 cells together with acidic MSC (Fig.50, panel C).

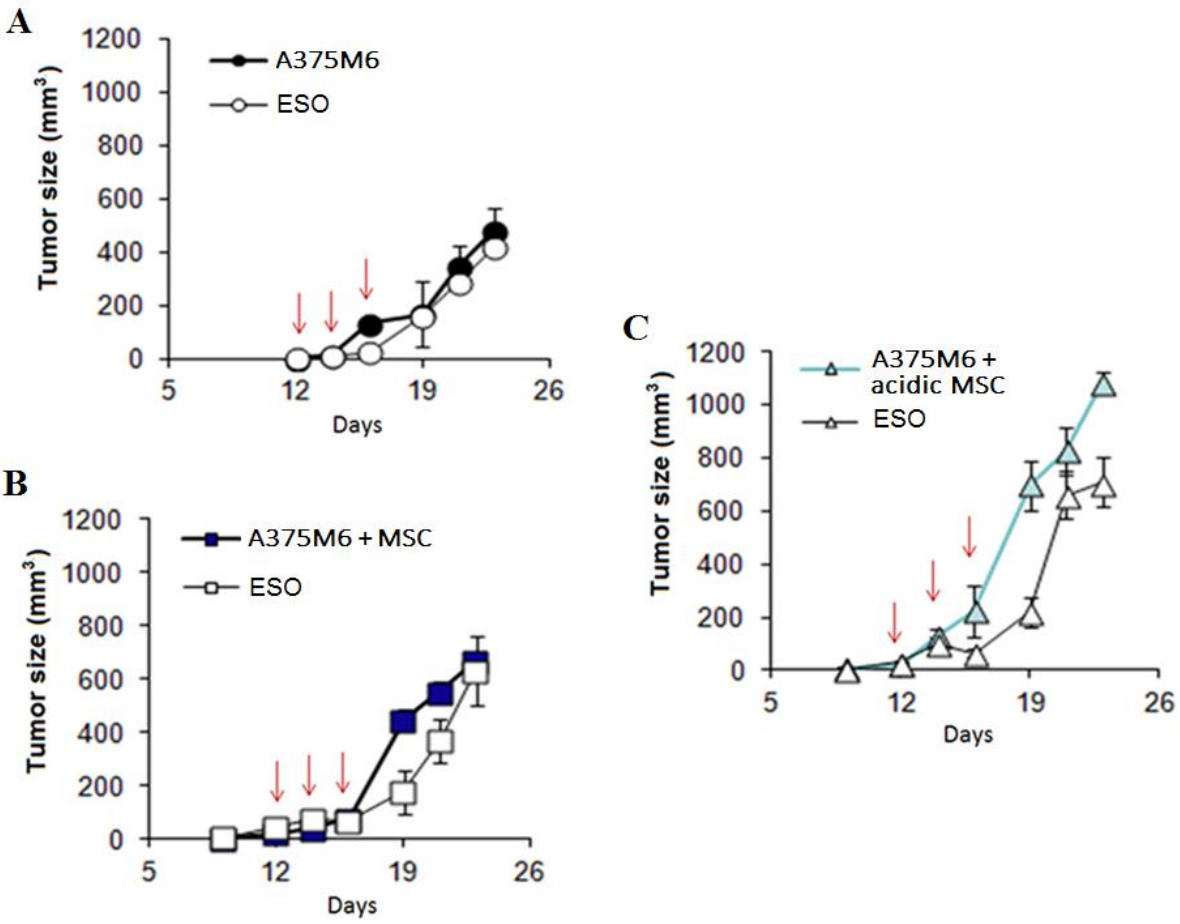

Figure 50- Effect of esomeprazole (ESO) on tumor growth. ESO was administered by intravenous injection at the 12, 14 and 16th day after tumor cell injection (see red arrows), at a dose of 25 mg/Kg. **A)** Growth of tumors obtained by injecting A375M6 cells alone, **B)** A375M6 with MSC or **C)** A375M6 with acidic MSC.

5. DISCUSSION

Tumor microenvironment is a complex system that consists of several important components including cancer cells, non-neoplastic stromal cells, signaling molecules, soluble factors and extracellular matrix (ECM). Each of these microenvironment components and the reciprocal dynamics interactions between them can regulate all aspects of tumorigenicity, promoting neoplastic transformation, tumor growth, migration, invasion and metastasis formation. Tumor microenvironment interactions influence a metabolic reprogramming, as well as secretion and activation of soluble factors and give rise to the abnormal organization, structure, and function of tumor blood vessels. In this way microenvironment acts as a coconspirator during carcinogenesis and neoplastic progression. Since significant regions of tumors are at a great distance from supporting blood vessels causing a gradient of oxygen tension, tumor cells respond to these conditions through the induction of HIF-1α transcription factor, which contributes to adapt their metabolism by increasing glycolysis and decreasing mitochondrial function. An important consequence of the switch to glycolytic metabolism is the increased lactic acid production and the resulting low extracellular pH (pHe). Therefore, the hostile metabolic microenvironment of tumors is often characterized by hypoxia and acidosis. Actually, the external pH of human melanomas has been reported to be within the range of 6.4 to 7.3 (Vaupel *et al*., 1989) or 6.6 to 7.0 (Wike-Hooley *et al.,* 1984), lower than those measured for human skin. In this study we investigated whether acidity is able to induce in melanoma cells different aspects of malignancy.

