UNIVERSITY OF SIENA

DEPARTMENT OF BIOTECHNOLOGY,
CHEMISTRY AND PHARMACY

DOCTORAL THESIS IN
BIOCHEMISTRY AND MOLECULAR BIOLOGY
Cycle XXXIII

Coordinator: Prof. Lorenza Trabalzini

TUMOR PROGRESSION: NEW TARGETS AND NUTRACEUTICAL APPROCHES

SCIENTIFIC-DISCIPLINARY SECTOR: BIO/10

TUTOR

Prof. LORENZA TRABALZINI

PhD STUDENT

JASMINE ERCOLI

Academic year 2019-2020

ABSTRACT:

Cancer development is a complicated and multistep process that drives the progressive transformation of normal human cells into highly malignant derivatives through genetic alterations. Successive mutations in oncogenes and tumor-suppressor genes virtually result in enhanced proliferation and resistance to cell death. These genes play a central role in the genesis and progression of human cancers, as their frequent alterations have been found in a variety of human cancers. Furthermore, inflammation is a key component of the tumor microenvironment that acts as a key regulator of tumor promotion and progression by several mechanisms.

Due to the complexity of the numerous pathways involved in cancer progression and the increasingly frequent onset of the phenomenon of resistance to anticancer drugs in some tumors, current research focuses on discover of new targets and new approaches for cancer treatment.

In this work, we analysed two key objectives of cancer research: anticancer activity of natural compounds and identification of new possible molecular targets for therapy and diagnosis.

In the first part of the work we studied the effects of two different natural compounds/extracts on cellular pathways altered in cancer. In particular, we evaluated the activity of oat-derivatives, the Avenanthramides, on cell proliferation, migration and EMT, focusing on EGFR signalling pathway, and the antioxidant, antinflammatory and antiproliferative effects of an extract of a rare variety of common bean (*Phaseolus Vulgaris* L. Var Venanzio) growth in South of Tuscany.

In the second part of the work we studied the involvement in cancer of KRIT1, a protein known for its involvement in Cerebral Cavernous Malformation (CCM), a rare vascular disease typically found in the central nervous system. As KRIT1 in CCM induces characteristic similar to cancer including loss of cell junctions, improvement of migration and proliferation and new vessel formation, a tumor-suppressor like behaviour of KRIT1 has been hypothesized. Starting from this hypothesis, we first performed a bio-bank data analysis to find a correlation between KRIT1 and cancer and then we evaluated the expression levels of KRIT1 in human cancer specimens and its function in cancer cell lines.

Taken together the data of this work contribute to increase the knowledge on the molecular mechanisms underlying the anticancer activities of two different natural derivatives and describe a new molecular pathway involved in cancer progression, thus providing scientific support for future therapies and new possible targets in the treatment of cancer.

LIST OF PUBLICATIONS:

1. Finetti F., Travelli C., Ercoli J., Colombo G., Buoso E., Trabalzini L. *Prostaglandin E2 and Cancer: Insight into Tumor Progression and Immunity.* Biology (Basel). 2020 Dec 1; 9(12):434. doi: 10.3390/biology9120434.

2. Finetti F., Biagi M., Ercoli J., Macrì G., Miraldi E., Trabalzini L. *Phaseolus vulgaris L. var. Venanzio Grown in Tuscany: Chemical Composition and In Vitro Investigation of Potential Effects on Colorectal Cancer.* Antioxidants (Basel). 2020 Nov 26; 9(12):1181. doi: 10.3390/antiox9121181.

3. Ercoli J., Finetti F., Woodby B., Belmonte G., Miracco C., Valacchi G., Trabalzini L. *KRIT1 as a possible new player in melanoma aggressiveness.* Arch Biochem Biophys. 2020 Sep 30; 691:108483. doi: 10.1016/j.abb.2020.108483.

4. Finetti F., Schiavo I., Ercoli J., Zotta A., Boda E., Retta SF., Trabalzini L. *KRIT1 loss-mediated upregulation of NOX1 in stromal cells promotes paracrine pro-angiogenic responses*. Cell Signal. 2020 Apr; 68:109527. doi: 10.1016/j.cellsig.2020.109527.

5. Schiavo I., Ercoli J., Finetti F., Trapani E., Retta SF., Trabalzini L. *NADPH oxidase regulates the expression of angiogenic growth factors in cerebral cavernous malformation cellular models.* Free Radical Biology and Medicine, 2018 May 20, 120:147. (Abstract) doi: 10.1016/j.freeradbiomed.2018.04.485

INDEX

1. INTRODUCTION

1.1 Hallmarks of cancer

The term neoplasm, or tumor, indicates an abnormal mass of tissue, whose growth exceeds and is uncoordinated with that of normal tissues and persists in the same excessive manner after cessation of the stimuli that evoke the change. Neoplasms may be benign (not cancer) or malignant (cancer) (Willis 1952; Arvind Babu et al. 2014).

Tumor is a genetic disease that involves dynamic changes in the genome. Its formation, called tumorigenesis, o carcinogenesis, is a multistep process that drive the progressive transformation of normal human cells into highly malignant derivatives through genetic alterations passing through an intermediate step involving lesion formation (Foulds 1954; Bertram 1984; Hanahan and Weinberg 2000).

Carcinogens can simultaneously affect many cells in a tissue even if the macroscopic tumor that ultimately develops usually represents the progeny of a single cell or a very few cells. This let presume that other neoplastic or preneoplastic cells in the exposed tissue don’t successfully proliferate or are destroyed before progressing to a fully developed tumor (Nowell 1976; Hanahan and Weinberg 2011).

In carcinogenesis two separate elements are distinguishable, tumor inception or initiation, that is irreversible, and tumor promotion. Initiating agents convert normal cells into "latent tumor cells," or in a "subthreshold neoplastic state," and promoting agents stimulate the latent tumor cells to proliferate to form a visible tumor. Tumor initiation occurs by an induced change in a single previously normal cell which makes it "neoplastic" and provides it with a selective growth advantage over adjacent normal cells. Neoplastic proliferation then proceeds immediately or after a latent period. Promotion on the contrary is reversible; when the promoting stimulus is denied, the tumor regresses and cells revert to the subthreshold neoplastic state (Friedewald and Rous 1944; Nowell 1976; Hanahan and Weinberg 2011).

In the initial neoplastic event both internal factors (such as inherited mutations, hormones, and immune conditions) and environmental/acquired factors (such as tobacco, diet, radiation, and infectious organisms) can influence one or more stages in the evolution of tumor cell populations and provoke genetic instability. Neoplastic populations have a

higher frequency of mitotic errors and other genetic alterations that may range from point mutations to major chromosomal aberrations, and changes in chromosome number (Gelboin 1967; Herceg and Ushijima 2010). These changes during tumor development, in most cases, bring to the acquisition of seven capabilities or “hallmarks” that characterise malignant growth of cells (Figure 1.1) (Hanahan and Weinberg 2000; Colotta et al. 2009):

- Self-sufficiency in growth signals
- Insensitivity to growth inhibitory signals
- Evasion of programmed cell death (apoptosis)
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis
- Inflammatory microenvironment

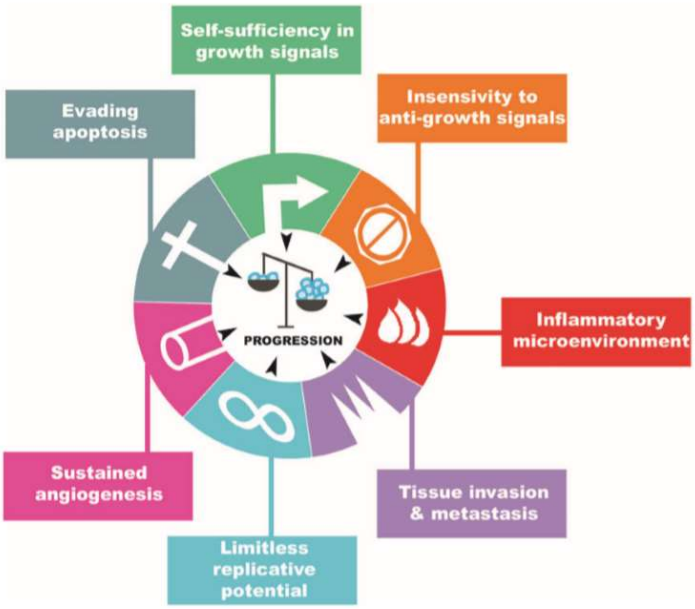


Figure 1.1. *The seven hallmarks of cancer [from Colotta et al. 2009].*

Most of the hallmarks mentioned above are linked to an important dysregulation in cancer cell signaling. In fact, normal cells require growth signals before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction

molecules (Hanahan and Weinberg 2000). Without these signals normal cells can't proliferate instead, tumor cells generate many of the stimuli they need, reducing their dependence from microenvironment and creating a positive feedback signaling loop (Fedi et al. 2000). There are three molecular strategies used by tumor to acquire growth-signals autonomy, involving alteration of extracellular growth signals, transcellular transducers or intracellular circuits that translate those signals into action (Hanahan and Weinberg 2000). For example, overexpression of receptors as EGFR (Epidermal Growth Factor Receptor) could enable the cancer cell to become hyperresponsive to ambient level of growth factors that normally would not trigger to proliferation (Fedi et al. 2000). This can also be achieved through structural alteration of receptors, as the EGFR lacking much of its cytoplasmic domain fires constitutively (Fedi et al. 2000). Cancer cells can also switch the type of extracellular matrix (ECM) receptor towards one that transmits pro-growth signals (Lukashev and Werb 1998; Giancotti and Ruoslahti 1999). Cell adhesion to the ECM modulates the expression and functional state of several cell-cycle regulators and is crucial for the progression through the G1–S cell-cycle checkpoint. In particular integrin-mediated adhesion to the ECM activates a p53-dependent apoptotic pathway in response to UV irradiation, whereas inhibition of integrins blocks this pathway and triggers p53-independent apoptosis (Assoian and Zhu 1997).

In a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. These signals include soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surface of nearby cells, which are received by transmembrane cell surface receptors coupled to intracellular signaling circuits and block proliferation forcing out the active proliferative cycle into quiescent (G_0) state form or inducing cells to enter into postmitotic states (Hanahan and Weinberg 2000). Cancer cells must evade these antiproliferative signals to growth.

Two classes of genes are involved in cell cycle regulation, oncogenes and tumor suppressor genes, that link cell cycle control to tumor formation and development (Kontomanolis et al. 2020). Oncogenes in their proto-oncogene state drive the cell cycle forward, allowing cells to proceed from one cell cycle stage to the next. This highly regulated process becomes dysregulated due to activating genetic alterations that lead to cellular transformation. Examples include receptors at the cell surface that bind to growth factors, proteins that interact with DNA to initiate replication, and signaling molecules that link the

receptors to the replication initiators through various pathways (Chow 2010). Tumor suppressor genes, on the other hand, restrict cell cycle progression, coding for proteins that normally operate to restrict cellular growth and division or promoting cell death. Their control over cell division is lost with genetic alterations leading to their inactivation. Yet the most commonly mutated gene in human tumors, is the p53 gene (Vogelstein and Kinzler 2004), that activates expression of proliferation-inhibiting and apoptosis-promoting proteins in response to DNA damage (Chow 2010; Kontomanolis et al. 2020).

Other than antigrowth signalling pathway and oncogene/oncosuppressor genes, the main process involved in maintenance and control of cells in a system is apoptosis, a form of programmed cell death in contrast with necrosis. Apoptosis is a process in which a cell is driven toward death upon receiving certain stimuli to remove damaged cells, such as those resulting from DNA damage or during development (Chen et al. 2001; Fuchs and Steller 2011). Apoptosis can be triggered in a cell through either the caspase-mediated extrinsic or intrinsic pathways. Both converge to activate the effector apoptotic caspases resulting in cellular alterations, characteristics of apoptosis, as disruption of cellular membranes, cytoplasmatic and nuclear skeleton, extrusion of cytosol, degradation of chromosomes and fragmentation of nucleus. In the end, the shrivelled cell corpse is engulfed by nearby cells in a tissue and disappears (Wyllie et al. 1980; Hanahan and Weinberg 2000; Wong 2011).

The apoptotic machinery can be divided into sensors and effectors. The sensors monitor the extracellular and intracellular environment and regulate the second class of components, that function as effectors of apoptotic death. The sentinels include cell surface receptors that bind survival or death factors (Hanahan and Weinberg 2000). Many of the signals that elicit apoptosis converge in the mitochondria, that respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis, whose release is induced by p53 tumor suppressor protein. The ultimate effectors of apoptosis include intracellular proteases termed caspases, which are activated by death receptors or by the cytochrome C released from mitochondria for example (Green and Reed 1998; Fuchs and Steller 2011).

Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. The most common include loss of a proapoptotic regulator through mutation of p53 tumor suppressor gene (50% of human cancers) that results in the removal of a key component

of the DNA damage sensor that can induce the apoptotic effector cascade (Harris 1996; Pistritto et al. 2016).

Many mammalian cells have an intrinsic, cell-autonomous program, called senescence, that limits their multiplication and operates independently of the cell-to-cell signaling pathways. By disabling p53 tumor suppressor protein, the cells are able to continue multiplying until they enter into a second state termed crisis that is characterized by massive cell death and occasional mutation of cells that acquire the ability to multiply without limit, in a process called immortalization. This process is fundamental for development and progression of cancer, suggesting that at a certain point of multistep process, tumor cells have to acquire unlimited replicative potential (Hanahan and Weinberg 2000).

Angiogenesis is the physiological process of formation of blood vessels from a pre-existing vascular network important for organogenesis. This process is transitory and carefully regulated. During tumorigenesis neoplastic cells develop the ability to promote angiogenesis through the increased production of angiogenic factors that results in the induction of an “angiogenic switch” from vascular quiescence (Nishida et al. 2006). During development, tissue repair or in disease conditions vascular growth involves sprouting, migration and proliferation of endothelial cells (ECs), which are regulated by multiple factors. Among these, VEGF plays a critical role (Hoeben et al. 2004). Compared to tissue blood vessels, the tumor vasculature shows atypical morphological features. The tumor vascular network is characterized by dilated, tortuous, disorganized blood vessels, vascular immaturity and lack of mural cells association that leads to excessive permeability, poor perfusion and increased hypoxia. Moreover, tumors present a high degree of vascular heterogeneity, with hypervascular sites and other regions of low vessel density (Viallard and Larrivée 2017).

Tumors appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and inhibitors (Hanahan and Folkman 1996; Nishida et al. 2006), through mechanisms that involve alteration of gene transcription.

Hypoxia is a major driver of tumor angiogenesis. Under hypoxic conditions, hypoxia-inducible factor 1-alpha (HIF-1α) binds hypoxia response elements (HREs) leading to the transcription of genes involved in cellular adaptation against chronic or acute hypoxia such as angiogenesis, survival, cell proliferation and glucose metabolism (Marin-Hernandez et

al. 2009). Recently, it was demonstrated that the ERK signaling cascade can result in the increased rate of mRNA translation of HIF-1α protein (Masoud and Li 2015). Furthermore, cAMP-dependent protein kinase A (PKA), deregulated in cancer, enhances HIF-1α transcriptional activity and decreases its degradation by the proteasome (Bullen et al. 2016).

During development of cancer, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues and travel to distant sites where they find new colonies. This process is known as tumor metastasis, a multistep process by which tumor cells disseminate from their primary site and form secondary tumors at a distant site through a series of steps: local invasion, intravasation, transport, extravasation and colonization. Metastases are the cause of 90% of human cancer deaths (Gilkes et al. 2014).

The capability for invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrains in the body where, at least initially, nutrients and space are not limiting, depending on both tumor cell and host properties.

As previously mentioned, the metastatic process is thought to consist of several steps (Figure 1.2):

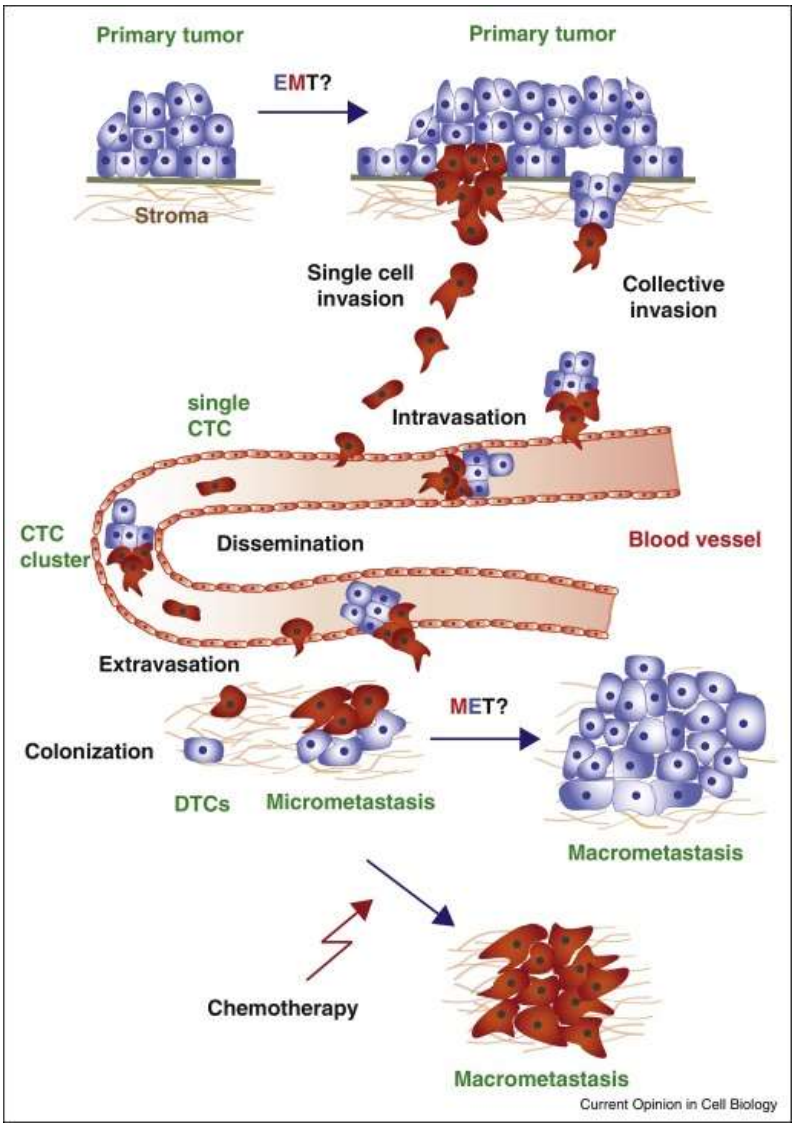


Figure 1.2. *Schematic representation of the multiple stages of metastatic dissemination of cancer cells from the primary tumor to a distant organ (from Diepenbruck and Christofori 2016).*

- Invasion. The initial escape from the primary site requires the epithelial tumor cells to become motile and degrade the underlying basement membrane and ECM; breakdown of these barriers initiates invasion into the nearby tissue parenchyma.
- Intravasation. Tumor cells invade across the endothelial lamina, penetrate into the blood or lymphatic vessels, and thereby enter the systemic circulation.
- Systemic transport. Once in the circulation, only a small number of the disseminated neoplastic cells appear to be capable to survive various insults within the circulation.

- Extravasation. Some of the surviving cells may arrest in the vascular lumen and extravasate through the capillary endothelium into the parenchyma of distant organs.
- Colonization. In the new stromal environment, an even smaller subset of tumor cells establish micrometastases with the potential to proliferate into fully malignant, secondary tumors that are clinically detectable and eventually life-threatening (Their 2002; Fidler 2003; Kalluri and Weinberg 2009; Fares et al. 2020).

It has been estimated that only 0.01% of tumor cells entering the systemic circulation are able to form secondary tumors (Chambers et al. 2002).

1.1.1 Epithelial-mesenchymal transition in cancer

Epithelial–mesenchymal transition (EMT) is a cellular process in which cells lose their epithelial characteristics and acquire mesenchymal features. Indeed during specific embryonic morphogenesis processes, cells lose their epithelial junctions and epithelial markers and switch to producing vimentin filaments, to allow stationary epithelial cells to gain the ability to migrate and invade (Boyer and Thiery 1993; Hay 1995; Nieto et al. 2016). EMT is seen in early embryogenesis, when it is involved in gastrulation and neural crest migration (type 1). Later in organogenesis, the expression of local developmental cytokines drives secondary epithelial cells and endothelial cells (specialized squamous epithelial cells) to become fibroblasts that close the cranial plates, facial bones and populate interstitial spaces with resident fibroblasts as part of the normal expansion of connective tissues (type 2) (Zeisberg and Neilson 2009). Moreover, EMT processes might be involved in the initial steps of the metastatic cascade, including tumor invasion, intravasation and micrometastases formation (type 3) (Diepenbruck and Christofori 2016).

The EMT program is not necessarily an irreversible commitment. These epithelial cells can activate a transitory EMT program and then undergo a reverse process called mesenchymal–epithelial transition (MET) to continue their differentiation paths (Lim and Thiery 2012). This reversibility might be an essential feature of the metastatic cascade. Indeed distant metastases arising from carcinomas usually present the same histology as the primary tumor, indicating the maintenance or reacquisition of the epithelial morphology by disseminated tumor cells at distant sites (Brabletz 2012).

The EMT program in tumor metastasis is highly dynamic and adapts to heterogeneity and constantly evolving microenvironment in human tumors to allow tumor cells to successfully metastasize. The complex morphological and cellular changes during EMT require the cooperation of numerous molecular signalling pathways and regulators, that in normal tissues is tightly controlled through a complex regulation of EMT transcription factors (EMT-TFs) (Nieto et al. 2016). The main one is Snail, a transcription factor common of various signaling pathways whose activation, particularly of Snail1 and Snail2, regulates EMT (Barrallo-Gimeno and Nieto 2005). Snail is most widely recognized as a suppressor of E-cadherin expression, but it regulates increased expression of mesenchymal cell/fibroblast markers (as fibronectin), decreased expression of various epithelial markers (claudins and occludins), inhibition of proliferation through suppression of cyclin D proteins and cyclin-dependent kinase 4 (CDK4), increased MMP expression, and protection from cell death (Lamouille et al. 2014). Snail transcriptional activity is regulated by control of its subcellular localization. Phosphorylation of Snail causes it to be exported from the nucleus into the cytoplasm, resulting in its inactivation as a transcription factor (Zeisberg and Neilson 2009). In addition to Snail, Twist is a protein that is transcriptionally active during cell differentiation. It is up-regulated during early embryonic morphogenesis (Castanon and Baylies 2002) and cancer metastasis. It can act independently of Snail to repress E-cadherin and to up-regulate fibronectin and N-cadherin (Yang et al. 2004, 2007).

Cadherins are cell surface glycoproteins with important functions in cell-cell adhesion, tissue pattering and cancer (Van Roy 2014). Through their extracellular domains, they interact with cadherins on adjacent cells in a Ca^{2+} dependent manner to form the adherens junctions. E-cadherin is a key component of the apical zonula adherens in epithelial monolayers. It is considered a master regulator of the epithelial phenotype (Kourtidis et al. 2017) and its expression is decreased during EMT (Loh et al. 2019). Normal cells inhibit their growth and migration when they adhere to each other. These properties are progressively lost in tumor cells, contributing to increased rates of cell proliferation and migration (Martin et al. 2014). Several studies demonstrated the role of E-cadherin as tumor suppressor and its depletion leads to mesenchymal morphology and increased cell migration and invasion (Vleminckx et al. 1991; Peri et al. 1998; Gheldof and Berx 2013) as consequence of EMT. In fact, a key event during EMT is the loss of strong cell-cell adhesion due to suppression of E-cadherin expression and the switch from E-cadherin to N-cadherin.

This is expressed in mesenchymal cells, fibroblasts, cancer cells and neural tissue and has often been used to monitor the progress of EMT during embryonic development and cancer progression (Hay and Zuk 1995; Kalluri and Neilson 2003; Huber et al. 2005; Loh et al. 2019), in particular in tumor of epithelial origin (Wijnhoven et al. 2000; Gravdal et al. 2007; Kourtidis et al. 2017). N-cadherin promotes anchorage-independent growth via loss of contact inhibition and enables stronger epithelial-endothelial cell interactions, suggesting a role in promoting invasion and metastasis via the vasculature (Hazan et al. 2000). Cumulatively, the role of cadherins is central during EMT and significantly determines intracellular and intercellular signaling, overall cell behaviour, and tumor progression (Kourtidis et al. 2017).

Classical cadherins contain a highly conserved cytoplasmic domain, which interacts with proteins that are collectively termed catenins. Catenins regulate adherens junction function and stability (Gloushankova et al. 2017); the main one is β-catenin, a cytoplasmic plaque protein that plays a dual role in EMT: it links cadherins to the cytoskeleton and serves as a co-transcriptional activator together with T cell factor (TCF)/LEF (Bienz 2005). β-catenin activity is mainly regulated by mechanisms controlling its level in the cytoplasm, through either its recruitment to cadherin-binding partners or ubiquitination and subsequent degradation (Gavert and Ben-Ze'ev 2007). The β-catenin/TCF/LEF complex directly controls gene expression associated with EMT, particularly Snail1 (Yook et al. 2006), and for this reason is used as a marker of EMT in various studies of embryonic development and cancer (Brabletz et al. 1998, 2018; Kalluri and Neilson 2003; Medici et al. 2006). Although β-catenin is localized to cell membranes in normal epithelial cells and non-invasive tumor cells, it is located either in the cytoplasm (something that is reflective of dissociation from E-cadherin) or in the nucleus (something that is reflective of its role as a transcriptional activator) in cells that undergo EMT (Brabletz et al. 2018).

The pleiotropic effects of the EMT program, when activated in tumor cells, favour the acquisition of a compendium of cellular abilities intimately linked to tumor progression and metastasis besides influencing tumor evolution and response to therapeutic treatments (Nieto et al. 2016; Lambert et al. 2017).

The microfilaments, intermediate filaments (IFs), and microtubules constitute the three major groups of non–muscle cell cytoskeletal proteins. Vimentin, a 57-kDa protein, is one of the most widely expressed and highly conserved proteins of the IF protein family and is

a controversial marker of EMT during embryonic development and in cancers (Boyer et al. 1989; Colucci-Guyon et al. 1994; Liu et al. 2015). Normally it is expressed in various cells, including fibroblasts, endothelial cells, cells of the hematopoietic lineages and glial cells; it participates both in the cytosolic and nucleic functions where mediate DNA- and RNA-mediated events (Franke et al. 1978; Satelli and Li 2011; Liu et al. 2015).

Evidences indicate that vimentin serves as a potential diagnostic tool in the detection of cancer and plays a key role in its development and progression. In particular there is a positive correlation of vimentin expression with increased invasiveness and metastasis (Raymond and Leong 1989; Satelli and Li 2011; Wei et al. 2019).

Since vimentin is overexpressed during the EMT process and NF-κB is one of the transcription factors binding to the vimentin promoter, it is tempting to speculate that this overexpression of vimentin results from the activation of NF-κB in cancer cells (Satelli and Li 2011). Also, the TGFβ1 response element was found within the activated protein complex-1 region of the vimentin promoter and was shown to be involved in the regulation of vimentin expression in myoblasts and myotubes.

The number of environmental factors that are known to induce EMT, including growth factors, growth factor receptor dysregulation, ECM constituents, proteases and hypoxia, is increasing (Zeisberg and Neilson 2009). Moreover, increasing literature data have emphasized a link between cancer-associated EMT and chronic inflammation. Indeed, inflammatory mediators can foster the acquisition of EMT-like features in cancer cells (Suarez-Carmona et al. 2017).

1.1.2 Receptor tyrosine kinases (RTKs) dysregulation and EGFR pathway in cancer cells

Receptor tyrosine kinases (RTKs) are a subclass of tyrosine kinases that are involved in mediating cell-to-cell communication and controlling a wide range of complex biological functions, including cell growth, motility, differentiation, and metabolism. There are 58 known RTKs in humans (Du and Lovly 2018) and all RTKs share a similar protein structure: an extracellular ligand binding domain, a single transmembrane helix, and an intracellular region containing a juxtamembrane regulatory region, a tyrosine kinase domain (TKD) and a carboxyl (C-) terminal tail (Hubbard 1999; Trenker and Jura 2020).

The RTK family mainly consists of endothelial growth factor receptors (ErbBs), fibroblast growth factor receptors (FGFRs), insulin-like growth factor receptors (IGFRs), vascular endothelial growth factor receptors (VEGFRs), and hepatocyte growth factor receptors (HGFRs) (Lemmon and Schlessinger 2010).

RTKs are generally activated by receptor-specific ligands. Growth factor ligands bind to extracellular regions of RTKs, and the receptor is activated by ligand-induced receptor dimerization and/or oligomerization, with a consequent autophosphorylation of each tyrosine kinase domain (TKD), that assumes an active conformation (Lemmon and Schlessinger 2010). Autophosphorylation of RTKs also recruits and activates a wide variety of downstream signaling proteins containing Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains, which bind to specific phosphotyrosine residues within the receptor and engage downstream mediators that propagate critical cellular signaling pathways (Du and Lovly 2018). Dysregulation of RTK signaling is fundamental for cancer development and progression (Du and Lovly 2018).

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of RTKs and exerts critical functions in epithelial cell physiology (Schlessinger 2014). Overactivation of EGFR signaling pathway is detected in various malignant tumors, including non-small cell lung cancer (NSCLC), breast cancer, head and neck cancer, colon cancer and ovarian cancer (Ellis 2004; Viloria-Petit and Kerbel 2004; Yarden and Pines 2012; Liu et al. 2018).

Early studies of the EGFR pathway started with the discovery of EGF in 1963 by Stanley Cohen and, later in the 1980s, of the EGFR gene (Sigismund et al. 2018).

The ErbB family is of particular interest in cancer biology. The extracellular regions of the ErbB receptors family include four subdomains (I-IV). In the absence of ligands, the intracellular TKD is inactive, and the extracellular region adopts a configuration in which the dimerization arm forms intra-molecular autoinhibitory interactions. Ligand simultaneously binds to two subdomains within the extracellular region of one receptor that induces a dramatic conformational change that “extends” the extracellular region and exposes the previously buried dimerization arm to an active conformation. With the dimerization arm exposed, the extracellular region of the receptor dimerizes, inducing intracellular conformational changes so that they can enable kinase activation (Zhang et al. 2006), with the consequent activation of the intracellular signaling cascade. This cascade consists in the activation of multiple pathways that deliver the information from the cell

surface and the intracellular vesicular compartments to the nucleus leading to the activation of genes responsible for cell proliferation, survival, and differentiation such as the PI3K/Akt and MAPK (Lemmon and Schlessinger 2010; Schlessinger 2014).

Two primary downstream signaling pathways of EGFR are the PI3K/Akt/PTEN/mTOR and the RAS/RAF/MEK/ERK. Phosphorylated tyrosine kinase of EGFR acts as a docking site for PI3K which can stimulate the generation of phosphatidylinositol-3,4,5-triphosphate (PIP-3) and promote the activation of Akt (Cully et al. 2006). Subsequently, mTOR, a downstream target of Akt, is activated and provokes the expression of associated proteins needed for the cell cycle progression from the G1 to the S phase (Liu et al. 2016). Accordingly, overactivation of this pathway suppresses apoptosis and stimulates tumor growth (Morgensztern and McLeod 2005). Moreover, the binding of ligand to EGFR induces the activation of MAPK signaling cascade. The dimerization of EGFR activates RAS leading to the phosphorylation of RAF-kinase, which in turn phosphorylates MEK, that could induce the production of cell cycle-associated transcription factors (Myc, c-Fos, CREB, NF-κB). Functional transcription factors may stimulate the cumulation of cyclin D catalysing the division of cells (Montalto and De Amicis 2020).

The best characterized functions of the EGFR are in the context of ligand- and kinase-dependent activation, that is, the ‘canonical’ EGFR signaling pathway (Lemmon and Schlessinger 2010) The noncanonical functions are generally induced by cellular and environmental stresses and are activated in cancer cells to provide them with a survival advantage and resistance to therapy (Jutten et al. 2013; Tan et al. 2016).

Monoclonal antibodies and small-molecule tyrosine kinase inhibitors (TKIs) are two clinically important pharmacological approaches in anti-EGFR therapies (Martinelli et al. 2009). Anti-EGFR monoclonal antibodies act as competitors for selective binding to the inactive extracellular domain of EGFR receptors and block ligand-induced EGFR tyrosine kinase activation (Ciardiello and Tortora 2001). Since the discovery of anti-EGFR therapies in cancer treatment, hundreds of small molecules have been synthesized and evaluated as EGFR TKIs, categorized from first- to third-generation of EGFR TKIs. First-generation (gefitinib, erlotinib) and second-generation (afatinib, dacomitinib) EGFR-TKIs have been standard-of-care first-line treatment in cancer therapy. Osimertinib, a third-generation EGFR-TKI, potently and selectively inhibits the most common EGFR resistance mutations (Gelatti et al. 2019). Nevertheless, most patients treated with first-line first- or second-

generation EGFR-TKIs develop resistance for the presence of mutations in EGFR, resulting in tumor progression and relapse (Viloria-Petit and Kerbel 2004). More recently, the fourth-generation of EGFR TKI (Thiazole amide derivative EAI001) have been introduced to the clinical evaluation to fight against resistance caused by T790M and C797S EGFR mutations (Ayati et al. 2020).

1.1.3 Inflammation and oxidative stress in cancer

In 1863 Rudolf Virchow described for the first time the involvement of inflammation in cancer progression. He observed that infiltrated immune cells reflect the place where cancer lesions appear in the inflamed tissue and hypothesized that chronic inflammation is a condition that predisposes to cancer development (Virchow 1989). Several years later Colotta et al., supposed that cancer-related inflammation is a key component of tumors and may represent the seventh hallmark of cancer proposed by Hanahan and Weinberg in 2000 (Colotta et al. 2009).

Inflammation is the immune system's response to infection and injury that leads to removal of offending factors and restoration of tissue structure and physiological function. In the acute phase, leukocytes, primarily granulocytes, migrate along a chemotactic gradient to the site of injury mediated by cytokines and acute-phase proteins. This process causes the cardinal signs of acute inflammation: rubor (redness), calor (heat), tumor (swelling) and dolor (pain) (Ricciotti and Fitzgerald 2011; Germolec et al. 2018).

The usual outcome of the acute inflammatory program is successful resolution and repair of tissue damage rather than persistence and dysfunction of the inflammatory response, which can lead to the chronic phase with scarring and loss of organ function, vasodilation (passive hyperemia), increasing blood flow (active hyperemia) and vascular permeability (Brown 2019). Chronic inflammation is reported to contribute to numerous diseases, including arthritis, asthma, atherosclerosis, autoimmune diseases, diabetes, and cancer (Gabay and Kushner 1999; Germolec et al. 2018).

Prostaglandins play a key role in the generation of the inflammatory response. In particular, Prostaglandin E2 (PGE2) is a bioactive lipid that can elicit a wide range of biological effects associated with inflammation and cancer (Nakanishi and Rosenberg 2013). PGE2 is synthesized by phospholipases (PLAs), a family of enzymes that catalyze the hydrolysis of

membrane phospholipids liberating arachidonic acid (AA). Membrane-released AA is rapidly oxidized into the relatively unstable metabolite, PGG2, which is subsequently reduced to PGH2, both steps sequentially catalysed by the cyclooxygenase (COXs) enzymes. There are two major COX isoforms, COX-1 and COX-2. COX-1 is constitutively active and present within most cells in the body, whereas constitutive COX-2 expression is insignificant in normal cells and largely restricted to the kidney as well as areas of the central nervous system (Finetti et al. 2020c). COX-2 levels are highly inducible in many tissues by pro-inflammatory and mitogenic stimuli, including cytokines and growth factors (Wang and Dubois 2006). Once synthesized, PGH2 is rapidly converted into prostanoids by a panel of terminal synthases, comprised of three isoforms that include microsomal PGE synthase-1 (mPGES-1), mPGES-2 and cytosolic PGE synthase (cPGES) (Funk 2001; Nakanishi and Rosenberg 2013). mPGES-1 is frequently induced concomitantly with COX-2 by various proinflammatory stimuli to generate a transient spike in PGE2 levels. On the other hand, mPGES-2 and cPGES are constitutively expressed and functionally coupled with COX-1 to maintain basal levels of PGE2 (Murakami et al. 2000; Finetti et al. 2008).

The physiological activity of PGE2 and related prostanoids is mediated by the activation of a diverse group of downstream signaling cascades via seven transmembrane G-protein coupled receptors (GPCR). In particular PGE2 binds to members of the EP family of receptors that consist of four isoforms (EP1-4) and play a major role during inflammation (Funk 2001). They are coupled to Gα proteins that could be stimulatory ($G\alpha_S$) or inhibitory ($G\alpha_i$) subunits that can activate divergent downstream signaling pathways (Sugimoto and Narumiya 2007). EP receptors activate a range of intracellular signaling pathways that mediate the effects of PGE2 on cell functions. The EP1 receptor is coupled to the Gq protein subunit that is linked to phosphoinositide-PLC activation. This signaling leads to an increase of intracellular Ca^{2+} and PKC activation that finally induces gene transcription through the activation of nuclear factor of activated T cells (NFAT), NF-κB, and the MAPK pathways (Finetti et al. 2020c).

Instead, both the EP2 and EP4 receptors are linked to Gαs proteins that activate adenylate cyclase (AC) and generate cAMP which in turn activates the PKA pathway (Narumiya et al. 1999; Finetti et al. 2020b). PGE2 is able to promote colon cancer cell growth through EP2 signaling that involves the activation of PI3K, the protein kinase Akt and the β-catenin

signaling pathway (Castellone et al. 2005). Several studies have demonstrated that the EP2 receptor controls the progression of lung, skin and breast cancer (Finetti et al. 2020c).

The human EP3 gene encodes at least eight distinct EP3 splice variants (Kotani et al. 1997). The major EP3 splice variant is thought to be coupled to an inhibitory (Gi) protein. Therefore, the primary outcome of EP3 receptor signaling is inhibition of AC and activation of the Ras/Raf and MAPK signaling pathway (Sugimoto and Narumiya 2007; Ma et al. 2013; Finetti et al. 2020c). EP3 has been reported to mediate the carcinogenesis in numerous tumors with conflicting effects (O'Callaghan and Houston 2015).

Inflammation is a key component of the tumor microenvironment and acts as a key regulator of tumor promotion and progression by several mechanisms including acceleration of cell cycle progression and cell proliferation, evasion from apoptotic cell death, and stimulation of tumor neovascularization (Philip et al. 2004). Tumor tissue is composed not only of tumor cells, but also of immune cells, endothelial cells, and fibroblasts (Hanahan and Coussens 2012).

The tumor microenvironment (TME) is fundamental for tumor progression induced by inflammation and is an inducer for COX-2 over-expression (Ohtsuka et al. 2018).

Several evidences show that COX-2 is overexpressed in many types of tumors, where enhanced levels of angiogenic factors, such as VEGF, fibroblast growth factor-2 (FGF-2), HIF-1α and MMPs have been detected. These factors are able to induce tumor angiogenesis together with PGE2, that promotes new vessel formation of its own (Finetti et al. 2009), or through phosphorylation of FGF-2 receptor (FGFR-1) (Finetti et al. 2008). Moreover, it has been previously described in prostate, breast and colon cancer cells that PGE2 itself up-regulates COX-2 in a feedback positive loop, enhancing the pro-angiogenic effect (Madrigal-Martínez et al. 2019).

To date, it has been demonstrated in many works that inflammation and oxidative stress are tightly related in a sort of vicious circle, contributing to the pathophysiology of several debilitating illnesses, such as cardiovascular diseases, diabetes, neurodegenerative processes or cancer.

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the ability of cellular antioxidant mechanisms to detoxify the reactive intermediates or repair the resulting damage (Ferro et al. 2014).

