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EICOSANOIDS AND CANCER: FOCUS ON MELANOMA

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ABSTRACT

Inflammation plays a key role in tumor promotion and development. Indeed, high levels of cyclooxygenase-2 (COX-2) expression are associated with worse prognosis in several types of cancer including melanoma. The aim of our project, divided in three sections, has been to investigate on the role of COX-2 in melanoma development and progression. In the first phase of the project, we evaluated the expression of both COX-1 and COX-2 in a large panel of human melanoma cells and assessed the effect of COX-2 ablation on cancer cell proliferation and invasiveness by the mean of siRNA technology and by selective inhibition of COX-2 activity. Translation of *in vitro* data to *in vivo* models of cutaneous melanoma showed that in COX-$2^{-/-}$ mice tumor development was almost blunted as compared to littermate control C57Bl/6J. Finally, we performed a retrospective clinical study on 45 human lymph node melanoma metastases and correlated COX-2 expression to progression free survival (PFS). Our results show an inverse correlation between PFS and COX-2 expression suggesting that COX-2 is a negative prognostic factor in metastatic melanoma.

In addition to COX enzymes, also hydrogen sulfide (H_2S), an endogenous gasotransmitter, has been recently demonstrated to be involved in human melanoma. Thus, the second phase of the project was focused on the evaluation of the efficacy of a new H_2S-releasing nonsteroidal anti-inflammatory drugs (H_2S-NSAIDs) named ATB-346, developed by combining naproxen with a chemical moiety that donates hydrogen sulfide. In particular, we used cell culture and a murine melanoma model to evaluate the effect of ATB-346 on: i) proliferation of human melanoma cells; ii) melanoma development in mice. Cell culture studies demonstrated that ATB-346 reduced the proliferation of human melanoma cells and this effect was associated to induction of apoptosis and inhibition of NF-κB activation. Moreover *in vivo* data showed that

ATB-346 significantly reduced melanoma development. In conclusion, by using this dual approach we propose that COXs and H_2S pathway could be innovative therapeutic targets to generate new treatment options based on “combination therapy” for melanoma.

Finally, in the third and last phase of the project, we decided to better define the role of COX-2 in melanoma development. The aim was to establish if this enzyme acts predominantly in the microenvironment rather than in tumor cells. For this purpose we deleted COX-2 in B16/F10 murine melanoma cells by the mean of CRISPR/Cas9 technology. We firstly investigated on the effect of COX-2 knockdown on proliferation, migration, invasion and colonie formation of B16/F10 murine melanoma cells. *In vitro* studies demonstrated that CRISPR/Cas9-mediated COX-2 knockdown decreased proliferation of B16/F10 cells and inhibited some features of metastatic melanoma such as motility, invasiveness and focus formation. Finally, subcutaneously injection of B16/F10 cells knocked down for COX-2 showed slightly reduced melanoma growth and reduced the CXCL1 chemokine plasma levels. In addition, we also investigated on the expression and role of miR-143-3p in human malignant melanoma that has been shown to be dysregulated in many cancers. Our results showed that the expression of miR-143-3p was lower in human melanoma cells, as well as human tumor biopsy specimens, when compared to normal human melanocytes. Ectopic expression of miR-143-3p in human melanoma cells inhibited proliferation, migration, invasion and promoted apoptosis acting through a molecular mechanism that, at least in part, is dependent on inhibition of the COX-2 gene. Collectively, our findings show that COX-2 has a critical role in modulating melanoma development and progression. Nonetheless, significant challenges still lie ahead for blocking the interactions between the microenvironment and tumors.

When you are forge, stay;

when you are hammer bang.

TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHODS

3.1 *IN VITRO* EXPERIMENTS

ABBREVIATIONS

15dPGJ2: 15-deoxy-Δ12,14-PGJ2
AA: Arachidonic acid
ACT: Adoptive cell transfer
AJCC: American Joint Committee on Cancer
APL: Acute promyelocytic leukemia
APC: Antigen presenting cells
ATB-346: 2-(6- methoxynapthalen-2-yl)-propionicacid 4 thiocarbamoyl phenyl ester
BCC: Basal cell carcinoma
CDK: Cyclin dependent kinase
CLRs: C-type lectin receptors
COX: Cyclooxygenase
COX-1: Cyclooxygenase-1
COX-2: Cyclooxygenase-2
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats associated nuclease 9
DCs: Dendritic cells
DMEM: Dulbecco's modified Eagle's medium
EMT: Epithelial-mesenchymal transition
FDA: Food and Drug Administration
Gr-MDSCs: Granulocytic MDSCs
H_2S: Hydrogen sulfide
H_2S-NSAIDs: H_2S-releasing nonsteroidal anti-inflammatory drugs
HMGB1: High-mobility group box 1 protein
HPGD: Hydroxyprostaglandin dehydrogenase
IAP: Inhibitor of apoptosis
IDO: Indoleamine 2,3-dioxygenase
IFNs: Type I interferons
ILCs: Innate lymphoid cells
IRF3: IFN regulatory factor 3
LGP-2: Laboratory of genetics and physiology-2
LPS: Lipopolysaccharides
LOX: Lypooxigenase
mAbs: Monoclonal antibodies
MAPK: Mitogen-activated protein kinase
MDA5: Melanoma differentiation factor-5
MDSC: Myeloid derived suppressor cells
miRNAs: microRNAs
Mo-MDSCs: Monocytic MDSC

MRP4: Multidrug resistance-associated protein 4
MTT: 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MyD88: Myeloid differentiation primary response 88
NALP3: Leucine-rich repeat-and pyrin-domain-containing protein
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEM: Normal Human Epidermal Melanocytes
NLRs: NOD-like receptors
NMSC: Non-melanoma skin cancers
NO: Nitric oxide
NSAIDs: Non-steroidal anti-inflammatory drugs
PAF: Platelet-activating factor
PAMPs: Pathogen-associated molecular patterns
PARP: Poly (adenosine diphosphate-ribose) polymerase
PFS: Progression free survival
PGG2: Prostaglandin G2
PGH2: Prostaglandin H2
PI3K/AKT: Phosphatidylinositol-3 kinases
PLA2: Phospholipase A2
PPARs: Peroxisomal proliferator activated receptors
PRRs: Pattern-recognition receptors
PS: Phosphatidylserine
PTEN: Phosphatase and tensin homolog
PTGS: Prostaglandin endoperoxide synthases
qPCR: Quantitative real-time PCR
RAF: Rapidly Accelerated Fibrosarcoma
RGP: Radial growth phase
RLRs: Retinoic acid-inducible gene I Retinoic acid-inducible gene (RIG)-I-like receptors
ROS: Reactive oxygen species
SCC: Squamous cell carcinoma
T-reg: Regulatory T cells
TBZ: 4-hydroxy-thiobenzamide
TGFβ: Transforming growth factor β
TILs: Tumor infiltrating lymphocytes
TLRs: Toll-like receptors
TMA: Tissue micro-array
TME: Tumor microenviroment
TNF-α: Tumour-necrosis factor-α
TRIF: TIR-domain-containing adapter-inducing interferon- β
VEGF: Vascular endothelial growth factor
VGP: Vertical growth phase
XIAP: X-chromosome-linked inhibitor of apoptosis protein

CHAPTER 1: INTRODUCTION

1.1 INFLAMMATION AND CANCER

1.1.1 The inflammatory pathway

Inflammation is a protective response involving host cells, blood vessels, proteins and other mediators that is intended to eliminate the initial cause of cell injury, as well as the necrotic cells and tissues resulting from the original insult, and to initiate the process of repair [1]. The first description of inflammation by Cornelius Celsus in the 1st century, defined the four cardinal signs of inflammation: heat *(calor),* redness *(rubor),* swelling *(tumor),* pain *(dolor),* and loss of function *(functio laesa)* then added by Rudolf Virchow in the 19th century [2]. Inflammation is often considered in terms of acute inflammation and chronic inflammation that differ in onset, cellular infiltrate and local and systemic signs [3]. The acute inflammatory response follows within seconds of the tissue injury and lasts for some minutes inducing vasodilation and increase of capillary permeability which allows to augment the blood flow and entry of fluids and diffusible components to the infected area inducing redness and *erythema* as consequence. This phase usually terminates after several tens minutes and is common in case of minor injuries such as bruising, scratching, cuts, and abrasions. If there has been sufficient damage to the tissues, or if

infection has occured, the acute inflammatory response takes place over the next few hours.

In this phase there is the recruitment of leukocytes, particularly neutrophils, in the tissues [4]. The sequence of events in the recruitment and activation of leukocytes consists of different phases: first leukocytes accumulate themselves to the endothelial cells (*margination*), then begin to tumble on the endothelial surface by the interaction between adhesion molecules expressed on the surface of both leucocytes and endothelial cells (*rolling and adhesion*) and then migrate through the vessels and move toward sites of infection or injury (*diapedesis and chemotaxis*). Finally leukocytes bind and ingest most microorganisms and dead cells (*phagocytosis*) and the subsequent resolution process begins restoring the normal tissue architecture [5]. If the acute inflammatory response fails to eliminate the pathogen, a chronic inflammatory state ensues where the inflammatory process persists and acquire new characteristics. Here the neutrophil infiltrate is replaced with macrophages, lymphocytes and plasma cells producing inflammatory cytokines, growth factors and enzymes which in turn contribute to the progression of tissue damage and secondary repair including fibrosis and granuloma formation.

There are two types of chronic inflammation:

Non specific proliferative: characterized by the presence of non-specific granulation tissue formed by infiltration of mononuclear cells

(lymphocytes, macrophages, plasma cells) and proliferation of fibroblasts, connective tissue, vessels and epithelial cells, for example, an inflammatory polyp-like nasal or cervical polyp and lung abscess.

Granulomatous inflammation: A specific type of chronic inflammation characterized by the presence of different nodular lesions or granulomas formed with a conglomeration of activated macrophages or its derived cells called epithelioid cells usually enclosed by lymphocytes. The macrophages or epithelioid cells inside the granulomas often combine to form Langhans or giant cells such as foreign body, Aschoff, Reed-Sternberg and Tumor giant cells.

There are two major types of granuloma:

Foreign body granuloma: that are formed to a foreign body or T-cell mediated immune response such as in sarcoidosis and Crohn's disease [6, 7].

Infectious granuloma: Granuloma that are formed from chronic infection such as in tuberculosis and leprosy [8].

Although chronic inflammation progresses silently, it is the cause of most chronic diseases including obesity, type 2 diabetes, atherosclerosis, asthma and neurodegenerative diseases [9].

1.1.2 Inducers and sensors of inflammation

A typical inflammatory response consists of four components: inducers, sensors, mediators and the effectors of inflammation. There are different inducers that trigger the inflammatory response. They activate specific sensors, which then stimulate the production of distinct sets of mediators. Inducers of inflammation can be divided in exogenous or endogenous [10].

Exogenus stimuli are both microbial and non-microbial, such as pathogen-associated molecular patterns (PAMPs) and virulence factors. PAMPs are a restricted and specific set of conserved molecular patterns that are carried by all microorganisms and are recognize by the host through the expression of a specific set of receptors known as pattern-recognition receptors (PRRs) [11]. The second class of microbial inducers are virulence factors that are not recognized by specific receptors but by specialized sensors such as NACHT-, leucine-richrepeat-and pyrin-domain-containing protein (NALP3) inflammasome [12]. Non-microbial exogenous stimuli are allergens, irritants, foreign bodies and toxic compounds that simulate the virulence activity of parasites or can act as irritants on the mucosal epithelia [1]. Others exogenous stimuli are: physical (such as extremely low or high temperature or ionizing radiations) and nutritive (e.g. deficiency of oxygen and vitamins). ***Endogenous stimuli*** are signals generated by damaged or stressed tissues. For example, the release of ATP and high-mobility group box 1

protein (HMGB1) during necrotic death cells, can cooperate with Toll-like receptors (TLRs) to induce an inflammatory response [13]. Or, the formation of urate and calcium crystals that are common in people-eating high purine foods and are responsible of periarticular diseases like gout and pseudogout [14]. These crystals are detected by macrophages activating the NALP3 inflammasome which in turn activates the production of caspase-1 substrates and members of the interleukin 1 (IL-1) family [15].

Pattern-recognition receptors are designed to sense the presence of infectious pathogens and substances released from dead cells. Currently, four different classes of pattern-recognition receptors families have been identified: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) [16].

TLRs are one of the best-characterized PRRs and are responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes [17]. TLRs are expressed by T cells, B cells neutrophils, mast cells, monocytes and macrophages. There are ten mammalian TLRs, TLR1 –TLR10, which recognize products of bacteria (such as endotoxin and bacterial DNA), viruses (such as double stranded RNA), and other products including lipoproteins and di- and triacyl lipopeptides, fungal zymosan, peptidoglycans, flagellin and different synthetic molecules such as imidazoquinolines and guanosine

analogues. These molecules are recognized by distinct TLRs in combination with coreceptors, or by TLR heterodimers [18]. TLR1 is closely related to TLR2 and TLR6 with which it forms heterodimers that recognize bacterial lipoproteins and lipopeptides [19]. TLR4 recognizes lipopolysaccharides (LPS), which are present in the cell wall of Gram-negative bacteria [20]. The TLR5 recognizes flagellin the dominant component of the bacterial flagellum and the structure responsible for motility in different bacterial species [21]. TLR3, TLR7, TLR8, and TLR9 are different from other TLRs since they are not expressed at the cell surface but located in endosomal compartment. TLR3, TLR7, and TLR8 detect double- and single-stranded RNA and TLR9 detects unmethylated CpG DNA [22]. Finally, TLR10 is the only remaining orphan receptor without a known ligand or signaling function. Moreover, it has been recently reported that TLR10 does not activate the immune system and has instead been shown to suppress inflammatory signaling on primary human cells [23].

TLRs signaling is divided into two distinct pathways: the myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (Figure 1.1). Those signaling pathways lead to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein, that induces transcription of a wide range of genes involved in the inflammatory response including cytokines, chemokines, and immunoreceptors [24].

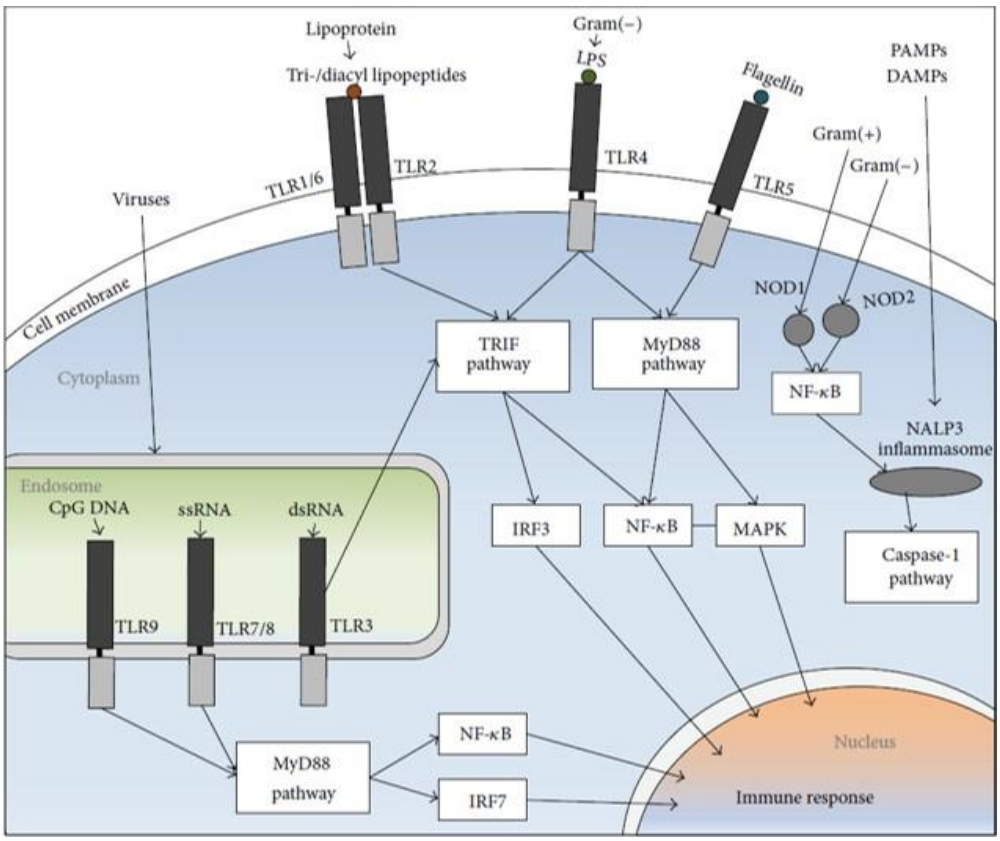

Figure 1.1: TLR and NLR signaling pathways [25]

MyD88 is utilized by all TLRs with the exception of TLR3 and drives NF-κB and mitogen-activated protein kinase (MAPK) activation to control inflammation. TRIF, also known as TICAM1, is utilized by TLR3 and TLR4 and is triggered by dsRNA and LPS, respectively [26].

CLRs are crucial for controlling both innate and adaptive immune response and are expressed by dendritic cells (DCs), monocytes and macrophages [16]. CLRs recognize carbohydrates, such as mannose, fucose, and glucan carbohydrate structures present in bacterial, viral, and fungal components.

RLRs are intracellular receptors for virus recognition. There are three major components of this family: retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor-5 (MDA5), and laboratory of genetics and physiology-2 (LGP-2) that bind to virus double-stranded RNA. Activation of RIG-I and MDA5 increase the secretion of type I interferons (IFNs), such as IFN-β, through the activation of the IFN regulatory factor 3 (IRF3) transcription factor [27]. Conversely, LGP-2 has a negative regulatory role by inhibiting IRF3 and the secretion of IFN-β.

In addition to sensing of microbial RNAs by RLRs, STING signaling is a recent identified pathway that recognize microbial and self-DNA [28]. STING (Stimulator of Interferon Genes, also known as MITA, ERIS, MPYS, or TMEM173) is thought to function as an adaptor protein, which links upstream DNA sensors to downstream IRF-3 and NF-κB pathway

activation inducing the expression of IFNs and inflammatory cytokines such as TNF, IL-1β, and IL-6 [29].

NLRs are intracellular sensors localized in the cytoplasm of DCs, macrophages and lymphocytes and are associated with cell stress. There are different members of this family, in particular NOD1, NOD2, and NALP3 [30]. NOD1 and NOD2 recognize intracellular bacterial cell products in both Gram-positive and Gram-negative bacteria driving the activation of NF-κB and MAPK pathways, leading to proinflammatory cytokine secretion [16]. NALP3 responds to a widest array of stimuli through the development, togheter with NLRP1 and NLRC4, of a multiprotein complex named NALP3 inflammasome (Figure 1.1) [30]. Inflammasome induces the cleavage and consequent activation of caspase-1 which in turn cleaves the precursor cytokines pro-IL-1β and pro-IL-18, producing the biologically active cytokines IL-1β and IL-18 and also inducing an inflammatory form of cell death known as pyroptosis [31]. IL-1β is a cytokine that promotes T helper 1 (Th1) and T helper 17 (Th17) differentiation. IL-18 is important for IL-17 expression by Th17 cells and for T cell polarization toward Th1 or Th2 profiles in combination with other cytokines [32].

1.1.3 Mediators of inflammation

Inflammatory mediators are soluble, diffusible molecules that can be produced locally by cells at the site of inflammation or derived from circulating inactive precursors to be then activated at the site of inflammation. Some mediators (such as histamine and serotonin) are normally stored in intracellular granules of mast cells, basophils and platelets and are rapidly released upon cellular activation. Others are synthesized de novo or circulate as inactive precursors in the plasma and require a proteolytic cleavage to acquire their biological activity [1]. Inflammatory mediators can be divided into seven groups according to their biochemical properties: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes.

The ***vasoactive amines*** are responsible for the immediate and short-lived responses in inflammation including vasodilation, increase of vascular permeability and smooth muscle contraction. Histamine and serotonin are the most important vasoactive amines and are secreted by mast cells and platelet degranulation [33].

Vasoactive peptides are small proteins, such as substance p that is stored in secretory vesicles and released by sensory neurons transmitting pain signals and regulating vessel tone and vascular permeability. Other vasoactive peptides are bradykinin, fibrinopeptide A, fibrinopeptide B and fibrin degradation products. Bradykinin is the

main product of the kallikrein–kinin cascade activated by the Hageman factor together to the clotting, fibrinolytic and the complement system. Like histamine, bradykinin increases vascular permeability, arteriolar dilation, and bronchial smooth muscle contraction [34].