It is known that during melanoma progression, malignant melanocytes are reprogrammed into mesenchymal-like cells through an epithelial-mesenchymal transition (EMT) process associated with the acquisition of an invasive and pro-metastatic phenotype. EMT, described as the developmental switch undergone by cells from a polarized epithelial to a motile mesenchymal phenotype during embryonic development, has emerged as a central process of cancer progression (Huber *et al*., 2005; Thiery and Sleeman, 2006; Kalluri and Weinberg, 2009). During EMT, epithelial cells lose contacts with neighboring cells and assume migratory characteristics. At the molecular level, the EMT transition is characterized by a series of coordinated changes including downregulation of epithelial markers (E-cadherin and a- and g-catenin), up-regulation of transcriptional repressors of

E-cadherin (Snail and Slug) and up-regulation of mesenchymal markers (fibronectin, vimentin, and N-cadherin). There are several growth factors, probably emanating from tumor-associated stromal cells, that induce the loss of E-cadherin expression/function and promote cancer cell migration and invasion, including interleukin-6 (IL-6) (Yadav *et al*., 2011), epidermal growth factor (EGF) family members (Lo *et al.*, 2007), fibroblast growth factors (FGF) (Acevado *et al.*, 2007), hepatocyte growth factor (HGF) (Savagner *et al.*, 1997), and insulin-like growth factor (IGF) (Graham *et al.*, 2008). Until now nothing was known about the role of acidity in the stimulation of EMT. Our study indicates that an acidic pH medium promotes in A375P human melanoma cells and in A375M6 melanoma cells (isolated in our laboratory from lung metastasis of SCID bg/bg mice i.v. injected with A375P cells) the expression of markers of EMT, such as N-cadherin, vimentin and Twist transcription factor, together with an elongated spindle-shaped morphology, similar to mesenchymal cells. Loss of E-cadherin was significant, but not as evident as the increase of mesenchymal markers; however, this behavior can be explained considering the fact that a reduced E-cadherin expression can be observed early in the nevus stage, and that vast types of melanoma express very low level of E-cadherin (Hsu *et al.*, 1996). Anyway, the evident increase in N-cadherin makes considerable the "cadherin switch" that characterizes the EMT (Hao *et al.*, 2012). The gain of N-cadherin was found to be associated in breast cancer cells with cell migration, invasion, and metastasis (Hazan *et al*., 2000) and recently, Alonso *et al*. (2007) showed that N-cadherin facilitates metastatic dissemination in human melanoma, acting as oncogene. Also the expression of vimentin is induced in invasive epithelial carcinoma cell lines including prostate (Zhao et al., 2008), breast (Sommers *et al*., 1989; Sommers *et al*., 1992), lung (Dauphin et al., 2013), gastrointestinal (Takemura *et al*., 1994) and cervical (Gillies *et al*., 1996) cancers and is correlated with tumor growth, metastasis and poor prognosis (Thomas *et al*., 1999; Jin *et al*., 2010; Hu *et al*., 2004). Twist transcription factor has been consistently associated with EMT in epithelial cancer cells (De Wever *et al.*, 2008) and a clear link between Twist 1 expression and tumor-initiating capabilities is well established: Bmi1, a polycomb protein that promotes self-renewal of certain stem-cell populations is a direct target of EMT inducer Twist1 (Yang *et al*., 2010). Besides the modification of the phenotype, EMT also results in the acquisition of other properties involved in carcinoma progression, such as an increased ability to migrate, a higher resistance to apoptosis and the acquisition of stemness properties (Savagner, 2010). We found that EMT profile of melanoma cells

grown in acidic environment was associated with higher resistance to apoptosis, reduction in cell proliferation, and increased invasiveness, while markers of stemness (such as CD133, CD20) were not affected. Also Ryder *et al.* (2012) showed that acidosis blocks apoptosis of lymphoma cells exposed to multiple cytotoxic metabolic stresses, such as deprivation of glucose or glutamine and treatment with dexamethasone.

We found that EMT of acidic A375M6 melanoma cells was transient and acidic cells after a 7 day-culture period in standard media recovered their original morphology, EMT marker expression and invasiveness. This process is called mesenchymal-to-epithelial transition (MET). The "seed and soil" theory states that EMT is pivotal to the former stages of cancer metastasis, and MET, which promotes conversion back to the parent cell morphology and growth of macrometastasis in the target organ, is critical to the latter stages. Reversibility of process and absence of mortality of melanoma cells incubated in an acidic medium, propose that acidity promotes EMT by an inductive mechanism.

In our experiments, acidity was not able to induce HIF-1α, a transcription factor involved in angiogenesis, cancer cell survival, invasion and important stimulatory factor of EMT (Zhang *et al.*, 2013), but was able to trigger in A375M6 cells the activation of the nuclear factor kappa B (NF-κB). Recent studies have demonstrated that the activation of NF-κB transcription factor is linked to various signal transduction pathways and to transcription activation events that mediate cell proliferation, cell migration, and angiogenesis (Gilmore, 2006; Haffner *et al.*, 2006). NF-κB is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, apoptosis and immune reaction (Baldwin, 1996) and different authors (Belloq *et al*., 1998; Shi *et al*., 1999; Xu and Fidler, 2000) demonstrated that acidic pH activates NF-κB. Our results indicate that the silencing of NF-κB inhibits invasiveness of acidic cells and up-regulation of N-cadherin. The association between NF-κB and melanoma progression is evident in many studies utilizing various inhibitors of NF-κB for melanoma treatment (Amiri *et al*., 2005). Huber *et al*. (2004) found that NF-κB inhibition prevents EMT in Ras-transformed epithelial cells, while activation of this pathway promotes the transition to a mesenchymal phenotype. Furthermore, inhibition of NF-κB activity in mesenchymal cells causes a reversal of EMT, suggesting that NF-κB is essential for both the induction and maintenance of EMT. We found that the increased invasiveness of acidic A375M6 was mediated by the increase of MMP-9 and MMP-2 and inhibited by both Parthenolide and

