The Emerging Role of p38 Alpha in Cancer Specific Metabolism and Therapy: Analysis of Autophagic and Apoptotic Pathways in Response to Its Inhibition

Thesis

The emerging role of p38 alpha in cancer specific metabolism and therapy: analysis of autophagic and apoptotic pathways in response to its inhibition

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ABSTRACT

Cancer is one of the leading cause of death in the world. Since tumorigenesis was described as a multistep mechanism in 1958 by Foulds, important progresses are obtained in the research field. Through these years many altered mechanisms were discovered revealing a very intricate picture of this disease. Mutations, epigenetic changes, aneuploidy imply severe modifications in naïve cellular pathways involved in every feature of cell life.

p38 MAPK is a family of kinases composed by four isoforms: alpha, beta, delta and gamma. These kinases are involved in several important cellular pathways, from the differentiation of muscle cells to inflammation and also in cancer progression. The pharmacologic and genetic inhibition of p38 alpha in colorectal cancer cells induced cell cycle arrest, autophagy and then cell death with autophagic features.

My PhD project is focused on the understanding the role of p38 alpha in the autophagic activation in colorectal cancer cell lines, finding that its inhibition induced the decrease of HIF-1 alpha protein levels and its glycolytic transcriptional program. Moreover, p38 alpha inhibition trigger the activation of FoxO3A-dependent transcription, which is related to cell cycle arrest, autopahgy and cell death. We checked for other HIF-1 alpha-dependent tumors which shown also overactivation of p38 alpha, such as ovarian cancer and prostate cancer. In these kind of tumors we obtained the same encouraging results.

However, in the DU145 prostate cancer cell line, the inhibition of p38 alpha failed to activate autophagic pathway due to the lack of LKB1 kinase, which is the upstream activator of AMPK. In this cell line the inhibition of p38 alpha triggered apoptotic pathway. Chemoresistance is one of the main obstacle in the treatment of cancer. A part of my PhD project is based on the study of p38 alpha role in cisplatin chemoresistance in colorectal cancer cells. Surprisingly, we found that its inhibition, together with the administration of cisplatin, induced apoptotic cell death in the resistant HT29 cell line and increased the effect of cell death in the responsive HCT116 cell line, through the activation of FoxO3A. All these evidences indicated that inhibition of p38 alpha could be used as a promising therapeutic approaches for colorectal cancer and other malignancy, through the activation of FoxO3A-dependent transcription program.

Chapter 1: INTRODUCTION

1.1 Aspect of cancer

Cancer is a major public health problem in the United States and many other parts of the world. One in 4 deaths in the United States is due to cancer (Siegel et al., 2012). Over the past 30 years, significant progress has been achieved in understanding the molecular basis of cancer. The accumulation of this basic knowledge has established that cancer is a variety of distinct diseases caused by different defective genes. Further, gene defects are diverse in nature and can involve either loss or gain of gene functions.

While cancer is clearly associated with an increase in cell number, alterations in mechanisms regulating new cell birth, or cell proliferation, are only one facet of the mechanisms of cancer. Decreased rates of cell death, i.e. apoptosis, are now known to contribute to certain types of cancer. At least five rate-limiting steps must be overcome before a clinically observable tumor could arise. These rate-limiting steps are genetic mutations that deregulate the activities of genes that control cell growth, regulate sensitivity to programmed cell death, and maintain genetic stability. Hence, tumorigenesis is a multistep process. Although the processes that occur during tumorigenesis are only incompletely understood, it is clear that the successive accumulation of mutations in key genes is the force that drives tumorigenesis. Each successive mutation is thought to provide the developing tumor cell with important growth advantages that allow cell clones to outgrow their normal neighboring cells. Importantly, it was found that no single

oncogene could confer all of the physiological traits of a transformed cell to a normal cell. Rather this required that at least two oncogenes acting cooperatively to give rise to cells with the fully transformed phenotype (Land et al., 1983). Carcinogenesis is the process that leads to genetic mutations induced by physical or chemical agents. Conceptually, this process can be divided into three distinct stages: initiation, promotion, and progression (Hennings et al., 1993). Initiation involves an irreversible genetic change, usually a mutation in a single gene. Promotion is generally associated with increased proliferation of initiated cells, leading to increase of this population. Progression is the accumulation of more genetic mutations which lead to the acquisition of the malignant or invasive phenotype. The initiation step, probably, is the most important step because it is an irreversible event that occurs when different causes produce DNA mutations. The different types of mutations that can occur include point mutations, deletions, insertions, chromosomal translocations, and amplifications. Three important steps involved in initiation are carcinogen metabolism, DNA repair, and cell proliferation. Hence, DNA repair is essential to reverse and to prevent DNA damage. Failure in the repair mechanisms, followed by cell proliferation, results in permanent alterations or mutation(s) in the genome that can lead to oncogene activation or inactivation of tumor suppressor genes.

1.1.1 Oncogenes and tumor suppressor

Oncogenes are derived from normal host genes, also called proto-oncogenes, that become deregulated as a consequence of mutation. Oncogenes contribute to the transformation process by driving cell proliferation or reducing sensitivity to cell death. Historically, oncogenes were identified in four major ways: chromosomal translocation, gene amplification, RNA tumor viruses, and gene transfer experiments. There are seven classes of oncogenes, classified by their location in the cell and their biochemical activity. All of these oncogenes have different properties that can lead to cancer. The classes of oncogenes are growth factors, growth factor receptors, membrane-associated guanine nucleotide-binding proteins, serine-threonine protein kinases, cytoplasmic tyrosine kinases, nuclear proteins, and cytoplasmic proteins that affect cell survival. Among these classes of oncogenes, of particular importance is the role of nuclear protein, which are also called transcription factors.

One of the transcription factor involved in tumorigenesis is CMYC (Henriksson and Luscher, 1996). Deregulation of *CMYC* often occurs either by gene rearrangement (Zhao et al., 2008b) or amplification in human cancers (Deming et al., 2000). The overproduction of CMYC results in uncontrolled cell proliferation. *CMYC* overexpression occurs both in solid and hematologic tumors such as Burkitt's lymphoma (Mitchell et al., 1984), and is usually the result of gene amplification (Koskinen and Alitalo, 1993). The oncogenic potential of CMYC has been studied most widely as it pertains to the development of colon

cancer (Rochlitz et al., 1996). Both CMYC RNA and protein are overexpressed at the early and late stages of colorectal tumorigenesis (Rochlitz et al., 1996).

Cell growth and proliferation are subject to regulation by external signals that are typically transmitted to the cell by growth factors (i.e. EGF (Cohen, 1983)) that bind to and activate specific receptors (i.e, EGFR (Yarden and Schlessinger, 1987)); In many cases, signaling that is initiated by growth factors activating their receptors passes next to membrane associated guanine nucleotide-binding proteins, which when activated by mutation, constitute another class of oncogenes (i.e. *HRAS, KRAS* and *NRAS*). Once activated, RAS then transmits the growth signal to a third class of signaling molecules that is comprised of the serine/thereonine kinases. The best studied of these serine-threonine protein kinases is the RAF oncogene (Stokoe et al., 1994). RAF then initiates a cascade of mitogen-induced protein kinases (MAPKs), Another RAS effector gene is phosphoinositol 3-kinase (PI3K), which initiates a signaling pathway for cell survival (Rodriguez-Viciana et al., 1994). This signalling pathway in cancer leads to the transduction of different stimuli to the nucleus, where transcription factor are able to transcribe all those genes usefull for proliferation, avoiding all systems for the control of cell growth.

In contrast to oncogenes, tumor suppressor genes can directly or indirectly inhibit cell growth. Those that directly inhibit cell growth or promote cell death are known as "gatekeepers". Both copies of gatekeeper tumor suppressors must be functionally eliminated for tumor development. Mutations that inactivate one allele of a gatekeeper gene can be inherited through the germline, leading to

cancer predisposition syndromes. Those tumor suppressor genes that do not directly suppress proliferation, but promote genetic stability are known as *caretakers*. *Caretakers* regulate DNA repair pathways and their inactivation results in increased mutation rates. Because numerous mutations are required for the full development of a tumor, elimination of caretaker tumor suppressors can greatly accelerate tumor progression. The most studied tumor suppressors are retinoblatoma protein (RB), p53, adenomatous polyposis coli (APC) and Phosphatase and tensin homolog-10 (PTEN) .

RB is related to retinoblastoma syndrome and is childhood disease. *RB* was the first human tumor suppressor gene identified, and the loss of RB protein function leads to malignancy and is dysfunctional in several major cancers (Murphree and Benedict, 1984). The RB protein is localized in the nucleus where it is either phosphorylated or unphosphorylated . When unphosphorylated, RB binds to the E2F transcription factor and prevents transcriptional activation of E2F target genes. When phosphorylated, RB can no longer bind to E2F. When loss of RB function occurs because of various mutations in the *RB*, the cell cycle becomes deregulated, and uncontrolled cell division results (De et al., 2006).

The tumor suppressor APC is mutated in almost 90% of human colorectal cancers (CRC) (Markowitz and Bertagnolli, 2009) and 30% of melanoma skin cancers (Worm et al., 2004). The inherited loss of APC tumor suppressor function results in familial adenomatous polyposis (FAP). Deletion of *APC* alleles, or mutations causing truncations in APC that influence its interaction with beta-catenin (CTNNB1), leading to its stabilization and activation of

Tcf/lymphoid enhancing factor (Lef)–dependent gene expression. One of the genes transcribed by this complex is the oncogene *CMYC*. However, *APC* could be wild type in CRC. Mutations in *CTNNB1* that abrogate its regulation by APC represent an alternative route to WNT activation and do occur more commonly in sporadic cancers outside of the gut. These mutations were first identified in sporadic CRC (Korinek et al., 1997; Morin et al., 1997) and in melanoma (Rubinfeld et al., 1997), but subsequent sequencing efforts indicated that they occur quite infrequently in these cancers (Demunter et al., 2002; Johnson et al., 2005; Luchtenborg et al., 2005; Thorstensen et al., 2005).

PTEN was first tumor suppressor gene identified in the most aggressive form of brain cancer, glioblastoma multiform (Simpson and Parsons, 2001). *PTEN* also is mutated in a significant fraction of endometrial carcinomas, prostate carcinomas , and melanomas (Tashiro et al., 1997; Gray et al., 1995; Celebi et al., 2000). Its tumor suppressor role is correlated with the induction of cell cycle arrest and apoptosis (Chu and Tarnawski, 2004). PTEN is a dual-specificity phosphatase, meaning that it can dephosphorylate proteins on serine, threonine, and tyrosine residues. It antagonizes the function of PI3K. PTEN, therefore, acts as a negative regulator of AKT activation (Maehama and Dixon, 1999), inducing apoptosis of mutated or stressed cells to prevent tumor formation. PTEN suppresses the PI3K-mediated induction of blood vessel growth factors like VEGF (Tian et al., 2010; Ma et al., 2009). PTEN also inhibits cell migration and formation of focal adhesions when overexpressed in glioblastoma cell lines, suggesting that it helps to inhibit metastasis as well (Tamura et al., 1998). The best known and studied

tumor suppressor is p53. This protein is activated in response to a wide variety of cellular stresses including DNA damage, ribonucleotide depletion, redox modulation, hypoxia, changes in cell adhesion, and the stresses created by activated oncogenes. The p53 protein functions as a transcription factor that, when activated, stimulates the expression of a variety of effectors that bring about growth arrest, promote DNA repair, and stimulate cell death by apoptosis. Collectively these activities act to maintain genomic stability. Elimination of p53 function leads to increased rates of mutation and resistance to apoptosis. Consequently, mutations in *p53* are the most frequent genetic change encountered in human cancers.

Since the main effect of tumor progression is uncontrolled proliferation, it is clear how one of the way to stop tumor formation is to block the cell division. However, the capacity of these cells to overcame cell cycle check points leads to find different strategies for the elimination of aberrant cells. The main way for this targeted elimination is the activation of cell death. Different types of cell deaths have been described (Galluzzi et al., 2012) but the most studied and characterized are Apoptosis and Autophagy.

Apoptosis is a tightly regulated multi-step pathway that is responsible for cell death not only during development, but also in adult multicellular organisms, in which it partly controls cell numbers. It is characterized by cell shrinkage, chromatin condensation, nuclear and cell fragmentation. These features result in the formation of apoptotic bodies that are then engulfed by neighbouring phagocytic cells. Autophagy is a very well regulated intracellular pathway and is

a process evolutionary conserved, from yeast to human. Its role consist of breaking macromolecules, membranes and entire organelles to obtain essential components. Autophagic degradation is used for three ends: elimination of old and not functional organelles, turnover of short-lived proteins and recovery of energetic balance due to particular environmental conditions, such as starvation. In addition, defects in autophagy have been linked to a wide range of diseases in mammalian cells indicating that autophagy is crucial in physiology (Liang et al., 1999).

1.2 COLORECTAL CANCER

1.2.1 Introduction to the pathology

Colorectal cancer (CRC) is a major health concern, with more than 1 000 000 new cases and 500 000 deaths expected worldwide per year (Samoha and Arber, 2005). Prognostic evaluation is currently based on histological appearance, and there are no molecular markers internationally recognized as standard predictor factors. The conventional therapy involving surgery and adjuvant therapy seems to give rise to improvements in progression-free and overall survival. Nevertheless about 50% of patients die within 5 years owing to metastasis or recurrent disease (McGartland et al., 2004). The prospects for further substantial advances in the management of CRC reside in a systematic genetic and functional dissection of cell cycle and cell death regulatory pathways in tumor cells in order to identify differential cellular effects of agents that may have a direct impact on cancer therapy. Tumorigenesis has long been thought to be a multistep process, since 1958 Foulds proposed is model (FOULDS, 1958). Fearon and Vogelstein were the first authors to propose a multistep model for CRC, in which were explained the genetic causes of progression from adenomas to cancer (Fearon and Vogelstein, 1990). For the authors this model is composed by four salient features: first, colorectal tumors appear to arise as a result of the mutational activation of tumor suppressor genes, the latter changes predominate; second, mutations in at least four to five genes are required for the formation of a

malignant tumor, fewer changes suffice for benign tumorigenesis; third, although the genetic alterations often occur according to a preferred sequence, the total accumulation of changes, rather than their order with respect to one another, is responsible for detemining the tumor's biologic properties; fourth, in some cases, mutant tumor suppressor genes may not be "recessive" at the cellular level (Fearon and Vogelstein, 1990).

Hence, according to the model developed by Vogelstein and coworkers, colorectal neoplasia evolves through a series of genetic alterations that includes the activation of oncogenes by mutations and the inactivation of tumor suppressor genes by mutation, loss, or methylation (Vogelstein et al., 1988) (Figure 1). These alterations occur in steps such that successive suBCLones gain a survival or growth advantage over their neighbors until a final suBCLone emerges with the potential for invasion and metastasis. The malignant suBCLone will grow and eventually efface all trace of the precursor lesion, but will nevertheless contain within its genome a permanent record of the underlying genetic changes. A surprising feature of this model is the relative rapidity of an evolutionary sequence that requires the accumulation of a multitude of genetic changes within a single cell. Key insights in explaining multistep oncogenesis were derived from the lessons of CRC (Kinzler and Vogelstein, 1996). FAP indicated how biallelic silencing of the tumor suppressor *APC* could initiate adenomas while Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) highlighted the feature of genetic instability as the explanation for rapid neoplastic progression. Genetic instability not only accounts for loss of fidelity with respect to genomic

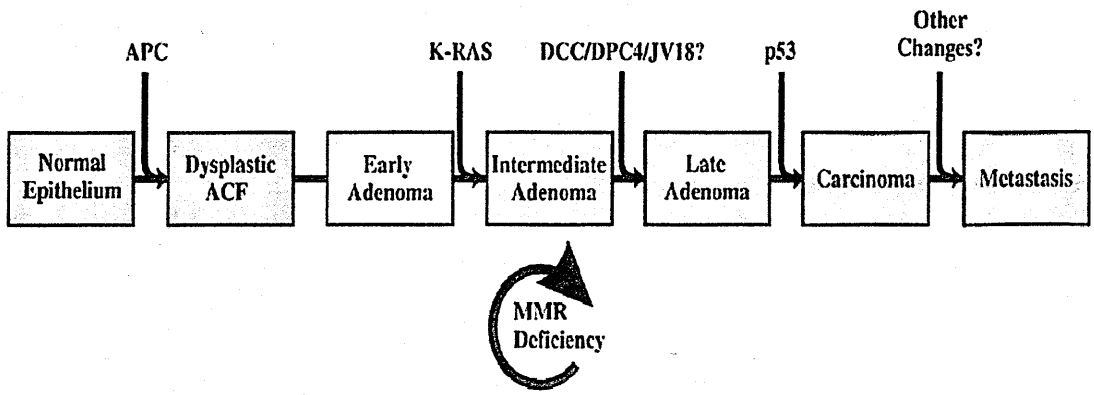

Figure 1. Genetic mutation in CRC progression. At least 4 mutations are necessary for the trasformation in cancer of normal epithelium (Vogelstein et al., 1988). In this model *APC* mutation is the leading mutation for cancer initiation.

replication during cell division, but also implicates the breakdown of cellular mechanisms that would normally detect DNA damage and trigger apoptosis.

1.2.2 The Wnt pathway in CRC

A large body of recent studies points the WNT signaling pathway as the unique player in the physiology of the intestine Figure 2. Several secreted WNT factors are secreted by the epithelial cells at the crypt bottom (Gregorieff et al., 2005), with the creation of a morphogen-like gradient of WNT signals along the crypt-villus axis (Logan and Nusse, 2004). Genetic studies in *Drosophila* and *Caenorhabditis elegans*, ectopic gene expression in *Xenopus*, and gene knockout mice have demonstrated the contribution of WNTs in very different processes such as segmentation, CNS patterning, and control of asymmetric cell divisions. The identification of Frizzled proteins as cell surface receptors for WNTs and the finding that CTNNB1, a downstream effector of the surface receptor, can translocate to the nucleus and function as a transcriptional activator led to a better understanding of WNT signaling (Wodarz and Nusse, 1998). *WNT* genes, of which the human and the mouse genome harbor almost 20 members, encode for cysteine-rich glycoproteins. Once secreted into intracellular environment from mesenchimal and intestinal pericryptal fibroblasts (Bienz and Clevers, 2000), WNT interact with other secreted proteins such as Frizzled-related proteins (SFRPs) and WNT inhibitory factor (WIF). Signaling is prompted when WNT ligands bind their cognate receptor complex, including the serpentine receptor of

Frizzled proteins and the single-span transmembrane protein Lrp5/6 of the LDL receptor family (Bhanot et al., 1996; Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). WNTs may simultaneously bind to Frizzled and LRP receptor, laying the first step of the so-called canonical pathway, with the formation of nuclear TCF/CTNNB1 complex. The cytoplasmatic protein CTNNB1, the stability of which is regulated by the destruction complex, is the key player of the WNT canonical cascade. When WNT receptors are not engaged, two scaffolding proteins in the destruction complex, the tumor suppressor APC and AXIN/AXIN2 sequester newly synthesized CTNNB1 allowing casein kinase I (CKI) to phosphorylate the N-terminus of CTNNB1 at Ser S45, a residue often mutated in CRC (Amit et al., 2002). Then, glycogen synthase kinase 3 beta (GSK3beta) phosphorylate additional serine and threonine residues N-terminal to S45 (Rubinfeld et al., 1996). The resulting phosphorylated footprint of CTNNB1 recruits a beta-TrCP-containing E3 ubiquitin ligase, which mediates ubiquitination of CTNNB1 and its proteasomal degradation (Hart et al., 1999; Kitagawa et al., 1999; Winston et al., 1999). Under physiological conditions, continued destruction of CTNNB1 is interrupted by receptor occupancy that inhibits the kinase activity by an incompletely understood mechanism involving the direct interaction of AXIN and Lrp5/6 or the actions of axin-binding adapter protein, Dishevelled, in association with Frizzled and GSK3beta-binding protein, Frat (Yost et al., 1998; Chen et al., 2003; Wong et al., 2003). Another mechanism proposed that WNTs induce phosphorylation of the cytoplasmatic tail of Lrp5/6, which allows the docking of AXIN (Tamai et al.,

2004). Thus, the recruitment of AXIN to the membrane is thought to avoid the formation of the destruction complex, with the subsequent release of the CTNNB1. Stabilized CTNNB1 is somehow released from the AXIN complex and translocates to the nucleus. Once in the nucleus, it associates with members of the TCF family of transcription factors (TCF1, LEF, TCF3, and TCF4) (Waterman, 2004) (Figure 2). Actually, TCFs target CTNNB1 to specific DNA elements present in promoters and enhancers of known target genes (Behrens et al., 1996; Molenaar et al., 1996), and in turn CTNNB1 serves as a coactivator of TCFs to stimulate transcription of WNT target genes recruiting a number of nuclear factors responsible for transactivating TCF target genes. Among these nuclear factors, two key members are the histone acetylase CBP/p300 and the SWI/SNF component BRG1 (Hecht et al., 2000; Takemaru and Moon, 2000; Barker et al., 2001; Simone, 2006). Activation of TCF target genes is dependent also by other nuclear proteins, Legless and Pygopus, that are proposed to be directly involved in the activation of transcription recruiting chromatin remodeling factors, and in the transport of CTNNB1 to the nucleus (Kramps et al., 2002; Parker et al., 2002; Townsley et al., 2004). Accumulation of CTNNB1/TCF complex imposes a proliferative phenotype by inducing the expression of cell cycle promoting proteins such as CyclinD1 (Shtutman et al., 1999) and CMYC (He et al., 1998). Mutational inactivation of *APC* leads to inappropriate high levels of free CTNNB1, whose stabilization is thought to be critical in tumorigenesis (Korinek et al., 1997). Indeed, it is well known that the tumor suppressor role of APC is mediated by its ability to destabilize free

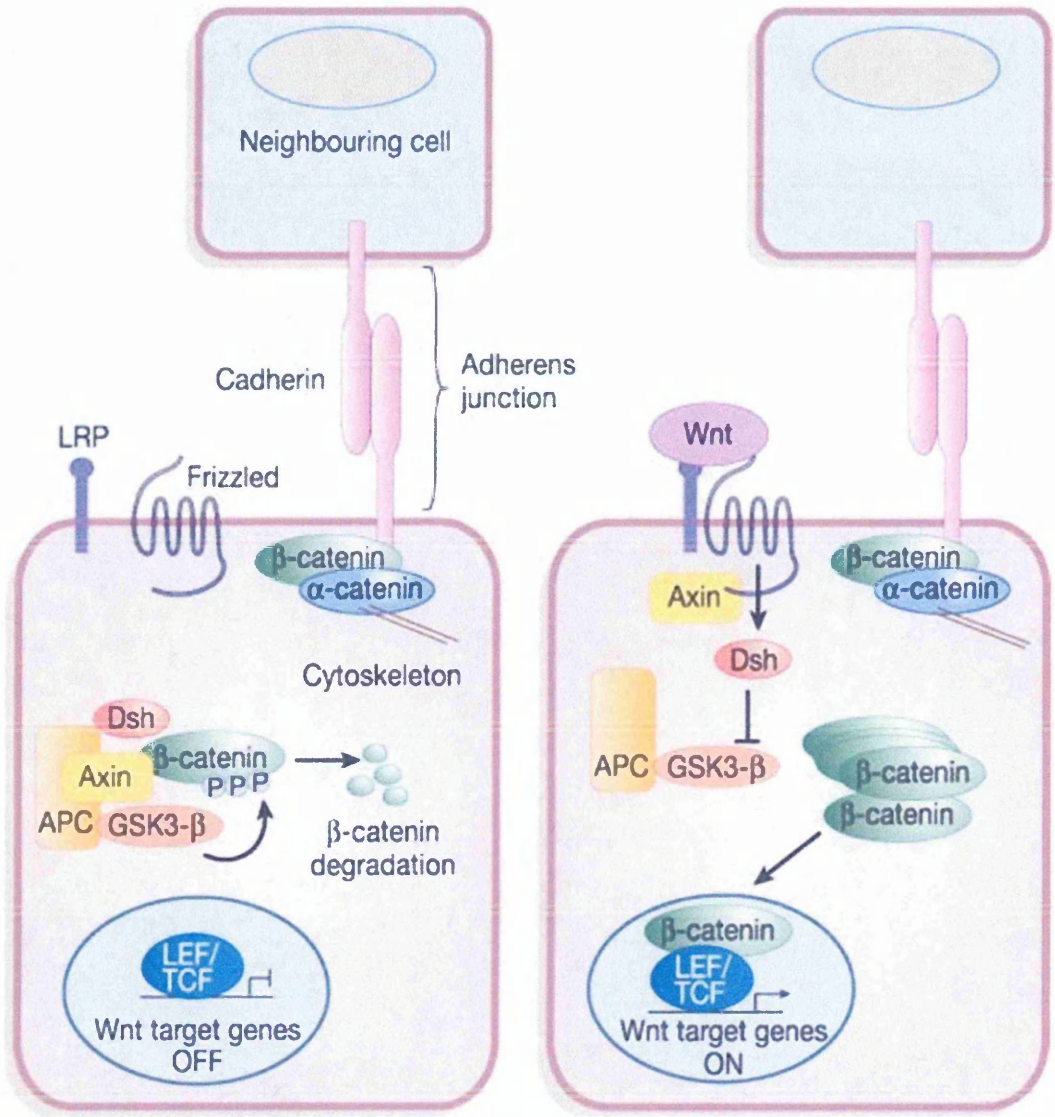

Figure 2. The canonical WNT signalling pathway. In the absence of WNT signalling (left panel), CTNNB1 is in a complex with axin, APC and GSK3-beta, and gets phosphorylated and targeted for degradation. CTNNB1 also exists in a cadherin bound form and regulates cell–cell adhesion. In the presence of WNT signalling (right panel), CTNNB1 is uncoupled from the degradation complex and translocates to the nucleus, where its binds Lef/Tcf transcription factors, thus activating target genes (Reya and Clevers, 2005).

CTNNB1 (Smits et al., 1999). Truncation of *APC* removes repetitive elements that allow APC to dock CTNNB1 and AXIN, with consequent impairment of GSK3beta mediated phosphorylation and degradation (Nathke, 2004). The evidence that CTNNB1 can be the first player in the initiation of malignant development was supported by the discoveries of other mutations in WNT signaling genes which cause free CTNNB1 accumulation. Moreover, in 15% of colon carcinomas that have conserved wild type *APC*, there are point mutations in one of the four serine/threonine residues located at the N-terminus of CTNNB1 that are the known targets of CK1/GSK3beta (Morin et al., 1997; Ilyas et al., 1997). Consequently, mutant CTNNB1 is no longer recognized by beta-TrCP and does not undergo proteosomal degradation.

1.2.3 Genetic CRC syndromes

CRC has one of the largest proportions of familial cases. Kindred and twin studies estimated that approximately 30% of all CRC cases are an inherited form of the disease (Lichtenstein et al., 2000; Grady, 2003). The syndromes involving CRC are defined on the basis of clinical, pathological, and more recently, genetic findings. Conditions that express adenomatous polyps include HNPCC, FAP, attenuated FAP, and MUTYH-associated polyposis (MAP). Hamartomatous polyps are the primary lesions in Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS). Finally, hyperplastic polyposis (HPP) is an unusual condition that has a substantial cancer risk and must be distinguished from the

other conditions. All of these conditions are inherited, autosomal dominant disorders, except MAP, which is an autosomal recessive, and HPP, which is rarely inherited.

1.2.3.1 Hereditary Non Polyposis Colorectal Cancer

Originally described in the early 20th century, elaborated on by Henry Lynch (Lynch, 1974), and refined through consensus conferences (Vasen et al., 1991; Vasen et al., 1999), this syndrome is marked by an autosomal dominant mode of inheritance, early onset of CRC often with a predilection for the right colon, and an 80% lifetime risk of CRC. The syndrome is noteworthy for a spectrum of extracolonic tumors, such as those originating from the endometrium, ovary, stomach, bile duct, kidney, bladder, ureter, and skin.

The syndrome accounts for 2%–4% of all CRCs (Hampel et al., 2008). Although affected individuals can develop colonic adenomas with greater frequency than the general population, polyposis is rare. The lifetime CRC risk is estimated to be 50%– 80% (Stoffel et al., 2009). CRCs and polyps arise in HNPCC at a younger age of onset and a more proximal location compared to sporadic neoplams. Histologically, cancers are often poorly differentiated, mucinous, and -have large numbers of tumor-infiltrating lymphocytes. Lynch syndrome is the result of a germline mutation in a class of genes involved in DNA mismatch repair (MMR), including *hMSH2*, *hMLH1*, *hMSH6*, and *hPMS2*. The MMR system is necessary for maintaining genomic stability by correcting single-base mismatches and

insertion-deletion loops that form during DNA replication. As a result, DNA replication errors occur in repeat sequences (Aaltonen et al., 1993); (Fishel et al., 1993; Ionov et al., 1993), typically in dinucleotide repeats, and are designated as the mutator phenotype. This phenotype can be revealed by PCR-based interrogation of genome-wide microsatellite sequences to evaluate for the possibility of length changes in the microsatellite sequences called microsatellite instability (MSI). The tumor is referred to as MSI-high or MSI-H when two or more markers of the recommended panel by the National Cancer Institute (BAT26, BAT25, D5S346, D2S123, and D17S250 markers) demonstrate MSI, MSI-low or MSI-L with one unstable marker, or MSstable or MSS when no microsatellite loci are unstable. Of note, MSI may be found in ~15% of sporadic colorectal tumors (Thibodeau et al., 1998). Target genes of MSI include *TGFbetaIIR*, *E2F4*, and *BAX*, among others. Of interest is that CRCs with MSI-H overall have a better prognosis compared to those without MSI (Ribic et al., 2003).

Mutations in *hMSH2* and *hMLH1* account for the up to 90% of Lynch syndrome cases; mutations in *hMSH6* account for approximately 10% and mutations in *hPMS2* are detected on rare occasions (Rustgi, 2007; Peltomaki and Vasen, 2004). Differences in cancer risks have been reported among the MMR genes, including *MSH6* where CRC risk may be slightly lower and endometrial cancer risk possibly higher compared to *hMSH2* and *hMLH1* carriers (Plaschke et al., 2004). The most striking difference is for *hPMS2* mutation carriers which have recently been shown to have a 15%–20% risk for CRC, 15% for endometrial

cancer, and 25%–32% for any Lynch syndrome-associated cancer to age 70 years (Senter et al., 2008).

1.2.3.2 Familial Adenomatous Polyposis

FAP is the second-most common inherited. Features of FAP include the development of hundreds to thousands of colonic adenomas, beginning in early adolescence, and inevitable CRC in untreated individuals. The average age of CRC diagnosis if untreated is 39 years; 7% develop CRC by age 21 and 95% by age 50. Attenuated FAP is a less-severe form of the disease, characterized by an average 69% lifetime risk of CRC, an average of approximately 30 colonic adenomatous polyps (range 0 to 100s), the tendency to develop proximal colonic neoplasms, and a later age of polyp and CRC development (Burt et al., 2004). Nevertheless, the inevitable course is the development of CRC unless this distinctive natural history is interrupted by surgical intervention, typically in the form of total proctocolectomy with ileoanal anastomosis. Certain situations may mandate a subtotal colectomy with ileorectal anastomosis, but the rectal stump or remnant must be monitored for polyp recurrence. Extraintestinal manifestations include gastric fundic polyps, small bowel (especially duodenal) adenomatous polyps, congenital hypertrophy of the retinal pigment epithelium (CHRPE), supernumerary teeth, osteomas, cutaneous lipomas and cysts, thyroid tumors, desmoid tumors, adrenal cortical adenomas, and hepatoblastomas. While many of these features are benign, FAP patients may develop thyroid cancer, gastric

adenocarcinoma (<1% lifetime risk), duodenal adenocarcinoma (5%–10% lifetime risk), and/or ampullary adenocarcinoma. Desmoid tumors, which are benign fibroblastic neoplasms (typically intra-abdominal), present a particularly vexing problem due to their proclivity to occur after colectomy and/or to recur after surgical removal of the desmoid tumor itself. Cytogenetic analysis associated FAP with an interstitial deletion on human chromosome 5q21 (Herrera et al., 1986), which was further expanded on by independent genetic linkage analyses to 5q21. Positional cloning verified the gene responsible for FAP to be the *APC* gene, a landmark effort (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991).

The presence of extra-colonic lesions can also contribute to the initial diagnosis. FAP, attenuated FAP, and Gardner syndrome (FAP with epidermoid cysts, osteomas, dental anomalies, and/or desmoid tumors) all result from germline mutations in *APC*. The identification of *APC* mutations in a proband confirms the diagnosis, allowing precise identification of other relatives who are at risk.

Attenuated FAP is suspected when 10 or more, but fewer than 100 adenomas, are found in a person over 40 or 50 years of age (Burt et al., 2004; Knudsen et al., 2003). A precise diagnosis is often difficult in a single patient; polyp numbers vary with this disorder; attenuated FAP can mimic typical FAP, MAP, or even sporadic polyp development. Examinations of multiple family members can often determine the phenotype. Genetic testing is useful in that specific *APC* mutations are associated with attenuated FAP. Attenuated FAP and MAP, respectively, account for 10% to 20% of persons with 10 to 100 adenomatous polyps who do

not have early classic FAP. It is common not to find a genetic etiology in this group. New or de novo *APC* mutations are responsible for approximately 25% of FAP cases. In addition, approximately 20% of individuals with an apparent de novo *APC* mutation have somatic mosaicism (Hes et al., 2008). The location of the mutation within *APC* has been associated with the severity of colonic polyposis, the degree of cancer risk, and the presence and/or frequency of non-malignant findings, including desmoids and CHRPE (genotype–phenotype correlations) (Nieuwenhuis and Vasen, 2007). Studies have failed to show a correlation between genotype and upper GI tumor development.