The ***complement system*** consists of plasma proteins that play an important role in inflammation. In particular, the complement-derived factors C3a, C4a and C5a (also called anaphylatoxins) increase vascular permeability and cause vasodilation by inducing mast cells to release histamine, activate leukocytes, increasing their adhesion to endothelium, and augment the phagocytosis by neutrophils and macrophages [35].

Lipid mediators are eicosanoids and platelet-activating factor (PAF). Eicosanoids derived from the metabolism of arachidonic acid (AA). AA is a component of cell membrane phospholipids released through the action of phospholipase A2 (PLA2) that is activated by other inflammatory mediators sush as the complement-derived factor C5a [36]. AA is processed by two different enzimes: Cyclooxygenase (COX) and Lypooxigenase (LOX), which in turn produce respectively prostaglandins, thromboxanes and leukotrienes and lipoxins. We will discuss about eicosanoids and their relationship with cancer later in this thesis. PAF is generated by the acetylation of lysophosphatidic acid and acts directly on target cells through the binding with a specific G protein–coupled receptor. PAF stimulates platelet aggregation, causes

bronchoconstriction and induces a potent vasodilatation and increase of vascular permeability.

Another class of inflammatory mediators are ***cytokines***. Inflammatory cytokines are produced by different cell types including in particular macrophages and mast cells. The major cytokines involved in inflammation are tumour-necrosis factor-α (TNF-α), IL-1, IL-6 and many others [37]. TNF-α and IL-1 stimulate the expression of adhesion molecules on endothelial cells, increasing leukocyte binding and recruitment, and augment the production of additional cytokines.

Chemokines are produced by different cell types and act as chemoattractants controlling extravasation and chemotaxis of monocytes, lymphocytes, neutrophils, eosinophils, basophils, natural killer cells, dendritic cells, and endothelial cells towards the site of inflammation [38].

Proteolytic enzymes have different roles in inflammation. In particular elastase, collagenase, and cathepsin, secreated by neutrophils and monocytes, destroy phagocytosed substances. Their activity is regulated by antiproteases present in the plasma and tissue fluids such as α1-antitrypsin and α2-macroglobulin.

In addition, other mediators of inflammation are: reactive oxygen species (ROS) and nitric oxide (NO).

ROS are released from neutrophils and macrophages and amplify the cascade of inflammatory mediators by inducing adhesion molecule expression, chemokines and cytokines promoting their migration across the endothelial barrier helping also in the clearence of phagocytosed microbes and necrotic cells [39].

NO is a soluble gasotransmitter that possesses cytotoxic properties serving as a killing mechanism against invading microbes. In particular, macrophages produce it as cytotoxic agent for killing microbes and tumor cells [40]. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in unusual conditions such as in inflammatory disorders of the joint, lungs and gut [41].

1.1.4 Cyclooxygenase

COX enzymes, also known as prostaglandin endoperoxide synthases (PTGS), are enzymes involved in the rate-limiting step of prostaglandin production from AA [42]. They exist in two isoforms: COX-1 (also known as PTGS1) and COX-2 (also known as PTGS2). COX-1 is a constitutively-expressed enzyme found in many tissues. This enzyme is often referred as the 'housekeeping' form of the COX enzymes and modulates aspects of routine functions such as platelet aggregation and gastric acid secretion by maintaining basal prostanoid levels [43]. By contrast, COX-2 is highly induced by a variety of endogenous and external stimuli that may include growth factors, tumor promoters, viral or bacterial infections as well as a widely range of proinflammatory cytokines [43, 44]. The gene structures of COX-1 and COX-2 are found at distinct genetic loci and differ mainly in size. While COX-1 maps to the long arm of chromosome 9 and measures 22 kb, COX-2 maps to the long arm of chromosome 1 and measures 8.3 kb [45]. By contrast, both COX isoforms share approximately 60% of their protein structure which differs in the substitution of a Valine residue in COX-1 for an Isoleucin at corresponding position 523 within the active site of COX-2. This change, together to the loss of a methyl group, increases the volume of the COX-2 active site by 25% allowing the accomodation of larger chemical structures (Figure 1.2) [46].

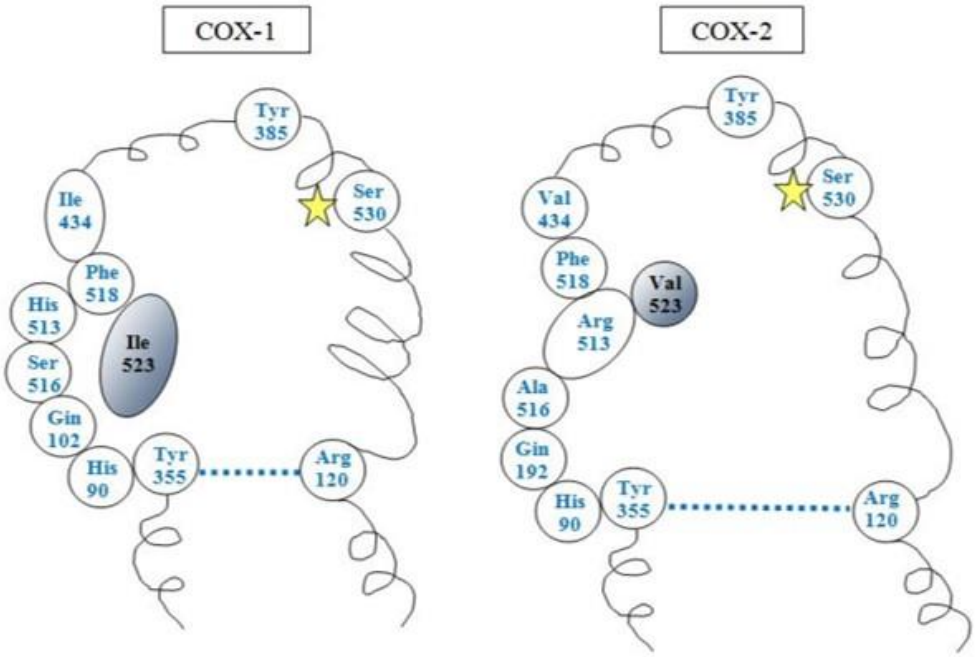

Figure 1.2: Structure of COX-1 and COX-2 isoforms [46]

These differences have been exploited for the development of COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs) that are widely used in clinical practice for the treatment of chronic inflammatory diseases such as osteoarthritis, rheumatoid arthritis and gout showing fewer gastrointestinal adverse effects than non-selective NSAIDs [47]. Cyclooxygenase enzymes catalyse the oxidation of arachidonic acid to form the peroxide intermediate prostaglandin G2 (PGG2) which in turn is reduced to prostaglandin H2 (PGH2) (Figure 1.3). The latter acts as a substrate for several prostaglandin synthases including PGE2, PGD2, PGF2α, PGI2, and TXA2 [48]. Prostaglandins exert their biological effects in an autocrine or paracrine fashion by binding to cognate membrane bound G-protein-coupled receptors such as DP for PGD2; EP1, EP2, EP3 and EP4 for PGE2; FP for PGF2α; IP for PGI2; and TP for TXA2. Moreover, some prostaglandins and their metabolites bind to intracellular peroxisomal proliferator activated receptors (PPARs) inducing their transcriptional activity in the nucleus [49]. For example, PGI2 and PGE2 can bind to PPARβ while, 15-deoxy-Δ12,14-PGJ2 (15dPGJ2), a PGD2 dehydration product, can bind to PPARγ.

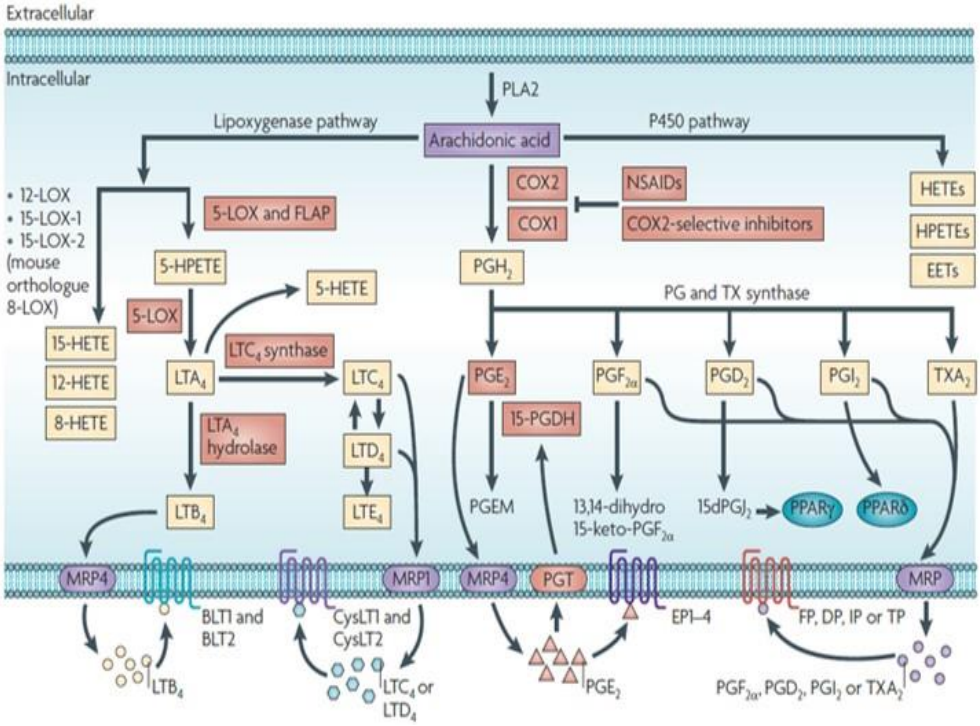

Figure 1.3: Eicosanoid synthesis pathway [50]

In addition to their synthesis, the extracellular levels and the inactivation of prostaglandins is regulated by different transporters and enzymes such as the influx prostaglandin transporter PGT; the efflux transporter multidrug resistance-associated protein 4 (MRP4); and the enzyme hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD; also known as 15-PGDH) that metabolize PGE2 and PGF2α, while other prostaglandins are metabolized in a non-enzymatic manner [51].

Prostaglandins regulate different biological processes in a wide range of tissues under both physiological (mainly ascribed to COX-1 activity) and pathological conditions (mainly ascribed to the induction of COX-2). For example, PGE2 and PGI2 exert cytoprotective effects in the gastrointestinal tract by reducing gastric acid and increasing the release of mucous, while in the kidneys these prostaglandins act as vasodilators that maintain renal homeostasis [48]. Moreover, prostaglandins control the homeostasis of other peripheral tissues such as the cardiovascular system, the lungs, reproductive system and the central nervous system.

1.1.5 Eicosanoids and cancer

The concept that inflammation is a critical component of tumour progression is not new. In 1863, Rudolph Virchow was the first to hypothesize that cancer occurred at sites of chronic inflammation, assuming that some classes of irritants, together with the tissue injury and consequent inflammation, release factors stimulating cancer cell proliferation [52]. In 1957, Burnet and Thomas postulated the existence of tumor immunosurveillance: the immunological resistance of the host against the development of cancer. Burnet and Thomas proposed that lymphocyte populations of the immune system continuously recognized and eliminated cancerous and/or precancerous cells arising in the host before they could cause harm. In the last years different data highlighted the concept that inflammation is a critical component of tumour progression and has been reported to be one of the newest "hallmarks of cancer" [53]. Different epidemiological data reported that about 20% of cancer are linked to chronic inflammation [52]. For example, inflammatory bowel diseases are linked to colorectal cancer; bronchitis to lung cancer and prostatitis to prostate cancer [54]. Most precancerous and cancerous lesions show signs of inflammation including innate immune cells, cytokines, and chemokines [55]. For example, macrophages in inflammatory bowel disease are phenotypically different from normal macrophages and produce different cytokines such as IL-1, IL-6, and TNF-α that can promote tumor

invasion and metastasis [56]. Among various inflammatory mediators, deregulation of prostaglandin mediated signaling as a result of aberrant COX expression, has been linked to different types of cancer. In 1994 it has been reported that COX-2 is overexpressed in human colorectal adenomas and adenocarcinomas and has a key role in inflammatory bowel disease and colorectal cancer [57]. Moreover COX-2 has been detected in a wide range of cancers including: breast cancer, lung cancer, ovarian cancer, colon cancer, colorectal cancer, gliomas, prostate cancer, esophageal carcinoma, pancreatic cancer, gastric carcinoma, Kaposi's sarcoma and melanoma [54, 58]. The importance of COX-2 in cancer has been demostrated by treatment with aspirin or other NSAIDs that inhibit prostaglandin synthesis decreasing the incidence of esophageal, colorectal, bladder, lung, and gastric cancer [54]. Moreover, COX-2 selective inhibitors (such as celecoxib) showed inhibition of adenoma growth in patients at high risk for colorectal carcinoma in three different clinical trials. Unfortunately, all three trials were aborted because of cardiovascular and gastrointestinal toxicity highlighting the importance to develop new classes of anti-inflammatory agents for cancer therapy [59-61]. Among prostaglandins, PGE2 is the most common prostaglandin that is found in various human malignancies, including colon, lung, breast, and head and neck cancer, and is often associated with a poor prognosis [50]. PGE2 appears to play essential roles in tumor cell proliferation, invasion, angiogenesis, and

epithelial-mesenchymal transition (EMT), all of which are associated with the established or emerging “hallmarks of cancer” [50, 62-64]. Another critical mechanism for the potent pro-tumorigenic activity of PGE2 is the local suppression of the immune responses [65]. PGE2 inhibits macrophages, natural killer cells and T cell activation, resulting in immunosuppressive and pro-tumorigenic activity [66]. Moreover, PGE2, also plays a key role in promoting myeloid derived suppressor cells (MDSC) differentiation from bone marrow stem cells that are responsible for inhibiting the antitumor immune response; this provides a therapeutic approach for anti-cancer therapy [67]. Another strategy to inhibit the pro-tumorigenic activity of PGE2 is the overexpression of 15-PGDH enzyme, which is responsible for the degradation of PGE2. In fact, this enzyme results to be down-regulated in human gastric cancer, breast cancer, lung cancer, and bladder cancer, suggesting its tumor suppressive role [68-71]. Another approach for cancer therapy is the inhibition of PGE2 receptors. Mice deficient for EP1, EP2 or EP4 receptors showed decreased tumorigenesis while administration of PGE2 receptor antagonists (such as ONO-8711 which is selective for EP1 receptor) inhibit tumor growth and can act as chemo-preventive agents [72-75].

In addition to PGE2, there are conflicted data about the role of PGD2 in cancer progression. Disruption or overexpression of hematopoietic prostaglandin D synthase, which synthesizes PGD2, accelerates or

suppresses intestinal tumour growth in mice [76]. By contrast, an immunosuppresive effect of PGD2 has been recently reported in human acute promyelocytic leukemia (APL) by involving a new class of innate immune cells named innate lymphoid cells (ILCs) which in turn activate MDSC [77].

While COX-2 has been the most extensively studied enzyme in cancer, few reports have indicated that LOX enzyme has also an important role in tumor progression and survival and is often constitutively expressed in various epithelial cancers [78]. However, due to its association with the inflammatory response under most physiological conditions as well as the overexpression of COX-2 in many tumors, including melanoma, the cyclooxygenase pathway will be given the principle focus throughout this thesis.

1.2 MELANOMA

1.2.1 Generalities and classification

Melanocytes are melanin-producing cells located in the basal layer of the skin's epidermis, the uvea, the inner ear, meninges, bones and heart. The word "melanin" comes from the ancient Greek melanos, meaning 'dark' and is the pigment that provides color and protection against ionizing radiations [79]. Cutaneous melanoma is a type of skin cancer that arises from abnormal melanocytes proliferation and can occur in any anatomic location containing melanocytes. There are four different types of skin cancer: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) identified as non-melanoma skin cancers (NMSC) or keratinocyte carcinoma; melanoma and other non-epithelial skin cancer. Moreover, there are also other uncommon types of skin cancer, such as Merkel Cell Carcinoma, Cutaneous T-cell lymphoma and Kaposi's sarcoma [80]. BCC and SCC represent approximately 80 % and 16 % of all NMSC, respectively. By contrast, melanoma represents less than 5% of the skin cancer cases, however, it far the most dangerous as it accounts for 80% of all the deaths related to skin cancer [81]. When diagnosed in the early stage, melanoma can be easily cured by surgical resection. However late metastatic stages are often fatal and, currently available or developing treatment strategies, are only able to prolong life for a few months, are expensive and may produce severe adverse effects [82]. In the past few years melanoma has steadily increased

worldwide becoming the 5th neoplasia in America [83]. Its incidence varies by geographic location and across different ethnic groups. This variation is partly related to decreased photoprotection to UV radiations that is well known to induce both cell death and malignant transformation of skin cells and are considered the major risk factor for melanoma [84]. In 2017 the American Cancer Society and the National Cancer Institute estimated 87,110 new cases of patients with invasive melanoma and 9,730 deaths only in USA. Melanoma incidence is also dependent on gender, infact in the U.S. is higher in women than in men before age 50, but by age 65, rates in men are double those in women, and by age 80 they are triple. In addition to UV radiations, genetic predisposition and environmental insults contribute to the genesis of melanoma. About 10% of melanoma patients report a family history of melanoma and are associated with germline mutations in cyclin dependent kinase (CDK) gene CDKN2A which is a potent cell cycle inhibitor through a direct negative interaction with CDK4. Moreover, also xeroderma pigmentosum and MC1R genes have been implicated in familial melanomas [85]. These initial observations opened the door to additional efforts in understanding the genetics of melanoma, and several other mutations have been identified since then. For example, the MAPK/ERK and phosphatidylinositol-3 kinase (PI3K/AKT) pathways are one of the first most frequently deregulated signaling pathways in the great majority of melanomas. In particular, the role of the Ras

oncogene family in the melanoma development and their effects on downstream signaling were one of the first mutations to be identified, then NRAS mutation which is mutated in 15-30% of tumor samples leading to serial activation of both the downstream components of the RAS effector pathways and the non-MAPK pathways such as PI3K/AKT [86]. In 2002 the mutation called 'Rapidly Accelerated Fibrosarcoma' (RAF) were observed and the V600E variant identified as a frequent mutation in cutaneous melanoma [87]. This mutation and the consequent constitutive activation of the MAPK pathway has become the target of multiple pharmaceutical trials of small molecule inhibitors resulting in several new Food and Drug Administration (FDA) approved therapies [88]. The other pathway involved in melanoma development and progression is PI3K/AKT. In particular, in 12% of melanoma patients a lack of the tumour suppressor phosphatase and tensin homolog (PTEN) that antagonizes PI3K activity has been detected. Lack of PTEN antagonism causes increased phosphorylation of AKT promoting cancer cell survival and proliferation [89]. In addition there are also other mutations affecting genes that control growth, such as those of Bcl2 family, production of growth factors and loss of adhesion molecules which favor the alteration of intracellular signaling allowing the melanocytes to escape from the keratinocyte control [90].

Pathological features of melanoma can be divided in 5 steps according to the Clark model: I) Nevi formation of structurally normal

melanocytes, II) dysplastic nevi formation, III) radial growth phase (RGP) primary melanoma, IV) vertical growth phase (VGP) involving cells that have invaded the dermis and cells with metastatic potential and V) metastatic melanoma to distant organs (Figure 1.4). The development of this illness can be seen as a disruption of normal homeostatic mechanisms in the skin. Disruption of the homeostatic control also leads to le loss of adhesion molecules such as E-cadherin and the increase of N-cadherin that favors the mobility and invasiveness of melanoma cells thus the development of metastasis [91].