siRNA for NF-κB. In murine melanoma cells, Kato *et al*. (2005) showed that NF-κB mediates acidic extracellular pH signaling to induce MMP-9 expression. Moellering *et al.* (2008) indicated that acidosis promotes the selection of a stable invasive phenotype suggesting that either chronic or acute acidity consistently drives to invasiveness of tumor cells. Our study also shows that media conditioned by acidic tumor cells stimulate invasiveness of non-acidic melanoma cells and a mixed population of acidic and non-acidic cells invades Matrigel filters better than acidic cells. The ability of non-acidic cells to cross Matrigel filters using an amoeboid style of migration was promoted by MMP released by acidic cells and might have a role in the highest migration rate expressed by a mixed population of acidic and non-acidic tumor cells. Indeed, cells using an amoeboid style of migration are usually faster than cells expressing the mesenchymal style of migration (Friedl *et al*., 2003).

When we tested the lung colonizing potential of acidic cells in immunodeficient mice, we found that these cells express a metastatic ability similar or even lower than cells grown in standard pH media. This behavior was probably associated with the reduction in cell proliferation, the cell cycle arrest, unchanged markers of stemness and unchanged cloning efficiency. Celia-Terrassa *et al*. (2012) sustained the hypothesis that an inverse correlation exists between *in vitro* invasiveness and *in vitro* cloning efficiency. To understand whether cooperation between acidic and non-acidic cells might have a role also in the formation of lung colonies, we co-injected acidic (non-GFP-expressing) and non-acidic (GFP-expressing) cells and we counted the developed lung micrometastases that were more numerous than those observed following injection of non-acidic (GFP-expressing) cells only. Analysis of GFP expression in lung micrometastases obtained by co-injecting acidic and non-acidic cells revealed that non-acidic cells represent the greater part of cells of lung colonies. This suggest that growth of lung colonies depends on proliferation of non-acidic tumor cells, while acidic cells might have a role in tumor cell implantation and/or niche formation. We do not, also, exclude that the non-GFP expressing cells of lung colonies might result from a MET of acidic tumor cells. Quite recently, Tsuji *et al.* (2009) and Celia-Terrassa *et al*. (2012) have demonstrated that EMT and non-EMT cells cooperate to complete the several steps of metastatic cascade. Celia-Terrassa *et al*. proposed a model in which the more epithelial/self-renewal tumor populations that leave their primary site either passively, aided by stromal or mesenchymal-like tumor cells, or actively, through their own transient EMT, can form metastases because they have

maintained their epithelial phenotypes or, if they have undergone EMT at the primary site, revert to an epithelial/self-renewal program at distant sites. Tsuji and colleagues hypothesized that EMT is necessary but not sufficient for metastasis showing that EMT and non-EMT cells cooperate to complete the spontaneous metastasis process: they hypothesized that EMT cells, with enhanced migratory and invasive phenotype are responsible for degrading the surrounding matrix to invade and intravasate; non-EMT cells then migrate together with EMT cells, enter the blood stream, survive in circulation and reestablish colonies in the secondary sites (Tsuji *et al.,* 2008 and 2009). This way they put forward the pivotal importance of cell cooperativity during the metastatic process and these concepts have clinical impact for future therapeutic strategies against metastasis: targeting EMT alone might be counterproductive because it actives proliferation of disseminated cells; it should be combined with therapy against cycling cells (standard chemotherapy) and inhibiting MET, thereby maintaining dormancy or directly targeting the stem cell phenotype (Brabletz, 2012). On the other hand, Rofstad *et al* (2006) showed that acidic pH promotes experimental pulmonary metastasis of A-07, D-12 and T-22 human melanoma cells, and that this effect is transient (the metastatic potential of acidic cells decrease with increasing recovery at standard pH) and correlates with a reduction of plating efficiency of tumor cells. Besides, it was demonstrated that B16 murine melanoma cells exposed to pH 6.5 for 48 hours, enhanced their lung colonization after exposure to a standard pH medium (Jang and Hill, 1997). It is possible that these various results could be related to the different capacity of tumor cells to sense and respond to pH deviation of their growth media (Casey *et al.,* 2009).

Finally, our study provides evidence that acidity triggers in melanoma cells a "partial" but "useful" EMT program, able to promote invasiveness and lung metastasis development of non-acidic tumor cells. These results sustain a cooperation model of cancer metastasis, and acidosis might be considered as a reasonable target to combat melanoma progression.