1.2.3.3 Peutz-Jeghers Syndrome

PJS is a hamartomatous polyposis syndrome with an autosomal dominant mode of inheritance. The incidence is about one in every 200,000 births, and onset is in early childhood. Clinically, patients have moderate–large sized, but few hamartomatous polyps, typically in the small bowel but also in the colon and/or in the stomach. These polyps may enlarge and result in hemorrhage or intussusception with obstruction. However, the pathogonomic features are revealed through histopathology with increased smooth muscle bands. There is increased risk for CRC and, rarely, small bowel cancer (Giardiello et al., 2001). Other phenotypic features include macules with broad but focal distribution: peroral, buccal, periocular, palmar/plantar surfaces, and anogenital surfaces. Earlier genetic linkage studies localized the gene locus responsible for PJS to

human chromosome 19p13.3 (Amos et al., 1997). The actual gene was discovered to be a novel serine-threonine kinase 11 (STK11), or LKB-1 (Hemminki et al., 1998; Resta et al., 1998). The tumors associated with PJS have 19p LOH or somatic mutations in *LKB1*. Patients suspected to have PJS are candidates for genetic testing with evaluation for germline *LKB1* mutations. Those identified patients, or patients at risk, should undergo periodic upper endoscopy, colonoscopy, and small bowel follow-through X-ray series; and meticulous attention to the risk of various cancers (especially pancreatic cancer).

1.2.3.4 Juvenile Polyposis Syndrome

JPS is an autosomal dominant disorder in which 10 or more juvenile polyps are observed in the GI tract. It affects one in 100,000 births, and the phenotypic manifestations are found in childhood to adolescence. The polyps, as with the polyps in PJS, may bleed or obstruct. The establishment of these hamartomatous polyps as juvenile polyps is predicated on histological interpretation and confirmation of microcysts in the epithelia. They are found in the colon, but also other parts of the GI tract. Germline mutations in *BMPR1A* (bone morphogenic protein receptor 1A), *SMAD4*, or *ENG* (endoglin, an accessory receptor for TGF-beta) are reported in FJP (Howe et al., 1998; Howe et al., 2001; Sweet et al., 2005), suggesting that the TGF-beta pathway is critical in the pathogenesis of FJP. Certain features seen in JPS, such gastric polyposis and HHT, are more common with *SMAD4* mutations than with *BMPR1A* and might guide genetic

testing strategies. Individuals with a clinical diagnosis of JPS but without an identifiable mutation in *SMAD4* or *BMPR1A* could have another hamartomatous polyposis condition or an unidentified mutation.

1.2.3.5 Actual therapy and drug resistance

Adjuvant chemotherapy improves overall and disease-free survival of patients with resected Dukes' stage C CRC. 5-Fluorouracil (5-FU)-based chemotherapeutic regimens are the standard treatment for these patients. However, response rates for 5-FU as a single first-line treatment in advanced CRC are only 10–15% (Johnston and Kaye, 2001). Combining 5-FU with the newer chemotherapies irinotecan (CPT-11) and oxaliplatin has improved response rates for advanced CRC to 40–50% (Giacchetti et al., 2000; Douillard et al., 2000). Despite these improvements, new therapeutic strategies are needed. The use of novel biological agents, such as the monoclonal antibodies, cetuximab (an EGFR inhibitor) and bevacizumab (BV) (a VEGF inhibitor), have recently been shown to provide additional clinical benefit for patients with metastatic CRC (Cunningham et al., 2004; Hurwitz et al., 2004). Cetuximab (Erbitux, Merck Serono) is a recombinant monoclonal antibody that blocks the human epidermal growth factor receptor (EGFR) and therefore inhibits the proliferation of cells that depend on EGFR activation for growth. Cetuximab is indicated for the treatment of patients with EGFR-expressing, Kirsten rat sarcoma (KRAS) wild-type metastatic CRC in combination with chemotherapy or as a single agent

in patients who have failed oxaliplatin- and irinotecan-based therapy and who are intolerant to irinotecan.

The addition of BV to irinotecan, bolus 5-FU, and leucovorin combination (IFL) was associated with a high response rate (RR) as well as a significant longer time to progression (TTP) and overall survival (OS) in CRC patients (Hurwitz et al., 2004). In a second-line setting, BV has also shown improved RR, progression-free survival (PFS), and OS, when combined with FOLFOX (oxaliplatin, 5-FU, and leucovorin). However, patients in the BV-only arm showed PFS rates lower than patients in the control (FOLFOX alone) arm (2.7 vs. 4.7 months) (Giantonio et al., 2007). Moreover, these agents are now under intense investigation in the adjuvant setting.

Resistance to chemotherapy limits the effectiveness of current cancer therapies, including those used to treat CRC. Drug resistance can be intrinsic or acquired during treatment and is believed to cause treatment failure in over 90% of patients with metastatic cancer. Furthermore, drug resistant micrometastic tumor cells are also likely to reduce the effectiveness of adjuvant chemotherapy following surgery. Overcoming drug resistance is one of the main challenges of current cancer research. Many factors affect drug sensitivity, such as the pharmacokinetic profile of the drug; drug activation and inactivation; alterations in the drug target; processing of drug- induced damage; and evasion of apoptosis. another problematic related to the chemotherapy is the presence of mutations in pathways targeted by chemotherapics; for example, cetuximab is effective only for those tumors wild type for *KRAS*, but this protein is usually mutated in CRC.

1.3 OVARIAN CANCER

1.3.1 The "silent" killer

In 2002, there were an estimated 204,000 new cases of Ovarian Cancer (OvCa) and 125,000 deaths due to this disease worldwide (Parkin et al., 2005). The incidence rates of OvCa are highest in the Western world, where it is the leading cause of death from gynecological malignancies (Parkin et al., 2005; Jemal et al., 2008). The 5 year survival rate of OvCa ranges from 30 to 92%, depending on the spread of disease at diagnosis (Jemal et al., 2008). OvCa is associated with high morbidity and mortality because approximately 75% of patients present with evidence of metastatic spread beyond the ovaries (FIGO stages (the International Federation of Gynecology and Obstetrics stages) III and IV) and require combined surgery and chemotherapy (Dinh et al., 2008).

The evolution of surgical techniques and chemotherapy regimens through multiple clinical trials over the past three decades has resulted in improvements in OvCa treatment (Agarwal and Kaye, 2003; Balvert-Locht et al., 1991). Debulking surgery remains key in OvCa treatment; a residual tumor size of greater than 2 cm is associated with a reduced survival of 12–16 months, compared with 40–45 months if the tumor is less than 2 cm (Mutch, 2002).

OvCa has a distinctive biology and behavior at the clinical, cellular and molecular levels. Clinically, OvCa is often present as a complex cystic mass in the pelvis. Although OvCa has been termed the 'silent killer', more than 80% of

patients have symptoms, even when the disease is still limited to the ovaries (Goff et al., 2000).

These symptoms are, however, shared with many more common gastrointestinal, genitourinary and gynecological conditions and have not yet proved useful for early diagnosis. Metastases can occur through lymphatics to nodes at the renal hilus or through blood vessels to the parenchyma of the liver or lung. Most frequently, small clusters of cancer cells are shed by the ovary and implant on the peritoneal surface, forming numerous nodules. For cancer in the ovary, unlike cancers at many other sites, no anatomical barrier exists to widespread metastasis throughout the peritoneal cavity.

Although OvCa risk is, at least in part, influenced by hormonal, environmental and racial factors, a major role is played by genetic factors. Indeed, a key advance in the study of OvCa etiology has been the identification of mutations in the *BRCA* genes. *BRCA1* and *BRCA2* genes act as tumor suppressor genes and, when mutated, are associated with the accumulation of chromosomal abnormalities and thus with a higher risk of developing cancer. Inheritance of mutations in *BRCA* genes is associated with a 27% to 44% lifetime risk of OvCa. A higher incidence of carcinomas of the ovary has also been detected in families affected by the HNPCC syndrome (Watson and Lynch, 1993), which is caused by mutations in DNA MMR genes. HNPCC carriers account for approximately 1% of OvCa patients, and their estimated lifetime risk of OvCa is 9% to 12% (Rubin et al., 1998). Mutations in *BRAF*, *KRAS* and *ERBB2* oncogenes and in the tumor suppressor *PTEN* have been found in a large subset of OvCa (Singer et al., 2003;

Nakayama et al., 2006). The inactivation of *PTEN* and an activating mutation of *KRAS* are sufficient to induce ovarian endometrioid carcinoma in a mouse model (Dinulescu et al., 2005). Furthermore, mutations of *CTNNB1* have been detected both in ovarian carcinomas and in their precursor lesions (Oliva et al., 2006). Indeed, inactivation of the WNT/CTNNB1 and the PI3K/PTEN pathways has been shown to induce the development of endometrioid carcinoma in an engineered mouse model (Wu et al., 2007). The small G-protein RAB25, which regulates motility, aggressiveness, apoptosis and autophagy, and mediates survival in response to stress, has also been found up-regulated in the majority of OvCa (Cheng et al., 2005). The Aurora-A kinase (Aurora-A) is associated with tumor initiation and progression and is overexpressed in various malignancies. Inhibition of Aurora-A induces cell cycle arrest and decreases proliferation of epithelial OvCa stem cells, which represent the chemoresistant population and act as a source of recurrence (Chefetz et al., 2011). All of these and several other amplified oncogenes are potential targets for OvCa therapy.

Recent attention has focused on the role that the surrounding microenvironment plays in the process of tumorigenesis as well as tumor progression and how it contributes to tumor biology (Harris, 2002; Hockel and Vaupel, 2001). Ovarian carcinoma cells leaving the primary tumor may therefore experience lower oxygen levels (Mutch and Williams, 1994). Evidences exist that microenvironmental hypoxia and aggressively invasive phenotypes are observed in ovarian carcinomas (Imai et al., 2003). *HIF-1 alpha* overexpression is associated with a poor prognosis in patients with OvCa (Osada et al., 2007),

suggesting that hypoxia is important for the acquisition of aggressive behavior in OvCa. In contrast with endometrial- and cervical carcinoma, a consistent significant correlation between tumor stage, grade and *HIF-1 alpha* expression has been described for ovarian carcinoma. *HIF-1 alpha* expression was significantly higher in tumors of FIGO stages III and IV than in those of stages I and II (Acs et al., 2004). With larger tumor bulk in the high stage tumors and subsequent areas of hypoxia this is as expected. *HIF-1 alpha* expression was also significantly higher in grade 1 OvCa compared with grade 3 tumors (Birner et al., 2001).

1.3.2 Actual therapy and drug resistence

Adjuvant chemotherapy improves both overall and progression-free survival in all patient subgroups, although it is expected to have a greater effect in patients who are optimally debulked. Evidence from several randomized controlled clinical trials has now established the platinum–paclitaxel combination regimen as first-line treatment for advanced OvCa, yielding response rates of over 80% and 40–60% complete responses (Agarwal and Kaye, 2003; Birner et al., 2001; Ozols et al., 2003; Sandercock et al., 2002). However, most of these patients will eventually relapse with a median progression-free survival of 18 months (Sandercock et al., 2002). At relapse, patients might be treated with the same drugs, with response rates that are proportional to their treatment-free interval (Gore et al., 1990). The length of the disease-free period following response to

platinum compounds can be used to categorize patients into groups with different prognoses: platinum-sensitive disease, platinum-resistant disease and platinum-refractory disease. Although the response rate of platinum-sensitive disease to the single agent carboplatin is greater than 50%, the response rate to carboplatin is only 10–20% for platinum-resistant disease and less for platinum-refractory disease. These two groups of patients are therefore usually treated with other agents, such as liposomal doxorubicin, gemcitabine, topotecan, etoposide and hormonal therapies. However, the response rates to treatment decrease with each subsequent relapse following the development of drug resistance. One of the major disappointments in the field of OvCa research is the failure of currently established therapies to induce a cure at diagnosis, even in chemosensitive tumors. Efforts have been made to cure OvCa over the past decade using different classes of chemotherapy agents in various combinations, dosages and schedules to overcome chemoresistance. Multiple studies in OvCa have established associations between both microvessel density and VEGFa levels in primary tumors and the extent of disease, time to progression and overall survival after initial anti-angiogenic therapy (Burger, 2007). In addition, the VEGF pathway is implicated in preclinical models of ascites formation (Nagy et al., 1995; Yoshiji et al., 2001). Anti-angiogenic agents are currently moving from Phase II to Phase III clinical trials in OvCa. In the recurrent disease setting, two Phase II studies of BV by Cannistra et al. and Burger et al. yielded response rates of 15% and 25% respectively (Cannistra et al., 2007; Burger et al., 2007). Progression-free survival rates at 6 months of 28% and 40% were particularly

encouraging; notably, 84% and 42% of the patients treated had platinum resistant disease. The EGFR is overexpressed in 70% of OvCa and associated with advanced disease at presentation, poor prognosis and chemoresistance (Bartlett et al., 1996; Fischer-Colbrie et al., 1997). Preclinical studies suggested that inhibiting this target might have anti-tumor activity and reverse chemoresistance (Sirotnak, 2003; Ciardiello et al., 2000).

1.4: PROSTATE CANCER

1.4.1 An asymptomatic disease

PCa is a multifocal disease that requires androgens for development and finally develops into solid tumors. In prostate tissue, androgens are involved in differentiation, development and normal functioning (Culig and Bartsch, 2006). Initial stages of erroneous PCa growth can be controlled by reducing the availability of androgens to the prostate cells (Huggins and Hodges, 2002). However, overtime PCa cells attain androgen independence.

Though there are many different ways leading to progression of cancer and ultimately to androgen-independent cancer (Asirvatham et al., 2006), mechanisms underlying the emergence to an androgen receptor (AR)-independent aggressive PCa are not completely understood.

Tumor microenvironment usually consists of disorganized and hemorrhagic vasculature (Carmeliet and Jain, 2000; McDonald and Choyke, 2003), which can lead to low oxygen (hypoxia) and nutrient supply to cells. Clinical studies with withdrawal of androgens have demonstrated reduction in hypoxia in tumor regions of PCa patients (Milosevic et al., 2007), suggesting that hypoxia may be involved in the development of androgen independence in these patients, however, mechanisms for this androgen independence have not been identified. Interestingly, *HIF-1alpha* has been found to be overexpressed in PCa even in non hypoxic conditions and signaling pathways, commonly induced in cellular stress

like JNK and MAPK have been implicated in the activation and control of *HIF-1 alpha* in cancer (Berra et al., 2000; Baek et al., 2001). In PCa, p38 has been implicated in androgen-independent progression of PCa (Shida et al., 2007).

Both p38 and its active form phospho p38, as well as some upstream kinases (PAK1, MKK6, MKK4), are overexpressed in human cancerous prostatic epithelium (Ricote et al., 2006; Royuela et al., 2002; Uzgare et al., 2003). Uzgare et al. using a transgenic mouse model for PCa, described that phospho p38 is overexpressed in prostatic intraepithelial neoplasia (PIN), well-differentiated and moderately differentiated cancers while was reduced or absent in late-stage adenocarcinomas and metastatic deposits. However, like in other tissues, studies focused on p38 function in the prostate malignancy reveal that this MAPK can elicit multiple and even opposite responses, which seem to vary depending on the cell system and context. A proapoptotic role for p38 has been established in a number of PCa in vitro models and conditions. p38 promotes apoptosis induced by 2-methoxyestradiol (Shimada et al., 2006), melatonin (Joo and Yoo, 2009), proanthocyanidins (Vayalil et al., 2004), raloxifene (Zhang and Kong, 2008), carprofen (Khwaja et al., 2008), or protoapigenone (Chang et al., 2008). By contrast, p38 exerts a protective effect in TNF-induced apoptosis in PCa cells (Ricote et al., 2006). In spite of having a prominent proapoptotic role p38 may contribute to PCa progression by promoting tumor growth, androgen independence acquisition, and metastasis. It has been proposed that IL-6 may support androgen-independent tumor growth by enhancing AR expression/activity. Lin and colleagues (Lin et al., 2001) demonstrated that, in

turn, the IL-6-induced androgen response depends on p38 activity. Huang and colleagues (Huang et al., 2005) showed in PC3 cells that p38 is necessary for TGF-beta-mediated activation of MMP-2 and cell invasion in PCa. Moreover, p38 has been involved in the invasion and migration abilities of the PCa DU145 cells, by enhancing the expression of *MMPs-2* and *-9*, and urokinase-type plasminogen activator (*u-PA*) (Shen et al., 2010; Xu et al., 2009) also described MKK4 as a regulator and activator of MMP-2. In agreement, Tang and Lu (Tang and Lu, 2009) found that p38 activity contributes to adiponectin induced integrin expression and migration capability of human PCa cells. Therefore, and in spite of displaying proapoptotic functions, p38 may constitute a target for PCa treatment given its demonstrated contribution to some PCa hallmarks, as androgen dependence and metastatic phenotype acquisition (Huang et al., 2005).

1.4.2 Actual therapy and drug resistance

Along with surgery or radiation therapy, hormonal therapy is a main mode of PCa treatment. For men with metastatic disease, chemotherapy provides a significant survival advantage. Therefore, new treatment options are being actively pursued to extend the survival of metastatic cancer patients. Androgen and AR are required for normal prostate development and carcinogenesis. Castration-resistant PCa tissues express AR and remain responsive to low levels of androgens. AR mutation, truncation and/or amplification may confer differential ligand and antagonist affinity and specificity. Thus, even low levels

of testosterone could still activate the AR and confer the growth and survival advantages for PCa cells. Several studies have demonstrated that low levels of testosterone are present in PCa cells. Mohler and colleagues studied testosterone levels in clinical specimens collected from castrated patients who underwent prostatectomy and found that intratumoral testosterone levels were elevated despite an overall reduction in serum testosterone (Petrylak, 2011). Abiraterone and orteronel are specific inhibitors of 17,20 lyase, which inhibit the conversion of 17-alpha-hydroxyprogesterone to androstenedione. Treatment with abiraterone was associated with a 35% reduction in death from PCa with an improvement in median survival from 10.9 to 14.8 months (de Bono et al., 2011), while in a phase I/II study of orteronel, of 43 patients with RECIST-evaluable disease, 6 showed a partial response, 23 had stable disease, and 9 showed progression (Massard and Fizazi, 2011). Phase III clinical trials with Orteronel are currently in progress in chemotherapy naïve and post-docetaxel settings. MDV3100 antagonizes AR action by preventing the translocation of the AR from cytoplasmic to nuclear compartment and by inhibiting DNA binding of AR and hence repressed the expression of androgen-regulated genes. In a phase I study of docetaxel-naïve and docetaxel-treated patients, 62% and 51% of patients, respectively, had at least a 50% PSA decline (Scher et al., 2010). One unique feature of the androgen-independent PCa cells is that the regression of prostate tumors still requires an activation of apoptotic machinery. In many cases, AR blocking is capable of inducing apoptosis. Clusterin is a antiapoptotic protein expressed in prostate, kidney, bladder, ovarian, lung, colorectal, and breast cancers. Clusterin

expression increases with Gleason score, and is upregulated after androgen blockade (July et al., 2002; Miyake et al., 2000). Clusterin modulates resistance to androgen blockade, radiation therapy, and chemotherapy. OGX-011 (Custirsen) is an investigational antisense compound that downregulates clusterin expression and enhances apoptotic death of PCa cells (Chi et al., 2008).

1.5: AUTOPHAGY AND APOPTOSIS IN CANCER

1.5.1 Two pathways linked together

Autophagy, a process long known to provide a survival advantage to cells undergoing nutrient deprivation or other stresses (Lum et al., 2005), has also been more recently linked to cell death process (Levine and Yuan, 2005). Thus, apoptosis is not the sole means by which the cell can undergo a genetically programmed regulated process leading to self-elimination (Ouyang et al., 2012). Cell death can occur by several mechanisms and the phenotypic changes that accompany cell death can vary depending on the stimulus and cell types. In any given death scenario, the cell decides which pathway to use, depending on the nature of the stimulus and the particulars of the cell environment. Furthermore, apoptosis and autophagy are not mutually exclusive pathways. They have been shown to act in synergy and also to counter each other. Three different types of interplay have come to light, each of which is equally valid for a particular cell type, stimulus and environment. Both apoptosis and autophagy can act as partners to induce cell death in a coordinated or cooperative manner; autophagy acts as an antagonist to block apoptotic cell death by promoting cell survival, or autophagy acts as enabler of apoptosis, participating in certain morphologic and cellular events that occur during apoptotic cell death, without leading to death in itself. One of the major clues today is the comprehension of the crosstalk between autophagy and apoptosis in tumor development (Todde et al., 2009). Recently, it

has been shown that both cFLIP and vFLIP of Kaposi's sarcoma-associated herpesvirus, in addition to inhibiting apoptosis mediated by death receptors, suppress autophagy by preventing ATG3 from binding and processing MAP1LC3 (LC3) (Lee et al.,2009). These findings identify another negative regulatory step common to autophagy and apoptosis, besides the antiapoptotic BCL2 family proteins, which can play a fundamental role facilitating the early stages of oncogenesis. In many cases, chemotherapy- and metabolic stress triggering apoptosis induced activation of the autophagic pathway, contributing to the survival of formed tumors, thereby favoring resistance. It is clear that the introduction in tumor therapy of autophagy inhibitors, in combination with therapies designed to induce apoptosis in human cancers, appears a rationale end in the tumor treatments.

1.5.2 Autophagy

Cellular homeostasis requires a constant balance between biosynthetic and catabolic processes. Eukaryotic cells primarily use two distinct mechanisms for large-scale degradation, the proteasome and autophagy; but only autophagy has the capacity to degrade entire organelles. The three types of autophagy are macroautophagy, microautophagy, and chaperone-mediated autophagy (Klionsky, 2004).

Autophagy plays an important physiological role in human health. During autophagic process, a double- or multimembrane–bound structure, called the

autophagosome or autophagic vacuole, is formed de novo to sequester cytoplasm. Then, the vacuole membrane fuses with the lysosome to deliver the contents into the organelle lumen, where they are degraded and the resulting macromolecules recycled (Nair and Klionsky, 2005) (Figure3).

Autophagy occurs at basal levels in most tissues and contributes to the routine turnover of cytoplasmic components. However, autophagy can be induced by a change of environmental conditions such as nutrient depletion. In addition to turnover of cellular components, autophagy is involved in development, differentiation, and tissue remodeling in various organisms (Levine and Klionsky, 2004).

Autophagy is also implicated in different human diseases. Paradoxically, autophagy can serve to protect cells (Boya et al., 2005) but may also contribute to cell damage. This pathway is involved in programmed cell death (PCD). Type I PCD, apoptosis, is characterized by condensation of cytoplasm and chromatin, DNA fragmentation, and cell fragmentation into apoptotic bodies, followed by removal and degradation of the dying cells by phagocytosis (Galluzzi et al., 2012). Type II PCD is characterized by the accumulation of autophagic vesicles (autophagosomes and autophagolysosomes) and is often observed when massive cell elimination is demanded or when phagocytes do not have easy access to the dying cells (Galluzzi et al., 2012). One feature that distinguishes apoptosis from autophagic cell death is the source of the lysosomal enzymes used for most of the dying cells' degradation. Apoptotic cells use phagocytic cell lysosomes for this process, whereas cells with autophagic morphology use the dying cells'

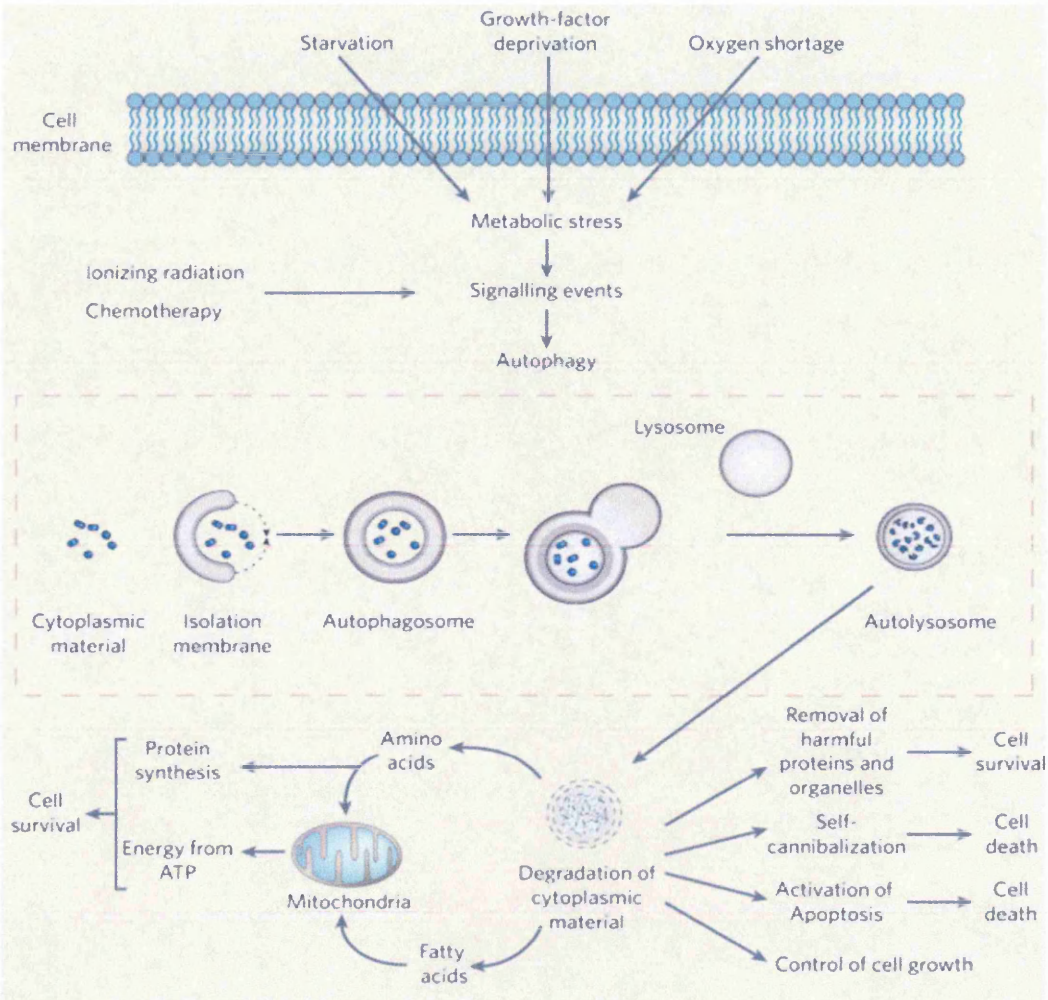

Figure 3. Autophagic pathway. Both metabolic stress and cancer therapies activate signalling pathways that stimulate autophagy. The process involves the sequestration of cytoplasmic material by a membrane-bound vesicle called the autophagosome, which then fuses with a lysosome to form an autolysosome. The degradation of cytoplasmic material within the autolysosome can promote cell survival either by generating free fatty acids and amino acids, which can be reused by the cell to maintain energy production and protein synthesis, or by removing harmful proteins and organelles. It can also promote cell death independently (presumably through self-cannibalization) or together with apoptosis. Furthermore, the turnover of proteins and organelles by autophagy may contribute to the control of cell growth (Levine, 2007).

lysosomal machinery. It has been unclear whether autophagy directly executes cell death or is the secondary effect of apoptosis. Recently has been shown that type I PCD and type II PCD are probably nonexclusive mechanisms in mammals, because they are sometimes observed in the same dying cell (Gonzalez-Polo et al., 2005).

Autophagy is controlled by molecules that fall into two categories: sensors and effectors. Effectors can be further divided into type-I and type-II effectors. Type-I effectors are involved in the steps (nucleation, expansion, uncoating and completion) leading to the formation of autophagosomes from the preautophagosomal membrane or phagophore (Nair and Klionsky, 2005). Type-I effectors are represented by the generally evolutionarily conserved ATG proteins and PI3K, the ortholog of yeast Vps34. The activity of type-I effectors is regulated by the formation of a class III PI3K-ATG6 (BECLIN1) complex, (Kihara et al., 2001a; Kihara et al., 2001b) and ubiquitin-like conjugation systems (the ATG8/LC3 and ATG12 systems) (Mizushima et al., 1998; Kabeya et al., 2000; Tanida et al., 2002). One type-I effector, BECLIN1, is a tumor suppressor gene product. Monoallelic deletion of this effector is observed in 40 to 75% of sporadic ovarian and breast cancers (Aita et al., 1999; Liang et al., 1999). Recently, transgenic mouse models have shown that monoallelic deletion of *BECLIN1* promotes tumor development in various tissues (Qu et al., 2003; Yue et al., 2003). Type-II effectors are involved in the maturation of autophagosomes (e.g., Lamp-2, RAB proteins, SNAREs, SKD1) (Darsow et al., 1997; Nara et al., 2002; Jager et al., 2004; Eskelinen et al., 2004; Ishihara et al., 2001). Sensors

comprise the diverse signaling pathways, second messengers and protein kinase complexes that respond to environmental changes, and regulate the steps in autophagosome formation that occur upstream of the effectors. Many sensors involved in autophagy signaling are tumor suppressor gene products [e.g., phosphatase and PTEN, TSC1-TSC2, p53, death associated protein kinase (DAPk) and oncogenes (e.g., AKT, Ras)]. These sensors are not specific for autophagy, but are also implicated in pathways that control a variety of cell functions. Thus, the signaling pathways that regulate autophagy form part of a cell program that is altered in cancer cells. Autophagy is believed to have an important role in tumor development. When baseline levels were compared, the amount of proteolysis or autophagic degradation in cancer cells was less than that of their normal counterparts (Gunn et al., 1977; Kisen et al., 1993; Kirkegaard et al., 2004). In these studies, the rate of degradation of long-lived proteins was considered the standard index for measuring autophagy. Other studies also reported that cells that were transformed by simian virus 40 (SV40) or treated with a carcinogen, underwent decreased levels of proteolysis compared with non-transformed or non-treated cells. This indicates a link between carcinogenesis and decreased levels of autophagy (Otsuka and Moskowitz, 1978; Schwarze and Seglen, 1985). Conversely, under conditions of nutrient deprivation, induction of the autophagic pathway is not downregulated in transformed epithelial cells compared with normal epithelial cells (Lee et al., 1992). Although autophagy is suppressed during the early stages of tumorigenesis, it seems to be upregulated during the later stages of tumor progression as a protective mechanism against

stressful conditions (Ogier-Denis and Codogno, 2003; Gozuacik and Kimchi, 2004; Cuervo, 2004). As the tumor grows, cancer cells at the periphery (close to blood vessels) continue to proliferate by maintaining anabolism (Cuervo, 2004). On the other hand, cancer cells that are located in the central areas of the tumor are poorly vascularized, so the induction of autophagy allows them to survive in these low-nutrient and low oxygen conditions (Cuervo, 2004). Autophagy can promote cell adaptation and survival, but under some conditions it leads to cell death. A number of studies have reported that autophagy, or autophagic cell death, is activated in cancer cells that are derived from tissues such as breast, colon, prostate and brain, in response to various anticancer therapies (Bursch et al., 1996; Paglin et al., 2001; Yao et al., 2003; Opipari, Jr. et al., 2004). Unfortunately, the autophagic response of cancer cells to therapeutics is not always an indication of cell death. Infact, inhibitors of autophagy can produce different outcomes, cell growth or cell death, that highlight the need to elucidate the mechanisms of autophagy, and its inhibition (Bursch et al., 1996; Yao et al., 2003; Bursch et al., 2000; Kanzawa et al., 2004; Bursch et al., 2000). It will be important for clinical oncologists and cancer researchers to determine which cancer cell types most commonly undergo autophagy in response to therapy, and whether increased autophagy is a sign of drug responsiveness or resistance.