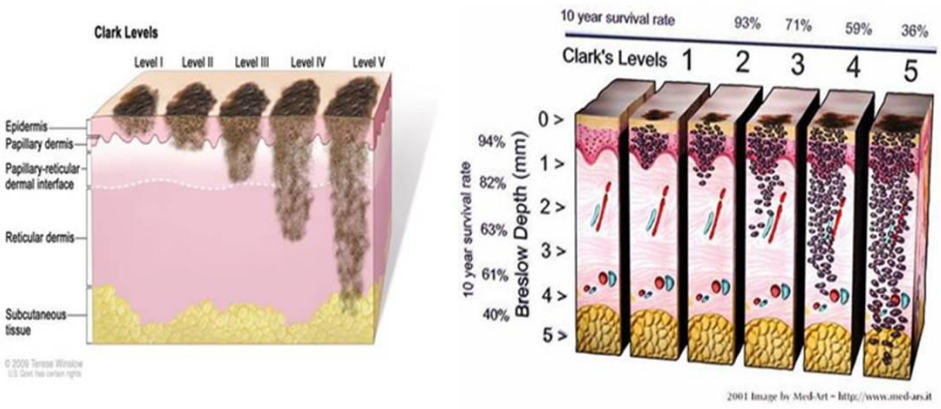

Figure 1.4: Clark and Breslow's model [92]

In addition, the American Joint Committee on Cancer (AJCC) considers the Breslow index as the most important prognosis factor in primary melanomas. This model measures the melanoma thickness (in millimetres) according to the distance between the upper layer of the epidermis and the deepest point of tumor penetration (Figure 1.4) [93]. According to AJCC the melanoma stages are classified into 4 groups: I and II as primary invasive melanomas and III and IV define local regional and distant metastasis respectively [94]. However, regular self-skin examinations by patients showed to decrease the risk of metastatic melanoma at the time of diagnosis expecially when coupled with regular visits to a dermatologist [95]. The A-B-C-D-E guide, devised by the american cancer society, highlights several clinical features of melanoma, including asymmetry, border, color, diameter and evolution (Figure 1.5).

A = asymmetry, when the two halves of the lesion do not match.

B = border, when the edge are irregular or uneven.

C = color, when the lesion doesn't have an uniform color but rather a combination of black, brown, blue, or even white.

D = diameter, when the lesion is larger than 6 mm.

E = evolution, when the lesion changes in size, color or shape.

Figure 1.5: The A-B-C-D-E guide

1.2.2 Immune response in melanoma

The concept of immune response in tumors was propose for the fist time by Paul Ehrlich in 1907 and later expanded when Coley obtained tumor reduction after injection of bacteria into tumors [96]. This kind of work revealed the importance of studying the relationship of host immune response to tumors and patients' survival. The work that followed showed that injection of melanoma cell lysates resulted in 2/26 patients with total remission and 5/26 with partial tumor regression. Furthermore, it was observed that patients injected with irradiated melanoma cells increased the index of lymphotoxicity. In all cases histology from regressing tumors showed high lymphocyte infiltration [96]. In fact, the importance of lymphocytes in tumors has been long recognized but their possible role in melanoma prognosis has been debated and only recognized in the past 40 years. Some groups showed increased survival and improved prognosis in patients with mild and high lymphocyte infiltration and finally established 3 categories: Brisk (dense infiltrate), No brisk (mild infiltrate) and absent [97, 98].On the contrary, other groups failed to show a positive correlation between tumor infiltrating lymphocytes (TILs) and prognosis or patients' survival [99-101]. Discrepancies in those results could be explained by the extreme diversity of the patients included in the studies and the fact that no phenotypic characterization of those TILs was done [102], in fact studies were such characterization was done, showed that $CD8^+$ T cell

infiltration improve prognosis [103]. More answers to this might be found in the understanding of the role of inflammatory cytokines in lymphocyte functions, and the immunosuppressive aspects of other cells in the tumor microenvironment [96]. Human tumors bear antigens that can be recognized by autologous T lymphocytes. Some of these antigens appear to be tumor specific (neo-antigens), whereas others are also present on normal tissues such as overexpressed antigens or viral-derived antigens. Specifically in melanoma, several tumor specific antigens have been described making easier the assessment of the anti-tumor immune response [104, 105]. After identification of melanoma antigens several groups reported evidence about the existence of a spontaneous anti-tumoral T cell response in patients. For example it has been reported that tumor-specific $CD8^+$ T lymphocytes from melanoma patients have a memory effector phenotype (TEM) ($CCR7^+$, $CD4^+45RO^+$) while healthy donors bear naive tumor-specific T lymphocytes ($CCR7^+$, $CD4^+45RA^+$) [106, 107]. In addition TILs of melanoma patients were shown to contain not only $CD8^+$ T cells but also $CD4^+$ T cells recognizing melanocyte differentiation antigens and cancer testis antigens [108]. In conclusion, spontaneous T cell response correlates with a better prognosis, even though the correlation may be obscured in some circumstances by the fact that most metastatic melanoma patients are progressing despite having mounted a quantitatively significant T cell response against their tumor [109]. T cell unresponsiveness does not

seem to be due to their failure to reach the tumor sites but the anti-tumoral T cell response must become ineffective at one point, either because the tumor cells have become insensitive to the effector cells or because the effector cells themselves have become unable to be stimulated by the antigen or to exert their function, a state also refered as “exhaustion”. This exhausted state could result from inhibitory processes elicited by tumor cells that were selected by the immune response (immunoediting), the loss of antigen or MHC molecules [110], the expression of immunosuppressive factors such as Indoleamine 2,3-dioxygenase (IDO) and prostaglandins [111, 112], the secretion of immunosuppressive cytokines such as IL-10 and TGFβ [113], arginase expression [114, 115], TREG infiltration [116] or the expression of PDL-1 molecule at the surface of tumor cells among others [117].

1.2.3 Treatment and immunotherapy in melanoma

In the last 30 years many drugs and combination of drugs have been introduced in melanoma therapy to improve patient survival for longer time. Cytotoxic chemotherapy was the main approach for metastatic melanoma while radiation therapy is used in 1–6% of patients with melanoma in the US in the setting of inoperable disease [118]. Common antineoplastic agents currently being used include dacarbazine and temozolomide that, in monotherapy, showed low overall response rates (approximately 10%-15%). In the past decades, the increasing knowledge in cellular immunology and tumor led to the development of immunotherapy approaches. One of the first approaches was the discovery of the T cell growth factor IL-2 that was the first agent used to modulate the immune system for the treatment of patients with metastatic melanoma [105]. It was approved by the FDA in 1998 but its response rate was low (approximately 16%) and showed marked secondary effects [119]. In fact, before 2010 none of the systemic therapies approved for metastatic melanoma treatment showed to increase patients' survival, and only little improvement was observed by using TNF-α as co-adjuvant. Currently, treatment with BRAF inhibitors, which are included in the "target therapy" drugs, have been used to treat melanoma patients bearing this mutation that constitutive activates MAPK pathway. However, BRAF inhibitor Vemurafenib, approved by the FDA in 2011, can only be used in approximately 50% of

melanoma patients where the V600E mutation is present [120]. There are also other BRAF inhibitors approved such as Dabrafenib and Trametinib but unfortunately, an important percentage of the patients acquire drug resistance loosing response to the therapy and tumor progression approximately after 6-8 months [121]. After the BRAF inhibitors' development, the interest about therapy continued to grow and the latest clinical advances in melanoma treatment have targeted immune response by blocking inhibitory checkpoint molecules. In particular, the two best characterized are CTLA-4 and PD-1 for which monoclonal antibodies have been developed and already approved by the FDA. For example, Tremelimumab was the first anti-CTLA-4 agent to be investigated in patients with metastatic melanoma. Although in phase I/II clinical trials, it showed antitumor activity in patients with stage III/IV melanoma, a phase III study failed because tremelimumab didn't demonstrate any benefit over chemotherapy [122]. More successes has had Ipillimumab (Yervoy®) approved by FDA in 2011. Ipilimumab blocks the CTLA-4 molecule favoring the activation state of T lymphocytes. CTLA-4 or CD152 works as an inhibitory receptor for the co-stimulatory signal delivered by the interaction with B7 expressed on the surface of antigen presenting cells (APC) [123] and has been also reported to be capable of depleting regulatory T cells (T-reg) [124]. In patients Ipillimumab showed an overall response of 11% [125] and, in

combination with dacarbazine, it improved patients' survival for 11,2 months with 24% reduction of patients' disease relapse [126].

Another inhibitory checkpoint is the PD-1/PDL-1 pathway. Physiologically, PD-1/PDL-1 interaction limits T cell activity in the setting of an inflammatory response. PD-1 is expressed in activated T, B and NK cells, whereas PDL-1 or PDL-2 are expressed on immune cells as well as tumor cells. PD-1 has been shown to be express on TILs while circulating melanoma antigen specific T cells and melanoma tumors express PD-L1 which induce T cell anergy and immunosuppression [105]. Nivolumab (Opdivo®) is directed against PD-1 and showed 28% of clinical response with a dosage of 3 mg/kg bodyweight and with a low rate of toxicity, infact the most common side effects are diarrhea, rush, and pruritus [127]. Pembrolizumab (formerly known as lambrolizumab) is a second anti-PD-1 agent approved by FDA on September 2014. It showed 25% of clinical response at low doses and 52% at high doses and 77% of the patients showed tumor regression [128].

Immunotherapy, based on adoptive cell transfer (ACT) of ex-vivo activated and expanded TILs, has shown promising clinical results in patients with metastatic melanoma. TIL therapy after lymphodepletion showed response rates of around 50% and significant survival benefit in refractory melanoma patients [129]. However TIL-based ACT is an individualized treatment that requires specialized laboratories and is more complex to perform than conventional therapies. Today, properly

designed clinical trials are required to formally demonstrate the advantages of this kind of therapies over the classic treatment modalities. In addition, identifying predictors of TIL efficacy and detection of TIL subsets with specific reactivity against tumors is necessary to improve the clinical responses and patients survival [130]. Other types of immunotherapies are cancer vaccines that have been also tested in the past decade and, in the last years, their development has been evolved in a significant manner in antigenic composition, delivery methods, adjuvants, treatment frequency and administration techniques [129]. Nonetheless, this approach has not been very successful in terms of objective response and patients survival. Current immunotherapy approaches for melanoma show encouraging results and the inefficacy of some of these therapies could be explain by different reasons such as the type of adjuvants used during the vaccination process [131], expression of exhaustion markers, loss of functionality, limited access to tumor site and the immunosuppressive tumor microenvironment [132].

1.2.4 The role of inflammation in melanoma and NMSC

As previously reported, inflammation is linked with several stages of tumor development, including initiation, promotion, invasion, and metastasis [133]. NF-κB protein is the main player in inflammation and, together with other molecules including cytokines, chemokines, ROS, oncogenes, inflammatory enzymes (COX-2 and LOX), anti-apoptotic proteins, transcription factors (STAT3, AP-1, CREB, NRF2) regulate tumor cell proliferation, transformation, and survival [134]. Cell lines from SCC showed high expression of activated NF-κB while, other cancer cells, including melanoma cells, showed higher expression of STAT-3 which in turn up-regulates anti-apoptotic proteins such as Bcl-2 and controls the expression of cyclins and the proto-oncogene c-Myc [135].

Different cytokines also control the inflammatory milieu promoting tumor progression. For example, IL-6 showed a direct effect on cancer cell growth and survival in melanoma [136]. The expression of inflammatory cytokines is induced by different pathways, including MAPK and in particular RAS, which is mutated in approximately 25 % of all malignancies, and has also been reported to be linked with tumor progression locus 2 (Tpl2) which in turn favors ERK phosphorylation and is associated with resistance to the Raf kinase inhibitor PLX4720 [137]. IL-6 is induced by UV radiation in keratinocytes [138] and promotes angiogenesis in human BCC line by inducing b-fibroblast growth factor (bFGF) via PI3-kinase/Akt pathway. Moreover, silencing of COX-2 by

siRNA reduced angiogenic activity of IL-6, suggesting that COX-2 also plays a role in IL-6-induced angiogenesis [139].

COX-2 has also a key role in keratinocyte differentiation and, chronic exposure to UV radiations, induces its over-expression that, together with the accumulation of DNA damage and mutations, causes malignant changes in epidermal keratinocytes and skin cancers [140]. Moreover, COX-2 up-regulation is also induced by different molecules such as TGFβ1 affecting the invasion and the metastatic spread of melanoma cells [141]. In addition, also IL-1α cytokine has been reported to induce the expression of COX-2 [142]. In fact, over-expression of the antagonist of IL-1α in mouse skin carcinoma cell line results in down-regulation of COX-2 expression and slower in vitro and in vivo growth [140]. The important role of COX-2 in tumor promotion has been also demonstrated in vivo by using transgenic mouse model, wherein COX-2 over-expressing transgenic mice are highly susceptible to develop spontaneous skin tumor [143], while COX-2 knockout mice are less inclined to induce tumor development [144].

Increase in COX-2 causes a consequent increase in the level of PGs, which are up-regulated in various premalignant and malignant tissues and are functionally related to mouse skin tumor promotion [145]. PGE2, which is engaged in normal skin homeostasis, has been shown to be a key player mediating the involvement of the COX-2 pathway to cancer development. Elevated levels of PGE2 were observed in

melanoma and have been correlated to cancer cell invasion and migration [146]. Several studies have shown increased expression of EP1 receptor in murine skin tumor cells and that this receptor is decisive for the mitogenic effects of PGE2 on these cells in vitro [147]. Moreover, even topical application of PGD2 dehydration product, 15dPGJ2, has been shown to potentiate mouse skin tumorigenesis [148].

Taken together, the relation between inflammation and tumor development and progression has been sustained supported by an extensive number of clinical studies in particular regarding the treatment with both COX-2 selective or not selective NSAIDs. However, these studies do not consent any acumen in the cellular and molecular mechanisms that are at the basis of the relation between tumor progression and inflammation in melanoma and NMSC.

CHAPTER 2: AIM

Inflammation has emerged as a major factor promoting cancer development. In the current literature there is an increasing interest for the role played by COX- 1 and COX-2, the key rate-limiting enzymes involved in regulation of PGE2 synthesis. In particular, the COX-2 isoform has been shown to be constitutively expressed in various cancers, predominantly by stromal cells [50]. In melanoma COX-2 expression has been detected in human specimens and murine models [149, 150] and has been proposed to be correlated with the development and progression of disease [151-153]. More recently this concept has been reinforced by the finding that increased levels of PGE2 are associated with enhanced cancer cell survival, growth, invasion, angiogenesis and immunesuppression [50]. In fact it is recognized that the consumption of certain anti-inflammatory drugs, including aspirin, can significantly reduce cancer risk, suggesting that common NSAID and more specific COX-2 inhibitors can be used in cancer prevention [154]. In addition, recent evidences demonstrated that microRNAs (miRNAs), that play a critical role in the post-transcriptional regulation of gene expression and regulate many aspects of tumor progression [155, 156] have a huge impact as important cancer prevention genes. In particular, several miRNAs have been demonstrated to be important direct regulators of COX-2 gene expression in cancer or non cancer cells [157].

Starting from this evidence, the aim of my PhD project was to investigate the role of the cyclooxygenase pathway in human melanoma development and progression.

To address this issue we:

1) Evaluated the correlation between COX-2 expression and disease progression in human melanoma.

2) Evaluated the role played by COX inhibitors in preventing melanoma progression.

3) Evaluated the effect of COX-2 deletion in the tumor cells rather than in the tumor microenvironment by using CRISPR/Cas9 technology and investigated the role of miR-143-3p in human malignant melanoma.

CHAPTER 3: MATERIALS AND METHODS

3.1 IN VITRO EXPERIMENTS

3.1.1 Patients and specimens

The retrospective study samples consisted of 45 metastatic lymph node samples obtained from melanoma patients who underwent surgical resection from September 2001 to January 2009 in Istituto Nazionale per lo Studio e la Cura dei Tumori “Fondazione G. Pascale”, Naples (Italy) and were enrolled in a specific clinical protocol where all the 45 patients were diagnosed with lymph node metastases after the first surgery (“in progress disease”). PFS was selected as primary outcome. The melanomas were divided according to the AJCC TNM classification for melanoma staging into four groups pT1 (n = 6 melanomas), pT2 (n = 12 melanomas), pT3 (n = 1 5 melanomas) and pT4 (n = 12 melanomas). The number of patients in the different sub-groups were: for levels of COX-2 expression <=9% (n=23); for levels of COX- 2 expression >10% (n=22).

3.1.2 Tissue micro-array

Tissue micro-array (TMA) was built using the two representative areas from each single case. All tumours areas were selected by two experienced pathologists. Finally, two tissue cylinders (diameter 1 mm) were punched from morphologically representative tissue areas of each donor tissue block and brought into one recipient paraffin block using a

semi-automated tissue arrayer (Galileo TMA CK3500, Integrated System Engineering srl, Milan, Italy).

3.1.3 Immunohistochemistry analysis

Immunohistochemical staining was carried out on TMA 4-µm section to evaluate the expression of COX-2 marker. Briefly, paraffin slides were deparaffinized in xylene and then rehydrated through alcohol gradient. Antigen retrieval was performed by decloaking chamber™ (Biocr Medical) in 0.01 M citrate buffer for 10 min. After peroxidase and protein block (BSA 5% in 1X PBS), the slides were incubated with primary antibody to human COX-2 (D5H5 XP® Cell Signaling). Antigen expression was evaluated independently and blindly by two experienced pathologists using light microscopy. The percentage of cancer cells with cytoplasmic staining was determined by counting the number of positive cells as a fraction of the total number of cancer cells in tissue cores at ×400 magnification as follow:

$$\%\ \text{cancer cells with cytoplasmic COX} - 2\ \text{staining} = \frac{\text{positive cells}}{\text{total n° cancer cells.}}$$

The median value of positive expression (9%) was used as the cut-off point for statistical analyses to distinguish tumours with negative or low COX-2 expression (≤9%; COX-2^{low}) from tumours with high COX-2 expression (≥10%; COX-2^{high}).

3.1.4 Cell culture and reagents

Normal Human Epidermal Melanocytes (NHEM) were purchased from Lonza (Walkersville, MD, USA) and were grown in Melanocyte growth medium 2 (Lonza). The melanoma cell lines B16/F10, Sk-Mel-5 and Sk-Mel-28 were purchased from IRCCS AOU San Martino – IST (Genova, Italy), A375 from Sigma-Aldrich (Milan, Italy) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 μmol/L non essential amino acids, penicillin (100 U/mL), streptomycin (100 μg/mL) and 1 mmol/L sodium pyruvate (all from Sigma-Aldrich, Milan, Italy). WM35, WM983A and WM983B were from Rockland (Limerick, Ireland) and were cultured in Tumor Specialized Media (1:5 Leibovitz's – MCDB153), containing 2% inactivated FBS and 1,68 mM CaCl2. The cell line PES43 was isolated from a lung metastases of a patient from the National Cancer Institute, G. Pascale Foundation and cultured in Iscove's modified Dulbecco's medium (Cambrex Bioscience, Verviers, Belgium) supplemented with heat-inactivated 10% FBS, penicillin, and streptomycin (100 units/mL each). Cells were grown at 37°C in a humidified incubator under 5% CO_2. All cell lines used in this study were characterized by the cell bank were they were purchased. Celecoxib (Selleck Chemicals, Munich, Germany) and naproxen (Sigma-Aldrich, USA) were solubilized in H_2O. ATB-346 and 4-hydroxy-thiobenzamide (TBZ) were synthesized by Antibe Therapeutics Inc. (Toronto, ON, Canada) and were solubilized in DMSO.

3.1.5 RNA purification and quantitative real-time PCR (qPCR)

Total RNA was isolated from cells by use of the TRI-Reagent (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions, followed by spectrophotometric quantization. Final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥ 1.7. Isolated mRNA was reverse-transcribed by use of iScript Reverse Transcription Supermix for RTqPCR (Bio-Rad, Milan, Italy). The quantitative real-time PCR was carried out in CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy) with specific primers (hCOX-1 5'-AAGGTGGCATTGACAAACTCC-3', 5'-CG CCAGTGATCCCTGTTGTT-3'; hCOX-2 5'-TAAGTGC GATTGTACCCGGAC-3', 5'-TTTGTAGCCATAGTCAGCATTGT-3'; mCOX-2 5'-TACCCTCCTCACATCCCTGA -3', 5'-CCTGCTTGAGTATGTCGCAC-3') by the use of SYBR Green master mix kit (Bio- Rad, Milan, Italy). Samples were amplified simultaneously in triplicate in one-assay run with a non-template control blank for each primer pair to control for contamination or primer dimer formation, and the ct value for each experimental group was determined. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the ct values, using the $2^{-\Delta Ct}$ formula.