A critical early step in the metastatic pathway is the invasion of cancer cells not only into surrounding stroma but also into peritumoral lymphatics (Howell and Grandis, 2005). Because of its proven association with the progression of metastasis and connection with lymphatic spread, the potent tumor lymphangiogenesis growth factor VEGF-C is one of the leading investigational molecular targets for metastatic signaling pathways. The expression of VEGF-C is correlated with increased lymphatic spread and distant metastasis in a number of human tumors, including melanoma (Skobe *et al.,* 2001). Skin

malignant melanoma primarily metastasizes to lymph nodes and the detection of sentinel lymph node metastases is considered as an important prognostic parameter (Rinderknecht and Detmar, 2008). It is well known that melanoma cells can promote growth of lymphatic vessels, mainly at the tumor-stroma interface and the size of peritumoral lymphatic vessels has been indicated as the most important factor that contributes to lymph node metastasis (Ji, 2006). Some typical structural features of lymphatic vessels, such as a large diameter, a discontinuous layer of basement membrane and absence in small lymphatic vessels of pericytes or vascular smooth muscle cells, make them more accessible for intravasation by tumor cells. Thus the so-called "lymphangiogenic switch" is crucial for tumor cell fate (Cao, 2005). Recent reports showed that VEGF-C promotes not only metastasis by inducing lymphangiogenesis but also tumour cell invasion and motility in cancer (Su *et al*. 2006; Bock *et al.,* 2008; Chen et al., 2010).

Our experiments conducted using A375P melanoma cells have indicated that VEGF-C expression is stimulated by inflammatory cytokines, IL-1β and TNFα, but not by severe hypoxia. These findings are in agreement with data reported by B. Enholm *et al.* (1997) using normal fibroblasts, rat C6 glioblastoma and human HT-1080 fibrosarcoma cells. Indeed, unlike the VEGF gene, the VEGF-C gene promoter does not contain putative binding sites for the hypoxia-regulated factors (TATA box), but contains conserved putative binding site for some transcription factors (Sp-1 and Ap-2), including NF-kB (Chilov *et al.,* 1997). Evidence that acidosis modifies the expression of members of the VEGF family are limited to the VEGF-A. It has been demonstrated, using endometrial carcinoma cells, that incubation in a low pH (8 hours at pH 5.5) stimulates the VEGF-A 121 isoform expression through p38 activation (Elias and Dias, 2008). Moreover, a transient acidosis (not more than 4 hours) was found to stimulate VEGF-A expression in human pancreatic, ovarian, colonic and prostatic cancers (Shi *et al*., 2001). In contrast, an acidic condition does not stimulate mRNA for VEGF-A in human breast carcinoma cell lines (Scott *et al*., 1998). We didn't find a VEGF-A regulation by acidity in melanoma cells, but our results identify for the first time acidity as a positive regulator of mRNA and protein VEGF-C synthesis in melanoma cells and thereby implicate low pH in the control of lymphangiogenesis. The results further suggest that NF-κB signaling, that we have already shown to be involved in the EMT induction in acidic A375M6 cells, plays a role in the up-regulation of VEGF-C in acidic A375P melanoma cells. In order to find a drug to control VEGF-C up-regulation by acidosis, we tested whether esomeprazole, a vacuolar

H+-ATPase (V-ATPase) inhibitor, might be effective. Several researchers have explored the inhibition of the membrane ion pumps, involved in the maintainance of an alkaline pHi, to decrease intracellular pH and impact the cancer cell behaviour (De Milito *et al.*, 2007; Yeo *et al*., 2008; Supino *et al*., 2009). Esomeprazole is used extensively to suppress gastric acidity, however V-ATPase is expressed also in the plasma membrane of several tumor cells (Sennoune *et al*., 2004; Fais et al., 2007; Pérez-Sayáns *et al.*, 2009), including A375 melanoma cells (Martínez-Zaguilán and Gillies, 1992). Fais *et al* (2007) demonstrated that this type of drugs is activated in the mildly acidic extracellular space of tumors, and used at a non-toxic dose, have been shown to suppress the growth of human melanoma in nude mice (De Milito *et al.,* 2010). Moreover, omeprazole the racemate form, from which the R- and S-isomers (esomeprazole) can be isolated, potentiates the growth-retard effect of cisplatin on human melanomas, probably facilitating the intracellular uptake of the chemotherapeutic drug (Luciani et al., 2004). Recent studies have elucidated that this type of proton pump inhibitor can exert anti-inflammatory effects unrelated to the inhibition of gastric acid production (Kedika *et al.,* 2009). We demonstrated that esopremazole, without modifying pH, abrogates NF-κB activation and VEGF-C expression in acidic A375P melanoma cells. In addition, melanoma cells isolated from a spontaneous metastatic lesion express a higher amount of VEGF-C when grown in low pH and esomeprazole abolishes this effect. This is an important confirmation, because during a long-stay in culture, tumor cells may acquire or lose some special characters becoming different from those freshly isolated from spontaneous tumors. Esomeprazole, activated by an acidic medium, was also effective to reduce VEGF-C expression in tumor cells exposed to IL-1β. IL-1β has been found to be a strong promoter of melanoma VEGF-C expression through NF-κB pathway. It is known that acidosis and a chronic inflammatory reaction are often associated in tumors. IL-1β stimulation of VEGF-C expression was showed in human lung fibroblasts and vascular endothelial cells (Ristimäki *et al*., 1998). In accordance with our findings, Handa *et al. (*2006) observed that omeprazole and lansoprazole blocked IL-8 production in human umbilical endothelial cells, possibly by interfering with NF-κB pathway. Hashioka *et al.* (2009) proved that these PPIs significantly reduced TNF-α secretion by stimulated monocytic THP-1 cells in a concentration dependent manner. On the whole, our results identify acidity as a positive regulator of VEGF-C expression and secretion in melanoma cells via NF-κB activation

and thereby implicate pH of tumor environment as lymphangiogenesis controller. Indeed, we proved that acidity is also affective in promoting VEGF-C expression in breast and prostate carcinoma cells. Our data further suggest that esomeprazole abolishes VEGF-C expression when tumor cells are exposed to acidity or both acidity and IL-1β. Thus, we may speculate that to inhibit lymphangiogenesis and interfere with metastatic dissemination through lymphatic vessels, might be considered for new therapeutic strategies able to regulate acidity and inflammatory cytokines.