ROS comprise of a family of short-lived molecules that can be produced by all vascular cell types, including endothelial cells, smooth muscle cells, adventitial fibroblasts and perivascular adipocytes (Kim and Byzova 2014). They are reactive molecules containing oxygen and most of them are produced at low levels by normal aerobic metabolism; in contrast, excessive accumulation of ROS leads to a condition of oxidative stress that may cause extensive oxidative damage to most cellular components, including proteins, lipids and DNA, conducing to pathophysiological consequences. Many reactive oxygen species, including superoxide (O_2•−), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), reactive nitrogen species (RNS), nitric oxide (NO) and peroxynitrite (ONOO-) are produced in biological systems. In particular, the superoxide anion (O_2•−) plays a key role in the overall effects of ROS. Even though O_2•− has a short half-life, it is highly reactive and it is the uppermost mediator in the propagation of detrimental oxidative chain reactions, being a precursor of all other major ROS found in biological systems (Ferro et al. 2014). In the vasculature, the most important enzyme system responsible for ROS production, in particular O_2•−, is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX). It is accepted that under physiological conditions, vascular NOXs have low activity, that can be increased in response to stimuli such as cytokines, growth factors, hyperlipidaemia and high glucose, thus leading to disruption of vascular homeostasis and resulting in pathology (Konior et al. 2014).

ROS have long been associated with cancer because different types of tumor cells have been shown to produce elevated levels of ROS that are thought to be oncogenic, causing damage to DNA, proteins and lipids, promoting genetic instability and tumorigenesis. ROS also act as signalling molecules in cancer, contributing to abnormal cell growth, metastasis, resistance to apoptosis and angiogenesis (Moloney and Cotter 2018).

Several evidences suggest that ROS are necessary for cell movement: when specific receptors located at cell surface bind to extracellular stimuli, such as growth factors and chemoattractants, ROS are generated at the cell surface and intracellular compartments and react with specific proteins to regulate their activity and function (Xu et al. 2017). ROS may affect migration also through regulation of Matrix Metalloproteinases (MMPs), whose activity is implicated in regulation of cell adhesion, migration, processing of growth factors and cytokines, and liberation of angiogenic factors (Tabouret et al. 2016).

Depending on the concentration and duration of ROS exposure, ROS can both promote and inhibit cell proliferation. Indeed, depletion of the ROS generator NOX1 reduces cell proliferation, while its overexpression enhances proliferation in an H_2O_2-dependent manner (Mu et al. 2010; Xian et al. 2019).

Neovascularization and angiogenesis are ROS-sensitive processes that combine many mechanisms including proliferation, migration and adhesion. VEGF is the most potent and primary endothelial specific angiogenic growth factor, in both physiological and pathological conditions, and its signalling pathway seems to be affected by ROS: it has been demonstrated that exogenous ROS stimulate the induction of VEGF expression in various cell types that in turn induces angiogenesis (Kim and Byzova 2014). In tumors, both tumor and stromal cells produce substantial amounts of ROS. In particular, it has been demonstrated that in prostate cancer NADPH oxidase-derived ROS cause HIF-1α activation with consequent release of VEGF (Masoud and Li 2015; Aggarwal et al. 2019).

The main function of antioxidant defences is to prevent the build-up of excessive ROS and maintain a redox balance. The antioxidant capacity of tumor cells scavenges excessive ROS, while maintaining pro-tumorigenic ROS levels, allowing the disease to progress and develop resistance to apoptosis (Peiris-Pagès et al. 2015). The overproduction of ROS in cancer has been shown to induce a variety of biological effects including enhanced cell proliferation, DNA damage and genetic instability, adaptation, cellular injury and cell death, autophagy and resistance to drugs. The outcome is dependent on the genetic background of the cancer, the types of ROS involved (O_2•-, H_2O_2, etc.) and the levels and duration of ROS exposure. However, toxic levels of ROS production in cancers are anti-tumorigenic resulting in an increase of oxidative stress and induction of tumor cell death (Storz 2005). For this reason, therapies used to eliminate ROS or to elevate ROS production may be potentially effective cancer therapies although it is a rather challenging concept (Moloney and Cotter 2018).

As mentioned before, ROS and oxidative stress originating from various sources, like chemicals, drugs or other agents, can lead to inflammation by activating a variety of redox-sensitive transcription factors, which drive the expression of pro-inflammatory genes, leading to induction of various cytokines and chemokines, including MAPK and NF-κB, the master regulator of cellular inflammatory response. NF-κB controls the activity of numerous genes crucial for immunity, inflammation and stress responses, including TNF-α,

iNOS, COX-2 and PI3K/Akt (Yamamoto and Gaynor 2004; Natarajan et al. 2018). In summary, ROS can induce post-translational modifications of proteins involved in important redox-sensitive signaling transduction pathways that modulate inflammatory and angiogenic responses (Figure 1.3). At the same time, ROS production can result from activation of immune and endothelial cells by pro-inflammatory stimuli, including COX-2 (Holmström and Finkel 2014). The dysfunction of ROS-generating organelles and enzymes leads to ROS over-production, which induces oxidative stress. The kinases and phosphatases can be activated or inhibited by ROS and subsequently regulate the expression of transcription factors. However, whether ROS promote or suppress cancer development depends on cell context (Gào and Schöttker 2017).

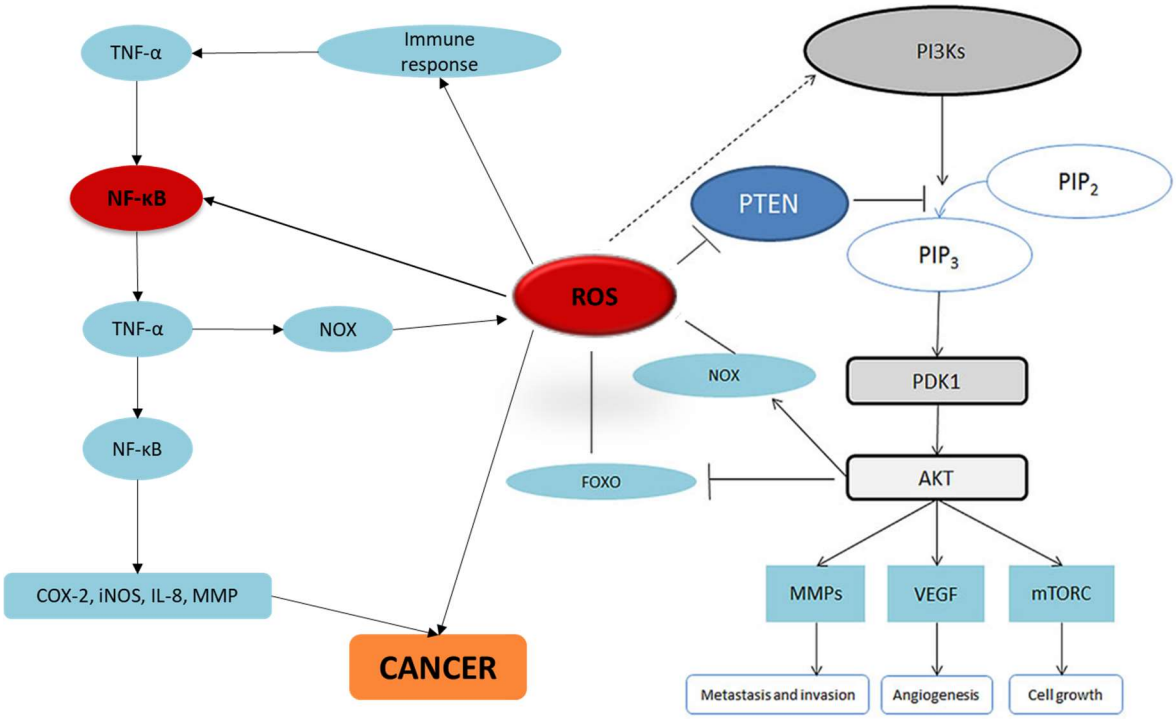


Figure 1.3. *Example of reduction–oxidation pathways involved in cancer development. (Figure modified from Gào and Schöttker 2017; Vallée et al. 2019)*

1.2 Natural compounds in cancer therapy

Natural compounds (sometimes called phytochemicals or phytonutrients) are biologically active substances present in plants (pigments) that can be classified in several groups such as carotenoids, flavonoids, anthocyanins or terpenoids based on their chemical structure, the pathway they activate, and their function. Some of these are found also in mushrooms, bacteria or marine organisms (Rejhová et al. 2018).

The main source of natural compounds is food, that is both a source of macronutrients (proteins, fats, carbohydrates) and micronutrients (vitamins, minerals), but it also contains a complex mixture of bioactive compounds of that our organism can benefit, even if usually the ingested dose may not represent an effective amount useful to obtain a real effect (Nobili et al. 2009).

Food items that are fortified with nutrients such as vitamins and minerals to ensure proper nutrient levels are “nutraceuticals”. The term arises from the integration of “nutrition” and “pharmaceutical” and refers to substances related to nutrition, endowed with physiological benefits and associated with prevention and/or protection against chronic diseases. Nutraceuticals include mixtures and pure compounds isolated from herbs, as well as dietary derivatives, transformed foods such as cereals, spices, condiments and beverages with beneficial effects on health (Koyande et al. 2019). Nutraceuticals can be used to improve health, prevent chronic diseases, increase life expectancy, delay aging processes or provide structural/functional support to the body (Adefegha 2018).

Several epidemiological and intervention studies reported the protective anti-inflammatory and antioxidant actions of bioactive compounds. Thus, a way to prevent inflammation which can lead to carcinogenesis is through the use of bioactive food compounds with both antioxidant and anti-inflammatory properties (Teodoro 2019). It is believed that, besides metabolic syndrome and obesity, the occurrence of two other of the major pathological conditions, cardiovascular diseases and cancer, are also strictly dependent on dietary and lifestyle factors (Koyande et al. 2019). We know that many tumors (almost 30%) can be prevented by lifestyle changes. Behavioural studies suggest that the promotion of healthy dietary habits and exercising belong to successful strategies and could help oncology through diverse actions (Albini et al. 2012). For this reason, since ancient time, natural compounds have occurred in the treatment and prevention of cancer

and inflammation disease, especially in traditional Chinese medicine or Indian Ayurveda. Many current anti-cancer drugs also originate from natural sources - irinotecan, vincristine, etoposide and paclitaxel are classic examples of plant-derived compounds (Nobili et al. 2009). Some have the ability to modulate signaling pathways and regulate the expression of genes involved in cell cycle regulation, cell differentiation and apoptosis (Pan et al. 2011), others have the function of ROS scavengers (Abdel lateif et al. 2016). Finally, since natural compounds target multiple signaling pathways, the combination of them with conventional therapy may overcome altered regulatory cell pathways, which may be responsible for drug resistance mechanisms. Therefore, the use of bioactive compounds could be a promising approach to minimize adverse effects associated with conventional chemotherapy.

1.2.1 Avenanthramides

Oats are cereal grain crops belonging to the family of Poaceae (or Gramineae) (Ben Halima et al. 2015). Two main species of oat grow naturally, namely, *Avena sativa*, known as common oat, and *Avena nuda*. Besides containing high levels of phenolic acids, tocopherols, and alk(en)ylresorcinol derivatives, oats are a unique source of avenanthramides (Avns) which are not present in other cereals (Mattila et al. 2005). Avenanthramides are low molecular weight phenolic amides (also known as N-cinnamoylanthranilate alkaloids or anthranilic acid amides), consisting of an anthranilic acid linked to a hydroxycinnamic acid with an amide bond. These secondary metabolites are constitutively expressed in the bran and outer layers of the oat kernel and were originally identified as phytoalexins involved in the plant defence mechanisms against certain pathogens, such as fungi (Collins 1989).

Oats contain a unique group of approximately 40 different types of Avns: the most abundant are Avn-A (N-(4’-hydroxycinnamoyl)-5-hydroxyanthranilic acid), Avn-B (N-(4’-hydroxy-3’-methoxycinnamoyl)-5-hydroxyanthranilic acid), and Avn-C (N-(3’-4’-dihydroxycinnamoyl)-5-hydroxyanthranilic acid) (Figure 1.4), which are amides of 5-hydroxyanthranilic acid with p-coumaric, ferulic, and caffeic hydroxycinnamic acids, respectively (Miyazawa et al. 1996; Okazaki et al. 2004; Meydani 2009).

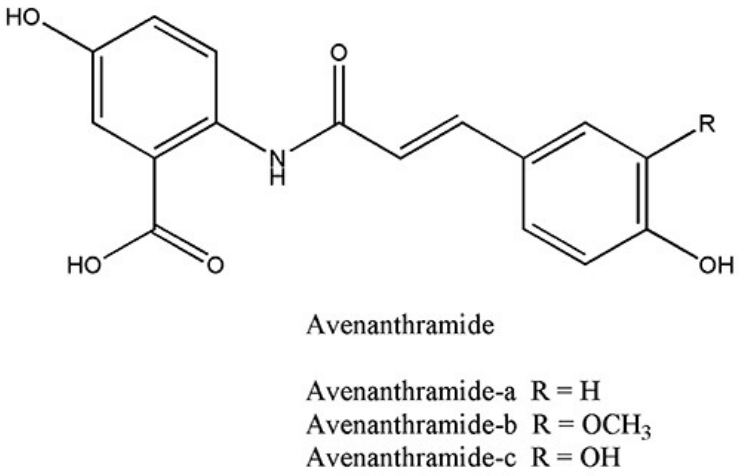


Figure 1.4. *Molecular structure of oat avenanthramides. The structure of Avns differs for the functional group in R position.*

Avns exhibit structural similarity with Tranilast, a derivative of the amino acid tryptophan (N-[3',4'-dimethoxycinnamoyl]-anthranilic acid), a well-known drug, identified as an anti-allergic agent, and used in the treatment of inflammatory diseases (Platten et al. 2005). Recently Tranilast was found to be effective in a variety of disease states, such as fibrosis, proliferative disorders, cancer, cardiovascular problems, autoimmune disorders, ocular diseases, diabetes and renal diseases. Moreover, several trials have shown that Tranilast has very low adverse effects and it is generally well tolerated by patients (Darakhshan and Pour 2015; Wang et al. 2020).

Although they are not essential nutrients, Avns have been found to possess pleiotropic bioactivities, including antioxidant, anti-inflammatory, anti-proliferative, anti-fibrotic, anti-itching and anti-atherogenic properties (Figure 1.5), with consequent major beneficial health effects. Avns may prevent or limit cellular oxidative dysfunctions and the development of oxidative stress-related diseases such as cardiovascular diseases, cancer and diabetes. In particular, accumulated evidence demonstrate that oat Avns have the potential to slow the progression of cancer by targeting and modulating different signaling pathways (Amawi et al. 2017; Finetti et al. 2018; Perrelli et al. 2018; Turrini et al. 2019). Avns extracted from oats and semi synthetic derivatives exhibit potent antioxidant properties *in vitro* and *in vivo*, 10-30 times greater than those of other oats-derived phenolic antioxidants (Dimburg et al. 1993). Avn-C often comprises about one-third of the

total concentration of Avns in oat grain (although the relative proportion of Avns is highly variable), and shows the highest antioxidant activity *in vitro* (Peterson et al. 2002).

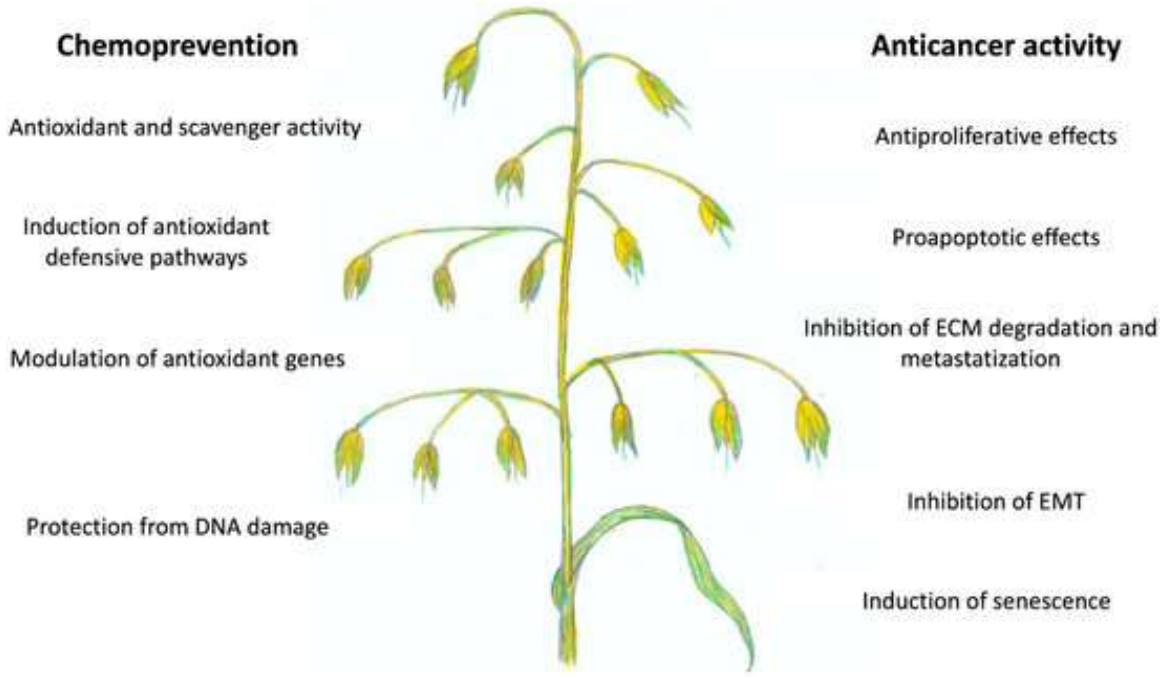


Figure 1.5. *Chemopreventive and anticancer activity of Avns from oat (from Turrini et al. 2019).*

The antioxidant activity of Avn-enriched extracts of oats has been correlated with increase of superoxide dismutase (SOD) and glutathione peroxidase activity (Ji et al. 2003) and attenuated the exercise-induced production of ROS (O'Moore et al. 2005). *In vivo* studies confirm the antioxidant and protective activity of Avns, suggesting an involvement in the *in vivo* defence against oxidative stress damage (Ji et al. 2003; Ren et al. 2011; Turrini et al. 2019).

In addition, Avn compounds may affect cellular components, not only through their antioxidant activity, but also through their interactions with specific molecular and signaling pathways that govern cellular responses during inflammation. In fact it have been shown that Avn-A possesses anti-inflammatory effects by inhibiting IL1β- and TNF-α-stimulated NF-κB activation in human aortic cells and keratinocytes, (Guo et al. 2008; Sur et al. 2008) decreasing the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in vascular endothelial cells, and suppressing the production of several inflammatory cytokines and chemokines including IL-6, IL-8, and

monocyte chemoattractant protein-1 (MCP-1) in skeletal muscle cells (Yeo et al. 2019). These effects of Avns are reported to be mediated through inhibition of NF-κB by up-regulation of NAD+ levels and the expression of COX-2 in lung tissues (Guo et al. 2008). Moreover, recent studies have shown that Avns supplementation in humans could attenuate exercise-induced inflammatory markers, including plasma TNF-α and IL-6 levels, NF-κB activation in neutrophils, and ROS generation in monocytes. Thus, Avns seem to be capable of acting on multiple cell types and inhibiting the NF-κB-induced inflammatory pathway. Several studies have also reported that Avns could inhibit NF-κB up-regulation under inflammatory and oxidative stresses (Ji et al. 2003; Guo et al. 2008). Avns suppressed TNF-α-induced muscle cell atrophy, along with inactivation of the NF-κB pathway and reduction of proinflammatory cytokines (Kang et al. 2018).

Avns are able to modulate several events specifically involved in cancer development, such as apoptosis, cell proliferation and metastatization, as proved by many mechanistic studies evaluating their anticancer activity in various cancer cell lines and in animal models (Turrini et al. 2019).

In particular, Avns inhibit cancer cell proliferation (Nie et al. 2006), through modulation of cell cycle regulatory proteins such as p53, p21, p27, cyclin-D1, and pRb at the G1 to S phase transition (Meydani 2009; Moglia et al. 2015; Finetti et al. 2018). Same results were obtained in colon cancer cells where Avns induce an antiproliferative effect independently of COX-2 expression and PGE2 production (Guo et al. 2010a). Moreover in mouse primary macrophages, Avns didn't affect the expression of COX-2, but down-regulated its activity and the production of PGE2 (Guo et al. 2010a; Turrini et al. 2019). Taken together, these results suggest that Avns exert their effect on colon cancer cells through mechanisms both dependent and independent of inflammation. Moreover, other studies confirmed the ability of Avns to inhibit proliferation in several colon cancer cell lines as Caco-2 and HepG2 (Scarpa et al. 2018) and HT29 and Widr (Finetti et al. 2018). Avns are also able to induce apoptosis by increasing Caspase 3, 8 and 9 and by activating the extrinsic apoptotic pathway via caspase-8 (Scarpa et al. 2018).

The induction of cellular senescence is another potential mechanism postulated for the tumor-suppressive activity of Avns in colon cancer cells via the activation of miR-129-3p/Pirh2/p53 signaling pathway (Fu et al. 2019b).

By engineering a *Saccharomyces cerevisiae* strain with two plant genes encoding key proteins involved in the biosynthesis of phenolic esters, two yeast-derived recombinant Avns (YAvns), namely N-(4'-hydroxycinnamoyl)-3-hydroxyanthranilic acid (YAvnI) and N-(3'-4'-dihydroxycinnamoyl)-3-hydroxyanthranilic acid (YAvnII) were recently produced (Moglia et al. 2015; Finetti et al. 2018). YAvns share structural similarity with two major oat Avns, respectively YAvnI with Avn-A and YAvnII with Avn-C (Figure 1.6), differing only in the position of the hydroxyl group in the anthranilic moiety (Moglia et al. 2010) suggesting enhanced functional and nutraceutical properties (Finetti et al. 2018).

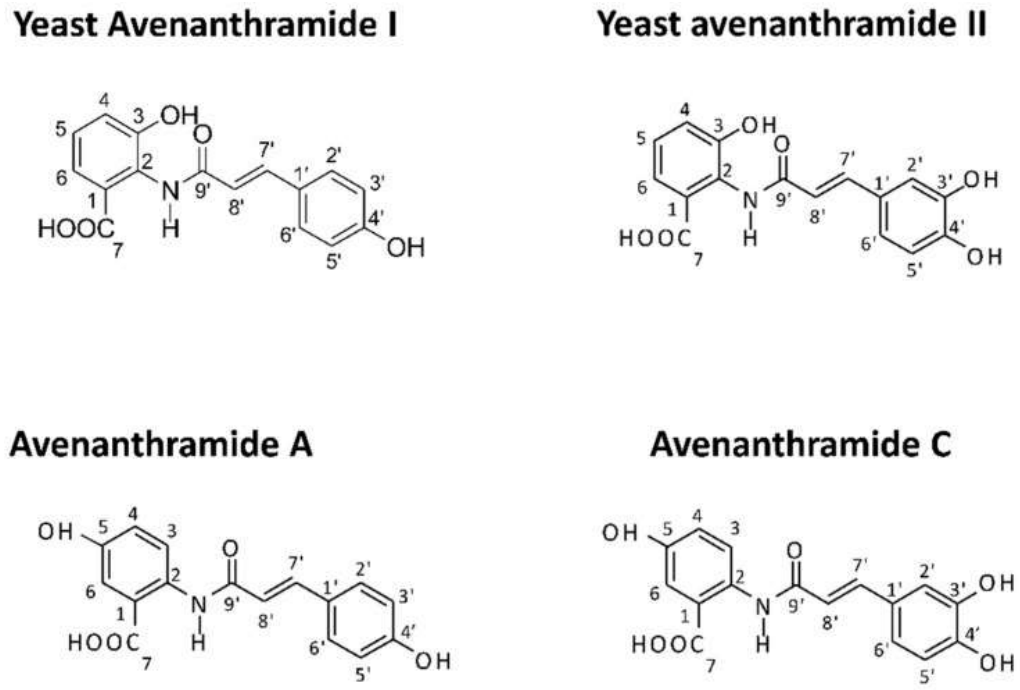


Figure 1.6. *Molecular structure of yeast (YAvns) and oat avenanthramides. The structure of YAvnI and YAvnII differ from Avn-A and Avn-C respectively in the position of the hydroxyl group relative to the amide bond. (Figure from Finetti et al. 2018)*

Both natural Avns and YAvns were effective in the inhibition of tumor cell growth and survival, even if YAvns resulted more effective in blocking EMT and reducing tumor cell migration, responsible of cancer metastatization (Figure 1.5) (Turrini et al. 2019).

Furthermore, they were shown to possess bioactive properties relevant to biomedical applications, including a potent antioxidant activity related to their capacity of stimulating master regulators of cellular antioxidant responses (Moglia et al. 2010; Lee et al. 2018), as well as putative anti-inflammatory and antiproliferative properties related to their capacity

of inhibiting NF-κB activation (Goitre et al. 2017) and Cyclin D1 expression (Moglia et al. 2010).

The main hallmark of EMT is the loss of epithelial surface markers, mainly caused, as previously reported, by the modification of proteins such as E-cadherin and vimentin (Suarez-Carmona et al. 2017). YAvns resulted more effective in down-regulating E-cadherin compared to natural Avns. The modulation occurred through the down-regulation of the protein Snail1 and the lymphoid-enhancing factor-1 (LEF-1), two transcriptional factors that facilitate the transition from the epithelial to the mesenchymal state via E-cadherin suppression (Onder et al. 2008; Finetti et al. 2018).

Recent studies suggested an inverse relationship between long term intake of oats diet and incidence of colon cancer demonstrating that Avns were active in reduction of tumor size (Guo et al. 2010b; Fu et al. 2019a). Tumor cells reside in abnormal microenvironments with hypoxia, nutrient depletion and low extracellular pH, which constitute a pathological barrier to the delivery of therapeutic agents to tumors (Dehne et al. 2017). Avns were effective in these stress conditions, thanks to good penetration characteristics (Fu et al. 2019a). Avns are active on the modulation of two pro-survival genes in cancer cells: the VEGF and HIF-1α. Both VEGF and HIF-1α allow cancer cells coping with oxidative stress and unbalanced redox status arising from the rapid growth and scarcity of oxygen and nutrients of advanced tumors. Cancer cells can contrast oxidative stress by the up-regulation of HIF-1α and VEGF, which provides more oxygen to cancer cells (Gorrini et al. 2013). Avns significantly down-regulated the expression of VEGF and HIF-1α on both Caco2 and HepG2 cancer cells, representing a convergent anti-survival effect (Scarpa et al. 2018; Turrini et al. 2019). Avn-C showed also a strong suppressive effect on COX-2 expression under hypoxia (Lim and Kang 2020) and Avns down-regulated the expression of HIF-1α gene in cancer cell lines (Kang et al. 2018). Avn-C inhibits hypoxia-induced COX-2-expression via SIRT1 in A549 cells. This inhibition of COX-2 probably contributes to the anti-inflammatory and chemopreventive properties of Avn-C under hypoxic conditions (Lim and Kang 2020).

Finally, it is well documented that tumor cells characteristically require more ATPs than cellular stress responses or harsh microenvironment, which is closely related to mitochondria (Nakazawa et al. 2016), a promising therapeutic target against tumors. Avns showed to induce mitochondrial swelling and bioenergetics imbalance, causing a reduction of expression of electron transport chain complexes subunits (Fu et al. 2019a).

In conclusion, Avns properties rely on their chemical structure that provides antioxidant activity and modulates cellular and molecular events involved in multiple stages of cancer development, potentially for both preventive and therapeutic intervention. However, clinical trials and safety studies with oat preparations or Avns are not yet sufficient to support their efficacy in patients at cancer risk and this aspect can be considered as an emerging science.

1.2.2 Beans

Phaseolus vulgaris L. (common bean) is the most important edible legume in the diet and gastronomy of many countries in the world. Numerous species of *Phaseolus* are cultivated and more than 12 million tons of dry beans are produced worldwide (Suárez-Martínez et al. 2016). *Phaseolus vulgaris* exists in many variations regarding growth characteristics, maturation and adaptation, accounting for more than 40,000 varieties. Common beans play an important role in human nutrition as a fundamental source of plant proteins, unsaturated fatty acids, minerals, dietary fibers and vitamins (Ombra et al. 2016; Ganesan and Xu 2017). Moreover, in the past years beneficial effects for human health have been described and have been associated to high content in phenolic compounds. In the majority of characterized bean extracts, the phenolic content is mainly represented by phenolic acids, hydroxycinnamic acids, flavones, flavanols, flavanones, isoflavonoids, anthocyanins, chalcones and dihydrochalcones (Ganesan and Xu 2017; Yang et al. 2018). The consumption of beans has received increased attention due to their beneficial health effects in the prevention and control of numerous chronic and degenerative diseases that are the main causes of mortality in the world. In particular, beans consumption has proven to be effective in reducing the risk of cardiovascular diseases for the antioxidant, anti-inflammatory and hypolipidemic properties (Mensack et al. 2012), in obesity and diabetes for the presence of α-amilase inhibitors and phytohemagglutinin and for the presence of starch (Thompson et al. 2012), and in cancer (Mensack et al. 2012; Hayat et al. 2014; Suárez-Martínez et al. 2016; Ganesan and Xu 2017).

Several epidemiological studies suggest a link between a diet rich in beans and reduced risk of numerous types of cancer. Bean consumption for two or more times per week reduced

the risk of colon cancer (up to 47%) (Correa 1981) and prostate cancer (about 22%) (Kolonel et al. 2000).

The anticarcinogenic activity of beans is related to the presence of resistant starch, soluble and insoluble dietary fibers, phenolic compounds, as well as other microconstituents including phytic acid, protease inhibitors, and saponins (Hayat et al. 2014). The fermentation of resistant starch results in the production of short chain fatty acids, principally butyrate, that have protective effect against colon cancer. Butyrate has been reported to induce apoptosis, growth arrest, and differentiation in colon cancer cell lines. These data were confirmed in *in vitro* cellular models (HT29, MCF-7 and HepG2) where bean extracts exert antiproliferative activity through activation of cytochrome c or by a caspase-independent path (Haydé et al. 2012; Chan et al. 2016; Moreno-Jiménez et al. 2019). It has also been demonstrated that beans modulate expression of genes related to growth inhibition and apoptosis mediated by p21 and JNK and induce the down-regulation of NF-κB in HT29 cells. p53 was down-regulated by common beans as well (Campos-Vega et al. 2010).

In breast cancer, bean feeding seems to affect several lipid metabolic pathways that in turn affect both PKC and mTOR signalling or modulate eicosanoid metabolism. This implicates involvement of COX-2, the rate-limiting step in eicosanoid degradation which is catalysed by prostaglandin dehydrogenase, a catabolic enzyme that exhibits potent tumor suppressor functions (Mensack et al. 2012).

In fact, *in vivo* models showed that in mice fed with basal diet and common beans there was an up-regulation or facilitation of apoptosis and cell cycle arrest during initial events of carcinogenesis on colorectal cancer that may increase the elimination of mutated cells (Hangen and Bennink 2002; Feregrino-Perez et al. 2014).

Regarding to antioxidant and antimutagenic activity of bean phenolic compounds, they have the ability to restrain the development of initiating free radical species by chelating metal ions or inhibiting enzymes that are involved in the radical production process (Hayat et al. 2014). In addition to this, phenolic compounds are also reported to act as antimutagenic agents by inhibiting the potential mutagens such as aflatoxins, nitroarenes, and polycyclic aromatic hydrocarbons. Common beans result to be antioxidant and antimutagenic in a dose and chemical structure dependant manner (Hayat et al. 2014).

In conclusion, *P. vulgaris* is a good source of bioactive compounds and recent evidence provide information of their impact and mechanism of action on several pathologies and in cancer progression. Further researches are required regarding the implications and the molecular mechanisms by which common beans and their bioactive compounds modulate the development of different types of cancer.

2. AIM OF THE THESIS

The term neoplasm or tumor indicates an abnormal mass of tissue, which growth is uncoordinated and persists after cessation of the stimuli that evoke the change (Willis 1952). Tumor formation, called tumorigenesis, or carcinogenesis, is a complicated and multistep process that drives the progressive transformation of normal human cells into highly malignant derivatives through genetic alterations (Bertram 1984), in which successive mutations in oncogenes and tumor-suppressor genes virtually result in enhanced proliferation and resistance to cell death (Hanahan and Weinberg 2011).

The multistep carcinogenesis involves activation of multiple cellular oncogene pathways (Vogelstein and Kinzler 1993). Normally, cellular proto-oncogenes function as positive regulators of cell growth, but minute alterations at the level of expression, subtle mutations or fusions can trigger increased cellular proliferation. The growth-promoting activities of protooncogenes are checked by a group of genes that act as negative growth regulators, the tumor suppressors. They activate expression of proliferation-inhibiting and apoptosis-promoting proteins in response to DNA damage (Chow 2010; Kontomanolis et al. 2020), and their control over cell division is lost with genetic alterations leading to their inactivation (Vogelstein and Kinzler 2004).

During the past decades, focus on cancer research has shifted from the malignant cancer cell itself to the tumor microenvironment, which consists of resident fibroblasts, endothelial cells, pericytes, leukocytes and extracellular matrix, and also contributes to the progression of cancer (Hanahan and Coussens 2012). Inflammation is a key component of the tumor microenvironment and acts as a key regulator of tumor promotion and progression by several mechanisms including acceleration of cell cycle progression and cell proliferation, evasion from apoptotic cell death, and stimulation of tumor neovascularization (Philip et al. 2004; Colotta et al. 2009).

To date, it has been demonstrated in many works that inflammation and oxidative stress are tightly related in a sort of vicious circle, contributing to the pathophysiology of several debilitating illnesses, such as cardiovascular diseases, diabetes, neurodegenerative processes or cancer (Aggarwal et al. 2019).

Seen the complexity of the several pathways involved in tumor progression and the well-known resistance to anticancer drug, current researches focus on discover of new targets and new approaches for cancer treatment (Ke and Shen 2017).

Natural and nutraceutical compounds have historically been used in the treatment of cancer and inflammation diseases (Adefegha 2018; Koyande et al. 2019). In combined therapy, the addition of natural compounds may overcome altered regulatory cell pathways that may be responsible for drug resistance mechanisms and could minimize adverse effects associated with conventional chemotherapy. Moreover, several epidemiological and intervention studies reported the protective anti-inflammatory and antioxidant actions of bioactive compounds.

The aim of this thesis has been to investigate the activity of natural compounds on tumor progression and the identification of new possible molecular targets for cancer therapy.

To accomplish this aim, the experimental work has been organized in two different tasks:

- **Task I: antitumoral activities of natural compounds**
    - Part I. **Effects of Avenanthramides in lung cancer progression induced by EGF**. Epidermal growth factor receptor (EGFR) and its ligands are frequently up-regulated in human cancers. The oncogenic effects of EGFR include enhanced cell growth, invasion and metastasis, and down-regulation or inhibition of EGFR signaling has therapeutic benefit in clinical practice. However, the clinical effects of EGFR inhibition may be lost after few months of patient treatment due to the onset of chemioresistence (Lui and Grandis 2002). In this work of thesis, we evaluated the anticancer activity of Avns focusing on EGFR signaling pathway. In particular lung cancer cellular models have been used to evaluate the activity of Avns on: tumor growth, migration, EMT and Anoikis induced by EGFR activation. In addition, the role of Avns on EGFR phosphorylation has been investigated.
    - Part II. ***Phaseolus vulgaris* L. Var. Venanzio and colon cancer**. We have studied for the first time an endangered Italian variety of *P. vulgaris*, grown in a restricted area of the municipality of Murlo (Siena, Tuscany) named “Fagiola di Venanzio” (FV). Here, to characterize the composition and the antitumor activities of *P. vulgaris* beans, we explored the chemical composition of FV extracts and then we studied antioxidant, anti-

inflammatory and antiproliferative activity on colorectal cancer cellular models (HT29 and HCT166) using endothelial cells (HUVEC) as comparative model of non-cancer cells.

- **Task II: identification of new molecular targets involved in tumor progression**. This task is based on the hypothesis that by studying non-cancer pathologies, it is possible to identify signalling molecules that are able to regulate cellular activities, as proliferation, migration and EMT, typical hallmarks of cancer. From this hypothesis came the idea of studying the involvement in cancer progression of KRIT1, a protein well known in our laboratory for its involvement in Cerebral Cavernous Malformations (CCM), a rare vascular disease typically found in the central nervous system (Couteulx et al. 1999; Sahoo et al. 1999). Based on its ability to regulate several signaling pathways and its ubiquitous expression, KRIT1 is likely to be involved in others pathological conditions than CCM. To date, very few studies have demonstrated that KRIT1 is implicated in other pathologies as cardiovascular disease (Sega et al. 2019), diabetes (Antognelli et al. 2018), intestinal epithelial barrier dysregulation (Wang et al. 2019) and cancer (Orso et al. 2013a; Abou-Fadel et al. 2020). In particular, the involvement of KRIT1 as tumor suppressor in cancer has been hypothesized since the oncogenic miR-21 expression anticorrelates with KRIT1 (Orso et al. 2013a). In fact, it is known that in CCM pathology loss of KRIT1 induces proliferation, migration, up-regulation of VEGF, reduction of apoptosis, loss of cell-cell junction, pro-inflammatory status, altered redox homeostasis and activation of growth factors receptor signalling (Finetti et al. 2020a). To this aim, we first analysed data banks to check the association score between *Krit1* gene and several types of cancer to found the best one in which study this protein.

  As Cutaneous Malignant Melanoma (CMM), showed to have one of the higher association score, we decided to test the role of KRIT1 in this tumor type starting from the analysis of protein expression in human samples by immunohistochemistry and Western blot analysis.

  To clarify the role of KRIT1 in cancer progression, we used A375 melanoma cell line silenced for KRIT1 in order to evaluate proliferation, invasion and migration and biomarkers of processes related to cell proliferation and migration, including matrix

metalloproteinases (MMPs, degradation of extracellular matrix), N-cadherin, vimentin and β-catenin (EMT markers) and Cyclin D1 and p27. To determine a link between inflammation, KRIT1 expression and cancer progression we measured the expression levels of COX-2 and mPGSE1.

3. MATERIALS AND METHODS

3.1 Cell culture

Adenocarcinomic human alveolar basal epithelial (A549), epidermoid squamous cell carcinoma (A431) human colon cancer (HCT116) and melanoma (A375) cell lines were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), with 4500 mg glucose/l and 100 U/ml penicillin/streptomycin (Euroclone, Milan, Italy).

Human colon cancer (HT29) cells were grown at 37 °C and in 5% CO_2 in RPMI-1640 (Euroclone) medium supplemented with 10% FBS with 100 U/ml penicillin/streptomycin.

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Lonza, Basel, Switzerland). All experiments were performed on low passage cell cultures. Cells were grown on gelatin-coated dishes in Endothelial Growth Medium (EGM-2) (EBM-2, FBS 10%, VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000) (Lonza) at 37 °C and in 5% CO_2 supplemented with 10% FBS with 100 U/ml penicillin/streptomycin.

3.2 Chemicals

Stock solutions of Avn-C and Avn-A (Sigma-Aldrich, Milano, Italy) were prepared by dissolving compounds in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a final concentration of 0.1 M.

3.3 Preparation of Extracts of FV

Phaseolus vulgaris L. var. “Fagiola di Venanzio” (FV) dried seeds (beans), were harvested in the municipality of Murlo (Siena, Tuscany, Italy) and were identified by botanists in the Siena University Botanical Garden. Four samples of FV provided by different growers were used (Table 1).

Grower	# Sample
Società Agricola Aiellino	1
Nicola Ulivieri	2
Burresi family	3
Azienda Agricola Podere Vignali	4

Table 1. *"Fagiola di Venanzio" samples*

In order to preserve the whole phytocomplex of beans and to extract polyphenols, proteins and carbohydrates at the same time, the extractive procedure was accomplished by briefly soaking 10g of manually grinded beans in water at 50°C, discarding the liquid and then performing a maceration at 35°C with 100 mL of distilled water for 48 hrs. The extract was adjusted to a 1:10 final drug:extract ratio.

3.4 MTT assay

MTT assay was performed with all cell lines using different protocols, described below.

- A549 and A431 were plated (2.5 x 10^3 cells/well) in 96 wells multiplates in medium with 10% FBS. After 24 hrs, the cells were starved and then treated with Avn-A and Avn-C (10, 50, 100 µM) (Sigma-Aldrich,St. Louis, MO, USA) with and without EGF (25 ng/mL) (Peprotech, London, UK) for 48 hrs in 0.1% FBS. Gefitinib (10 µM) was used as negative control.
- HCT116 and HT29 were plated (respectively 2.5 x 10^3 and 3.5 x 10^3 cells/well) in 96 wells multiplates in medium with 10% FBS. After 24 hrs, the cells were starved and then treated in medium 0,1% FBS with different concentrations of the bean extract (5, 10, 50 and 100 µM) and then exposed to IL1β (Reliatech GmBH, Wolfenbüttel, DE) (10 ng/ml) for 24 hrs.
- A375 cells were plated (2.0 x 10^4 cells/well) in 96 wells multiplates in DMEM 10% FBS. After 24 hrs, the medium was removed.