For the miR-143-3p study, total RNA was isolated from 20-µm sections from formalin-fixed, paraffin-embedded tissue blocks according to the protocol of miRNeasy FFPE Kit (Qiagen, Valencia, CA). Total RNA was

reversely transcribed using miScript II RT Kit (Qiagen, Valencia, CA). Quantitative real-time PCR was carried out in CFX384, as described above, by the use of miScript SYBR Green PCR Kit (Qiagen). The U6 expression level was used as an internal control miRNAs.

3.1.6 Proliferation assay

Cell proliferation was measured by the 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). Briefly, the human melanoma cells and the NHEM cells were seeded on 96-well plates (2 x 10^3 cells/well) and treated with celecoxib (10-100 µM) or naproxen (10-100 µM) for 24-48-72 h before adding 25 µL of MTT (Sigma, Milan, Italy) (5 mg/mL in saline). Cells were thus incubated for an additional 3 h at 37°C. After this time interval, cells were lysed, and dark blue crystals were solubilized with a solution containing 50% N,N-dimethyl formamide and 20% sodium dodecylsulfate with an adjusted pH of 4.5. The optical density of each well was measured with a microplate spectrophotometer (TitertekMultiskan MCC/340), equipped with a 620 nm filter.

For the pharmacological modulation study with ATB-346, cells were treated with ATB-346 (10–100 µM), TBZ (10–100 µM) or naproxen (10–100 µM) for 24–48–72 h, then we followed the same method described above.

For the CRISPR/Cas9 study, B16/F10, B16/PTGS2Δ and B16/Scramble cells were seeded on 96-well plates (2 x 10^3 cells/well) and then we

evaluated the growth rate at 24-48 and 72 h by following the same method described above.

For the miR-143-3p study, the human melanoma cells A375 were seeded on 96-well plates (3×10^3 cells/well) and the next day transfected with miR-143-3p mimics, scramble or pCMV6-AC-PTGS2. MTT was added to the cells at 24-48-72 h post transfection, then we followed the same method described above.

3.1.7 COX-2 small interfering RNA transfection of SK-Mel-5

For the silencing experiments SK-Mel-5 were seeded onto 96-well plates (2×10^3 cell/well) and transfected the next day, according to the manufacturer's instruction, with PTGS2 Trilencer-27 Human siRNA (OriGene, Rockville, MD, USA) (rCrCrArArUrUrGrUrC rArUrArCrGrArCrUrUrGrCrArGrUGA;rGrGrCrUrArArUrArCrUrGrArUrArGr GrArGrArGrArCrUAT;rGrCrArGrCrUrUrCrCrUrGrArUrUrCrArArArUrGrArG rATT). The final concentration of the siRNA pool was 10 nM. Forty-eight hours after transfection, cell proliferation was evaluated by MTT assay (see Proliferation assays). The knockdown of COX-2 expression in cells after transfection was confirmed using western blot analysis. The Universal scrambled negative control siRNA duplex was used as negative control.

3.1.8 Preparation of cell lysates and western blot analysis

Melanoma cells were harvested, washed with cold phosphate-buffered saline and lysed with ice-cold lysis buffer supplemented with protease inhibitors. Equal amounts of proteins were resolved on 10% Tris–Glycine gels and transferred onto a nitrocellulose membrane. After blocking the nonspecific binding sites, the membrane was incubated with the primary antibody (COX-2; cod: 12282; batch 2; diluited 1:1000, Cell signaling, MA, USA) at 4°C overnight. The membrane was then incubated with the appropriate peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence reagents. To verify equal protein loading, the membrane was stripped and reprobed with anti-b actin antibody.

For the pharmacological modulation study with ATB-346, A375 cells were treated with ATB-346, TBZ and naproxen, 100 μM for 6 and 24 h. Whole-cell or nuclear extracts were prepared as described above, then the filters were incubated with the following primary antibodies: IκBα (sc-1643 Cruz Biotech-nology, Santa Cruz, CA; diluted 1:200); Bcl-2 (2876, Cell Signaling,USA; diluited 1:1000), caspase 3 (9662, Cell Signaling, USA; diluited1:1000), PARP (9542, Cell Signaling, USA; diluited 1:1000), Akt (9272, Cell Signaling, USA; diluited 1:1000), Phospho-Akt (Ser473)XP (4060, Cell Signaling, USA; diluited1:2000), XIAP (R&D Sys-tem, Minneapolis; 1 μg/mL); NF-κB p65 (F-6): (sc-8008 Santa

CruzBiotechnology, Santa Cruz, CA; diluted 1:200); β-actin (Santa CruzBiotechnology.

For the CRISPR/Cas9 study, B16/F10, B16/PTGS2Δ and B16/Scramble whole-cell extracts were prepared as described above, then the filters were incubated with the primary antibody (COX-2; cod: 12282; batch 2; diluited 1:1000, Cell signaling, MA, USA) at 4°C overnight. To verify equal protein loading, the membrane was stripped and reprobed with anti-GAPDH antibody.

For the miR-143-3p study, whole-cell extracts were prepared as described above, then the filters were incubated with the primary antibody (COX-2; cod: 12282; batch 2; diluited 1:1000, Cell signaling, MA, USA). To verify equal protein loading, the membrane was stripped and reprobed with anti-GAPDH antibody.

3.1.9 Cell invasion assay

The assay was performed using chambers with polycarbonate filters with 8-µm nominal pore size (Millipore, USA) coated on the upper side with Matrigel (Becton Dickinson Labware, USA). The chambers were placed into a 24-well plate. Two groups of melanoma cells ($2.5x10^5$/mL) were harvested and placed in the upper chamber in serum-free DMEM: SK-Mel-5 CTL and siRNA COX-2 transfected SKMel- 5. The bottom chamber contained DMEM with 10% FBS. After the incubation period (16h), the filter was removed, and non-invaded cells on the upper side of the filter were detached with the use of a cotton swab. Filters were

fixed with 4% formaldehyde for 15 min, and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and then washed with PBS. The filters were examined microscopically and cellular invasion was determined by counting the number of stained cells on each filter in at least 4–5 randomly selected fields. Resultant data are presented as a mean of invaded cells ± SD/microscopic field of three independent experiments.

For the CRISPR/Cas9 study, B16/F10, B16/PTGS2Δ and B16/Scramble (2.5×10^5/mL) were plated in the upper chamber in serum-free DMEM, then we followed the same method described above.

For the miR-143-3p study, A375 cells (2.5×10^5) transfected with miR-143-

3p mimics or scramble, were plated to the upper chamber in serum-free DMEM, then we followed the same method described above.

3.1.10 Annexin V-FITC/PI Flow Cytometry

Annexin V and Propidium Iodide were used for the pharmacological modulation study with ATB-346. A375 cells were seeded in 35 mm culture dishes and allowed to attach overnight. The cells were treated with ATB-346 (100 µM), TBZ (100 µM) or naproxen (100 µM) for 24–48, collected and washed twice with PBS. Samples were then taken to determine baseline and drug-induced apoptosis by Annexin V-FITC/Propidium Iodide(PI) (Beckman Coulter; Brea, CA) double staining or PI staining and flow cytometry analysis using a FACS Canto II 6-colour

flow cytometer (Becton Dickinson Biosciences, San Jose, CA). To detect early and late apoptosis, both adherent and floating cells were harvested together and resuspended in annexin V binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a concentration of 10^6 cells/mL. Subsequently, 5 µL of FITC-conjugated Annexin V and 5 µL of PI were added to 100 µL of the cell suspension (10^5 cells). The cells were incubated for 15 min at room temperature in the dark. Finally, 400 µL of annexin V binding buffer was added to each tube. A minimum of 50,000 events for each sample were collected and data were analyzed using FacsDivasoftware (Becton Dickinson Biosciences).

3.1.11 Elisa

CXCL1/KC plasma concentrations were evaluated for both pharmacological modulation study with ATB-346 and CRISPR/Cas9 study by using ELISA kit according to the manufacturer's instruction (DuoSet ELISA, R&Dsystems, Minneapolis, MN, USA).

3.1.12 Knockdown of PTGS2 with CRISPR-Cas9

A CRISPR knock-down kit against mouse PTGS2 was purchased from OriGene (USA, Cat# KN314183). Transfections were performed as recommended by the manufacturer. Briefly, 3×10^5 B16/F10 cells were seeded into 6 well plates and maintained for 24 hours. TransIT-X2 (Mirus, Cat# MIR6003) was used at a final concentration of 2.4%

together with a total of 2 µg plasmid (1 µg gRNA or Scrambled control with 1 µg donor) per well. TransIT-DNA complexes were made up in serum-free growth medium. Cells were maintained for 48 hours before cells were returned to growth medium. Transfected cells were sub-cultured 7 times before puromycin selection (1 µg/ml, SantaCruz, Cat#sc-108071). Selected cells (puromycin resistant) were screened for expression of PTGS2 by quantitative real-time PCR (qPCR) and Western blot analysis.

3.1.13 Wound healing assay

Wound healing assay was used for the CRISPR/Cas9 study. B16/F10, B16/PTGS2Δ and B16/Scramble were seeded in 12-well plates (2×10^5 cells/well). Once the cells reached 90% confluency, a wound area was carefully created by scraping the cell monolayer with a sterile 200 µl pipette tip. After being washed three times with PBS, scratches including the flanking front lines of cells, were photographed (20-fold magnification). Subsequently, the cells were incubated at 37°C in 5% CO2. The width of the wound area was monitored with an inverted microscope at various time points. Wound closing was compared between B16/PTGS2Δ cells and B16/Scramble cells after measuring the wound area and evaluated using Image J software (LASV3.8, Germany). Differences between the data points were determined by Student's t test where $p < 0.05$ was considered significant. Experiments were

performed independently two times, evaluating 4 – 8 scratches in each experiment.

3.1.14 Clonogenic assay

For the CRISPR/Cas9 study, B16/F10, B16/PTGS2Δ and B16/Scramble ($1x10^3$ cells/well) were seeded in 6-well plates. The medium was changed every 2 days. Cells cultured for 14 days were washed twice with 1X PBS, fixed by 4% paraformaldehyde, and stained with 0.5% crystal violet and colonies containing more than 50 cells (established by microscopy) were counted manually. Images of the colonies were obtained using a digital camera. The experiments were done in duplicate at least three times.

For the miR-143-3p study, miR-143-3p mimics or control (scramble) – transfected A375 cells were seeded onto a 6-well plate ($1×10^3$ cells/well) and allowed to grow for two weeks, then we followed the same method described above.

3.1.15 miR-143-3p mimics transfection

miR-143-3p mimics and negative control (scramble) were purchased from Qiagen, (Valencia, CA), pCMV6-AC-PTGS2 was purchased from Origene Technologies Inc. (Rockville, MD, USA). The human melanoma cells A375 were transfected with miR-143-3p mimics (25 nM), scramble or pCMV6-AC-PTGS2 using TransIT Transfection Reagent (Mirus Bio LLC, Madison, WI USA) according to the manufacturer's instruction.

3.1.16 Apoptosis assay

Apoptosis assay was used for the miR-143-3p study. Human melanoma cells A375 were seeded onto 96-well plates (3×10^3 cells/well) and transfected with miR-143-3p mimics (25 nM) and control (scramble) sequences. Apoptosis assay was performed 48 h post transfection using the ApoTox-Glo™ Triplex Assay according to the manufacturer's instructions (Promega, Madison, WI).

3.1.17 Cell migration assay

Migration assay was used for the miR-143-3p study. Cell migration was performed by use of a transwell chamber (8 µm, 24-well insert; Corning, Lowell, MA, USA). A375 cells (2.5×10^5) transfected with miR-143- 3p mimics or scramble, in serum-free medium were added to the upper chamber and medium containing 10% FBS was added to the lower chamber. Cells were then incubated for 18 h. Finally, cells that migrated into the lower chambers were fixed with methanol, stained with crystal violet, and counted in six random fields using ImageJ (MD, USA).

3.1.18 miRNA target prediction

The prediction of putative miRNA target sites on COX-2 mRNA was performed using three commonly used free softwares: TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org/) and PicTar (http://pictar.mdc-berlin.de/).

3.1.19 Luciferase reporter activity assay

The pMirTarget-PTGS2-3'-UTR luciferase construct was obtained from Origene Technologies Inc. (Rockville, MD, USA). The A375 cells were transfected with 100 ng of pMirTarget-PTGS2-3'-UTR and miR-143-3p mimics (25 nM) or scramble using TransIT Transfection Reagent (Mirus Bio LLC, Madison, WI) according to the manufacturer's instruction. After 48 h, the cells were harvested and assayed using the Dual-Glo Luciferase Assay (Promega, Madison, WI).

3.1.20 Prostaglandin E_2 assay

PGE2 concentrations was evaluated in cell culture supernatants obtained from miR-143-3p mimics or scramble transfected A375 cells. Prostaglandin E2 EIA kit (Cayman Chemicals, Ann Arbor, MI)) was used according to the manufacturer's instruction.

3.2 *IN VIVO* EXPERIMENTS

3.2.1 Animals

The experimental procedures, according to Italian (DL 26/2014) and European (n. 63/2010/UE) regulations on the protection of animals used for experimental and other scientific purposes, were approved by the Italian Ministry. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [158]. Mice were observed daily and humanely euthanized by CO_2 inhalation if a solitary subcutaneous tumor exceeded 1.5 cm in diameter or mice showed signs referable to metastatic cancer. All efforts were made to minimize suffering. Male or female C57BL/6 mice (18−20 g) were purchased from Charles River Laboratories, Inc. Male COX-$2^{-/-}$ mice, kindly supplied by Dr Jane A. Mitchell, back-crossed for >7 generations onto a C57Bl/6J background were used at 10 to 12 weeks of age. Animals were genotyped before use [159]. Mice were housed at the Animal Research Facility of the Department of Pharmacy of the University of Naples Federico II.

3.2.2 Induction of subcutaneous melanoma

Mice were subcutaneously (s.c.) injected in the right flank with B16-F10 murine melanoma cells (1×10^5/0.1ml). Tumour size was measured using a digital caliper, and tumour volume was calculated using the following

equation: tumour volume = π/6(D1xD2xD3) where D1=length; D2=width; D3= height and expressed as cm^3.

For the pharmacological modulation study with ATB-346, groups of mice (n = 8 each) were treated orally, twice daily beginning on day 1, with equimolar doses (43 μmol/kg) of ATB-346, naproxen or TBZ. The control group was treated with an equal volume of vehicle. All tested drugs were suspended in dimethylsulfoxide/1% carboxymethylcellulose (5:95 ratio) then we evaluated tumor volume as described above.

For the CRISPR/Cas9 study, mice were s.c. injected with B16/F10, B16/PTGS2Δ and B16/Scramble cells (1×10^5/0.1 ml) then we evaluated tumor volume as described above.

3.2.3 Statistical analysis

To analyze the correlation between COX-2 expression and BRAF or NRAS mutation the Mann-Whitney non-parametric test was used. Progression free survival (PFS) curve was calculated with the Kaplan- Meier method and analyzed with the log-rank test. P values less than 0.05 were considered to be statistically significant. Data from both in vitro and in vivo experiments are reported as the mean ± SEM. Data were analyzed using GraphPad Prism software (GraphPad). Significance was determined using a Student's two-tailed t test. Results were considered significant at P value less than 0.05 and are labeled with a single asterisk. In addition, P values less than 0.01 and 0.001 are designated with double and triple asterisks, respectively.

CHAPTER 4: RESULTS

4.1 DIFFERENTIAL EXPRESSION OF CYCLOOXYGENASE-2 IN METASTATIC MELANOMA AFFECTS PROGRESSION FREE SURVIVAL

4.1.1 Expression of COX-1 and COX-2 in human melanoma cell lines

In order to gain further insights into the role of COXs in human melanoma we decided to evaluate the expression levels of both COX-1 and COX-2 genes in normal human epidermal melanocytes (NHEM) and in different melanoma cell lines namely A375, SK-MEL-5, SK-MEL-28, WM35, WM983A, WM983B by using quantitative realt-time pcr analysis (qPCR). All cell lines showed an increased expression of COX-1 and COX-2 as compared to NHEM. Indeed, COX-1 or COX-2 expression was always as minimum triplicated in all cell lines examined. What is of particular interest is the finding that the expression level of both COX-1 and COX-2 appears to be reciprocal compensate within the melanoma cell lines (Figure 4.1.1A, 4.1.1B). Indeed, by looking at the Figure 4.1.1A and 4.1.1B it appears that within each single cell line analyzed the ratio between the two isoforms is always about 1:2. The highest level of COX-2 expression was exhibited by SK-Mel-5 and thus this cell line was selected for the silencing experiments.

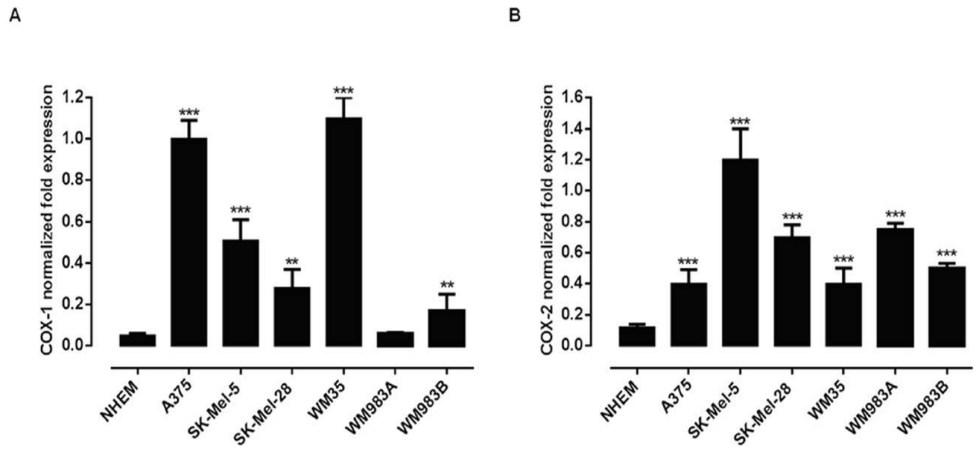

Figure 4.1.1: Expression of COX-1 and COX-2 in human melanoma cell lines.

A. quantitative real-time PCR (qPCR) analysis to evaluate the expression levels of both COX-1 A. and COX-2 B. genes was performed on normal human epidermal melanocytes (NHEM) and on the melanoma cell lines A375, Sk-Mel-5, Sk-Mel-28, WM35, WM983A and WM983B. All human melanoma cell lines expressed both enzymes but with different level of expression. **P<0.01; ***P<0.001 vs NHEM. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the ct values.

4.1.2 Selective inhibition of COX-2 activity and expression reduces human melanoma cell proliferation and invasiveness

To investigate on the effect of COX-2 inhibition on melanoma cell proliferation we chose celecoxib, a selective COX-2 inhibitor, and compared the effect versus naproxen, a non selective COX inhibitor. Usually, concentrations of celecoxib required to induce apoptosis of cultured cells range from 25–100 μmol/L, thus we selected these concentrations to run our proliferation assays. As shown in Table 4.1.1 celecoxib, but not naproxen, inhibited the growth of all cell lines tested in a time- and concentration-dependent manner. Celecoxib and naproxen did not inhibit proliferation of NHEM (data not shown). To further address the role of COX-2 versus COX-1 we transfected SK-Mel-5 cells with siRNA for COX-2. The knockdown of COX-2 expression in cells after silencing was confirmed by western blot analysis (Figure 4.1.2A). As expected, COX-2 silencing significantly reduced cell invasiveness as compared to control (Figure 4.1.2B-4.1.2C).

(A) 24h			
Cell line	**CTL**	**celecoxib 100µM**	**naproxen 100µM**
Sk-Mel28	0.295±0.002	0.155±0.005***	0.293±0.01
Sk-Mel5	0.317±0.009	0.186±0.01**	0.321±0.01
A375	0.331±0.01	0.206±0.009*	0.342±0.02
(B) 48h			
Sk-Mel28	0.503±0.01	0.182±0.01***	0.509±0.01
Sk-Mel5	0.504±0.03	0.421±0.01**	0.691±0.04
A375	0.569±0.01	0.458±0.005	0.621±0.02
(C) 72h			
Sk-Mel28	0.798±0.02	0.235±0.01***	0.781±0.005
Sk-Mel5	0.660±0.02	0.413±0.02**	0.686±0.08
A375	0.779±0.03	0.478±0.001**	0.714±0.03

Table 4.1.1: Effect of celecoxib and naproxen on A375, Sk-Mel-5 and Sk-Mel-28 melanoma cell proliferation

Growth inhibition was measured using the MTT assay and is expressed as O.D. values at 24-48-72h. Celecoxib, but not naproxen, inhibited the growth of all melanoma cells at all times considered. Control (CTL). Experiments were run in triplicate. (**P<0.01; ***P<0.001 vs CTL).