1.5.3 Apoptosis

Apoptosis is a genetically controlled form of cell death that is essential for tissue remodeling during embryogenesis and for maintenance of the homeostatic balance of cell numbers later in adult life. Although multiple forms of cell death have been described (Galluzzi et al., 2012), apoptosis is characterized by morphological changes including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation, loss of microvilli, and extensive degradation of chromosomal DNA (Galluzzi et al., 2012) (Figure 4). In general, the apoptotic program can be subdivided into three phases: the initiation phase, the decision/effector phase, and the degradation/execution phase. In the initiation phase, signal transduction pathways that are responsive to external stimuli, such as death receptor ligands, or to internal conditions, such as that produced by DNA damage, are activated (Lazebnik et al., 1993; Solary et al., 1993). During the ensuing decision/effector phase, changes in the mitochondrial membrane occur that result in disruption of the mitochondrial membrane potential and ultimately loss of mitochondrial membrane integrity (Wang, 2001). A key event in the decision/effector phase is the release of cytochrome c into the cytoplasm (Wang, 2001) and activation of proteases and nucleases that signal the onset of the final degradation/execution phase. An important concept in understanding apoptosis is that the mitochrondrion is a key target of apoptotic stimuli and disruption of mitochondrial function is central to subsequent events that lead to degradation of vital cellular components (Wang, 2001). When the apoptotic machinery is

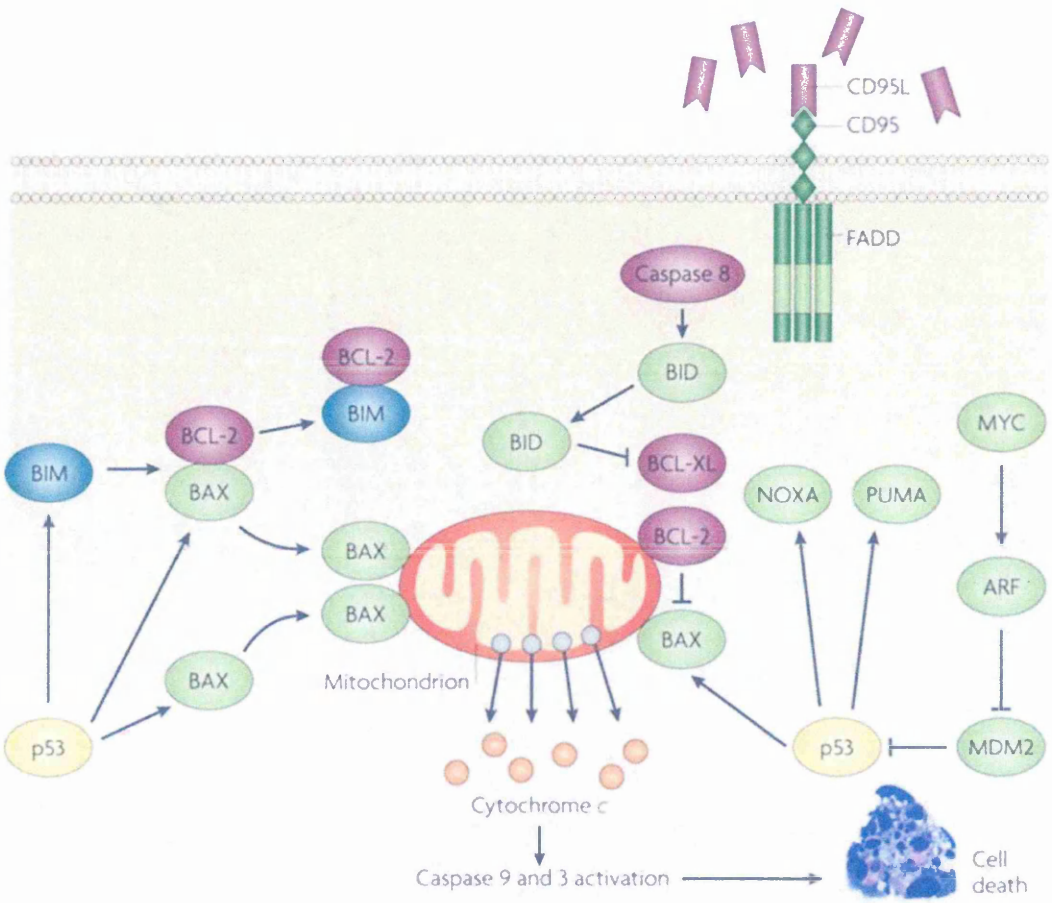

Figure 4.Signalling pathways in apoptosis. A simplified scheme of apoptotic pathway. Here are indicated both intrinsic and extrinsic pathways. However, both are convergent on mitochondria, with the final result of cytochrome C extrusion from the membranes (Cotter, 2009).

triggered in response to a wide range of death stimuli that are generated from within the cell, such as oncogene activation and DNA damage, the authors refer to this event as "intrinsic pathway". The inactivation of this pathway is generally regarded as a hallmark of cancer (Hanahan and Weinberg, 2000). Conversely, the extrinsic pathway is initiated by the binding of an extracellular death ligand, such as FASL, to its cell-surface death receptor, such as FAS (Nagata, 1999). In both cases, the critical downstream effectors are the caspases, and these are considered the engine of apoptotic cell death (Mittl et al., 1997). Although the first mammalian caspase (caspase-1) was identified as an important regulator of the inflammatory response (Thornberry et al., 1992; Cerretti et al., 1992) at least 7 of the 14 known mammalian caspases have important roles in apoptosis (Shi, 2002; Earnshaw et al., 1999). The apoptotic caspases are generally divided into two classes: the initiator caspases, which include caspase- 2, -8, -9 and -10 and the effector caspases, which include caspases-3, -6 and -7. They are synthesized in the cells as inactive enzymes that must be processed by proteolytic cleavage at aspartic acid residues (Shi, 2002). The activated proteases cleave other proteins by recognizing an aspartic acid residue at the cleavage site and are consistent with an auto- or transcleavage processing mechanism for activation when recruited to activated death receptors. Importantly, biochemical studies support the notion of a caspase hierarchy that consists of initiators and effectors that are activated in a cascade fashion. Initiator caspases such as caspase-8 and -9 are activated directly by apoptotic stimuli and function, in part, by activating effector caspases such as caspase-3, -6, and -7 by proteolytic cleavage (Shi, 2002). It is

the effector caspases that result in highly specific cleavage of various cellular proteins and the biochemical and morphological degradation associated with apoptosis. In 1996 Liu and colleagues (Liu et al., 1996) showed that the release of cytochrome c from the mitochondrion was crucial for the execution of the apoptotic pathway because it activated caspase 9, which in turn activated caspase 3. Yang and colleagues showed that BCL2 suppressed this release of cytochrome c30 and therefore prevented caspase activation. BCL2 was the first protein involved in the regulation of apoptosis to be molecularly characterized. The *BCL2* gene was originally identified by Yoshide Tsujimoto (Tsujimoto et al., 1984). During the 1990s, membership of the BCL2 family grew and the proteins discovered fell into one of three subfamilies. Proteins such as BCL-XL (also known as BCL2L1) (Boise et al., 1993), BCL-W (Gibson et al., 1996) and MCL1 (Kozopas et al., 1993) all contain three or four BCL2 homology (BH) domains, which are required for their antiapoptotic function. These domains drive interactions with other BCL2 family members, particularly at the level of the mitochondrion, where they regulate the release of proapoptotic mediators, such as SMAC (also known as DIABLO) (Du et al., 2000; Verhagen et al., 2000). The second subfamily consists of proapoptotic members, such as BAX (Oltvai et al., 1993) and BAK (Chittenden et al., 1995), which can form pores or interact with pore forming proteins at the level of the mitochondrial membrane, a function that is antagonized by BCL2. The discovery of a third BCL2 subfamily, which consists of proteins that have only a short BH3 domain, was important because these proteins provided a link between the terminal effector process and the

signalling network that informs the cell of its status regarding events such as growth factor stimulation, redox balance, DNA integrity, cell attachment and microtubule function. Although BCL2 was originally identified as a crucial factor in the development of B cell lymphomas, it also plays an important part in other tumors. In addition to activation of BCL2 by chromosomal translocations in cancer, increased *BCL2* expression is found in other tumors, such as non Hodgkin's lymphomas and certain lung cancers (Monni et al., 1997; Ikegaki et al., 1994). Since these discoveries, several other mechanisms have been identified that can lead to increased *BCL2* expression, including hypermethylation of *BCL2* and loss of micro rRNAs that normally downregulate *BCL2* expression (Hanada et al., 1993; Cimmino et al., 2005). For example, the transcription factor p53 is mutated in most human cancers, and some of its direct targets include BAX, BID and the BH3-only proteins PUMA and NOXA, all of which are pro-apoptotic members of the BCL2 family (Miyashita and Reed, 1995; Oda et al., 2000; Sax et al., 2002; Nakano and Vousden, 2001). Any impairment of p53 function leads to deregulation of apoptosis signalling pathways and increases tumorigenesis.

1.6: SIGNAL-TRANSDUCTION PATHWAYS REGULATING THE AUTOPHAGY/APOPTOSIS BALANCE: THE ROLE OF p38 ALPHA

1.6.1 p38 family

Cellular behavior in response to extracellular stimuli is mediated through intracellular signaling pathways such as MAPK (Rouse et al., 1994). MAPK are members of discrete signaling cascades and serve as focal points in response to a variety of extracellular stimuli. Four distinct subgroups within the MAPK family have been described: (1) extracellular signal-regulated kinases (ERKs), (2) c-JUN N-terminal or stress-activated protein kinases (JNK/SAPK), (3) ERK/ big MAP kinase 1 (BMK1), and (4) the p38 group of protein kinases. p38 alpha (p38) was first isolated as a 38-kDa protein rapidly tyrosine phosphorylated in response to lipopolysaccharide (LPS) stimulation (Han et al., 1993; Han et al., 1994). p38 cDNA was also cloned as a molecule that binds puridinyl imidazole derivatives which are known to inhibit biosynthesis of inflammatory cytokines such as IL1 and TNF in LPS stimulated monocytes (Lee et al., 1994). To date, four splice variants of the p38 family have been identified: p38 alpha, p38 beta (Jiang et al., 1996), p38 gamma (ERK6, SAPK3) (Lechner et al., 1996; Li et al., 1996), and p38 delta (SAPK4) (Jiang et al., 1997; Kumar et al., 1997). Sequence comparisons have revealed that each *p38* isoform shares ~60% identity within the *p38* group but only 40-45% to the other three MAPK family members.

The four *p38* isoforms are encoded by different genes and have significant levels in most cell types, whereas the others seem to be expressed in a more tissue-specific manner; for example, *p38 beta* in brain, *p38 gamma* in skeletal muscle and *p38 delta* in endocrine glands. p38 family members have overlapping substrate specificities, albeit some differences have been reported, with particular substrates being better phosphorylated by p38 alpha and p38 beta than p38 gamma and p38 delta or vice versa (Cuenda and Rousseau, 2007). The genetic ablation of specific p38 members has also demonstrated the existence of functional redundancy.

1.6.2 Activation of p38

First described in 1994, the p38 cascade regulates a variety of cellular responses to stress, inflammation and other signals (Han et al., 1994;(Lee et al., 1994). As in many other protein kinases, the activation of p38 isoforms requires phosphorylation on a flexible loop termed the phosphorylation lip or activation loop. These phosphorylations induce conformational reorganizations that relieve steric blocking and stabilize the activation loop in an open and extended conformation, facilitating substrate binding. p38 members are activated by dual phosphorylation in the activation loop sequence Thr-Gly-Tyr. In response to appropriate stimuli, threonine and tyrosine residues can be phosphorylated by three dual specificity MKKs. MKK6 can phosphorylate the four p38 members, whereas MKK3 activates p38 alpha, p38 gamma and p38 delta, but not p38 beta.

Both MKK3 and MKK6 are highly specific for p38 (Enslen et al., 1998; Alonso et al., 2000). Furthermore, p38 alpha can be also phophorylated by MKK4, an activator of the JNK pathway (Doza et al., 1995; Brancho et al., 2003). In addition, several studies including genetic analysis in mice have demonstrated functional differences between MKK3 and MKK6 (Zhang et al., 2007). Depending on the stress stimulus, MKK3 and MKK6 also contribute to different extents to the activation of other p38 members (Remy et al., 2010). In addition to the activation by upstream kinases, there is a MKK independent mechanism of p38 activation involving TGF beta-activated protein kinase 1 (TAK1)-binding protein (TAB1) (Ge et al., 2002). The activation of p38 in this pathway is achieved by the autophosphorylation of p38 alpha after interaction with TAB1. Although there is an indication that TAB1-dependent p38 phosphorylation occurs in LPS, TNF, and CpG treated B cell lines, a study using *MKK3/6* knockout mouse embryonic fibroblast (MEF) cells showed that TNF-induced p38 activation is solely dependent on MKKs (Brancho et al., 2003).

1.6.3 Inactivation of p38

Under physiological conditions, p38 activation is often transient despite the unchanging level of p38 throughout the course of stimulation. Dephosphorylation, then, would seem to play a major role in the downregulation of p38 activity. Many dual-specificity phosphatases have been identified that act upon various members of the p38 pathway and are grouped as the MAPK

phosphatase (MKP) family (Sun et al., 1993). Several members can efficiently dephosphorylate p38 alpha and p38 beta (Camps et al., 1998; Muda et al., 1996); however, p38 gamma and p38 delta are resistant to all known MKP family members. In addition, other types of phosphatases such as serine/threonine protein phosphotase type 2C (PP2C) has been shown to have a role in downregulating the MAPK HOG1 pathway as well as negatively regulating human MKK6 and MKK4 levels *in vitro* and *in vivo* (Maeda et al., 1994; Posas et al., 1996; Takekawa et al., 1998). Taken together, these results suggest a mechanism by which p38 isoforms are differentially regulated depending on phosphatase levels and specificity.

1.6.4 Targets of p38

It has been estimated that p38 may have approx. 200–300 substrates each. Accordingly, p38 have been reported to phosphorylate a broad range of proteins, both *in vitro* and *in vivo*. Much of the information about p38 substrates comes from the use of chemical inhibitors. On the basis of their functions, p38 alpha substrates comprise protein kinases implicated in different processes, nuclear proteins, including transcription factors and regulators of chromatin remodelling, and a heterogeneous collection of cytosolic proteins that regulate processes as diverse as protein degradation and localization, mRNA stability, endocytosis, apoptosis, cytoskeleton dynamics or cell migration (Figure 5). The first p38 alpha substrate identified was the MAPK-activated protein kinase 2 (MAPKAPK2 or

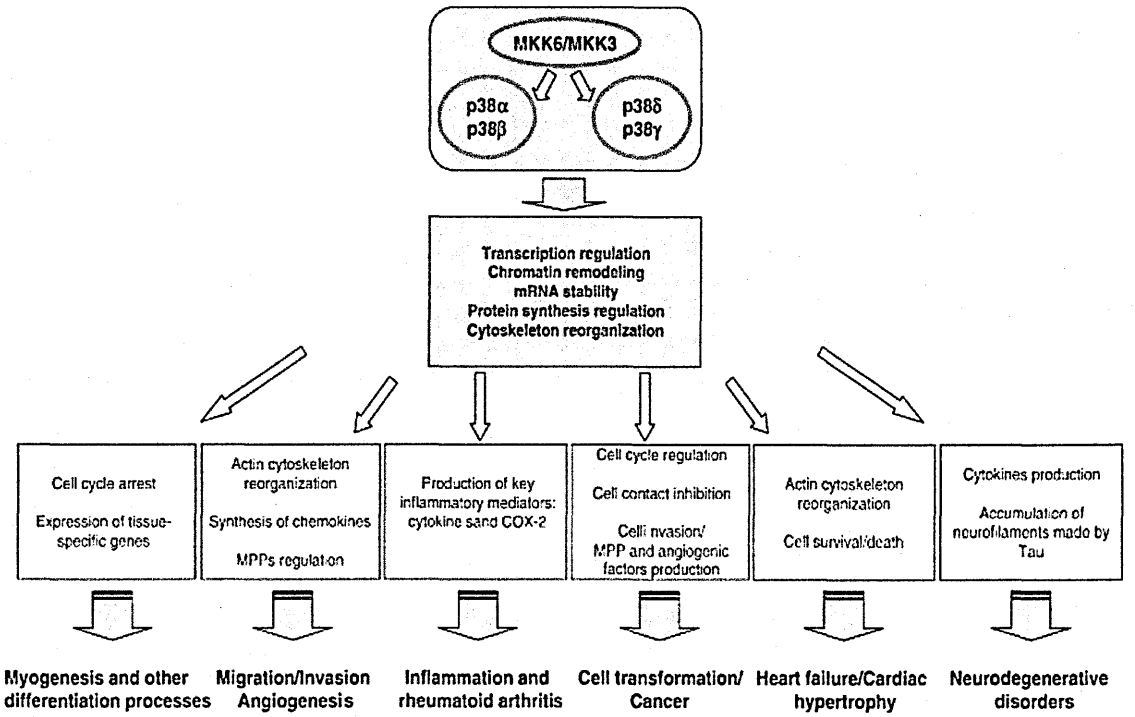

Figure 5. p38 family plays multiple roles in cell behavior. Once activated, depending on cell specific stimuli, p38 members are involved in myogenesis, inflammation, angiogenesis and also in cancer progression and cell transformation (Cuenda and Rousseau, 2007).

MK2) (Rouse et al., 1994; Freshney et al., 1994; McLaughlin et al., 1996). This substrate, along with its closely related family member MK3, were both shown to activate various substrates including small heat shock protein 27 (HSP27) (Stokoe et al., 1992), lymphocyte-specific protein 1 (LSP1) (Huang et al., 1997), CREB (Tan et al., 1996), transcription factor ATF1 (Tan et al., 1996), serum response factor (SRF) (Heidenreich et al., 1999), and tyrosine hydroxylase (Thomas et al., 1997). More recently, MK2 has been found to phosphorylate tristetraprolin (TTP), a protein that is known to destabilize mRNA hinting at a role for p38 in mRNA stability (Mahtani et al., 2001). p38 regulated/activated kinase (PRAK) is a p38 alpha and/or p38 beta activated kinase that shares 20-30% sequence identity to MK2 and is thought to regulate HSP27 (New et al., 1998). Mitogen- and stress-activated protein kinase-1 (MSK1) can be directly activated by p38 and ERK, and may mediate activation of CREB (Deak et al., 1998; New et al., 1999). A large number of cytosolic proteins can be phosphorylated by p38 members, including phospholipase A2, the microtubuleassociated protein tau, Na+/H+ exchanger 1 (NHE-1), cyclin D1, cyclin-dependent kinase inhibitors (CDKI), BCL2 family proteins, growth factor receptors or keratins (Ono and Han, 2000; Cuenda and Rousseau, 2007; Shi and Gaestel, 2002). The p38 pathway is an important regulator of protein turnover. For example, short isoform of FLIP is an inhibitor of TNF alpha-induced apoptosis whose proteasome-mediated degradation is regulated by p38 phosphorylation. A recent report has proposed that p38 alpha inhibits the lysosomal degradation pathway of autophagy by interfering with the intracellular

trafficking of the transmembrane protein ATG9. This would be mediated by p38 alpha-interacting protein (p38IP), which was found to bind to ATG9, facilitating starvation-induced ATG9 trafficking and autophagosome formation (Webber and Tooze, 2010). How exactly p38 alpha might regulate the p38IP–ATG9 interaction and whether this is the only link between the p38 pathway and autophagy remains to be elucidated. Additional examples of p38 substrates are the fibroblast growth factor receptor 1 (FGFR1) and the ARE-binding and mRNA-stabilizing protein human antigen R HuR. FGFR1 can be translocated from the extracellular space into the cytosol and nucleus of target cells, and regulates processes such as rRNA synthesis and cell growth. FGFR1 translocation requires p38 activation, which phosphorylates the C-terminal tail of FGFR1 on Ser777. Interestingly, the mutation S777A abolishes FGFR1 translocation, whereas phosphomimetic mutants bypass the requirement for active p38 for translocation (Sorensen et al., 2008). In the case of HuR, a new direct link with p38 family has been established recently in the G1 cell-cycle arrest induced by gamma-radiation (Lafarga et al., 2009). When cells are irradiated, p38 alpha is transiently activated and phosphorylates HuR on Thr118, which results in HuR cytoplasmic accumulation and enhanced binding to the p21Cip1 mRNA. HuR binding increases p21Cip1 mRNA stability, therefore allowing the expression of the appropriate p21Cip1 protein levels required for G1 cell-cycle arrest (Lafarga et al., 2009). Many transcription factors are phosphorylated and activated by p38 isoformss in response to different stimuli. Classical examples include ATF1, 2 and 6, SRF accessory protein 1 (Sap1),

CHOP [C/EBP (CCAAT/enhancer-binding protein)-homologous protein], p53 and myocyte enhancer factor 2 (MEF2C and MEF2A) (Shi and Gaestel, 2002). Recent results have established a role for p38 alpha in the regulation of lineage choices during myelopoiesis through phosphorylation of C/EBP alpha on Ser21 (Geest et al., 2009). A core network of 16 transcription factors has also been recently proposed to mediate the regulation by p38 alpha of human squamous carcinoma cell quiescence (Adam et al., 2009). In addition, p38 dependent phosphorylation of the transcription factor upstream stimulatory factor 1 (USF1) on Thr153 has been reported to facilitate acetylation, which in turn changes the gene regulatory properties of USF1 (Corre et al., 2009). p38 isoforms are emerging as important modulators of gene expression by regulating chromatin modifiers and remodellers (Simone, 2006). The promoters of several genes involved in the inflammatory response, such as IL-6, IL-8, IL-12p40 and monocyte chemoattractant protein 1 (MCP-1), display a p38 dependent enrichment of histone H3 phosphorylation on Ser10 in LPS stimulated myeloid cells. This phosphorylation enhances the accessibility of the cryptic NF-κB-binding sites marking promoters for increased NF-κB recruitment. Importantly, H3 phosphorylation is not carried out directly by p38, but more likely through MSK1 (Saccani et al., 2002). In addition, phosphorylation of histone H3 by the p38 pathway also contributes to the chromatin relaxation status necessary for nuclear excision repair factor assembly in response to UV-induced DNA lesions. Rapid activation of the p38 pathway in response to UV radiation helps to enhance the damaged-DNA-binding complex 2 (DDB2) ubiquitination, probably through

DDB2 phosphorylation. Consequent DDB2 degradation facilitates the recruitment of xeroderma pigmentosum complementation group C (XPC) needed for continuation of the repair process (Zhao et al., 2008a).

In *S. cerevisiae*, the p38 homolog Hog1 is recruited to the promoters of regulated genes through its association with transcription factors, which is important to stimulate RNA polymerase II as well as for recruitment of the Rpd3 histone deacetylase and SAGA (Spt–Ada–Gcn5–acetyltransferase) histone acetylase complexes, resulting in transcription initiation. Hog1 also mediates the recruitment of the chromatin structure remodelling complex of the SWI/SNF family to osmoresponsive genes, which is required for the nucleosome rearrangements found in osmostress genes in response to high osmolarity (de and Posas, 2010). The importance of p38 signalling for the function of SWI/SNF chromatin remodellers has also been demonstrated in higher eukaryotes. Thus, during muscle differentiation, p38 is required for the association between myogenic differentiation factor D (MyoD) and the ATPase subunits of the SWI/SNF complex BRG and BRM. Moreover, the SWI/SNF subunit BRG1-associated factor 60 (BAF60) is phosphorylated by p38 in vitro, and the functional relevance of this phosphorylation has been established (Simone et al., 2004). It is worth mentioning that several BAF60 isoforms have been implicated in the interactions between the SWI/SNF complex and transcription factors. In addition, BRG1 can interact with both MyoD and Pbx on the myogenin promoter (Lluis et al., 2006).

1.6.5 Cellular localization

In contrast with other MAPKs, p38 alpha has no nuclear localization signal and has been detected in both the nucleus and the cytoplasm of non-stimulated cells. However, the subcellular localization upon activation is controversial. Some evidence indicates that, following activation, p38 alpha translocates from the cytoplasm to the nucleus (Raingeaud et al., 1995), but there is also evidence showing that, in response to specific stimuli, p38 alpha preferentially accumulates in the cytosol (Ben-Levy et al., 1998). The discrepancy could be due to the analysis of different pools of p38 alpha, as it is conceivable that p38 alpha molecules may be located in different subcellular compartments as well as bound to different partners. For example, the re-localization of activated p38 alpha to the cytoplasm has been ascribed to nuclear export in association with its substrate MK2. A recent study postulates that p38 alpha nuclear translocation could be relevant for the regulation of G2/M cell-cycle arrest and to promote DNA repair, since p38 alpha translocates to the nucleus upon activation by stimuli that induce DNA double-strand breaks, but not other stimuli (Wood et al., 2009). Such translocation does not require p38 alpha catalytic activity, but it is induced by a conformational change triggered by the phosphorylation on Thr180 and Tyr182 at the activation loop (Wood et al., 2009). Since phosphorylation of p38alpha in response to DNA damage, but not in response to other stimuli, promotes nuclear accumulation, it is plausible that nuclear shuttling is also specifically induced by DNA damage. Therefore selective nuclear transport of p38alpha would require

both its phosphorylation and active nuclear shuttling. Alternatively, DNA damage signals could release p38 alpha from docking molecules such as MK2 or TAB1 that are known to retain p38 alpha in the cytosol (Engel et al., 1998; Lu et al., 2006), perhaps due to conformational changes induced upon DNA damage.

1.7: SIGNALING PATHWAY IN CANCER-SPECIFIC METABOLISM: HIF-1 ALPHA REGULATION

Biological processes require inevitably availability of ATP produced by mitochondrial respiration and cytosolic glycolysis. In mammals, organisms and their cells modify energy supplies, substrates availability and metabolic demands by balancing the amount of ATP generated by the different systems of the cell.

There are two major metabolic processes by which cells may obtain energy: lactic fermentation and aerobic respiration. In lactic fermentation, which occurs only in the cytosol, glucose is initially converted to pyruvate through glycolysis. Pyruvate is subsequently reduced to lactate, which is eliminated in the bloodstream. This process generates two ATPs per glucose molecule. In aerobic respiration glucose and other substrates are completely oxidized to carbon dioxide and water. It includes glycolysis, Krebs cycle and oxidative phosphorylation (OXPHOS), the last two processes occurring within mitochondria. Aerobic respiration is characterized by higher energy yield than lactic fermentation (ca. 15 times), but it can only occur in the presence of oxigen, which acts as electron acceptor, whereas fermentation can occur in its absence. Rapidly proliferating cells, key feature of embryogenesis, growth and tumorigenesis, exhibit a canonical anabolic profile, counterbalancing increased proliferative energy demands by exploiting the accelerated production of ATP by glycolysis (Chung et al., 2007). Cancer cells produce large amounts of lactate regardless of the availability of oxygen and for this reason their metabolism is

often reported as "aerobic glycolysis." This hypothesis, originally postulated by the Nobel Laureate Otto Warburg, came from the observation of a specific metabolic pattern in slices of living tissues. Thus, while in normal tissues lactate production occurs almost exclusively during oxygen absence, the so called Pasteur effect, this production is not eliminated in tumor slices by the presence of oxygen (WARBURG, 1956). Thus, he found that even in presence of oxygen, cancer cells prefer to metabolize glucose by glycolysis, respect to the more efficient oxidative phosphorylation. Glycolysis has the capacity to generate ATP at a higher rate than oxidative phosphorylation and so would be of advantage as long as glucose supplies are unlimited. Alternatively, it has been proposed that glycolytic metabolism arises as an adaptation to hypoxic conditions during the early phase of tumor development during which the vascularization is not complete, as it allows ATP production in the absence of oxygen (Gillies et al., 2008). An important player in the stimulation of glucose consumption and production of lactate is the HIF-1, a heterodimer composed of the constitutively expressed HIF-1 beta and HIF-1alpha subunit. HIF-1 alpha activation is highly associated with cancer cell growth and survival, tumor development, tumor angiogenesis, and poor clinical prognosis (Zhong et al., 1999; Birner et al., 2000; Akakura et al., 2001; Kung et al., 2000). In histopathological studies, HIF-1 alpha expression has been detected in most primary tumors of the brain, breast, colon, lung, ovary, and prostate and their metastases but not in the corresponding normal tissues (Zhong et al., 1999). In brain tumors, HIF-1 alpha expression correlated with histological grade (Zagzag et al., 2000). The expression of *EPO*,

another HIF-1 alpha targeted gene as well as the *EPO* receptor, is also markedly increased in gliomas and breast cancers (Acs et al., 2001). HIF-1 beta and HIF-1alpha subunits (Wang et al., 1995) contain basic helix-loop-helix domains that mediate dimerization and DNA binding, as well as a second dimerization motif referred to as the PAS domain based upon its original identification in the PER, ARNT, and SIM proteins. A superfamily of PAS domain proteins has been identified, the majority of which are prokaryotic signal transduction molecules involved in responding to environmental stimuli such as light, oxigen concentration, and redox state (Taylor and Zhulin, 1999). This finding suggested that HIF-1 alpha might be directly regulated by oxigen because the PAS domains of several prokaryotic proteins bind prosthetic groups such as heme. This complex is rapidly stabilized in absence of oxygen (Huang et al., 1998). *HIF-1 alpha* is expressed under the control of growth-factor signaling, in particular the PI3K/AKT/mTOR pathway (Cramer et al., 2003; Jiang et al., 2001). While cells are in normoxic conditions, HIF-1 alpha goes through a posttranslational modification by prolyl hydroxylation, In well oxygenated cells (normoxia), the hydroxylation of two proline residues (P402 and P564 in human HIF-1 alpha) by the HIF-prolyl hydroxylases [prolyl hydroxylases domains (PHDs)] allows the specific recognition and polyubiquitination by the von Hippel-Lindau protein (pVHL) E3–ligase complex, leading to proteasomal degradation (Maxwell et al., 1999) (Figure 6). Moreover, the hydroxylation of an asparagine residue (N803 in human HIF-1 alpha) by the factor inhibiting HIF (FIH) prevents binding of the

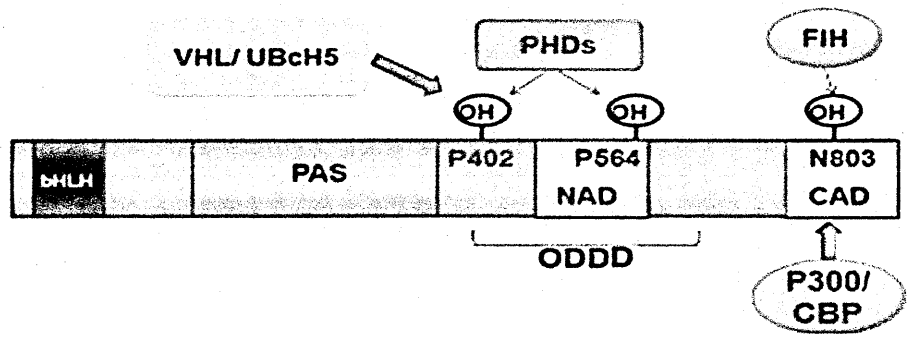

Figure 6. Structure and critical aminoacids of HIF-1 alpha. In this picture taken from (Kizaka-Kondoh et al., 2009) are indicated the domains of HIF-1 alpha (bHLH, PAS and ODDD) and its aminoacids targeted for regulation, such as the prolin P402 and P564 and asparagine N803.

coactivator p300/CBP and hence blocks HIF-1 alpha transcriptional activity (Lando et al., 2002) (Figure 6). In contrast, restricted availability of oxygen, by relaxing HIF-1 alpha hydroxylation, results in HIF-1 alpha stabilization and activation of the HIF transcriptional complex. Like FIH, the PHDs belong to the super family of iron- and 2-oxoglutarate-dependent dioxygenases, which, by using oxigen as a cosubstrate, provide the molecular basis for their oxigen-sensing function (Berra et al., 2006). In mammalian cells, three *PHDs* isoforms have been identified (*PHD1*, *PHD2*, and *PHD3*) and shown to hydroxylate HIF-1 alpha in cellulo depending on their relative abundance (Appelhoff et al., 2004). PHD2 has a dominant role, as it is the rate-limiting enzyme that sets the low steady-state level of HIF-1 alpha in normoxia (Berra et al., 2003). Constitutive cellular stabilization of HIF-1 alpha during normoxia is possible only in presence of mutations in the tumor suppressor *VHL* that occur in tumors. VHL disease is a hereditary cancer syndrome characterized by the development of highly vascular tumors that overproduce hypoxia-inducible mRNAs such as VEGF (Ivan and Kaelin, Jr., 2001). The product of the *VHL* tumor suppressor gene, pVHL, is a component of a multiprotein complex that bears structural and functional similarity to SCF (Skp1/ Cdc53 or Cullin/F-box) ubiquitin ligases (Stebbins et al., 1999; Ohh et al., 2000; Deshaies, 1999). In the presence of oxygen, pVHL, in association with elongin B and elongin C, binds directly to HIF-1 alpha subunits and targets them for polyubiquitination and destruction (Ohh et al., 2000; Tanimoto et al., 2000). Cells lacking functional pVHL cannot degrade HIF-1 alpha and thus overproduce mRNAs encoded by HIF target genes (Maxwell et

al., 1999). pVHL binds to a region of HIF-1 alpha called the oxygen-dependent degradation domain (ODDD) (Ohh et al., 2000), driving HIF-1 alpha toward ubiquitination and degradation. Dimethyloxalylglycine (DMOG), which is a competitive antagonist of alpha-ketoglutarate, inhibits hydroxylases and induces HIF-1-dependent transcription (Epstein et al., 2001). HIF-1 alpha activity is also induced by iron chelators, e.g., desferrioxamine (DFO) and cobalt chloride, which inhibit hydroxylases by displacing Fe(II) from the catalytic center (Epstein et al., 2001). During hypoxia, prolylhydroxylation is prevented by reactive oxygen species (ROS) generated in the mitochondria, stabilizing HIF-1alpha accumulation in the nucleus where it forms a complex with the constitutively expressed HIF-1 beta favoring the recruiment of transcriptional coactivator proteins, such as p300/CBP, and bind to the hypoxia response elements present in >70 known genes, enhanching their transcription (Brunelle et al., 2005; Mansfield et al., 2005; Guzy et al., 2005). HIF-1alpha induces genes that control crucial features of cancer biology, embracing angiogenesis, glucose metabolism, cell proliferation and invasion. Under hypoxic conditions, HIF-1 alpha mediates a transition from oxidative to glycolytic metabolism through its regulation of four factors: pyruvate dehydrogenase (PDH) kinase 1 (PDK1), lactate dehydrogenase A (LDHa), BNIP3, and BNIP3L. PDK1 inhibits the conversion of pyruvate to acetyl coenzyme A for entry into the tricarboxylic acid cycle (Kim et al., 2006; Papandreou et al., 2006). LDHa converts pyruvate to lactate (Semenza et al., 1996). Finally, BNIP3 (Zhang et al., 2008) and BNIP3L (Bellot et al., 2009) mediate selective mitochondrial autophagy. HIF-1 alpha also mediates a subunit

switch in cytochrome c oxidase (COX) that improves the efficiency of electron transfer under hypoxic conditions (Fukuda et al., 2007). HIF-1 alpha does not only stimulate glycolysis in tumor cells. Indeed, it has been shown that it is able to suppress the respiratory activity of mitochondria, suggesting that it can act as a master switch between glycolysis and oxidative phosphorylation (Kim et al., 2006).

1.8: METABOLIC EFFECTORS MEET CHROMATIN: THE FOXO3A FAMILY

In molecular biology and genetics, a transcription factor is a protein that binds to specific DNA sequences, thereby controlling the transcription of genetic information from DNA to mRNA (Karin, 1990; Latchman, 1993). Transcription factors perform this function alone or with other proteins in a complex, by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase to specific genes (Roeder, 1996; Nikolov and Burley, 1997; Lee and Young, 2000).

A defining feature of transcription factors is that they contain one or more DNA-binding domains (DBDs), which attach to specific sequences of DNA adjacent to the genes that they regulate (Mitchell and Tjian, 1989; Ptashne and Gann, 1997). Additional proteins such as coactivators, chromatin remodelers, histone acetylases, deacetylases, kinases, and methylases, while also playing crucial roles in gene regulation, lack DNA-binding domains, and, therefore, are not classified as transcription factors (Brivanlou and Darnell, Jr., 2002). A class of transcription factors possesses a particular DBD, the "forkhead domain", called in this way after the isolation of the first member. This "forkhead domain" is an approximate 100-amino acid, monomeric DBD and represents a variant of the helix–turn–helix motif. It is made up of three helices and two characteristic large loops or butterfly-like 'wings'. The high degree of sequence homology within the DBD is in contrast with an almost complete lack of similarity in the N-terminal and C-

terminal transactivation domains. Over the last decade, more than 100 members have been identified, with roles in development, differentiation, proliferation, apoptosis, stress resistance and metabolism (Carlsson and Mahlapuu, 2002) (Figure 7). During evolution, the number of forkhead genes appears to have increased, with greater numbers identified in vertebrates than in invertebrates; the human forkhead-box gene family consists of at least 43 members. Several years ago, a standard nomenclature for this family of proteins was introduced (Kaestner et al., 2000), and Fox (Forkhead box) was adopted as a unified symbol for all chordate forkhead/winged helix transcription factors. Subclasses are designated by a letter, and within each subclass proteins are given a number.