Cells were then incubated for 4 hrs with fresh medium in the presence of 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich). The MTT solution was removed and 50 µL of DMSO were added to each well to dissolve the blue formazan crystals. The absorbance of the formazan dye was measured at 570 nm with a

microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Data were expressed as a percentage of the basal control.

The experiment was replicated three times. Each experiment was performed in triplicate.

3.5 Clonogenic assay

A549 were plated (2.5 x 10^2 cells/well) in 6-well multiplate in medium with 10% FBS. After 24 hrs cells were starved and then treated with Avn-A and Avn-C (10 and 100 µM) with and without EGF (2,5 ng/mL) for ten days in 0.1% FBS. Colonies (>50 cells) were fixed and stained with Diff-Quik, counted and photographed. Data were expressed as a percentage of the basal control.

The experiment was replicated three times. Each experiment was performed in triplicate.

3.6 Western Blotting

Western blot was performed with all cell lines by using different protocols, described below.

- A549 and A431 were plated (2.5 x 10^5 cells/well) in 6-well multiplates in medium with 10% FBS. After 24 hrs, cells were treated with Avn-A and Avn-C (10 and 100 µM) with and without EGF (25 ng/mL) for 48 hrs or for 15 minutes in 0.1% FBS.
- HT29, HUVEC and HCT116 (2.5 and 3.0 x 10^5 cells/well) were seeded in 6-well multiplates in medium added with 10% serum. After 6 hrs in medium (0.1% serum), cells were treated with extracts for 18 hrs (10 and 50 µM) and then exposed to IL1β (10 ng/ml) for 24 hrs.
- Cells silenced with siKRIT1 and siCTRL were seeded (5.0 x 10^5 cells/well) in 6-well multiplates in DMEM with 10% FBS for 24 hrs.

Then cells were lysated and briefly centrifuged at 15,000×g for 15 min at 4 °C. Protein concentration in cell extracts was determined spectrophotometrically using the BCA protein assay kit (Euroclone). For WB, cell extract supernatants containing an equal amount of proteins (50 µg) were treated with Laemmli buffer, boiled for 5 min, resolved on 4-20% stain-free gel and then blotted onto a nitrocellulose membrane using Novablot Semidry System (GE Healthcare Bio-Sciences). The blots were blocked with 5% nonfat dry milk (Euroclone) in Tris-buffered saline (TBS) containing 0.5% Tween 20 for 1hr at room temperature and incubated with appropriate dilutions of primary antibodies overnight at 4°C and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibodies

for 1 hr at room temperature. Proteins were then visualized by an enhanced chemiluminescence detection system (EMD Millipore, Burlington, MA, USA). The following antibodies were used: anti-β-actin (Sigma Aldrich), anti-β-catenin (Santa Cruz, Dallas, TX, USA), anti-COX-2 (Cell Signaling Technology, Danvers, MA, USA), anti-cyclin D1 (BioRad, Hercules, CA, USA), anti-E-cadherin (Cell Signaling Technology), anti-GAPDH (EMD Millipore), KRIT1 (EMD Millipore), anti-mPGES-1 (Santa Cruz), anti-N-cadherin (Bioss, Woburn, MA, USA), anti-p-Akt (Santa Cruz), anti-p-EGFR1068 (Genetex, Irvine, CA, USA), anti-p-Tyr (Cell Signaling Technology), anti-VEGF (EMD Millipore) or anti-vimentin (Cell Signaling Technology). Primary antibodies were detected using affinity-purified HRP-conjugated secondary antibodies (Sigma-Aldrich). Protein bands from Western blots were quantified by densitometry using the ImageJ software, and their relative amounts were normalized to the levels of housekeeping proteins serving as internal loading controls. Data were expressed as fold increase compared with control.

The experiment was replicated three times.

3.7 Migration assay

Scratch assay was performed with A549 and A375 cells using different protocols, described below.

- A549 cells were seeded (8.0 x 10^5 cells/well) in 24-well multiplate in DMEM 10% FBS. After adhesion, cells were starved and after 24 hrs cells were treated with Avn-A and Avn-C (100 μM) (Sigma-Aldrich) with and without EGF (25 ng/mL) (Peprotech) in DMEM 0.1% FBS.
- A375 cells were seeded (1.0 x 10^6 cells/well) in 24-well multiplate in DMEM 10% FBS.

Cell monolayers were scored vertically down the centre of each well with a sterile tip. Each well was washed with PBS to remove detached cells. Fresh medium (1% serum) with ARA C (Sigma–Aldrich, St. Louis, MO, USA) (2.5 μg/ml) to inhibit cell proliferation was added. Images of the wound in each well were acquired at time 0 and after 6, 12 and 24 hrs (magnification of 10X) as reported in section *Results.*

Results were expressed as arbitrary units of wound and percentage of healing taking as reference the area at time 0 (Finetti et al. 2012).

The experiment was replicated respectively three and two times. Each experiment was performed in triplicate.

3.8 Polyphenols Content

Total polyphenols content (TPP) of FV extracts was evaluated by Folin–Ciocalteau (FC) colorimetric

Assay. Briefly, 100 µL of extract were diluted to 3 mL with distilled water; 500 µL of 1:10 FC reactive in water (Sigma-Aldrich) were added and the mixture was gently shaken for 1 min. A quantity of 1000 µL of 30% w/v sodium carbonate water solution was added and, after incubation for 1 hr in the dark at RT, absorbance of samples was read at 750 nm, using distilled water as blank. Gallic acid (Sigma-Aldrich) was used as reference standard. A calibration curve was created using gallic acid 5000 to 78 mg/L.

3.9 Soluble Carbohydrates Content

Total soluble carbohydrates of extract were quantified using the acid phenol assay described for the first time by Dubois et al. (Dubois et al. 1951). A quantity of 100 µL of the supernatant was added to 190 µL of water and 100 µL of a 6% w/v phenol (Sigma-Aldrich) water solution. The solution was gently shaken for 30 s and 500 µL of concentrated sulfuric acid (Sigma-Aldrich) were added. The mixture was heated at 80° C for 15 min and cooled to RT. Absorbance was read at 490 nm. D-glucose (Sigma-Aldrich) was used from 80 to 1.25 mg/L as reference standard.

Total saccharide content was calculated interpolating the data on the calibration curve of D-glucose.

3.10 Protein Content

Total proteins of extracts were determined spectrophotometrically using the BCA protein assay kit (Euroclone). Briefly, 2 µL of different dilutions of FV extract were added to 100 µL of bicinchoninic acid and, after incubation at 37°C for 30 min, the absorbance was measured at 562 nm with a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Protein concentration was determined and reported with reference to standards of bovine serum albumin (BSA).

3.11 HPLC-DAD Analysis on Main Polyphenolic Constituents

HPLC-DAD analysis was performed by using a Shimadzu Prominence LC 2030 3D instrument equipped with a Bondapak® C18 column, 10 µm, 125 Å, 3.9mm300mmcolumn (Waters Corporation, Milford, MA, USA).

Water solutions containing 0.1 % (v/v) formic acid (A) and 0.1% (v/v) acetonitrile (B) were used as mobile phase. The following program was applied: B from 10% at 0 min to 35% at 20 min, then B 50% at 25 min; flux was set at 0.8 mL/min. Chromatograms were recorded at 254, 280, 330 and 350 nm. Analyses were performed using 10 µL of FV extract; gallic acid, chlorogenic acid, caffeic acid, catechin, genistein, daidzein, quercetin and kaempferol (Sigma-Aldrich) were used as external standards. Calibration curves were established using reference standards ranging from 0.008 mg/mL to 0.500 mg/mL. The correlation coefficient (R^2) of each curve was >0.99.

3.12 HPLC-DAD-DPPH (2,2-Diphenyl-1-picrylhydrazyl)

To evaluate the different role of FV polyphenols in exerting antiradical activity, the HPLC-DAD run described above was repeated after having incubated the FV extract with a 1×10^{-2} M DPPH (2,2-diphenyl-1-picrylhydrazyl) methanolic solution for 15 min. Each chromatogram peak area was compared before and after DPPH reaction. Ascorbic acid was used to validate the test.

3.13 ROS measurement

3.5 and 2.5 $\times 10^3$ cells/well (HT29, HCT116 and HUVEC) were seeded in 96-multiwell plates and, after adherence, maintained for 18 hrs in medium without phenol red (0.1% serum) with different concentrations of the extract of beans (10 and 50 µM) and then exposed to IL1β (10 ng/ml) for 24 hrs. DCFH2-DA (2,-7-dichlorodihydrofluorescein diacetate) (Invitrogen, Milan, Italy) was added (10 µM, 1hr) and intracellular levels of ROS were evaluated photometrically with a microplate reader (excitation/emission 495/527) (EnVision, PerkinElmer). Data were expressed as relative fluorescence units.

The experiment was replicated three times. Each experiment was performed in triplicate.

3.14 Human melanocytic samples analysis

Taking advantage of the rich collection of samples kept at the Section of Pathological Anatomy of the Azienda Ospedaliera Universitaria Senese, we choose 57 samples of melanocytic lesions with a 5-year minimum follow up. They included 5 benign common nevi (BCN); 5 dysplastic nevi (DN); 5 radial growth phase melanomas (RGPMM), 23 vertical growth phase malignant melanomas (VGPMM) and 11 melanoma metastases (MelMet). As inclusion criteria, we chose frozen samples with mutations for BRAF, NRAS, and C-KIT. Control samples were represented by healthy skin from the lesion margins.

3.15 Immunohistochemistry

As previously described (Ferrara et al. 2019), tissue slides were deparaffinised in xylene and dehydrated in ethanol. Microwave pre-treatment in 10 mM citrate buffer (pH 6.0) was performed for 30 min. Non-specific binding was blocked for 20 min with 2.5% normal horse serum (NHS). The slides were incubated with primary antibodies targeting KRIT1 (1:100; ThermoFisher, Waltham, MA, USA) followed by chromogenic visualization using ImmPress-AP (Vector). Sections were incubated for 15 min with NBT-BCIP added with Levamisole. After counterstaining with nuclear fast red (NFR), slides were washed thoroughly, dehydrated, cleared in xylene and mounted. Staining intensity was scored as negative (no staining) or positive (blue colour).

The experiment was replicated three times. Each experiment was performed in triplicate.

3.16 siRNA transfection

A375 cells were plated on 6 well multiplates and after adhesion transfected with 50 nM of control or KRIT1 siRNA (Qiagen, Hilden, Germania) using 4 µL of lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. 6 hrs post-transfection, cells were washed with PBS, and medium was changed. Cells were used for experiments 24 hrs post-transfection.

3.17 Proliferation assay

A375 cells were seeded in 96-well microplates (2.0 x 10^4 cells/well) in DMEM 10% FBS and after 12, 18 and 24 hrs fixed for 10 min with EtOH 70% and coloured with Coomassie Blue solution (10% Acetic acid, 10% ethanol and 0,25% Blue Coomassie).

The number of proliferated cells present in five fields/well was counted at 20X magnification. Data were reported as percentage of proliferating cells (Finetti et al. 2012). The experiment was replicated three times. Each experiment was performed in triplicate.

3.18 BrdU assay

A375 cells were seeded (5.0×10^3 cells/well) in a 96-well plate and incubated overnight. The next day, cells were transfected with 50 nM of control or KRIT1 siRNA using 0.3 µL of lipofectamine 3000 in 200 µL total volume of media, using the same method as previously mentioned. BrdU experiments were performed 24 hrs post-transfection, using a BrdU assay kit (Roche), and BrdU was added to the cells for 1 hr (as suggested by the manufacturer for this cell line). Next, cell culture medium was removed, the cells were fixed and the DNA was denatured by adding FixDenat (from kit). Then, anti-BrdU-POD, an anti-BrdU antibody conjugated with peroxidase, was added to the samples. This antibody binds to newly synthesized cellular DNA containing BrdU. Next, the cells were washed with PBS, and the peroxidase substrate was added. Absorbance was measured at λ 370 nm (reference wavelength: 492 nm) (Muresan et al. 2019).
The experiment was replicated three times. Each experiment was performed in triplicate.

3.19 Invasion assay

Chemotaxis experiments were performed using a trans-well system. The day before, the trans-well was coated with gelatin 0.25% (100 µL /well) and maintained overnight at 4°C. A375 cells were plated (1.0×10^5 cells/well) in coated trans-well in DMEM without FBS, and 650 µL of DMEM 10% FBS were added in the lower chamber. After 12, 18 and 24 hrs cells were fixed in EtOH 70%, coloured with Coomassie Blue solution (10% Acetic acid, 10% ethanol and 0,25% Blue Coomassie) and washed with DDI water. The number of migrated cells present in five fields/well was counted at 20X magnification. Data were reported as percentage of migrated cells above the control.
The experiment was replicated three times. Each experiment was performed in triplicate.

3.20 MMP activation assay

A375 (5×10^4 cells/well) were cultured in 96-well cell culture plates in 10% fetal bovine serum medium. After adhesion, cells were incubated with 50 µL of serum-free media. After

48 hrs, media were collected, clarified by centrifugation and assayed for zymography. Media were subjected to electrophoresis in 8% SDS-PAGE containing 1 mg/ml gelatin under non-denaturing conditions, by using Sample Buffer w/o β-ME. After electrophoresis, gel was washed with 2.5% Triton X-100 to remove SDS and incubated for 48 hrs at 37°C in 50 mM Tris buffer containing 200 mM NaCl and 20 mM $CaCl_2$, pH 7.4. Gels were stained with 0.05% Coomassie brilliant blue R-250 in 10% acetic acid and 10% ethanol and destained with 10% acetic acid and 10% ethanol. Bands of gelatinase activity appeared as transparent areas against a blue background. Gelatinase activity was then evaluated by quantitative densitometry (Finetti et al. 2008, 2020a) and normalized to the number of cells/well. The experiment was replicated three times.

3.21 Immunofluorescence Analysis

A375 cells (2.5 x 10^5 cells/well) were seeded on glass cover-slips. After 24 hrs cells were fixed with formalin for 10 min and permeabilized with PBS 0.25% Triton x100 for 10 min, incubated with 1% BSA for 30 min and stained overnight at 4 °C with primary antibody for β-catenin, Vimentin or N-cadherin (Santa Cruz). Slips were washed three times with PBS and then incubated 1 hr at room temperature with Alexa Fluor 568 or 488 secondary antibodies (ThermoFisher Scientific, Waltham, MA, USA). Nuclei were stained with 1 µg/ml DAPI (D1306 Invitrogen, USA) for 1 min after removal of secondary antibody. Microscopy imaging was performed on Olympus IX71/X51 (Olympus Life science) inverted microscope using a 60X objective. Data were expressed as fold increase compared with control.
The experiment was replicated three times. Each experiment was performed in duplicate.

3.22 Statistical analysis

Data were generated from three independent experiments and expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's t test for unpaired data; $p<0.05$ was considered statistically significant.

4. RESULTS AND DISCUSSION

4.1 Task I: Antitumoral activity of natural compounds

4.1.1 Part I: Effects of Avenanthramides in lung cancer progression induced by EGF

Avns have been found to possess pleiotropic bioactivities, including antioxidant, anti-inflammatory, anti-proliferative, anti-fibrotic, anti-itching, anti-atherogenic properties. In particular, accumulated evidence demonstrate that oat Avns have the potential to slow the progression of cancer by targeting and modulating different signaling pathways (Meydani 2009; Guo et al. 2010b).

Previous studies of our lab investigated the anticancer activity of Avns in colon cancer models (Finetti et al. 2018). In this work of thesis we further explored the Avns properties by analysing their effects on lung cancer (Liu et al. 2017).

EGFR activation is a key step in lung cancer progression (Liu et al. 2017) and its inhibition appears to be an important strategy in clinical setting (Liu et al. 2018). However, patients harbouring EGFR mutations become resistant to TKR inhibitors (Nagano et al. 2018). The development of new EGFR targeting drugs to combine with the existing drugs could be a promising strategy to counteract the onset of resistance phenomena.

To evaluate the effects of Avns on EGF signalling, we determined the activity of Avns on the viability of lung cancer cells. We treated A549 and H1299 cells with different concentrations of Avn-A and Avn-C (10, 50, 100 µM) with and without EGF (25 ng/mL) and we performed the MTT assay after 72 hrs of exposure. In all the assays, the anti-EGFR drug Gefitinib (10 µM) was used as positive control.

As shown in Figure 4.1.1, Avn-C was able to reduce growth of both A549 (A) and H1299 (B) cells. Even though the MTT assay does not distinguish between decreased levels of proliferation or increased cytotoxicity, previous evidence demonstrates that Avns can both inhibit cell proliferation and stimulate apoptosis (Guo et al. 2010b; Moglia et al. 2015). Interestingly, when lung cancer cells were treated with EGF, we observed an increased proliferation that was reduced in a dose-dependent manner by both Avn-A and Avn-C (Figure 4.1.1), indicating a possible inhibitory role of Avns in EGF signalling pathway.

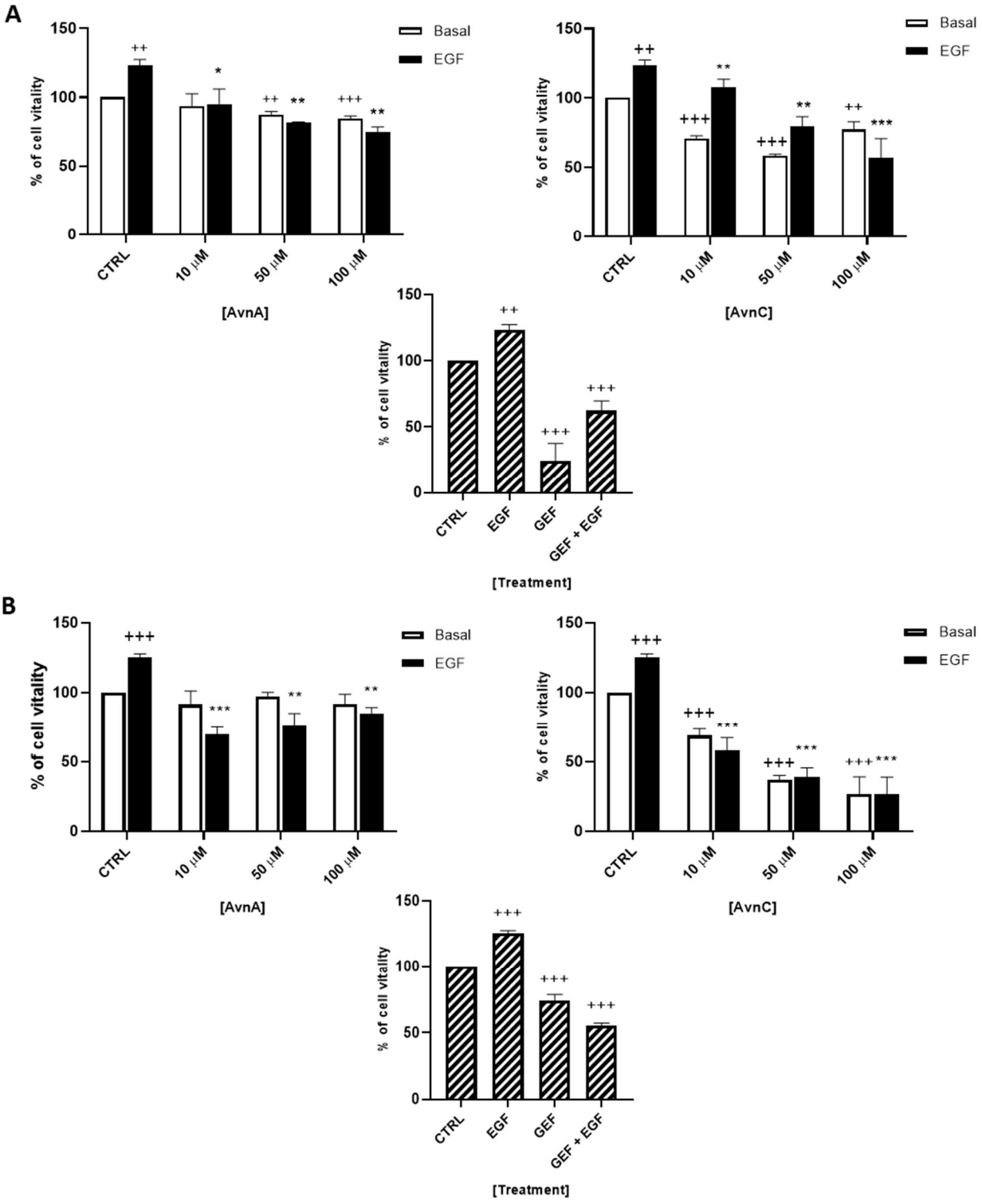


Figure 4.1.1. *Effects of natural Avns on lung cancer cell vitality. A549 (A) and H1299 (B) vitality was evaluated by the MTT assay. Cells were exposed to EGF in presence of increasing concentration of Avns (10, 50 and 100 μM) for 3 days. Data are expressed as % over basal control and are representative of three independent experiments run in triplicate.*
*Statistical analysis: +++ p<0,01; ++ p<0,1 and +p<0,1 vs CTRL; ***p< 0,01; ** p< 0,1 and *p<0,5 vs EGF.*

To confirm the antiproliferative role of Avns, we performed a clonogenic assay. To this end, cells were incubated with Avn-A and Avn-C, at different concentrations (10 and 100 µM) with and without EGF (25 ng/mL) for 10 days. Experimental outcomes showed that Avn-C was able to significantly restrain the colony formation of cancer cells with results comparable with Gefitinib, indicating that this compound is indeed effective in inhibiting proliferation and colony formation ability of lung cancer cells. Interestingly both Avns are able to inhibit the EGF-induced colony formation, indicating a possible activity on EGF signaling pathway (Figure 4.1.2 A and B).

Both data on cell growth and colony formation clearly indicate that Avn-A and Avn-C are able to inhibit lung cell growth induced by EGF.

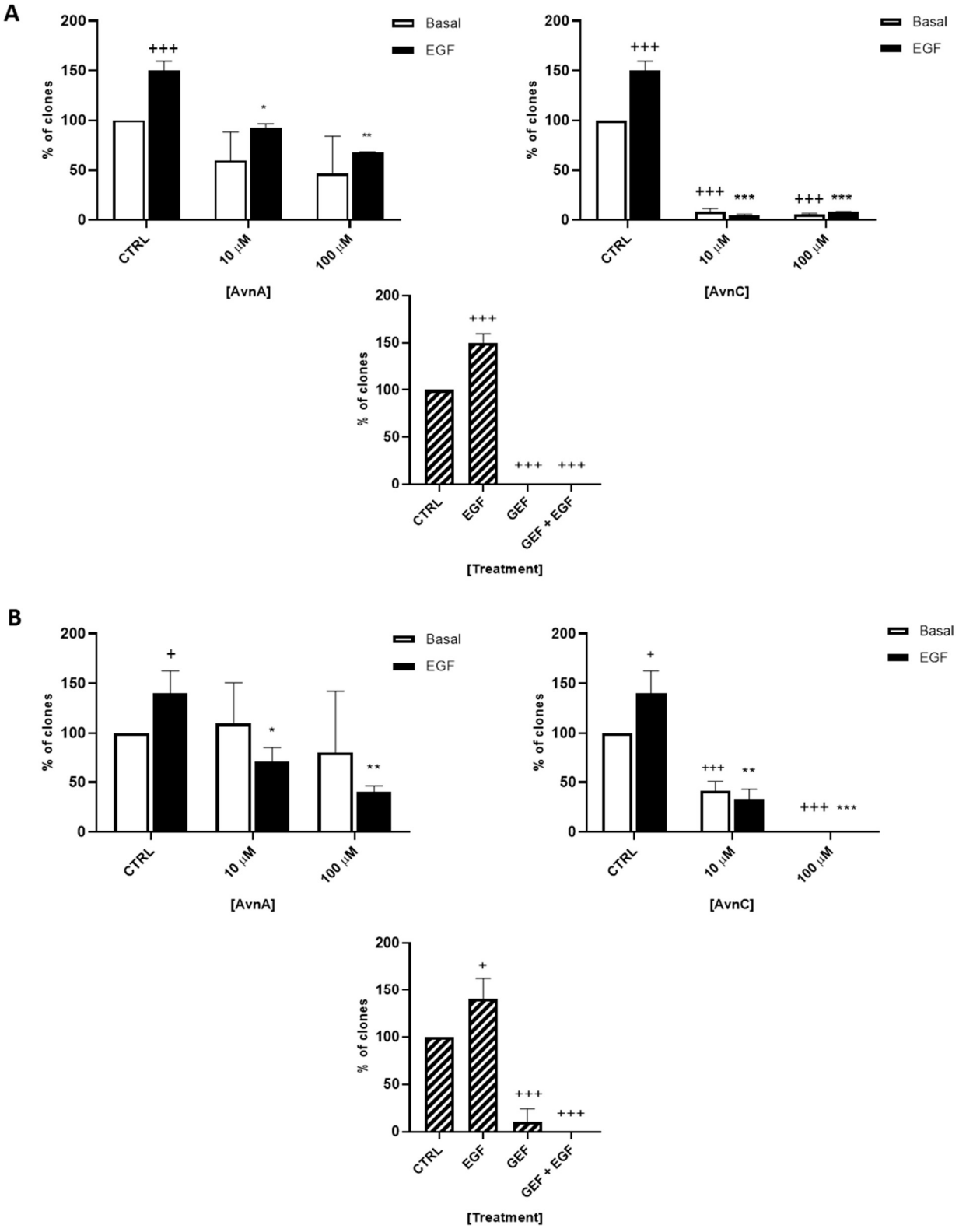


Figure 4.1.2. *Natural avenanthramides reduce clonogenicity of lung cancer cells promoted by EGF. Percentage of colonies of A 549 (A) and H1299 (B) cells in response to EGF in presence or absence of Avns. Data are expressed as % over basal control and are representative of three independent experiments run in triplicate.*

*Statistical analysis: +++ p<0,01; ++ p<0,1 and + p<0,1 vs CTRL; *** p< 0,01; ** p< 0,1 and * p<0,5 vs EGF.*

It is well known that normal cells die through apoptosis when detached from extracellular matrix (Vaquero et al. 2003). However, cancer cells undergo phenotypic changes, acquiring the ability to survive and grow under anchorage-independent conditions, as well as to leave the original tumor site, migrate through surrounding tissues and establish metastasis to a distant site (Finetti et al. 2018). To further investigate the putative anticancer properties of Avns, we analysed the effects of these compounds on anchorage-independent growth. A549 cells were maintained for 24 or 48 hrs in suspension and then the number of dead cells were counted. Figure 4.1.3 A and B show that treatment with 100 µM Avn-A or Avn-C caused an increase in the number of dead cells, both in basal condition and EGF-treated cells, indicating that Avns reduce the possibility of lung cancer cells to survive in anchorage-independent conditions and inhibit the EGF activity.

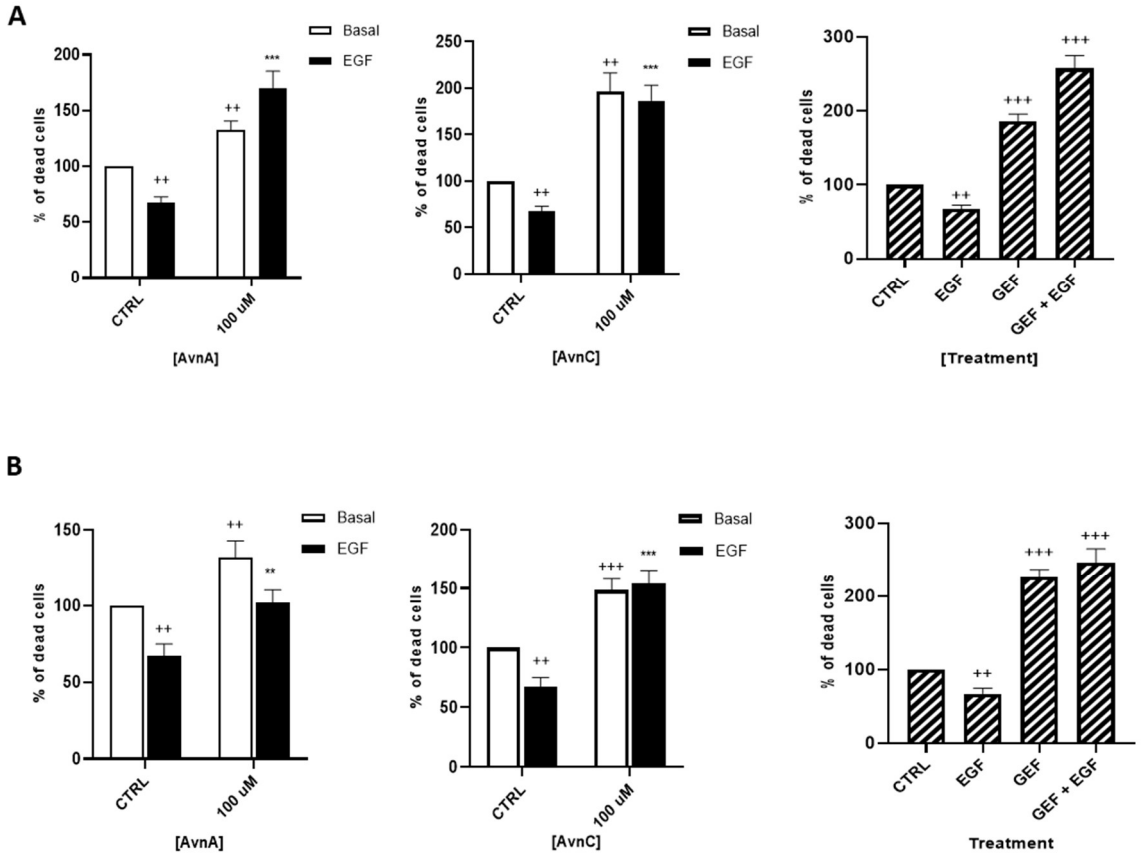


Figure 4.1.3. *Effects on Anoikis by Avns after 24 hrs (A) and 48 hrs (B). Cell vitality of A549 in suspension treated with Avns (100 µM) in 0.1% of serum. Results are expressed as % of dead cells and are representative of three independent experiments run in triplicate.*
*Statistical analysis: +++ p<0,01 and ++ p<0,1 vs CTRL; ***p< 0,01 and ** p< 0,1 vs EGF.*

In addition, we evaluated the role of Avns on cell mobility and metastatization by studying cell migration and EMT. As shown in Figure 4.1.4, both Avn-A and Avn-C did not affect the migration in basal conditions but were effective in reducing A549 cell migration induced by EGF, suggesting that these compounds possess an anti-migratory activity on lung cancer cells.

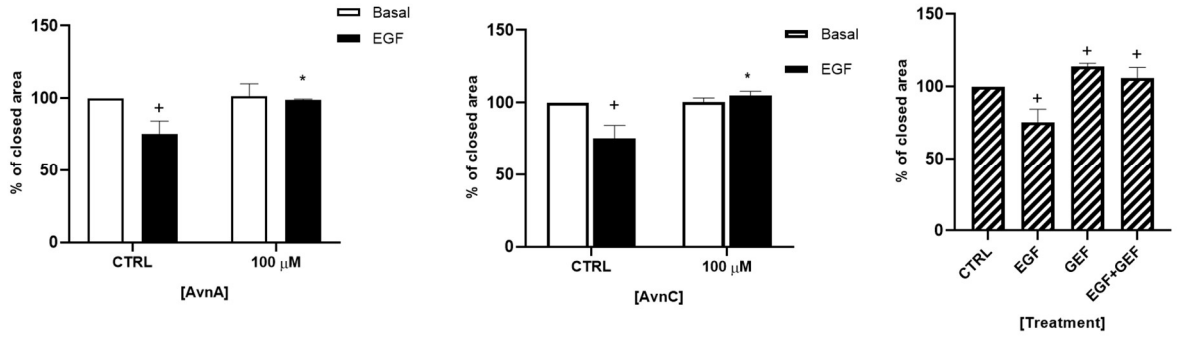


Figure 4.1.4. *Avns reduce migration of tumor cells promoted by EGF. A549 migration was evaluated by scratch assay. Data are reported as % of closed area over basal control and are representative of three independent experiments run in triplicate.*
*Statistical analysis: + p<0,1 vs CTRL and * p< 0,1 vs EGF.*

Given the important role of E-cadherin down-regulation and vimentin up-regulation in phenotypic changes associated to EMT (Their 2002; Brabletz et al. 2018), we analysed the expression levels of these proteins on A549 cells after 48 hrs treatment with Avns in presence/absence of EGF (25 ng/ml). Western blot analysis showed an up-regulation of E-cadherin and a down-regulation of vimentin protein levels upon cell treatment with Avn-C (Figure 4.1.5). Interestingly, as expected EGF was able to both reduce E-cadherin levels and increase vimentin expression, but these effects were reverted by Avn-C. Notably, while Avn-A induced E-cadherin expression after EGF treatment, we did not observe a reduction in vimentin expression (Figure 4.1.5). Overall, our data demonstrate the activity of Avns in inhibiting EMT and migration induced by EGF, although with different extent.

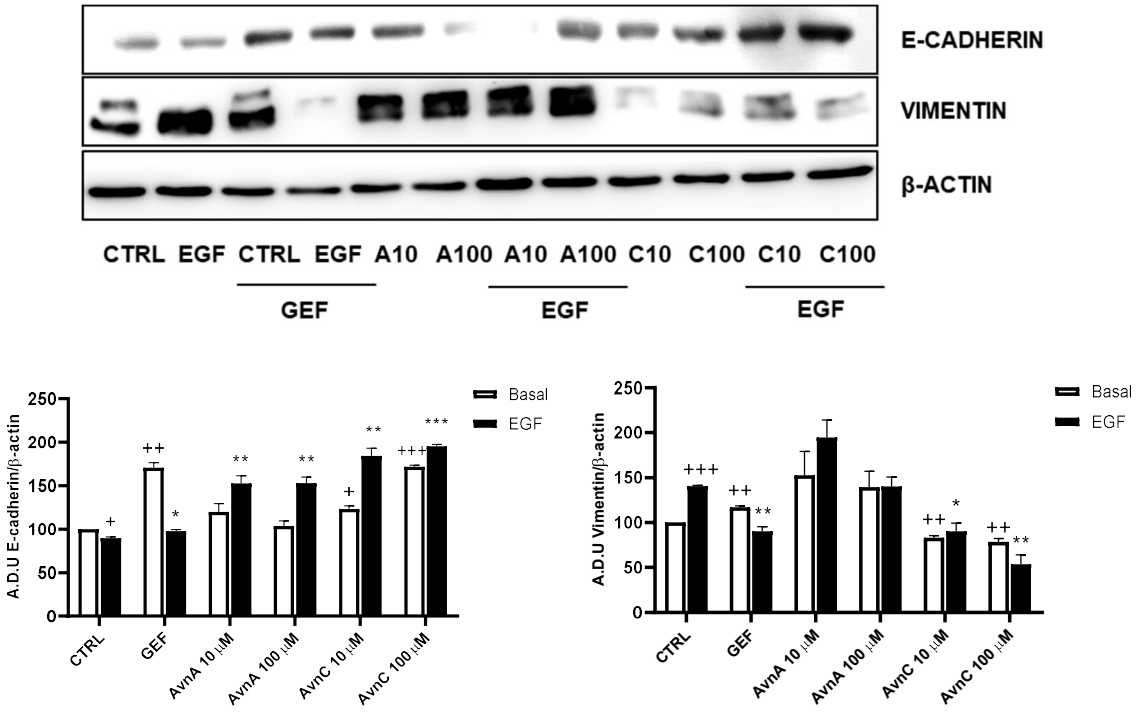


Figure 4.1.5: *Avenanthramides regulate E-cadherin and Vimentin expression. Representative images of Western blot analysis of A549 cells exposed to Avns (10 and 1000 µM, 48 hrs) and its quantification (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments.*
*Statistical analysis: +++ p<0,01 and + p<0,5 vs CTRL; ***p< 0,01; ** p< 0,1 and *p<0,5 vs EGF.*

Many works reported that inflammatory pathways are connected with cancer development, and that up-regulation of COX-2 plays an important role in tumorigenesis (Finetti et al., 2020, Hashemi Goradel et al. 2019). For this reason, we decided to evaluate the activity of Avns on COX-2 expression after exposure to EGF. A549 cells were treated for 48 hrs with Avns (10 and 100 µM) with and without EGF (25 ng/mL) and the expression levels of COX-2 were analysed by Western blot. As reported in Figure 4.1.6, Avns inhibited the COX-2 up-regulation induced by EGF.

These data clearly indicate that Avns may inhibit EGF signalling also through the inhibition of COX-2 up-regulation promoted by EGF.

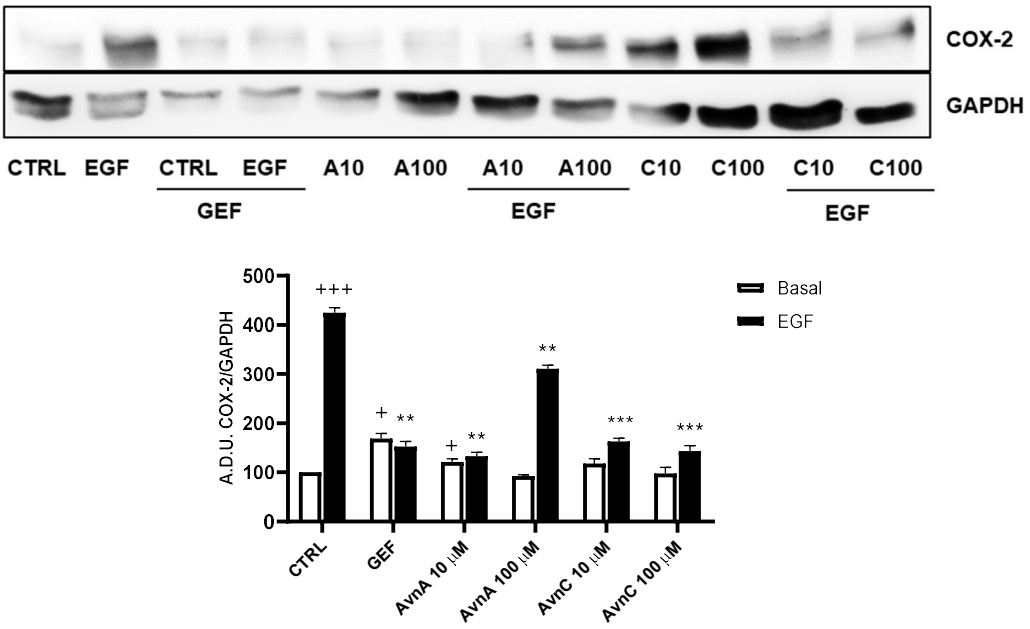


Figure 4.1.6. *Regulation of inflammation markers by Avns. Representative images and quantification of Western blot analysis of COX-2 expression in A549 cells treated with Avns (10 and 100 µM, 48 hrs). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments.*
*Statistical analysis: +++ p < 0,001 and + p <0,05 vs CTRL; *** p <0,001 and ** p< 0,1 vs EGF*

PI3K/mTOR/Akt and MEK/ERK are two essential EGFR downstream signaling pathways that play key roles in the transmission of proliferative signals from membrane bound receptors (Cappuzzo et al. 2004) and consist of kinases cascades that are regulated by phosphorylation and de-phosphorylation by specific kinases (Steelman et al. 2011).

To verify whether Avns are able to interfere with specific components of EGF pathways we measured the phosphorylation levels of ERK 1/2 and Akt by Western blot, using A549 cells pre-treated for 24 hrs with Avn-A and Avn-C (10 and 100 µM) and 15 minutes with EGF (25 ng/mL). The results indicate that both Avns were able to inhibit Akt and ERK 1/2 phosphorylation induced by EGF. However, Avn-A showed to be less effective than Avn-C (Figure 4.1.7).