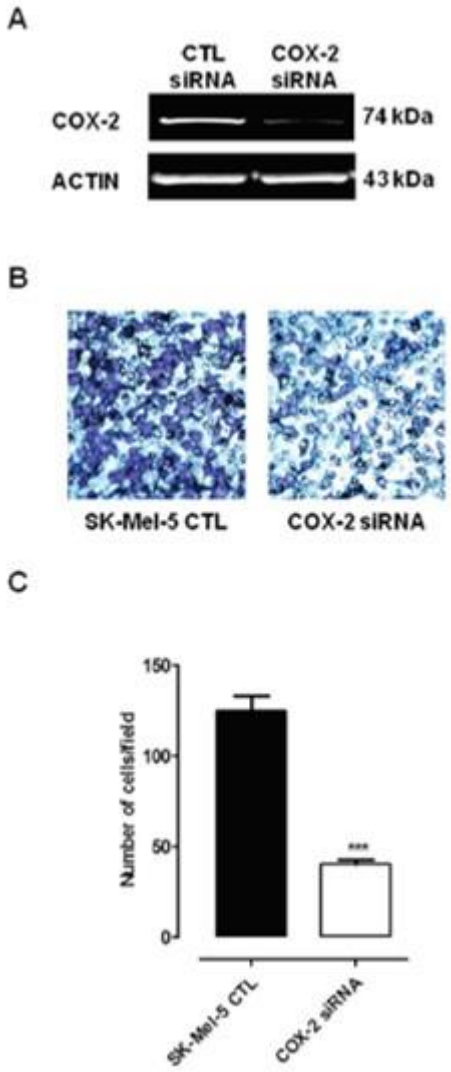

Figure 4.1.2: Silencing of COX-2 gene in Sk-Mel-5 cells significantly decreases melanoma cell invasiveness.

Sk-Mel-5 cells were transfected with COX-2 siRNA to knockdown COX-2 expression. A. transfection of Sk-Mel-5 cells by COX-2 siRNA resulted in marked reduction in the levels of COX-2 protein in cells as confirmed by western blot analysis. A significant reduction of cell invasiveness B, C. versus control siRNA-treated cells was observed. Each column is the mean ± SEM of three independent experiments, each performed in quadruplicate $P<0.001$ vs CTL.

4.1.3 B16-F10 murine cell induced melanoma is blunted in COX-2$^{-/-}$ mice

To investigate the role of COX-2 also in melanoma development, we performed a reverse translational approach using COX-2$^{-/-}$ mice. Toward this aim, we used the most widely acknowledged experimental model to study melanoma development in vivo [160]. The tumour was implanted by subcutaneous injection of B16-F10 murine cells in the right flank of COX-2$^{-/-}$ mice and littermate controls C57Bl/6J. Tumour development in COX-2$^{-/-}$ mice was reduced in volume by 91% and in wet weight by 87% ($P<0,001$; n=10) (Figure 4.1.3A-C).

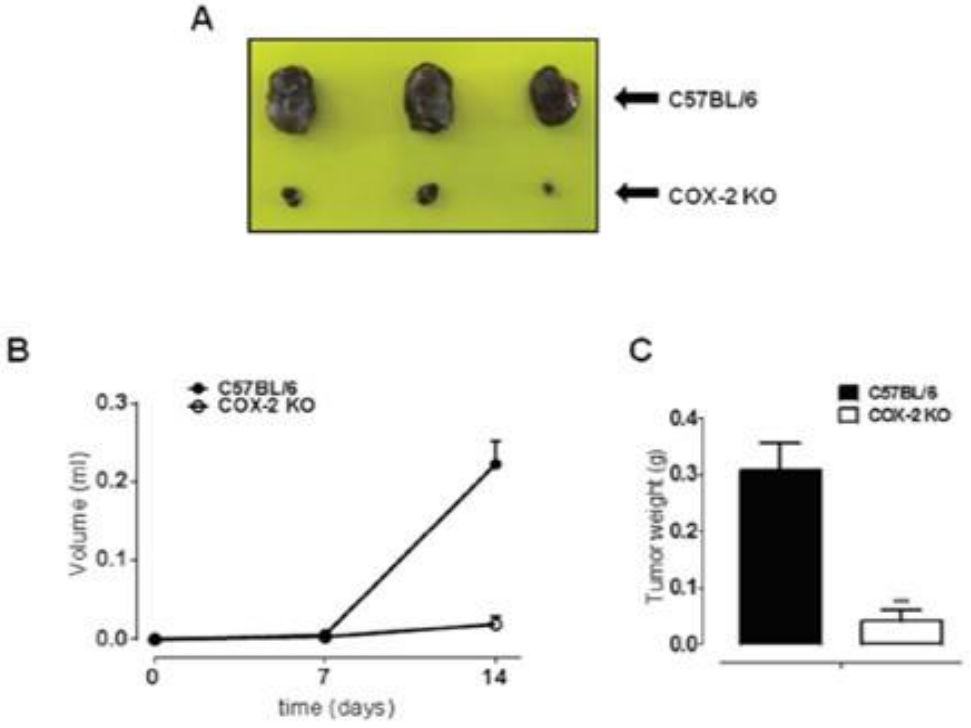

Figure 4.1.3: COX-2 is essential for melanoma development in mice.

B16-F10 murine cells were subcutaneously injected into the right flank of COX-$2^{-/-}$ mice (n=10). C57Bl/6J mice were used as littermate control (n=10). A. representative image of tumour sizes. B. tumour development expressed as tumour volume. A marked and significant reduction in tumour volume by 91% was observed in COX-$2^{-/-}$ mice (○) ($P < 0.001$) as compared to littermate mice (•). C. tumour weight is significant reduced by 87% ($P<0.001$) in COX-$2^{-/-}$ mice (open square) as compared to littermate control (filled square).

4.1.4 Human melanoma samples

The histological samples analysed were obtained from 45 lymph node melanoma metastases from the Biobank of the National Cancer Institute G. Pascale. The median age of patients was 50 years, female patients represented 44% (20/45). Primary tumour (pT) grade was distributed as follows: grade 1, 13.3% (6/45); grade 2, 26.7% (12/45); grade 3, 33.3% (15/45); grade 4, 26.7% (12/45). Ulceration was present in 35.6% (16/45) of the samples analyzed (Figure 4.1.4A).

4.1.5 Higher COX-2 expression percentage in lymph node metastases correlates with negative progression free survival (PFS) outcome

COX-2 expression was evaluated in all 45 samples. COX-2 immunoreactivity was detected in 23 out of 45 (51%) lymph node metastases samples. A representative image of COX-2 negative staining is reported in Figure 4.1.4 (panel B, left) vs the positive staining (panel B, right). In order to verify if the percent of expression of COX-2 does play a role in melanoma malignancy evaluated as PFS, we compared negative samples vs samples with low COX-2 expression (COX-2^{low}) set as cut off at up to ≤9%. As it can be seen in Figure 4.1.4 panel C, plotting samples with null COX-2 expression (blue line) vs COX-2^{low} expression (≤9%; green line) there was no significant trend in PFS reduction.

Next, positive samples were separated into two new sub-groups, one where COX-2 expression was ≥10%, defined as COX-2^{high} expression (green line), and a second where COX-2 expression was ≤9%, defined as COX-2^{low} (blue line). When we plotted the data we found that the COX-2^{high} expression group showed a striking negative correlation with PFS. Indeed, patient with COX-2^{high} had a reduction in PFS of 35 months (almost 3 years).

4.1.6 BRAF and NRAS mutational status does not correlate with COX-2 expression in lymph node metastases

In order to verify if melanoma most frequent mutations could influence the data outcome we characterized the $NRAS^{Q61}$ and $BRAF^{V600E}$ mutations. $NRAS^{Q61}$ was present in 8.9% of patients (n = 4/45), while activating $BRAF^{V600E}$ mutations, as expected, was more represented and found in 64.4% of patients (n = 29/45). Interestingly COX-2 expression did not correlate with $BRAF^{V600E}$ (p=0,768) or with $NRAS^{Q61}$ (p=0,934) mutational status (Figure 4.1.4 E-F).

A

		Tot	COX2_Median_Expression (-)	(+)	P value
Age	<= 50	23(51,1)	12(26,7)	11(24,4)	0,768
	51+	22(49,9)	10(22,2)	12(26,7)	
Gender	M	25(55,6)	13(28,9)	12(26,7)	0,767
	F	20(44,6)	9(20)	11(24,4)	
pT	1,00	6(13,3)	3(6,7)	3(6,7)	0,212
	2,00	12(26,7)	6(13,3)	6(13,3)	
	3,00	15(33,3)	10(22,2)	511,1)	
	4,00	12(26,7)	3(6,7)	9(20)	
Ulceration	N	29(64,4)	15(33,3)	14(31,1)	0,758
	Y	16(35,6)	7(15,6)	9(20)	

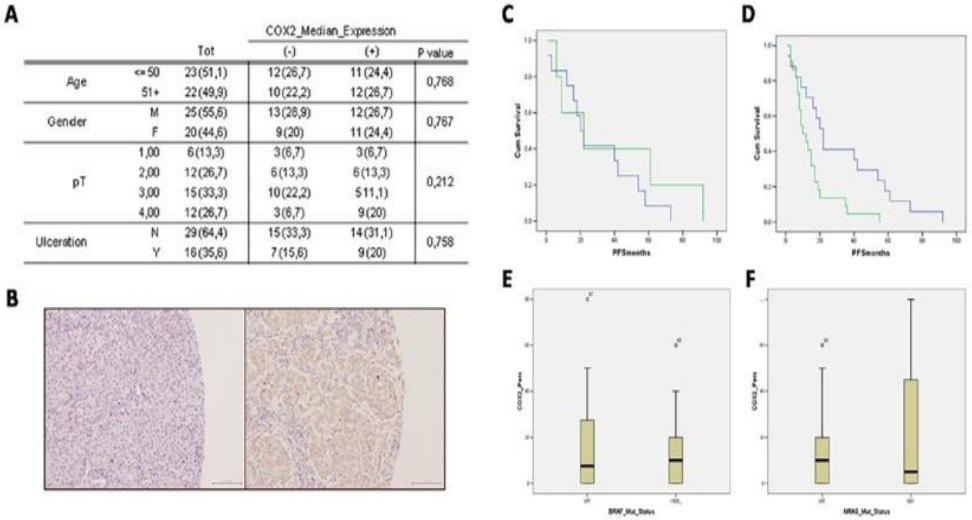

Figure 4.14: Clinical-pathological characteristics of melanoma patients and tumours and relation to COX-2 expression.

Frequencies are indicated in absolute values (percentage). COX-2 median expression values, where indicated with (-), include negative samples and samples with low COX-2 expression (≤9%); where indicated a (+) include samples with high COX-2 expression (≥10%). Abbreviations: pT (primary tumour), M (male), F (female), Y (present), N (not present). B-D. Immunohistochemistry staining of COX-2 and Kaplan–Meier progression free survival curve stratified by median of COX-2 expression. B. Immunohistochemistry staining of COX-2: left panel COX-2 negative expression; right panel COX-2 positive expression. Images are 20x. C. Kaplan–Meier progression free survival (PFS) curve of cases under the median of COX-2 expression stratified by not expressed (0%; n=12; blue line) and low COX- 2 expression (≤9%; n=5 green line) shows a not significant trend in PFS reduction. D. Kaplan–Meier PFS curve of cases stratified by low COX-2 expression (≤9%; n= 17; blue line), and

high COX-2 expression (≥10%; n=22; green line). Kaplan–Meier curve illustrate a significant correlation between COX-2 high expression and PFS. E-F. COX-2 expression and $BRAF^{V600E}$ and $NRAS^{Q61}$ mutational status. (E) Absence of correlation between COX-2 expression and $BRAF^{V600E}$ mutational status. (F) Absence of correlation between COX-2 expression and $NRAS^{Q61}$ mutational status. Data are expressed as BOX-plot distribution.

4.2 ATB-346, A NOVEL HYDROGEN SULFIDE-RELEASING ANTINFLAMMATORY DRUG, INDUCES APOPTOSIS OF HUMAN MELANOMA CELLS AND INHIBITS MELANOMA DEVELOPMENT IN VIVO

4.2.1 ATB-346 inhibits human melanoma cell proliferation

In the last few years, numerous physiological and pathophysiological roles have been proposed for the gasotransmitter hydrogen sulfide (H_2S), along with a plethora of cellular and molecular targets [161, 162]. Recent studies indicate that H_2S is also involved in the regulation of cancer biological processes and both pro- and anti-cancer effects have been described for this molecule [163-165]. Moreover, we have shown that the metabolic pathway leading to H_2S endogenous production is involved in human melanoma progression [163]. An interesting class of new compounds has been developed in the last few years combining traditional NSAIDs with a chemical moiety that donates hydrogen sulfide. These molecules have been demonstrated to exhibit few if any damaging effects in the GI tract, despite producing suppression of prostaglandin synthesis and reduction of inflammation at least as effectively as the parent NSAID [166]. In the second phase of the project, we addressed the role played by COXs and H_2S in preventing melanoma progression through a pharmacological modulation approach by using a new H_2S-releasing derivative of naproxen, 2-(6-

methoxynapthalen-2-yl)-propionicacid 4-thiocarbamoyl phenyl ester (ATB-346), that inhibits COXs but also releases H_2S [167].

In order to investigate on the potential anti-proliferative effect of ATB-346 an MTT assay was carried out with ATB-346, TBZ (the H_2S releasing moiety) and naproxen on a panel of human melanoma cells and on NHEM. In preliminary, dose-response experiments, the concentration of 100 μM was selected. As shown in Table 4.2.1, only ATB-346 inhibited the growth of all cell lines tested in a time-dependent manner. Since the A375 cell line resulted to be the most sensitive to the anti-proliferative effect of ATB-346 it was selected as a tool. Incubation of A375 cells with ATB-346 (100 μM) for 24, 48 or 72 h caused an inhibition of cell proliferation by 38.2%, 63.2% and 66%, respectively ($P < 0.001$) (Table 4.2.1). Neither TBZ nor naproxen reduced A375 cell proliferation significantly at any time or concentration tested. None of the drugs inhibited the proliferation of NHEM up to 100 μM (Table 4.2.1).

(A) 24h

Cell line	CTL	ATB 100µM	TBZ 100µM	NAP 100µM
NHEM	0.191±0.004	0.182±0.001	0.176±0.003	0.182±0.002
A375	0.300±0.02	0.185±0.005***	0.316±0.005	0.293±0.01
Sk-Mel-5	0.366±0.009	0.286±0.01**	0.396±0.01	0.431±0.01
Sk-Mel-28	0.351±0.01	0.346±0.009	0.438±0.009	0.442±0.02
PES 43	0.216±0.01	0.200±0.008	0.224±0.02	0.163±0.02

(B) 48h

Cell line	CTL	ATB 100µM	TBZ 100µM	NAP 100µM
NHEM	0.211±0.002	0.212±0.002	0.212±0.003	0.200±0.002
A375	0.503±0.009	0.182±0.01***	0.520±0.01	0.509±0.01
Sk-Mel-5	0.554±0.03	0.421±0.01**	0.587±0.04	0.691±0.04
Sk-Mel-28	0.499±0.01	0.458±0.005	0.617±0.001	0.621±0.02
PES 43	0.319±0.006	0.259±0.05**	0.366±0.01	0.342±0.01

(C) 72h

Cell line	CTL	ATB 100µM	TBZ 100µM	NAP 100µM
NHEM	0.230±0.002	0.219±0.007	0.212±0.006	0.213±0.001
A375	0.810±0.02	0.275±0.01***	0.721±0.03	0.751±0.005
Sk-Mel-5	0.560±0.009	0.403±0.02**	0.627±0.01	0.626±0.08
Sk-Mel-28	0.579±0.03	0.458±0.001**	0.769±0.02	0.814±0.03
PES 43	0.385±0.01	0.234±0.01***	0.531±0.02	0.459±0.03

***P<0.01; ***P<0.001 vs Control (CTL)*

Table 4.2.1: Effect of ATB-346, Naproxen and TBZ on NHEM and on A375, Sk-Mel-5, Sk-Mel-28 and PES43 melanoma cells proliferation.

Growth inhibition was measured using the MTT assay and is expressed as O.D. values at 24–48–72 h. Only ATB-346 inhibited the growth of all melanoma cells at all times that were examined. Experiments were run in triplicate, each performed in quadruplicate (**P < 0.01; ***P < 0.001 vs CTL).

4.2.2 ATB-346 induces apoptosis of human melanoma cells

A375 human melanoma cells were treated with ATB-346, TBZ or naproxen (100 μM for 24 and 48 h) and apoptosis was determined by annexinV/PI staining, which detects the externalization of phosphatidylserine (PS), a characteristic feature of cells entering apoptosis. This dual staining distinguishes between unaffected cells (unlabeled; quadrant 3, Fig. 4.2.1A), early apoptotic cells (annexinV positive; quadrant 4, Fig. 4.2.1A), late apoptotic cells (annexin V positive, PI positive; quadrant 2, Fig. 4.2.1A), and necrotic (PI positive; quadrant 1, Fig. 4.2.1A). As shown in Fig. 4.2.1B only ATB-346 induced apoptosis of A375 cells in a time-dependent manner (18% and 24% at24 and 48 h respectively). The pro-apoptotic effect of ATB-346 was confirmed by the time-dependent cleavage of caspase 3, the main effector caspase, and of its substrate poly (adenosine diphosphate-ribose) polymerase (PARP) (Fig. 4.2.1C).

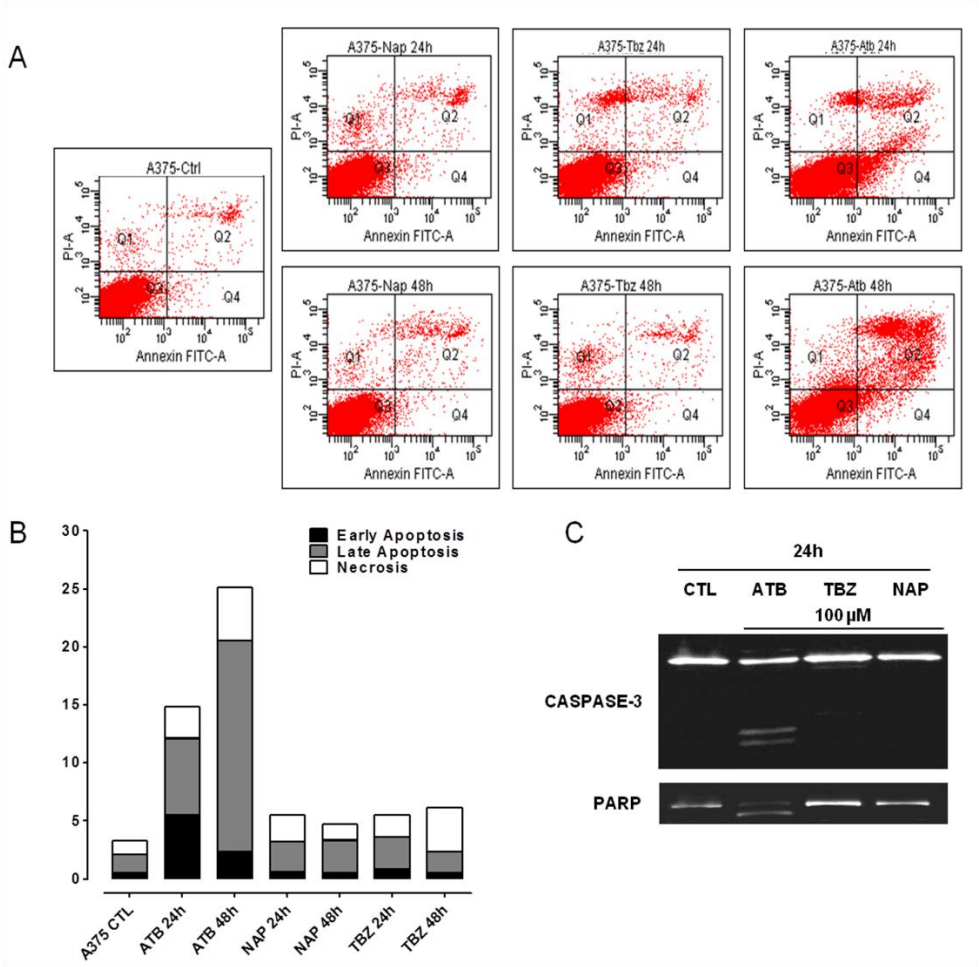

Figure 4.2.1: ATB-346 induces apoptosis of human melanoma cells.