The FoxO class of transcription factors consists of four members: FoxO1, 3A, 4 and 6. The alternative names for these genes used in earlier studies were *FKHR* (*FoxO1*), *FKHRL1*(*FoxO3A*) and *AFX* or *Mllt7* (*FoxO4*). *FoxO2*, originally named *AF6q21* and cloned as a novel fusion partner for the AF6 protein, is homologous to *FoxO3a* and likely not a separate FoxO. *FoxO5* is expressed in *Danio rerio* only. *FoxO1*, *3A* and *4* are ubiquitously expressed, but between different cell types or organs, the expression level of these FoxOs can differ considerably. For example, *FoxO1* is highly expressed in adipose tissue, whereas *FoxO4* is highly expressed in muscle and *FoxO3A* in liver (Furuyama et al., 2000). *FoxO6* expression appears restricted to brain (Jacobs et al., 2003). Splice variants have been described for *FoxO1*, *3A* and *4* (Yang et al., 2002). Because of the shared DBD, FoxOs are expected to bind to similar sequences within the

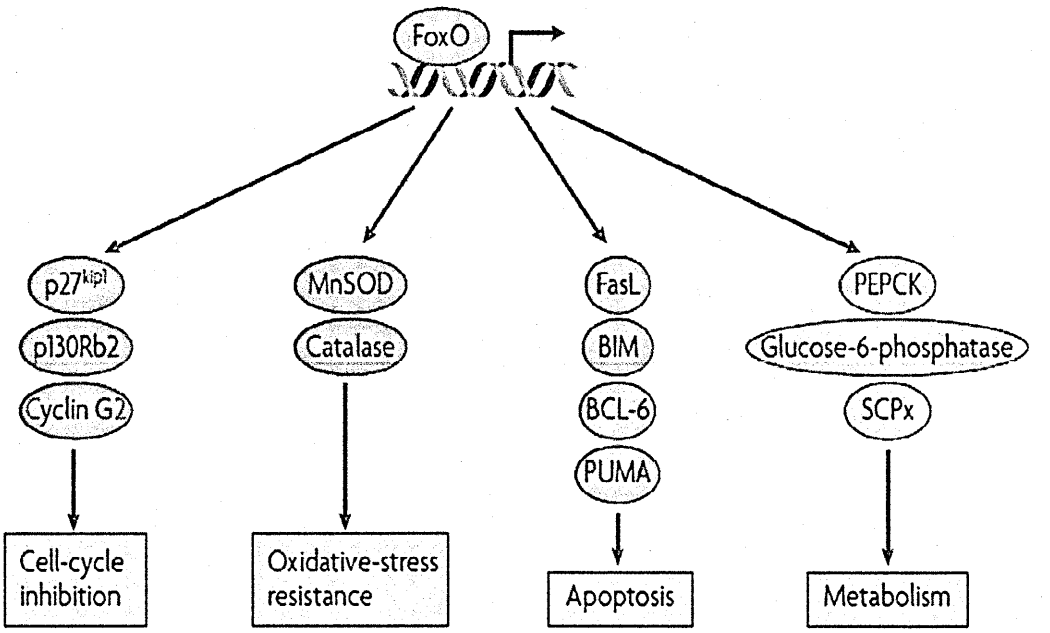

Figure 7. Transcriptional outputs of FoxO familyactivity. Increased class O forkhead box transcription factor (FoxO) activity participates in several cellular processes, such as inhibition of the cell cycle, regulation of cell death, protection from cellular (oxidative) stress, by the upregulation of catalase and regulation of cellular metabolism (gluconeogenesis and fatty-acid oxidation) trough phosphoenolpyruvate carboxykinase (PEPCK). The picture is taken from (van der Horst and Burgering, 2007).

DNA and a core consensus DNA sequence for FoxO binding has been determined (5'TTGTTTAC3') (Furuyama et al., 2000). However, the details of DNA binding of the FoxOs are still elusive.

Initial interest in the FoxO transcription factors stemmed from the fact that they were identified as translocation partners in a number of cancers. The best studied example being the t(2;13) and t(1;13) translocations present in a percentage of alveolar rhabdomyosarcomas resulting in a PAX3–FOXO1 (Galili et al., 1993) and PAX7–FOXO1 (Davis et al., 1994) fusion protein, respectively. Although these observations did not directly imply a role for FoxOs in tumorigenesis, it has become clear by now that FoxOs are genuine tumor-suppressors. An evolutionary conserved feature of FoxO transcription factors is that they function as downstream effectors of the PI3K/AKT (Burgering and Kops, 2002). The PI3K/AKT signalling cascade is a key pathway by which cells may respond to a wide range of stimuli, which may be generated intrinsically (for example, growth factors, cytokines and cell–cell contact) or from an external source (for example, irradiation, and physical or genotoxic stress) (Leevers et al., 1999; Hennessy et al., 2005; Wymann and Marone, 2005; Samuels and Ericson, 2006). At the molecular level, many of the PI3K/AKT-mediated mitogenic responses are achieved through the direct repression of FoxO transcription factors by AKT-mediated phosphorylation, promoting nuclear export, proteosomal degradation and a decrease in transactivation activity (Brunet et al., 1999; Brunet et al., 2001; Kops et al., 2002b; Jacobs et al., 2003). Oxidative stress induces translocation of FoxO from the cytosol to the nucleus (Henderson and Johnson, 2001; Brunet et

al., 2004; Essers et al., 2004) and peroxide-activated JNK phosphorylates FoxO4 at sites that are important for FoxO4 activity (Essers et al., 2004). AKT-mediated phosphorylation of FoxO induces binding of 14-3-3 proteins and this correlates with inhibition of FoxO through nuclear exclusion. JNK has been shown to phosphorylate 14-3-3, which results in reduced 14-3-3 binding to partner proteins (Sunayama et al., 2005). Another important factor in the activation of FOXO family members is the energy sensor AMP-activated Protein Kinase (AMPK). AMPK directly phosphorylates human FOXO3 at six previously unidentified residues in vitro (Greer et al., 2007). AMPK is necessary and sufficient for the phosphorylation of FoxO3A at these sites in cells. Mutation in these phosphorilation sites impairs FoxO3A-dependent transcription but does not affect FoxO3A localization. A genome-wide microarray analysis indicates that mutation of AMPK phosphorylation sites in FoxO3A specifically impairs the expression of a subset of target genes, including oxidative stress resistance and energy metabolism genes (Greer et al., 2007). Recently, we demonstrated that the activity of AMPK is necessary for FoxO3A-dependent transcription (Chiacchiera et al., 2009).

Cell cycle checkpoints are activated in response to genotoxic stress induced by most chemotherapeutic drugs (Lukas et al., 2004; Bartkova et al., 2005). Thus, in addition to preceding differentiation, cell cycle arrest may also be induced by genotoxic stress and lead to apoptosis. Indeed, most chemotherapeutic drugs are reported to arrest or delay cell cycle progression, and if the DNA damage or other

cellular defects are extensive, the cell will undergo either apoptosis or cellular senescence (Lukas et al., 2004; Bartkova et al., 2005).

Moreover, cancer cells may be considered to display an enhanced or higher level of basal stress, such as increased levels of ROS or stress kinase activity, and it is precisely the deregulation of cell cycle checkpoints that allow cancer cells to tolerate such cellular conditions, while simultaneously promoting genomic instability and tumor progression (Giles, 2006; Wu, 2006; Fruehauf and Meyskens, Jr., 2007). One such mechanism by which cancer cells can tolerate cellular stress is the deactivation of FoxO3A, which would be anticipated to be activated by oxidative stress and stress kinases, both of which may converge through JNK-mediated phosphorylation of FoxO3A (Essers et al., 2005; Essers et al., 2004; Vogt et al., 2005; Huang and Tindall, 2007). Thus, it is not perhaps surprising that the re-introduction of FoxO3A can induce tumor cell kill in cancer cells that have repressed FoxO3A activity (Modur et al., 2002), and also that activation of FoxO transcription factors has a role in mediating the cytostatic and cytotoxic functions of chemotherapeutic drugs in some cancer types. However, the cell cycle arrest induced by FoxO can have an influence on the eventual outcome in terms of cell fate and may also promote DNA damage repair and stress resistance (Tran et al., 2002). FoxO family members have also been shown to promote mammalian cell survival by inducing cell cycle arrest and quiescence in response to oxidative stress (Brunet et al., 2004; Kops et al., 2002a; Essers et al., 2004; Medema et al., 2000). At the same time, the CDKI p27Kip1, the prominent downstream target of FoxO, which blocks progression of cells from

late G1 to S phase, has also been implicated directly in the induction of apoptosis by FoxO (Wang et al., 1997; Wu et al., 1999; Dijkers et al., 2000).

Chapter 2: MATERIAL AND METHODS

2.1 Cell Culture

HT29, HCT116, OVCAR-3, A2780, SKOV-3 and DU145 cells were maintained in DMEM while LS174T and PC3 in RPMI 1640, each supplemented with 10% FBS, penicillin, and streptomycin, avoiding confluence at any time. All the cell lines were purchased from ATCC.

2.2 Reagents

SB202190 (SB; 10 mM stock) , Propidium Iodide (P.I.), trypan blue, Compound C (CC; 10 mM stock), AICAR (50mM stock), Deferoxamine (DFO; 100 mM stock), Cisplatin (CDDP; 30 mM stock) were purchased from Sigma. Z-IEDT-FMK from BD Bioscience. Dimethyl sulfoxide (DMSO) was purchased from SIGMA.

2.3 Microscopic Quantization of Viability, Cell Death, and Autophagic Cells

The viability and cell death of the reported cell lines were scored by cell counting. Briefly, at the indicated time points, the supernatants (containing dead/floating cells) were collected, and the remaining adherent cells were detached with Trypsin/EDTA (Sigma). Then, both cell suspensions were mixed and centrifuged. Cell pellets were suspended in 1M phosphate-buffered saline (PBS), and 10 μl were mixed with an equal volume of 0.01% trypan blue solution. The mixture was then transferred to the hemacytometer. Viable cells

(unstained cells) and dead cells (stained cells) were counted with a phase contrast microscope (x40). The percentage of viable cells (V) (% = 100 x V / [V + D]) and the percentage of dead cells (D) (% = 100 x D / [V + D]) were determined. The percentage of autophagic cells was measured by optical microscopy by measuring the number of vacuolated cells (A) against the total number of cells (% = 100 x A / [NA + A], where NA means "non autophagic". The data shown in Results are representative of three or more independent sets of experiments.

2.4 Proliferation Assay

Proliferation assays were performed using the colorimetric WST-1 assay (Roche). Briefly, $2x10^3$ cells for each cell line were cultured in 96-well plate in the presence or the absence of SB for up to 72 hours (OvCa cells experiments), 96 hours (PCa cells experiments) or 144 hours (CRC cells experiments). In the case of CDDP experiment CRC cells were treated also with CDDP alone or in combination with SB. In each experiment, DMSO was used as a vehicle. At the indicated time points, the WST-1 reagent was added with a final dilution of 1/10 for 30 minutes. The plate was shaken thoroughly for 60 seconds on a shaker, and then the absorbance of the samples was measured against a background control using a spectrophotometer at 450 nm, the reference wavelength being 650 nm. Each experiment was performed three times.

2.5 Colony Formation Assay

CRC, OvCa and PCa cells were cultured in 60-mm dishes in the presence or the absence of SB and/or CDDP (CDDP experiment). After 72 hours, the medium was discarded and the cells were washed 2 times with 1X phosphate-buffered saline. Two milliliters of Coomassie brilliant blue (Bradford) dye were added in each dish for 5 minutes. The cells were then washed with 70% ethanol to remove the excess of dye. The plates were dried at room temperature.

2.6 Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted with TRI reagent (Sigma) following the manufacturer's instructions. The samples were then treated with DNAase-1 (Ambion). 1 to 4 μg of total RNA were retrotranscribed using the High-Capacity DNA Archive Kit (Applied Biosystem) following the manufacturer's instructions. Polymerase chain reactions (PCRs) were performed in triplicate using the ABI 7500HT machine (Applied Biosystem). For all experiments, the following PCR conditions were used: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 45 seconds. The quantitative normalization of the cDNA in each sample was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin amplifications as internal control. Relative quantification was done using the ΔΔCT method. Each experiment were repeated three times. Each sample was loaded on the real time plate in duplicate.

2.7 Multiplex Real-Time PCR

Multiplex real-time PCRs (RT-PCRs) were performed with 5-U/ µl Taq DNA polymerase, 25 mmol/L MgCl2, 10x Taq buffer, and 10 mmol/L deoxyribonucleotide triphosphates (dNTP) purchased from 5PRIME. The following PCR conditions were used: 95°C for 15 minutes, followed by 28 cycles at 94°C for 30 seconds, 59°C for 90 seconds, 72°C for 60 seconds, and in the final step, 72°C for 10 minutes. The samples were loaded on a 2% agarose gel and pictures were taken.

2.8 Immunoblot analysis

Immunoblots were performed according to the instructions of Cell Signaling Technology, Inc. Briefly, cells were homogenized in 1X lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 5 mmol/L EDTA; 250 mmol/L NaCl; 0.1% Triton X- 100) supplemented with protease and phosphatase inhibitors (1 mmol/L phenylmethanesulphonylfluoride (PMSF), 1.5 mmol/L pepstatin A, 2 mmol/L leupeptin, 10 µg/mL aprotinin, 5 mmol/L NaF, and 1 mmol/L Na3VO4). From 20 to 40 µg of protein extracts from each sample were denatured in 5X Laemmli sample buffer and used for Western blot analysis. Western blots were executed using antibodies directed against alpha-actin (Sigma), pospho AKT (Ser473), AKT, phospho AMPK (Thr172), AMPK, p38 alpha, p38 total, phospho p38, hexokinase 2 (HK2; all from Cell Signaling Technology, Inc), FoxO3A total, phospho FoxO3A (Thr32; Santa Cruz Biotechnology), HIF-1 alpha (Abcam), activated caspase 3-8, PARP-1 cleaved fragment (p85), MAP1LC3 (LC3), PTEN,

phospho MK2, LKB1, phospho ACC. Western blots were developed with the ECL Plus chemiluminescence reagent (GE Healthcare) as per manufacturer's instructions. The densitometric evaluation was performed by the ImageJ software.

2.9 Immunofluorescence assay

CRC cells were seeded on glass coverslips and treated for 48 hours with SB, CDDP alone or in combination. At the end of the treatment cells were fixed in 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. Coverslips were incubated with the indicated primary antibodies. Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 594 from Invitrogen; nuclei were counterstained using P.I. Slides were sealed using Vectashield mounting medium (Vector Laboratories). Images were acquired using a Zeiss LSM-5 Pascal confocal microscopy.

2.10 ATP assay

HT29 cells were treated with SB or DMSO, as control, for up 8 hours. At the indicated time, Cells were collected, counted and lysed and ATP levels were determined by luciferine–luciferase assay; using the ATP Bioluminescence Assay kit (Roche) according to standard protocol. The results were normalized to the number of cells. The experiment were done five times.

2.11 FACS analysis

HT29 cells and HCT116 cells were plated and treated as indicated in the text. At the indicated time point cells were collected and counted to obtain the same number of cells. the samples were wash with PBS 1x two times risospende with 1X Binding Buffer (Sigma). 5µl of Annexin V FITC conjugate (Sigma) and 10µl of P.I. were added and incubate for 10 minutes. 20 thousands of events were counted by a BD FACS Advantage (BD Bioscience)

2.12 RNA interference

CRC cells were seeded in a 30-mm dish and transfected with 100 nM siRNA using RNAi MAX (Invitrogen) following manufacturer's instructions. siRNA against FoxO3A was added to HT29 cells and HCT116 cells for 12 hours. Then medium was replaced with fresh one. After 36 hours the cells were treated with CDDP alone (HCT116 cells) or SB plus CDDP (HT29 cells) for further 48 hours. Immunoblotting of cell lysate was performed to evaluate the protein levels of FoxO3A, p85 and actin. A scramble siRNA (Invitrogen) was used as control.

2.13 Glucose and Lactate assay

HT29 cells were plated in complete medium and treated or not with SB. At the indicated time points 50µl for each sample were mixed with 50µl of Glucose Reaction Mix (BioVision Research Products). After 30 minutes of incubation at 37°, protected from the light, the O.D at 570 nm was measured using a microplate reader. The concentration of glucose was estimated using a standard curve and

normalized on cell number. For lactate assay HT29 cells were treated as previously described. At the indicated time points 50µl of medium was mixed with 50µl of Lactate Reaction Mix (BioVision Research Product). After 30 minute at room temperature the O.D. at 450nm was measured using a microplate reader. The amount of lactate in the medium was estimated using a standard curve and normalized on cell number. Each experiment was performed three times and samples were plated in duplicate.

2.14. Statistical analysis.

All results are expressed as mean ± SEM. Gene expression statistical analysis were performed using NCSS statistical and power analysis software 2007. Multiple groups were tested by one-way ANOVA or two ways ANOVA repeated measures where appropriate, followed by Fisher's least significant difference test for unpaired data. Comparisons of two groups were performed using a Student's t test followed by Mann-Whitney U test where appropriate. A $P < 0.05$ was considered significant.

2.15. CONFAC software

The conserved transcription factor binding site (CONFAC) software enables the high-throughput identification of conserved transcription factor binding sites (TFBSs) in the regulatory regions of hundreds of genes at a time. The CONFAC software compares non-coding regulatory sequences between human and mouse genomes to enable identification of conserved TFBSs that are significantly

enriched in promoters of gene clusters from microarray analyses compared to sets of unchanging control genes using a Mann–Whitney *U*-test. For analysis go to http://morenolab.whitehead.emory.edu/cgi-bin/confac/login.pl (Karanam and Moreno, 2004)

2.16. Densitometry

Densitometry evaluation of western blotting were performed using Photoshop software (Adobe). Briefly, rectangular section of the same area of the immunoblot bands were created. For each band was analyzed the mean value obtained from the command "Histogram" (*a*). To avoid overestimation, for each band is subtracted the mean of the same rectangular section taken from a zone right above the band (*b*). The subtraction *a-b* gives the "normalized" value (*c*). This operation was done for each band, included the untreated one. Finally, each *c* is divided by the *c* of control (c_c). The ratio of the untreated was 1.

Example:

band 1 (control): a=150, b= 30, c_c=(a-b)= 150-30=120;

band 2 (compound X): a=120, b= 60; c_x=(a-b)= 120-60=60

Result:

c_c/c_c= 1; c_X/c_c= 60/120= 0,5.

Chapter 3: BACKGROUND

Since the beginning of my undergraduate studies, I worked with Dr. Simone and colleagues to elucidate the role of p38 alpha in CRC homeostasis, in which we demonstrated that the inhibition of this kinase led to the induction of autophagy, cell cycle arrest and cell death with autophagic features. Indeed, we induced pharmacological blockade of the p38 cascade using SB and analyzed the gene expression profile of HT29 cells growing logarithmically, confluent in culture or induced to differentiate. Five genes were first considered, three of them being cell cycle-related: *cyclin E*, *cyclin A* and *p21*. The other two genes codify for tissue-specific proteins: *sucrose isomaltase* (*SI*) and *villin*. At the onset of differentiation, SB treatment inhibits the expression of *p21*, *SI* and *villin* confirming the role of the p38 pathway in the differentiation program of distinct cell types (Comes et al., 2007). Surprisingly, we discovered that p38 alpha is required for CRC cell proliferation and survival as pharmacological blockade or genetic depletion (by RNAi) of its kinase activity induced growth arrest, autophagy and cell death in a cell type-specific manner (Comes et al., 2007). Of note, p38 alpha blockade in HT29 cells induced the formation of large cytoplasmic vacuoles regardless of culture conditions (Comes et al., 2007). To investigate the nature of these vacuoles, we employed different cytological methods such as periodic-acid Schiff (PAS), Papanicolaou, Sudan black B and Oil red O. Among them only PAS demonstrated a dot-like staining within vacuoles suggesting the presence of glycoproteins (Comes et al., 2007).

Numerous large vacuoles filled up with dense material, autophagic vacuoles containing cell organelle debris and double-membrane autophagosomes were recognized by Transmission Electron Microscopy (TEM). The detection of these structures, termed as the 'golden standard' for assessing autophagic activity, clearly indicated that pharmacological blockade of p38 alpha induced an autophagic response in HT29 cells (Comes et al., 2007). To better characterize this process, we evaluated the expression of the *GABARAP* gene, one of the human homologs of the yeast *ATG8* gene. *ATG8* is a member of a novel ubiquitin-like protein family and an essential component of the autophagic machinery. In yeast, it is upregulated by rapamycin-dependent Tor inhibition and nitrogen starvation. In HT29 cells, *GABARAP* expression levels were significantly augmented by p38 alpha blockade and correlated with autophagic vacuoles and autophagosomes. Notably, pharmacological blockade of SB-dependent autophagy by 3-methyladenine (3MA), a specific inhibitor of autophagy, not only failed to increase viability, but even increased cell death by inducing a switch from autophagic cell death to apoptosis (Comes et al., 2007). To get further insight into the involvement of p38 alpha in the autophagic response, we extended our investigation to other 4 human CRC cell lines: CACO2, SW480, LS174T and HCT116. Cytological and morphological evaluation confirmed the SB-mediated induction of growth arrest and autophagic cell death in all CRC lines. Consistently, SB-mediated upregulation of *GABARAP* was detected in all cell lines tested (Comes et al., 2007). To investigate whether p38 alpha inhibition induced PCD type II in a cell type-

specific manner, Hep3B liver carcinoma cells, HeLa cervical carcinoma cells, HEK293 embryonic kidney cells, and human fetal skin fibroblasts (hFSF) were exposed to SB for 24 hours. Pharmacological inhibition of p38 alpha failed to induce cell cycle arrest, autophagy or cell death, nor did it promote *GABARAP* upregulation in any of the cell lines tested (Comes et al., 2007).

Furthermore, my colleagues demonstrated that SB treatment is protective upon tumor development in a mouse model of CRC xenograft. They injected $5x10^6$ LS174T cells into each flank of athymic nude mice. Once the tumors reached a measurable volume (mean = 61.6 mm^3), p38 alpha was continuously inhibited by daily intraperitoneal injection of SB. After 10 days mice were sacrificed since control tumors attained a mean volume of 500 mm^3. We found that SB treatment can inhibit tumor growth in nude mice in a dose-dependent manner. To further investigate the efect of p38 alpha inhibition *in vivo*, we used the murine model of colorectal cancer ($APC^{min/+}$) and , once induced the tumor formation, we treated these mice with SB for two weeks. This experiment showed that SB treatment in this model induced a strong reduction both of the volume and of the number of tumors. It is worth noting that no signs of liver damage were detected in mice serum (Chiacchiera et al.,2009).

In conclusion, the inhibition of p38 alpha in CRCs provoked the induction of cell growth arrest, the activation of autophagy and cell death with autophagic features (Comes et al., 2007). Moreover, the inactivation of the p38 alpha greatly affected the cell growth of xenografts and induced a strong reduction in tumor volume and number in *in vivo* experiments (Chiacchiera et al., 2009).

3.1 AIM OF STUDY

As discussed before, the pharmacological inhibition of p38 alpha with SB and its genetic ablation induced autophagy, cell cycle arrest and cell death with autophagic features in CRC cells (Comes et al., 2007). Moreover, this inhibition increased the mRNA levels of different metabolic genes (Chiacchiera et al., 2009).

Since autophagy is activated by energy demand, due to the high levels of intracellular AMP (Hardie, 2011) and, recently, p38 alpha was associated to the inhibition of autophagy, by the sequestering of protein involved in autophagosomes formation (Webber and Tooze, 2010), the aim of my studies was to understand the possible causes of this autophagic activation during the pharmacological inhibition of p38 alpha and the putative role of this protein in this scenario.

To this purpose we used three CRC cell lines, HT29, HCT116 and LS174T and we focused our attention on the glycolytic pathway, since it is the fastest way to obtain energy by cancer cells (Warburg, 1956). Then we extend our analysis to other malignancies, such as OvCa and PCa, evaluating whether the inhibition of p38 alpha could be useful also for other types of cancer. However, we checked whether there were implied other regulator proteins and whether the inhibition of p38 alpha could modify other pathways different from autophagy. Moreover, this kinase is linked also with the resistance to chemotherapeutics, such as CPT-11 and 5-FU (Paillas et al.,2011; Yang et al.,2011) and its inhibition or genetic

ablation was linked to the overcome of the resistance to these compounds. We also decided to evaluate the role of p38 alpha in the treatment with CDDP of two CRC cell lines, HCT116 and HT29, which showed two different outcomes to this treatment. The inhibition of p38 alpha in this scenario could be useful to understand the mechanisms related to the resistance to CDDP in CRC cell lines

Chapter 4: RESULTS

4.1 p38 alpha inhibition in colorectal cancer

One of the effect of p38 alpha inhibition in CRC is the activation of autophagic pathway (Comes et al., 2007). This pathway is activated by energy demand (Hardie, 2011) so we measured the intracellular levels of ATP during p38 alpha inhibition. We cultured HT29 cells for up to 8 hours in the presence of SB or DMSO, used as a control. Then, we measured the amounts of ATP with a luciferin-luciferase assay (Figure 8). The results showed a decrease in the levels of ATP in the first hours of treatment in SB-treated cells.

Since cancer cells predominantly produce ATP by constitutively activated aerobic glycolisys (WARBURG, 1956; Christofk et al., 2008), we determined whether this decrease could be attributed to variations in this pathway. To support the hypothesis that p38 alpha inhibition could produce a variation in glycolytic pathway, we treated HT29 cells with SB or DMSO for up two hours and we measured by real time PCR the levels of mRNA of the most important genes involved in glycolysis such as glucose transporter 1 (*GLUT1*), the rate-limiting enzymes *HK1* and *HK2*, pyruvate kinase isoform M2 (*PKM2*), and *LDHa*. Moreover, it has been shown that tumor cells express exclusively the PKM2, which is necessary for aerobic glycolysis, and that provides a selective growth advantage for tumor cells *in vivo* (Christofk et al., 2008). It is worth noting that knockdown of LDHa has been proven to severely diminish tumorigenicity of

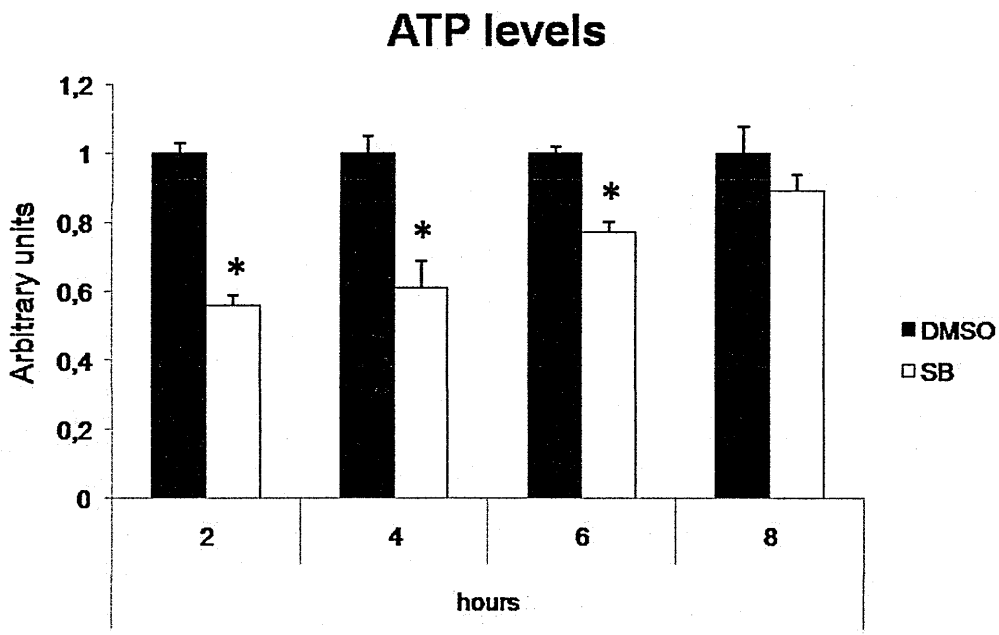

Figure 8. Effect of SB treatment on ATP levels in HT29 cell line. HT29 were cultured with or without 10μM of SB for 8 hours and intracellular ATP levels were measured by luciferin-luciferase assay. ATP levels reduced in the first hours of SB treatment and then increased probably due the activation of autophagy. ATP levels of DMSO treated cells were used as control. Results are expressed as mean ±SEM (n=5). Comparison between ATP levels of SB-treated and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*= $p<0.05$).

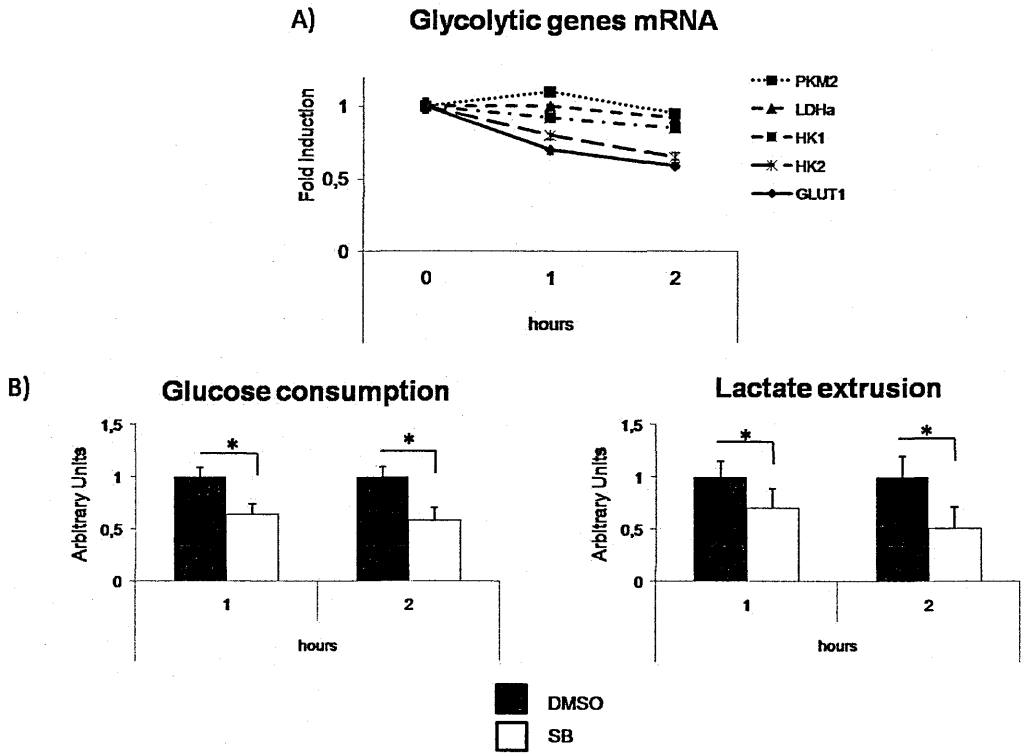

Figure 9. Evaluation of p38 alpha inhibition on Glycolytic pathway. HT29 cells were treated with SB (10μM) or DMSO for up to 2 hours. A) mRNA levels of *PKM2*, *LDHa*, *HK1*, *HK2* and *GLUT1* were analyzed by real-time PCR at the indicated time points. All these genes decrease in a time dependent manner, indicating that glycolytic genes expression is affected by p38 alpha blockade. beta-actin was used as calibrator of mRNA quantity. The relative mRNA levels are expressed as fold induction. Results are expressed as mean ±SEM (n=3). B) Glucose consumption was measured as the difference of glucose amount in the medium of HT29 cells SB-treated versus DMSO-treated at the indicated time points. The consumption of glucose by SB-treated cells was lower than DMSO-treated cells. The measures were normalized on cells number. Results are expressed as mean ±SEM (n=3). Comparison of glucose amount in the medium between SB-treated and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$). Lactate production was measured comparing lactate amount in the medium of SB-treated and DMSO-treated cells at the indicated time points. The amount of lactate in the medium of SB-treated cells decreased in a time dependent manner. The measures were normalized on cells number. Results are expressed as mean ±SEM (n=3). Comparison of glucose amount in the medium between SB-treated and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

cancer cells in mice (Fantin et al., 2006) (Figure 9A). As shown in the figure, these genes resulted downregulated in SB-treated cells, especially *GLUT1* and *HK2,* which are the transporter of glucose in to the cells and first enzime in its metabolism. To have a wider view in the glucose metabolism, we treated HT29 cells with SB for up to two hours and we quantified the levels of extracellular glucose and the lactate amount in the medium, using a colorimetric assay (Figure 9B,C). Both the glucose consumption and the lactate extrusion decreased during the use of SB in this cell line. These results indicate that p38 alpha inhibition could trigger a modification of the glucose metabolism. To better evaluate the glycolytic pathway, we treated HT29 cells and LS154T cells with SB for up to 72 hours and we performed a real time PCR to evaluate the expression levels of genes involved in this pathway (Figure 10). Worthy of note, among the genes used for our analysis only *HK1* was not downregulated, probably because is not the main isoform used by CRC cell lines to metabolize the glucose, while the other genes were rapidly downregulated and mainteined at a decreased expression levels for longer time during p38 alpha inhibition. Given the fact that all these genes are HIF-1 alpha targets genes (Schofield and Ratcliffe, 2004), we decided to investigate whether a relationship could exist between p38 alpha and this transcription factor. We treated HT29 cells and LS174T cells with SB for up to 96 hours and we carried out a western blot analysis, to detect the protein levels of HIF-1 alpha (Figure 11A). As shown in the figure, in both cell lines HIF-1 alpha protein levels decreased in a time dependent manner. To confirm these

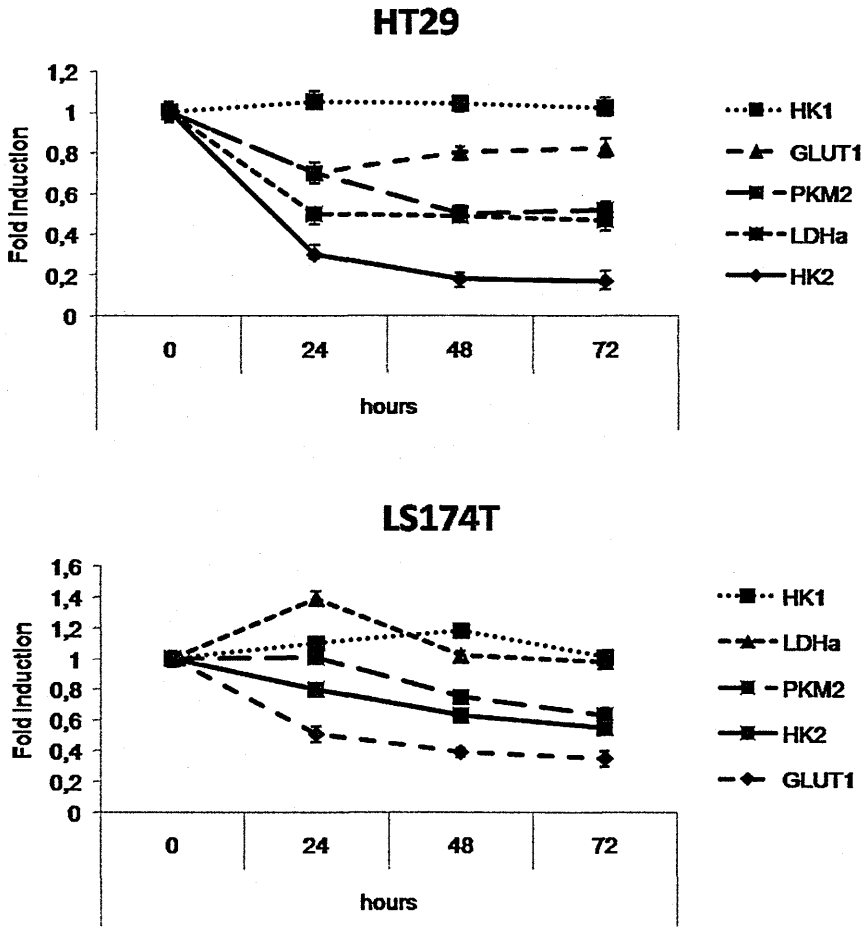

Figure 10. Effect of SB treatment on Glycolytic genes expression. HT29 cells and LS174T cells were treated for up to 72 hours with SB (10μM). The glycolytic genes (*GLUT1*, *HK1/2*, *PKM2* and *LHDa*) were analyzed by real time PCR. The prolonged p38 alpha inhibition affected the transcription of these genes in both cell lines, indicating that this kinase could sustain glycolytic pathway. The relative mRNA levels are expressed as fold induction. beta-actin was used as calibrator of mRNA quantity. The relative mRNA levels are expressed as fold induction. Results are expressed as mean ±SEM (n=3).