After observing the stimulation of VEGF-C and the induction of the EMT in melanoma cells by acid environment, we investigated if acidity could modify also the metabolic program of melanoma cells. Indeed, it is known that acidity in tumor microenvironment is a direct consequence of the metabolism of cancer cells, which use glycolysis even in the areas of ample oxygen supply (the Warburg effect) (Vander Heiden *et al*., 2009), but on the contrary, the effect of the acidity on cell metabolism is less known. Calderon-Montano *et al.* (2011) discussed the role of the intracellular pH in the metabolic switch between oxidative phosphorylation and aerobic glycolysis in a review. They proposed that, in the presence of adequate oxygen levels, the intracellular pH may play a key role in determining the way cells obtain energy, an alkaline pH driving aerobic glycolysis and an acidic pH driving oxidative phosphorylation.

Our experiments conducted using A375M6 melanoma cells indicate that acidity reduces the glycolytic metabolism, as evidenced by the decreased expression of glucose transporters (GLUT1 and GLUT3), the reduced lactate efflux (reduced MCT4 expression and lactate released in media) and the increased lactate uptake (increased MCT1 expression), all inverse features compared to those detected in A375M6 grown under hypoxic condition. Indeed, the induction of HIF1α by hypoxia is responsible for glucose transport and glycolytic metabolism of tumor cells, resulting in the production of lactate. High expression of MCT4 was found in glycolytic tissue, including several hypoxic and rapidly growing tumors (Pinheiro *et al*., 2012). The result that acidic cells are not associated with a glycolytic metabolism is in agreement with the absent HIF-1α stimulation by acidity. Moreover, NF-κB, the transcription factor activated by acidity which we have shown to mediate the acidic-associated increase of VEGF-C and invasiveness, has been identified as a physiological regulator of mitochondrial respiration with a crucial role in metabolic adaptation of normal cells and cancer (Mauro *et al*., 2011).

We also found that the pyruvate kinase M2 (PKM2), a key player in the Warburg effect on cancer cells, is reduced in acidic cells. Tumour cells have been shown to express exclusively the embryonic M2 isoform of pyruvate kinase and Christofk and collegues (2008) demonstrated that M2 expression is necessary for aerobic glycolysis and that this metabolic phenotype provides a selective growth advantage for tumour cells in vivo. Since acidity acts on melanoma cells not only promoting EMT and resistance to apoptosis but also inducing a metabolic reprogramming shifting cell metabolism to oxidative phosphorylation, cell metabolism of acidic cells may represent a potential therapeutic end point. In order to target oxidative phosphorylation of acidic melanoma cells we used metformin, the most widely prescribed oral hypoglycemic agent used in type 2 diabetes, which acts through inhibition of mitochondrial respiration by inhibiting complex I of the electron transport chain (El-Mir *et al.,* 2000). Metformin has recently received increased attention for its potential antitumorigenic effects. We investigated whether metformin, acting at the metabolic level, could have an effect on the malignant characteristics of acidic cells and we found that the enhanced invasiveness acquired by melanoma cells exposed to an acidic culture medium was reduced by metformin treatment. Actually, metformin has been found able to inhibit the growth of breast cancer cells and to suppress the development of breast, colon and other tumors in transgenic mice (Anisimov *et al*., 2005; Tomimoto *et al*., 2008; Hirsch *et al.,* 2009). Interestingly, metformin may also be involved in regulating breast cancer-initiating cell ontogeny by transcriptionally repressing the EMT process (Vazquez-Martin *et al.,* 2010).

For much of the 20th century, lactate was largely considered a dead-end waste product of glycolysis due to hypoxia and his biological contribution has been largely ignored. Interestingly, however, lactate accumulation in human tumors was shown to be associated with metastasis, tumor recurrence, and poor survival (Brizel *et al*., 2001; Walenta *et al*., 2000). Sonveaux *et al.* (2008) identifies lactate as a prominent fuel for the oxidative metabolism of oxygenated tumor cells and MCT1 as a crucial component of a metabolic symbiosis based on lactate exchange in tumors. For example, lactate released as the end-product of glycolysis in the hypoxic tumor cell compartment, prominently fuels the oxidative metabolism of the oxygenated tumor cell subpopulation, thereby sparing glucose for glycolytic cells (Sonveaux *et al*., 2008). Another example of symbiosis in which glycolytic and oxidative tumor cells mutually regulate their access to energy metabolites was demonstrated by Fiaschi and collegues: fibroblasts in contact with epithelial cancer