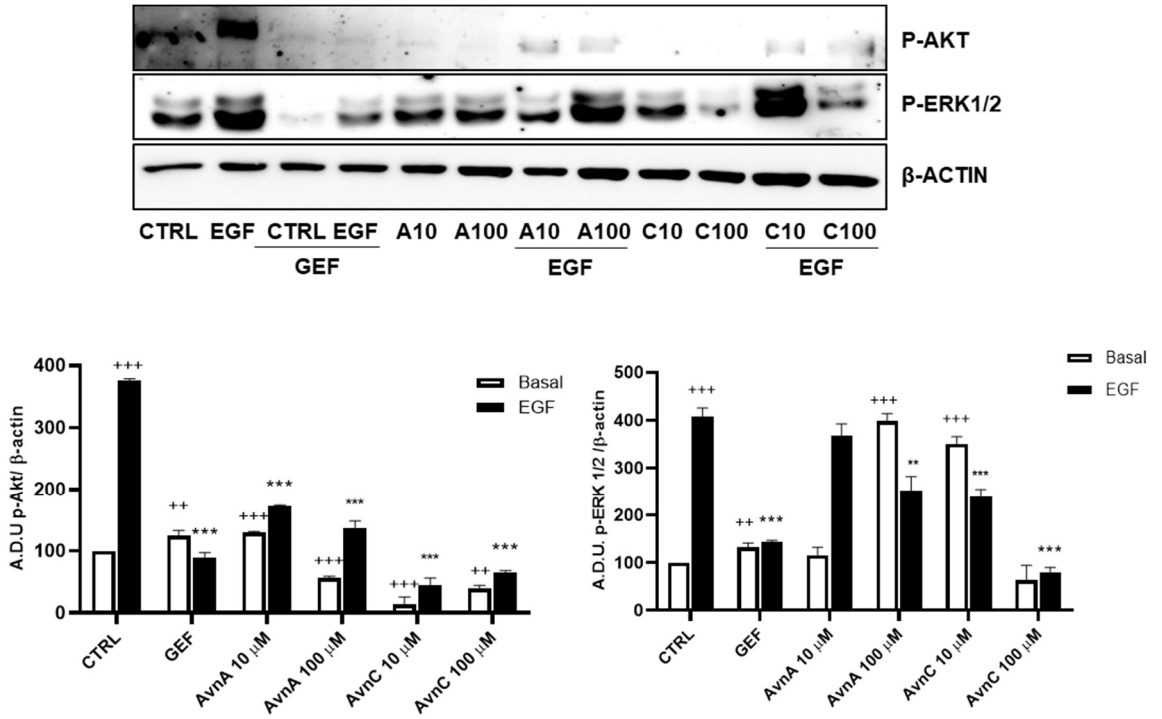


Figure 4.1.7. *Induction of phosphorylated proteins by Avns. Representative images and quantification of Western blot analysis of p-Akt and p-ERK 1/2 expression in A549 cells pre-treated with Avns (10 and 100 µM, 24 hrs) and 15 minutes with EGF (25 ng/mL). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of four independent experiments. Statistical analysis: +++ p<0,01; ++ p<0,1 and + p<0,5 vs CTRL; *** p< 0,01; ** p< 0,1 and * p<0,5 vs EGF*

It has been reported that mutations that can induce constitutive activation of EGFR have been found in many types of cancer (Libermann et al. 1985). Dysregulation of this receptor is an unfavourable prognostic factor in patients affected by a variety of tumors including lung cancer where the EGFR represents a fundamental target for the therapy (Liu et al. 2017).

In this work we verified if Avns are able to interfere with EGFR phosphorylation induced by EGF. A549 cells were treated with Avns and analysed by Western blotting as reported in the section *Materials and Methods*. The results reported in Figure 4.1.8 show that both Avn-A and Avn-C were able to significantly reduce the phosphorylation of EGFR induced by EGF, being Avn-C more potent that Avn-A, as partially suggested by previous data. These data indicate that Avns are able to inhibit EGFR phosphorylation induced by EGF and its signalling pathway thus reducing lung cancer cells proliferation and migration.

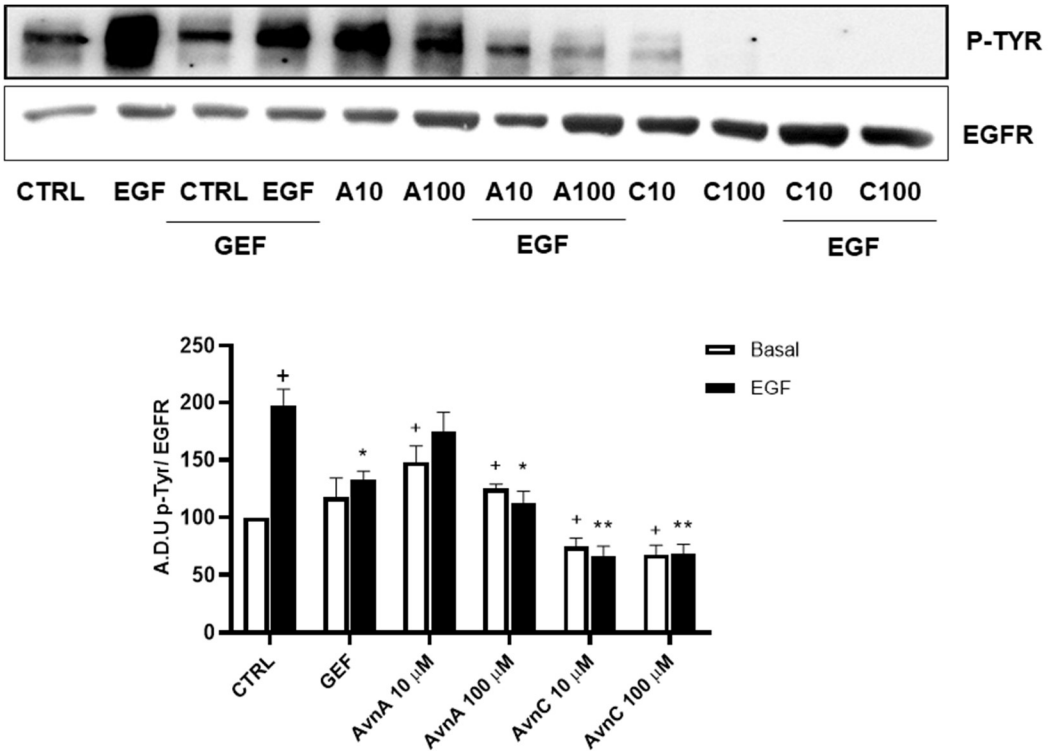


Figure 4.1.8. *Regulation of EGFR phosphorylation by Avns. Representative images and quantification of Western blot analysis of EGFR and p-Tyr in A549 cells treated with Avns (10 and 100 µM, 48 hrs). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments.*
*Statistical analysis: + p<0,5 vs CTRL; ** p< 0,1 and *p<0,5 vs EGF*

EGFR is overexpressed in a wide range of solid tumors. A431 cells are used in studies of the cell cycle and cancer-associated cell signalling pathways since they express abnormally high levels of this receptor and they are often used as a positive control for EGFR expression. They contain no functional p53, a potent tumor suppressor gene, and are highly sensitive to mitogenic stimuli. For this reason, to confirm the results produced in lung cancer cell lines, we repeated some experiments on A431 cell line (Xu et al. 1984).

First, we performed the MTT assay and we observed that also in this model, both Avn-A and Avn-C (10, 50 and 100 µM) reduced cell growth promoted by EGF (Figure 4.1.9), confirming the above reported results.

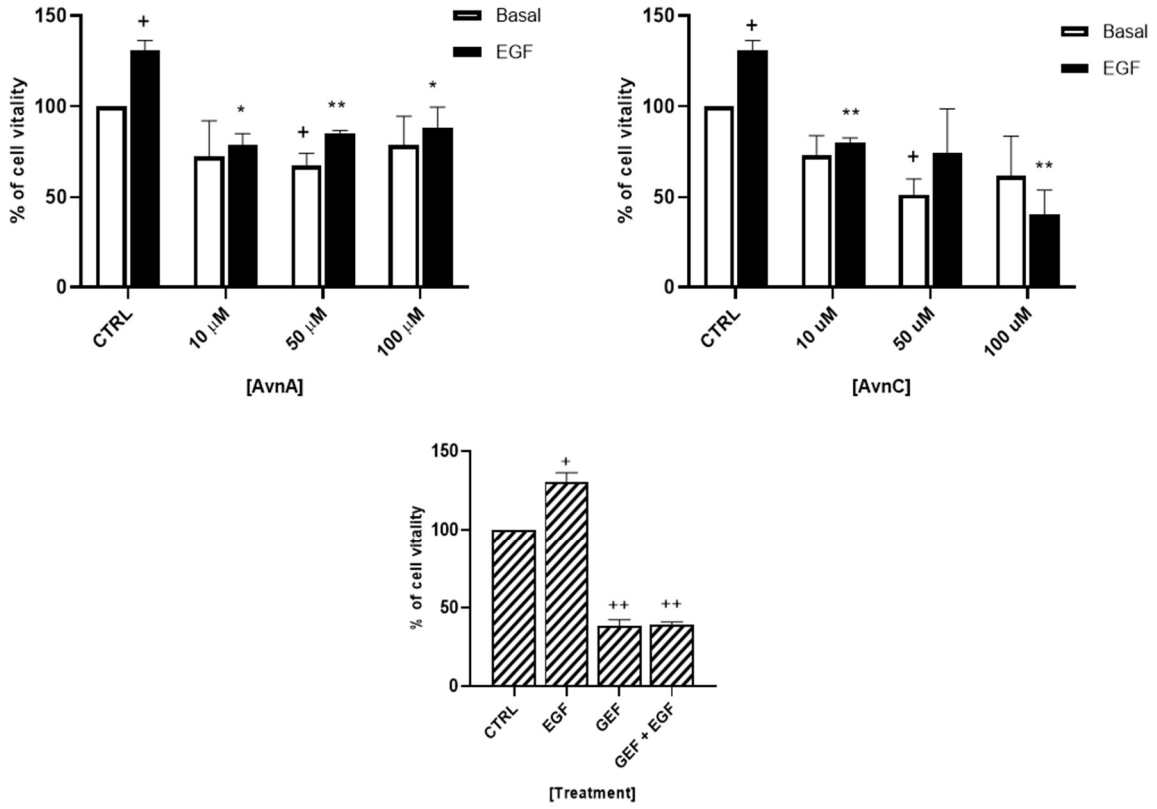


Figure 4.1.9. *Effects of Avns on A431 cells vitality evaluated by the MTT assay. Cells were exposed to EGF in presence of increasing concentration of Avns (10, 50 and 100 µM) for 3 days. Data are expressed as % over basal control and are representative of three independent experiments run in triplicate. Statistical analysis: ++ p<0,1 and +p<0,1 vs CTRL; ** p< 0,1 and *p<0,5 vs EGF.*

We then analysed the role of Avns on EGFR activation induced by EGF in A431 cells. As showed in Figure 4.1.10, both Avns were able to reduce the EGF-induced EGFR phosphorylation, being Avn-C more active than Avn-A, and confirming the results obtained in A549 cell line.

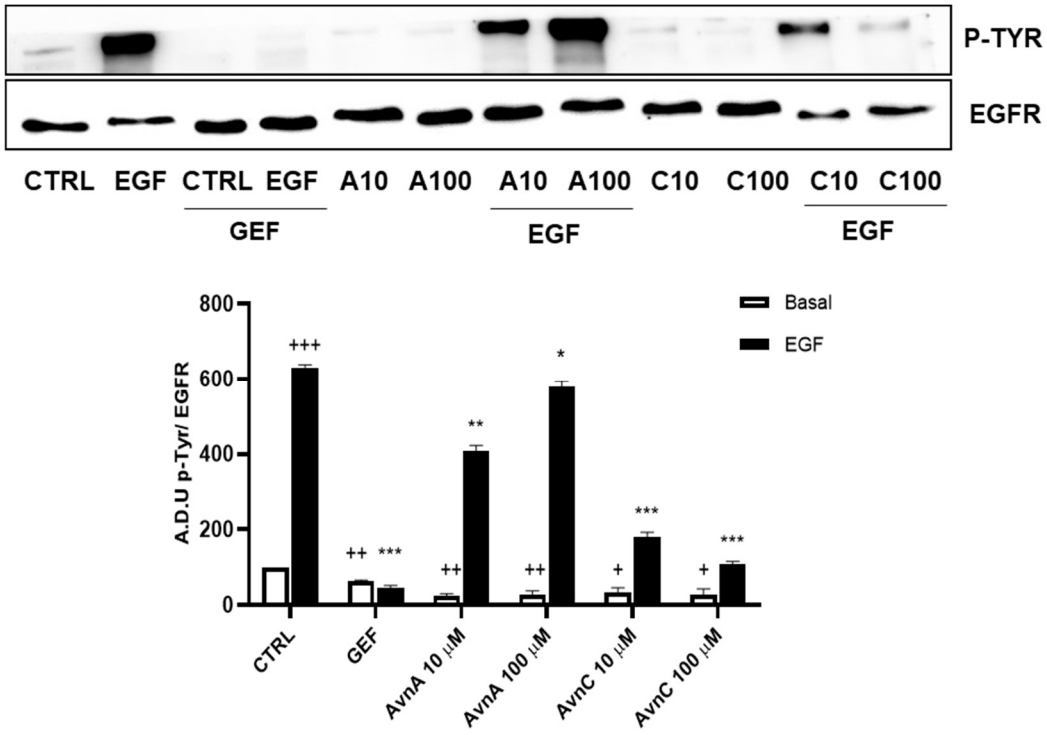


Figure 4.1.10. *Regulation of EGFR phosphorylation by Avns. Representative images and quantification of Western blot analysis of EGFR and p-Tyr in A431 cells treated with Avns (10 and 100 µM, 48 hrs). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments.*
*Statistical analysis: +++ p<0,01; ++ p<0,1 and + p<0,5 vs CTRL; ***p< 0,01; ** p< 0,1 and *p<0,5 vs EGF.*

In conclusion, the data reported in this part of the thesis indicate that Avns are able to inhibit EGFR phosphorylation and EGF signaling pathway. In particular, Avns inhibit tumor growth, migration, EMT and Anoikis induced by EGFR activation. Avn-C showed to be more active than Avn-A in most of the experiments performed, confirming the previously published data were an higher efficacy of Avn-C was demonstrated (Ji et al. 2003; Guo et al. 2010a; Moglia et al. 2015; Scarpa et al. 2018; Turrini et al. 2019). Actually Avn-C is able to better modulate several events related to cancer progression such as apoptosis, cell proliferation and metastatization in various cell lines and in animal models. These results could be explained by the highest cell permeability and bioavailability characterizing Avn-C and demonstrated *in vivo* (Scarpa et al. 2018; Turrini et al. 2019). Moreover, the presence of the second hydroxyl group in the phenolic unit of Avn-C could improve the binding affinity through non-covalent intermolecular interactions between the molecule and its receptor.

In conclusion, Avns and in particular Avn-C seem to have analogous properties of Gefitinib, suggesting a possible use in therapy thanks to their very low adverse effects and good toleration by patients.

Further studies will be necessary to better understand the mechanism of action of the Avns and the molecular bases underlying the different activity of the two different types of Avn.

4.1.2 Part II: *Phaseolus Vulgaris* L. Var. Venanzio and colon cancer

Phaseolus vulgaris is cultivated all over the world and is the most important edible legume for direct consumption. However, beans are more than a foodstuff since they are rich in many compounds with antioxidant, anti-inflammatory and hypolipidemic properties that reduce cardiovascular diseases (Ombra et al. 2016), possess amilase inhibitors and phytohemagglutinin, and thanks to the presence of starch they are active on obesity and diabetes (Thompson et al. 2012) and cancer (Feregrino-Perez et al. 2014; Moreno-Jiménez et al. 2019).

"Fagiola di Venanzio" (FV) is an endangered and recently identified Italian variety of *P. vulgaris* grown in a restricted area of Murlo, a small municipality in the south of Tuscany. In this work we analysed for the first time this new variety of bean with the aim to characterize its chemical composition and potential biological activities.

We initially carried on chemical analysis of FV extracts obtained from four different samples provided by different growers (see Table 1 in *Material and Methods*). To investigate the polyphenolic fraction of FV extracts and to identify the main chemical constituents, a high-performance liquid chromatography-diode array detection (HPLC-DAD) analysis was carried out. FV was found to be rich in polyphenols (table 2), in accordance with other published papers on the species (Ganesan and Xu 2017).

Components	**Quantification (mg/g)**			
	#1	**#2**	**#3**	**#4**
Total polyphenols	0.142 ± 0.018	0.123 ± 0.011	0.129 ± 0.016	0.120 ± 0.015
Total hydroxycinnamic derivatives	0.054 ± 0.004	0.051 ± 0.003	0.046 ± 0.005	0.052 ± 0.005
Isoflavones	<0.005	<0.005	<0.005	<0.005

Table 2. *Chemical composition of FV extracts.*

HPLC-DAD analysis (Figure 4.1.11) revealed that two main polyphenols subclasses could be identified in FV extracts, namely simple phenolic acids and hydroxycinnamic derivatives. As a water extraction was performed, the prevalence of hydrophilic compounds in the extract was expected and consistent with a previous work on common beans endemic of Southern

Italy (Ombra et al. 2016). Gallic acid (Retention Time, RT = 4.41 min) and chlorogenic acid (RT = 7.94 min.) resulted the main phenolic and hydroxycinnamic acid, respectively. The other main peaks before gallic acid (RT =3.88–4.18 min) could be assigned to phenolic acids by monitoring UV spectra for their typical λ_{max} at 270–280 nm. Other hydroxycinnamic derivatives, different from caffeic acid, were recognized by UV spectra (λ_{max} at 270–280 nm and 320–330 nm) and linked with the peak at 6.47 min. Flavonoids were found only in small amounts. At RT = 11.33, 11.71 and 12.40 min, the zone of the chromatogram where isoflavones are recorded, two constituents with UV spectrum similar to genistein and daidzein (λ_{max} at 250–255 nm), but with different RTs, were found. Other flavonoids referable to used standards and their derivatives were not detected as present in concentrations below the detection limits of the method. Table 3 shows the quantification of gallic and chlorogenic acid identified in FV extracts and total phenolic and hydroxycinnamic derivatives, expressed as gallic and chlorogenic acid, respectively.

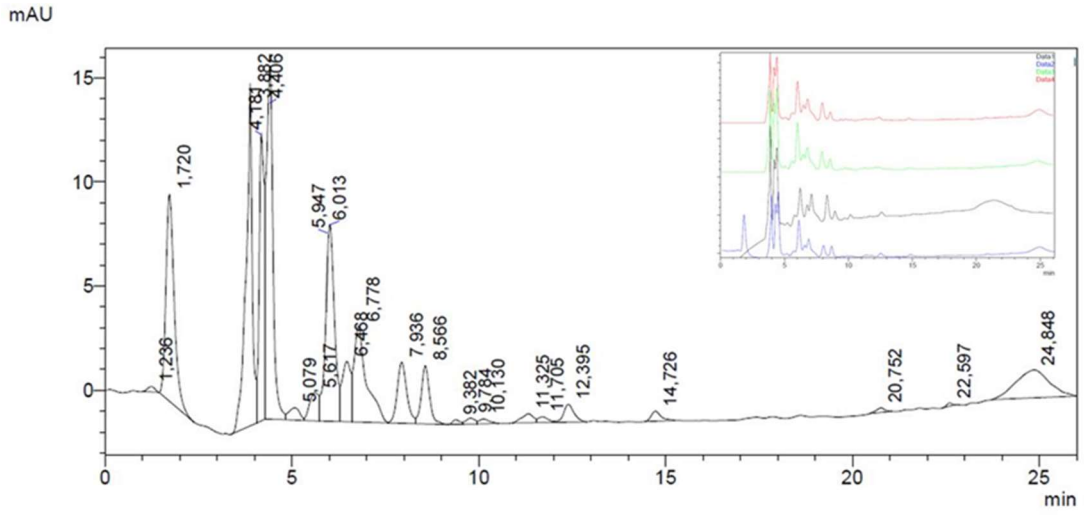


Figure 4.1.11. *HPLC-DAD profile of polyphenols present in FV extract. Chromatogram of sample #1. In the top box, chromatograms of all samples: blue = sample #1, grey = sample #2, green = sample #3 and red = sample #4.*

The four samples of beans initially analysed were cultivated in four different areas within Murlo, that are quite close to each other, but differ in terms of soil composition and exposure. Nevetheless, the four samples did not demonstrate significant differences in chemical composition.

Due to the minimum variance in polyphenols content and a strong similarity in chromatogram profiles of the four different samples (Figure 4.1.11), only the extract obtained from sample #1, available in highest amount, was further analysed and investigated in chemical and biological tests.

Table 3 summarizes the chemical composition of the selected FV extract of sample #1. In accordance with known nutritional data and literature on *P. vulgaris*, carbohydrates represent the main class of metabolites: the concentration of soluble carbohydrates in the FV extract was shown to be 10.032 mg/g. The concentration of total proteins was 15.190 mg/g.

Composition	Quantification
Total polyphenols	0.131 ± 0.016 mg/g
Total hydroxycinnamic derivatives	0.046 ± 0.004 mg/g
Gallic acid	0.052 ± 0.005 mg/g
Chlorogenic acid	0.011 ± 0.002 mg/g
Isoflavones	<0.005 mg/g
Total soluble carbohydrates	10.032 ± 0.820 mg/g
Total proteins	15.190 ± 2.020 mg/g

Table 3. *Chemical composition of #1 FV extract.*

As previously reported (Ombra et al. 2016), extracts of common beans obtained from different Italian varieties of *P. vulgaris* possess antioxidant and antiradicalic properties. According to that, the FV extract showed antiradicalic capacity, monitored by DPPH reduction. The comparison between the HPLC-DAD chromatograms obtained before and after adding DPPH to FV extract showed that differences in peak areas related to polyphenols occur, thus demonstrating that some constituents of FV extract were able to react with DPPH and underwent oxidative degradation.

Chlorogenic acid was the most degraded molecule, more than 75.0% after DPPH reaction, meaning that this hydroxycinnamic derivative primarily contributed to the antiradicalic activity of FV extract. The other main hydroxycinnamic derivative displayed a minor degradation (−39.4%); among phenolic acids, only gallic acid seemed to participate in DPPH

reaction and its recorded degradation was 37.6%. Other polyphenolic constituents of FV extract did not show a significant degradation after DPPH reaction (Figure 4.1.12).

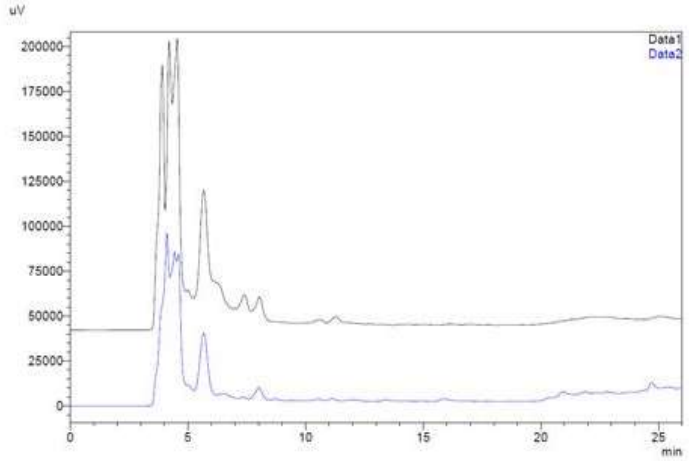


Figure 4.1.12. *Antioxidant activity of FV extract. HPLC-DAD-DPPH test. Chromatograms showed the differences in peak areas between the chromatograms obtained before (black) and after (blue) mixing DPPH and FV extract.*

To further analyse the antioxidant properties of FV extract, we performed *in vitro* experiments on different cellular models. Due to the high nutraceutical impact and to the findings that common bean consumption is associated with *in vivo* chemoprotective effects at the early stages of colon cancer (Haydé et al. 2012) and pro-apoptotic and anti-proliferative activities *in vitro* (Moreno-Jiménez et al. 2019), we selected two different cellular models of colorectal adenocarcinoma, HT29 and HCT116 cells. To mimic a pro-oxidant and pro-inflammatory milieu, we stimulated colon cancer cells with interleukin 1β (IL1β, 10 ng/mL, 48 hrs) in the presence and in the absence of different concentrations of FV extract (10, 50 µg/mL), and we measured ROS levels by means of DCFH2-DA assay. As reported in Figure 4.1.13, the ability of IL1β to promote ROS production was inhibited by the FV extract, at both concentrations and in both cell lines.

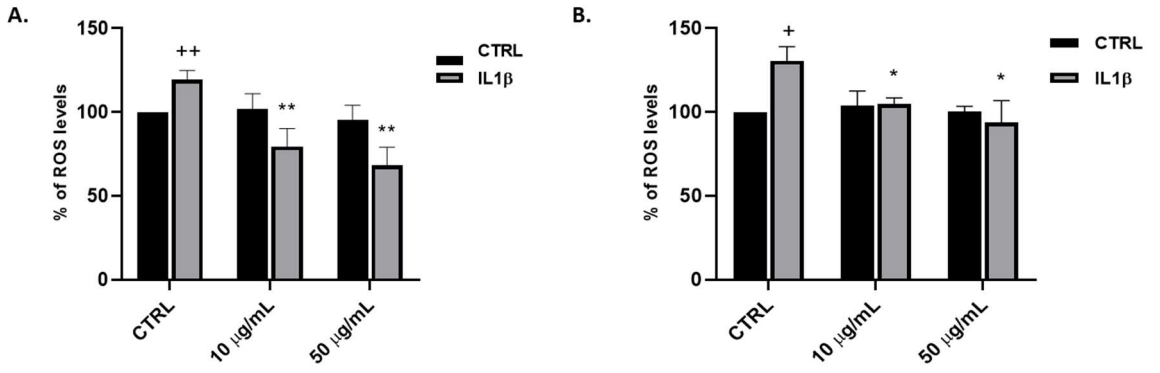


Figure 4.1.13. *Antioxidant activity of FV extract. ROS measurement in HT29 (A) and HCT116 (B) cells after 18 hrs of exposure with the different concentrations of FV extract (10–50 µg/mL) followed by a 24 hrs incubation with IL1ϐ (10 ng/mL). Data are expressed as relative fluorescence units and are representative of three independent experiments run in triplicate.*
*Statistical analysis: ++ p < 0.01 and + p < 0.05 vs. CTRL; ** p < 0.01 and * p < 0.05 vs.IL1ϐ.*

It is known that, in addition to triggering traditional post-translational protein modifications (including phosphorylation, acetylation, ubiquitination, etc.), ROS can directly modify cellular proteins, adding another layer of protein regulation to the proteome classified as oxidative post-translational modifications (OPTMs). In particular, ROS may cause various types of chemical modifications of proteins, including glutathionylation (Dalle-Donne et al. 2009; Hsieh et al. 2014). To investigate whether FV extract could affect these mechanisms, we treated colon cancer cells with the FV extract for 36 hrs and we measured the levels of glutathion–protein complexes by Western blotting. Figure 4.1.14 shows that the FV extract was able to reduce significantly the levels of total glutathionylated proteins, indicating the antioxidant activity of the FV extract.

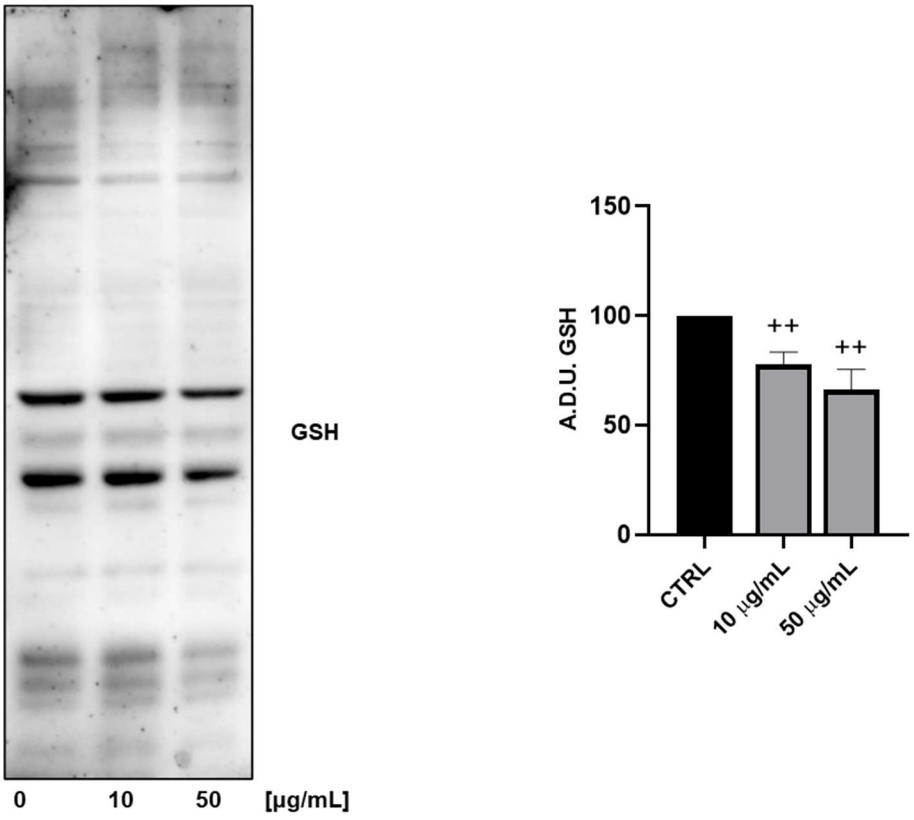


Figure 4.1.14. *Antioxidant activity of FV extract. Evaluation of the levels of glutathionylated proteins. HT29 cells were treated with different concentrations of FV extract (10–50 µg/mL) for 36 hrs and then analysed by Western blot under non reducing conditions using an anti-GSH primary antibody. Data are expressed as fold increase compared with control and are representative of three independent experiments (A.D.U.: arbitrary densitometry units).*
Statistical analysis: ++ $p < 0.01$ vs. CTRL.

All these results indicate that FV possesses antiradicalic activity *in vitro* and reduces ROS production promoted by IL1β in two different models of colon cancer cells.

To further explore the biological properties of the FV extract, we investigated its ability to reduce the inflammation related to cancer. It is well known that inflammation is a key component in colon cancer onset and progression and that the cyclooxygenase 2 (COX-2) pathways play a major role in modulating cell growth, apoptosis and epithelial mesenchymal transition (EMT) (Donnini et al. 2012; Hashemi Goradel et al. 2019). Recent reports indicate that a direct interplay exists between inflammation and carcinogenesis. In fact, the risk of developing colon cancer is increased by chronic inflammatory diseases (such as inflammatory bowel disease), chronic infections or inflammations caused by environmental exposures. In addition, administration of COX inhibitors, such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), is connected with a lower risk of

developing colon cancer and its recurrence (Patrignani and Patrono 2016; Piotrowski et al. 2020).

It has been demonstrated that IL1β, a pro-inflammatory cytokine, induces COX-2 expression in colorectal cells and that COX-2 drives colon cancer progression (Liu et al. 2003). In this context, we evaluated the activity of FV extract on COX-2 expression induced by IL1β. We stimulated HT29 cells with IL1β (10 ng/mL, 48 hrs), in presence of 10 and 50 µg/mL of FV extract and we observed that at the higher concentration FV extract was able to strongly inhibit IL1β-induced COX-2 expression. These data clearly indicate that FV extract could reduce the inflammation related to cancer (Figure 4.1.15).

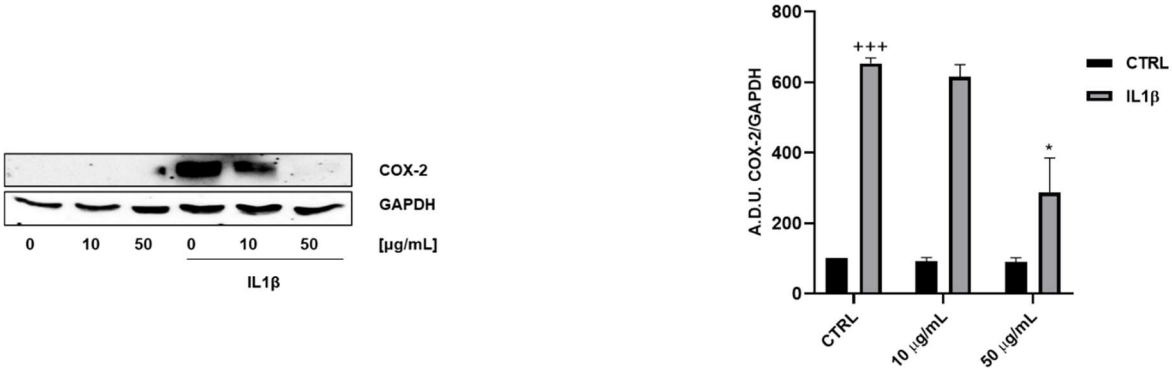


Figure 4.1.15. *Anti-inflammatory activity of the FV extract. Western blot analysis and quantification of COX-2 expression in HT29 cells after 18 hrs of exposure with different concentrations of FV extract (10–50 µg/mL) followed by a 24 hrs incubation with IL 1β (10 ng/mL). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three experiments. Statistical analysis: +++ p < 0.001 vs. CTRL, * p < 0.05 vs. IL1β.*

It has been described that natural compounds, and in particular dietary polyphenols, exhibit a relevant anti-inflammatory activity linked to the inhibition of NF-κB, MAPK and iNOS signalling.

In this light, we investigated the activity of the FV extract on NF-κB and MAPK activation, by analysing the phosphorylation levels of NF-κB and ERK1/2. As reported in Figure 4.1.16 FV extract was able to reduce both p-NF-κB (A) and p-ERK1/2 (B) levels in IL1β-stimulated colon cancer cells, indicating once again an anti-inflammatory activity of FV components.

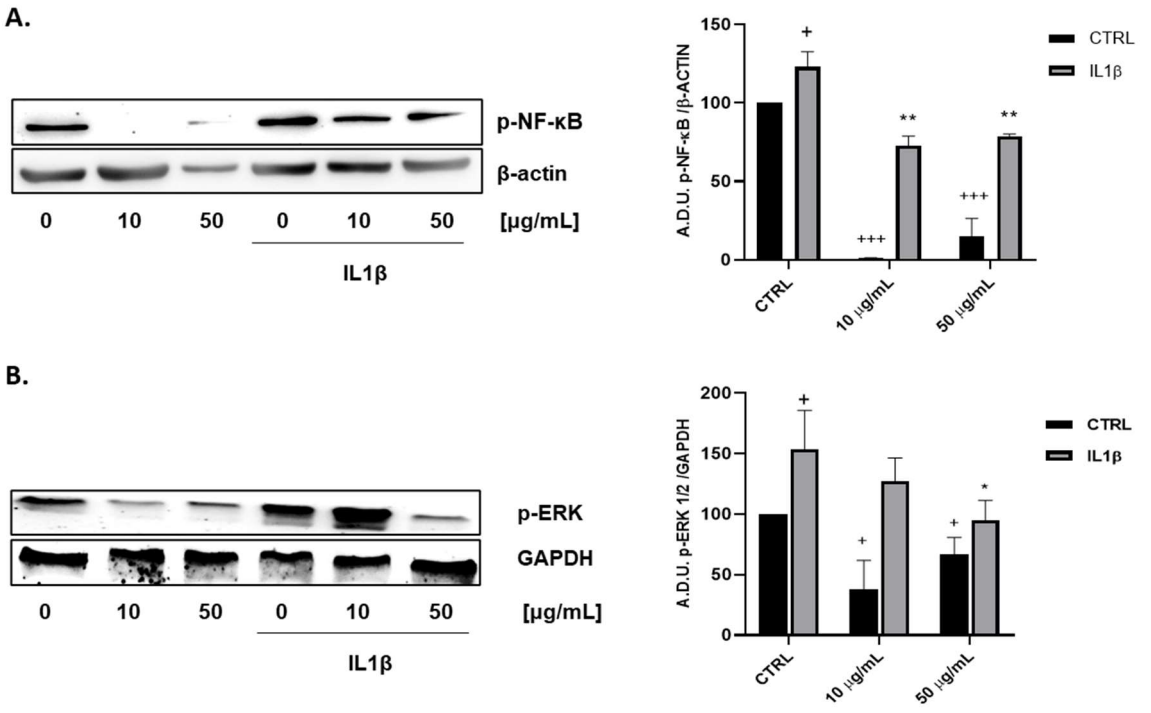


Figure 4.1.16. *Anti-inflammatory activity of the FV extract. Western blot analysis and quantification of NF-κB (A) and ERK 1/2 (B) phosphorylation in HT29 cells after exposure with FV extract (10–50 µg/mL) followed by incubation with IL 1ß (10 ng/mL). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three experiments.*
*Statistical analysis: +++ p<0,001 and + p < 0.05 vs. CTRL; **p < 0.01 and * p < 0.05 vs. IL1ß.*

In order to further investigate the activity of FV extract, we measured the ROS levels and COX-2 expression in non-cancer cells. To this aim we used Human Umbilical Vein Endothelial Cells (HUVEC) as a model in which it is well known the activity of IL1β in inducing ROS production and COX-2 expression (Monti et al. 2016). The results show that while IL1β treatment induced both ROS production (Figure 4.1.17) and COX-2 expression (Figure 4.1.18), FV extract was inactive, indicating a potential selective role of FV extract on cancer cells.

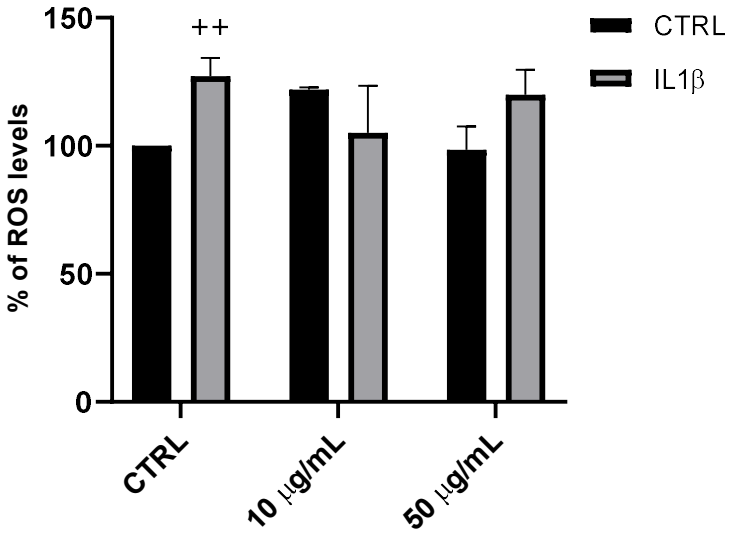


Figure 4.1.17. *FV extract activity in endothelial cells. ROS measurement in HUVEC cells after 18 hrs of exposure with the different concentrations of FV extract (10–50 µg/mL) followed by a 24 hrs incubation with IL1ϐ (10 ng/mL). Data are expressed as relative fluorescence units and are representative of three independent experiments run in triplicate.*
Statistical analysis: ++ p<0,01 vs CTRL.

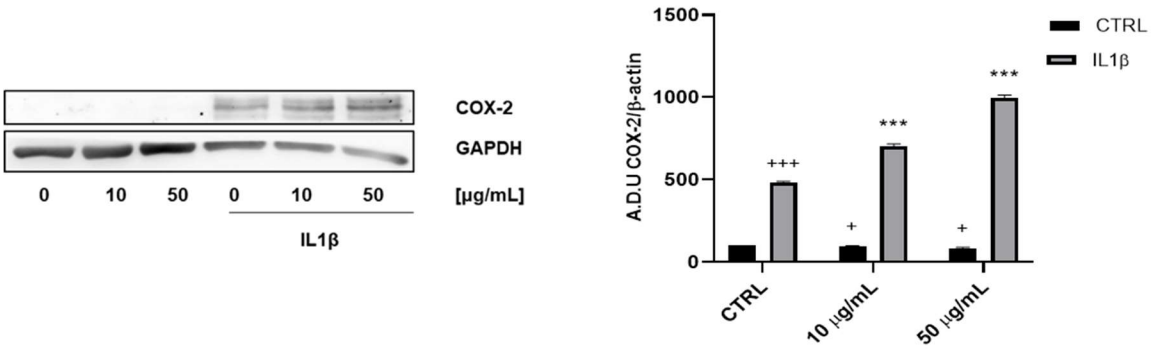


Figure 4.1.18. *FV extract activity in endothelial cells. Western blot analysis of COX-2 expression in HUVEC cells after 18 hrs of exposure with different concentrations of FV extract (10–50 µg/mL) followed by a 24 hrs incubation with IL 1ϐ (10 ng/mL). (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control.*
*Statistical analysis: +++ p<0,001 and + p<0,05 vs CTRL; *** p<0,001 vs IL1ϐ.*

In order to evaluate whether the FV extract could modulate tumor progression, we studied the proliferation of colon cancer cells by MTT test. HT29 and HCT116 were treated with 10 ng/mL IL1β for 48 hrs in the presence of increasing concentrations of the FV extract (from 5 to 100 µg/mL). According to previous studies conducted on white beans (Ombra et al. 2016), the FV extract did not modify cancer cells' growth in basal conditions. However,

when the cells were exposed to an inflammatory milieu (IL1β), FV extract strongly reduced cell growth in a concentration-dependent manner (Figure 4.1.19).