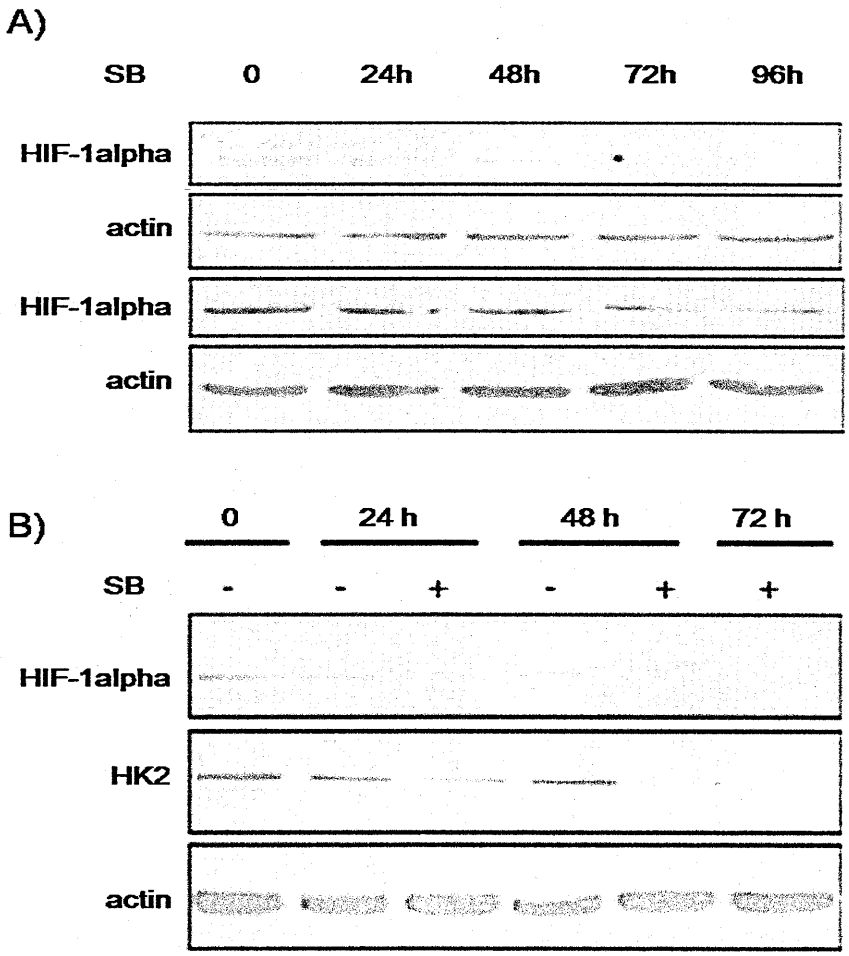

Figure 11. Relationship between SB-treatment and HIF-1 alpha protein levels and its transcriptional activity. A) HT29 cells and LS174T cells were treated for up to 96 hours with SB (10μM) and were collected at the indicated time points to analyze HIF-1 alpha protein levels by immunoblotting. The inhibition of p38 alpha induced a reduction of HIF-1 alpha protein in a time dependent manner, indicating that exist a relationship between the kinase and this transcription factor. Actin was used as loading control. The pictures shown are representative of three replicates. B) HT29 cells were treated for up to 72 hours with SB (10μM) or DMSO and were collected at the indicated time points. HIF-1 alpha and HK2 protein levels were evaluated by immunoblotting. This experiment elucidated that the absence of HIF-1 alpha it is related to its target gene protein. Actin was used as loading control. The pictures shown are representative of three replicates.

data, we treated HT29 cells for up to 72 hours with SB and we also checked for the protein levels of the HK2 (Figure 11B). The figure shows that during SB treatment there is a strong decrease of the protein level of HK2, which is well related to the decrease of HIF-1 alpha protein levels.

Given these results, we investigated whether p38 alpha and HIF-1 alpha could be linked. Two papers have established a positive role of p38 alpha in the stabilization of this transcription factor, and these authors used MEFs (Emerling et al., 2005) and pancreatic cancer cells (Kwon et al., 2005) as model. We took advantage of deferoxamine (DFO), a well known drug that mimics the effects of hypoxia (Wang and Semenza, 1993), to stabilized HIF-1 alpha and we treated HT29 cells for 24 hours with this compound in presence or not of SB. We evaluated the protein levels of HIF-1 alpha by immunoblot analysis (Figure 12A). As shown by densitometric analysis, DFO treatment was not able to completely rescue the degradation effect of SB on HIF-1 alpha protein, indicating that p38 alpha could play a role in HIF-1 alpha stabilization in CRC cells. Then, we evaluated whether p38 alpha also plays a role on the transcriptional activity of HIF-1 alpha. We performed the same previous experiment in HT29 cells and we analyzed the mRNA levels of *HK2* and *LDHa* (Figure 12B). When the drugs are used together, mRNA levels of these genes were similar to those in which DFO is used alone, indicating that p38 alpha could be not involved in this process. This result is in agreement with Emerling and colleagues that used a luciferase assay to demonstrate that p38 alpha was not involved in the transcriptional activity of

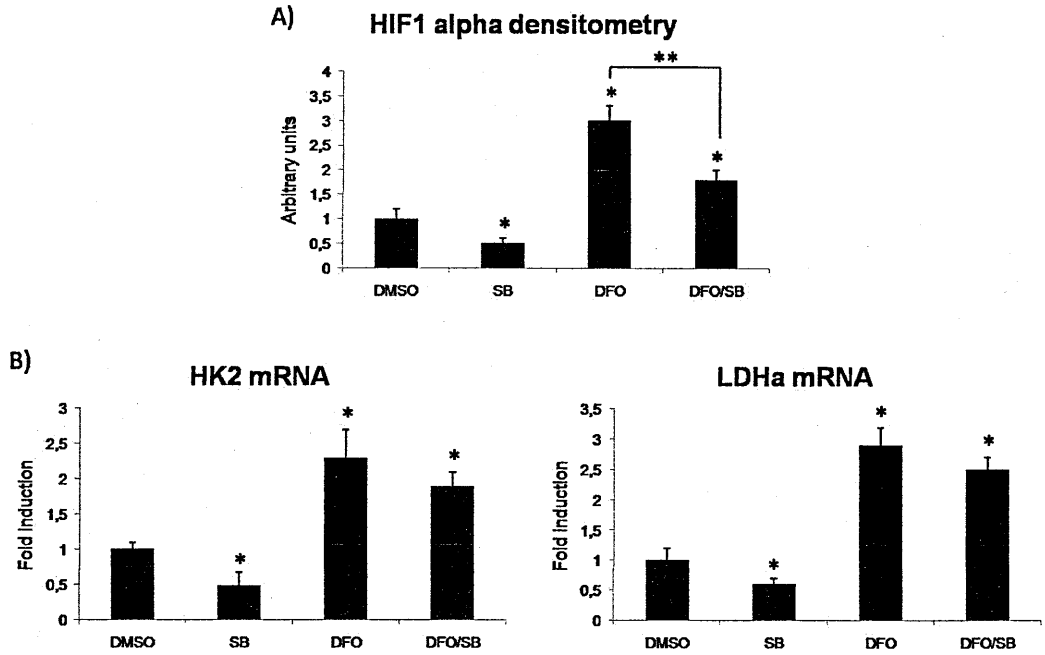

Figure 12. p38 alpha inhibition affected HIF-1 alpha protein stability but not its dependent transcription. A) HT29 cells were treated for 24 hours with DMSO, SB (10μM), DFO (100μM) and DFO plus SB. The graph indicates the ratio between the band intensity of each experimental point (SB, DFO and DFO/SB) and control (DMSO) for HIF-1 alpha. The stabilization by DFO of HIF-1 alpha protein is disrupted during p38 alpha inhibition. Actin was used as loading control. Comparison between densitometry of each treatment and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$). Results are expressed as mean ±SEM (n=3). B) HT29 cells were treated for 24 hours with DMSO, SB (10μM), DFO (100μM) and DFO plus SB. The graph shows mRNA levels of two HIF-1 alpha target genes (*HK2* and *LDHa*). HIF-1 alpha transcriptional activity was not affected when DFO and SB are used together, indicating that p38 alpha is not involved in HIF-1 alpha dependent transcription. The relative mRNA levels are expressed as fold induction. beta-actin was used as calibrator of mRNA quantity. Comparison between densitometry of each treatment and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*/**=$p<0.05$). Results are expressed as mean ±SEM (n=3).

HIF-1 alpha but only with its stabilization (Emrling et al., 2005). All these experiments indicated that the inhibition of p38 alpha in CRC cell lines induces a decrease of the glycolityc pathway. Indeed, the inhibition of this kinase produces a strong reduction of HIF-1 alpha protein and the transcription of the most important glycolityc genes, depriving these cells of the fastest "fuel" for survival. In CRC cells SB treatment promotes a significant upregulation of genes that regulate autophagy, cell cycle progression and cell death (Comes et al., 2007). Starting from this observation we decided to analyzed a greater number of genes related to these pathways and we treated HT29 cells for 96 hours with SB and we performed a real time PCR to detected the changes of their mRNA levels (Figure 13A-C). Interestingly, we found that upon p38 alpha blockade the autophagic genes homolog of *yeast ATG8*, such as *GABARAP*, *GABARABPL1* and *MAP1LC3*, and other *ATG* genes were upregulated in a time-dependent manner, indicating that the inhibition of p38 alpha induced a strong energy demand, probably due to the blockade of glycolysis. Moreover, also the genes related to cell cycle arrest, such as *p21* and *p27,* and to cell death, such as *BIM* and *GADD45* were upregulated in a time dependent manner. Worth of note is that the genes involved in the progression of cell cycle, such as *CYCA2* and *CYCD1*, were downregulated, confirming that the inhibition of p38 alpha induced autophagy, cell cycle arrest and cell death (Comes et al., 2007). Since this analysis revealed a strong reprogramming in CRC cell lines during p38 alpha inhibition, we decided to explore the possibility that one or more transcription factors might be triggered

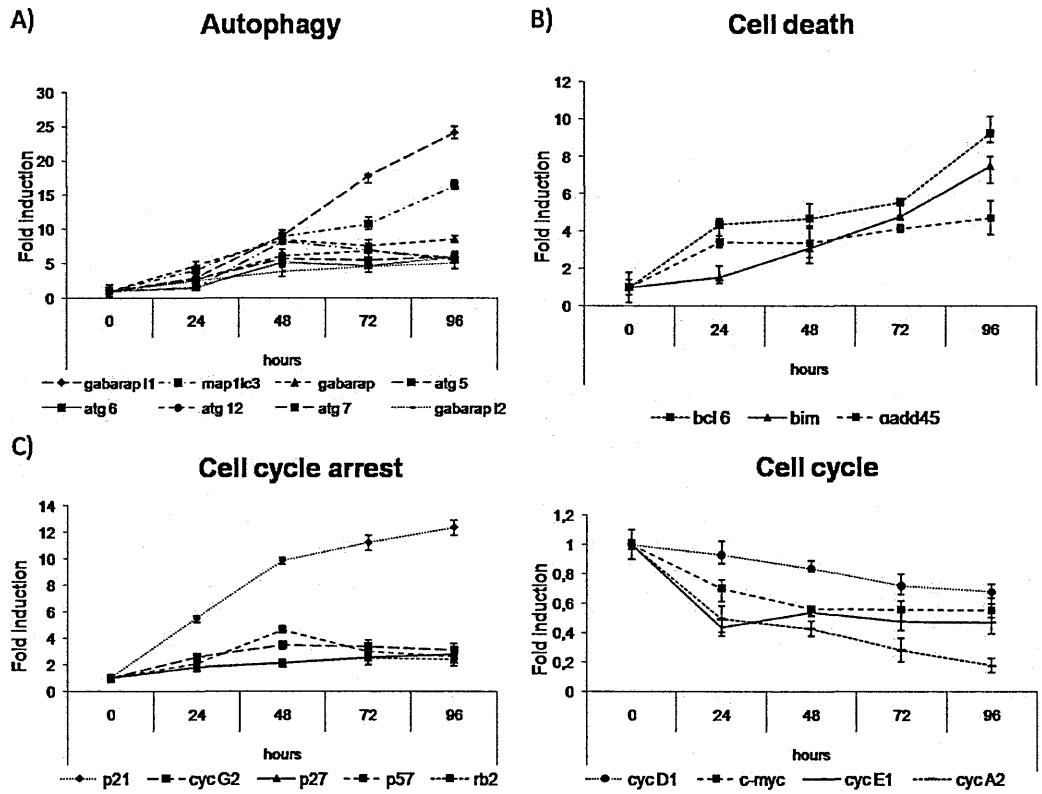

Figure 13. SB-treatment in HT29 cells induced upregulation of genes involved in autophagy, cell cycle arrest and cell death. A-C) HT29 cells were treated with SB (10μM) for up to 96 hours. During SB treatment, mRNA levels of genes related to autophagy, cell cycle arrest and cell death are upregulated. Interestingly, gene related to cell cycle progression were downregulated during p38 alpha inhibition. The relative mRNA levels are expressed as fold induction. beta-actin was used as calibrator of mRNA quantity. Results are expressed as mean ±SEM (n=3).

in this scenario. Thus, my colleagues made use of the "CONFAC" software (Karanam and Moreno, 2004) to perform an *in silico* analysis of the promoters of these genes and they found that the SB treatment-responsive genes were significantly enriched in the conserved consensus core recognition motif FHRE, which is specific for FoxO3A, indicating that this transcription factor could play a role during SB treatment of CRC cells. To evaluate whether FoxO3A is activated in this scenario, we decided to analyzed the pathways that control the localization of this protein in the cells. It is worth noting that AKT is a negative regulator of FoxO family, while AMPK has a positive role in its regulation, recruiting the members in the nucleus (Greer et al., 2007). To ascertain whether these pathways were involved in FoxO3A activation during p38 alpha inhibition, we treated HT29 cells for up 96 hours and we performed an immunoblot analysis to verify the activation status of these upstream regulators of FoxO3A (Figure 14). Significantly, we found that throughout the inhibition of p38 alpha, AKT is not phosphorylated while AMPK is phosphorylated in a time-dependent manner. Moreover, the protein levels of PTEN, a negative regulator of PI3K/AKT pathway (Maehama and Dixon, 1999), resulted increased. This experiment showed that FoxO3A regulating pathways were modified during SB treatment triggering its activation. To better understand the role of AMPK in this scenario, we treated HT29 cells with SB with or without the AMPK inhibitor Compound C (CC) for 24 hours (Figure 15). The inhibition of AMPK abrogates the induction of FoxO3A-target genes induced by p38 alpha inhibition, indicating that this kinase plays a role in the FoxO3A-dependent transcription during SB treatment.

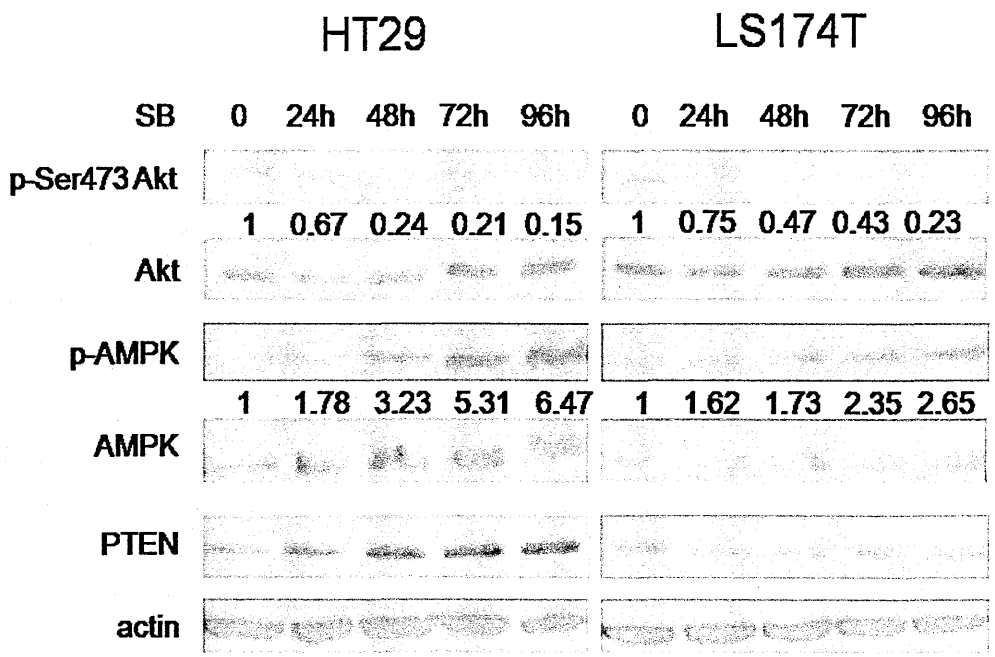

Figure 14. p38 alpha inhibition modulated FoxO regulating pathways . HT29 cells and LS174T cells were treated with 10μM of SB for up to 96 hours. Cells were collected at the indicated time points and analyzed for the amount of protein levels of phosphorylated and total form of AKT, AMPK and PTEN. The numbers indicate the ration of phosphorylated form versus the total amount of protein. As indicated by the ratio, the inhibition of p38 alpha induced modification in the phosphorylation status of AKT and AMPK kinases and the accumulation of the tumor suppressor PTEN. This immunoblot indicates that during SB-treatment AKT is inhibited while AMPK is activated. Actin was used as loading control. The pictures shown are representative of three replicates.

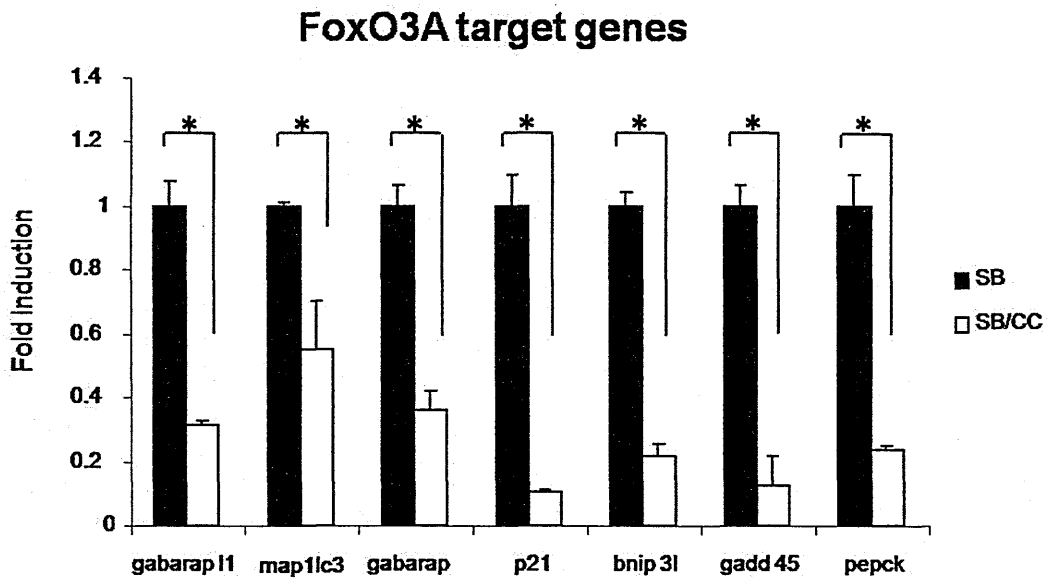

Figure 15. SB-treatment induced upregulation of FoxO3A-target genes in HT29 cells in AMPK-dependent manner. HT29 cells were treated for 24 hours with SB (10μM) with or without Compound C (CC, 10μM), to relate AMPK activation to FoxO3A-dependent transcription. When AMPK is inhibited during SB treatment, this kinase failed to activate FoxO3A-dependent transcription. beta-actin was used as calibrator of mRNA quantity. Comparison of mRNA levels of genes indicated in the graph between SB-treated and SB/CC- treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$). Results are expressed as mean ±SEM (n=3).

Moreover, my colleagues demonstrated that the silencing of AMPK by siRNA assay abolished the nuclear localization of FoxO3A in HT29 cell line during SB treatment (Chiacchiera et al., 2009). All these data indicated that p38 alpha inhibition in CRC cellsproduced an impairment in the glycolytic pathway. This is related with the decrease of HIF-1 alpha protein level and its related transcriptional activity. Moreover, SB treatment positively regulates the FoxO3A-dependent transcription of genes involved in autophagy, cell cycle arrest and cell death by acting on the AMPK and PI3K/AKT pathways

4.2 The role of p38 alpha in ovarian cancer

The inhibition of p38 alpha impairs key metabolic functions of CRC cells, inducing growth arrest, autophagy, and cell death both *in vivo* and *in vitro* (Comes et al., 2007; Chiacchiera et al., 2009). These effects are mediated by a switch from HIF-1 alpha to FoxO3A-dependent transcription (Chiacchiera et al., 2009). These results prompted us to ascertain whether other tumors are sensitive to p38 alpha inhibition. To this end, we tested SB activity in other types of malignancies, focusing on cancers in which the role of HIF-1 alpha has been well documented, such as those originating in the ovary (Osada et al., 2007).

For this purpose, we first evaluated the expression pattern of *p38* isoforms in OvCa cell lines, evaluating the mRNA of the four *p38* isoforms in OVCAR-3, A2780, and SKOV-3 OvCa cell lines by a semiquantitative analysis (Figure 16A). This experiment revealed that *p38 alpha* represents the main isoform in these cells, such as in CRC cells. Because SB specifically inhibits p38 alpha and p38 beta, we analyzed the relative abundance of the mRNA of these two isoforms by quantitative RT-PCR in OVCAR3 cells (Figure 16B). We confirmed that *p38 alpha* is highly expressed, whereas *p38 beta* is not detectable. Then, we measured the basal levels of the activated form of p38 in these cells by immunoblot analysis (Figure 16C). When compared with primary human fibroblasts, which express both the alpha and beta isoforms of p38 at levels comparable to those detected in cancer cells but are insensitive to SB (Comes et al., 2007), OVCAR-3 cells were found to display an aberrant activation of p38, similar to that detected

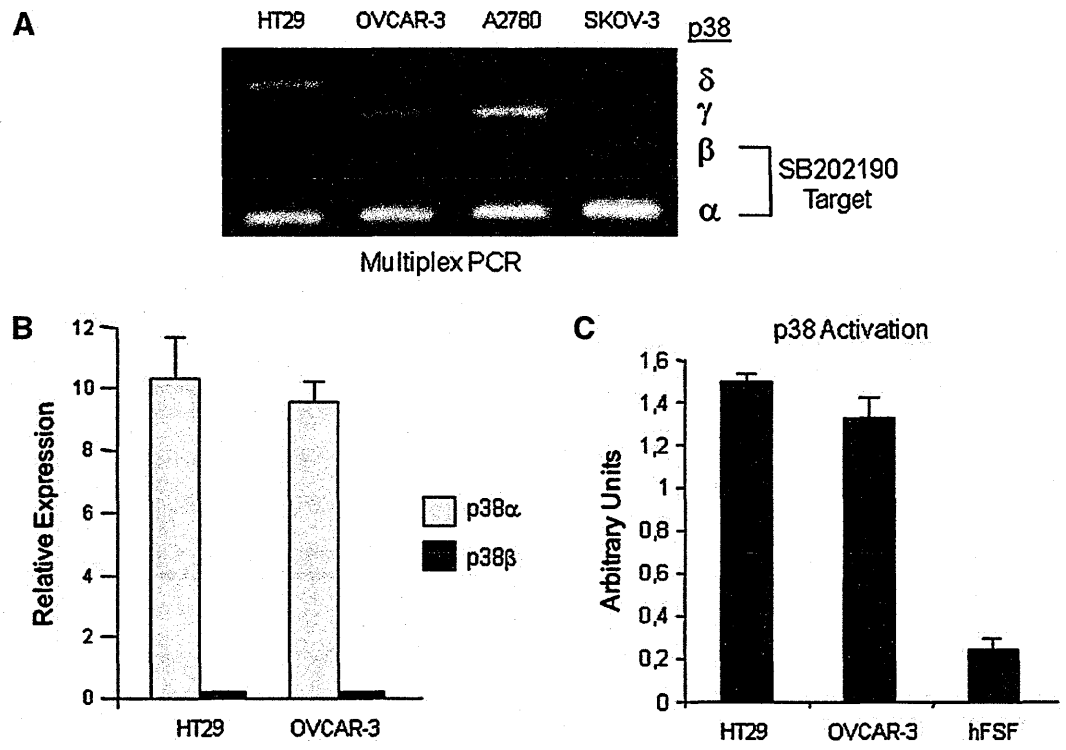

Figure 16. p38 alpha is required for OvCa cell homeostasis. Characterization of the expressions of the p38 alpha, beta, gamma and delta isoforms in HT29, OVCAR-3, A2780, and SKOV-3 cell lines. A) Multiplex RT-PCR analysis was performed to evaluate the amount of each p38 isoforms. beta-actin was used as calibrator of mRNA quantity. B) The relative levels of expression of the p38 alpha and beta isoforms were measured by quantitative real-time PCR analysis in HT29 cells and OVACR-3 cells. beta-actin was used as calibrator of mRNA quantity. Results are expressed as mean ±SEM (n=3). C) Activation state of p38 in HT29 cells, OVCAR-3 cells and human fibroblasts (hFSF). The values are the results of the densitometric analysis of the phosphorylated form of p38 normalized against p38 total. Actin was used as loading controls. Results are expressed as mean ±SEM (n=3).

in HT29 cells. These results prompted us to inhibit p38 alpha in the three OvCa cell lines.To test the effects of SB treatment in OvCa cells, we treated for up to 72 hours with SB or DMSO OVCAR-3, A2780, and SKOV-3 cells and we evaluated the morphological changes in these cell lines by optical microscope (Figure 17). Significantly, the exposure to SB induced the formation of large intracellular vacuoles and reduced the number of cells compared with DMSO-treated cells. As in CRC, we decide to explore the possibility that the inhibition of this kinase could affect the protein levels of HIF-1 alpha. To this purpose we treated OVCAR-3 cells with SB for up to 48 hours and we analyze the protein levels of HIF-1 alpha and of HK2 (Figure 18A). We found that HIF-1 alpha and HK2 protein levels decreased in a time-dependent manner, thus indicating that also in OvCa cells the inhibition of this kinase could play a role in HIF-1 alpha stabilization. We also treated OVCAR-3 cells with SB for up to 72 hours to evaluate the expression profile of HIF-1 alpha targets genes *HK2* and *PKM2* (Figure 18B). These genes resulted down-regulated by p38 alpha blockade in OVCAR-3 cells. These data, similar to CRC cell lines, suggest that p38 alpha could be involved in HIF-1 alpha stabilty also in OvCa cells. Since in CRC cell lines the inhibition of p38 alpha induced a block in HIF-1 alpha-dependent transcription triggering a FoxO3A-dependent transcription, after nuclear localization and activation of this trascription factor (Chiacchiera et al., 2009). We treated OVCAR-3 cells with SB for up to 72 hours and we analyzed the activation status of FoxO3A regulators and FoxO3A transcriptional activity (Figure A-D). SB treatment significantly reduced the inhibitory phosphorylation

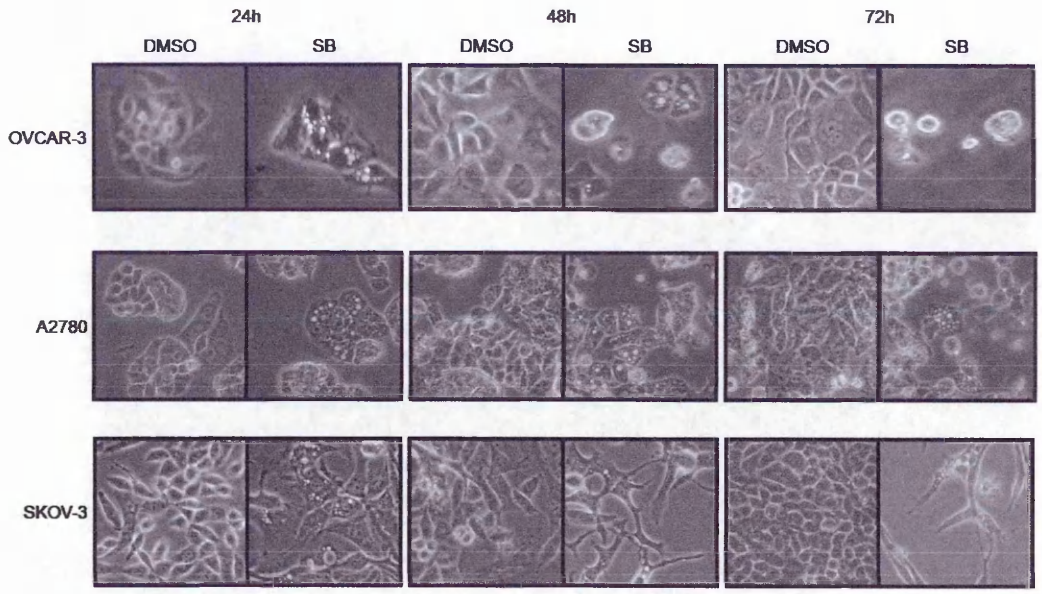

Figure 17. Morphological evaluation of three OvCa cell lines. OVCAR-3, A2780, and SKOV-3 cells were treated or not (DMSO) with SB (10μM) for up to 72 hours. SB treatment reduces the number of cells and increased the number of vacuolated cells. Representative pictures of three replicates were taken at the time points indicated.

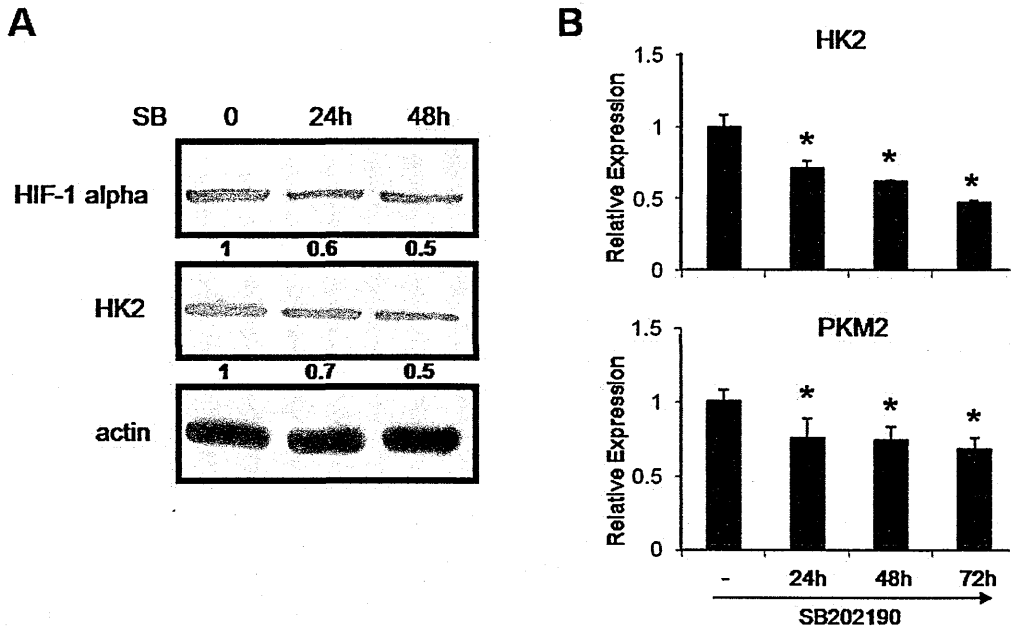

Figure 18. p38 alpha sustains HIF-1 alpha protein expression and its dependent transcription. A) OVCAR-3 cells were treated with SB (10μM) for up to 48 hours. HIF-1 alpha and HK2 protein levels were analyzed by immunoblot. The inhibition of p38 alpha reduced the amount of HIF-1 alpha and HK2 protein levels. The numbers are the ratio of the densitometric analysis of the protein levels in DMSO treated cells and in SB-treated cells and actin. Actin was used for loading control. The pictures shown are representative of three replicates. B) OVCAR-3 cells were cultured with SB (10μM) for up to 72 hours. Quantitative real-time PCR analysis of HIF-1 alpha target genes *HK2* and *PKM2* involved in glycolysis was performed. beta-actin was used as calibrator of mRNA quantity. Results are expressed as mean ±SEM (n=3). Comparison of mRNA levels of genes evaluated during the treatment with SB was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

by AKT of FoxO3A. Importantly, this time-dependent reduction in phosphorylated

FoxO3A is related to the reduction in AKT phosphorylation. Conversely, AMPK was significantly phosphorylated in OVCAR-3 cells. These findings depicted a scenario very similar to CRC cells during SB treatment. Consistently, all FoxO3A target genes tested, some of which code for proteins playing a role in autophagy such as *MAP1LC3*, *GABARAPL1*, and *GABARAP*, and others genes involved in cell cycle control and cell death, were significantly up-regulated by p38 alpha blockade Among the cell cycle regulators, we detected a significant induction of the cyclin-dependent kinase inhibitors *p21* and *p27*, the retinoblastoma family protein retinoblastoma-like 2 (*RBL2*), and the transcriptional repressor *BCL-6*. Moreover, we detected the down-regulation of the genes coding for *Cyclins E* and *A2*.