cells undergo myofibroblast differentiation and produce lactate through aerobic glycolysis and Warburg metabolism which is used by cancer cells for respiration (Fiaschi *et al*., 2012). We therefore thought that tumor microenvironment might facilitate a symbiosis between non-acidic/glycolytic cells that use glucose and produce lactate, and acidic cells that capture the lactate produced by non-acidic cells (a process mediated by MCT1) and oxidize it to produce energy to increase their aggressiveness. Indeed exogenous lactate (at the concentration corresponding to the range of lactate detected in tumors), used by acidic cells for respiration, does not induce an increase of cell proliferation, but on the contrary induces an arrest, and stimulates motility of acidic cells, maintaining their high invasiveness and the EMT profile. Lactate also acts on non-acidic cells increasing their motility, invasiveness, and inducing the EMT. Manipulation of lactate uptake was accomplished with use of the α-cyano-4-hydroxycinnamate (CHC), a drug known to reversibly inhibit MCT1 (Manning *et al.,* 2000). We found that 10 mM CHC kills acidic cells, while has only an antiproliferative effect on non-acid cells, suggesting that glycolytic cells can better tolerate the effects of MCT1 inhibition. The use of lower doses of CHC showed that MCT1 inhibition represses not only the lactate-dependent stimulation of motility, but also the invasiveness of acidic or non-acidic cells treated with lactate and even that of untreated acidic cells. This result could be explained by considering that MCT1 inhibition has recently been found to provide antitumor effects in human osteosarcoma cells by suppressing the NF-κB pathway (Zhao *et al.,* 2014), which we have shown to be involved in the increased invasiveness of acidic cells.

In the last experiment on the metabolic investigation, which actually opens the door to many others, we used the medium conditioned by control (non-acidic) cells, probably containing lactate, to grow acidic cells. In this way we tested the hypothesis on the presence of a symbiosis between acidic and non-acidic cells, useful to obtain an increased aggressiveness, already observed both *in vitro* and *in vivo* (invasiveness of the mixture of acidic and non-acidic cells and the formation of lung micrometastases). Medium conditioned by control cells is able to stimulate the motility of acidic cells and CHC blocks this effect, confirming the hypothesis that the lactate produced by non-acidic cells may stimulate acidic cells and that this cross-talk could be inhibited by CHC. MCT1 is expressed in a variety of human cancer cell lines and in primary human tumors, including breast, head and neck, and lung cancers (Sonveaux *et al.,* 2008) as well as neuroblastoma (Fang *et al*., 2006), brain (Froberg *et al.,* 2001), and colon (Pinheiro *et al.,* 2008) cancers

and many studies have documented that the reduction of MCT1 markedly delays tumor growth and reduces tumor metastasis, indicating that MCT1 is important for tumor progression (Mathupala *et al.,* 2004; Sonveaux *et al.,* 2008) and that targeting MCT1 offers the possibility to inhibit several protumoral effects with a single therapeutic molecule.

In addition to observing the direct effect of the acidic environment on cancer cells, we also studied how acidity influences the interactions between mesenchymal stem cells (MSC) and tumor cells. It is well known that tumors are composed of malignant tumor cells and non-malignant benign cells, including endothelial cells, peri-vascular cells, fibroblasts, myofibroblasts, macrophages, lymphocytes, dendritic cells, and mast cells. Currently, it is thought that tumor stromas play a significant regulatory role in the balance of the tumor-host interface microenvironment. Cancer-associated fibroblasts (CAF), also termed "myofibroblasts" or "activated fibroblasts", are the main cellular component of tumor stromas and participate at all stages of tumor progression. Previous research has suggested that CAF secrete a series of cytokine or growth factors to enhance angiogenesis, stimulate the tumor growth and metastatic potential of tumors (Orimo *et al*., 2005; Guo et al., 2008); indeed, tumor growth and metastasis is significantly reduced in fibroblast-deficient mice, and injection of wild-type fibroblasts into these mice partially reversed the observed phenotype, providing further evidence for the involvement of fibroblasts in the emergence of metastasis (Witz, 2008; Rose *et al.*, 1991; Rose *et al.*, 1994). Current evidence suggests that at least a proportion of CAF are bone marrow derived, especially derived from bone marrow MSC (Quante *et al.*, 2011). MSC are multipotent precursors endowed with the ability to differentiate into a variety of mesenchymal cells, such as osteoblasts, chondrocytes, adipocytes, muscle cells, pericytes, reticular fibroblasts, and even neural cells (Chamberlain *et al.*, 2007). Large number of MSC is recruited into the stroma of developing tumors through the action of chemokines/cytokines such as VEGF, transforming growth factors (TGFs), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF) and interleukin-8 (IL-8) (Maxson *et al*., 2012). Mishra and colleagues (2008) have demonstrated that, by prolonged exposure to tumor cell conditioned medium, MSC could be activated, differentiate into CAF and become part of the tumor microenvironment. Moreover, recent evidences suggest that these cells play a role in facilitating cancer progression, influencing the behavior and aggressiveness of

tumor cells (Karnoub *et al.*, 2007; Jing *et al.*, 2012). Indeed, the relationship between MSC and tumor cells appears dual: primary and metastatic tumors attract MSC in their microenvironment where they affect tumor cell survival, angiogenesis, motility and invasiveness (Feng and Chen, 2009; Zhang *et al.*, 2013); vice versa in the bone marrow MSC attracts tumor cells and contribute to a microenvironment that promotes osteolysis, tumor growth, survival, and drug resistance (Bergfeld and DeClerck, 2010). Moreover, MSC are an important source of inflammatory cytokines that affect tumor and immune cells, thus have an immunomodulatory function (Sotiropoulou and Papamichail, 2007).