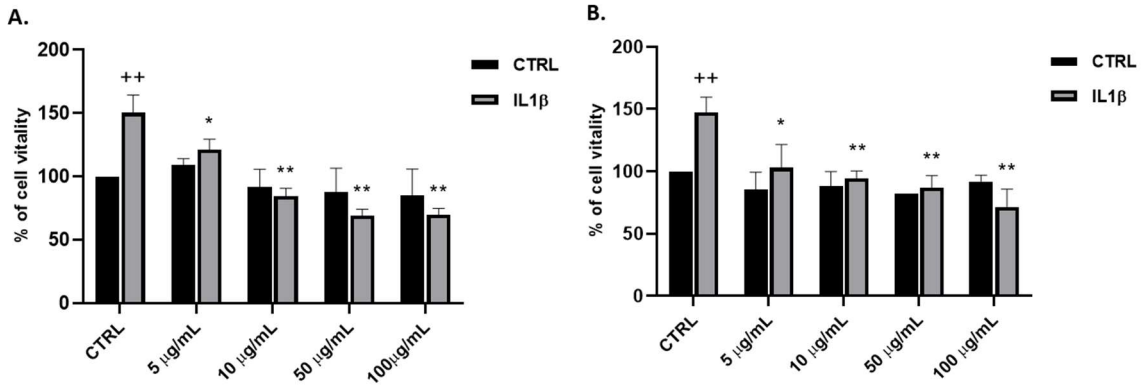


Figure 4.1.19. *FV extract inhibits colon cancer cell growth. Cell proliferation induced by IL 1β (10 ng/mL) in the presence or absence of FV extract (5, 10, 50 and 100 µg/mL) was measured by MTT assay. HT29 (A) and HCT116 (B) cells were exposed to IL 1β for 48hrs. Data are reported as % of cell viability and are the means of 4 experiments run in triplicate.*
*Statistical analysis: ++ $p < 0.01$ vs. CTRL, ** $p < 0.01$ and * $p < 0.05$ vs. IL 1β.*

It is known that IL1β mimics inflammatory conditions that occur in intestinal tract and that may drive the development of inflammatory chronic disease as cancer or inflammatory bowel diseases (IBD) as ulcerative colitis and Crohn's disease. Our results, showing that FV extract is able to reverse the effects of IL1β on colon cancer cells, strongly suggest that FV may play an important role in preventing the alteration of molecular processes characterizing the inflammatory microenvironment that leads to cancer and chronic diseases. In this scenario, these data suggest that bean consumption may be helpful in the prevention or treatment of inflammatory diseases of intestinal tract, and outline once again the importance of nutrition for human health.

4.2 Task II: Identification of new molecular targets involved in tumor progression

4.2.1 KRIT1 as a possible new player in melanoma aggressiveness

As previously reported, carcinogenesis is a complicated multistep process in which numerous signaling pathways are involved. Seen the complexity of all these pathways involved in tumor progression and the well-known resistance to anticancer drugs, researches focus on discover of new specific targets of the cancer disease and new approaches for cancer treatment (Ke and Shen 2017). Molecular targeted therapies are revolutionary approaches which interfere with specific molecules to block cancer growth, progression, and metastasis. Specifically, they act on altered oncogenes and oncosuppressors that promote cancer development.

In this work, we investigated the possible role of a new signalling pathway never described for cancer in order to identify new specific antitumoral molecular targets.

KRIT1 protein is known for its involvement in Cerebral Cavernous Malformations (CCM), a rare pathology of the central nervous system. CCMs are characterized by thin-walled capillaries lacking of normal vessel structure (pericytes and astrocytes), surrounded by hemosiderin and appearing as mulberry-like vascular sinusoids (Antognelli et al. 2018). Among the three genes, *CCM1, CCM2* and *CCM3,* found to be mutated in the CCM disease, *CCM1/KRIT1* is by far the most involved in CCM onset and progression.

In the CCM disease, loss of KRIT1 induces in endothelial cells of brain vessels characteristics comparable to tumors, including increased growth and migration, reduced apoptosis, endothelial mesenchymal transition, loss of cell-cell junctions, pro-inflammatory status, altered redox homeostasis and activation of growth factors receptor signalling (Lampugnani et al. 2010; Wüstehube et al. 2010; DiStefano et al. 2014; Goitre et al. 2017). All these evidences suggest a possible role as oncosuppressor for KRIT1.

Starting from this hypothesis we investigated if KRIT1 might have a role in cancer progression.

To this purpose we first consulted several oncologic data banks to identify an association score between KRIT1 and cancer and to find the best one to analyse. Association score is a novel analysis that correlates a gene with different cancer types based on clinical studies, patient mutation, copy number alteration, gene expression and cell line dependency data

(Coker et al. 2019). The higher is the resulting score, the higher is the correlation with the tumor. We used EGFR as control gene because it is generally mutated and/or overespressed in cancer, as previously reported (Sigismund et al. 2018). As showed in table 4, the association score of EGFR in cancer where is known its involvement is between 0.1 and 0.66. Association score of KRIT1 is between 0.12 and 0.26, and although it is lower than that reported for EGFR, indicates that KRIT1 could be involved in the progression of several tumors. In particular, skin cancer exhibits one of the highest scores.

	Association score	
	KRIT1	**EGFR**
Mouth and oropharyngeal Cancer	0.26	0.28
Stomach Cancer	0.24	0.2
Endometrial Cancer	0.23	0.21
Female Cancers	0.2	0.12
Breast Cancer	0.19	0.37
Head and Neck Cancer	0.17	0.28
Skin Cancer	0.17	0.45
Bowel Cancer	0.14	0.1
Lung Cancer	0.14	0.34
Lymphoma	0.14	0.24
Bladder Cancer	0.13	0.24
Brain Cancer	0.13	0.11
Cervical Cancer	0.13	0.19
Pancreatic Cancer	0.13	0.11
Soft Tissue Cancer	0.13	0.11
Bile duct Cancer	0.12	0.12
Liver Cancer	0.12	0.19
Prostate Cancer	0.12	0.14

Table 4. *Association score between Krit1 and EGFR genes and several localised tumors (canSAR BLACK, www.cansarblack.icr.ac.uk)*

Based on this data, and given the importance to find new therapeutic approaches for melanoma, we decided to initially use this tumor type as a model for the study of the role of KRIT1 in cancer. Moreover, a deep analysis of data banks documented an incidence of mutated *Krit1* gene in melanoma cancer between 3% and 12.5% (Table 5).

	canSAR Black			Cosmic			cBIOPortal		
Gene	Number of patients	Number of mutations	%	Number of patients	Number of mutations	%	Number of patients	Number of mutations	%
Krit1	448	56	12,5	815	25	3,07	367	11	3
Malcavernin	448	4	0,9	815	24	2,94	367	5	1,36
PDCD10	448	25	5,58	815	9	1,1	367	6	1,63

Table 5. *Incidence of CCM gene mutations in melanoma patients*

To investigate the role of KRIT1 in melanoma progression, we evaluated the expression levels of KRIT1 in 57 samples of human specimens by immunohistochemical and Western blot analysis (Figure 4.2.1). The samples have been provided by Prof. Clelia Miracco, Section of Pathological Anatomy of the Azienda Ospedaliera Universitaria Senese. In all sections, the endothelium was intensely positive to KRIT1, and it represented our internal positive control. KRIT1 immunopositivity was also observed in the luminal side of the sweat glands epithelium. Normal melanocytes, as well as benign common nevi (BCN), were strongly decorated by KRIT1 (Figure 4.2.1, panel A). In dysplastic nevi (DN) and in radial growth phase melanomas (RGPM), single melanocytes and groups of negative melanocytes were observed, disorderly mixed with positive melanocytes.

The quantification of immunohistochemical images shows a significant and progressive decrease of KRIT1 protein expression in DN, vertical growth phase melanomas (VGPM), and melanoma metastasis (MelMet), when compared to BCN, with the lowest values registered in MelMet. These data were confirmed by Western blot analysis of the corresponding frozen samples (Figure 4.2.1, panel B), and indicated a possible regulatory role of KRIT1 in melanoma metastasis.

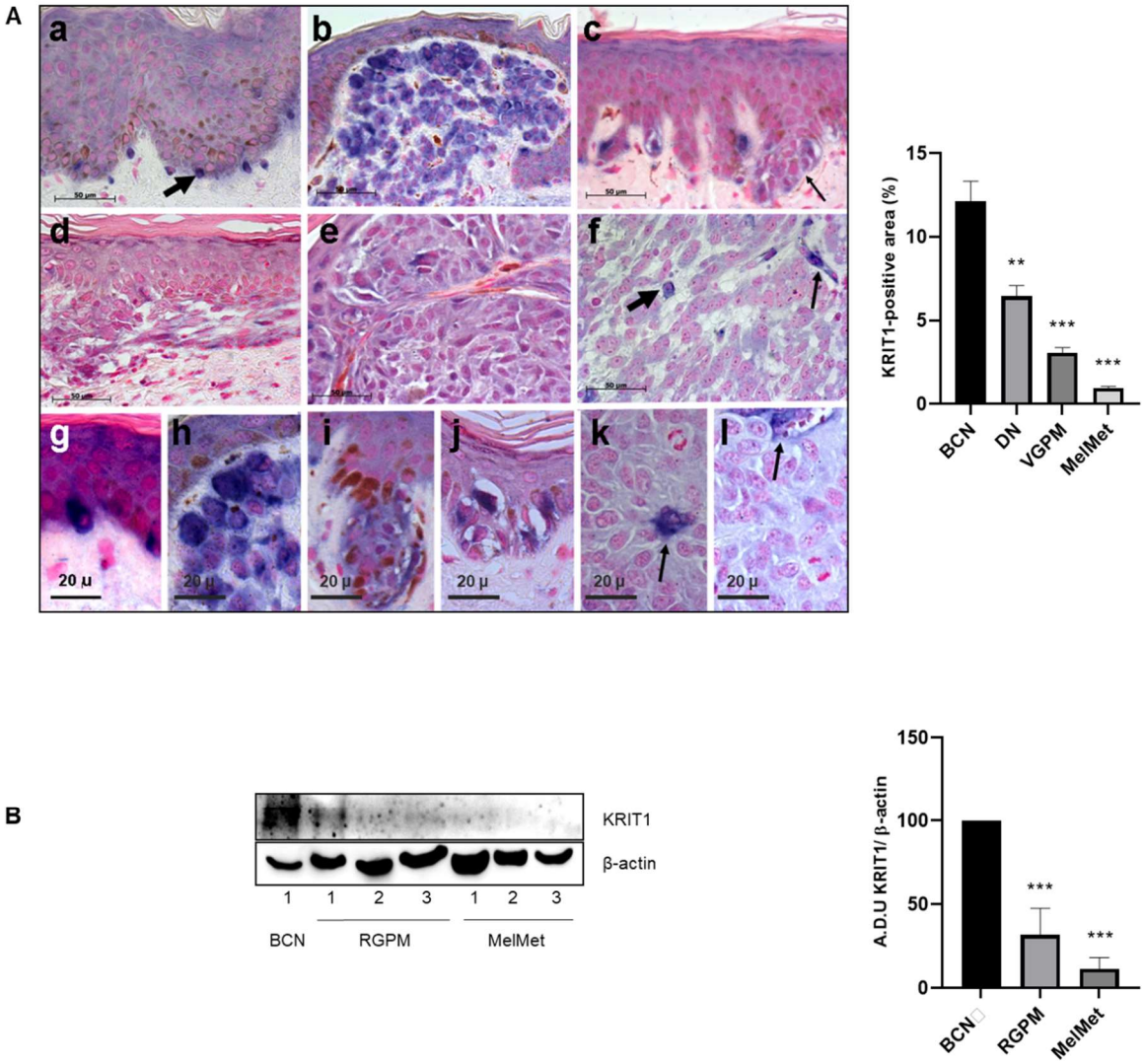


Figure 4.2.1. *KRIT1 expression in melanoma and healthy tissues. (A) Strongly immunopositive melanocytes (as the one indicated by the arrow) in normal skin (a), and in a benign common nevus, BCN (b). Marked decrease in immunopositivity in a group of melanocytes of a dysplastic nevus, DN (c, arrow), and in a radial growth phase melanoma, RGPM (d). Almost completely immunonegative melanocytes in a vertical growth phase melanoma, VGPM (e) and in a melanoma metastasis, MelMet (f; the thick arrow points on an isolated immunopositive melanocyte, and the thin arrow on immunopositive vessels). Details of KRIT1 immunohistochemistry: normal skin (g); benign common nevus (h); dysplastic nevus (i); early growth phase melanoma (j); vertical growth phase melanoma (k), and melanoma metastases (l). The arrows in "k" and "l" boxes indicate immunopositive vessels. (B) Western blot analysis of KRIT1 levels in patient samples. (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments. The scale bar in figures (a–f) represents 50 µm; in figures (g–l) represents 20 µm.*
*Statistical analysis: ***p < 0.001 and **p < 0.01 vs siCTRL.*

To further investigate the role of KRIT1 in melanoma progression, we first knocked down KRIT1 in A375 melanoma cells by siRNA transfection (siKRIT1 A375). Then we used siKRIT1 A375 cells to evaluate cell vitality and proliferation by MTT and BrdU assay. Figure 4.2.2

shows that KRIT1 knockdown increased both cell vitality (Figure 4.2.2 A) and cell proliferation (Figure 4.2.2 B).

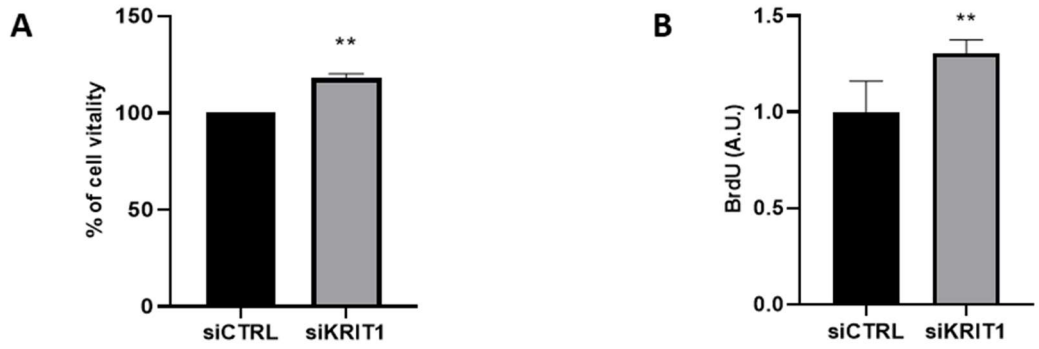


Figure 4.2.2. *KRIT1 knockdown stimulates cellular proliferation in A375 cells. (A) Cell vitality of siKRIT1 and siCTRL A375 cells by MTT assays. These results are representative of three independent experiments perfomed in triplicate. (B) BrdU incorporation assay was performed in A375 cells transfected with either control siRNA (siCTRL) or KRIT1-targeting siRNA (siKRIT1) 24 hrs post-transfection. Data shown are the averages of results from three independent experiments.*
*Statistical analysis: **p < 0.01 vs siCTRL.*

To further investigate the role of KRIT1 in cellular growth we performed a proliferation assay. After KRIT1 silencing we incubated siKRIT1 and siCTRL A375 for 24 hrs in DMEM 1% FBS, then we fixed and counted cells, observing increased proliferation of siKRIT1 compared to siCTRL cells (Figure 4.2.3). All these data indicate that KRIT1 protein loss induces melanoma cell growth.

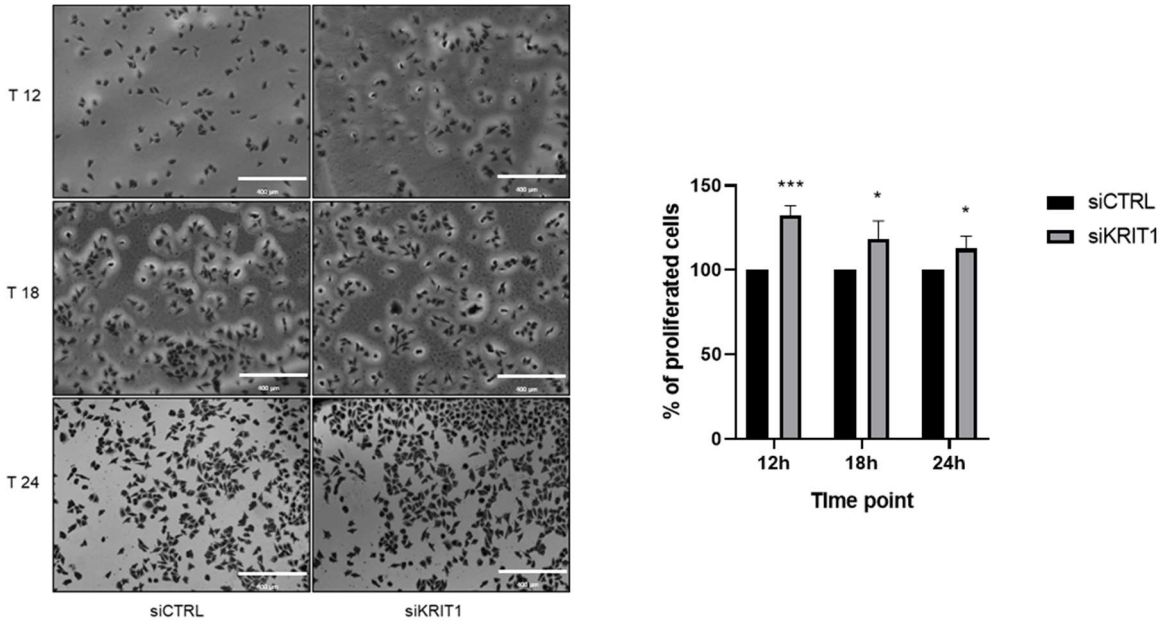


Figure 4.2.3. *Cellular proliferation in siKRIT1 and siCTRL A375 cells. Images are obtained at 20× magnification. Results are reported as % of proliferating cells above the control and are representative of three independent experiment performed in triplicate. The scale bar in figure above represents 400 µm. Statistical analysis: ***p < 0.001 and *p < 0.05 vs siCTRL.*

To confirm these data we measured the expression levels of proteins involved in cell cycle regulation by Western blotting analysis. Indeed, we observed increased cyclin D1 levels and decreases p27 levels in siKRIT1 A375 cells respect to siCTRL A375 cells (Figure 4.2.4).

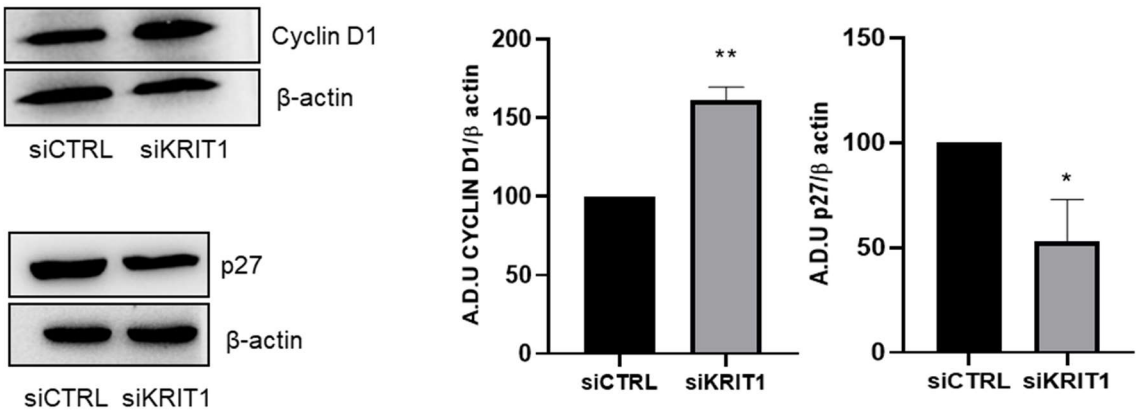


Figure 4.2.4. *Western blot analysis of cyclin D1 and p27 in siKRIT1 and siCTRL A375 cells. The histogram represents the relative expression, normalized to β-actin expression. (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments.*
*Statistical analysis: **p < 0.01 and *p < 0.05 vs siCTRL.*

We also evaluated whether loss of KRIT1 affected cell migration and invasion by using a wound closure *in vitro* scratch assay. As reported in Figure 4.2.5, the rate of wound closure in siKRIT1 A375 cells was faster than in siCTRL A375 cells, indicating that KRIT1 loss increases melanoma cell migration.

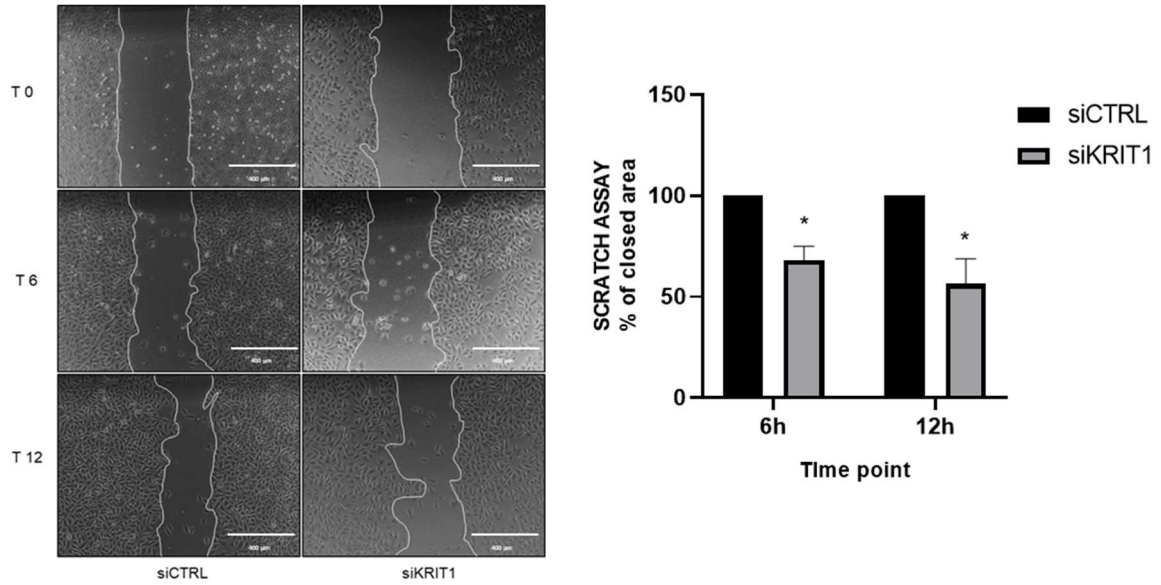


Figure 4.2.5. *KRIT1 down-regulation increases migration in A375 melanoma cells. Confluent monolayers of siKRIT1 and siCTRL A375 cells were scratched, and images were taken 6 and 12 hrs post-wounding at 20× magnification. Data are reported as % of wound closed area for well and are representative of two independent experiments. The scale bar in figure represents 400 µm.*
*Statistical analysis: *p<0.05 vs siCTRL.*

Additionally, we performed a migratory assay to assess whether knockdown of KRIT1 affected the ability of the cells to directionally move towards a selected chemoattractant. Silenced cells were plated in a trans-well system in DMEM without FBS. As chemoattractant, we used DMEM with 10% of FBS. Cells were fixed and coloured at several time points. The results indicate that knockdown of KRIT1 promoted cell invasion (Figure 4.2.6).

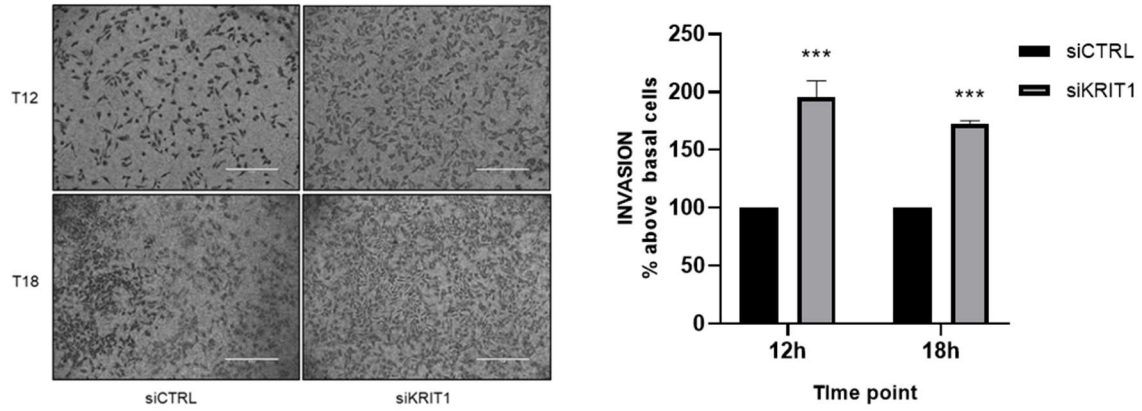


Figure 4.2.6. *KRIT1 down-regulation increases migration in A375 melanoma cells. Invasion assay. Image (20× magnification) of siKRIT1 and siCTRL A375 cells. Data are reported as % of migrated cells for well and are representative of three independent experiments. The scale bar in figure represents 400 µm. Statistical analysis: ***p < 0.01 vs siCTRL.*

It is known that the invasion process is linked to activation of MMPs, key enzymes involved in ECM degradation and in the regulation of cancer cells invasion into surrounding tissue/circulation (Radisky and Radisky 2010). For this reason, we evaluated the ability of KRIT1 to affect MMP2 activity by zymography technique. As shown in Figure 4.2.7, silencing KRIT1 significantly increased MMP-2 activity in melanoma cells.

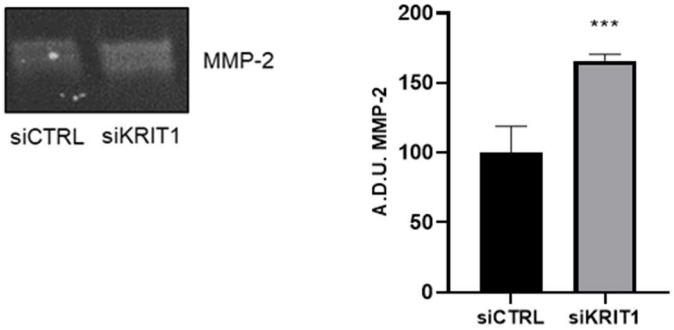


Figure 4.2.7. *KRIT1 down-regulation increases MMP2 in A375 melanoma cells. Enzymatic activity of MMP-2 in siKRIT1 and siCTRL A375 cells. Percentage of MMP-2 band was evaluated by quantitative densitometry and normalized to the number of cells/well (A.D.U: arbitrary densitometry units). Data are representative of three independent experiments Statistical analysis: *** p < 0.001.*

Since we observed increased migration and invasion in siKRIT1 cells, we hypothesized that knockdown of KRIT1 may affect also phenotype plasticity through regulation of the Wnt/β-catenin signalling pathway (Reya and Clevers 2005). Perturbations in Wnt-β-catenin signalling and elevated β-catenin levels are positively correlated with melanoma aggressiveness and are associated with melanoma malignant phenotype (Prabhakar et al. 2019). In addition, it has been already demonstrated that knockdown of KRIT1 increases nuclear β-catenin localization and activation of β-catenin dependent transcription (DiStefano et al. 2014). As shown in Figure 4.2.8, results obtained by Western blot (A) indicate that siKRIT1 A375 cells have higher levels of β-catenin expression in respect to siCTRL cells. Moreover, immunofluorescence analysis (B) of β-catenin localization indicated a shift of the protein from cytoplasm to nucleus (Figure 4.2.8).

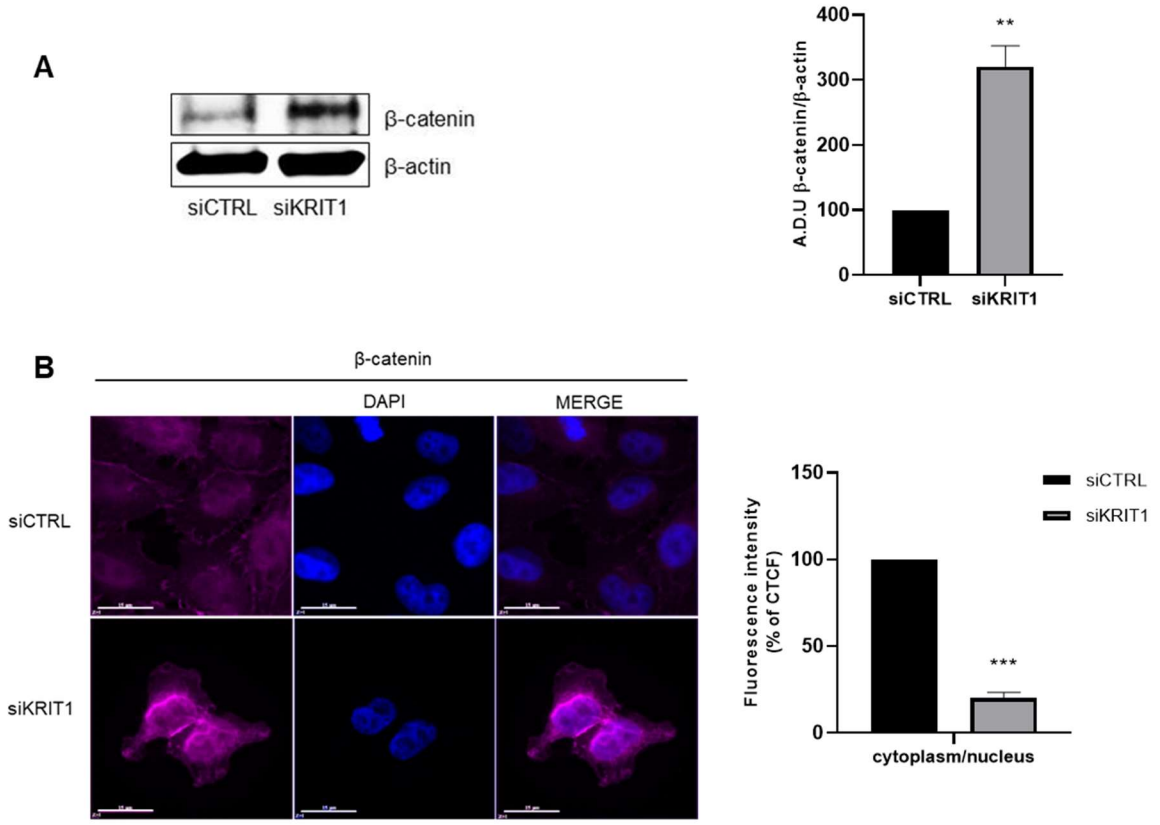


Figure 4.2.8. *KRIT1 silencing induces β-catenin overexpression and nuclear translocation of A375 melanoma cells. (A) Western blot analysis and quantification of β-catenin levels in siKRIT1 and siCTRL A375 cells (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments. (B) Immunofluorescence images and quantification of nuclear and cytoplasmatic β-catenin (40× magnification) in siKRIT1 and siCTRL A375 cells (CTCF: corrected total cell fluorescence). The scale bar in figures above represents 15 µm. Data are representative of three independent experiments performed in duplicate.*
*Statistical analysis: *** p< 0,001 and **p < 0.1 vs siCTRL.*

As nuclear translocation of β-catenin leads to transcription of β-catenin genes (Cadigan 2008), which are involved in EMT associated protein network and in melanoma plasticity, we measured the levels of EMT proteins as vimentin and N-cadherin in siKRIT1 and siCTRL A375 cells (Liu et al. 2015). As shown in Figure 4.2.9, silencing KRIT1 increased the levels of both mesenchymal markers, evaluated by both Western blot (A) and immunofluorescence (B).

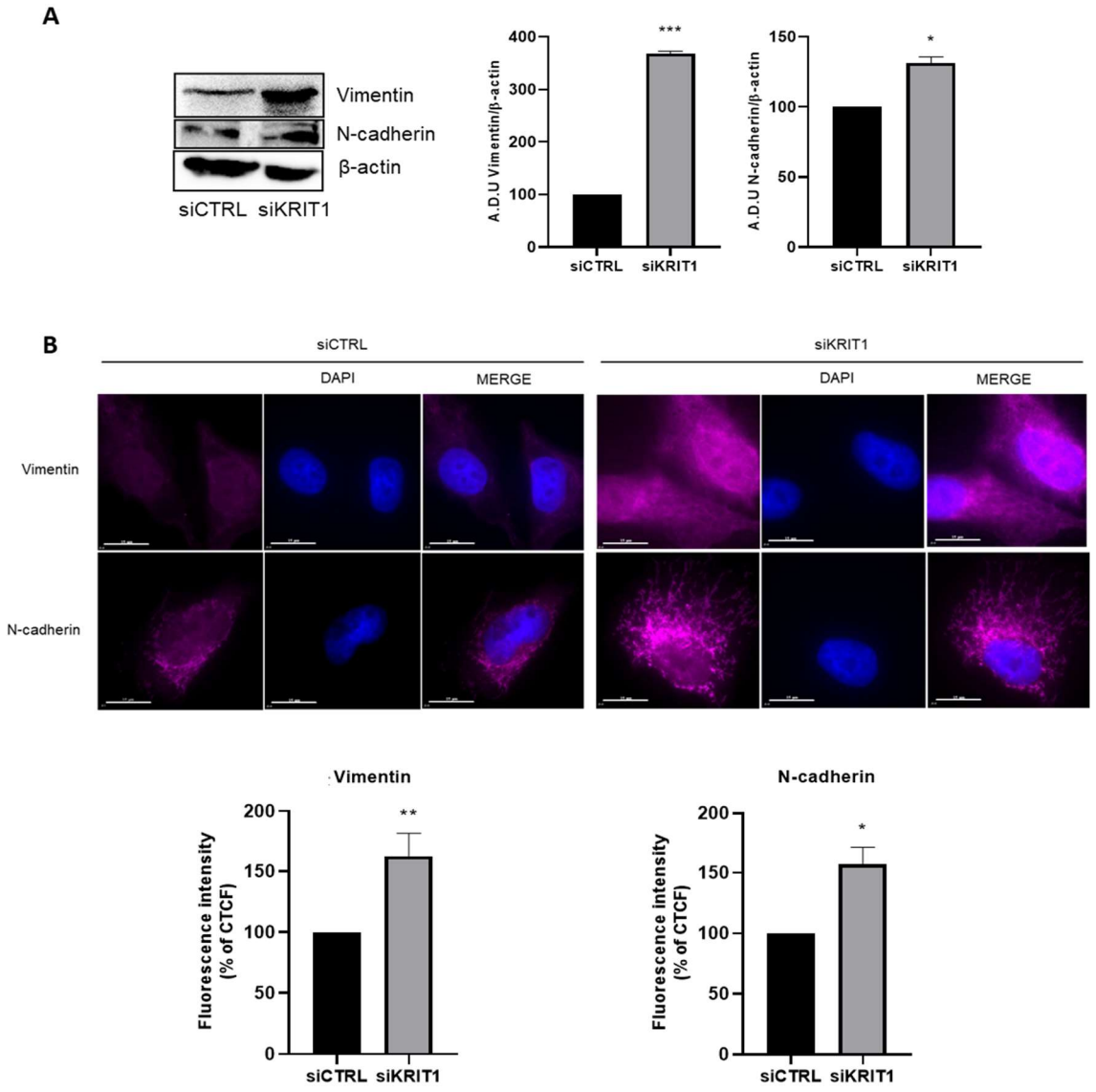


Figure 4.2.9. *KRIT1 silencing induces epithelial mesenchymal transition of A375 melanoma cells. (A) Western blot analysis and quantification of vimentin and N-cadherin expression in siKRIT1 and siCTRL A375 cells (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments. (B) Immunofluorescence images and quantification of vimentin and N-cadherin (40× magnification) in siKRIT1 and siCTRL A375 cells. (CTCF: corrected total cell fluorescence). Data are representative of three independent experiments performed in duplicate. The scale bar in figures above represents 15 µm.*

*Statistical analysis: ***p < 0.001, **p < 0.01 and * p < 0.05 vs siCTRL.*

Recently it has been reported that melanoma progression and metastatization is linked to inflammatory pathways (Panza et al. 2016). In addition, elevated expression of COX-2 plays an important role in tumorigenesis as mediating the progression and metastasis of many epithelial cancer and melanoma (Hashemi Goradel et al. 2019). As reported in Figure 4.2.10 siKRIT1 A375 expressed higher COX-2 levels than control cells.

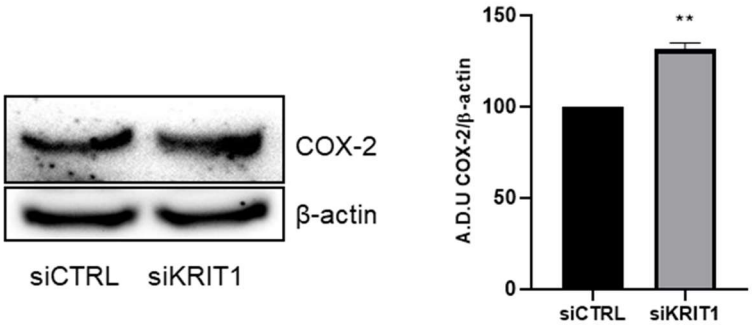


Figure 4.2.10. *KRIT1 silencing induces COX-2 expression. Western blot analysis and quantification of COX-2 expression in siKRIT1 and siCTRL A375 cells. (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments*
*Statistical analysis: **p < 0.01 vs siCTRL.*

Moreover, we also measured the expression levels of mPGES-1, the enzyme responsible of prostaglandin E2 synthesis and downstream to COX-2, and we observed that loss of KRIT1 promoted mPGES-1 up-regulation (Figure 4.2.11), indicating that KRIT1 is able to regulate the inflammatory status in melanoma cells possibly contributing to melanoma malignancy.

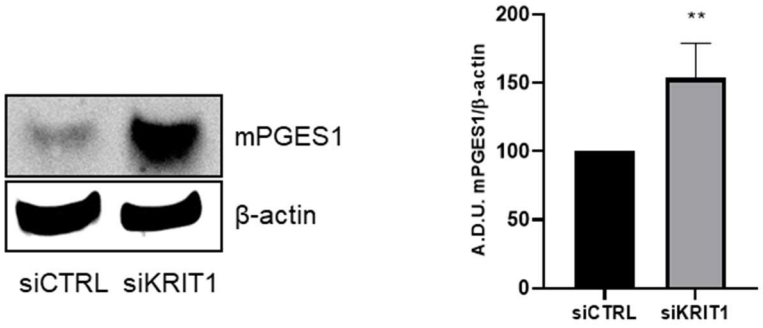


Figure 4.2.11. *KRIT1 silencing induces mPGES-1 expression. Western blot analysis and quantification of mPGES-1 expression in siKRIT1 and siCTRL A375 cells. (A.D.U: arbitrary densitometry units). Data are expressed as fold increase compared with control and are representative of three independent experiments.*
*Statistical analysis: **p < 0.01 vs siCTRL.*

In conclusion, the data reported in this work indicate the anti-proliferative, anti-migration and anti-EMT function of KRIT1 in melanoma cells supporting a possible role for this protein as tumor suppressor. Moreover, the data indicate that KRIT1 loss increases melanoma aggressiveness, suggesting a potential future of this protein as therapeutic target and diagnostic marker.

Further studies will be necessary to sustain and improve this hypothesis in other cancer lines.

5. CONCLUSIONS

Cancer is a group of diseases involving uncontrolled growth and spread of abnormal cells. It represents the most common cause of death and accounts for nearly 1 of every 4 deaths (Miller et al. 2016). There are several stages in cancer progression that are generally established with tumor size, extent of primary tumor and spreading capability to nearby lymph nodes or other organs. Diagnosis and staging are essential elements to initiate therapy (Mitra et al. 2015). Targeted therapy based on distinct tumor type aiming to maximize efficacy and minimize toxicity has remained extremely challenging. For effective cancer therapy, it is necessary to improve and develop novel therapeutical approaches.