(A) Cells were treated with ATB-346, naproxen or TBZ (100 µM) at different time points and apoptosis was determined by flow cytometry analysis. Apoptosis was determined by annexin V/propidium iodide (PI) staining, which detects the externalization of phosphatidylserine (PS). This dual staining distinguishes between unaffected cells (unlabeled; quadrant 3, Q3), early apoptotic cells (annexin V positive; quadrant 4, Q4), late apoptotic cells (annexin V positive, PI positive; quadrant 2, Q2), and necrotic (PI positive; quadrant 1, Q1). Treatment of A375 cells for 24–48 h with ATB-346 (100 µM) resulted in a time-dependent induction

of apoptosis. (B) Quantitative analysis of apoptosis at various time points of exposure to ATB-346, naproxen or TBZ, showing that at 48 h almost 25% of cells treated with ATB-346 exhibit markers of late apoptosis. Experiments (n = 3) were performed in triplicate. (C) Western blot analysis of caspase 3 and PARP in A375 whole-cell lysates. A375 cells were incubated with ATB-346, naproxen or TBZ 100 μM for 24 h and a time-dependent cleavage of caspase 3 and of its substrate PARP were observed.

4.2.3 ATB-346 inhibits NF-κB activation and down-regulates NF-κB dependent anti-apoptotic genes

In malignant melanoma NF-κB is constitutively activated following IκB inhibitor protein degradation. Indeed, the active NF-κB complex containing the p65 subunit promotes the expression of several anti-apoptotic proteins such as inhibitor of apoptosis (IAP) and Bcl-2 family contributing to cell survival and apoptosis escape [168]. Incubation of A375 cells with ATB-346 (100 μM) caused inhibition of IκBα degradation (Fig. 4.2.2A) and of NF-κB nuclear translocation as demonstrated by a reduction in band intensity of the p65 subunit (Fig. 4.2.2B). We next evaluated the expression of two NF-κB-dependent anti-apoptotic proteins, X-chromosome-linked inhibitor of apoptosis protein (XIAP) and Bcl-2. As shown in Fig. 4.2.2C-D ATB-346 reduced the expression of the anti-apoptotic genes considered, thus confirming the involvement of NF-κB. PI3K/Akt is one of the most frequently deregulated pathways in melanoma [169]. This pathway plays an important role in melanoma development and progression and is involved in the mechanism of resistance to targeted therapy [170]. In addition, it has been reported that Akt phosphorylates the NF-κB subunit p65, increasing the binding of the NF-κB complex to DNA [171]. Treatment of A375 cells with ATB-346 (100 μM) significantly ($P < 0.01$) reduced p-Akt band intensity (Fig. 4.2.2E), suggesting a specificity of the effect on the activation of the Akt/p-Akt/NF-κB signaling pathway.

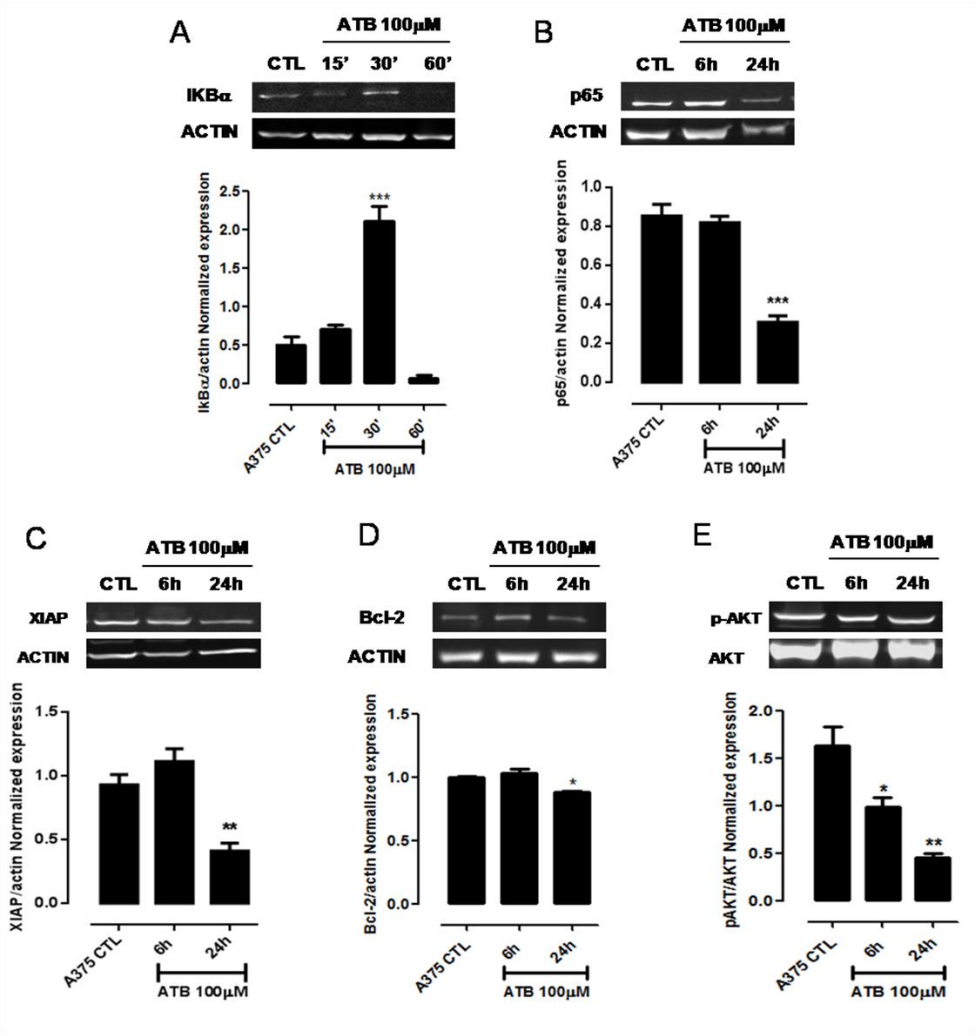

Figure 4.2.2: ATB-346 inhibits NF-κB activation, down-regulates NF-κB-dependent anti-apoptotic genes and pro-survival pathways in melanoma cells.

(A) Western blot analysis carried out on the cytosolic extracts obtained from A375 cells treated with ATB-346 (100 μM) for 15, 30, or 60 min shows an inhibition of IκBα degradation at the earliest time points. (B) Nuclear extracts from control-treated and ATB-346-treated A375 cells collected at 6–24 h were analyzed by Western blot for NF-κB activation. The A375 cell line displayed a constitutively high nuclear translocation of p65, that was reduced by ATB-346 at 24 h. (C–E) Western blot

analysis and relative densitometry of XIAP, Bcl-2 and phospho- and total Akt carried out on A375 cells treated with ATB-346 (100 μM) for 6–24 h. ATB-346 decreased the expression of both the anti-apoptotic genes analyzed as well as p-Akt band intensity; ***P < 0.001; **P < 0.01; *P < 0.05 vs control (CTL). Actin was detected as a loading control. Experiments (n = 3) were performed in triplicate.

4.2.4 ATB-346 inhibits growth of melanoma tumors in vivo and reduce plasma levels of melanoma-associated chemokines

In order to define if the effects described above translate in an in vivo setting we subcutaneously implanted B16-F10 murine melanoma cells in C57BL/6 mice. Tumor-bearing mice were treated twice daily with ATB-346, naproxen or TBZ, all drugs were administered orally at an equimolar dose of 43 µmol/kg. As shown in Fig. 4.2.3A, mice receiving ATB-346 displayed a reduction of tumor size by 61% (0.311 ± 0.05 cm3; $P < 0.01$) as compared to control mice (0.806 ± 0.1 cm3) 14 days after tumor implantation. Naproxen and TBZ reduced tumor size by 14% and 34% respectively (0.691 ± 0.156 cm3and 0.531 ± 0.086 mean tumor volume). Also tumor wet weight was significantly reduced by ATB-346 by 62% (379.5 ± 71 mg mean weight; $P < 0.01$) as compared to control mice (987.3 ± 190.3 mg mean weight). Naproxen and TBZ also reduced tumor wet weight by 24% and 37% respectively (753.8 ± 160.5 and 620.4 ± 87.5 mg mean weight; Fig. 4.2.3B). Among chemokines CXCL1, a member of the CXC chemokine subfamily, has been associated with metastatic melanoma [172]. As shown in Fig. 4.2.3C, only ATB-346 (43 µmol/kg) induced a significant reduction of CXCL1 plasma levels by 67% (83 ± 12 pg/mL; $P < 0.01$) as compared to control mice (255 ± 48.4). On the other hand, neither naproxen nor TBZ, at equimolar doses, reduced CXCL1 plasma levels (135 ± 65 and129 ± 30 pg/mL respectively).

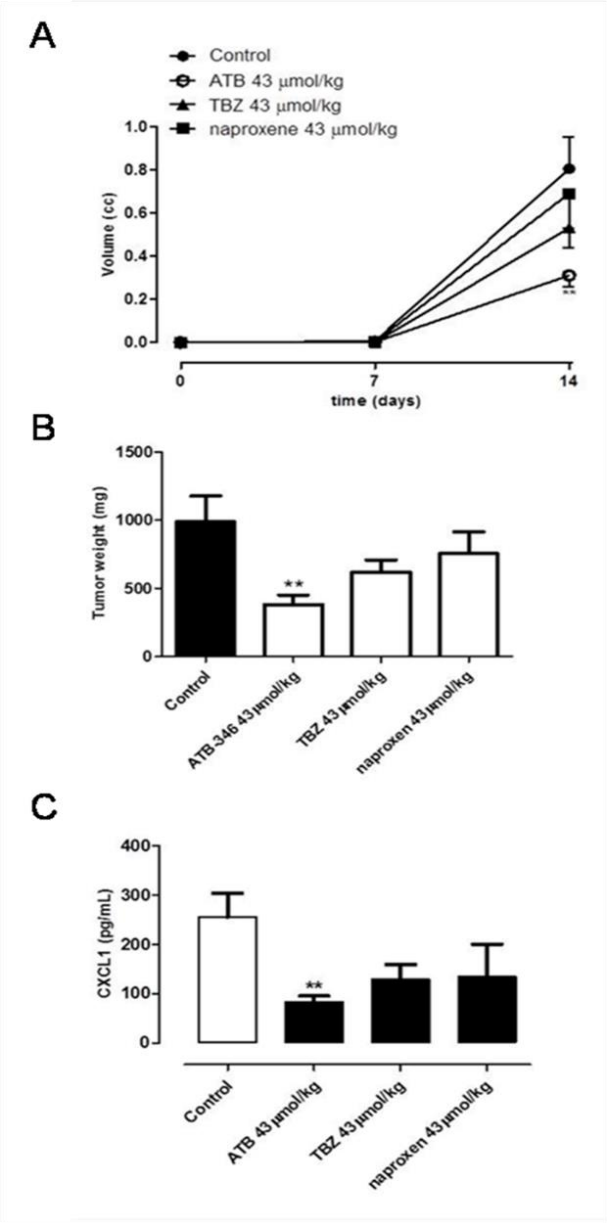

Figure 4.2.3: ATB-346 inhibits tumor growth in vivo and reduces plasma levels of thechemokine CXCL1.

(A) ATB-346, naproxen or TBZ (43 µmol/kg) were given orally to mice. Control mice received vehicle only. Tumor volume was monitored on the indicated days. The average tumor volume with standard error is plotted against the days after tumor implant. ATB-346 significantly reduced tumor volume (**P < 0.01 vs control, n = 8; day 14).

(B) ATB-346 significantly inhibited tumor weight (**P < 0.01 vs control, n = 8).

(C) ATB-346 induced a significant reduction of CXCL1 plasma levels (**P < 0.01 vs control, n = 8).

4.3 PTGS2 KNOCKDOWN BY CRISPR/CAS9 TECHNOLOGY INHIBITS AGGRESSIVENESS AND PROGRESSION OF MELANOMA

4.3.1 Generating a Knockdown PTGS2 Melanoma Cell Line Using the CRISPR/Cas9 System

For the last phase of the project we decided to establish if the role of COX-2 was played predominantly in the microenvironment rather than in tumor cells. For this purpose we deleted COX-2 in B16/F10 murine melanoma cells by using clustered regularly interspaced short palindromic repeats-CRISPR associated nuclease 9 (CRISPR/Cas9) technology, a natural bacterial adaptive immune mechanism which is now being utilized as a novel innovative technology to quickly perform genetic manipulations [173]. We co-trasfected B16/F10 murine melanoma cells with guide-RNAs targeting the PTGS2 gene or scramble negative control together with a Cas-9 and a donor construct containing the puromycin resistance gene. We chose to delete one allele to assure a knockdown rather than a complete knockout which might have been noxious. After clonal selection with puromycin, PTGS2 knockdown was detected by quantitative real-time PCR (qPCR), which was confirmed at the protein level by Western blotting (Figure 4.3.1A-B). Cells were then selected and used for next experiments.

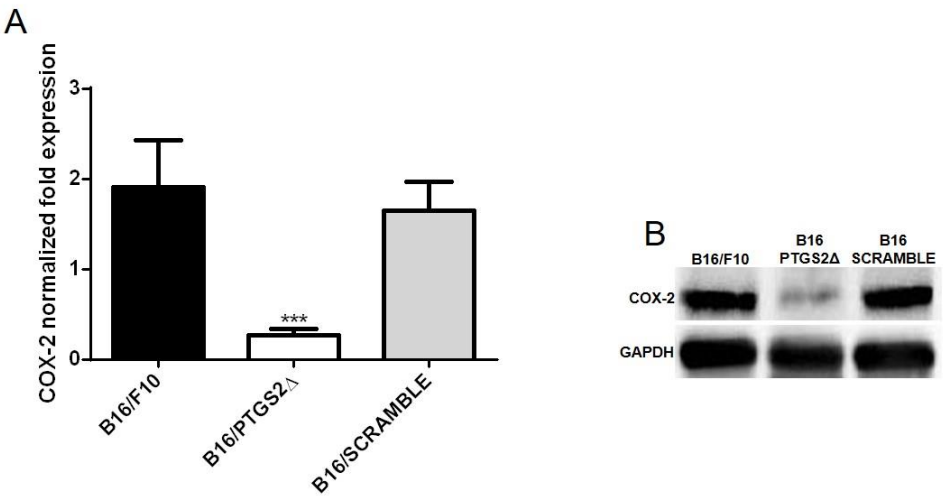

Figure 4.3.1: Knockdown effect of PTGS2 CRISPR/Cas9 in B16/F10 murine melanoma cells.

A. B16/F10 cells were transfected with PTGS2 CRISPR/Cas9 (B16/PTGS2Δ) and Scrambe (B16/Scramble) at a concentration of 2 µg (1 µg gRNA or Scrambled control with 1 µg donor). Cells were harvested 48 hours after the transfection, and were sub-cultured 5–7 times before puromycin selection. The knockdown effect at the PTGS2 mRNA level was determined using quantitative real-time PCR (qPCR). Data are shown as mean ± SEM (***P<0.001 vs B16/Scramble). B16/F10, negative control without plasmid. B. The silencing effect of PTGS2 CRISPR/Cas9 at the protein level was determined using western blot analysis.

4.3.2 PTGS2 Knockdown downregulates the proliferative potential in melanoma cells

In order to address whether PTGS2 is essential for the proliferation of melanoma cells, we next examined the proliferation rate of B16/F10 wild-type, B16/PTGS2Δ and B16/Scramble melanoma cells. Cell growth was determined at 24, 48 and 72 hours. B16/PTGS2Δ cells demonstrated slower growth rate (Table 4.3.1) compared with B16/F10 and B16/Scramble. This result suggested the crucial role of PTGS2 in the proliferation of melanoma cancer cells, and that suppression of PTGS2 lead to downregulation of cell proliferation.

TIME	B16/F10	B16/PTGS2Δ	B16/SCRAMBLE
24h	0.263 ±0.006	0.228 ±0.006 **	0.254 ±0.005
48h	0.362 ±0.006	0.248 ±0.006 ***	0.343 ±0.007
72h	0.478 ±0.01	0.406 ±0.008 ***	0.463 ±0.007

Table 4.3.1: PTGS2 knockdown downregulates proliferation rate.

Proliferation rate was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay and is expressed as OD values at 24-48-72h. B16/PTGS2Δ showed a slower growth rate at all times considered. Experiments were run in triplicate, each performed in quadruplicate. (**P<0.01; ***P<0.001 vs B16/Scramble). B16/F10, negative control without plasmid.

4.3.3 PTGS2 Knockdown thwarts cell motility and invasion

To gain further insight on the role of PTGS2 in vitro we decided to use a classic wound healing assay in order to analyze the cell motility of melanoma cancer cells. Thus, the cell monolayer was scratched and cells migrating to the wound area were monitored at different time points. B16/PTGS2Δ melanoma cells showed a wider wound area 24 hours after wound generation compared to B16/F10 wild-type and B16/Scramble and required a longer time to fill in the wound area, demonstrating a defect in migration (Figure 4.3.2A-B). Since both cell migration and invasion have important role in the diffusion of cancer cells and the development of metastases, we further investigated the cell invasiveness using in vitro invasion assay that was done by using Boyden chambers coated with matrigel. Cells that invaded to the bottom of the chamber were fixed, stained and counted. The results demonstrated that B16/PTGS2Δ were less invasive than B16/F10 and B16/Scramble (Figure 4.3.2C-D). Together, these data indicate that knockdown of PTGS2 decreases the invasiveness and the migration of melanoma cancer cells.

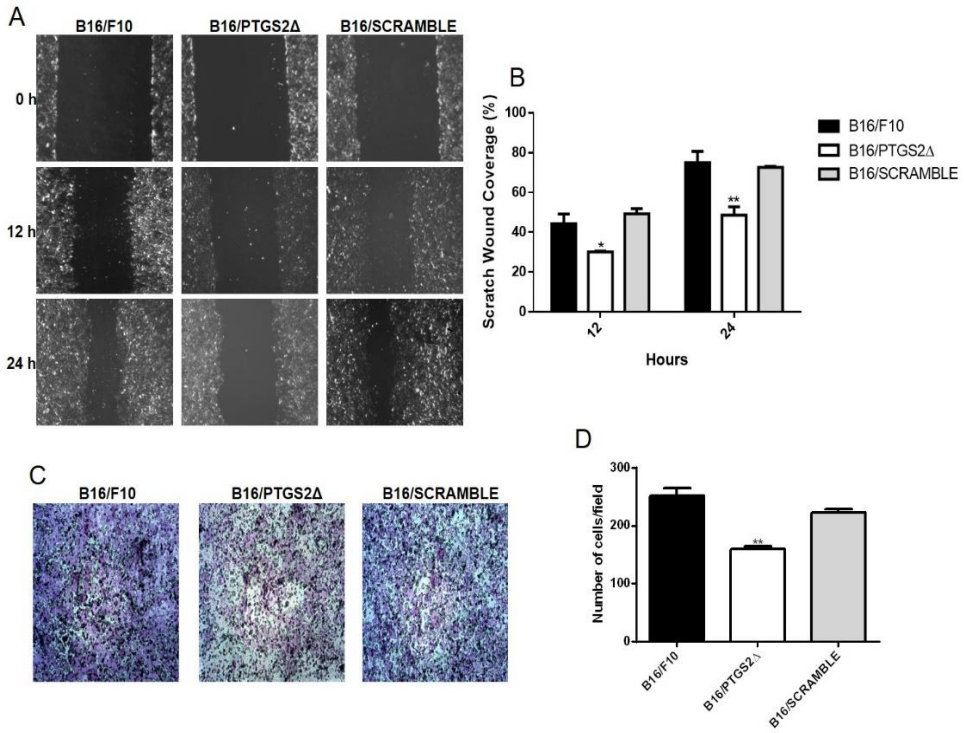

Figure 4.3.2: Knockdown of PTGS2 inhibited motility and invasion ability of B16/F10 melanoma cells.