Since the inhibition of p38 alpha in CRC cell lines induced a perturbation in the viability and an increase of cell death with autophagic features (Comes et al., 2007), our purpose was to analyze whether also in OvCa cells SB treatment produced the same results. We treated OVCAR-3, A2780, and SKOV-3 cells for 72 hours with SB and we performed a colony formation assay (Figure 20A). As shown in the picture, we found that there was a strong reduction in number of colonies in each cell line. After this result we treated OVCAR-3 cells for up to 72 hours with SB to analyzed the cause of this decrease and we performed a WST-1 analysis and a cellular count with trypan blu (Figure 30B-D). SB treatment

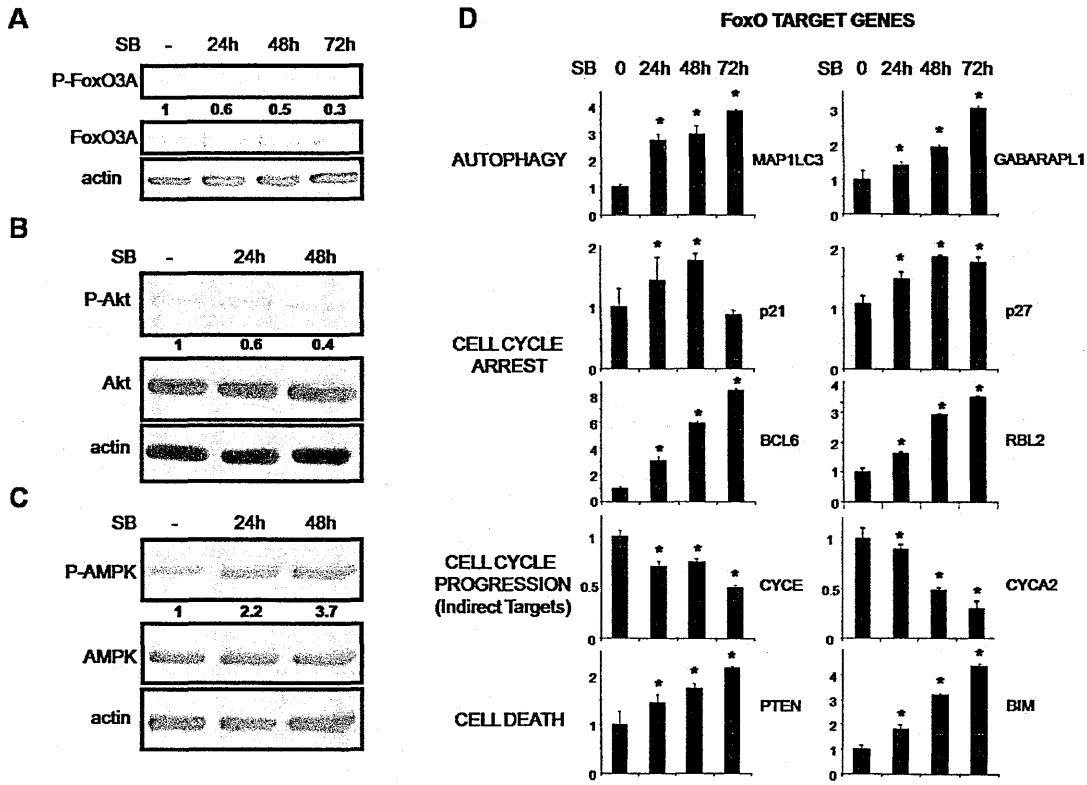

Figure 19. SB switches the activation of signaling pathways promoting FoxO-dependent transcription. OVCAR-3 cells were treated with SB (10μM) for up 72 hours (A) and for up to 48 hours (B,C). Immunoblot analysis was performed to evaluate the phosphorylation status of FoxO3A (A), AKT (B) and AMPK (C). The inhibition of p38 alpha induced the decrease of inhibitory phosphorylation of FoxO3A (A) and the decrease of AKT phosphorylation (B). Conversely, AMPK is activated (C). The numbers are the ratio of the densitometric analysis of the phosphorylated amount of the indicated proteins normalized against their total amount and actin. Actin was used for loading control. The pictures shown are representative of three replicates. D) OVCAR-3 cells were cultured in the absence or the presence of SB (10μM) for up to 72 hours. Quantitative real-time PCR analysis of FoxO3A target genes involved in autophagy, cell cycle arrest, and cell death was performed. p38 alpha blockade induces the expression of FoxO3A target genes involved in these pathways. Cell cycle progression genes (*cyc E* and *cyc A2*) are indirect targets. beta-actin was used as calibrator of mRNA quantity. Results are expressed as mean ±SEM (n=3). Comparison of mRNA levels of genes evaluated during the treatment with SB was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

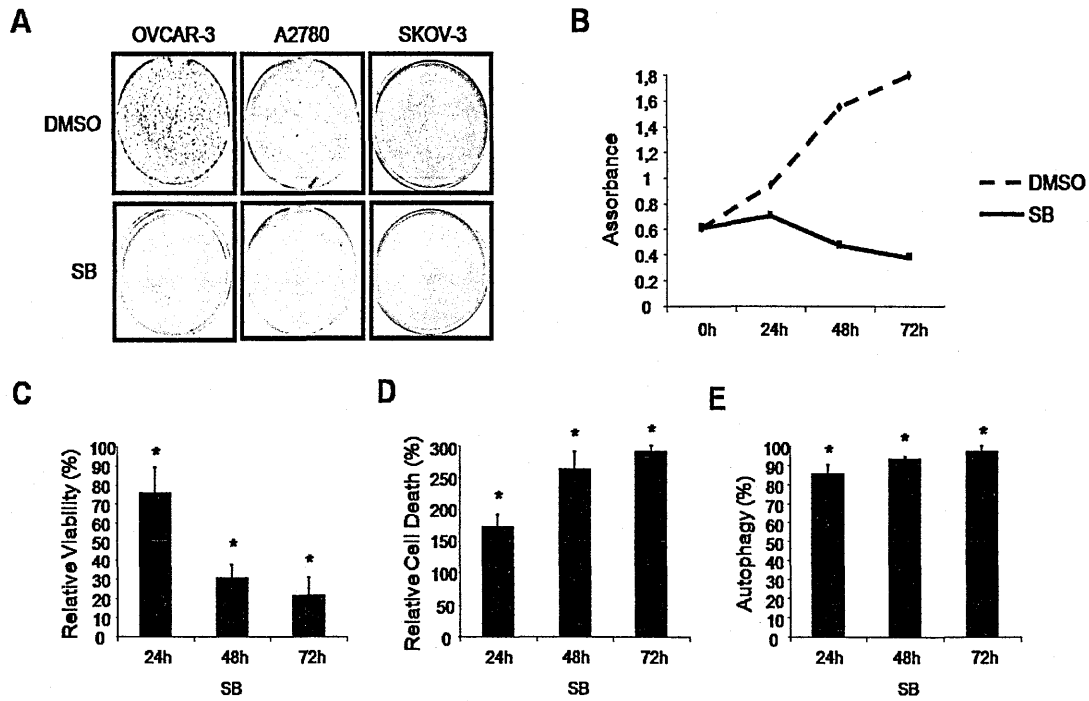

Figure 20. p38 alpha inhibition affects OVCAR-3 proliferation and survival. A) OVCAR-3, A2780, and SKOV-3 cell lines were treated with or without SB (10μM) for 72 hours. Colony formation assay was performed. Cell were stained with Coomassie blue. Colored cells are living cells. The inhibition of p38 alpha affected the cell number of these three OvCa cell lines. Representative pictures of three replicates were taken. B) OVCAR-3 cells were treated with or without SB (10μM) for up to 72 hours. OVCAR-3 cell growth was quantified using the WST-1 cell proliferation assay. The inhibition of proliferation during SB treatment was confirmed in OVCAR-3 cells. Results are expressed as mean ±SEM. C-D-E) OVCAR-3 cells were treated with or without SB (10μM) for up to 72 hours. C) Relative viability (percent) was estimated by calculating the ratio between the viable cells of treated cells (SB) versus those of DMSO treated cells. Results are expressed as mean ±SEM (n=3). Comparison of viable cells between treated (SB) and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$). D) Relative cell death (percent) represents the ratio between the dead cells in the treated cells versus those in the untreated OVCAR-3 cells. Results are expressed as mean ±SEM (n=3). Comparison of dead cells between treated (SB) and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$). E) The percentage of autophagic cells was estimated by phase-contrast microscopy. More than 100 cells were used for this evaluation. Results are expressed as mean ±SEM (n=3). Comparison of vacuolated cells between SB-treated and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

induced a strong reduction of cell number, as indicated by the low value of absorbance of the WST-1 assay and this is related to a decrease in viability and to an increase of cell death in a time-dependent manner. We checked also whether SB treatment induced the same autophagic vacuolation of CRC cells. To this purpose we treated OVCAR-3 cell for up to 72 hours and we counted the number of vacuolated cells (Figure 20E). This experiment showed that there was a time-dependent autophagic vacuolation, similar to CRC cells, from the first hours of SB treatment, affecting more that 80% of cells at 24 hours and reaching almost 100% at 48 and 72 hours. These results, together with the AMPK activation and the transcription of FoxO3A-dependent autophagic genes could suggest that inhibition of p38 alpha caused autophagic cell death also in OvCa cells.

All these data confirmed once again that inhibiting p38 alpha, in tumors which displayed an overactivation of this kinase, induced autophagy, cell cycle arrest and cell death. This scenario is due to the degradation of HIF-1 alpha and the activation of FoxO3A-dependent gene transcription.

4.3 Prostate cancer and p38 alpha inhibition

Besides CRC and OvCa, we decided to test the efficacy of the p38 alpha inhibition also on prostate cancer, which is another tumor that depends on HIF-1 alpha for sustaining (Khandrika et al., 2009). We employed two widely used PCa cell lines, DU145 and PC3. As for CRC cells and OvCa cells, to determine which was the most abundant isoform among the members of *p38* family we performed a multiplex PCR for all four isoforms in these cell lines (Figure 21A). Once verified that *p38 alpha* was the most abundant one, we treated DU145 cells and PC3 cells for up to 72 hours with SB and we performed an immunoblot analysis to check the phosphorylation status of MK2 (Figure 21B). This experiment showed that MK2 was not phosphorylated in both cell line when p38 alpha was inhibited, indicating that this kinase is inhibited. However, we can not explain the differences in the MK2 basal phosphorylation status between the two cell lines. Further analysis are necessary. Since in CRC cells and OvCa cells the inhibition of p38 alpha affected HIF-1 alpha protein levels and its dependent transcription, DU145 cells were cultured for up to 72 hours with SB to evaluate HIF-1 alpha protein levels and its transcriptional activity (Figure 22A and B). This experiment showed a decrease of HIF-1 alpha protein level which came together with the decreased expression of three glycolytic genes in a time-dependent manner.

Next step of our analysis was to evaluate the citotoxic effect of p38 alpha inhibition on PCa cell lines. To this purpose, we treated both cell lines with SB or not for up to 96 hours and we performed a cellular count using trypan blue dye

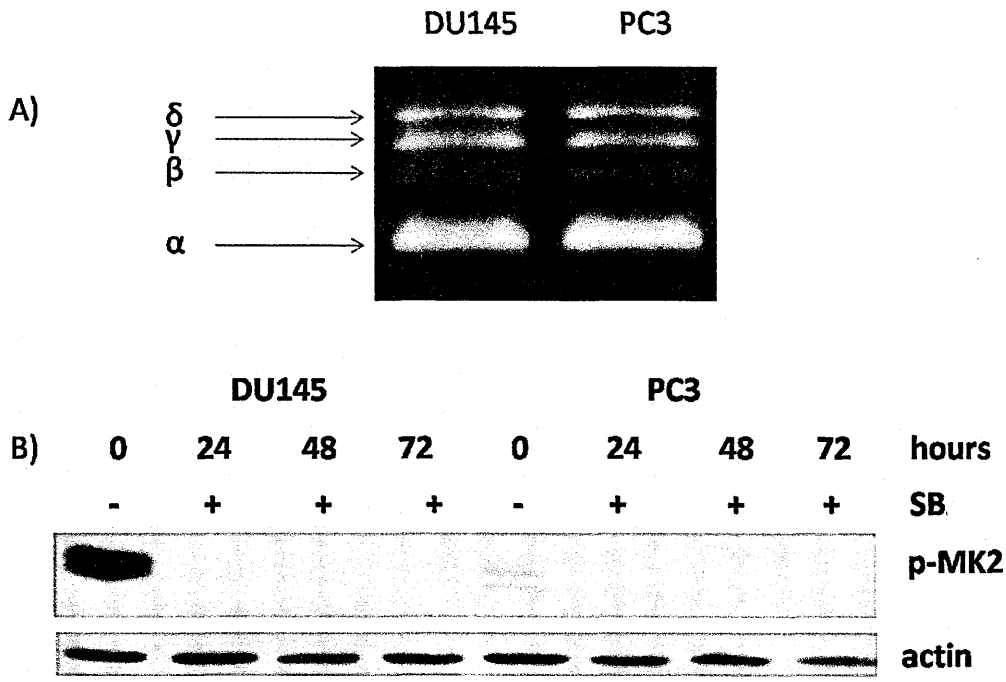

Figure 21. Analysis of isoforms and activity of p38 alpha in PCa cells. A) A multiplex PCR for the four isoforms of p38 in PCa cells was performed. The abundant isoform in these two prostatic cancer cell lines is alpha. The pictures shown are representative of three replicates. B) PCa cell lines were treated with SB (10μM) for up to 72 hours. Immunoblot for the activation status of p38 alpha was performed. In both cell lines p38 alpha is blocked by SB administration, as shown by the not phosphorylated status of MK2 during this treatment. Actin was used as loading control. The pictures shown are representative of three replicates.

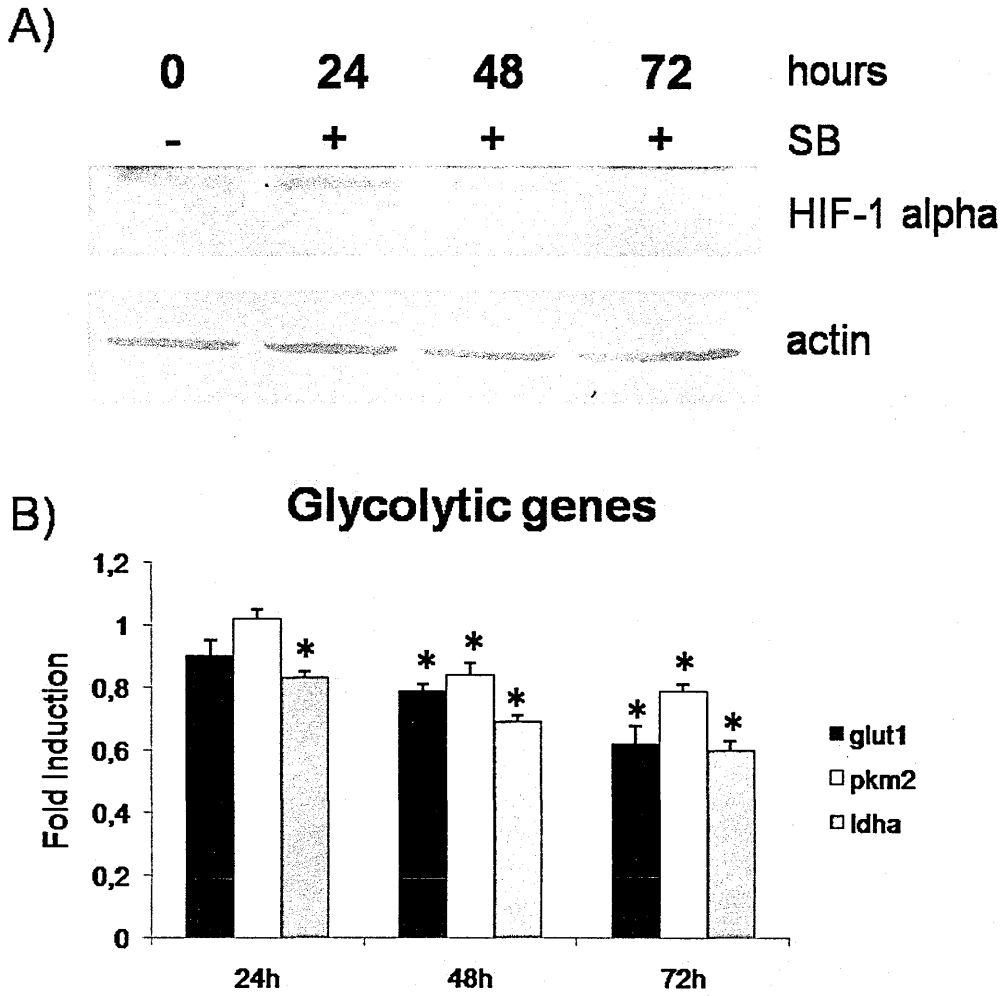

Figure 22. p38 alpha inhibition affects HIF-1 alpha protein levels and its transcriptional activity. A) DU145 cells were treated with SB (10μM) for up to 72 hours. Immunoblot analysis of HIF-1 alpha protein was performed. As in CRC and OvCa cell lines, p38 alpha inhibition affects the protein levels of HIF-1 alpha protein. Actin was used as loading control. The pictures shown are representative of three replicates. B) DU145 cells were treated with SB (10μM) for up to 72 hours. Real time PCR of the most important glycolytic genes (*GLUT1*, *PKM2* and *LDHa*) was performed. The decrease of HIF-1 alpha protein levels is reflected by the downregulation of its glycolytic target genes. beta-actin was used as calibrator of mRNA quantity. Results are expressed as mean ±SEM (n=3). Comparison of mRNA levels of these genes between treated (SB) and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

(Figure 23A,B). Surprisingly, the treatment had different outcomes between the two cell lines; indeed, DU145 cells revealed a strong reduction in cell number after 48 hours of treatment, while PC3 cells were still able to grow, but at a reduced rate compared to DMSO-treated cells. This result was confirmed by the percentage of cell death, which increased in DU145 cells, especially after 48 hours of SB treatment, but remained unchanged in SB-treated PC3 cells. Moreover, we treated these two cell lines with SB or DMSO for up to 96 hours and we performed a WST-1 analysis (Figure 24). This experiment confirmed that p38 alpha inhibition induced different effects in these two cell lines, as demonstrated by the diverse value of absorbance. Indeed, DU145 cells showed a decreased of this value, indicating a reduction of cell number, while in PC3 cells this value slowly increased, indicating that they are proliferating. To investigate the causes of these different responses in PCa cells to p38 alpha inhibition, we performed immunoblot analysis of du145 cells and PC3 cells treated with SB for up to 72 hours and we checked for the modulation of relevant pathways altered in SB-treated CRC cells and OvCa cells (Figure 25A). Importantly, we detected a significant difference in AMPK activation between the two cell lines, while the modulation of AKT pathway is almost similar. Even if the basal levels of total-AKT are different in DU145 and PC3 cells, possibly due to PTEN mutations in PC3 cells, its phosphorylation decreased in a time-dependent manner upon p38 alpha inhibition in both cell lines while AMPK was not activated by SB treatment in DU145 cell line. The strong difference in AMPK phosphorylation status led us to check known modifications in the AMPK pathway and we decided to

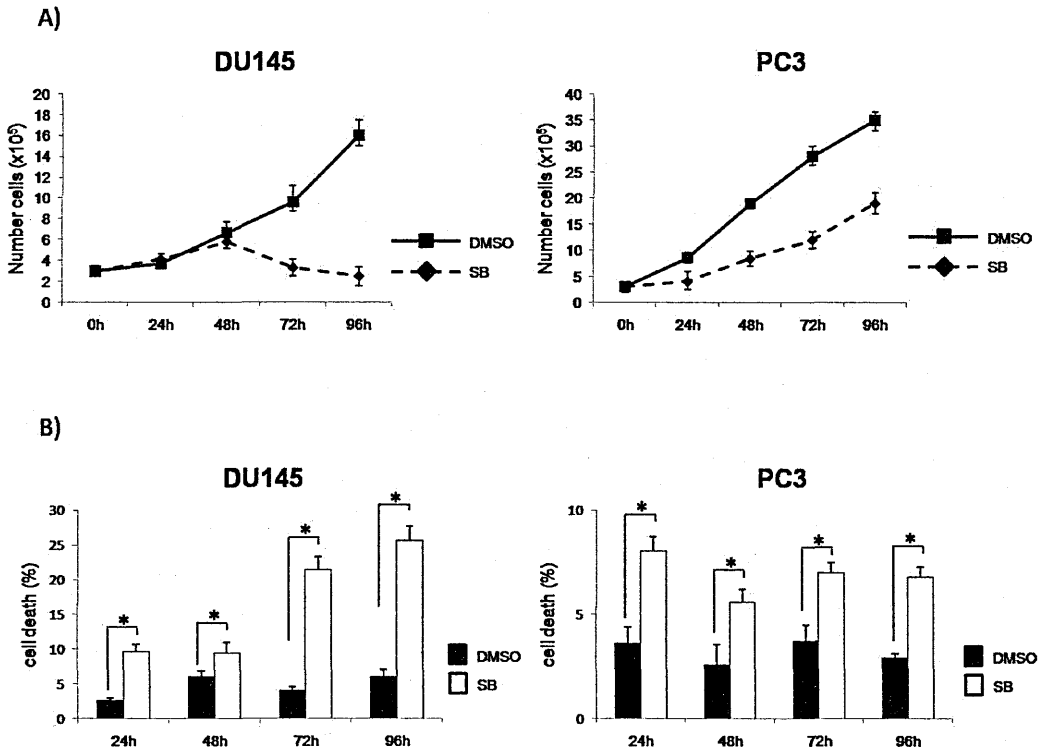

Figure 23. Evaluation of p38 alpha inhibition on cell proliferation and viability of PCa cells. DU145 cells and PC3 cells were treated for up to 96 hours with SB (10μM) and every 24 hours a cellular count was performed using trypan blue assay. A) p38 alpha inhibition exerted two different outcomes, the decrease in cell number in DU145 cells especially after 48 hours of incubation, while a slow proliferation in PC3 cells. Results are expressed as mean ±SEM (n=3). B) The differences in cell number is due to a strong cell death in DU145 cells while there is only a slight cell death in PC3 cells. Results are expressed as mean ±SEM (n=3). Comparison of dead cells between treated (SB) and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

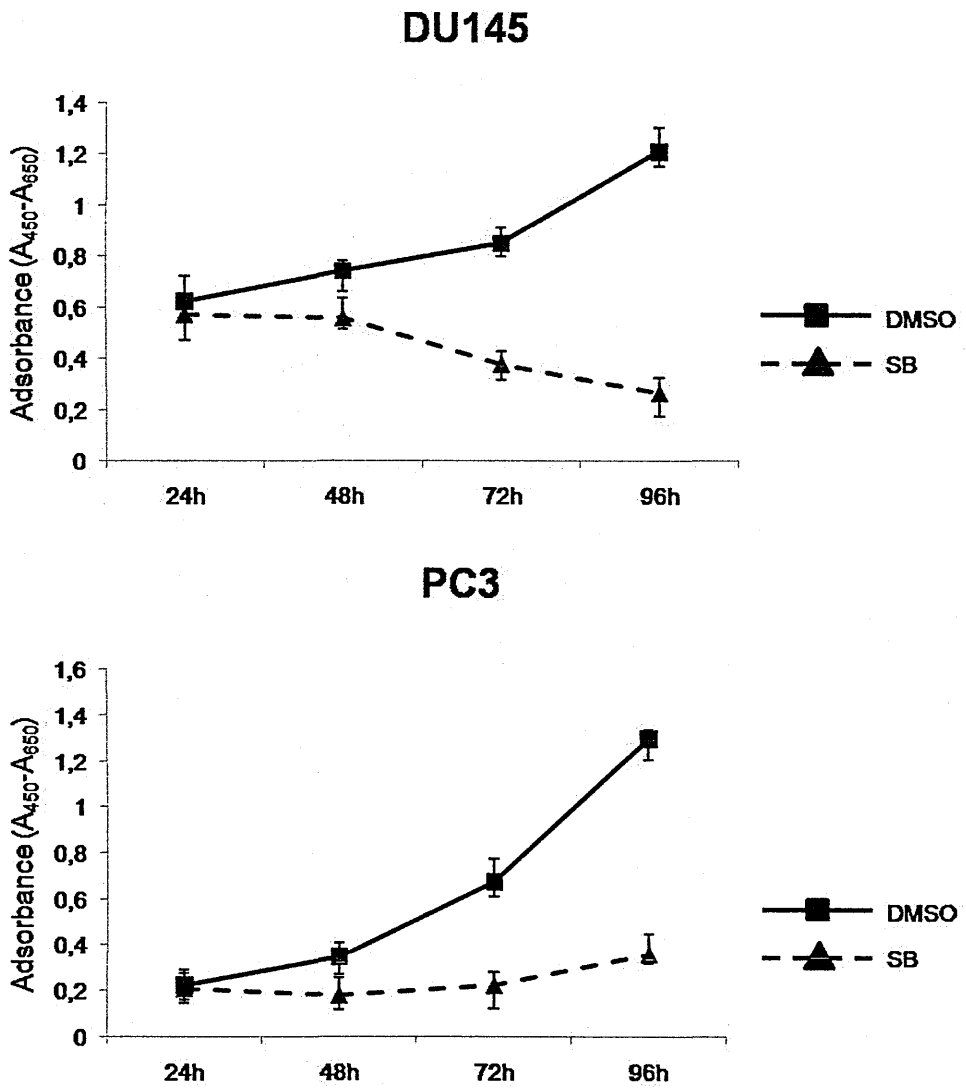

Figure 34. SB treatment induced cell death in DU145 cells and growth arrest in PC3 cells. DU145 cells and PC3 cells were treated for up to 96 hours with SB (10μM) or DMSO. Cell growth was quantified using WST-1 cell proliferation assay. This assay confirmed the two different outcomes of SB treatment in these cell lines on cell proliferation. The data shown are the mean of three different experimental settings. Results are expressed as mean ±SEM.

investigate whether DU145 and PC3 cells differ in LKB1 status. LKB1 is a well known AMPK upstream kinase (Shaw et al., 2004; Sakamoto et al., 2005), it is mutated in several cancer types (Wingo et al., 2009; Ji et al., 2007) and inactivated in the PJS (Giardiello et al., 2000). To this purpose, we performed an immunoblot analysis for LKB1 protein in PCa cells and HT29 cells, wild type for LKB1 protein (Figure 35B). The picture clearly shows that DU145 cells are null for this kinase. Thus, to induce the activation of AMPK in DU145 cells we decided to use its activator AICAR (Merrill et al., 1997). We treated DU145 cells and PC3 cells with AICAR and SB for 72 hours and we analyzed by immunoblot analysis the activation status of AMPK (Figure 36). The picture shows that AICAR failed to activate AMPK in DU145 cells, as indicated also by the fact that Acetyl CoA Carboxilase (ACC), target of AMPK (Witters et al., 1991), is not phosphorylated. Conversely, in PC3 cells AMPK is activated by AICAR, such as during SB treatment, since ACC was phosphorylated, indicating that LKB1 is necessary to activate AMPK in PCa cells. Since in CRC and OvCa cells SB treatment induced activation of autophagic pathway (Comes et al., 2007;(Matrone et al., 2010), and AMPK is a key regulator of autophagy (Meley et al., 2006), we decided to investigate whether there were changes in autophagic pathway activation. We treated DU145 cells and PC3 cells with SB for 72 hours and we performed an immunoblot for the marker of autophagy LC3. Contemporaneously, we decided to analyzed also the apoptotic pathway and we checked for the protein levels of p85 (Figure 27). This immunoblot analysis shows that PC3 cells (wild type for LKB1) were able to induce autophagy, as revealed by the increase

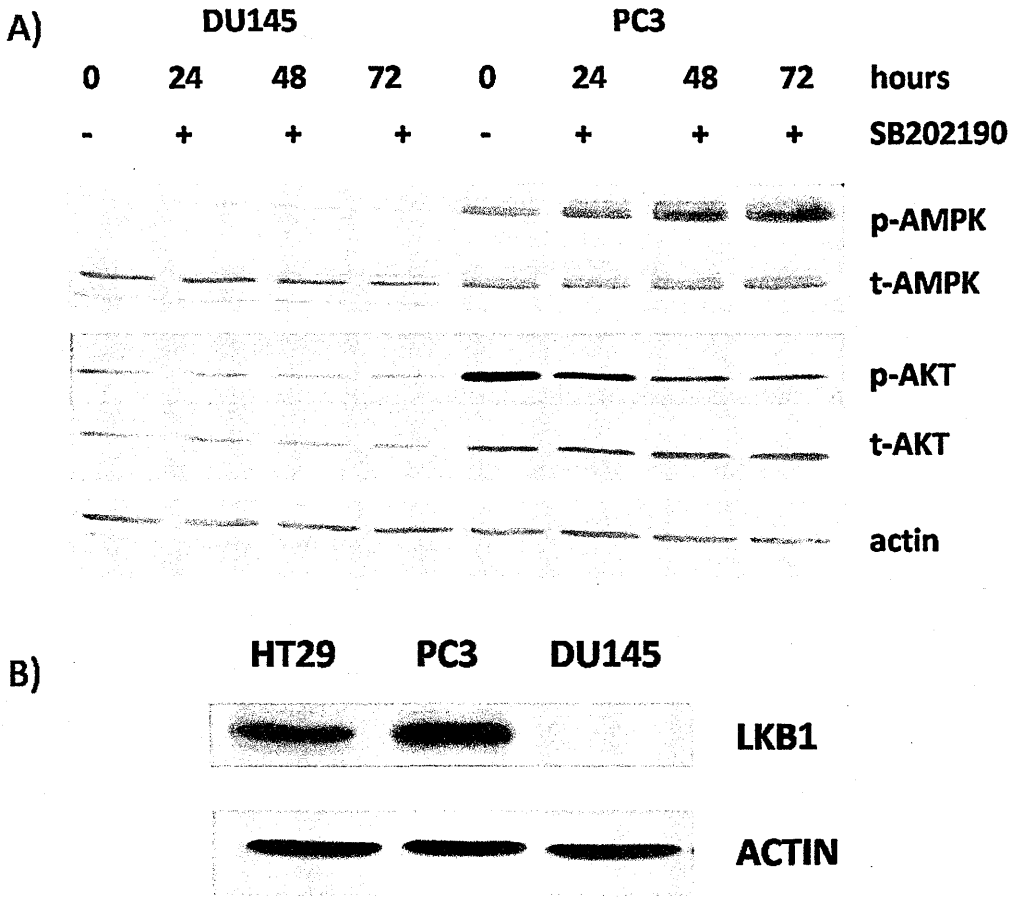

Figure 25. The inhibition of p38 alpha modulated AMPK and AKT pathways in PCa cells. A) PCa cell lines were treated for up to 72 hours with SB (10μM) and a immunoblot of phosphorylation status of AKT and AMPK was performed. The inhibition of p38 alpha in DU145 and PC3 exerted different outcomes regarding the phosphorylation status of AMPK while the decrease in the phosphorylation status of AKT resulted similar between the two cell lines. Actin was used as loading control. The pictures shown are representative of three replicates. B) Immunoblot of LKB1 in different cancer cell lines. As shown in the figure, DU145 are null for the upstream kinase of AMPK. Actin was used as loading control. The pictures shown are representative of three replicates.