In this work we evaluated two fundamental challenges of the research on cancer: the possibility to use natural compounds in cancer therapy and the research of new molecular targets for personalised therapy.

EGFR expression and activation are frequently up-regulated in human cancers. The oncogenic effects of EGFR include enhanced cell growth, invasion and metastasis, and inhibition of its activation and signalling has therapeutic benefit in preclinical and clinical studies (Lui and Grandis 2002).

In this work, we first investigated the role of Avns, an important class of molecules of natural source, as anti-cancer compounds in EGFR-dependent lung cancer progression. We demonstrated anti-proliferative, anti-migratory and anti-EMT activities of Avns, suggesting enhanced functional properties as bioactive nutraceuticals. Importantly, Avns are able to interact with a specific target, regulating the activation of EGFR. These data were confirmed on different cellular models of lung cancer and in EGFR overexpressing cell lines, demonstrating a possible role of Avns as anticancer compounds active on EGFR pathway. Further studies will be necessary to better understand the Avn/EGFR interaction and the possibility to apply our observations in clinical practices.

In addition, we reported new data on the “Fagiola di Venanzio” (FV) bean. FV is a recently recognized novel variety of *P. vulgaris* never studied before. Here we showed for the first time its chemical composition and potential biological activity. We demonstrated that FV reduced inflammation and oxidative stress induced by IL1β on colon cancer cells. This initial work on FV has been preparatory to a comparative work aimed at determining differences and similarities with other varieties of *P. vulgaris*, which is currently in progress. FV is

cultivated in a very restricted area of a small municipality in the South of Tuscany, by a very restricted number of growers, and for this reason is in danger of extinction due to the spread of commercial varieties. As FV appears to be a promising source of bioactive compounds and rich in nutraceutical properties, more in-depth studies aimed to further elucidate its biological and nutraceutical potential will be fundamental to safeguard and promote this specific variety of *Phaseolus*, and to better understand the implications of its consumption for public health.

Finally, in order to evaluate new possible molecular targets for cancer therapy we have identified a novel pathway that could play a role in melanoma progression.

KRIT1 protein is known for its involvement in Cerebral Cavernous Malformation (CCM), where endothelial cells of brain vessels acquire characteristics comparable to tumors including increased growth and migration, reduced apoptosis, endothelial mesenchymal transition, loss of cell-cell junction, pro-inflammatory status, altered redox homeostasis and activation of growth factors receptor signalling. Indeed, several studies demonstrated that KRIT1 is an antiangiogenic protein able to keep the human endothelium quiescent and to inhibit proliferation, migration, lumen formation and sprouting angiogenesis (Lampugnani et al. 2010; Wüstehube et al. 2010; DiStefano et al. 2014; Goitre et al. 2017). Based on its ability to regulate several signalling pathways and its ubiquitous expression, KRIT1 is likely to be involved in others pathological conditions than CCM. Very few studies have demonstrated that KRIT1 is implicated in other pathologies as cardiovascular diseases (Sega et al. 2019), diabetes (Antognelli et al. 2018), intestinal epithelial barrier dysregulation (Wang et al. 2019) and cancer (Orso et al. 2013b; Abou-Fadel et al. 2020).

Based on these hypotheses, we demonstrated that KRIT1 is mutated in melanoma cancer with a ranging between 3% and 12.5% versus a prevalence of 0% in benign melanocytic nevi, supporting our hypothesis that KRIT1 mutations play a role in melanoma development. Its depletion increased cells growth, migration and invasion of melanoma cells, improved the β-catenin expression and its translocation into the nucleus, and induced the expression of markers of inflammation and melanoma plasticity.

Among the three mutated genes in the CCM disease, *KRIT1* is by far the most involved in melanoma. Furthermore, data from the Cavernous Angioma patient registry showed that the incidence of melanoma in CCM patients is about 1.2% versus an incidence of about 0.02% in general population. Thus, even though specific information about the type of CCM

gene (*KRIT1/CCM1*, *CCM2* or *CCM3*) involved in CCM patients with melanoma is not available to date, the analysis of the different databases strongly suggest that patients bearing mutations in any of the three CCM genes are more likely to develop melanoma. The greater involvement of mutations of the *KRIT1* gene in melanoma compared to the *CCM2* and *CCM3* genes, however, suggests that CCM patients bearing *KRIT1* heterozygous mutations might be at higher risk of developing melanomas, possibly due to local effects in skin cells. The data reported here strongly support the hypothesis that KRIT1 acts as a tumor suppressor in melanoma and its loss increases melanoma aggressiveness. Thank to this fundamental function, KRIT1 has the potentiality to be a future therapeutic target and diagnostic marker for melanoma. Even though more in-depth epidemiological studies will be necessary to sustain and better elucidate these data, they will have important implications and relapses for both CCM and melanoma patients.

In conclusion, taken together the data of this work contribute to increase the knowledge on the molecular mechanisms underlying the anticancer activities of two different class of natural compounds and provide scientific support for future treatments besides the discovery of a new possible target in cancer progression.

Part of the data reported in this thesis have been the object of two recent publications (Ercoli et al., Archives Biochem. Biophys., 2020; Finetti et al., Antioxidants, 2020).

6. BIBLIOGRAPHY

Abdel lateif, K.S., Maghrabi, I.A., and Eldeab, H.A. 2016. The Plant Natural Products: Their Antioxidants, Free Radical Scavengers, DNA Protection and Antimicrobial Activities. J. Bioprocess. Biotech. doi:10.4172/2155-9821.1000293.

Abou-Fadel, J., Qu, Y., Gonzalez, E.M., Smith, M., and Zhang, J. 2020. Emerging roles of CCM genes during tumorigenesis with potential application as novel biomarkers across major types of cancers. Oncol. Rep. doi:10.3892/or.2020.7550.

Adefegha, S.A. 2018. Functional Foods and Nutraceuticals as Dietary Intervention in Chronic Diseases; Novel Perspectives for Health Promotion and Disease Prevention. doi:10.1080/19390211.2017.1401573.

Aggarwal, V., Tuli, H.S., Varol, A., Thakral, F., Yerer, M.B., Sak, K., Varol, M., Jain, A., Khan, M.A., and Sethi, G. 2019. Role of reactive oxygen species in cancer progression: Molecular mechanisms and recent advancements. doi:10.3390/biom9110735.

Albini, A., Tosetti, F., Li, V.W., Noonan, D.M., and Li, W.W. 2012. Cancer prevention by targeting angiogenesis. doi:10.1038/nrclinonc.2012.120.

Amawi, H., Ashby, C.R., Samuel, T., Peraman, R., and Tiwari, A.K. 2017. Polyphenolic nutrients in cancer chemoprevention and metastasis: Role of the epithelial-to-mesenchymal (EMT) pathway. doi:10.3390/nu9080911.

Antognelli, C., Trapani, E., Delle Monache, S., Perrelli, A., Daga, M., Pizzimenti, S., Barrera, G., Cassoni, P., Angelucci, A., Trabalzini, L., Talesa, V.N., Goitre, L., and Retta, S.F. 2018. KRIT1 loss-of-function induces a chronic Nrf2-mediated adaptive homeostasis that sensitizes cells to oxidative stress: Implication for Cerebral Cavernous Malformation disease. Free Radic. Biol. Med. doi:10.1016/j.freeradbiomed.2017.11.014.

Arvind Babu, R.S., Ramana Reddy, B. V., and Anuradha, C.H. 2014. Histogenetic concepts, terminology and categorization of biphasic tumours of the oral and maxillofacial region. doi:10.7860/JCDR/2014/7506.4078.

Assoian, R.K., and Zhu, X. 1997. Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. Curr. Opin. Cell Biol. 9(1): 93–98. Elsevier Ltd. doi:10.1016/S0955-0674(97)80157-3.

Ayati, A., Moghimi, S., Salarinejad, S., Safavi, M., Pouramiri, B., and Foroumadi, A. 2020. A review on progression of epidermal growth factor receptor (EGFR) inhibitors as an

efficient approach in cancer targeted therapy. doi:10.1016/j.bioorg.2020.103811.

Barrallo-Gimeno, A., and Nieto, M.A. 2005. The Snail genes as inducers of cell movement and survival: Implications in development and cancer. doi:10.1242/dev.01907.

Bertram, J.S. 1984. Sequential aspects of chemical carcinogenesis. Transplant. Proc.

Bienz, M. 2005. β-catenin: A pivot between cell adhesion and Wnt signalling. doi:10.1016/j.cub.2004.12.058.

BOYER, B., and THIERY, J.P. 1993. Epithelium-mesenchyme interconversion as example of epithelial plasticity. doi:10.1111/j.1699-0463.1993.tb00109.x.

Boyer, B., Tucker, G.C., Vallés, A.M., Gavrilovic, J., and Thiery, J.P. 1989. Reversible transition towards a fibroblastic phenotype in a rat carcinoma cell line. Int. J. Cancer. doi:10.1002/ijc.2910440719.

Brabletz, T. 2012. EMT and MET in Metastasis: Where Are the Cancer Stem Cells? doi:10.1016/j.ccr.2012.11.009.

Brabletz, T., Jung, A., Hermann, K., Günther, K., Hohenberger, W., and Kirchner, T. 1998. Nuclear overexpression of the oncoprotein β-Catenin in colorectal cancer is localized predominantly at the invasion front. Pathol. Res. Pract. doi:10.1016/S0344-0338(98)80129-5.

Brabletz, T., Kalluri, R., Nieto, M.A., and Weinberg, R.A. 2018. EMT in cancer. Nat. Rev. Cancer 18(2): 128–134. doi:10.1038/nrc.2017.118.

Brown, R.B. 2019. Potential interaction of inflammatory hyperemia and hyperphosphatemia in tumorigenesis. doi:10.2217/fon-2019-0213.

Bullen, J.W., Tchernyshyov, I., Holewinski, R.J., Devine, L., Wu, F., Venkatraman, V., Kass, D.L., Cole, R.N., Van Eyk, J., and Semenza, G.L. 2016. Protein kinase A-dependent phosphorylation stimulates the transcriptional activity of hypoxia-inducible factor. Sci. Signal. doi:10.1126/scisignal.aaf0583.

Cadigan, K.M. 2008. Wnt-β-catenin signaling. doi:10.1016/j.cub.2008.08.017.

Campos-Vega, R., Guevara-Gonzalez, R.G., Guevara-Olvera, B.L., Dave Oomah, B., and Loarca-Piña, G. 2010. Bean (Phaseolus vulgaris L.) polysaccharides modulate gene expression in human colon cancer cells (HT-29). Food Res. Int. doi:10.1016/j.foodres.2010.01.017.

Cappuzzo, F., Magrini, E., Ceresoli, G.L., Bartolini, S., Rossi, E., Ludovini, V., Gregorc, V., Ligorio, C., Cancellieri, A., Damiani, S., Spreafico, A., Paties, C.T., Lombardo, L.,

Calandri, C., Bellezza, G., Tonato, M., and Crino, L. 2004. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J. Natl. Cancer Inst. doi:10.1093/jnci/djh217.

Castanon, I., and Baylies, M.K. 2002. A Twist in fate: Evolutionary comparison of Twist structure and function. Gene. doi:10.1016/S0378-1119(01)00893-9.

Castellone, M.D., Teramoto, H., Williams, B.O., Druey, K.M., and Gutkind, J.S. 2005. Prostaglandin E 2 Promotes Colon Cancer Cell Growth Through a G s -Axin- b -Catenin Signaling Axis. Science (80-.).

Chambers, A.F., Groom, A.C., and MacDonald, I.C. 2002. Dissemination and growth of cancer cells in metastatic sites. doi:10.1038/nrc865.

Chan, Y.S., Xia, L., and Ng, T.B. 2016. White kidney bean lectin exerts anti-proliferative and apoptotic effects on cancer cells. Int. J. Biol. Macromol. doi:10.1016/j.ijbiomac.2015.12.094.

Chen, A.L., Hsu, C.H., Lin, J.K., Hsu, M.M., Ho, Y.F., She, T.S., Ko, J.Y., Lin, J.T., Lin, B.R., Wu, M.S., Yu, H.S., Jee, S.H., Chen, G.S., Chen, T.M., Chen, C.A., Lai, M.K., Pu, Y.S., Pan, M.H., Wang, Y.J., Tsai, C.C., and Hsieh, C.Y. 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res.

Chow, A.Y. 2010. Cell Cycle Control by Oncogenes and Tumor Suppressors: Driving the Transformation of Normal Cells into Cancerous Cells. Nat. Educ.

Ciardiello, F., and Tortora, G. 2001. A novel approach in the treatment of cancer: Targeting the epidermal growth factor receptor.

Coker, E.A., Mitsopoulos, C., Tym, J.E., Komianou, A., Kannas, C., Di Micco, P., Villasclaras Fernandez, E., Ozer, B., Antolin, A.A., Workman, P., and Al-Lazikani, B. 2019. CanSAR: Update to the cancer translational research and drug discovery knowledgebase. Nucleic Acids Res. doi:10.1093/nar/gky1129.

Collins, F.W. 1989. Oat Phenolics: Avenanthramides, Novel Substituted N-Cinnamoylanthranilate Alkaloids from Oat Groats and Hulls. J. Agric. Food Chem. doi:10.1021/jf00085a015.

Colotta, F., Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. 2009. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. Carcinogenesis 30(7): 1073–1081. doi:10.1093/carcin/bgp127.

Colucci-Guyon, E., Portier, M.M., Dunia, I., Paulin, D., Pournin, S., and Babinet, C. 1994. Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell. doi:10.1016/0092-8674(94)90553-3.

Correa, P. 1981. Epidemiological Correlations Between Diet and Cancer Frequency. Cancer Res.

Couteulx, S.L., Jung, H.H., Labauge, P., Houtteville, J., Lescoat, C., Cecillon, M., Marechal, E., Joutel, A., Bach, J., and Tournier-lasserve, E. 1999. Cause Hereditary Cavernous Angiomas. Nat. Genet. doi:10.1038/13815.

Cully, M., You, H., Levine, A.J., and Mak, T.W. 2006. Beyond PTEN mutations: The PI3K pathway as an integrator of multiple inputs during tumorigenesis. doi:10.1038/nrc1819.

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., and Milzani, A. 2009. Protein S-glutathionylation: a regulatory device from bacteria to humans. doi:10.1016/j.tibs.2008.11.002.

Darakhshan, S., and Pour, A.B. 2015. Tranilast: A review of its therapeutic applications. doi:10.1016/j.phrs.2014.10.009.

Dehne, N., Mora, J., Namgaladze, D., Weigert, A., and Brüne, B. 2017. Cancer cell and macrophage cross-talk in the tumor microenvironment. doi:10.1016/j.coph.2017.04.007.

Diepenbruck, M., and Christofori, G. 2016. Epithelial-mesenchymal transition (EMT) and metastasis: Yes, no, maybe? doi:10.1016/j.ceb.2016.06.002.

Dimburg, L.H., Theander, O., and Lingnert, H. 1993. Avenanthramides-A group of phenolic antioxidants in oats. Cereal Chem.

DiStefano, P. V., Kuebel, J.M., Sarelius, I.H., and Glading, A.J. 2014. KRIT1 protein depletion modifies endothelial cell behavior via increased vascular endothelial growth factor (VEGF) Signaling. J. Biol. Chem. doi:10.1074/jbc.M114.582304.

Donnini, S., Finetti, F., Terzuoli, E., Giachetti, A., Ĩiguez, M.A., Hanaka, H., Fresno, M., Rådmark, O., and Ziche, M. 2012. EGFR signaling upregulates expression of microsomal prostaglandin e synthase-1 in cancer cells leading to enhanced tumorigenicity. Oncogene. doi:10.1038/onc.2011.503.

Du, Z., and Lovly, C.M. 2018. Mechanisms of receptor tyrosine kinase activation in cancer. doi:10.1186/s12943-018-0782-4.

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., and Smith, F. 1951. A colorimetric method for the determination of sugars. doi:10.1038/168167a0.

Ellis, L.M. 2004. Epidermal growth factor receptor in tumor angiogenesis. doi:10.1016/j.hoc.2004.06.002.

Fares, J., Fares, M.Y., Khachfe, H.H., Salhab, H.A., and Fares, Y. 2020. Molecular principles of metastasis: a hallmark of cancer revisited. doi:10.1038/s41392-020-0134-x.

Fedi, P., Kimmelman, A., and Aaronson, S. a. 2000. Growth Factor Signal Transduction in Cancer. Holland-Frei Cancer Med.

Feregrino-Perez, A.A. ngelic., Piñol-Felis, C., Gomez-Arbones, X., Guevara-González, R.G., Campos-Vega, R., Acosta-Gallegos, J., and Loarca-Piña, G. 2014. A non-digestible fraction of the common bean (Phaseolus vulgaris L.) induces cell cycle arrest and apoptosis during early carcinogenesis. Plant Foods Hum. Nutr. doi:10.1007/s11130-014-0428-7.

Ferrara, F., Pambianchi, E., Pecorelli, A., Woodby, B., Messano, N., Therrien, J.P., Lila, M.A., and Valacchi, G. 2019. Redox regulation of cutaneous inflammasome by ozone exposure. Free Radic. Biol. Med. doi:10.1016/j.freeradbiomed.2019.11.031.

Ferro, E., Goitre, L., Baldini, E., Retta, S.F., and Trabalzini, L. 2014. Ras GTPases are both regulators and effectors of redox agents. doi:10.1007/978-1-62703-791-4_5.

Fidler, I.J. 2003. The pathogenesis of cancer metastasis: The “seed and soil” hypothesis revisited. doi:10.1038/nrc1098.

Finetti, F., Donnini, S., Giachetti, A., Morbidelli, L., and Ziche, M. 2009. Prostaglandin E2 primes the angiogenic switch via a synergic interaction with the fibroblast growth factor-2 pathway. Circ. Res. doi:10.1161/CIRCRESAHA.109.203760.

Finetti, F., Moglia, A., Schiavo, I., Donnini, S., Berta, G.N., Di Scipio, F., Perrelli, A., Fornelli, C., Trabalzini, L., and Retta, S.F. 2018. Yeast-derived recombinant avenanthramides inhibit proliferation, migration and epithelial mesenchymal transition of colon cancer cells. Nutrients. doi:10.3390/nu10091159.

Finetti, F., Schiavo, I., Ercoli, J., Zotta, A., Boda, E., Retta, S.F., and Trabalzini, L. 2020a. KRIT1 loss-mediated upregulation of NOX1 in stromal cells promotes paracrine pro-angiogenic responses. Cell. Signal. 68: 109527. doi:10.1016/j.cellsig.2020.109527.

Finetti, F., Solito, R., Morbidelli, L., Giachetti, A., Ziche, M., and Donnini, S. 2008. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor

receptor-1. J. Biol. Chem. 283(4): 2139–2146. doi:10.1074/jbc.M703090200.

Finetti, F., Terzuoli, E., Bocci, E., Coletta, I., Polenzani, L., Mangano, G., Alisi, M.A., Cazzolla, N., Giachetti, A., Ziche, M., and Donnini, S. 2012. Pharmacological inhibition of microsomal prostaglandin E synthase-1 suppresses epidermal growth factor receptor-mediated tumor growth and angiogenesis. PLoS One 7(7). doi:10.1371/journal.pone.0040576.

Finetti, F., Travelli, C., Ercoli, J., Colombo, G., Buoso, E., and Trabalzini, L. 2020b. Prostaglandin E2 and Cancer: Insight into Tumor Progression and Immunity. Biology (Basel). doi:10.3390/biology9120434.

Finetti, F., Travelli, C., Ercoli, J., Colombo, G., Buoso, E., and Trabalzini, L. 2020c. Prostaglandin E2 and Cancer: Insight into Tumor Progression and Immunity. Biology (Basel). 9(12): 434. doi:10.3390/biology9120434.

Foulds, L. 1954. The Experimental Study of Tumor Progression: A Review. Cancer Res. 14(5): 327–339.

Franke, W.W., Schmid, E., Osborn, M., and Weber, K. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.75.10.5034.

Friedewald, W.F., and Rous, P. 1944. THE INITIATING AND PROMOTING ELEMENTS IN TUMOR PRODUCTION. J. Exp. Med. doi:10.1084/jem.80.2.101.

Fu, R., Yang, P., Li, Z., Liu, W., Amin, S., and Li, Z. 2019a. Avenanthramide A triggers potent ROS-mediated anti-tumor effects in colorectal cancer by directly targeting DDX3. Cell Death Dis. doi:10.1038/s41419-019-1825-5.

Fu, R., Yang, P., Sajid, A., and Li, Z. 2019b. Avenanthramide A Induces Cellular Senescence via miR-129-3p/Pirh2/p53 Signaling Pathway To Suppress Colon Cancer Growth. J. Agric. Food Chem. doi:10.1021/acs.jafc.9b00833.

Fuchs, Y., and Steller, H. 2011. Programmed cell death in animal development and disease. doi:10.1016/j.cell.2011.10.033.

Funk, C.D. 2001. Prostaglandins and leukotrienes: Advances in eicosanoid biology. doi:10.1126/science.294.5548.1871.

Gabay, C., and Kushner, I. 1999. Acute-Phase Proteins and Other Systemic Responses to Inflammation. N. Engl. J. Med. doi:10.1056/nejm199902113400607.

Ganesan, K., and Xu, B. 2017. Polyphenol-rich dry common beans (Phaseolus vulgaris L.)

and their health benefits. doi:10.3390/ijms18112331.

Gào, X., and Schöttker, B. 2017. Reduction-oxidation pathways involved in cancer development: A systematic review of literature reviews. doi:10.18632/oncotarget.17128.

Gavert, N., and Ben-Ze'ev, A. 2007. β-Catenin signaling in biological control and cancer. doi:10.1002/jcb.21505.

Gelatti, A.C.Z., Drilon, A., and Santini, F.C. 2019. Optimizing the sequencing of tyrosine kinase inhibitors (TKIs) in epidermal growth factor receptor (EGFR) mutation-positive non-small cell lung cancer (NSCLC). doi:10.1016/j.lungcan.2019.09.017.

Gelboin, H. V. 1967. Carcinogens, Enzyme Induction, and Gene Action. Adv. Cancer Res. 10(C): 1–81. Academic Press. doi:10.1016/S0065-230X(08)60076-7.

Germolec, D.R., Shipkowski, K.A., Frawley, R.P., and Evans, E. 2018. Markers of inflammation. In Methods in Molecular Biology. doi:10.1007/978-1-4939-8549-4_5.

Gheldof, A., and Berx, G. 2013. Cadherins and epithelial-to-mesenchymal transition. In Progress in Molecular Biology and Translational Science. doi:10.1016/B978-0-12-394311-8.00014-5.

Giancotti, F.G., and Ruoslahti, E. 1999. Integrin signaling. doi:10.1126/science.285.5430.1028.

Gilkes, D.M., Semenza, G.L., and Wirtz, D. 2014. Hypoxia and the extracellular matrix: Drivers of tumour metastasis. doi:10.1038/nrc3726.

Gloushankova, N.A., Rubtsova, S.N., and Zhitnyak, I.Y. 2017. Cadherin-mediated cell-cell interactions in normal and cancer cells. doi:10.1080/21688370.2017.1356900.

Goitre, L., Distefano, P. V., Moglia, A., Nobiletti, N., Baldini, E., Trabalzini, L., Keubel, J., Trapani, E., Shuvaev, V. V., Muzykantov, V.R., Sarelius, I.H., Retta, S.F., and Glading, A.J. 2017. Up-regulation of NADPH oxidase-mediated redox signaling contributes to the loss of barrier function in KRIT1 deficient endothelium. Sci. Rep. 7(1): 1–12. Springer US. doi:10.1038/s41598-017-08373-4.

Gorrini, C., Harris, I.S., and Mak, T.W. 2013. Modulation of oxidative stress as an anticancer strategy. doi:10.1038/nrd4002.

Gravdal, K., Halvorsen, O.J., Haukaas, S.A., and Akslen, L.A. 2007. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin. Cancer

Res. doi:10.1158/1078-0432.CCR-07-1263.

Green, D.R., and Reed, J.C. 1998. Mitochondria and apoptosis. (Cover story). Science (80-.).

Guo, W., Nie, L., Wu, D., Wise, M.L., Collins, F.W., Meydani, S.N., and Meydani, M. 2010a. Avenanthramides inhibit proliferation of human colon cancer cell lines in vitro. Nutr. Cancer. doi:10.1080/01635581.2010.492090.

Guo, W., Nie, L., Wu, D., Wise, M.L., Collins, F.W., Meydani, S.N., and Meydani, M. 2010b. Avenanthramides inhibit proliferation of human colon cancer cell lines in vitro. Nutr. Cancer 62(8): 1007–1016. doi:10.1080/01635581.2010.492090.

Guo, W., Wise, M.L., Collins, F.W., and Meydani, M. 2008. Avenanthramides, polyphenols from oats, inhibit IL-1β-induced NF-κB activation in endothelial cells. Free Radic. Biol. Med. doi:10.1016/j.freeradbiomed.2007.10.036.

Ben Halima, N., Ben Saad, R., Khemakhem, B., Fendri, I., and Abdelkafi, S. 2015. Oat (Avena sativa l.): Oil and nutriment compounds valorization for potential use in industrial applications. doi:10.5650/jos.ess15074.

Hanahan, D., and Coussens, L.M. 2012. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. doi:10.1016/j.ccr.2012.02.022.

Hanahan, D., and Folkman, J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. doi:10.1016/S0092-8674(00)80108-7.

Hanahan, D., and Weinberg, R.A. 2000. The Hallmarks of Cancer Review Douglas. Cell 100(7): 57–70. doi:10.1007/s00262-010-0968-0.

Hanahan, D., and Weinberg, R.A. 2011. Hallmarks of cancer: The next generation. Cell 144(5): 646–674. Elsevier Inc. doi:10.1016/j.cell.2011.02.013.

Hangen, L., and Bennink, M.R. 2002. Consumption of black beans and navy beans (Phaseolus vulgaris) reduced azoxymethane-induced colon cancer in rats. Nutr. Cancer. doi:10.1207/s15327914nc441_8.

Harris, C.C. 1996. p53 tumor suppressor gene: From the basic research laboratory to the clinic - An abridged historical perspective. doi:10.1093/carcin/17.6.1187.

Hashemi Goradel, N., Najafi, M., Salehi, E., Farhood, B., and Mortezaee, K. 2019. Cyclooxygenase-2 in cancer: A review. J. Cell. Physiol. 234(5): 5683–5699. doi:10.1002/jcp.27411.

Hay, E.D. 1995. An overview of epithelio-mesenchymal transformation.

doi:10.1159/000147748.

Hay, E.D., and Zuk, A. 1995. Transformations between epithelium and mesenchyme: Normal, pathological, and experimentally induced. Am. J. Kidney Dis. doi:10.1016/0272-6386(95)90610-X.

Hayat, I., Ahmad, A., Masud, T., Ahmed, A., and Bashir, S. 2014. Nutritional and Health Perspectives of Beans (Phaseolus vulgaris L.): An Overview. Crit. Rev. Food Sci. Nutr. doi:10.1080/10408398.2011.596639.

Haydé, V.C., Ramón, G.G., Lorenzo, G.O., Dave, O.B., Rosalía, R.C., Paul, W., and Guadalupe, L.P. 2012. Non-digestible fraction of beans (Phaseolus vulgaris L.) modulates signalling pathway genes at an early stage of colon cancer in Sprague-Dawley rats. Br. J. Nutr. doi:10.1017/S0007114512000785.

Hazan, R.B., Phillips, G.R., Qiao, R.F., Norton, L., and Aaronson, S.A. 2000. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J. Cell Biol. doi:10.1083/jcb.148.4.779.

Herceg, Z., and Ushijima, T. 2010. Introduction. Epigenetics and cancer. In Advances in Genetics. Academic Press. doi:10.1016/B978-0-12-380866-0.60001-0.

Hoeben, A., Landuyt, B., Highley, M.S., Wildiers, H., Van Oosterom, A.T., and De Bruijn, E.A. 2004. Vascular endothelial growth factor and angiogenesis. doi:10.1124/pr.56.4.3.

Holmström, K.M., and Finkel, T. 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. doi:10.1038/nrm3801.

Hsieh, H.J., Liu, C.A., Huang, B., Tseng, A.H., and Wang, D.L. 2014. Shear-induced endothelial mechanotransduction: The interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. doi:10.1186/1423-0127-21-3.

Hubbard, S.R. 1999. Structural analysis of receptor tyrosine kinases. Prog. Biophys. Mol. Biol. doi:10.1016/S0079-6107(98)00047-9.

Huber, M.A., Kraut, N., and Beug, H. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. doi:10.1016/j.ceb.2005.08.001.

Ji, L.L., Lay, D., Chung, E., Fu, Y., and Peterson, D.M. 2003. Effects of avenanthramides on oxidant generation and antioxidant enzyme activity in exercised rats. Nutr. Res. doi:10.1016/S0271-5317(03)00165-9.

Jutten, B., Keulers, T.G., Schaaf, M.B.E., Savelkouls, K., Theys, J., Span, P.N., Vooijs, M.A.,

Bussink, J., and Rouschop, K.M.A. 2013. EGFR overexpressing cells and tumors are dependent on autophagy for growth and survival. Radiother. Oncol. doi:10.1016/j.radonc.2013.06.033.

Kalluri, R., and Neilson, E.G. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. doi:10.1172/JCI200320530.

Kalluri, R., and Weinberg, R.A. 2009. The basics of epithelial-mesenchymal transition. doi:10.1172/JCI39104.

Kang, C., Shin, W.S., Yeo, D., Lim, W., and Ji, L.L. 2018. Anti-inflammatory effect of avenanthramides via NF-κB pathways in C2C12 skeletal muscle cells. Free Radic. Biol. Med. doi:10.1016/j.freeradbiomed.2018.01.020.

Ke, X., and Shen, L. 2017. Molecular targeted therapy of cancer: The progress and future prospect. Front. Lab. Med. doi:10.1016/j.flm.2017.06.001.

Kim, Y.W., and Byzova, T. V. 2014. Oxidative stress in angiogenesis and vascular disease. doi:10.1182/blood-2013-09-512749.

Kolonel, L.N., Hankin, J.H., Whittemore, A.S., Wu, A.H., Gallagher, R.P., Wilkens, L.R., John, E.M., Howe, G.R., Dreon, D.M., West, D.W., and Paffenbarger, J. 2000. Vegetables, fruits, legumes and prostate cancer: A multiethnic case-control study. Cancer Epidemiol. Biomarkers Prev.

Konior, A., Schramm, A., Czesnikiewicz-Guzik, M., and Guzik, T.J. 2014. NADPH oxidases in vascular pathology. doi:10.1089/ars.2013.5607.

Kontomanolis, E.N., Koutras, A., Syllaios, A., Schizas, D., Mastoraki, A., Garmpis, N., Diakosavvas, M., Angelou, K., Tsatsaris, G., Pagkalos, A., Ntounis, T., and Fasoulakis, Z. 2020. Role of oncogenes and tumor-suppressor genes in carcinogenesis: A review. doi:10.21873/anticanres.14622.

Kotani, M., Tanaka, I., Ogawa, Y., Usui, T., Tamura, N., Mori, K., Narumiya, S., Yoshimi, T., and Nakao, K. 1997. Structural organization of the human prostaglandin EP3 receptor subtype gene (PTGER3). Genomics. doi:10.1006/geno.1996.4585.

Kourtidis, A., Lu, R., Pence, L.J., and Anastasiadis, P.Z. 2017. A central role for cadherin signaling in cancer. doi:10.1016/j.yexcr.2017.04.006.

Koyande, A.K., Chew, K.W., Rambabu, K., Tao, Y., Chu, D.T., and Show, P.L. 2019. Microalgae: A potential alternative to health supplementation for humans. doi:10.1016/j.fshw.2019.03.001.

Lambert, A.W., Pattabiraman, D.R., and Weinberg, R.A. 2017. Emerging Biological Principles of Metastasis. doi:10.1016/j.cell.2016.11.037.

Lamouille, S., Xu, J., and Derynck, R. 2014. Molecular mechanisms of epithelial-mesenchymal transition. doi:10.1038/nrm3758.

Lampugnani, M.G., Orsenigo, F., Rudini, N., Maddaluno, L., Boulday, G., Chapon, F., and Dejana, E. 2010. CCM1 regulates vascular-lumen organization by inducing endothelial polarity. J. Cell Sci. doi:10.1242/jcs.059329.

Lee, S.J., Sim, G.Y., Kang, H., Yeo, W.S., Kim, B.G., and Ahn, J.H. 2018. Synthesis of avenanthramides using engineered Escherichia coli. Microb. Cell Fact. doi:10.1186/s12934-018-0896-9.

Lemmon, M.A., and Schlessinger, J. 2010. Cell signaling by receptor tyrosine kinases. doi:10.1016/j.cell.2010.06.011.

Libermann, T.A., Nusbaum, H.R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M.D., Ullrich, A., and Schlessinger, J. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature. doi:10.1038/313144a0.

Lim, J., and Thiery, J.P. 2012. Epithelial-mesenchymal transitions: Insights from development. doi:10.1242/dev.071209.

Lim, W., and Kang, C. 2020. Avenanthramide C suppresses hypoxia-induced cyclooxygenase-2 expression through sirtuin1 activation in non-small-cell lung cancer cells. Animal Cells Syst. (Seoul). 24(2): 79–83. Taylor and Francis Ltd. doi:10.1080/19768354.2020.1748108.

Liu, C.Y., Lin, H.H., Tang, M.J., and Wang, Y.K. 2015. Vimentin contributes to epithelial-mesenchymal transition ancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget. doi:10.18632/oncotarget.3862.

Liu, Q., Li, A., Tian, Y., Wu, J.D., Liu, Y., Li, T., Chen, Y., Han, X., and Wu, K. 2016. The CXCL8-CXCR1/2 pathways in cancer. doi:10.1016/j.cytogfr.2016.08.002.

Liu, Q., Yu, S., Zhao, W., Qin, S., Chu, Q., and Wu, K. 2018. EGFR-TKIs resistance via EGFR-independent signaling pathways. doi:10.1186/s12943-018-0793-1.

Liu, T.C., Jin, X., Wang, Y., and Wang, K. 2017. Role of epidermal growth factor receptor in lung cancer and targeted therapies.

Liu, W., Reinmuth, N., Stoeltzing, O., Parikh, A.A., Tellez, C., Williams, S., Jung, Y.D., Fan, F.,

Takeda, A., Akagi, M., Bar-Eli, M., Gailick, G.E., and Ellis, L.M. 2003. Cyclooxygenase-2 is up-regulated by interleukin-1β in human colorectal cancer cells via multiple signaling pathways. Cancer Res.

Loh, C.Y., Chai, J.Y., Tang, T.F., Wong, W.F., Sethi, G., Shanmugam, M.K., Chong, P.P., and Looi, C.Y. 2019. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. doi:10.3390/cells8101118.

Lui, V.W.Y., and Grandis, J.R. 2002. EGFR-mediated cell cycle regulation.

Lukashev, M.E., and Werb, Z. 1998. ECM signalling: Orchestrating cell behaviour and misbehaviour. doi:10.1016/S0962-8924(98)01362-2.

Ma, J., Chen, M., Xia, S.K., Shu, W., Guo, Y., Wang, Y.H., Xu, Y., Bai, X.M., Zhang, L., Zhang, H., Zhang, M., Wang, Y.P., and Leng, J. 2013. Prostaglandin E2 promotes liver cancer cell growth by the upregulation of FUSE-binding protein 1 expression. Int. J. Oncol. doi:10.3892/ijo.2013.1782.

Madrigal-Martínez, A., Constâncio, V., Lucio-Cazaña, F.J., and Fernández-Martínez, A.B. 2019. PROSTAGLANDIN E 2 stimulates cancer-related phenotypes in prostate cancer PC3 cells through cyclooxygenase-2. J. Cell. Physiol. doi:10.1002/jcp.27515.

Marin-Hernandez, A., Gallardo-Perez, J., Ralph, S., Rodriguez-Enriquez, S., and Moreno-Sanchez, R. 2009. HIF-1α Modulates Energy Metabolism in Cancer Cells by Inducing Over-Expression of Specific Glycolytic Isoforms. Mini-Reviews Med. Chem. doi:10.2174/138955709788922610.

Martin, T., Ye, L., Sanders, A., Lane, J., and Jiang, W. 2014. Cancer Invasion and Metastasis: Molecular and Cellular Perspective. Metastatic Cancer Clin. Biol. Perspect.

Martinelli, E., De Palma, R., Orditura, M., De Vita, F., and Ciardiello, F. 2009. Anti-epidermal growth factor receptor monoclonal antibodies in cancer therapy. doi:10.1111/j.1365-2249.2009.03992.x.

Masoud, G.N., and Li, W. 2015. HIF-1α pathway: Role, regulation and intervention for cancer therapy. doi:10.1016/j.apsb.2015.05.007.

Mattila, P., Pihlava, J.M., and Hellström, J. 2005. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. J. Agric. Food Chem. doi:10.1021/jf051437z.

Medici, D., Hay, E.D., and Goodenough, D.A. 2006. Cooperation between snail and LEF-1

transcription factors is essential for TGF-β1-induced epithelial-mesenchymal transition. Mol. Biol. Cell. doi:10.1091/mbc.E05-08-0767.

Mensack, M.M., McGinley, J.N., and Thompson, H.J. 2012. Metabolomic analysis of the effects of edible dry beans (Phaseolus vulgaris L.) on tissue lipid metabolism and carcinogenesis in rats. Br. J. Nutr. doi:10.1017/S0007114512000827.

Meydani, M. 2009. Potential health benefits of avenanthramides of oats. doi:10.1111/j.1753-4887.2009.00256.x.

Miller, K.D., Siegel, R.L., Lin, C.C., Mariotto, A.B., Kramer, J.L., Rowland, J.H., Stein, K.D., Alteri, R., and Jemal, A. 2016. Cancer treatment and survivorship statistics, 2016. CA. Cancer J. Clin. doi:10.3322/caac.21349.

Mitra, A.K., Agrahari, V., Mandal, A., Cholkar, K., Natarajan, C., Shah, S., Joseph, M., Trinh, H.M., Vaishya, R., Yang, X., Hao, Y., Khurana, V., and Pal, D. 2015. Novel delivery approaches for cancer therapeutics. J. Control. Release. doi:10.1016/j.jconrel.2015.09.067.

Miyazawa, K., Hamano, S., and Ujiie, A. 1996. Antiproliferative and c-myc mRNA suppressive effects of tranilast on newborn human vascular smooth muscle cells in culture. Br. J. Pharmacol. doi:10.1111/j.1476-5381.1996.tb15486.x.

Moglia, A., Comino, C., Lanteri, S., De Vos, R., De Waard, P., Van Beek, T.A., Goitre, L., Retta, S.F., and Beekwilder, J. 2010. Production of novel antioxidative phenolic amides through heterologous expression of the plant's chlorogenic acid biosynthesis genes in yeast. Metab. Eng. doi:10.1016/j.ymben.2009.11.003.

Moglia, A., Goitre, L., Gianoglio, S., Baldini, E., Trapani, E., Genre, A., Scattina, A., Dondo, G., Trabalzini, L., Beekwilder, J., and Retta, S.F. 2015. Evaluation of the bioactive properties of avenanthramide analogs produced in recombinant yeast. BioFactors 41(1): 15–27. doi:10.1002/biof.1197.

Moloney, J.N., and Cotter, T.G. 2018. ROS signalling in the biology of cancer. doi:10.1016/j.semcdb.2017.05.023.

Montalto, F.I., and De Amicis, F. 2020. Cyclin D1 in Cancer: A Molecular Connection for Cell Cycle Control, Adhesion and Invasion in Tumor and Stroma. doi:10.3390/cells9122648.

Monti, M., Terzuoli, E., Ziche, M., and Morbidelli, L. 2016. H2S dependent and independent anti-inflammatory activity of zofenoprilat in cells of the vascular wall. Pharmacol. Res.

doi:10.1016/j.phrs.2016.09.017.