A. The migration potential of B16/PTGS2Δ was evaluated by Wound healing assay. Cells were wounded and monitored with a microscope for 12 and 24 hours.

B. The migration was determined by the rate of cells filling the scratched area and the result represented as mean ± SEM (n = 3). (*P < 0.05; **P<0.01 vs B16/Scramble).

C. Cell invasion was determined using Boyden chambers coated with Matrigel. Cells were plated in the upper chamber in serum-free medium. Cells migrated through pores were fixed with 4% formaldehyde and stained with 0.1% crystal violet. The filters were examined microscopically and cellular invasion was determined by

counting the number of stained cells on each filter in at least 4– 5 randomly selected fields.

D. Resultant data are presented as mean of invaded cells ± SEM /microscopic field of three independent experiments (**P<0.01 vs B16/Scramble).

4.3.4 PTGS2 Knockdown inhibits focus formation of melanoma cells

To test whether PTGS2 knockdown in B16/F10 melanoma cells affects the clonogenic potential which correlates with the capacity of cells to produce progeny and tumor formation in vivo [174], we used a focus formation assay. Thus, B16/F10, B16/PTGS2Δ and B16/Scramble cells were seeded into six-well plates and incubated for 14 days to allow focus formation. The cells were fixed, stained with crystal violet, and counted. As shown in Figure 4.3.3, B16/PTGS2Δ cells exhibited smaller focus diameter as well as focus numbers compared to B16/F10 and B16/Scramble. These data indicated that inhibition of PTGS2 significantly decreases the cell focus formation potential and thus the development and progression of tumors *in vivo*.

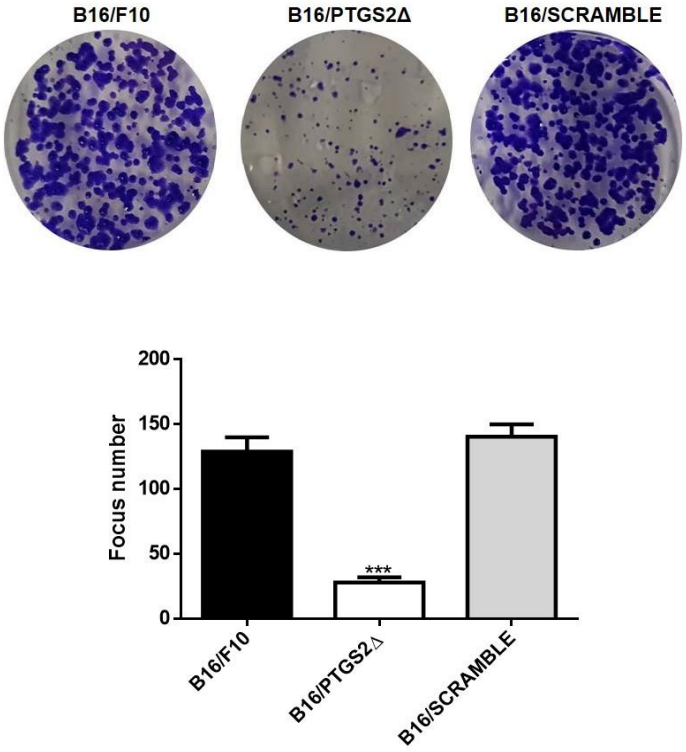

Figure 4.3.3: PTGS2 knockdown inhibits clonogenic growth.

A. Cells were seeded in six-well plate, and the medium was changed every two days. Cells cultured for 14 days were washed twice with 1xPBS, fixed by 4% paraformaldehyde, and stained with 0.5% crystal violet. Images of the colonies were obtained with a digital camera.

B. Foci containing ≥50 cells were counted and the result represented as mean ± SEM (n = 3). (***P<0.001 vs B16/scramble). B16/F10 negative control without plasmid.

4.3.5 PTGS2 Knockdown inhibits the development of cutaneous melanoma *In Vivo* and reduces CXCL1 plasma levels.

In order to define if the effects described above translate in an in vivo setting we subcutaneously implanted B16/F10 wildtype, B16/PTGS2Δ and B16/Scramble melanoma cells in C57BL/6 mice. As show in Figure 4.3.4A mice implanted with B16/PTGS2Δ displayed a slighted reduction of tumor size by ~ 30% (0.357 ± 0.04 cm3; $P < 0.05$) as compared to B16/F10 and B16/Scramble implanted mice (0.500 ± 0.02 cm3; 0.520 ± 0.03 cm3 respectively) 14 days after tumor implantation. Also tumor weight were slightly reduced in B16/PTGS2Δ implanted mice by 20 - 30% (469 ± 15.8 mg mean weight; $P <0.05$) (Figure 4.3.4B) as compared to B16/F10 and B16/Scramble implanted mice (669 ± 68 mg mean weight; 589 ± 53 mg mean weight respectively). Moreover, we also evaluated CXCL1 plasma levels. As show in Figure 4.3.4C only B16/PTGS2Δ implanted mice showed a significant reduction of CXCL1 plasma levels by 48% and 37% (359 ± 31 pg/ml) as compared to B16/F10 and B16/Scramble mice (689 ± 131 pg/ml; 568 ± 59 pg/ml respectively). Taken together, the in vivo data suggests that inhibition of PTGS2 reduces the development and aggressiveness of cutaneous melanoma by downstream pathways involving chemokines. Nonetheless, our results demonstrate that PTGS2 is relevant but not unique in determine the complex network between the microenvironment and tumors.

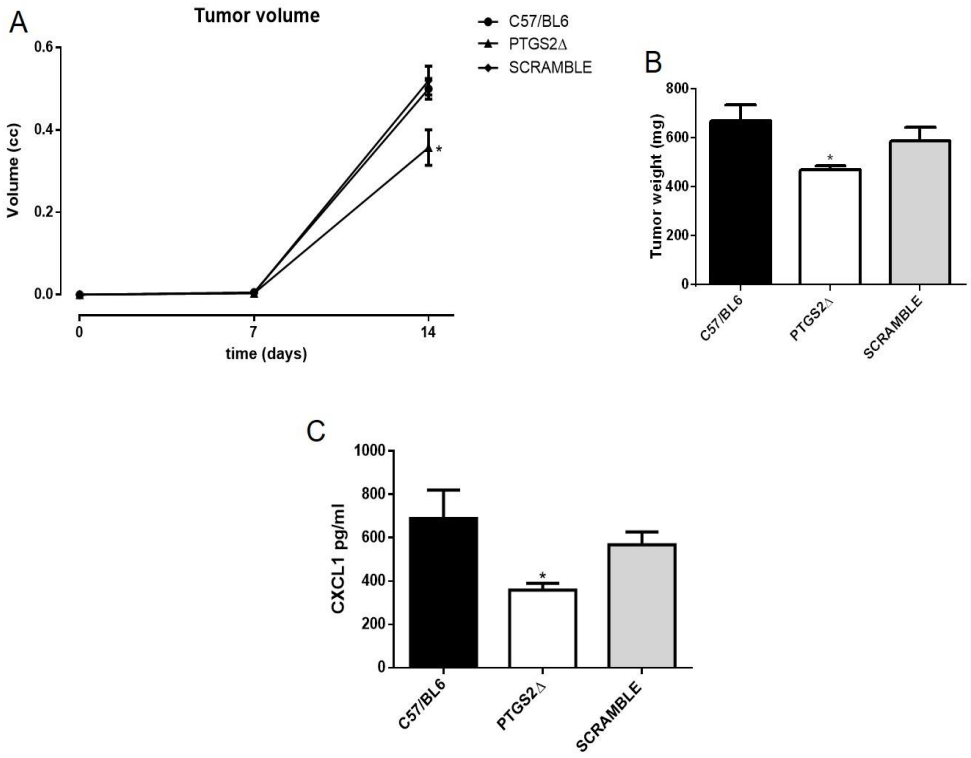

Figure 4.3.4: Knockdown of PTGS2 inhibits tumor growth in vivo and reduces plasma levels of the chemokine CXCL1.

A. B16/F10 (●), B16/PTGS2Δ (▲) or B16/Scramble (□) were subcutaneously implanted in mice. Tumor volume was monitored on the indicated days. The average tumor volume with standard error is plotted against the days after tumor implant. B16/PTGS2Δ significantly reduced tumor volume (*P < 0.05 vs B16/Scramble, n = 8; day 14).

B. B16/PTGS2Δ significantly inhibited tumor weight (*P < 0.05 vs B16/Scramble, n = 8; day 14). C. B16/PTGS2Δ induced a significant reduction of CXCL1 plasma levels (*P < 0.05 vs B16/Scramble, n = 8; day 14). Data are shown as mean ± SEM.

4.4 MicroRNA-143-3p INHIBITS GROWTH AND INVASIVENESS OF MELANOMA CELLS BY TARGETING CYCLOOXYGENASE-2 AND INVERSELY CORRELATES WITH MALIGNANT MELANOMA PROGRESSION

4.4.1 miR-143-3p is downregulated in melanoma cell lines

Recent evidences demonstrated that microRNAs (miRNAs), that play a critical role in the post-transcriptional regulation of gene expression, also regulate many aspects of tumor progression including cellular migration, proliferation and angiogenesis [155, 156]. Significant deregulation of miRNAs expression profiling between tumor cells and cells derived from normal tissue has been found, indicating that they function as either oncogenes or tumor suppressors in various cancers including melanoma [175-177]. Over the last years, several miRNAs have been demonstrated to be important direct regulators of COX-2 gene expression in cancer or non cancer cells [157]. In this respect, it has been shown that miR-101 inhibits the prostate cancer cell invasiveness by negatively regulating the COX-2 pathway [178]; miR-26b regulates COX-2 expression both in desferrioxamine-treated carcinoma of nasopharyngeal epithelial cells [179] and in breast cancer [180]. Another interesting candidate targeting COX-2 is miR-143-3p that is dysregulated in many cancers [181, 182]. In particular, its decreased expression is a marker of high malignancy and poor prognosis. However, the potential role of miR-143-3p in human melanoma remains unknown. Thus, following our results achieved during the first phases of the project we

decided to investigate on miR-143-3p-mediated COX-2 regulation of expression in human melanoma cells. In order to investigate the potential role of miR-143-3p in melanoma cells, we measured its expression levels in different human melanoma cell lines (WM1862, WM983A, A375, SKMel 28). Normal Human Epidermal Melanocytes (NHEM) were used as control. By means of quantitative PCR, we found that in melanoma cell lines the expression levels of miR-143-3p was significantly lower than control (NHEM) cells ($p < 0.001$) (Fig. 4.4.1A).

To gain further inside on the role of miR-143-3p in melanoma cells, we transiently transfected A375 cells with control (scramble) or miR-143-3p sequences. After transfection, in both control and miR-143-3p-transfected cells, we evaluated the cell proliferation and apoptosis. Firstly, using qPCR, we examined the expression of miR-143-3p in transfected cells, and as expected it was significantly higher than control cells (Fig. 4.4.1B). Moreover, in miR-143-3p-transfected A375 cells, we found a reduced proliferation in a time dependent manner (12% and 30% at 48 and 72 h, respectively; Fig. 4.4.1C) associated with an increased apoptosis (43%; Fig. 4.4.1D) as compared to the scramble-transfected cells.

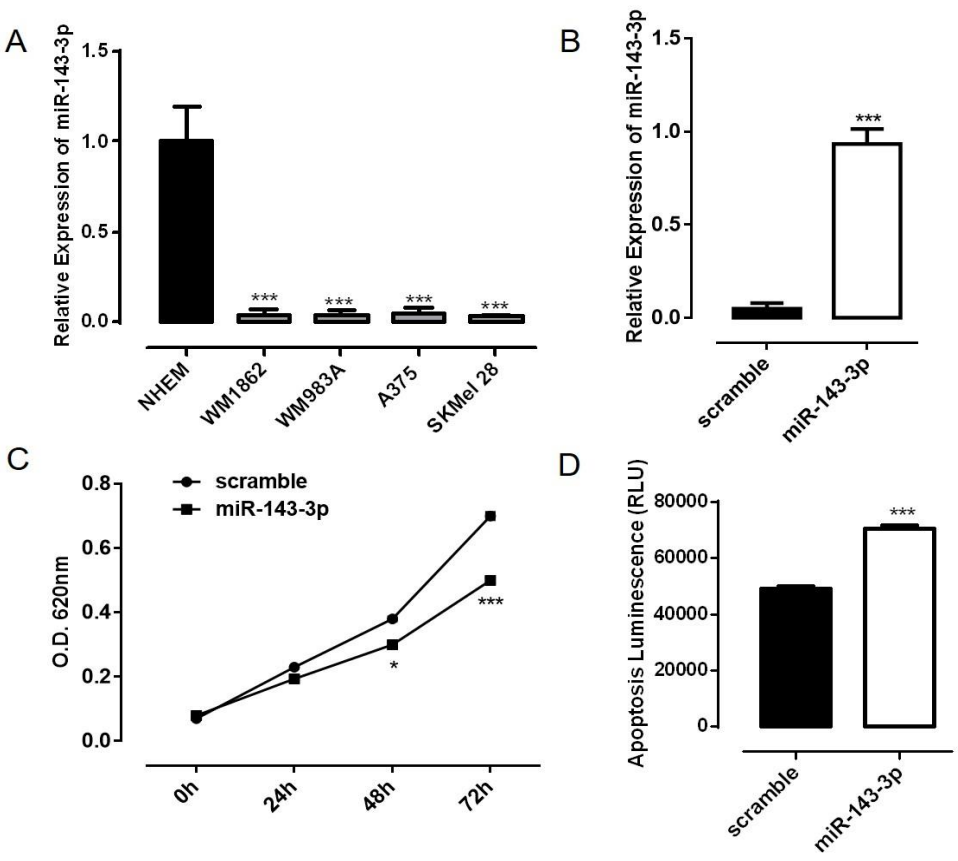

Figure 4.4.1: Expression levels of miR-143-3p in human melanoma cell lines and its effect on melanoma cell proliferation and apoptosis.

(A) Relative expression of miR-143-3p in four human melanoma cell lines (WM1862, WM983A, A375, SKMel28) compared with NHEM. (B) A375 cells were transfected with scramble or miR-143-3p mimics for 48 h. The successful overexpression of miR-143-3p was detect by qRT-PCR analysis. (C) MTT assay was performed to analyze the effect of miR-143-3p transfection on A375 cell proliferation at the indicated time points. (D) The influence of miR-143-3p on cell apoptosis was analyzed using ApoTox-Glo Triplex Assay. Data are shown as mean ± SEM (*P < 0.05, ***P < 0.001).

4.4.2 miR-143-3p suppresses migration and invasion of human melanoma cells

In addition to the findings described above, we evaluated the clonogenic ability, migratory potential and invasiveness of A375 cells following miR-143-3p-mimics transfection. As shown in Fig. 4.4.2A–B the clonogenic ability of A375 cells was markedly reduced following miR-143-3p-mimics transfection. Moreover, we found that the number of migratory (Fig. 4.4.2C and D; $p < 0.01$) and invasive (Fig. 4.4.2E and F; $p < 0.001$) miR-143-3p-transfected A375 cells, was significantly reduced compared to scramble-transfected cells.

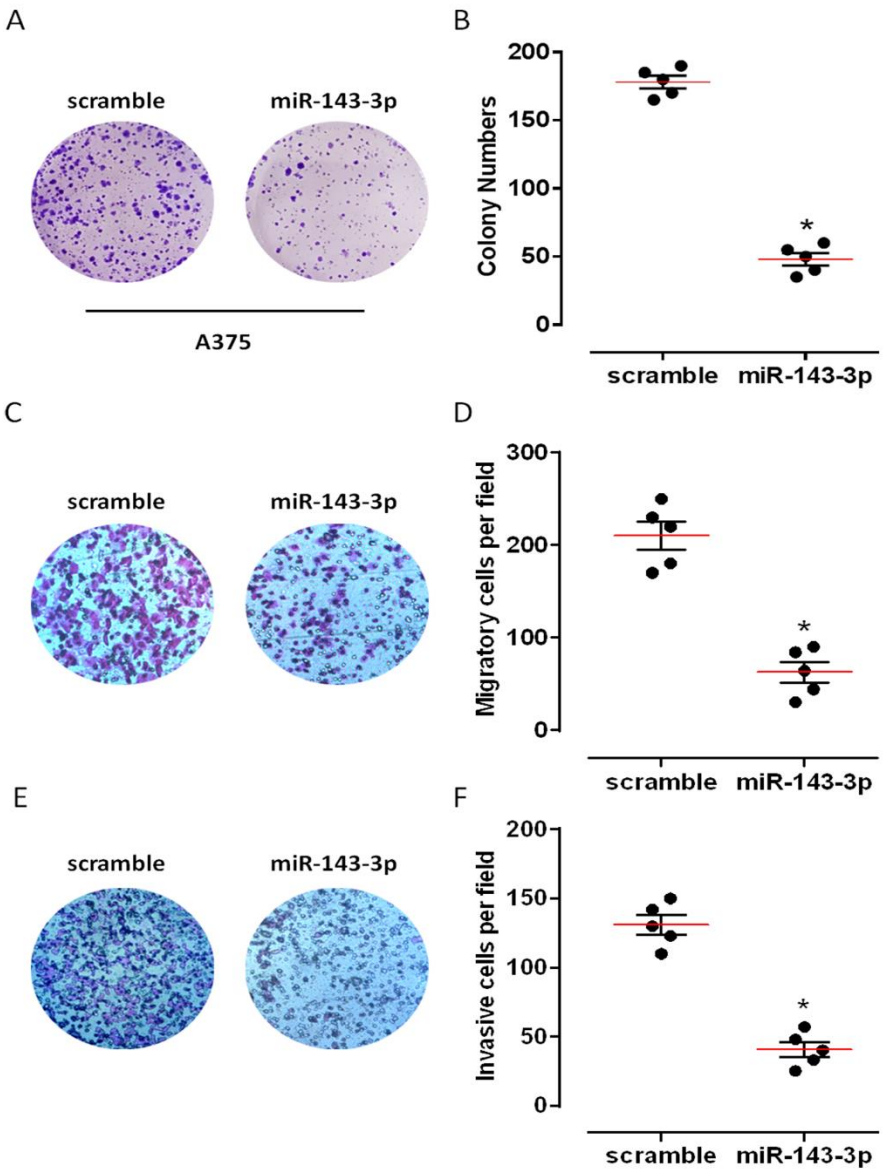

Figure 4.4.2: Effect of miR-143-3p on melanoma cell colony formation, migration and invasion.

A375 cells were transfected with scramble or miR-143-3p mimics. (A) Representative photographs and average number (B) of A375 cell colony formation. (C) Representative photographs and average number (D) of migratory A375 cells. (E) Representative photographs and average number (F) of invasive A375 cells. Data are shown as mean ± SEM (**P < 0.01, ***P < 0.001).

4.4.3 miR-143-3p targets the 3'-UTR of COX-2

To explore the mechanism(s) involved in miR-143-3p-mediated tumor suppression, putative downstream gene targets of miR-143-3p were searched using prediction programs TargetScan, miRanda, PicTar. Therefore, by means of bioinformatics analysis, we found putative binding sites of miR-143-3p in the three prime untranslated region (3'-UTR) of PTGS2 gene (position 1711/1717) (Fig. 4.4.3A–B). Luciferase reporter assay was then performed to prove the functional binding of miR-143-3p and COX2 gene at the predicted sites. In particular, luciferase reporters carrying a wild-type PTGS2 3'UTR sequence including the miR-143-3p binding site (PTGS2- 3'UTRwt) or a mutated 3'UTR (PTGS2-3'UTRmut) were used (Fig. 4.4.3C). Our results indicated that in A375 cells co-transfected with PTGS2- 3'UTRwt and miR-143-3p mimics there was a significant reduction of luciferase activity compared with that of PTGS2-3'UTRmut-transfected cells (Fig. 4.4.3C). In addition, in miR-143-3p-transfected A375 cells, we observed a decreased of COX-2 mRNA and protein expression (Fig. 4.4.3D–E). In conclusion, these results clearly demonstrated that miR-143-3p functionally suppresses the transcriptional activity of COX-2 gene.