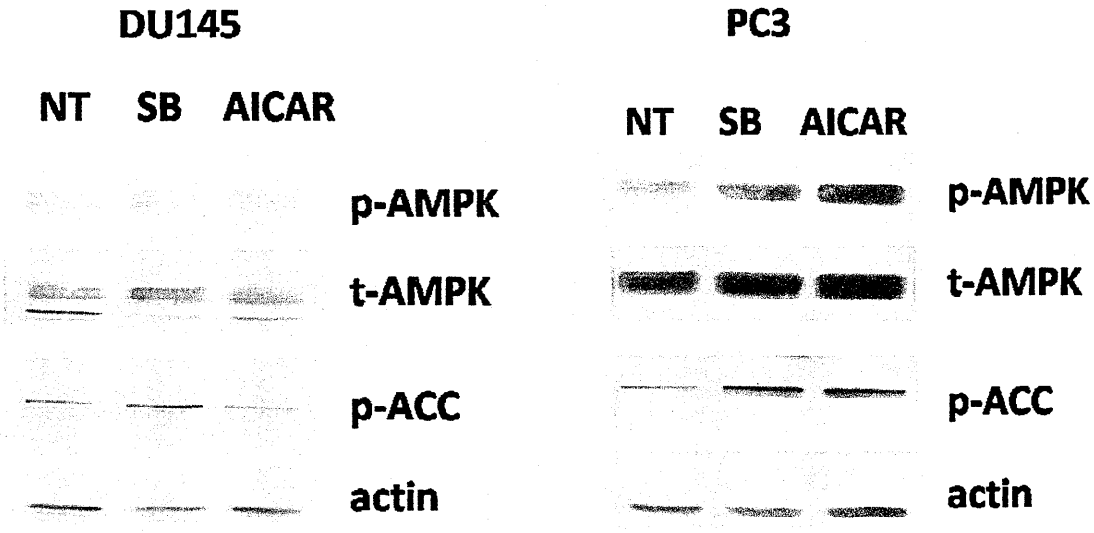

Figure 26. AICAR failed to phosphorylate AMPK protein in DU145 cells. DU145 cells and PC3 cells were incubated for 24 hours with DMSO, SB (10μM) and AICAR (500μM). Neither SB or AICAR were able to induce AMPK phosphorylation in DU145 cells (LKB1 null). This is confirmed by the not phosphorylated status of AMPK target protein AcetylCoA Carboxylase (ACC). Conversely, these two drugs activated AMPK in PC3 cells (LKB1 wild type). Actin was used as loading control. The pictures shown are representative of three replicates.

of the lipidated form of LC3 (II), but not apoptosis; conversely, DU145 cells (LKB1 null) were not proficient for autophagy but underwent apoptosis, as indicated by the increase of p85. This experiment suggests that AMPK plays a role in the activation of autophagy in PCa cells during SB-treatment. Moreover, p38 alpha inhibition in PCa cell lines lacking AMPK activation seemed to trigger apoptosis, that could be the causes of cell death in DU145 cells. Since the LKB1 status could be important to induce cell death in prostate cancer with SB treatment, we tested this molecule on primary cells taken from biopsies of one patient null for LKB1 and another with a LKB1 wild type, after the evaluation of LKB1 status by an immunohistochemistry (IHC) (data not shown). These cells were treated for up to 96 hours with SB and a cell count with trypan blue was performed (Figure 28). LKB1 null primary cells showed a percentage of cell death (60% circa) higher than LKB1 positive cells (30%).

For this experiment we thank our colleagues of the Policlinico of Bari, which made biopsies, the IHC of LKB1 and the cell count.

p38 alpha inhibition in PCa did not showed the same results of CRC and OvCa cell lines. This is a very interesting point because revealed that in PCa cells LKB1 plays an important role in this tumor. Indeed, this kinase seemed to be involved in the autophagic activation in these cell lines but, also, seemed to be protective, since its absence induced apoptotic cell death during SB treatment.

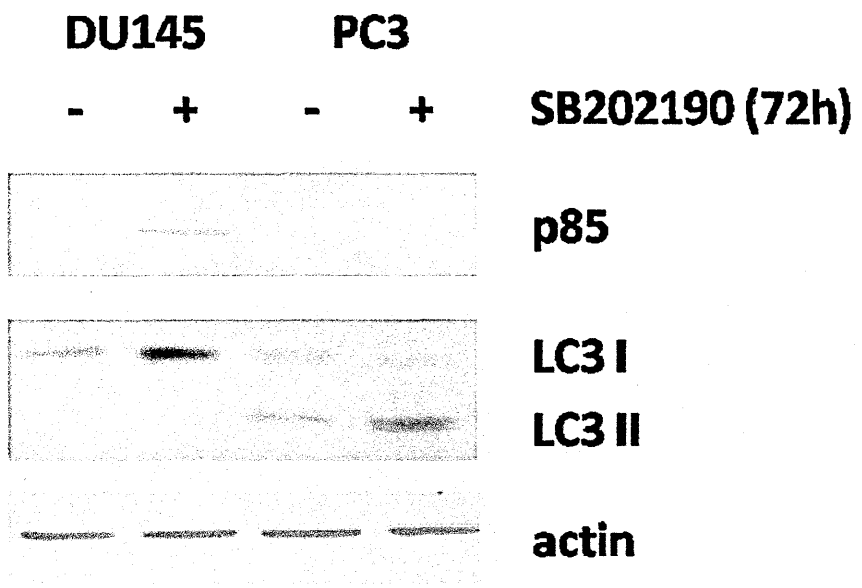

Figure 27. SB treatment increased apoptotic pathway in DU145 cells and autophagic pathway in PC3 cells. DU145 cells and PC3 cells were cultured for 72 hours with or without SB (10μM). Immunoblot for p85 and LC3 was performed. The inhibition of p38 alpha increased the protein levels of p85 only in DU145 cells, indicating that the cell death described before (see Figures 33 and 34) is apoptotic death. Interestingly, in DU145 LC3 II, the marker of autophagy, did not accumulate, excluding this pathway in SB treatment outcomes. Conversely, PC3 cells shown the characteristic pattern of SB treatment similar to CRC cells and OvCa cells. Actin was used as loading control. The pictures shown are representative of three replicates.

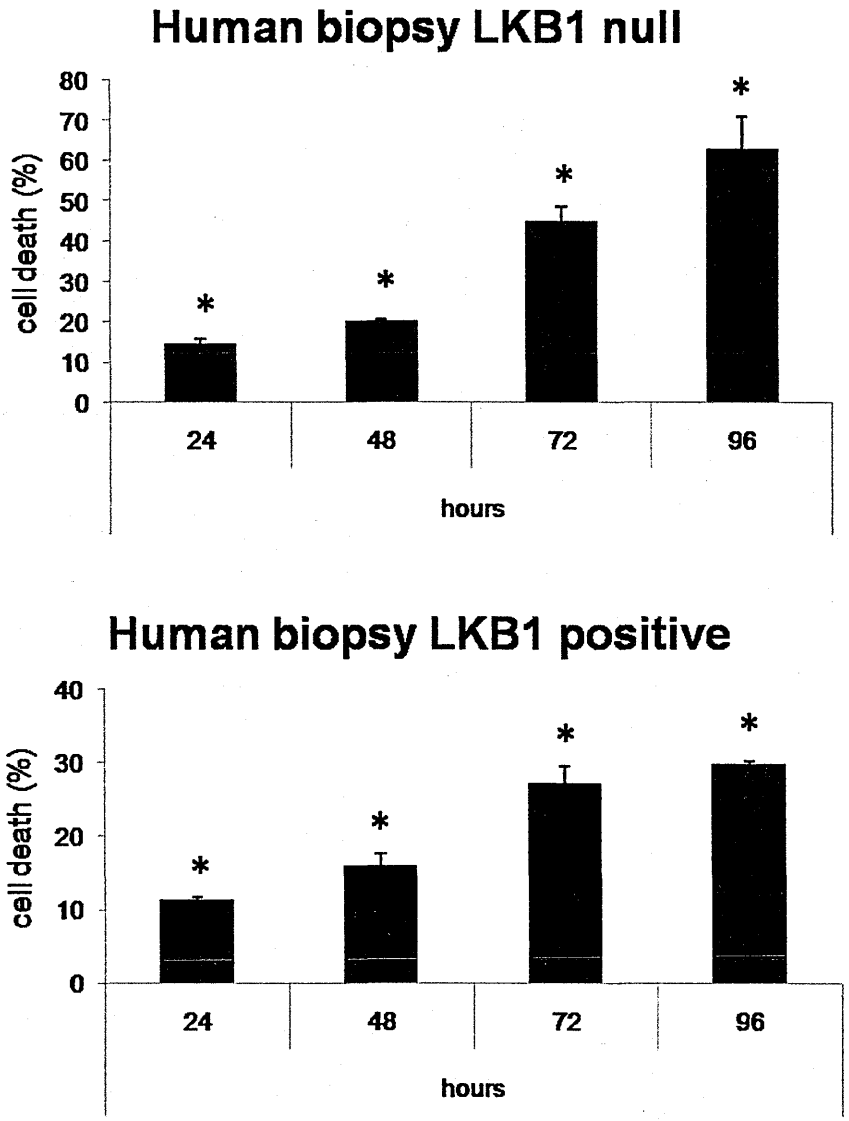

Figure 28. Primary cells of human biopsies treated with SB shown the similar pattern of PCa cells. For this experiment were used primary human cells taken from biopsies of patients affected by PCa, which were positive or null for LKB1 protein by immunohistochemistry, to replicate the genetic condition of PCa cell lines. These cells were treated with SB (10μM) for up to 96 hours. At the indicated time points cell were counted using trypan blue assay to ascertain the cell death. As indicated in the figure, the primary cells taken from human prostatic biopsy LKB1 null shown a percentage of cell death greater than human biopsy cells LKB1 positive, indicating how LKB1 could play a role in the SB-dependent cell death. Normal prostatic cells were used as control. Results are expressed as mean ±SEM (n=3). Comparison of dead cells between treated (SB) and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

4.4 Colorectal cancer and chemotherapy

Cisplatin (CDDP) is one of the most effective and commonly used chemotherapeutics for the treatment of many solid tumors, including ovarian, testicular, bladder, lung, head, neck and colorectal tumors (Siddik, 2003; Boulikas and Vougiouka, 2003). Unfortunately, parallel to high efficacy in the treatment of the above tumors, the development of resistance to CDDP-based therapy is a major obstacle for its successful clinical application. In the majority of investigated tumors, resistance to CDDP is generally multifactorial. CDDP, such as other most common chemotherapeutics, results ineffective due to chemoresistance, which could be acquired or dependent by genetic mutations. Recently, different groups demonstrated that chemotherapeutics such as 5-FU (Yang et al., 2011) or CPT-11 (Paillas et al., 2011) in CRC are able to induce p38 alpha phosphorylation. In both cases, its inhibition led to cell death, suggesting that p38 alpha could overcome chemorestistance.

In this study we evaluated the role of p38 alpha in CDDP cellular response in two CRC cell lines. We chose HT29 cells and HCT116 cells as model for our investigation, since several studies (Fernandez de et al., 2008; Arango et al., 2004) showed that these two CRC cell lines have different outcomes when treated with platinum-compounds, reveling that HT29 cells are less sensitive than HCT116 cells, probably due to the different status of *p53* gene. Indeed, this gene is mutated in HT29 cells and wild type in HCT116 cells. First at all, we checked whether CDDP was able to induce activation of p38 alpha. For this reason the

two CRC cell lines were treated for 48 hours with 30uM of CDDP and p38 phosphorylation status was evaluated by immunoblot (Figure 29). CDDP induced p38 phospho-activation in both cell lines, and this activation is underlined by the phosphorylation of its well known direct target MK2.

Once verified that CDDP was able to induce p38 alpha activation in both cell lines, next step of our analysis was to understand the effect of its inhibition on cell behavior. We treated HCT116 cells and HT29 cells for 48 hours with SB and CDDP alone and in combination.

To evaluate the effect of these treatments the cells were stained with Comassie brilliant blue, directly in the colture dishes. Only live cells remained attached and were blue stained. Assuming that DMSO-treated cells were 100% of stained cell, it is possible to compare the stained cells between the various treatments (Figure 30). As shown in the figure, the percentage of stained HCT116 cells was 35% with the only CDDP while during the co-treatment decreased to 18%. Conversely, stained HT29 cells were 87% with CDDP alone while surprisingly during the co-treatment were only the 16%. These data confirmed that CDDP has two different outcomes in these CRC cell lines while the co-treatment greatly reduce the number of stained HCT116 cells, and of stained HT29 cells. These results indicated that the inhibition of p38 alpha during the administration of CDDP enhanced its effect in HCT116 cells while made HT29 cells sensitive to this chemotherapeutic. The low percentage of relative stained cells during co-treatment led us to understand whether the combination of SB with CDDP induced an impact on cell cycle or an increase of cell death, To answer this

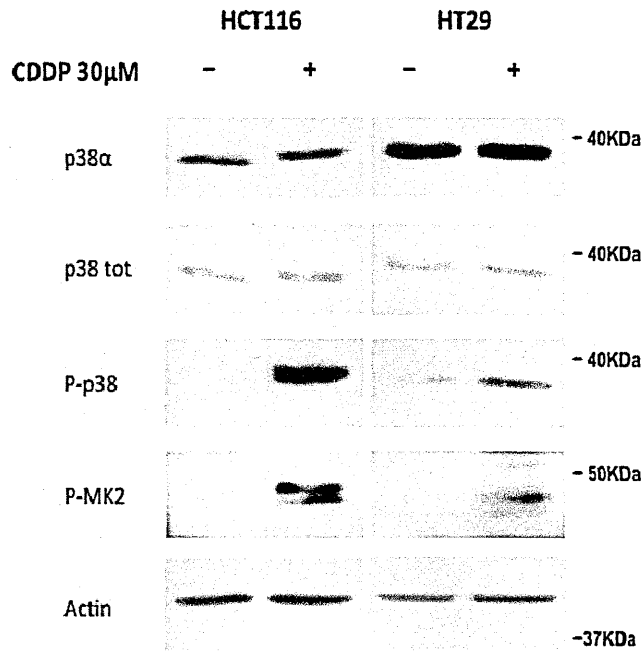

Figure 29. CDDP treatment activates p38 alpha kinase. HCT116 cells and HT29 cells were treated for 48 hours with CDDP and cells were collected and analyzed by immunoblotting to evaluate the activation of p38 alpha. The phosphorylation status of p38 alpha increased after CDDP treatment and its activity is underlined by the phosphorylation status of its downstream target MK2. The pictures shown are representative of three replicates. Actin was used as loading control.

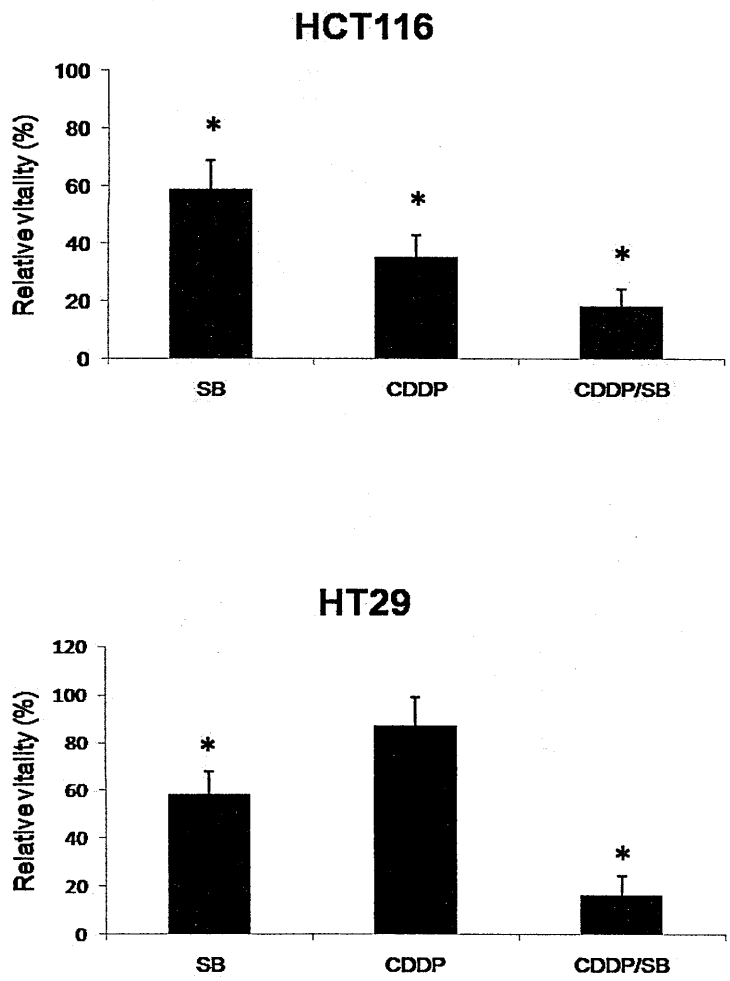

Figure 30. Macroscopic effect of CDDP and SB compounds on HCT116 and HT29 cell lines. HCT116 cells and HT29 cells were treated for 48 hours with DMSO, SB (10μM), CDDP (30μM) and CDDP plus SB. After 48 hours of treatment, cells were washed with PBS and then stained with Comassie brilliant blue. Only the live cells were blue stained. The graphs indicate that HT29 cells were less responsive to CDDP than HCT116 cells, while when CDDP is used together with SB both cell lines shown a strong reduction of live cells. The graphs indicate the ratio of blue cells between DMSO and each treatment. Untreated cells were considered 100%. Results are expressed as mean ±SEM (n=3). Comparison of percentage of blue cells between SB-treated and DMSO treated cells was performed using a student's *t* test followed by Mann-Witney U test (*=$p<0.05$).

question we treated HCT116 cells and HT29 cells for up to 96 hours with SB and CDDP alone or in combination and we performed a cellular count, using trypan blue assay. In this case this dye stained the dead cells, turning them blue at the optical microscopy. At the indicated times, we counted the number of blue cells (dead cells) and the number of non-blue cells (living cells) for each treatment (Figure 31A,B). As regard the percentage of viable cells, the combination of SB and CDDP was able to reduce it dramatically in both cell lines and in a time-dependent manner, compared with the two compounds alone; similarly, we counted a very strong increase in the percentage of cell death in HCT116 cells and in HT29 cells during the co-treatment. These data indicated that the combination of SB and CDDP affected the number of cells in both cell lines through the induction of cell death.

Starting from this observation, the next step of our analysis was to investigate the type of cell death. It is well known that CDDP induces apoptotic cell death (Fisher, 1994) while SB is a molecule which causes cell death with autophagic features (Comes et al.,2007). We cultured HCT116 cells and HT29 cells for 48 and 72 hours with CDDP and SB alone or in combination and we performed a protein analysis to investigate whether the cell death was apoptotic cell death or autophagic cell death (Figure 32). We detected the levels of the two most known caspases: caspase 8 and caspase 3. Caspase 8, one of the initiator caspases, is the protein upstream of caspase 3, which is considered the "effector" of apoptotic pathway (Boatright and Salvesen, 2003). To underline the activity of these caspases, we analyzed the levels of cleaved PARP-1 (p85). When the apoptotic

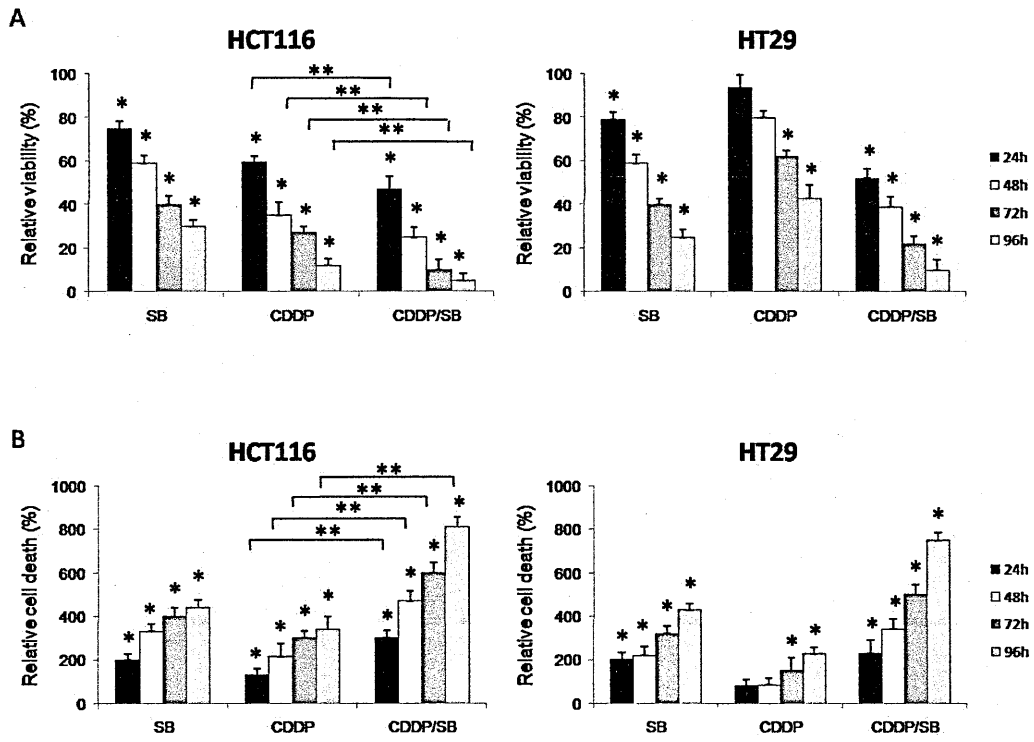

Figure 31. The co-treatment of HCT116 cells and HT29 cells induces the increase of cell death. HCT116 cells and HT29 cells were treated with DMSO, SB (10μM), CDDP (30μM) alone or in combination with SB for up to 96 hours. Every 24 hours, cells were collected and counted using trypan blue dye to evaluate the number of live and dead cells. A) In the graphs is shown that co-treatment induced a strong reduction of viable cells in both cell lines. This decrease is bigger than the decrease of compounds alone. Relative viability (%) was evaluated with the ratio between viable cells in treated (SB, CDDP, CDDP and SB) versus DMSO treated cells. Results are expressed as mean ±SEM (n=3). Comparison of percentage of live cells among each treatment was performed using a student's *t* test followed by Mann-Witney U test (*/**=$p<0.05$). B) In the graphs is shown that co-treatment induced an increase of cell death in both cell lines. This increase is bigger than the increase of compounds alone. Relative cell death (%) represents the ratio between dead cells in treated (SB, CDDP, CDDP and SB) versus DMSO treated cells. Results are expressed as mean ±SEM (n=3). Comparison of percentage of dead cells among each treatment was performed using a student's *t* test followed by Mann-Witney U test (*/**$p<0.05$).

pathway is activated, this protein is cleaved by caspase 3, producing a fragment of 85 KDa (Kaufmann et al., 1993; Casiano et al., 1996). To evaluate the activation of autophagic pathway, we chose LC3 (I/II) as marker. The accumulation of LC3II indicate that autophagy is activated. (Figure 32A,B). In both cell lines, during the co-treatment it is possible to note that the protein levels of activated caspase 8 (44-42 KDa and 18 KDa) and activated caspase 3 (19-17 KDa) were elevated if compared with the compounds alone. This accumulation is more evident at 72 hours of administration, confirming the data of cellular count. Moreover, it is evident that p85 is accumulated both in HCT116 cells and HT29 cells during the co-treatment. Conversely, it seems that during the co-treatment, the accumulation of the LC3II band was similar to that of SB alone in both cell lines. These results indicated that the co-treatment induced the activation of the apoptotic pathway in both cell lines, while autophagy seemed to be not involved in the cell death. To confirm the activation of the apoptotic pathway, these two CRC cell lines were treated for up to 96 hours with SB, CDDP alone and the two compounds together. Every 24 hours the activation of apoptotic pathway was evaluated using the standard marker of apoptosis annexin V. Annexin V is used as a probe to detect cells that have expressed phosphatidylserine (PS) on the cell surface, an event found in apoptosis (Koopman et al., 1994; Vermes et al., 1995) (FIG). As shown in the figure, CDDP alone strongly activated apoptotic pathway in HCT116 cells while had no effect on the in HT29 cells, since the percentage of Annexin V was similar to untreated cells and SB-treated cells. It is worth of note that SB does not activate apoptosis (Comes et al., 2007).

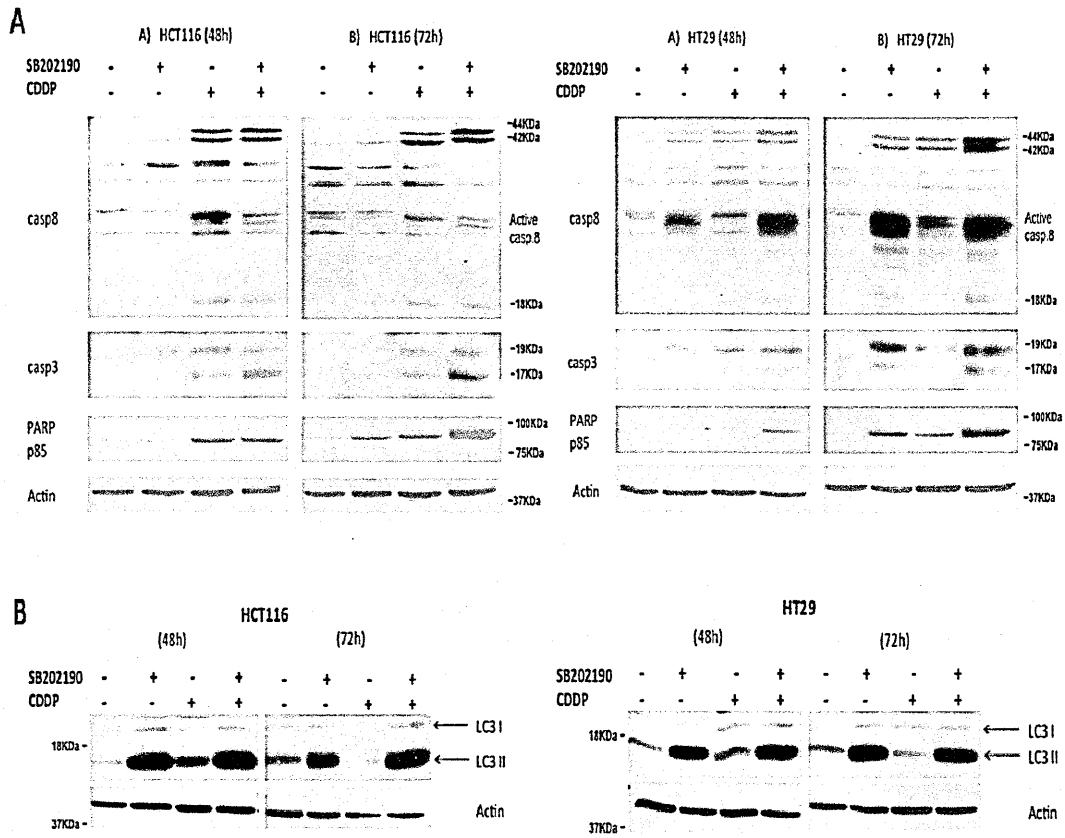

Figure 32. The cell death induced by co-treatment is apoptotic cell death. HCT116 cells and HT29 cells were treated for 48 and 72 hours with DMSO, SB (10μM), CDDP (30μM) and CDDP plus SB. A) At the indicated time points, cells were collected and proteins were analyzed by immunoblot to evaluate the activation of apoptotic pathway, using apoptotic markers caspase 3, caspase 8 and the cleavage fragment of PARP-1 (p85). The co-treatment (+/+) activated the apoptotic pathway in both cell lines in a time-dependent manner, as underlined by the accumulation of caspase 3 and caspase 8 and p85 proteins. Actin was used as loading control. The pictures shown are representative of three replicates. B) At the indicated time points, cells were collected and proteins were analyzed by immunoblot to evaluate the activation of autophagic pathway, using the marker of autophagy MAP1LC3 (LC3). Worth of note is that cell death is not associated to an increase of autophagy, since the accumulation of the lipidated form of LC3 (LC3II) in co-treated cells is equal to the samples treated only with SB, indicating that cell death is due only to the activation of apoptotic pathway. Actin was used as loading control. The pictures shown are representative of three replicates.

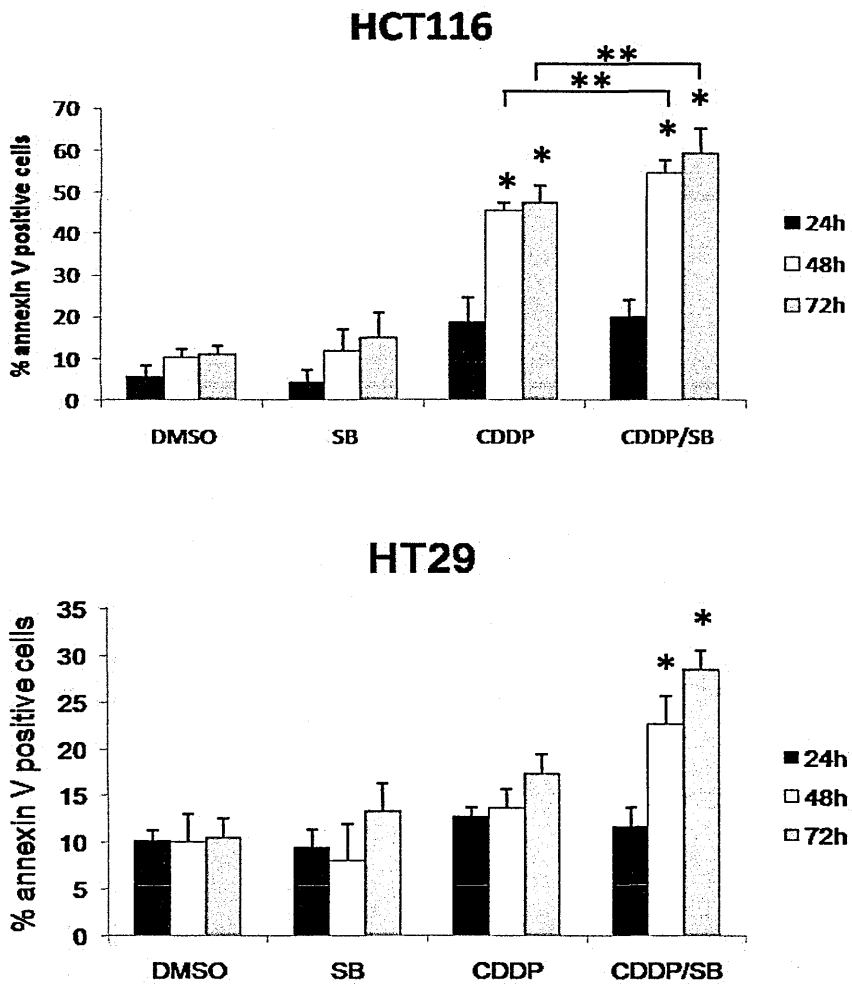

Figure 33. FACS analysis confirmed the activation of apoptotic pathway. The two CRC cell lines were treated for up to 96 hours with DMSO, SB (10μM), CDDP (30μM) and CDDP plus SB. At the indicated time, cells were analyzed for the presence of annexin V on the surface of the citoplasmic membrane. This effect is a particular feature of the apoptotic pathway. The two graphs show the percentage of positive cells to the annexin V, indicating that the co-treatment induced apoptosis in both cell lines. Here is shown also that CDDP did not activate apoptosis in HT29 cells. Results are expressed as mean ±SEM (n=3). Comparison of percentage of annexin V among each treatment was performed using a student's *t* test followed by Mann-Witney U test (*/**=$p<0.05$).

Interestingly, apoptosis is enhanced in HCT116 cells while is induced in HT29 cells when SB and CDDP are used in combination, highlighting the effect of p38 alpha inhibition in the reinforcement of apoptosis in the responsive cell line and in the induction of apoptosis in the insensitive cell line. To better evaluate the role of caspase 8 in the apoptotic cell death, we treated HT29 cells with CDDP and SB for 48 hours and then we added in the medium of cells its specific inhibitor Z-IEDT-FMK. We performed a protein analysis for p85 to evaluate apoptosis activation (Figure 34). The inactivation of caspase 8 led to a decrease of p85 fragment accumulation, indicating that caspase 8 is involved in the activation of apoptosis during the use of CDDP and SB simultaneously in HT29 cells. These data suggest that the co-treatment with CDDP and the inhibitor of p38 alpha in HT29 cells and HCT116 cells caused apoptotic cell death. In the last years an increase number of papers demonstrated that the mechanisms of resistance can be overcome through the activation of the transcription factor FoxO3A (De Mattos. et al., 2008). Moreover, our previous works demonstrated that the inhibition of p38 alpha in CRC cells and in OvCa cells induced the activation of the transcriptional pattern of FoxO3A (Chiacchiera et al., 2009; Matrone et al.2009). Given these observations, we evaluated whether FoxO3A plays a role in the capacity to enhance the cell death in HCT116 cell line and to sensitize the resistant HT29 cell line.

We treated both cell lines with the compounds alone or in combination for 48 hours, and we analyzed at confocal microscopy by an immunofluorescence assay the distribution of this transcription factor (Figures 35 and 36). Worth of noting,

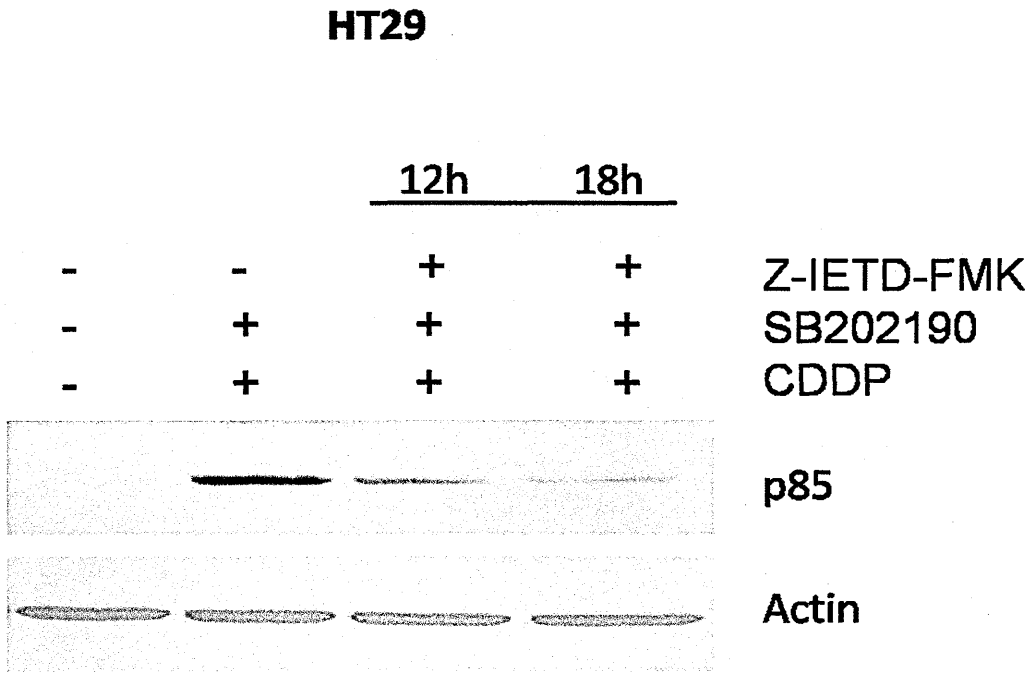

Figure 34. The activation of apoptosis is caspase 8-dependent. HT29 cells were treated with CDDP (30μM) plus SB (10μM) and after 24 hours was added to the medium the inhibitor of caspase 8 (Z-IEDT-FMK) for the indicated time. Analysis of p85 fragment was performed to evaluate the role of caspase 8 in apoptotic pathway activation in these cells. As demonstrated in the figure, the inhibition of caspase 8 decrease the accumulation of p85, indicating that this caspase plays a role in the apoptotic pathway triggered by the co-treatment in HT29 cells. Actin was used as loading control. The pictures shown are representative of three replicates.