Whereas many experiments demonstrating the ability of MSC to promote aggressiveness of tumor cells have been done, the effect of acidity on the interactions between MSC and tumor cells is still unknown. Our experiments, conducted using A375M6 human melanoma cells and human MSC derived from bone marrow, demonstrated that condition medium collected from acidic MSC promoted the migratory and invasive abilities of melanoma cells as well as the expression of EMT related genes in a way much more evident compared to the effect of the media conditioned by non-acidic MSC. Whereas the medium conditioned by non-acidic MSC elicited a marked increase in tumor cell proliferation, a result recently obtained also by Zhang *et al*. (2013) and Suzuki *et al*. (2011), the medium conditioned by acidic MSC had no significant effects on tumor cell growth; both types of conditioned media (from acidic or non-acidic MSC) induced a mesenchymal morphology of melanoma cells, but neither of them was able to induce resistance to apoptosis. A375M6 cells grown in medium conditioned by acidic MSC showed also a stimulated expression of TGF-β and TGF-β receptors. We then investigated which soluble factor secreted by acidic MSC could be responsible for stimulating a more aggressive phenotype of A375M6 melanoma cells. Some papers have described a pro-tumorigenic effect of MSC on tumor cells due to the secretion of soluble factors acting in a paracrine manner. Several cytokines usually involved during MSC mediated tissue regeneration (e.g. IL-6, TGF-β, VEGF) are secreted at elevated levels by MSC upon recruitment by cancer cells and support actively growth or invasion of cancer cells. MSC-derived IL-6 has been reported to increase tumor formation capacity of various cancers (Sansone *et al*., 2007), either by favoring cell proliferation and survival (Tsai *et al*., 2011) or by inducing in tumor cells the production of pro-angiogenic factors (Huang *et al*., 2012). MSC have also been shown to release elevated levels of TGF-β upon interaction with breast and prostate cancer (Ye *et al*., 2012), resulting into stimulation of the

proliferative and migratory capacities of cancer cells. Moreover, MSC-mediated immunosuppression activity has been shown to be modulated via tumor necrosis factor-alpha (TNFα) (Djouad *et al*., 2005). We evaluated the expression of several cytokines in MSC grown in acidic medium for 24 hours and the only one factor with enhanced expression in acidic MSC was the TGF-β, that we assumed to be the factor secreted by acidic MSC and involved in the changes of melanoma cell behaviors. The implication of TGF-β signaling pathway in regulation of cell growth, differentiation, tissue repair and in promotion of tumor invasion, cancer progression and metastasis (Dunn *et al.,* 2009; Massagué *et al*., 2000) via EMT (Xu et al., 2009) is well established and we confirmed its role in increasing the invasiveness of melanoma cells, in stimulating the expression of TGF-β and of EMT markers. Recent reports identify TGF-β as a novel mechanism enabling MSC (Hung *et al.,* 2013; Mele *et al.,* 2014) or CAF (Calon *et al.,* 2014; Shintani *et al*., 2013) to enhance aggressiveness of tumor cells and our results support the hypothesis that acidity upregulates TGF-β expression in MSC which is involved in the MSC conditioned medium induction of melanoma cell aggressiveness.

After having obtained good results with esomeprazole in inhibiting VEGF-C induced by acidity, we investigated whether esomeprazole was able to inhibit the effect of acidity on MSC and consequently on tumor cells grown in conditioned media. Our experiments indicate that esomeprazole prevents the increase of TGF-β expression and the increase of MMP2 activity in acidic MSC. Moreover, A375M6 cells grown in media conditioned by acidic MSC treated by esomeprazole show invasiveness and motility comparable with that of cells grown in media conditioned by non-acidic MSC, much lower than the invasiveness and motility induced by media conditioned by untreated acidic MSC. Esomeprazole was also able to block the stimulation of EMT in cancer cells grown in media conditioned by acidic MSC. Probably, acidic microenvironment acts on MSC activating them and producing CAF that, through the TGF-β secretion, stimulates aggressiveness of the neighboring tumor cells. Esomeprazole, counteracting the effect of acidity on MSC, prevents the release of TGF-β and the consequent activation of tumor cells. This study also provides evidence that acidity-induced TGF-β secreted by MSC is able to promote tumor growth *in vivo*. Subcutaneous co-injection of melanoma cells and acidic/non-acidic MSC in immunodeficient mice resulted in more rapid tumor growth compared with injection with tumor cells alone, but acidic MSC maximally promoted

growth of melanoma tumors. When we treated mice with esomeprazole, tumor growth was attenuated, especially in mice injected with acidic MSC. Also Shintani *et al*. (2013) showed the inhibition of CAF-induced changes in tumor cells (*in vitro)* or in tumor growth (*in vivo*) by addition of a TGF-β inhibitor. Our findings are in agreement with a large number of results indicating that MSC promote tumor growth *in vivo* (Suzuki *et al*., 2011; Zhu *et al*., 2005); furthermore, our study explores the relationship between MSC and tumor cells in an acidic microenvironment, proposing esomeprazole, which make acidic MSC incapable of communicating with tumor cells, as therapeutic strategy against cancer.