Moreno-Jiménez, M.R., López-Barraza, R., Cervantes-Cardoza, V., Pérez-Ramírez, I.F., Reyna-Rojas, J.A., Gallegos-Infante, J.A., Estrella, I., Rojas-Contreras, J.A., González-Laredo, R.F., and Rocha-Guzmán, N.E. 2019. Mechanisms associated to apoptosis of cancer cells by phenolic extracts from two canned common beans varieties (Phaseolus vulgaris L.). J. Food Biochem. doi:10.1111/jfbc.12680.

Morgensztern, D., and McLeod, H.L. 2005. PI3K/Akt/mTOR pathway as a target for cancer therapy. doi:10.1097/01.cad.0000173476.67239.3b.

Mu, P., Liu, Q., and Zheng, R. 2010. Biphasic regulation of H 2 O 2 on angiogenesis implicated NADPH oxidase . Cell Biol. Int. doi:10.1042/cbi20090092.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh-ishi, S., and Kudo, I. 2000. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J. Biol. Chem. doi:10.1074/jbc.M003505200.

Muresan, X.M., Narzt, M.S., Woodby, B., Ferrara, F., Gruber, F., and Valacchi, G. 2019. Involvement of cutaneous SR-B1 in skin lipid homeostasis. Arch. Biochem. Biophys. doi:10.1016/j.abb.2019.03.005.

Nagano, T., Tachihara, M., and Nishimura, Y. 2018. Mechanism of Resistance to Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors and a Potential Treatment Strategy. Cells. doi:10.3390/cells7110212.

Nakanishi, M., and Rosenberg, D.W. 2013. Multifaceted roles of PGE2 in inflammation and cancer. doi:10.1007/s00281-012-0342-8.

Nakazawa, M.S., Keith, B., and Simon, M.C. 2016. Oxygen availability and metabolic adaptations. doi:10.1038/nrc.2016.84.

Narumiya, S., Sugimoto, Y., and Ushikubi, F. 1999. Prostanoid receptors: Structures, properties, and functions. doi:10.1152/physrev.1999.79.4.1193.

Natarajan, K., Abraham, P., Kota, R., and Isaac, B. 2018. NF-κB-iNOS-COX2-TNF α inflammatory signaling pathway plays an important role in methotrexate induced small intestinal injury in rats. Food Chem. Toxicol. doi:10.1016/j.fct.2018.06.040.

Nie, L., Wise, M.L., Peterson, D.M., and Meydani, M. 2006. Avenanthramide, a polyphenol from oats, inhibits vascular smooth muscle cell proliferation and enhances nitric oxide production. Atherosclerosis. doi:10.1016/j.atherosclerosis.2005.07.027.

Nieto, M.A., Huang, R.Y.Y.J., Jackson, R.A.A., and Thiery, J.P.P. 2016. EMT: 2016. doi:10.1016/j.cell.2016.06.028.

Nishida, N., Yano, H., Nishida, T., Kamura, T., and Kojiro, M. 2006. Angiogenesis in cancer. doi:10.2147/vhrm.2006.2.3.213.

Nobili, S., Lippi, D., Witort, E., Donnini, M., Bausi, L., Mini, E., and Capaccioli, S. 2009. Natural compounds for cancer treatment and prevention. doi:10.1016/j.phrs.2009.01.017.

Nowell, P.C. 1976. The clonal evolution of tumor cell populations. Science (80-.). 194(4260): 23–28. doi:10.1126/science.959840.

O'Callaghan, G., and Houston, A. 2015. Prostaglandin E2 and the EP receptors in malignancy: Possible therapeutic targets? doi:10.1111/bph.13331.

Ohtsuka, J., Oshima, H., Ezawa, I., Abe, R., Oshima, M., and Ohki, R. 2018. Functional loss of p53 cooperates with the in vivo microenvironment to promote malignant progression of gastric cancers. Sci. Rep. doi:10.1038/s41598-018-20572-1.

Okazaki, Y., Isobe, T., Iwata, Y., Matsukawa, T., Matsuda, F., Miyagawa, H., Ishihara, A., Nishioka, T., and Iwamura, H. 2004. Metabolism of avenanthramide phytoalexins in oats. Plant J. doi:10.1111/j.1365-313X.2004.02163.x.

Ombra, M.N., D'Acierno, A., Nazzaro, F., Riccardi, R., Spigno, P., Zaccardelli, M., Pane, C., Maione, M., and Fratianni, F. 2016. Phenolic Composition and Antioxidant and Antiproliferative Activities of the Extracts of Twelve Common Bean (Phaseolus vulgaris L.) Endemic Ecotypes of Southern Italy before and after Cooking. Oxid. Med. Cell. Longev. doi:10.1155/2016/1398298.

Onder, T.T., Gupta, P.B., Mani, S.A., Yang, J., Lander, E.S., and Weinberg, R.A. 2008. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res. doi:10.1158/0008-5472.CAN-07-2938.

Orso, F., Balzac, F., Marino, M., Lembo, A., Retta, S.F., and Taverna, D. 2013a. MiR-21 coordinates tumor growth and modulates KRIT1 levels. Biochem. Biophys. Res. Commun. 438(1): 90–96. Elsevier Inc. doi:10.1016/j.bbrc.2013.07.031.

Orso, F., Balzac, F., Marino, M., Lembo, A., Retta, S.F., and Taverna, D. 2013b. MiR-21 coordinates tumor growth and modulates KRIT1 levels. Biochem. Biophys. Res. Commun. doi:10.1016/j.bbrc.2013.07.031.

O'Moore, K.M., Vanlandschoot, C.M., Dickman, J.R., Figi, A.R., Rothert, A.M., and Ji, L.L. 2005. Effect Of Avenathramide On Rat Skeletal Muscle Injury Induced By Lengthening

Contraction. Med. Sci. Sport. Exerc. doi:10.1249/00005768-200505001-02435.

Pan, M.H., Lai, C.S., Wu, J.C., and Ho, C.T. 2011. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. doi:10.1002/mnfr.201000412.

Panza, E., De Cicco, P., Ercolano, G., Armogida, C., Scognamiglio, G., Anniciello, A.M., Botti, G., Cirino, G., and Ianaro, A. 2016. Differential expression of cyclooxygenase-2 in metastatic melanoma affects progression free survival. Oncotarget. doi:10.18632/oncotarget.10976.

Patrignani, P., and Patrono, C. 2016. Aspirin and Cancer. doi:10.1016/j.jacc.2016.05.083.

Peiris-Pagès, M., Martinez-Outschoorn, U.E., Sotgia, F., and Lisanti, M.P. 2015. Metastasis and Oxidative Stress: Are Antioxidants a Metabolic Driver of Progression? doi:10.1016/j.cmet.2015.11.008.

Peri, A.K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. 1998. A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature. doi:10.1038/32433.

Perrelli, A., Goitre, L., Salzano, A.M., Moglia, A., Scaloni, A., and Retta, S.F. 2018. Biological activities, health benefits, and therapeutic properties of avenanthramides: From skin protection to prevention and treatment of cerebrovascular diseases. doi:10.1155/2018/6015351.

Peterson, D.M., Hahn, M.J., and Emmons, C.L. 2002. Oat avenanthramides exhibit antioxidant activities in vitro. Food Chem. doi:10.1016/S0308-8146(02)00219-4.

Philip, M., Rowley, D.A., and Schreiber, H. 2004. Inflammation as a tumor promoter in cancer induction. Semin. Cancer Biol. doi:10.1016/j.semcancer.2004.06.006.

Piotrowski, I., Kulcenty, K., and Suchorska, W. 2020. Interplay between inflammation and cancer. doi:10.1016/j.rpor.2020.04.004.

Pistritto, G., Trisciuoglio, D., Ceci, C., Alessia Garufi, and D'Orazi, G. 2016. Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted therapeutic strategies. doi:10.18632/aging.100934.

Platten, M., Ho, P.P., Youssef, S., Fontoura, P., Garren, H., Hur, E.M., Gupta, R., Lee, L.Y., Kidd, B.A., Robinson, W.H., Sobel, R.A., Selley, M.L., and Steinman, L. 2005. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. Science (80-.). doi:10.1126/science.1117634.

Prabhakar, K., Rodríguez, C.I., Jayanthy, A.S., Mikheil, D.M., Bhasker, A.I., Perera, R.J., and

Setaluri, V. 2019. Role of miR-214 in regulation of β-catenin and the malignant phenotype of melanoma. Mol. Carcinog. doi:10.1002/mc.23089.

Radisky, E.S., and Radisky, D.C. 2010. Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. doi:10.1007/s10911-010-9177-x.

Raymond, W.A., and Leong, A.S.-. 1989. Vimentin—a new prognostic parameter in breast carcinoma? J. Pathol. doi:10.1002/path.1711580205.

Rejhová, A., Opattová, A., Čumová, A., Slíva, D., and Vodička, P. 2018. Natural compounds and combination therapy in colorectal cancer treatment. doi:10.1016/j.ejmech.2017.12.039.

Ren, Y., Yang, X., Niu, X., Liu, S., and Ren, G. 2011. Chemical characterization of the avenanthramide-rich extract from oat and its effect on d -galactose-induced oxidative stress in mice. J. Agric. Food Chem. doi:10.1021/jf103938e.

Reya, T., and Clevers, H. 2005. Wnt signalling in stem cells and cancer. 434, 843–850 (2005). In Nature.

Ricciotti, E., and Fitzgerald, G.A. 2011. Prostaglandins and inflammation. Arterioscler. Thromb. Vasc. Biol. doi:10.1161/ATVBAHA.110.207449.

Van Roy, F. 2014. Beyond E-cadherin: Roles of other cadherin superfamily members in cancer. doi:10.1038/nrc3647.

Sahoo, T., Johnson, E.W., Thomas, J.W., Kuehl, P.M., Jones, T.L., Dokken, C.G., Touchman, J.W., Gallione, C.J., Lee-Lin, S.Q., Kosofsky, B., Kurth, J.H., Louis, D.N., Mettler, G., Morrison, L., Gil-Nagel, A., Rich, S.S., Zabramski, J.M., Boguski, M.S., Green, E.D., and Marchuk, D.A. 1999. Mutations in the gene encoding KRIT1, a Krev-1/rap1a binding protein, cause cerebral cavernous malformations (CCM1). Hum. Mol. Genet. doi:10.1093/hmg/8.12.2325.

Satelli, A., and Li, S. 2011. Vimentin in cancer and its potential as a molecular target for cancer therapy. doi:10.1007/s00018-011-0735-1.

Scarpa, E.S., Antonini, E., Palma, F., Mari, M., and Ninfali, P. 2018. Antiproliferative activity of vitexin-2-O-xyloside and avenanthramides on CaCo-2 and HepG2 cancer cells occurs through apoptosis induction and reduction of pro-survival mechanisms. Eur. J. Nutr. doi:10.1007/s00394-017-1418-y.

Schlessinger, J. 2014. Receptor tyrosine kinases: Legacy of the first two decades. Cold Spring Harb. Perspect. Biol. doi:10.1101/cshperspect.a008912.

Sega, F.V.D., Mastrocola, R., Aquila, G., Fortini, F., Fornelli, C., Zotta, A., Cento, A.S., Perrelli, A., Boda, E., Pannuti, A., Marchi, S., Pinton, P., Ferrari, R., Rizzo, P., and Retta, S.F. 2019. KRIT1 deficiency promotes aortic endothelial dysfunction. Int. J. Mol. Sci. doi:10.3390/ijms20194930.

Sigismund, S., Avanzato, D., and Lanzetti, L. 2018. Emerging functions of the EGFR in cancer. doi:10.1002/1878-0261.12155.

Steelman, L.S., Chappell, W.H., Abrams, S.L., Kempf, C.R., Long, J., Laidler, P., Mijatovic, S., Maksimovic-Ivanic, D., Stivala, F., Mazzarino, M.C., Donia, M., Fagone, P., Malaponte, G., Nicoletti, F., Libra, M., Milella, M., Tafuri, A., Bonati, A., Bäsecke, J., Cocco, L., Evangelisti, C., Martelli, A.M., Montalto, G., Cervello, M., and McCubrey, J.A. 2011. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mtor pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. doi:10.18632/aging.100296.

Storz, P. 2005. Reactive oxygen species in tumor progression. doi:10.2741/1667.

Suarez-Carmona, M., Lesage, J., Cataldo, D., and Gilles, C. 2017. EMT and inflammation: inseparable actors of cancer progression. doi:10.1002/1878-0261.12095.

Suárez-Martínez, S.E., Ferriz-Martínez, R.A., Campos-Vega, R., Elton-Puente, J.E., De La Torre Carbot, K., and García-Gasca, T. 2016. Bean seeds: Leading nutraceutical source for human health. doi:10.1080/19476337.2015.1063548.

Sugimoto, Y., and Narumiya, S. 2007. Prostaglandin E receptors. doi:10.1074/jbc.R600038200.

Sur, R., Nigam, A., Grote, D., Liebel, F., and Southall, M.D. 2008. Avenanthramides, polyphenols from oats, exhibit anti-inflammatory and anti-itch activity. Arch. Dermatol. Res. doi:10.1007/s00403-008-0858-x.

Tabouret, E., Bertucci, F., Pierga, J.Y., Petit, T., Levy, C., Ferrero, J.M., Campone, M., Gligorov, J., Lerebours, F., Roché, H., Bachelot, T., van Laere, S., Ueno, N.T., Toiron, Y., Finetti, P., Birnbaum, D., Borg, J.P., Viens, P., Chinot, O., and Gonçalves, A. 2016. MMP2 and MMP9 serum levels are associated with favorable outcome in patients with inflammatory breast cancer treated with bevacizumab-based neoadjuvant chemotherapy in the BEVERLY-2 study. Oncotarget. doi:10.18632/oncotarget.7612.

Tan, D.S.W., Yom, S.S., Tsao, M.S., Pass, H.I., Kelly, K., Peled, N., Yung, R.C., Wistuba, I.I., Yatabe, Y., Unger, M., Mack, P.C., Wynes, M.W., Mitsudomi, T., Weder, W.,

Yankelevitz, D., Herbst, R.S., Gandara, D.R., Carbone, D.P., Bunn, P.A., Mok, T.S.K., and Hirsch, F.R. 2016. The international association for the study of lung cancer consensus statement on optimizing management of EGFR mutation-positive non-small cell lung cancer: Status in 2016. doi:10.1016/j.jtho.2016.05.008.

Teodoro, A.J. 2019. Bioactive compounds of food: Their role in the prevention and treatment of diseases. doi:10.1155/2019/3765986.

Their, J.P. 2002. Epithelial-mesenchymal transitions in tumor progression. doi:10.1038/nrc822.

Thompson, S. V., Winham, D.M., and Hutchins, A.M. 2012. Bean and rice meals reduce postprandial glycemic response in adults with type 2 diabetes: A cross-over study. Nutr. J. doi:10.1186/1475-2891-11-23.

Trenker, R., and Jura, N. 2020. Receptor tyrosine kinase activation: From the ligand perspective. doi:10.1016/j.ceb.2020.01.016.

Turrini, E., Maffei, F., Milelli, A., Calcabrini, C., and Fimognari, C. 2019. Overview of the anticancer profile of avenanthramides from oat. doi:10.3390/ijms20184536.

Vallée, A., Lecarpentier, Y., and Vallée, J.-N. 2019. Targeting the Canonical WNT/β-Catenin Pathway in Cancer Treatment Using Non-Steroidal Anti-Inflammatory Drugs. Cells. doi:10.3390/cells8070726.

Vaquero, E.C., Edderkaoui, M., Nam, K.J., Gukovsky, I., Pandol, S.J., and Gukovskaya, A.S. 2003. Extracellular matrix proteins protect pancreatic cancer cells from death via mitochondrial and nonmitochondrial pathways. Gastroenterology. doi:10.1016/S0016-5085(03)01203-4.

Viallard, C., and Larrivée, B. 2017. Tumor angiogenesis and vascular normalization: alternative therapeutic targets. doi:10.1007/s10456-017-9562-9.

Viloria-Petit, A.M., and Kerbel, R.S. 2004. Acquired resistance to EGFR inhibitors: Mechanisms and prevention strategies. In International Journal of Radiation Oncology Biology Physics. doi:10.1016/j.ijrobp.2003.09.091.

VIRCHOW, R. 1989. As Based upon Physiological and Pathological Histology: CELLULAR PATHOLOGY. Nutr. Rev. doi:10.1111/j.1753-4887.1989.tb02747.x.

Vleminckx, K., Vakaet, L., Mareel, M., Fiers, W., and Van Roy, F. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell. doi:10.1016/0092-8674(91)90143-M.

Vogelstein, B., and Kinzler, K.W. 1993. The multistep nature of cancer. doi:10.1016/0168-9525(93)90209-Z.

Vogelstein, B., and Kinzler, K.W. 2004. Cancer genes and the pathways they control. doi:10.1038/nm1087.

Wang, D., and Dubois, R.N. 2006. Prostaglandins and cancer. doi:10.1136/gut.2004.047100.

Wang, W., Snooks, H.D., and Sang, S. 2020. The Chemistry and Health Benefits of Dietary Phenolamides. doi:10.1021/acs.jafc.0c02605.

Wang, Y., Li, Y., Zou, J., Polster, S.P., Lightle, R., Moore, T., Dimaano, M., He, T.C., Weber, C.R., Awad, I.A., and Shen, L. 2019. The cerebral cavernous malformation disease causing gene KRIT1 participates in intestinal epithelial barrier maintenance and regulation. FASEB J. doi:10.1096/fj.201800343R.

Wei, T., Zhang, X., Zhang, Q., Yang, J., Chen, Q., Wang, J., Li, X., Chen, J., Ma, T., Li, G., Gao, S., Lou, J., Que, R., Wang, Y., Dang, X., Zheng, L., Liang, T., and Bai, X. 2019. Vimentin-positive circulating tumor cells as a biomarker for diagnosis and treatment monitoring in patients with pancreatic cancer. Cancer Lett. doi:10.1016/j.canlet.2019.03.009.

Wijnhoven, B.P.L., Dinjens, W.N.M., and Pignatelli, M. 2000. E-cadherin-catenin cell-cell adhesion complex and human cancer. Br. J. Surg. doi:10.1046/j.1365-2168.2000.01513.x.

Willis, R. 1952. The Spread of Tumours in the Human Body. Med. J. Aust. 1(8): 265–266. BMJ Publishing Group. doi:10.5694/j.1326-5377.1953.tb81696.x.

Wong, R.S.Y. 2011. Apoptosis in cancer: From pathogenesis to treatment. doi:10.1186/1756-9966-30-87.

Wüstehube, J., Bartol, A., Liebler, S.S., Brütsch, R., Zhu, Y., Felbor, U., Sure, U., Augustin, H.G., and Fischer, A. 2010. Cerebral cavernous malformation protein CCM1 inhibits sprouting angiogenesis by activating DELTA-NOTCH signaling. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.1000132107.

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. 1980. Cell Death: The Significance of Apoptosis. Int. Rev. Cytol. doi:10.1016/S0074-7696(08)62312-8.

Xian, D., Song, J., Yang, L., Xiong, X., Lai, R., and Zhong, J. 2019. Emerging roles of redox-mediated angiogenesis and oxidative stress in dermatoses. doi:10.1155/2019/2304018.

Xu, Q., Huff, L.P., Fujii, M., and Griendling, K.K. 2017. Redox regulation of the actin cytoskeleton and its role in the vascular system. doi:10.1016/j.freeradbiomed.2017.03.004.

Xu, Y.H., Richert, N., Ito, S., Merlino, G.T., and Pastan, I. 1984. Characterization of epidermal growth factor receptor gene expression of malignant and normal human cell lines. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.81.23.7308.

Yamamoto, Y., and Gaynor, R.B. 2004. IκB kinases: Key regulators of the NF-κB pathway. doi:10.1016/j.tibs.2003.12.003.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. doi:10.1016/j.cell.2004.06.006.

Yang, Q.Q., Gan, R.Y., Ge, Y.Y., Zhang, D., and Corke, H. 2018. Polyphenols in Common Beans (Phaseolus vulgaris L.): Chemistry, Analysis, and Factors Affecting Composition. doi:10.1111/1541-4337.12391.

Yang, Z., Zhang, X., Gang, H., Li, X., Li, Z., Wang, T., Han, J., Luo, T., Wen, F., and Wu, X. 2007. Up-regulation of gastric cancer cell invasion by Twist is accompanied by N-cadherin and fibronectin expression. Biochem. Biophys. Res. Commun. doi:10.1016/j.bbrc.2007.05.023.

Yarden, Y., and Pines, G. 2012. The ERBB network: At last, cancer therapy meets systems biology. doi:10.1038/nrc3309.

Yeo, D., Kang, C., Zhang, T., and Ji, L.L. 2019. Avenanthramides attenuate inflammation and atrophy in muscle cells. J. Sport Heal. Sci. doi:10.1016/j.jshs.2018.08.002.

Yook, J.I., Li, X.Y., Ota, I., Hu, C., Kim, H.S., Kim, N.H., Cha, S.Y., Ryu, J.K., Choi, Y.J., Kim, J., Fearon, E.R., and Weiss, S.J. 2006. A Wnt-Axin2-GSK3β cascade regulates Snail1 activity in breast cancer cells. Nat. Cell Biol. doi:10.1038/ncb1508.

Zeisberg, M., and Neilson, E.G. 2009. Biomarkers for epithelial-mesenchymal transitions. doi:10.1172/JCI36183.

Zhang, X., Gureasko, J., Shen, K., Cole, P.A., and Kuriyan, J. 2006. An Allosteric Mechanism for Activation of the Kinase Domain of Epidermal Growth Factor Receptor. Cell. doi:10.1016/j.cell.2006.05.013.

Cellular Signalling 68 (2020) 109527

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

KRIT1 loss-mediated upregulation of NOX1 in stromal cells promotes paracrine pro-angiogenic responses

Federica Finetti[a,*,1,2], Irene Schiavo[a,1,2], Jasmine Ercoli[a,2], Alessia Zotta[b], Enrica Boda[c,2], Saverio Francesco Retta[b,2], Lorenza Trabalzini[a,*,2]

[a] Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Italy
[b] Department of Clinical and Biological Sciences, University of Torino, Italy
[c] Department of Neuroscience Rita Levi-Montalcini, Neuroscience Institute Cavalieri Ottolenghi, University of Torino, Italy

ARTICLE INFO

Keywords:
Cerebrovascular disease
CCM
KRIT1
NOX1
COX-2
PGE2

ABSTRACT

Cerebral cavernous malformation (CCM) is a cerebrovascular disorder of proven genetic origin characterized by abnormally dilated and leaky capillaries occurring mainly in the central nervous system, with a prevalence of 0.3–0.5% in the general population. Genetic studies have identified causative mutations in three genes, *CCM1/KRIT1*, *CCM2* and *CCM3*, which are involved in the maintenance of vascular homeostasis. However, distinct studies in animal models have clearly shown that CCM gene mutations alone are not sufficient to cause CCM disease, but require additional contributing factors, including stochastic events of increased oxidative stress and inflammation. Consistently, previous studies have shown that up-regulation of NADPH oxidase-mediated production of reactive oxygen species (ROS) in KRIT1 deficient endothelium contributes to the loss of microvessel barrier function.

In this study, we demonstrate that KRIT1 loss-of-function in stromal cells, such as fibroblasts, causes the up-regulation of NADPH oxidase isoform 1 (NOX1) and the activation of inflammatory pathways, which in turn promote an enhanced production of proangiogenic factors, including vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2). Furthermore and importantly, we show that conditioned media from KRIT1 null fibroblasts induce proliferation, migration, matrix metalloproteinase 2 (MMP2) activation and VE-cadherin re-distribution in wild type human endothelial cells.

Taken together, our results demonstrate that KRIT1 loss-of-function in stromal cells affects the surrounding microenvironment through a NOX1-mediated induction and release of angiogenic factors that are able to promote paracrine proangiogenic responses in human endothelial cells, thus pointing to a novel role for endothelial cell-nonautonomous effects of KRIT1 mutations in CCM pathogenesis, and opening new perspectives for disease prevention and treatment.

1. Introduction

Cerebral cavernous malformations (CCMs), also known as cavernous angioma or cavernoma, are vascular anomalies typically found in the brain and spinal cord with a prevalence of 0.3%–0.5%. CCMs are mulberry-like, thin-walled sinusoidal capillaries lacking normal vessel structural components, including pericytes and astrocytes, and often surrounded by hemosiderin deposits and gliosis [1–3]. These vascular lesions can develop anywhere in the body, but signs and symptoms generally appear only when they occur in brain and spinal cord, where they account for 5–15% of all vascular malformations.

CCMs can occur as single or multiple lesions (even in the hundreds order), with size ranging from a few millimeters to a few centimeters. Despite the high prevalence of CCM lesions, approximately only 30% of

Abbreviations: CCM, Cerebral Cavernous Malformation; VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase 2; ICH, intracerebral hemorrhage; fCCM, familial cerebral cavernous malformation; sCCM, sporadic cerebral cavernous malformation; CNS, central nervous system; NVU, neurovascular unit; ROS, reactive oxygen species; SOD, superoxide dismutase; FoxO1, Forkhead box protein O1; GKT, GKT137831; ML, ML171; NS, NS398; COX-2, cyclooxygenase 2; PGE2, Prostaglandin E2

* Corresponding authors.
E-mail addresses: finetti2@unisi.it (F. Finetti), lorenza.trabalzini@unisi.it (L. Trabalzini).
[1] These authors contributed equally to this work.
[2] CCM Italia research network (www.ccmitalia.unito.it).

https://doi.org/10.1016/j.cellsig.2020.109527
Received 1 August 2019; Received in revised form 20 December 2019; Accepted 3 January 2020
Available online 07 January 2020
0898-6568/

affected people develop clinical symptoms, including recurrent headaches, neurological deficits, seizures, stroke, and intracerebral hemorrhage (ICH); however, the majority of CCM lesions remain clinically silent during most of the host's lifetime [3].

CCM is a disease of proven genetic origin that can arise sporadically or may be inherited as an autosomal dominant condition with incomplete penetrance and variable clinical expressivity [4]. The sporadic form (sCCM) accounts for up to 80% of cases, whereas the familial form (fCCM) accounts for at least 20% of cases. Genetic studies have identified three genes associated to CCMs: *KRIT1* (*CCM1*), *MGC4607* (*CCM2*) and *PDCD10* (*CCM3*), which account for about 50%, 20% and 10% of the cases, respectively. The remaining 20% are likely associated to undetected genetic alterations of CCM genes [5]. Many different CCM mutations have been identified, most of which typical of a single family. A different clinical penetrance between the CCM genes (60–88% for *KRIT1,* up to 100% for *CCM2*, and 63% for *PDCD10*) [6], and a large variability of severity of CCM lesions even among family members carrying the same germline mutation have been observed. However, distinct studies in animal models have clearly shown that mutations of CCM genes are not sufficient to cause CCM disease, suggesting that additional factors can contribute to CCM disease pathogenesis [5,7]. Consistently, DNA sequencing analysis of surgically-resected CCM lesions from autosomal dominant CCM patients have identified somatic mutations at very low frequencies, suggesting that the minority of cells in the mature CCM harbor these mutations [8,9].

In the central nervous system (CNS) the endothelium is part of complex units, called neurovascular units (NVU), where it is in close contact with other cell types (pericytes, astrocytes and neurons). All components of this unit interact with each other in a multidimensional process in which mediators released from multiple cells engage distinct signaling pathways and effector systems in a highly orchestrated manner and safeguard the integrity of the structure itself by regulating immune response, angiogenesis, vasculogenesis, oligodendrogenesis, neuroprotection and neuroplasticity [10–12].

To date, the effect of the KRIT1 loss on NVU is not known. However, recent observations outline the importance of microenvironment in the development of vascular lesions observed in fCCM. For instance, Louvi et al., demonstrated in an animal model that *CCM3* neural deletion has cell nonautonomous effects resulting in the formation of multiple vascular lesions that closely resemble human cavernomas. Consistently, in a very recent paper Malinverno et al., showed that vascular lesions originate from clonal expansion of few *CCM3* KO endothelial cells that attract sourrounding wild-type endothelial cells thus contributing to cavernoma growth [3,13,14].

Although the effective mechanisms through which loss of CCM proteins leads to vascular malformations remain to be comprehensively defined, in recent years it has been demonstrated that these proteins exert pleiotropic effects, related to their role in the regulation of multiple molecules and mechanisms involved in angiogenesis, cellular response to oxidative stress, inflammation, cell-cell and cell-matrix adhesion, and cytoskeleton dynamics [3,15].

Reactive oxygen species (ROS) are produced by the activity of a wide array of cellular enzymes, including NADPH oxidases (NOX), enzymes of the mitochondrial respiratory chain, xanthine oxidases, cytochrome *p*450 monooxygenases, lipoxygenases and cyclooxygenases, which can be induced by a variety of endogenous and exogenous chemical and physical stimuli [16]. The NOX family of enzymes produces ROS as their sole function, and are becoming recognized as key modulators of signal transduction pathways with a physiological role under acute stress and a pathological role after excessive activation under chronic stress. ROS produced by NOX proteins are now recognized to play essential roles in the regulation of cytoskeletal remodeling, gene expression, proliferation, differentiation, migration, and cell death. The NOX isoforms (NOX1–5, and DUOX1/2) differ in their regulation, tissue and subcellular localization and even ROS products [17,18]. NOX1, NOX2, NOX4, and NOX5 are expressed in endothelium, vascular smooth muscle cells, fibroblasts, or perivascular adipocytes. While NOX1/NOX2 promote the development of endothelial dysfunction, hypertension, and inflammation, NOX4 may play a role in protecting the vasculature during stress; however, when its activity is increased, it may be detrimental [19]. Recently NOX1 has been involved in several brain diseases [20], and has been also described to play a role in cancer by inducing tumor progression and angiogenesis through the regulation of vascular endothelial growth factor (VEGF) expression [21–23].

Previously, we demonstrated that KRIT1 loss affects the intracellular redox homeostasis and results in increased ROS production through distinct mechanisms, including Forkhead box protein O1 (FoxO1) and superoxide dismutase (SOD) downregulation, NOX4 upregulation, and abnormal antioxidant responses, suggesting a novel pathogenetic mechanism whereby CCM disease may result from impaired endothelial cell defenses to microenvironmental oxidative stress events [4,16,24–30].

Herein, we show that KRIT1 loss-of-function in fibroblasts induces the upregulation of NOX1, which in turn can trigger a paracrine proangiogenic response in wild type endothelial cells through increased production and release of angiogenic growth factors, suggesting a novel important role for endothelial cell-nonautonomous effects of KRIT1 mutations in CCM disease pathogenesis, and pointing to NOX1 as a major regulator of these effects and as a new potential therapeutic target.

2. Material and methods

2.1. Cell culture

Wild-type (K+/+) and KRIT1 knock-out (K−/−) mouse embryonic fibroblast (MEF) cell lines were established from KRIT1+/+ and KRIT1−/− E8.5 mouse embryos, respectively [28]. KRIT1−/− MEFs re-expressing KRIT1 (K9/6) were obtained as previously reported [28]. Cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), with 4500 mg/l glucose and 100 U/ml penicillin/streptomycin (Euroclone, Milan, Italy).

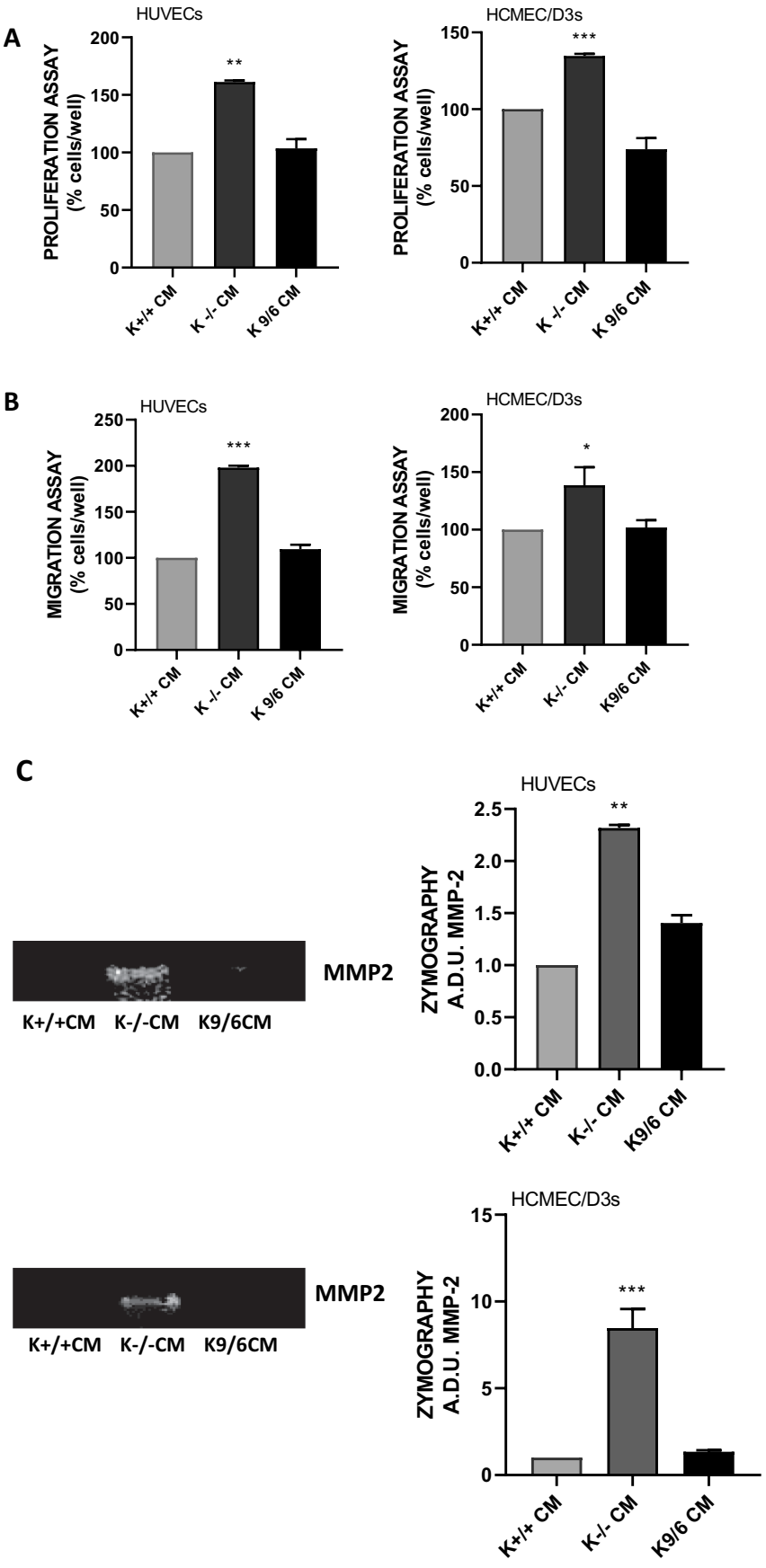
Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Lonza, Basel, Switzerlans) and Human Cerebral Microvascular Endothelial Cells (HCMEC/d3) were from MERK (MERK, Darmstadt, Germany). All experiments were performed on low passage cell cultures. Cells were grown on gelatin-coated dishes in Endothelial Growth Medium (EGM-2) (EBM-2, FBS 10%, VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000) (Lonza) at 37 °C and 5% CO_2.

2.2. Conditioned media (CMs) preparation

MEFs were seeded on 24-well plates (4×10^4 cells/well) in DMEM 10% FBS. After 24 h the medium was replaced with DMEM 0.5% serum and collected after 48 h. When reported, cells were treated for 24 h with NOX1/4 inhibitor GKT137831 (GKT, 5 μM) (Cayman Chemical, Ann Arbor, MI, USA), NOX1 inhibitor ML171 (ML, 5 μM) (EMD Millipore, Darmstadt, Germany) or COX-2 inhibitor NS398 (NS, 5 μM) (Cayman Chemical). The medium was then replaced with DMEM, 0.5% serum, and conditioned media (CM) were collected after 48 h and stored at −80 °C for further analysis. For the measurement of MMPs activity (gelatin zymography) CM were prepared without serum.

2.3. Proliferation assay

HUVEC and HCMEC/d3 cells were plated at density of 1.5×10^3 cells/well in 96-well microplates in EGM-2 with 10% serum for 24 h. After incubation for 24 h with medium added with 0.1% serum, cells were treated with MEF CMs for 72 h and then fixed and stained with



(caption on next page)

Fig. 1. Conditioned media from KRIT1 K−/− MEFs induce a pro-angiogenic response in HUVEC cells. (A) HUVEC and HCMEC/d3 proliferation. Starved endothelial cells were incubated with conditioned media (CM) obtained from K+/+, K−/− and K9/6 MEFs. Cells were then fixed, stained with PanReac and counted; results are expressed as percentage of proliferating cells and are representative of three independent experiments (** $p < .01$ vs K+/+ MEF). (B) HUVEC and HCMEC/d3 migration. The migration assay was performed by using the Boyden chamber as detailed in Materials and Methods. Data are reported as percentage of migrated cells per well and are representative of three independent experiments (*** $p < .001$ vs K+/+ MEF). (C) MMP-2 enzymatic activity through gelatin zymography. Endothelial cells were treated for 24 h with serum-free CM obtained from K+/+, K−/− and K9/6 MEF cells. MMP-2 band was evaluated with quantitative densitometry and normalized to number of cells/well. Data are representative of three independent experiments (A.D.U.: arbitrary densitometry units) (*** $p < .001$ vs K+/+ MEF). (D) Immunofluorescence analysis of VE-cadherin and β-catenin localization in HUVEC cells. HUVECs were cultured on glass cover-slips, starved and treated with CM from K+/+ or K−/− cells. HUVECs were then fixed and stained with anti-VE-cadherin or anti-β-catenin. Images are representative of three different experiments.

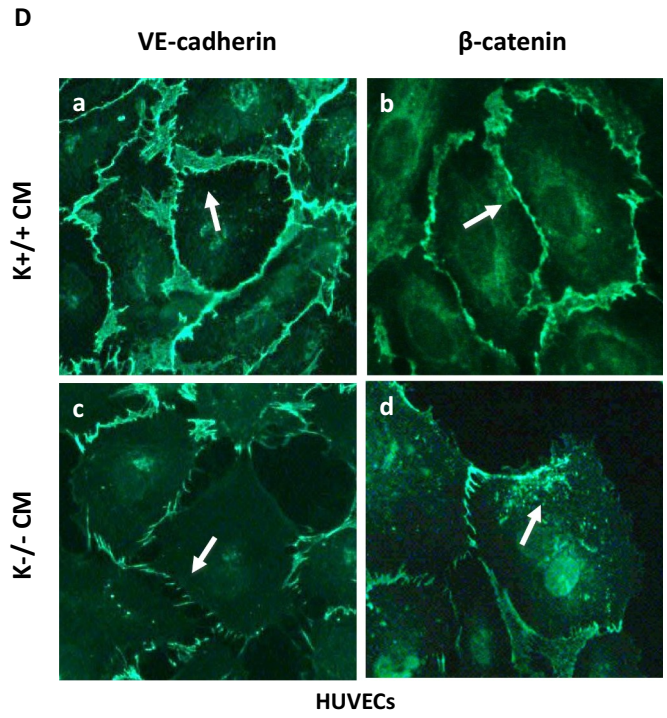


Fig. 1. (*continued*)

PanReac (Biomap, Applichem Gmbh, Darmstadt, Germany). The number of proliferated cells present in five fields/well was counted at 10× magnification. Data were reported as percentage of proliferating cells [31].

2.4. Migration assay

Chemotaxis experiments were performed using the Boyden chamber technique (Neuroprobe 48-well microchemotaxis chamber), with the filter coated with 1% gelatin (Sigma–Aldrich, Milan, Italy) [31,32]. HUVEC or HCMEC/d3 were added to the upper wells of the chamber (1.25×10^4 cells/well) suspended in 0.1% serum, and chemoattractant (0.5% serum) was placed in the lower wells. After 6 h incubation the cells were fixed and stained with PanReac. The number of migrated cells present in five fields/well was counted at 40× magnification. Data were reported as percentage of migrating cell.

2.5. Western blotting

K+/+, K−/− and K9/6 MEFs (1.5×10^5 cells/well) were seeded in 6-well multiplates in DMEM added with 10% serum. Cells were then treated with GKT (5 μM), ML (5 μM) or NS (5 μM) in DMEM with 0.5% serum. After 48 h, extraction of total proteins was performed by lysing cells in precooled radioimmunoprecipitation assay (RIPA) lysis buffer.