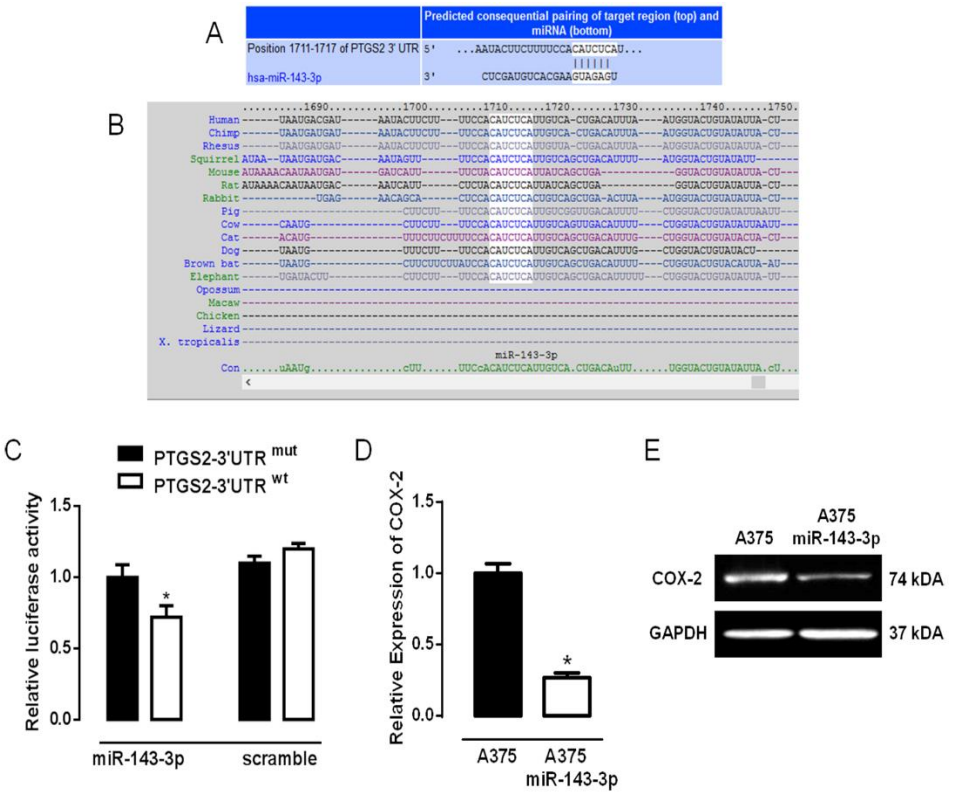

Figure 4.4.3: Prediction of PTGS2 as a target of miR-143-3p.

(A) Complementary 7mer-A1 seed match between miR-143-3p and the 3'UTR of PTGS2 as predicted by TargetScan software. (B) Conservation of the 7mer-A1 seed of miR-143-3p in the 3'UTR of PTGS2 in indicated organisms. (C) A dual-luciferase reporter system analysis was performed on A375 cells co-transfected with the wild-type/mutant 3'UTR sequences of PTGS2 and scramble or miR-143-3p mimics. (D–E) qRT-PCR and western blot were performed to detect the expression of COX-2 upon transfection with miR-143-3p mimics. Data are shown as mean ± SEM (*P < 0.05, ***P < 0.001).

4.4.4 miR-143-3p transfection decreases PGE2 levels in A375 cells

To further confirm that COX-2 is directly targeted by miR-143-3p we evaluated PGE2 levels in cell culture supernatants. Transfecting A375 cells with miR-143-3p mimics significantly decreased the concentration of PGE2 by about 50% compared with scramble (Fig. 4.4.4).

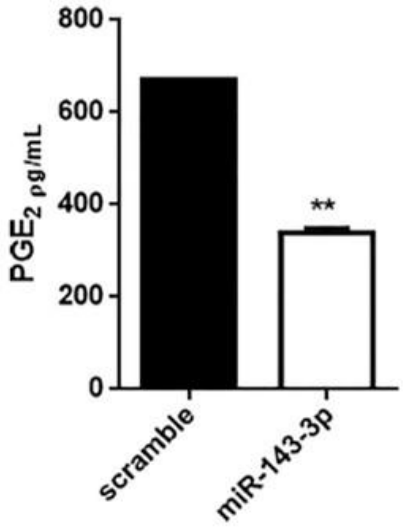

Figure 4.4.4: Effect of miR-143-3p on PGE2 secretion

PGE2 concentrations in A375 cell culture supernatants upon transfection

with miR-143-3p mimics or scramble. Data are presented as the mean ± SEM (**P < 0.01).

4.4.5 Expression of miR-143-3p in human primary melanoma lesions

Since we showed in the first phase of the project a correlation between high levels of COX-2 expression and poor survival in melanoma patients, in this last phase we analyzed the expression of miR-143-3p in human melanoma surgical specimens. We selected ten primary lesions and twenty metastasis showing high levels of COX-2 expression. Our results revealed a robust reduction of miR-143-3p expression between human melanoma samples vs healthy melanocytes (Fig. 4.4.5A). Moreover we demonstrated that COX-2 expression correlates with miR-143-3p expression level in each subject analyzed (Fig. 4.4.5B).

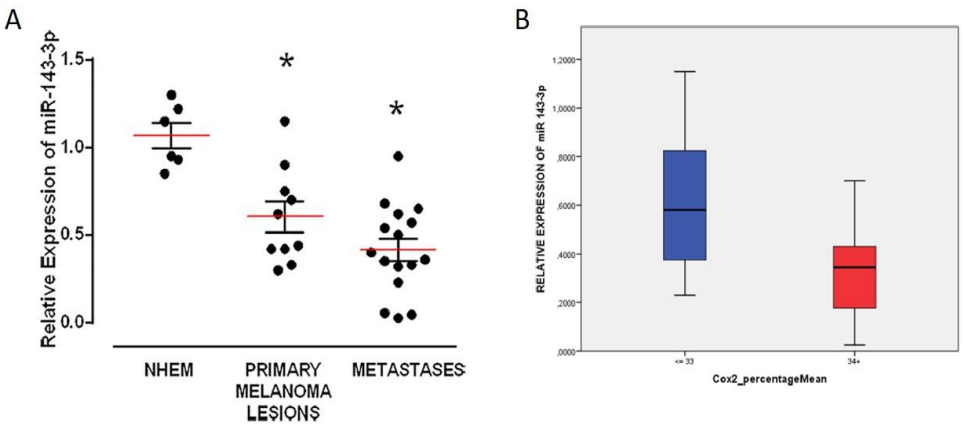

Figure 4.4.5: Expression levels of miR-143-3p in human melanoma specimens and correlation with COX-2 expression

(A) Relative expression of miR-143-3p in primary melanoma lesions and metastases compared with normal human epidermal melanocytes. Data are shown as mean ± SEM (*P < 0.05). (B) Cutoff of COX-2 expression was established according to the mean of expression (33%). Data are shown as mean ± SEM (**P < 0.01).

CHAPTER 5: DISCUSSION

Melanoma remains one of the most invasive and drug-resistant cancers with a consistently increasing of incidence and mortality rates [183]. A better understanding of the molecular mechanisms behind melanoma development and progression is needed to develop more effective therapies and to identify new markers to predict disease behavior. The effectiveness of the novel treatments for cancer patients are based on targeting the pro-tumorigenic factors instead of the single tumor cell. In the TME secreted factors like cytokines, growth factors, growth modulators, extracellular matrix proteins and matrix proteases as well as mesenchymal and inflammatory cells interact with tumor cells to increase tumor formation and metastasis [184]. Inflammation underlies this microenvironment and it is considered the strongest supporter in tumorigenesis. In particular, it has been shown that COX-2 and its product PGE2 are expressed at high levels in a variety of cancers promoting their growth [57, 185, 186]. Several lines of evidence indicate that COX-2 is strongly expressed also in malignant melanoma and it may be correlated with the development and progression of disease [150-153]. Based on these preliminary data we decided, for the first phase of the project, to gain further insights into the role of COXs in melanoma by using different human melanoma cell lines. All cell lines showed an increased expression of COX-1 and COX-2 as compared to NHEM.

The level of expression of both enzymes varied among the cell lines studied suggesting that expression of COX-1 vs COX-2 appears to be reciprocal compensate within melanoma cells. In other words, when COX-1 expression is lower COX-2 is doubled and viceversa. However, in order to further define the major role of COX-2 vs COX-1 we evaluated the effect of COX-2 inhibition on melanoma cell proliferation. We choose as selective COX-2 inhibitor celecoxib and compared its effect to naproxen, a NSAID less than 5-fold selective for COX-2 [187, 188]. Celecoxib was selected since it is known to exhibit the greatest potency among COX inhibitors for growth inhibition [189]. Celecoxib, but not naproxen, inhibited the growth of all cell lines tested in a time- and concentration-dependent manner further supporting a major role for COX-2 in melanoma development. We next transfected SK-Mel-5 cells with siRNA for COX-2. As expected COX-2 silencing significantly reduced both cell proliferation and invasiveness as compared to control siRNA. Thus, in melanoma cell lines, COX-2 increased expression appears to be a “cellular tool” to increase their ability to invade the host. In order to investigate on the role of COX-2 in melanoma development also *in vivo*, we performed a reverse translational approach by using COX-$2^{-/-}$ mice. The lack of COX-2 almost blunted tumour development as opposite to background mice. This finding, attained with mice with a competent immune system, supports a key role for COX-2 vs COX-1 in melanoma development.

However, since the removal of COX-2 gene does not completely abolish tumour development, COX-1 residual contribute cannot be ruled out. In conclusion we evaluated the expression of COX-2 on 45 human lymph node metastases from different primary melanoma sites, indicating that the COX-2 expression level influences human melanoma malignancy. However, if data are analyzed to search a close correlation between COX-2 expression and PFS, the results obtained do not give a clear outcome. Indeed, if the samples analyzed are simply divided into COX-2 negative- vs COX-2 positive the correlation with PFS is present but it is not remarkable. By performing a series of differential analysis on the percent of expression of COX-2 we found that when samples analyzed were separated into two sub-groups, namely COX-2^{high} where COX-2 expression was ≥10%, and COX-2^{low} where COX-2 expression was ≤9%, a striking difference was evident. Actually, the COX-2^{high} expression group showed a significant negative correlation with PFS which was reduced by 35 months (almost 3 years) in COX-2^{high} patients. Therefore, the level of COX-2 expression, determined as COX-2^{high}, represents a trade off versus a COX-2 negative significant contribute to melanoma malignancy. It is possible to hypothesize that COX-2^{high} tumour promoting effect may be driven by COX-2 derived PGE2 as a potent tumour immune escape mechanism associated with enhancement of cancer cell survival, growth, migration, invasion, angiogenesis, and immunosuppression [50].

Recently, Zelenay et al., by using an arbitrary cut off of microarray expression data in melanoma biopsies, demonstrated a positive correlation among COX-2 and the levels of some specific tumour promoting factor [190]. The same mechanism may apply to our COX-2^{high} samples. In fact, we can speculate that, the overcoming of a specific threshold in COX-2 expression, allows the tumour to become more 'aggressive' with a higher probability of malignant metastasis. In order to verify if melanoma most frequent mutations could influence the data outcome we characterized the $NRAS^{Q61}$ and $BRAF^{V600E}$ mutations in the samples analyzed. COX-2 expression neither correlated with $BRAF^{V600E}$ nor with $NRAS^{Q61}$ mutational status. Therefore, since COX-2^{high}/PFS correlation is affected by neither $NRAS^{Q61}$ nor $BRAF^{V600E}$ mutations it appears that COX-2^{high} is a variant non affected by these mutations, at least in our study. In conclusion, the first phase of the project, showed that COX-2 plays a role also in melanoma development as demonstrated *in vivo* by using COX-$2^{-/-}$ mice. Moreover, its expression may become an useful diagnostic tool in defining melanoma malignancy as well as argue for a possible therapeutic use of NSAID as *add on* therapy in selected cases.

The current clinical approach to melanoma is focused on reducing the probability of resistance that is the main therapeutic problem. Indeed, it is now widely accepted that multiple important targets will need to be simultaneously besieged in order to ensure an enduring effect.

To achieve this purpose different approaches have been used and are currently in clinical development as the “combo therapy” where different checkpoints of the melanoma development are targeted. ATB-346 is a novel hydrogen sulfide-releasing derivative of naproxen with reduced GI toxicity [167]. It has been recently shown that ATB-346 exerts superior chemopreventive effects to those of naproxen, while sparing the gastrointestinal tract of the injury normally associated with use of the parent drug in experimental colorectal cancer [191, 192]. Thus, for the second phase of the project, we further broaden the role played by COXs in preventing melanoma progression by using this new drug demonstrating, both *in vitro* and *in vivo*, its ability to inhibit melanoma progression. In this study, naproxen and TBZ (the H_2S releasing moiety) were used at equimolar doses. The dose of ATB-346 and naproxen employed have been previously shown to produce equivalent inhibition of prostaglandin and thromboxane synthesis [167]. To test ATB-346 activity we used a panel of human melanoma cells. We found that only ATB-346, but neither naproxen nor TBZ, inhibited the proliferation of human melanoma cell lines. In addition, ATB-346 did not modify NHEM proliferation, thus indicating a selectivity of action toward melanoma cells. Among the cell lines used the A375 was the most sensitive to the anti-proliferative effect of ATB-346 and for this reason was selected to carry on the in vitro characterization of ATB-346.

The anti-proliferative effect of ATB-346 involved apoptosis of human melanoma cells as defined in the cytofluorimetric studies. Indeed, ATB-346 induced apoptosis of A375 cells and this effect implicated the cleavage of caspase-3, the main "effector" caspase in the apoptotic pathway. Conversely, TBZ and naproxen, at the equimolar concentration tested, did not induce apoptosis of human melanoma cells, suggesting that the effect observed was due to ATB-346 rather than to the parent drug or to the hydrogen sulfide-releasing moiety (TBZ). Constitutive NF-κB and activated Akt expression have been described in melanoma and have been associated with increased survival of cancer cells and resistance to chemotherapy [193, 194]. Indeed, it is well known that the PI3K/Akt is one of the most frequently deregulated pathways in melanoma [169] playing an important role in development and progression of this deadly form of cancer, as well as being involved in the mechanism of resistance to targeted therapy [170]. Therefore following on characterizing ATB-346 we have investigated on the possible involvement of these two pathways. We found that ATB-346 inhibited IκBα degradation and this effect was associated to the inhibition of NF-κB nuclear translocation and activation in line with our previous finding obtained using H_2S donors [163]. ATB-346 inhibited Akt phosphorylation and activation, too. These data are of particular interest considering that a growing body of evidence indicates that Akt,

along with its downstream targets, constitute the major cell survival pathway in melanoma.

Since ATB-346 also greatly decreased the expression of the anti-apoptotic proteins XIAP and Bcl-2, we can speculate that the effect observed with ATB-346 is driven by inhibition of NF-κB activation and Akt phosphorylation that in turn down-regulates XIAP and Bcl-2 expression and activation [195]. The lack of effect of TBZ and naproxen, at the doses used in this study, strongly suggests that the ATB-346 effect relies on the simultaneous targeting of multiple key signaling pathways implicated in melanoma development, such as NF-κB activation and Akt phosphorylation, obtained through the synchronized release of H_2S and COX inhibition. In order to support this evidence, we designed a translational *in vivo* pre-clinical approach by using an animal model of cutaneous melanoma. Treatment of mice with ATB-346 significantly reduced tumor volume and weight. Naproxen and TBZ at the equimolar doses only slightly reduced tumor volume. Chemokines play a vital role in tumor progression and metastasis and are involved in the growth of many cancers including melanoma [196]. In particular, CXCL1 is up-regulated in melanoma cells, and it is involved in pathogenesis of melanoma [172]. When we measured CXCL1 plasma levels of tumor-bearing mice treated with ATB-346 we found that they were significantly down-regulated as compared to control mice. In conclusion, the second phase of the project demonstrates that the combination of

inhibition of cyclooxygenases and delivery of H_2S by ATB-346 may offer a promising alternative to existing therapies for melanoma.

Our study demonstrates that in human melanoma cells, ATB-346 1) inhibits cell proliferation; 2) induces apoptosis; 3) this apoptotic effect involves: a) the suppression of pro-survival pathways associated to NF-κB transcription activity as demonstrated by the decreased expression of, XIAP and Bcl-2 and b) inhibition of the activation of Akt downstream signaling pathways. Mechanistically, ATB-346 inhibited melanoma cell survival by targeting the COX and PI3K/Akt pathways, which decreased cellular proliferation and triggered apoptosis. Oral administration of ATB-346 retarded the growth of melanoma tumors by up to 70% without affecting animal body weight. Thus, a more effective agent has been developed that can decrease melanoma development by targeting key signaling pathways without causing major organ-related toxicity.

Finally, the last phase of the project, was focused on the effect of COX-2 deletion in the tumor cells rather than in the tumor microenviroment, that has already been studied in the first phase of the project, on melanoma development and progression. The analysis was conducted both *in vitro* and *in vivo* by using CRISPR/Cas9 technology to delete COX-2 gene in B16/F10 murine melanoma cells (B16/PTGS2Δ). Our results show that B16/PTGS2Δ cells had a slower growth rate as compared to wild-type B16/F10. Furthermore, we found that down-regulation of

COX-2 could inhibit cell migration, invasion and focus formation that are the major features of metastatic melanoma.

Taken together our results prove that COX-2 knockdown can affect melanoma growth and progression by reducing the proliferative potential of B16/F10 and their migration, invasion and colony formation ability. Nonetheless, subcutaneously injection of B16/PTGS2Δ cells in mice, slightly inhibited both melanoma growth and CXCL1 plasma levels confirming the relevance of the tumor microenvironment in promoting the switching of the immune system from anti-tumor to pro-tumor activity and *viceversa*, and finally in determining tumor outcome. Collectively, our findings show that COX-2 knockdown in melanoma cells is able to inhibit cell proliferation, migration and invasiveness. However, our *in vivo* results demonstrate that COX-2 is relevant but not unique in determine the complex network between the microenvironment and tumors. Understanding the molecular mechanisms of COX-2 and its downstream targets will help to implement stromal targeting strategies in clinical practice for future therapeutic approaches.

For this purpose, we focused our attention on miR-143-3p whose deregulation has been reported in various cancer [182, 197-200] but so far has not been explored in malignant melanoma. miRNAs represent a group of endogenous, small, non-coding RNAs that are currently emerging as key post-transcriptional regulators of gene expression,

affecting a multitude of biological processes including cell proliferation, differentiation, survival and motility [201].

Therefore, changes in the activity of miRNAs caused by chromosomal aberrations, genetic mutations and epigenetic changes, have been found in several diseases, including cancer [202]. Furthermore, there are also several studies demonstrating the important regulatory role of miRNAs during the melanoma progression [203-205]. In our study we demonstrate that miR-143-3p expression is downregulated in melanoma cell lines as compared to melanocytes suggesting that it might function as a tumor suppressor.

In addition, ectopic miR-143-3p expression into metastatic melanoma cells suppresses proliferation, migration and invasiveness while it promotes apoptosis. To explore the mechanism involved in miR-143-3p-mediated tumor suppression, we searched, and found, a putative binding site of miR-143-3p in the 3'UTR of COX-2 gene. The luciferase reporter assay determined that the binding site identified was the functional site predicted. Indeed, miR-143-3p suppressed the transcription activity of COX-2 gene, as well as reduced the expression of COX-2 mRNA and protein upon transfection with miR-143-3p mimics. Finally, we measured the expression of miR-143-3p within the same cluster of human samples analysed during the first phase of the project. We found an inverse correlation between miR-143-3p expression and COX-2 expression. Indeed, the expression of miR-143-3p is significantly

decreased in melanoma specimens (primary melanoma lesions and metastases) as compared to human epidermal melanocytes.

Our results demonstrated that miR-143-3p could function as tumor suppressor targeting and repressing COX-2 expression and activity suggesting that the miR-143-3p/COX-2 balance is an important factor in malignant melanoma tumorigenesis. These data suggest that miR-143-3p has all the features of a novel diagnostic marker and/or therapeutic tool in malignant melanoma.

In conclusion, all the results collected in these years point toward the definition of a prominent role for COX-2 in human melanoma progression and open new and exciting perspectives for the therapy of this still uncurable disease.

CHAPTER 6: REFERENCES

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