6. CONCLUSIONS

The data collected during this period of work, directed to study the role of the acidic microenvironment in the process of melanoma progression, suggest that:

- Acidity triggers in melanoma cells a NF-κB-dependent EMT program, able to promote invasiveness and lung metastasis development of non-acidic tumor cells, suggesting the existence of a cooperation between acidic/EMT cells and non-acidic/ non-EMT cells during the metastatic process.

- Acidity induces a NF-κB-dependent VEGF-C expression in melanoma cells and esopremazole, activated by acidity, is able to abrogate it. Thereby low pH is implicated in the control of lymphangiogenesis and PPI might be considered as a new therapeutic strategy to regulate lymph node colonization.

- Acidity drives a metabolic reprogramming of acidic melanoma cells, shifting cell metabolism to oxidative phosphorylation. This effect could contribute to the malignant features of acidic melanoma cells, disclosing a new potential therapeutic end point.

- Acidity activates MSC which, in turn, through the TGF-β production, induce in melanoma cells a more aggressive phenotype and facilitate the growth of tumors *in vivo*. Esomeprazole prevents the release of TGF-β by MSC and the consequent activation of tumor cells.

On the whole, these findings suggest that acidosis of tumor environment represents a key factor in modulating melanoma phenotype and strategies directed to target acidic environment and/or the intrinsic cell metabolism of low pH-adapted cells might be proposed as a new and prominent issue in tumor therapy (Figure 51). Particularly, due to his chemical properties, esomeprazole may really represent a model of a new anti-cancer drug for at least two important properties: (1) the ability to act as anti-inflammatory agent, inhibiting the transcription factor NF-κB (2) the ability to target tumor acidity and only there it is transformed in the active drug.

Not far from now, in 2006 Takahashi and Yamanaka found as is it possible to induce, almost into any cell type, a pluripotent stem cell phenotype. Now another Japanese team indicates as a simple exposure to a low pH is also able to promote a pluripotent stem phenotype in T cells (Obokata *et al*. 2014). Whether different stressors are able to induce stemness, that means that every time we treat tumors, we stress the cells and some of the surviving cells are likely to acquire stem-cell character. We have to reconsider the approaches to tumor treatment and evaluate a new biological strategy.

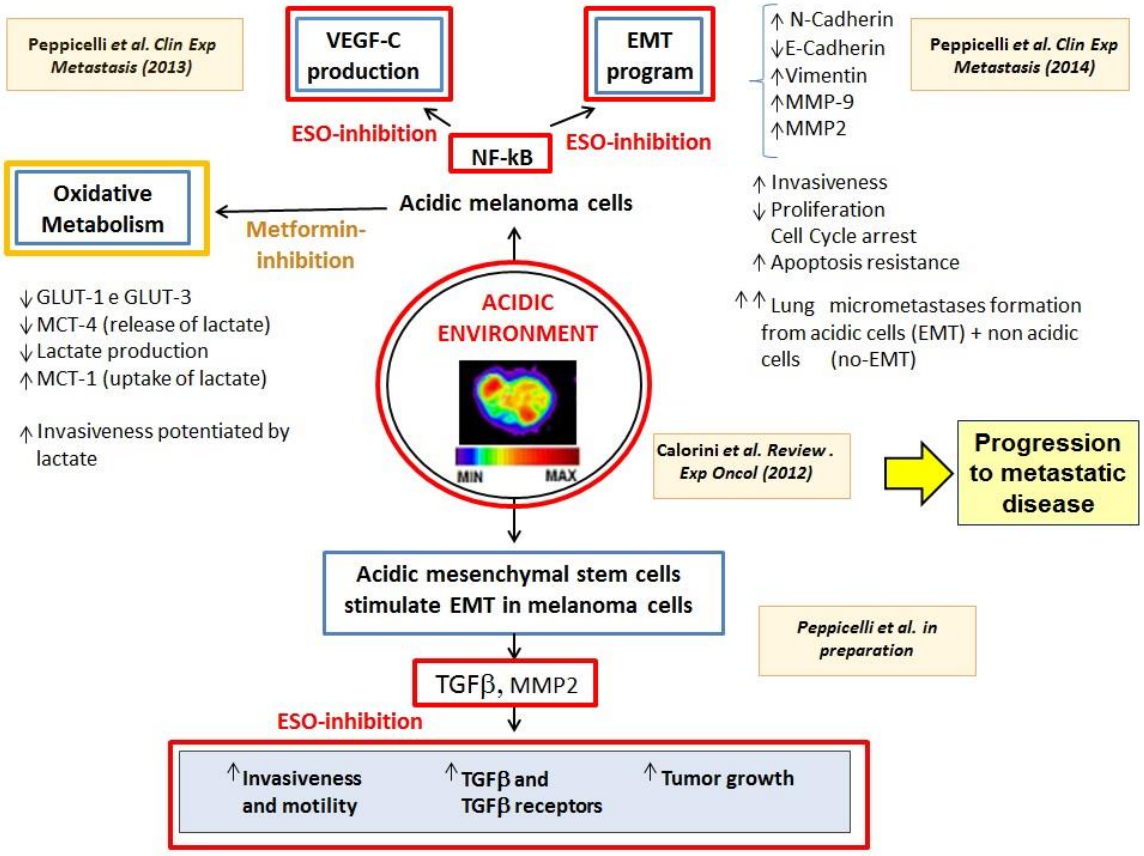

Figure 51- Summary of our results regarding the effect of acidic microenvironment on melanoma cells.

Esomeprazole-inhibition

Metformin-inhibition

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