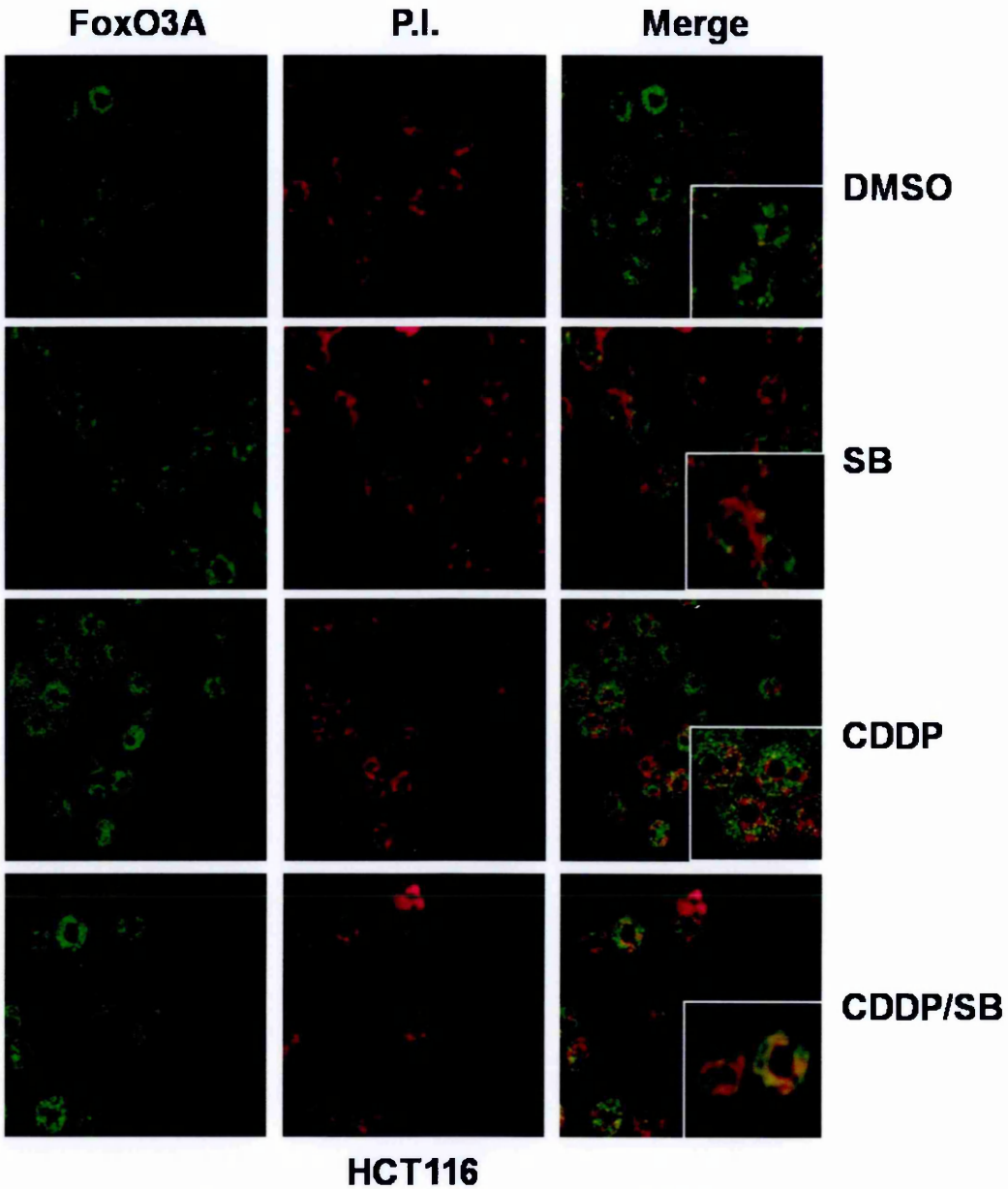

Figure 35. FoxO3A is present in the nuclei of HCT116 cells when treated with CDDP. HCT116 cells were treated for 48 hours with DMSO, SB (10μM), CDDP (30μM), CDDP plus SB. Immunofluorescence analysis of FoxO3A (green) localization was performed to elucidate the role of this transcription factor in CDDP treatment. As shown in the figures, FoxO3A accumulated in the nuclei of CDDP-treated HCT116 cells, indicating that this transcription factor could play a positive role in the response to CDDP. Propidium Iodide (P.I., red) was used to stain the nuclei. The pictures shown are representative of three replicates. The pictures were taken by confocal microscope. Magnification 60X.

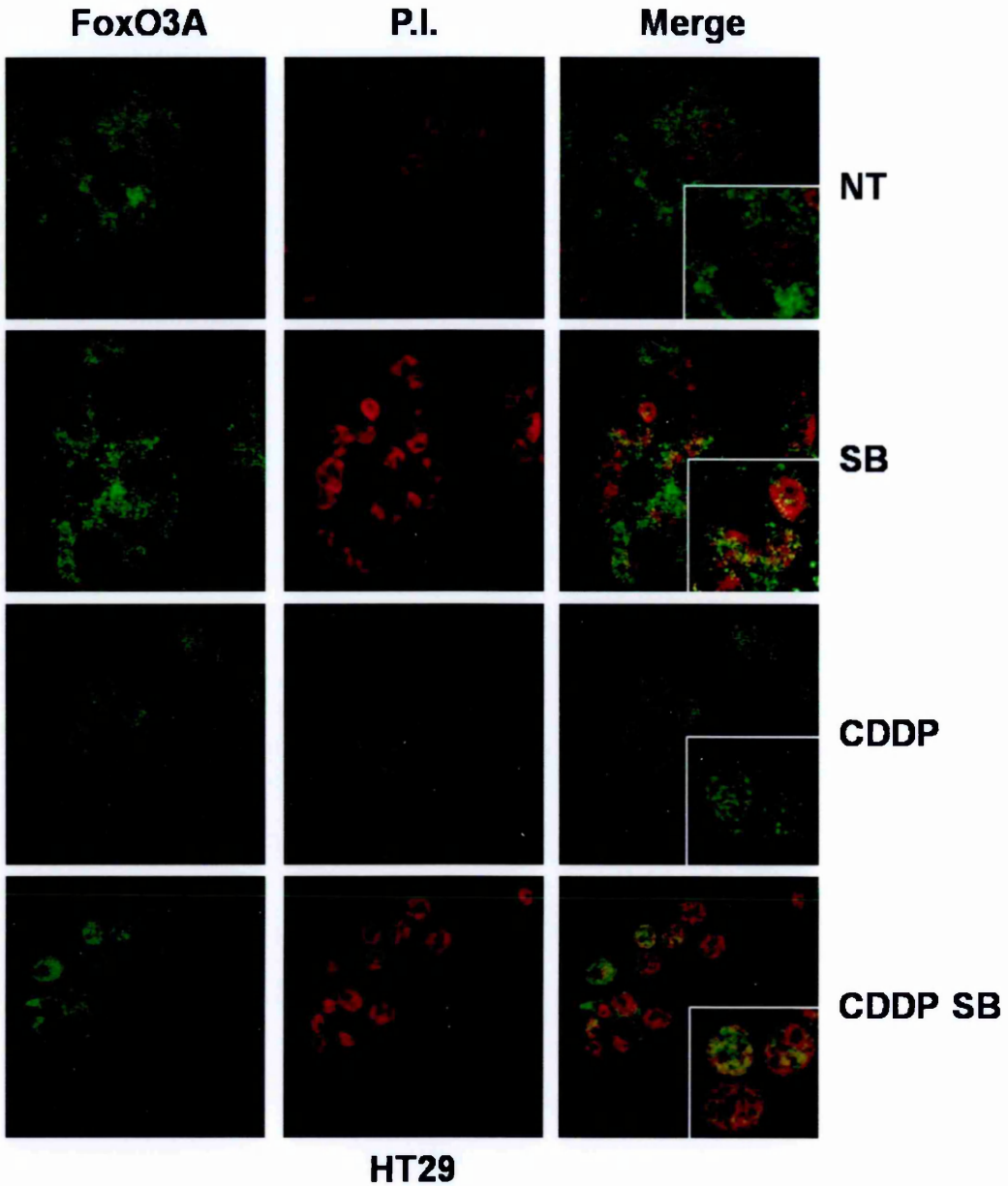

Figure 36. FoxO3A accumulated in the nuclei of HCT29 cells when treated with CDDP plus SB. HT29 cells were treated for 48 hours with DMSO, SB (10μM), CDDP (30μM), CDDP plus SB. Immunofluorescence analysis of FoxO3A (green) localization was performed to elucidate the role of this transcription factor in CDDP treatment. As shown in the figures, interestingly FoxO3A did not accumulate in the nuclei of CDDP-treated HT29 cells, while it accumulated in the nuclei of co-treated cells. This could indicate that the inhibition of p38 alpha is important for FoxO3A translocation in the nuclei of CDDP-treated HT29 cells. Propidium Iodide (P.I., red) was used to stain the nuclei. The pictures shown are representative of three replicates. The pictures were taken by confocal microscope. Magnification 60X.

HCT116 cells shown FoxO3A predominantly in the nucleus of DMSO-treated cells while HT29 cells presented a homogeneous distribution in the cells. Moreover, it seems that in some cells FoxO3A is not present in the nuclei of HT29 cells. Very interesting was also that when the cells were treated with CDDP alone the localization of FoxO3A was strongly nuclear in HCT116 cells while it seemed that in HT29 cells it was homogeneous yet. However, when SB is used together with CDDP, the nuclear localization in both these cell lines appeared more pronounced. This experiment given to us a reason to speculate about the different response to CDDP between these two cell lines; infact, it seems that the different localization of FoxO3A protein in the cell during CDDP treatment alone could indicate that this transcription factor plays a role in the response to this chemotherapeutic. To link this nuclear accumulation with a functional role of FoxO3A, we treated both cell lines with SB and CDDP alone or in combination for 48 hours and we made a quantitative real time PCR analysis of its targets genes, involved in cell death, such as *PTEN* and *BIM*, cell growth arrest, such as *p21*, and damage-activated gene, such as *GADD45* (Figure 37). As it is indicated in the graphs, in HCT116 cells these genes were upregulated when treated with CDDP alone and they were more upregulated when treated together with SB. Conversely, in HT29 cells the expression of these genes during CDDP treatment is similar to the DMSO-treated cells, while is upregulated when CDDP was used together with SB. The mRNA levels of these genes seemed to be in line with the localization of FoxO3A in the nuclei (see Figure 35 and 36). Indeed, the upregulation of these genes during the co-treatment with CDDP and SB seemed

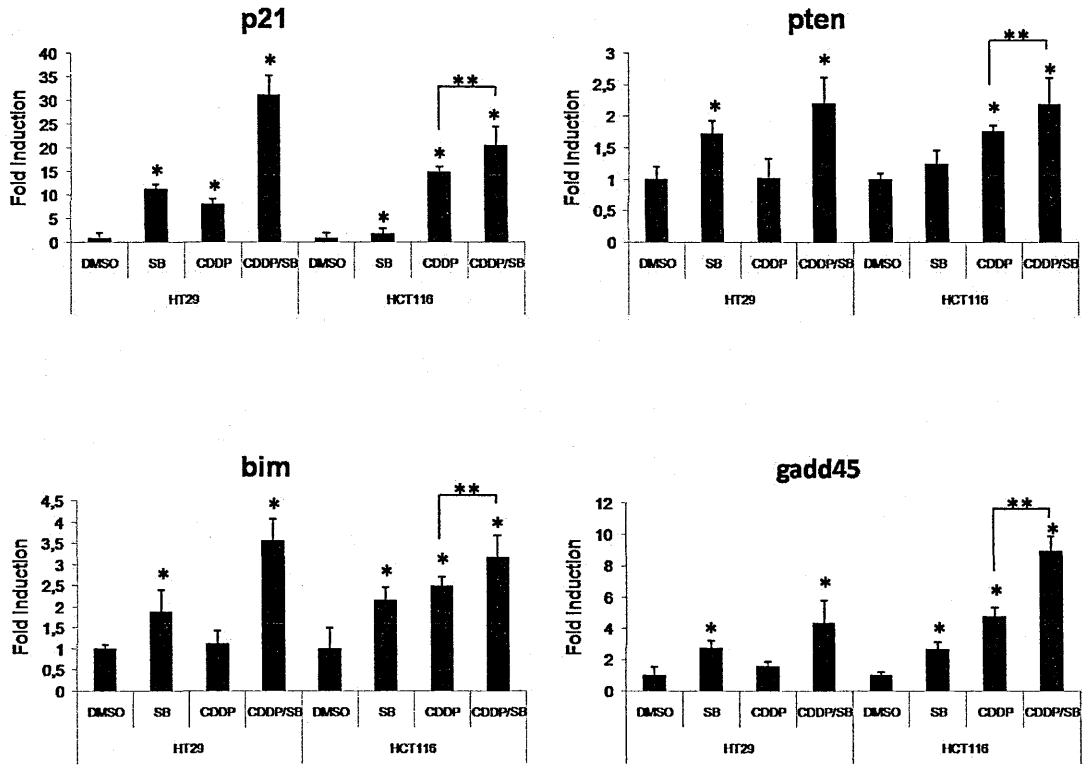

Figure 37. FoxO3A target genes are upregulated during co-treatment in both CRC cell lines. CRC cells were treated for 48 hours with DMSO, SB (10μM), CDDP (30μM) and CDDP plus SB. Real time PCR of putative FoxO3A target genes was performed to correlate the nuclear localization of this transcription factor (see Figures 22 and 23) with its transcriptional activity. Worth of note is that HT29 cells did not upregulate these genes during CDDP treatment, while it happened when the two drugs were added together. Moreover, in HCT116 cells treated with CDDP alone are able to upregulate these genes and during the co-treatment this upregulation is higher than the compounds alone. This different outcomes in CDDP treatment of the two CRC cell lines reflected the differences of the nuclear localization of FoxO3A (see. Figures 22 and 23, CDDP panels), indicating a possible positive role in CDDP sensitiveness in CRC cell lines. beta-actin was used as calibrator of mRNA quantity. The relative mRNA levels are expressed as fold induction. Results are expressed as mean ±SEM (n=3). Comparison of mRNA levels of genes evaluated among each treatment was performed using a student's *t* test followed by Mann-Witney U test (*/**=$p<0.05$).

to match with the strong accumulation of FoxO3A in the nuclei during the co-treatment in both cell lines. To confirm the role of FoxO3A in the induction of apoptosis, we decided to silence *FoxO3A* gene with siRNA technology in both cell lines for 36 hours. After this period we treated HCT116 cells with CDDP alone and HT29 cells with CDDP plus SB for 48 hours and we performed a blot analysis of p85 (Figure 38). The absence of FoxO3A affected the cleavage of PARP-1 in both cell lines, suggesting that FoxO3A could play a role in the activation of the apoptotic pathway.

Taken all these evidences, we may hypothesize that the inhibition of p38 alpha in CRC could be helpful in the treatment of this pathology with CDDP. Indeed, not only this inhibition improved the response to CDDP in a cell line sensitive (HCT116) but made a non responsive cell line (HT29) sensitive to this compound. These interesting results seemed to be driven by FoxO3A. Indeed, the different localization of this protein in the two CRC cell lines could explain the different outcomes in response to CDDP treatment. However, its nuclear localization could be associated with its dependent gene transcription and its absence affect the cleavage of PARP-1, suggesting that this transcription factor could play an important role in the activation of apoptotic response.

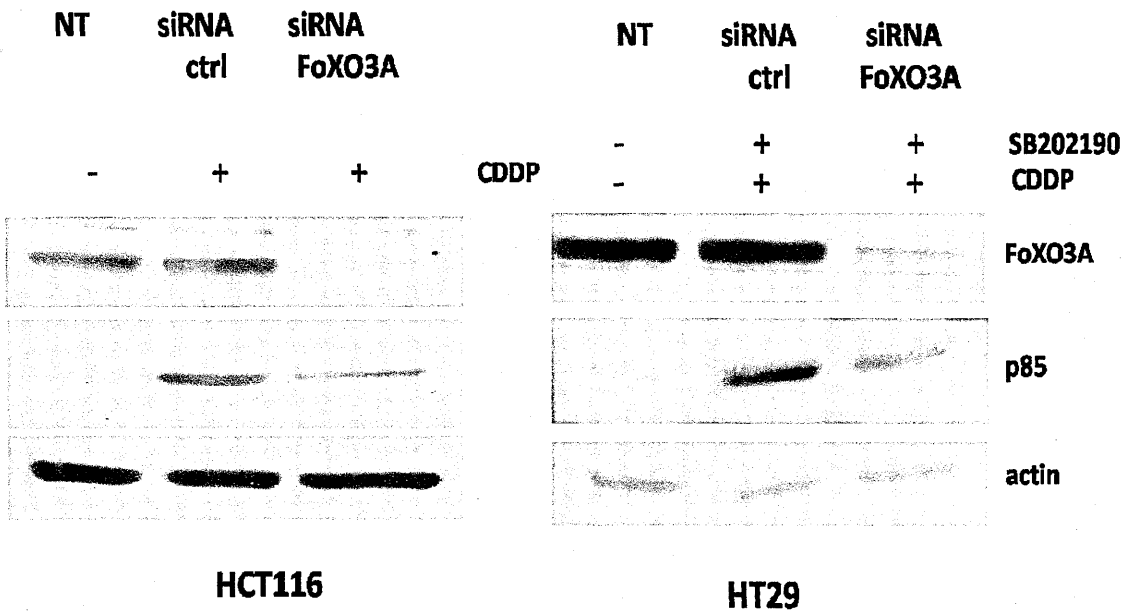

Figure 38. FoxO3A is necessary for apoptotic pathway. HCT116 cells and HT29 cells were silenced for FoxO3A for 36 hours. After 36 hours HCT116 cells were treated with CDDP (30μM) for 48 hours while HT29 cells were treated with CDDP (30μM) and SB (10μM) for 48 hours. Immunoblot for FoxO3A and p85 proteins was performed. As shown in the picture, the silencing of FoxO3A induced a decrease of p85 protein levels, indicating that the apoptosis triggered by CDDP in HCT116 cells and by the co-treatment in HT29 cells is driven by this transcription factor. This experiment enhanced the positive role of FoxO3A in the sensitization of CRC cells to CDDP treatment. Actin was used for loading control. The pictures shown are representative of three replicates.

Chapter 5: DISCUSSION

Cancer is a major public health problem in the United States and in many other parts of the world. In the United States 25% of deaths are due to cancer (Siegel et al., 2012). Over the past 30 years, significant progress has been achieved in understanding the molecular basis of cancer. The accumulation of this basic knowledge has established that cancer is a variety of distinct diseases and that defective genes cause these diseases.

CRC, OvCa and PCa are very common neoplasia in western countries. Different approaches have been studied in these years to counteract the progression of these malignancies; therapies were initially based on the use of chemotherapeutics alone, then on the concomitant administration of traditional chemotherapeutics and biological molecules and lastly on the administration of antibodies targeting specific membrane receptors overexpressed in tumors.

All these strategies, however, revealed many weaknesses due to side effects on the patient's health (chemotherapeutics) or mutations in the downstream enzymes of therapy targets (antibodies), making the treatment of these types of cancer ineffective. Moreover, chemoresistance is the cause of negative response to treatment and even if the improvement of new strategies is a hopeful signal for the future, much has still to be done.

Deregulation of the pathways targeted by chemotherapy is the cause of the common treatment failure, making necessary the use of new therapeutic approaches.

p38 alpha is a member of a group of four kinases that are activated by external stimuli, and are involved in different pathways such as myogenesis, inflammation, cancer, neurodegenerative disorders (Cuenda and Rousseau, 2007), as well as, in the regulation of autophagy (Webber and Tooze, 2010). Moreover, its inhibition in CRC cell lines induced cell cycle arrest, autophagy and then cell death, with autophagic features (Comes et al.,2007).

The aim of my PhD project was to investigate the possible causes of this autophagic activation in CRC cell lines during SB treatment. For this purpose we evaluated whether could exist variation of ATP levels, finding that they decreased during p38 alpha inhibition. The best established mechanism to produce ATP in tumors is glycolysis (Warburg, 1956), and for this reason we decided to analyze whether this pathway was affected by p38 alpha inhibition. A body of glycolytic genes was downregulated during SB treatment.

The decrease of glucose consumption for the production of ATP was confirmed also by the reduction of glucose uptake and lactate release in the medium of SB treated cells. Given the fact that all the glycolitic genes taken in consideration and downregulated by SB treatment are well known target genes of HIF-1 alpha transcription factor we decided to focus our attention on this transcription factor..

The analysis of protein levels and transcriptional activity of HIF-1 alpha during SB treatment confirmed that p38 alpha inhibition was able to negatively affected HIF-1 alpha stabilization and to induce downregulation of its target genes. While glycolytic genes and glycolysis were downregulated by SB treatment, other pathways seemed to be induced by p38 alpha inhibition, as suggested by the

overexpression of several genes involved in cell cycle, autophagy, and cell death (Comes et al., 2007). An *in silico* analysis performed by our group, indicated FoxO3A as another important transcription factor that could be involved in the pharmacological inhibition of p38 alpha.

p38 alpha inhibition in CRC cell lines induced the activation of this transcription factor with the consequent shift from a HIF-1 alpha to a FoxO3A-dependent transcription pattern. Indeed, AMPK that is an upstream regulator of FoxO3A was activated with SB treatment, whereas AKT, a negative regulator of this protein, was inhibited. Moreover, the SB dependent-induction of Foxo3A target genes involved in autophagy and cell death was avoided by AMPK inhibition via Compound C, suggesting a link between this kinases and p38 alpha inhibition. Similar encouraging results were obtained also in OvCa cell lines, where HIF-1 alpha plays an important role for tumor progression and it is a marker for prognosis (Osada et al., 2007). Indeed, in OvCa cells SB treatment reduced the mRNA levels of HIF-1 alpha targets genes, while Foxo3A pathway was upregulated leading to cell death with autophagic features (Matrone et al., 2010).

We also decided to evaluate the effects of SB treatment in PCa cell lines, where HIF-1 alpha is a key player for survival. Worth of note, the inhibition of p38 alpha in PCa cells exhibited two different; in DU145 cells SB-treatment induced apoptosis while in PC3 cells growth arrest. The analysis of molecular differences between these cell lines revealed that AMPK is not phosphorylated during SB treatment in DU145 cells, since this cell line is lacking for LKB1 tumor suppressor protein. In this condition, autophagy is not activated and the metabolic

impairment created during p38 alpha inhibition induces apoptotic cell death in DU145 cells. Conversely, in PC3 cells SB treatment induced growth arrest and the activation of autophagy, showing that p38 alpha plays a central role in PCa progression. The different effects on the two cell lines were confirmed using some human biopsies samples, which differed for LKB1 presence. In human biopsies that showed the absence of LKB1, SB treatment caused cell death, revealing how p38 alpha could be used as a marker for a targeted therapy, especially when the biopsy reveals the loss of LKB1.

Different papers underlined in these years the role of p38 alpha in chemoresistance in CRC cell lines. Paillas and colleagues (Paillas et al., 2011) and Yang and colleagues (Yang et al., 2011) showed that the inhibition of this kinase decreased chemoresistance of CPT-11 and 5-FU treatment in CRC, respectively, suggesting an important role of this kinase during chemotherapy. For this reason, we decided to focus our attention on the possibility that p38 alpha could play a role also in CDDP resistance in CRC cell lines. In our experimental settings, we focused on two CRC cell lines HT29, which is considered resistant to CDDP treatment, and HCT116, which is considered sensitive to this compound. The inhibition of p38 alpha together with CDDP administration induced a strong cell death in both cell lines. Co-treated cells underwent apoptotic cell death, as shown by caspases activation and the cleavage of PARP-1. De Mattos and colleagues (Fernandez de et al., 2008) demonstrated also that FoxO3A played an important role in chemoresistance in CRC cell lines, and our previous papers indicated that the inhibition of p38 alpha induced the transcription activity of this

protein (Chiacchiera et al., 2009; Matrone et al., 2010). Finally, we showed that during the co-treatment of CRC cell lines FoxO3A translocated in to the nuclei and activated its dependent transcription. Moreover, its absence affected the activation of apoptotic pathway.

In this study, p38 alpha seems to be linked to HIF-1 alpha stability, whose absence produces a decrease of glycolytic genes transcription, preventing the production of ATP. This impairment of the energetic reservoir activated different pathway such as cell cycle arrest, autophagy and then cell death, driven by FoxO3A transcription factor. Indeed, targeting cancer metabolism is one of the main goal of cancer research and an increasing number of researchers is focusing its attention on this topic. The identification of p38 alpha as one of the actors in this scenario could open to new strategies to fight these malignancies, especially when it is overactive. Moreover, the inhibition of this kinase seems to be fundamental to avoid resistance in CDDP treatment of CRC suggesting that this effect could be very helpful for future therapies especially for the chance to decrease the amount of chemotherapeutics used, overcoming all side effects for patient health. Worthy of note is that the effect of SB treatment on these cancer cell lines seemed to be linked to the possibility to activate FoxO3A transcription factor. This is a very important point because this transcription factor is been linked to the activation of cell cycle arrest and autophagy and in the last years it is emerging its role as tumor suppressor. Moreover, the possibility to modify its regulator pathways by the use of a biological compound as SB, strongly makes FoxO3A an instrument for the creation of new approaches in cancer therapy. The

inhibition of p38 alpha represents a very hopeful instrument for treatment of cancer, and this promising result is validated through in vivo $APC^{+/-}$ mouse model; indeed, SB treatment is able to cause a great reduction both in number and volume of intestinal tumors (Chiacchiera et al., 2009) without damaging the whole body homeostasis. Another interesting point, is that p38 alpha inhibition could be used to exacerbate the activation of autophagy. Indeed, when the activation of p38 alpha is restored by 48 hours, CRC cells are able to survive and to growth (Comes et al., 2007), indicating that autophagy could be used as a mechanism to survive in these cells. This is in agreement with the fact that CRC cells when treated with the inhibitor of autophagy 3-MA plus SB underwent apoptosis (Comes et al., 2007). This speculative conclusion could explain why PC3 cells dead when autophagy cannot be activated (DU145 cells) and are able to growth when autophagy is activated by SB treatment (PC3 cells). However, to better evaluate the role of autophagy in PCa cells are necessary more experiments, such as use 3-MA with SB or the silencing of *AMPK* gene. Autophagy plays a very crucial point in this scenario because, in some cases, mutations or epigenetic changes in the most important effectors of apoptotic pathway, such as p53, make ineffective the induction of the apoptotic cell death, undermining all efforts to trigger it. The strategy of autophagy activation to counteract cancer would give the possibility to overcome these obstacles and also would give the opportunity to ameliorate patient's health avoiding toxic compounds. Finally, the use of a biological compound in CRC such as SB together with a widely used chemotherapeutic could overcome many of the

problems related to chemoresistance, making this kinase a putative target molecule for cancer therapy.

ABBREVIATIONS

5-FU:	(5-fluorouracil)
ACC:	(Acetyl CoA Carboxylase)
AMPK:	(AMP-activated Protein Kinase)
APC:	(adenomatous polyposis coli)
AR:	(androgen receptor)
ATF1:	(activating transcription factor 1)
BAF60:	(BRG1-associated factor 60)
BAK:	(BCL2-antagonist/killer 1)
BAX:	(BCL2-associated X protein)
BCL2:	(B-cell CLL/lymphoma 2)
BCL-W:	(BCL2-like 2)
BCL-XL:	(BCL2-like 1)
BH:	(BCL2 homology)
BID:	(BH3 interacting domain death agonist)
BRCA:	(breast cancer)
BRG1:	(brahma-related gene 1)
BV:	(bevacizumab)
CBP:	(CREB binding protein)
CC:	(Compound C)
CDDP:	(cisplatin)
CDKI:	(cyclin-dependent kinase inhibitors)
cDNA:	(DNA complementary)
cFLIP:	(cellular FLICE-like inhibitory protein)

CKI:	(casein kinase 1)
CNS:	(central nervous system)
CPT-11:	(irinotecan)
CRC:	(colorectal cancer)
CREB:	(cAMP responsive element binding protein)
CTNNB1:	(beta catenin)
DAPk:	(death associated protein kinase)
DBD:	(DNA-binding domain)
DDB2:	(damaged-DNA-binding complex 2)
DFO:	(desferrioxamine)
DMSO:	(Dimethyl sulfoxide)
EGF:	(epidermal growth factor)
EGFR:	(epidermal growth factor receptor)
ERK:	(extracellular signal-regulated kinase)
FAP:	(familial adenomatous polyposis)
FGFR1:	(fibroblast growth factor receptor 1)
FIGO:	(international federation of gynecology and obstretics)
FIH:	(factor inhibiting HIF)
FOLFOX:	(oxaliplatin, 5-FU and leucovorin combination)
Fox:	(Forkhead box)
GAPDH:	(glyceraldehyde-3-phosphate dehydrogenase)
GI:	(gastrointestinal)
GLUT1:	(Glucose transporter 1)
GSK3b:	(glycogen synthase kinase 3 beta)
HIF-1 alpha:	(hypoxia-inducible factor 1 alpha)

HK2:	(hexokinase 2)
HNPCC:	(hereditary nonpolyposis colorectal cancer)
HPP:	(hyperplastic polyposis)
H-RAS:	(Harvey rat sarcomas)
HSP:	(heat shock protein)
HuR:	(human antigen R)
IFL:	(irinotecan, 5-FU and leucovorin combination)
IL:	(interleukin)
JNK:	(c-jun N-terminal kinase)
JPS:	(juvenile polyposis syndrome)
K-RAS:	(Kirsten rat sarcoma)
LDHa:	(lactate dehydrogenase A)
LDL:	(low density lipoprotein)
LKB1:	(liver kinase B1)
LOH:	(loss of heterozygosity)
LPS:	(Lipopolysaccharide)
LRP:	(low density lipoprotein receptor-related protein)
MAP:	(MUTYH-associated polyposis)
MAP1LC3 or LC3:	(microtubule-associated protein 1 light chain 3)
MAPK:	(mitogen-activated protein kinases)
MCL1:	(myeloid cell leukemia sequence 1)
MCP-1:	(monocyte chemoattractant protein 1)
MEF:	(mouse embryonic fibroblast)
MEF2:	(myocyte enhancer factor)
MK2:	(MAPK-activated protein kinase 2)

MKK:	(MAPK kinase)
MKP:	(MAPK phosphatase)
MLH:	(mutL homolog)
MMP:	(matrix metalloproteinase)
MMR:	(DNA mismatch repair)
MSH:	(mutS homolog)
MSI:	(microsatellite instability)
MSK1:	(Mitogen- and stress-activated protein kinase-1)
MUTYH:	(mutY Homolog)
MYC:	(myelocytomatosis viral oncogene homolog)
NHE-1:	(microtubuleassociated protein tau, Na+/H+ exchanger 1)
NOXA:	(Phorbol-12-myristate-13-acetate-induced protein 1)
N-RAS:	(neuroblastoma RAS)
ODDD:	(oxygen-dependent degradation domain)
OS:	(overall survival)
OvCa:	(ovarian cancer)
OXPHOS:	(oxidative phosphorylation)
P.I.:	(propidium iodide)
p38IP:	(p38 alpha-interacting protein)
PAK:	(p21 protein (Cdc42/Rac)-activated kinase 1)
PARP-1:	(Poly (ADP-ribose) polymerase-1)
PBS:	(phosphate-buffered saline)
PCa:	(prostate cancer)
PCD:	(programmed cell death)
PCR:	(polymerase chain reaction)

PDH: (pyruvate dehydrogenase)

PDK1: (pyruvate dehydrogenase kinase 1)

PFS: (progression-free survival)

PHD: (prolyl hydroxylases domain)

PI3K: (phosphoinositol 3-kinase)

PIN: (prostatic intraepithelial neoplasia)

PJS: (Peutz-Jeghers syndrome)

PKM2: (pyruvate kinase isoform M2)

PMS: (postmeiotic segregation increased)

PP2C: (serine/threonine protein phosphotase type 2C)

PRAK: (p38 regulated/activated kinase)

PTEN: (phosphatase and tensin homolog 10)

PUMA: (BCL2 binding component 3)

pVHL: (von Hippel-Lindau protein)

RAF: (v-raf-1 murine leukemia viral oncogene homolog)

RAS: (rat sarcomas)

Rb: (retinoblastoma protein9)

ROS: (reactive oxygen species)

RR: (response rate)

SAPK: (stress-activated protein kinases)

SB: (SB202190)

SFRP: (secreted frizzled-related protein)

SMAC: (second mitochondria-derived activator of caspase)

SRF: (serum response factor)

STK11: (serine-threonine kinase 11)

SV40:	(simian virus 40)
SWI/SNF:	(switch/sucrose nonfermentable)
TAB:	(TAK binding protein)
TAK:	(TGF-beta-activated protein kinase)
TCF:	(T-cell factor)
TGFbetaIIR:	(transforming growth factor, beta receptor II)
TNF:	(tumor necrosis factor)
TSC:	(tuberous sclerosis)
TTP:	(time to progression)
USF1:	(upstream stimulatory factor 1)
VEGF:	(vascular endothelial growth factor)
vFLIP:	(viral FLICE-like inhibitory protein)
WIF:	(WNT inhibitor factor)
WNT:	(wingless-type MMTV integration site family)

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