HUVECs (1.5×10^5 cells/well) were seeded in 6-well multiplates in EGM-2 added with 10% serum for 24 h. After starvation with EBM-2 (0.1% serum), cells were treated with CMs for 15 min.

Protein concentration of cell extracts was determined spectrophotometrically using the BCA protein assay kit (Euroclone S.P.A., Pero, MI, Italy). For western blotting analysis, aliquots of cell extract supernatants containing an equal amount of proteins (50 μg) were treated with Laemmli buffer, boiled for 10 min, resolved on 4–20% stain-free gel and then blotted onto a nitrocellulose membrane using Novablot Semidry System (GE Healthcare s.r.l., Milan, Italy). The blots were blocked with 5% nonfat dry milk (Euroclone) in Tris-buffered saline (TBS) containing 0.5% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with appropriate dilutions of primary antibodies. Subsequently membranes were incubated for 2 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies. Proteins were then visualized by an enhanced chemiluminescence detection system (EMD Millipore). The primary antibodies used in the present study included anti-COX-2 (Cell Signaling Technology, Leiden, The Netherlands), anti-GAPDH (EMD Millipore), anti-NOX1 (GeneTex, Irvine, CA, USA), anti-VEGF (EMD Millipore), anti-β-catenin and anti-P-β-catenin (Cell Signaling Technology). Affinity-purified HRP-conjugated secondary antibodies were from Sigma-Aldrich. Protein bands from western blots were quantified by densitometry using the ImageJ software, and their relative amounts were normalized to the levels of housekeeping proteins serving as internal loading controls.

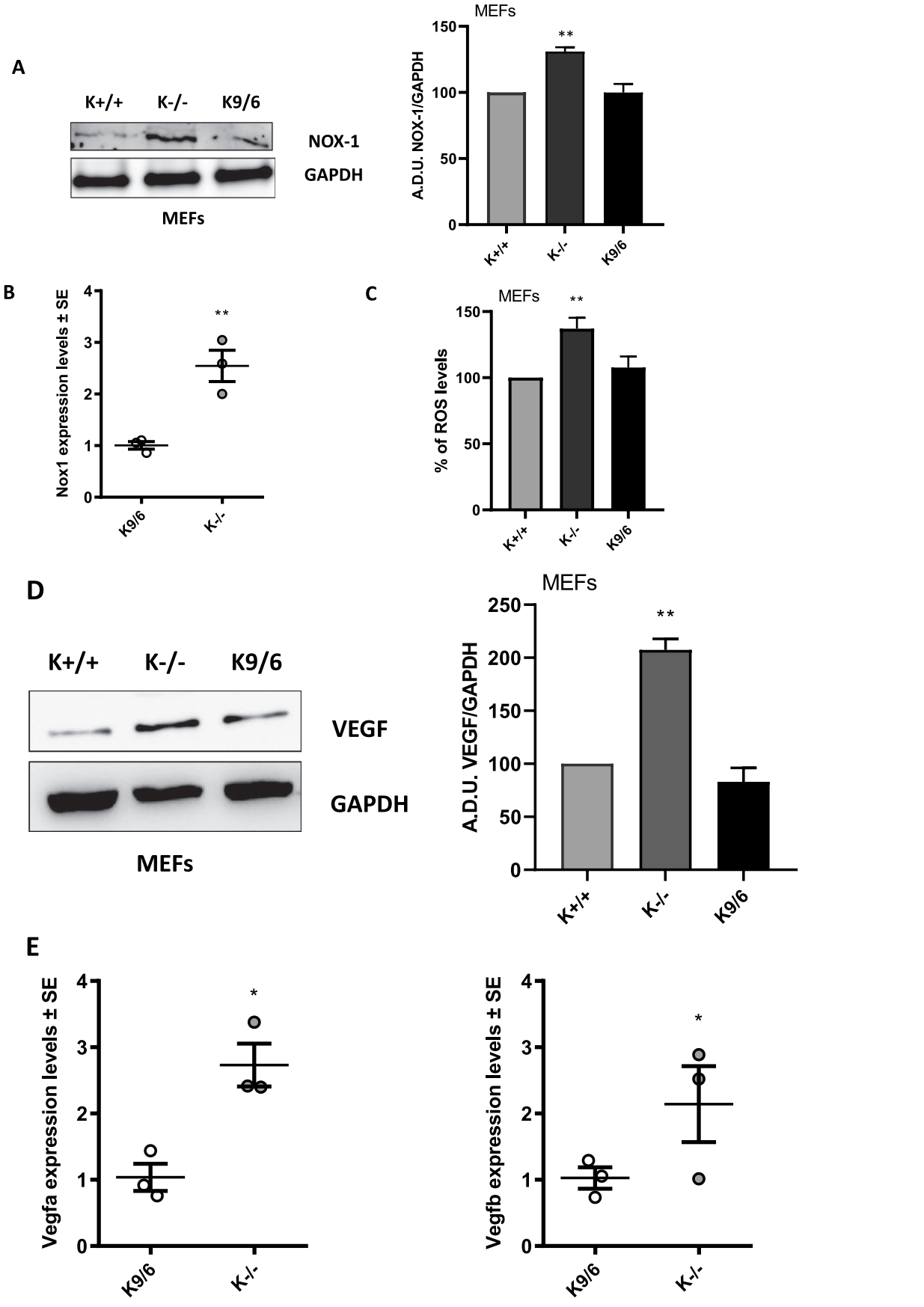
2.6. Gelatin zymography

5×10^3 cells/well (HUVECs and HCMEC/d3) were cultured in 96-well cell culture plates in 10% FBS medium. After adhesion, cells were incubated with 100 μl of serum-free MEF CMs for 48 h. The CMs were substituted with 50 μl of EBM and, after 48 h, media were collected, clarified by centrifugation and assayed for zymography. Media were subjected to electrophoresis in 8% SDS-PAGE containing 1 mg/ml gelatin under non-denaturing conditions, by using Sample Buffer w/o β-ME and sample boiling. After electrophoresis, gels were washed with 2.5% Triton X-100 to remove SDS and incubated for 48 h at 37 °C in 50 mM Tris buffer containing 200 mM NaCl and 20 mM $CaCl_2$, pH 7.4. Gels were stained with 0.05% Coomassie brilliant blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 45% methanol. Bands of gelatinase activity appeared as transparent areas against a blue background. Gelatinase activity was then evaluated by quantitative densitometry [32].

2.7. Immunofluorescence analysis

HUVEC cells (1×10^5 cells/well) were seeded on glass cover-slips pre-coated with 1% gelatin. After starvation, cells were treated with MEF conditioned media for 24 h.

Cells were fixed with acetone for 5 min, incubated with 3% BSA for 40 min and stained overnight at 4 °C with primary antibody. Slips were washed three times with PBS and then incubated 1 h at room temperature with Alexa Fluor 488 or 555 secondary antibody (ThermoFisher Scientific, Waltham, MA, USA). The anti β-catenin and anti VE-cadherin primary antibodies were from Cell Signaling Technology. Microscopy imaging was performed on Axio Lab A1 microscope (Carl Zeiss S.P.A., Milan, Italy) using a 40× objective.



(caption on next page)

Fig. 2. KRIT1 loss in stromal cells affects NOX1 and COX-2 expression. (A, D, F) Representative images and quantification of western blot analysis of NOX1, VEGF and COX-2 expression in K+/+, K−/− and K9/6 MEFs. The gels are representative of three independent experiments (A.D.U.: arbitrary densitometry units). (B, E, G) mRNA levels of Nox1, Vegf a and b and Cox-2 in K+/+, K−/− and K9/6 MEFs measured by RT-PCR (C) ROS measurement after 24 h of incubation in DMEM containing 0.5% serum. Data are expressed % of ROS levels. (H) PGE2 levels of K+/+, K−/− and K9/6 MEFs measured by EIA assay. Data are expressed as pg/ml. (***p < .001,** p < .01 and *p < .05 vs K+/+ MEF).

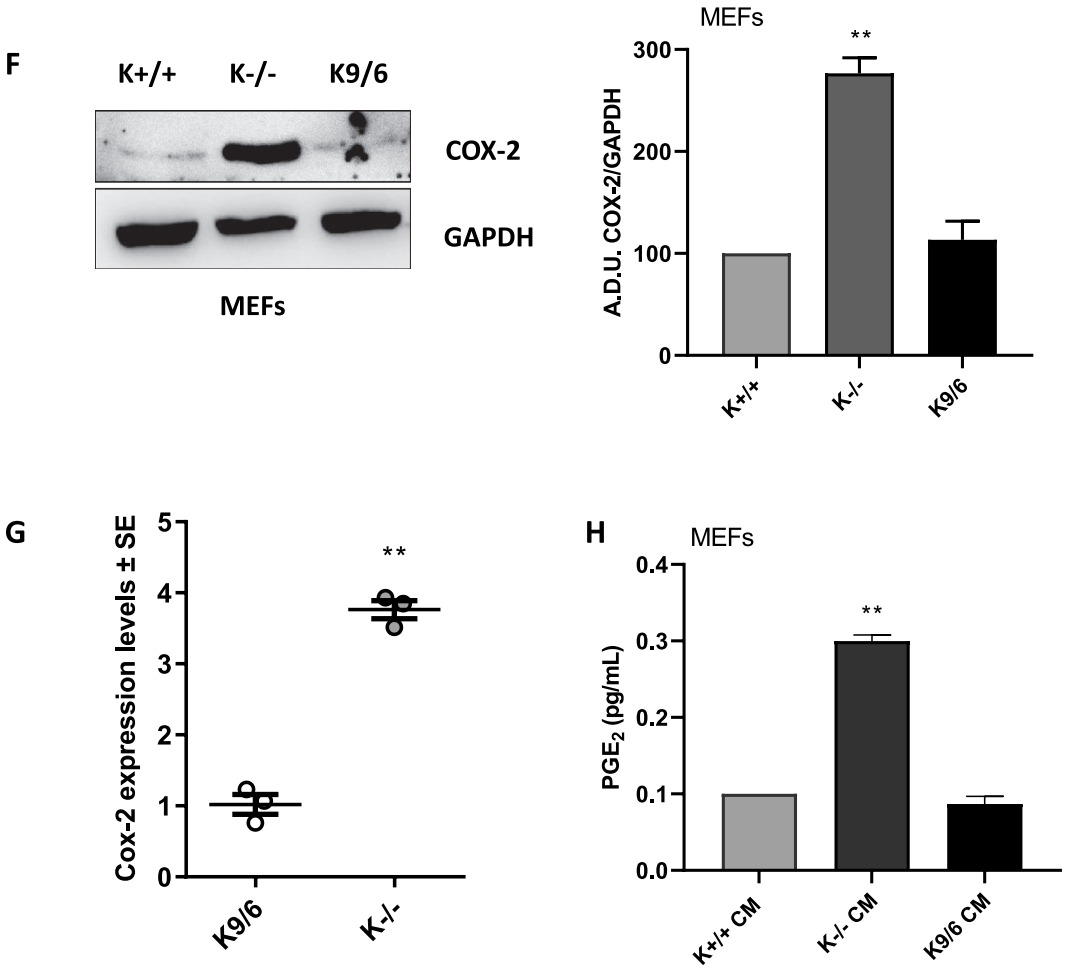


Fig. 2. (*continued*)

2.8. PGE2 immuno-assay

PGE2 was measured by an enzyme immunoassay (EIA) kit (Prostaglandin E2 EIA kit-Monoclonal, Cayman Chemical). MEFs were seeded on 24-well plates (4×10^4 cells/well) in DMEM 10% FBS. After 24 h the medium was replaced with DMEM without serum and, when reported, cells were treated with NOX1 inhibitor, ML (5 μM), or COX-2 inhibitor, NS (5 μM). Media were collected after 48 h and stored at −80 °C.

2.9. Quantitative RT-PCR

Total RNA was extracted with the RNeasy micro kit (Qiagen GmbH, Hilden, DE), and reverse transcribed to cDNA with the High-Capacity cDNA Archive kit (Applied Biosystems, Thermofisher, Waltham, USA). Quantitative Real Time RT-PCR was performed as described [33], either with predeveloped Taqman assays (Applied Biosystems, Thermofisher, Waltham, USA) or by combining the RealTime Ready Universal Probe Library (UPL, Roche Diagnostics, Monza, Italy) with the primers listed below. A relative quantification approach was used, according to the 2-ddCT method [34]. To normalize expression levels, GAPDH was used for quantifications in MEFs.

List of the predeveloped Taqman assays or combinations of primers + UPL probes used in the present study:

Gene	*Taqman assay (Applied Biosystems)*	*Primers + UPL probe (Roche Diagnostics)*
Ptgs2 (Cox2) - mouse		FW: gatgctcttccgagctgtg RV: ggattggaacagcaaggattt Probe #45
Vegfa - mouse	Cat. N: #Mm0437304_m1	
Vegfb - mouse		FW: gctcaacccagacacctgtag RV: aggaggttcgcctgtgct Probe #7
Nox1 - mouse		FW: atccttgcaccgattgctt RV: cattagatgggtgcatgacaa Probe #3
GAPDH - mouse	Cat. N: # Mm99999915_g1	

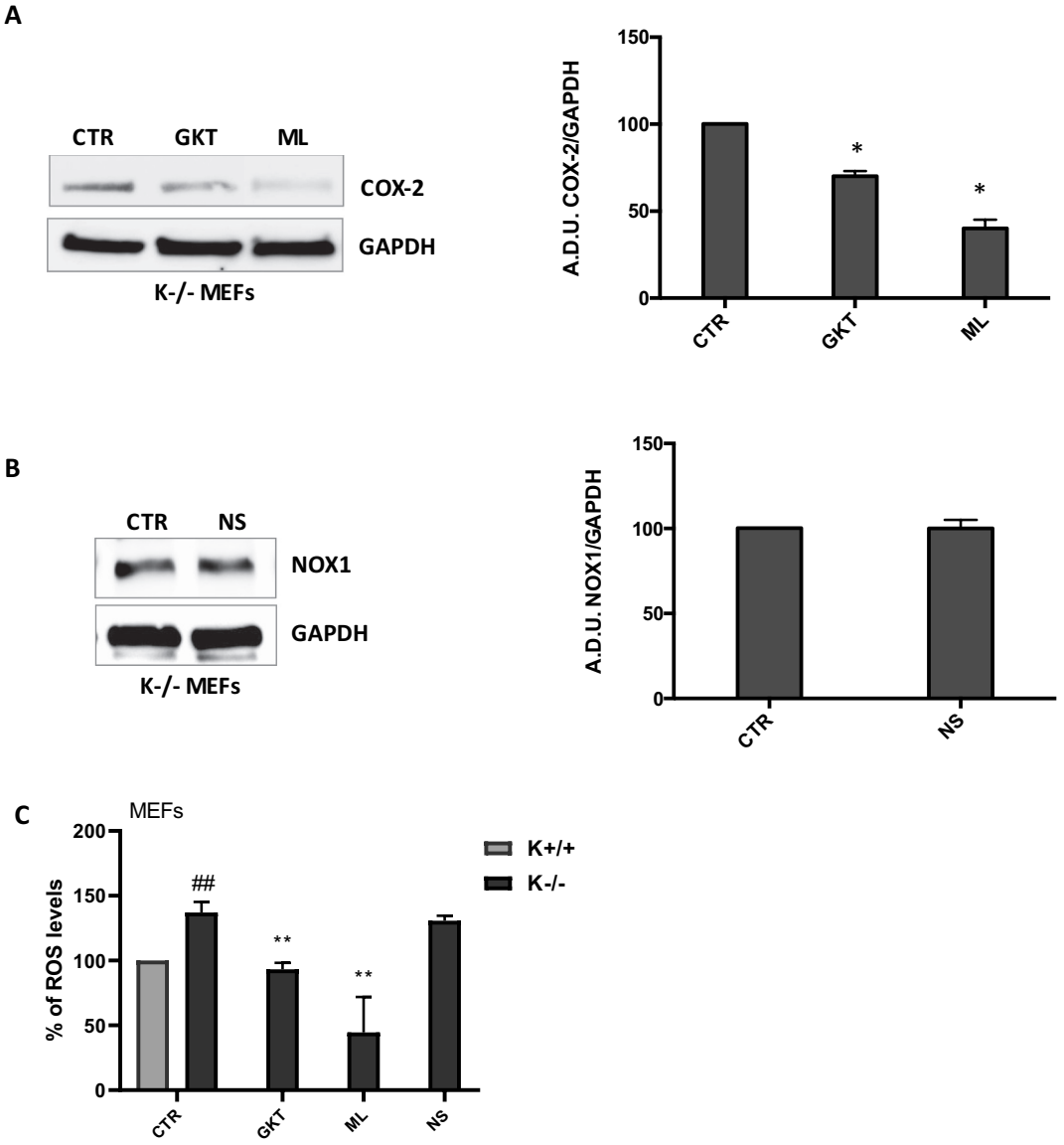


Fig. 3. NOX1 controls COX-2 expression in K−/− MEFs. (A) Western blot analysis of COX-2 expression in K−/− MEFs incubated for 48 h with a NOX1/NOX4 inhibitor (GKT, 5 μM) or a selective NOX1 inhibitor (ML, 5 μM) (B) Western blot analysis of NOX1 expression in K−/− MEFs incubated for 48 h with a COX-2 specific inhibitor (NS, 5 μM). Untreated K−/− MEFs (CTR) were analyzed as controls. The gels shown in the figure are representative of three independent experiments (A.D.U.: arbitrary densitometry units). (C) (*p < .05 vs CTR).

2.10. ROS measurement

ROS levels were evaluated as previously reported [35]. 1.5 × 10^3 cells (K+/+, K−/− or K9/6 MEFs) were seeded in 96-multiwell plates and, after adherence, were maintained for 24 h in medium without phenol red (0.5% serum) or treated with NOX1/4 inhibitor GKT137831 (GKT, 5 μM), NOX1 inhibitor ML171 (ML, 5 μM) or COX-2 inhibitor NS398 (NS, 5 μM) for 24 h. DCFH2-DA (2,-7-dichlorodihydrofluorescein diacetate) (Invitrogen, Milan, Italy) was added (10 μM, 30 min) and intracellular levels of ROS were evaluated photometrically with a microplate reader (excitation/emission 495/527) (EnVision, PerkinElmer).

2.11. Statistical analysis

Data were generated from three independent experiments and expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test for unpaired data; $p < .05$ was considered statistically significant.

3. Results

3.1. Conditioned medium from KRIT1−/− fibroblasts induces pro-angiogenic responses in wild type human endothelial cells

Accumulated evidences demonstrate that KRIT1 protein plays an important role in modulating different molecular pathways involved in the angiogenic process, whereas KRIT1 loss induces alteration of endothelial cell-cell and cell-extracellular matrix (ECM) adhesion, and enhanced vascular permeability [3,36,37]. However, recent evidence in conditional knockout (cKO) animal models has clearly shown that loss-of-function mutations in CCM genes alone are not sufficient for the development of CCM lesions, suggesting the potential contribution of endothelial cell-nonautonomous mechanisms and paracrine cues that

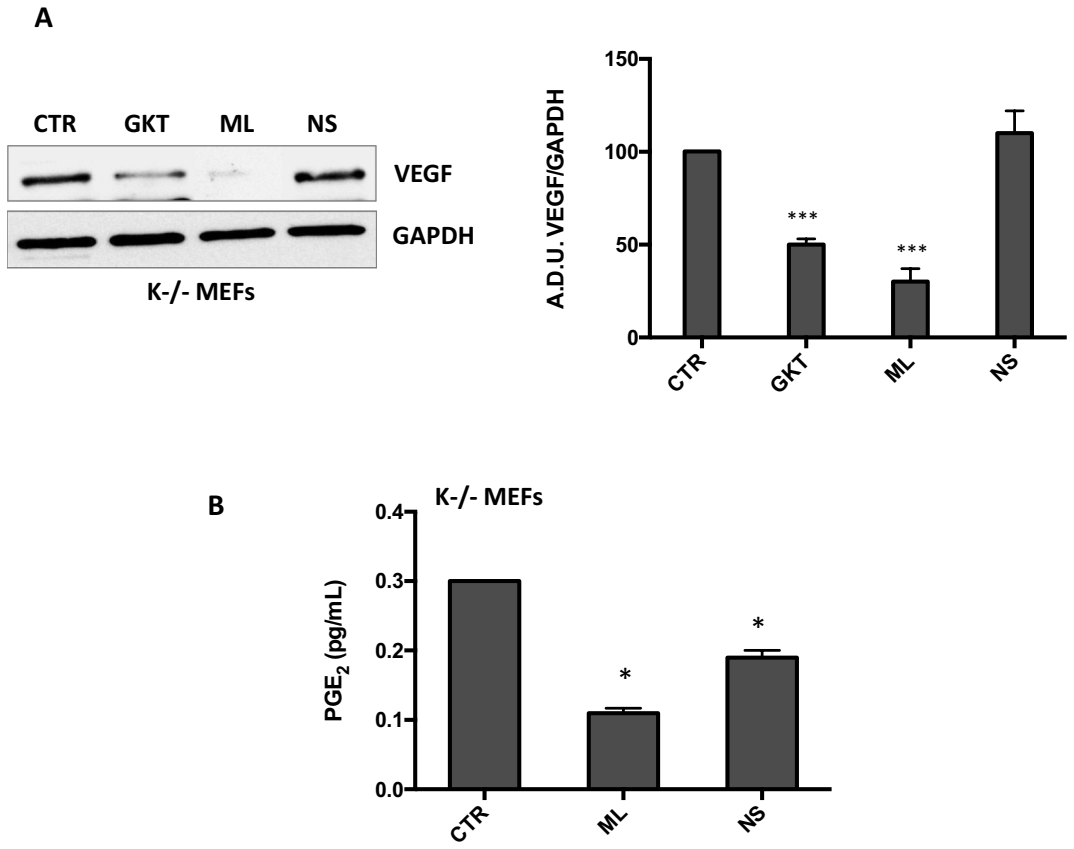


Fig. 4. NOX1 regulates VEGF expression and PGE2 production in K−/− MEFs. (A) Western blot analysis of VEGF expression in K−/− MEFs treated for 48 h with 5 μM NOX1/NOX4 inhibitor (GKT) or selective NOX1 inhibitor (ML). Untreated K−/− MEFs (CTR) were analyzed as controls. The gels shown are representative of three independent experiments (A.D.U.: arbitrary densitometry units) (*p < .05 and ***p < .001 vs CTR). (B) PGE2 levels measured in K−/− MEFs treated for 48 h with 5 μM NOX1 inhibitor (ML) and in untreated K−/− MEFs (CTR) by EIA assay. K−/− MEFs treated with the COX-2 inhibitor (NS), were used as internal control. Data are expressed as pg/ml (*p < .05 vs CTR).

affect the vascular microenvironment [3].

To address this possibility, we investigated the ability of conditioned media from KRIT1−/− fibroblasts to induce a proangiogenic response in wild type human endothelial cells. To this end, we took advantage of wild-type (K+/+) and KRIT1 knock-out (K−/−) Mouse Embryonic Fibroblasts (MEFs), a well-established cellular model of CCM disease [4,22,24,26,28,37–40], to produce conditioned media (CM) and test their effects on Human Umbilical Vein Endothelial Cells (HUVECs) and Human Cerebral Microvascular Endothelial Cells (HCMEC/d3). First we compared proliferation and migration of HUVECs and HCMEC/d3 treated for 48 h with CM from either K+/+ or K−/− MEFs, observing that CM from K−/− MEFs (K−/− CM) increased proliferation (Fig. 1A) and migration (Fig. 1B) of both endothelial cell lines compared to CM from K+/+ MEFs (K+/+ CM). In order to exclude that the differences observed could be caused by the different genetic background of K+/+ and K−/− MEF, we performed the same experiments by using KRIT1−/− MEFs re-expressing KRIT1 (K9/6 MEF) [28]. As reported in Fig. 1A and B, K9/6 CM was not able to induce neither proliferation or migration of both endothelial cell lines, suggesting that the activation of endothelial cells is specifically dependent to the levels of KRIT1 expression in stromal cells.

It is known that endothelial cell migration during angiogenesis is an invasive process that involves proteolytic activities required for the degradation of the endothelial basement membrane [41]. To investigate whether the increased migration of HUVECs cultured with K−/− CM was related to the activation of specific metalloproteinases (MMPs), we measured MMP-2 activity by gelatin zymography. As shown in Fig. 1C, MMP-2 resulted significantly activated in HUVECs and HCMEC/d3 treated with K−/− CM as compared to controls or K9/6 MEF. Accordingly, the mRNA levels of MMP2 in HUVEC and HCMEC/d3 treated with K−/− CM resulted higher compared to endothelial cells treated with K+/+ or K9/6 MEF (data not shown).

These data clearly indicate that CM derived from K−/− MEFs promotes endothelial cell proliferation and migration, suggesting a switch of endothelial cells from quiescent to active state.

Finally, in order to evaluate whether KRIT1 loss in fibroblasts could affect endothelial cell-cell interactions, we treated confluent HUVECs with either K+/+ or K−/− CM, and analyzed VE-cadherin and β-catenin expression by immunofluorescent staining. As showed in Fig. 1D, in HUVECs treated with K+/+ CM VE-cadherin expression was detected at junctional regions with a continuous zigzag pattern (panel a), while in cells cultured with K−/− CM VE-cadherin staining appeared intermittent, showing frequent gaps (panel c). Similarly, we detected a different pattern of β-catenin expression in HUVECs treated with K−/− CM, including increased protein internalization (panel d), as compared to cells treated with K+/+ CM (panel b). These results show that the CM generated by KRIT1-null fibroblasts is able to modulate VE-cadherin and β-catenin localization in wild type human endothelial cells, suggesting that KRIT1 loss in stromal cells may modulate cell-cell interactions in normal endothelium via paracrine mechanisms.

3.2. KRIT1 loss-of-function in stromal cells affects NOX1 and COX-2 expression

In recent years, it has been demonstrated that KRIT1 plays an important role in the manteinance of cellular redox homeostasis by

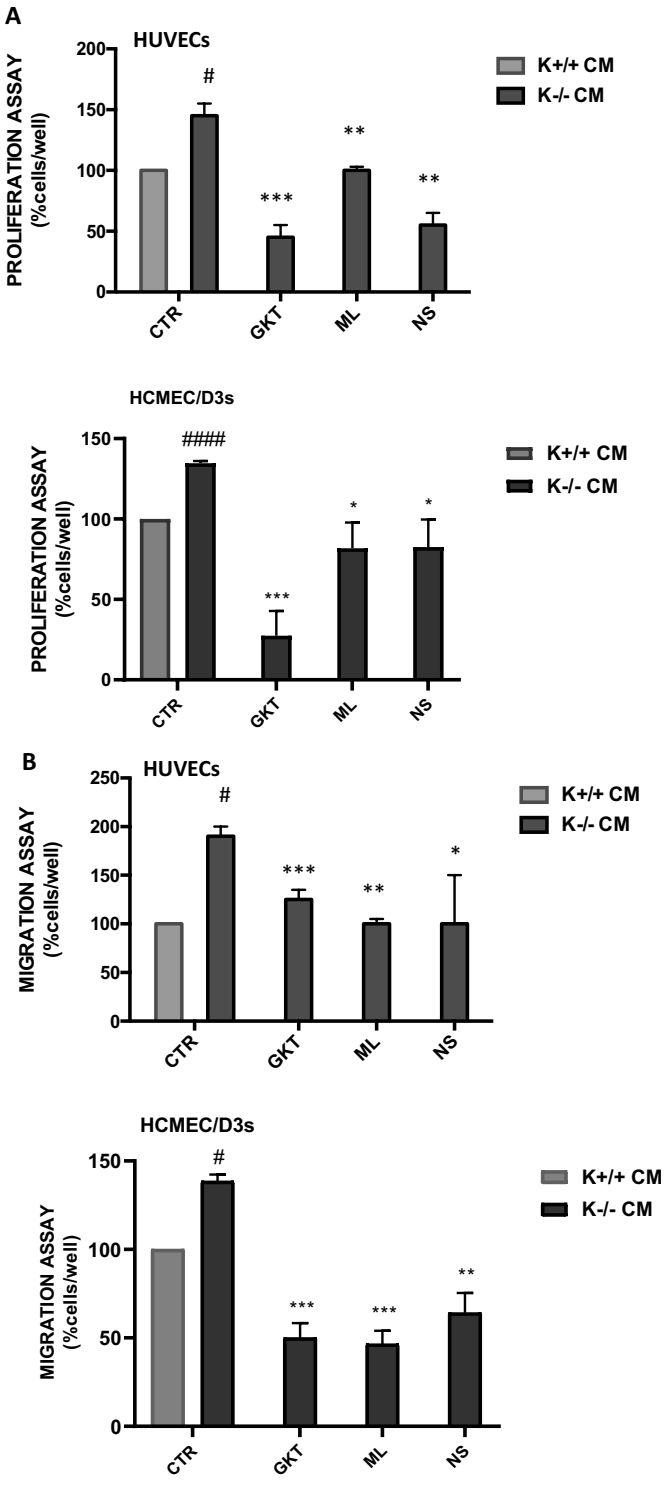
modulating master regulators of intracellular ROS levels and cell responses to oxidative stress, including SOD2, FOXO1 and NOX4 [26,28,29]. Conversely, KRIT1 loss-of-function has been clearly associated with the alteration of distinct redox-sensitive mechanisms, including the upregulation of major pro-oxidant and proinflammatory proteins such as c-Jun and cyclooxygenase 2 (COX-2) [26]. On the other hand, consistent with a possible significant involvement in CCM pathogenesis, oxidative stress and inflammation have been clearly implicated in vascular remodeling and endothelial dysfunction associated with cerebrovascular diseases [28]. Indeed, whereas it is known that NADPH oxidases are major enzymes responsable for ROS production [19,42,43], we previously demostrated that NOX4 is involved in CCM disease [29].

To address the putative mechanisms involved in the paracrine signaling between KRIT1-null fibroblasts and wild type human endothelial cells, we considered the possible involvement of NADPH oxidases, as these enzymes are known to play major regulatory roles both in ROS production and in the modulation of proinflammatory and proangiogenic responses [19,29,42,43]. In particular, very recent papers showed that NOX1 upregulation in tumor cells is able to induce the production of angiogenic factors, which in turn activate endothelial cells and trigger an angiogenic response [21–23]. To investigate the potential effects of KRIT1 loss-of-function on NOX1, we evaluated NOX1 expression levels in K+/+, K−/− and K9/6 MEFs both by western blot analysis and RT-PCR. As showed in Fig. 2A and B, NOX1 levels were significantly higher in K−/− cells compared to wild type cells (K+/+) and KRIT1−/− MEFs re-expressing KRIT1 (K9/6), suggesting a functional relathionship. In addition K−/− MEF expressed higher levels of ROS when compared to K+/+ and K 9/6 cells (Fig. 2C). Moreover, to address the putative correlation between NOX1 overexpression in KRIT1-null stromal cells and the observed paracrine effects on wild type human endothelial cells, we firstly investigated the expression levels of VEGF, a major angiogenic growth factor whose expression and signaling have been previously shown to be modulated by NADPH oxidase-derived ROS [44–46]. Western blot and RT-PCR analysis of total cell extracts showed that K−/− MEF cells expressed significantly higher levels of VEGF when compared to K+/+ and K9/6 cells (Fig. 2D and E). Furthermore, consistently with our previous findings that KRIT1 loss is associated with increased activation of inflammatory pathways [26], as well as that the main prostaglandin produced by COX-2, prostaglandin E2 (PGE2), exerts a potent proangiogenic activity [32,47,48], we found higher COX-2 expression levels (Fig. 2F and G) and PGE2 production (Fig. 2H) in K−/− MEF compared to K+/+ and K9/6 MEF.

In the attempt to determine if there was any hierarchy among major pro-oxidant and pro-inflammatory proteins affected by KRIT1 loss-of-function, including NOX1 and COX-2, we inhibited alternatively their activity with specific pharmacological inhibitors, namely ML (NOX1 inhibitor), GKT (NOX1/NOX4 inhibitor) and NS (COX-2 inhibitor). Interestingly, NOX1 inhibition in K−/− MEFs resulted in reduced COX-2 expression levels (Fig. 3A), whereas COX-2 inhibition did not affect NOX1 expression (Fig. 3B) or ROS production (Fig. 3C), suggesting that COX-2 activation induced by KRIT1 loss-of-function occurs via NOX1. Moreover, since both oxidative stress and inflammation can induce the upregulation of VEGF [45], we analyzed its expression levels in K−/− cells after either NOX1 or COX-2 inhibition. As reported in Fig. 4A, we observed a significant reduction of VEGF expression in K−/− cells treated with NOX1 inhibitor, while VEGF expression was not impaired by treatment with the COX-2 inhibitor. As expected, both NOX1 and COX-2 inhibition reduced PGE2 production (Fig. 4B). These data clearly indicate that NOX1 upregulation in K−/− MEFs controls both VEGF and COX-2/PGE2 production.

3.3. NOX1 upregulation in stromal cells regulates the paracrine activation of endothelial cells

Combining our findings that treatment of HUVECs with K−/− CM induces a pro-angiogenic phenotype and that NOX1 upregulation in K−/− MEFs induces overproduction of pro-angiogenic factors, we speculated that NOX1 inhibition in K−/− fibroblasts could revert the activation of endothelial cells triggered by K−/− CM. To test this



(caption on next page)

Fig. 5. NOX1 overexpression in stromal cells controls proliferation, migration and MMP-2 activation in endothelial cells. (A) Proliferation assay. HUVEC and HCMEC/d3 cells were cultured for 48 h with CM isolated from K+/+ or K−/− MEFs pre-treated with NOX1 or COX-2 inhibitor as previously described. Data are reported as percentage of number of cells per well (#p < .05 vs K+/+ MEF; *** p < .001 and **p < .01 vs K−/− MEF). (B) Migration assay. HUVEC and HCMEC/d3 cells were treated with CM isolated from K+/+ MEFs or K−/− MEF cells pre-treated with NOX1 or COX-2 inhibitor. The migration assay was performed by using the Boyden chamber as detailed in Materials and Methods. Data are reported as percentage of number of cells per well (#p < .05 vs K+/+ MEF; *** p < .001, **p < .01 and *p < .05 vs K−/− MEF). (C) Determination of MMP-2 enzymatic activity by gelatin zymography. Representative image of MMP-2 activity of HUVECs and HCMEC/d3 cultured with CM from K−/− MEFs pre-treated with NOX1 or COX-2 inhibitor. MMP-2 bands were evaluated by quantitative densitometry and normalized to the number of cells/well (A.D.U.: arbitrary densitometry units). Data are representative of three independent experiments (*p < .05 vs CTR).

possibility, we performed proliferation and migration assays on HUVECs and HCMEC/d3 treated with CM from K−/− MEFs pre-treated with NOX1 inhibitors. Moreover, as COX-2 has been implicated in PGE2-dependent neovascularization, we also pre-treated K−/− MEFs with the COX-2 inhibitor in order to assess whether either NOX1 or COX-2 inactivation could revert the HUVEC angiogenic phenotype triggered by K−/− CM. Interestingly, we observed a significant reduction in proliferation (Fig. 5A) and migration (Fig. 5B) when endothelial cells were cultured with CM from K−/− MEFs pre-treated with either NOX1 (GKT and ML) or COX-2 (NS) inhibitors. Consistently, MMP-2 activity decreased in HUVEC and HCMEC/d3 cells incubated with CM from K−/− pre-treated with ML or NS (Fig. 5C). These results indicate that NOX1 and COX-2 overexpressed in K−/− fibroblasts are able to induce paracrine effects that regulate proliferation and migration of endothelial cells, suggesting that NOX1 upregulation and activation of downstream pro-oxidative and proinflammatory pathways in stromal cells play a key role in the paracrine modulation of angiogenic phenotypes of HUVEC cells.

In addition, we performed immunofluorescence analysis of VE-cadherin and β-catenin to assess whether NOX1 and COX-2 inhibition could rescue also the altered cell-cell adhesion phenotype detected in HUVECs treated with CM from K−/− MEFs. As shown in Fig. 6A and B, HUVECs cultured with CM from K−/− MEFs pre-treated with either GKT, ML or NS (panels c, d and e respectively) exhibited a VE-cadherin (Fig. 6A) and β-catenin (Fig. 6B) distribution comparable to cells cultured with K+/+ CM (panels a), suggesting that inhibition of NOX1 and COX-2 rescues the altered cell-cell adhesion phenotype induced by KRIT1 loss (panels b). To further confirm these observations, we analyzed the phosphorylation levels of β-catenin in HUVECs treated for 15 min with CM from K+/+ or K−/− MEFs pre-treated or not with inhibitors. We observed consistent β-catenin phosphorylation in HUVECs treated with CM from K−/− MEFs, which was reverted by K−/− MEF pre-treatment with NOX1 and COX-2 inhibitors (Fig. 6C).

These results suggest that either NOX1 or COX-2 inhibition in K−/− MEFs can rescue the altered cell-cell adhesion phenotype observed in HUVECs treated with K−/− CM.

4. Discussion

CCM lesions are typically found in the CNS and are characterized by dilated and leaky capillaries that are devoid of normal vessel structural components.

Several lines of evidence show that the KRIT1 protein is involved in different physiological aspects of endothelial biology, including vascular development, modulation of different redox-sensitive signaling pathways, and maintenance of endothelial barrier homeostasis, as well as that the absence of KRIT1 in endothelial cells induces an angiogenic

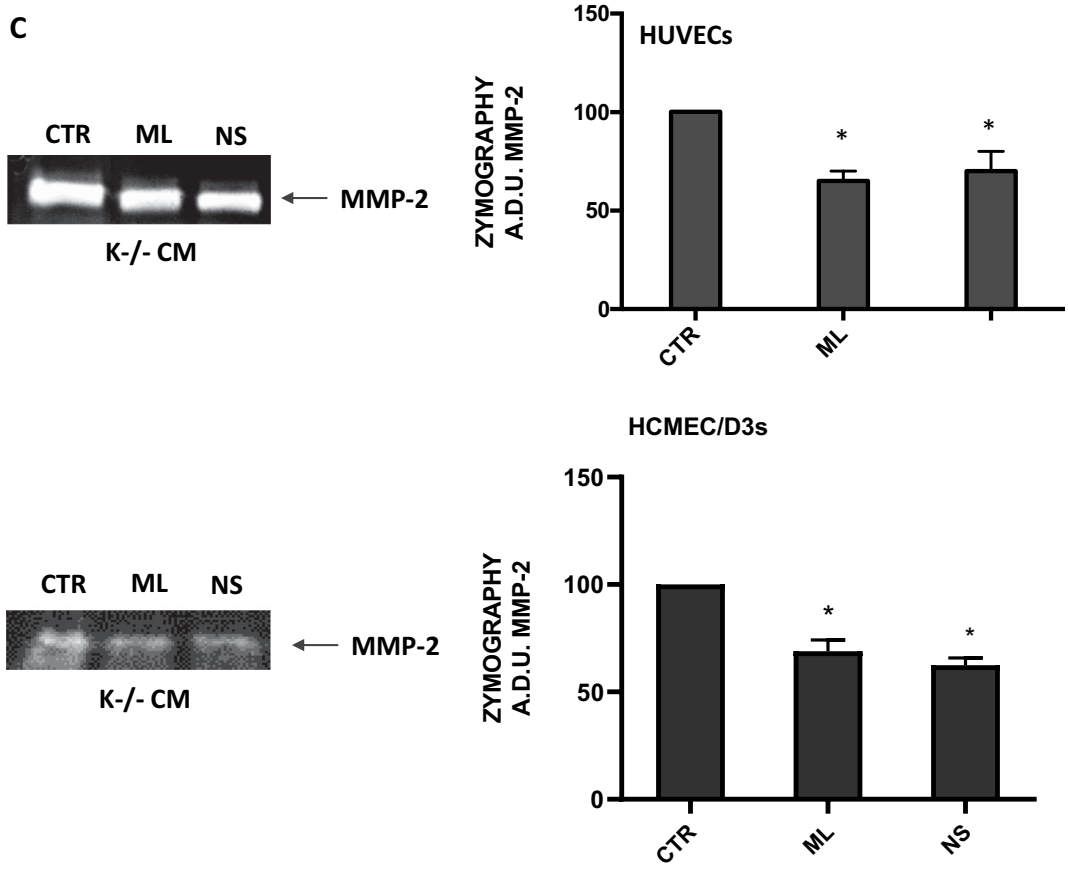


Fig. 5. (*continued*)

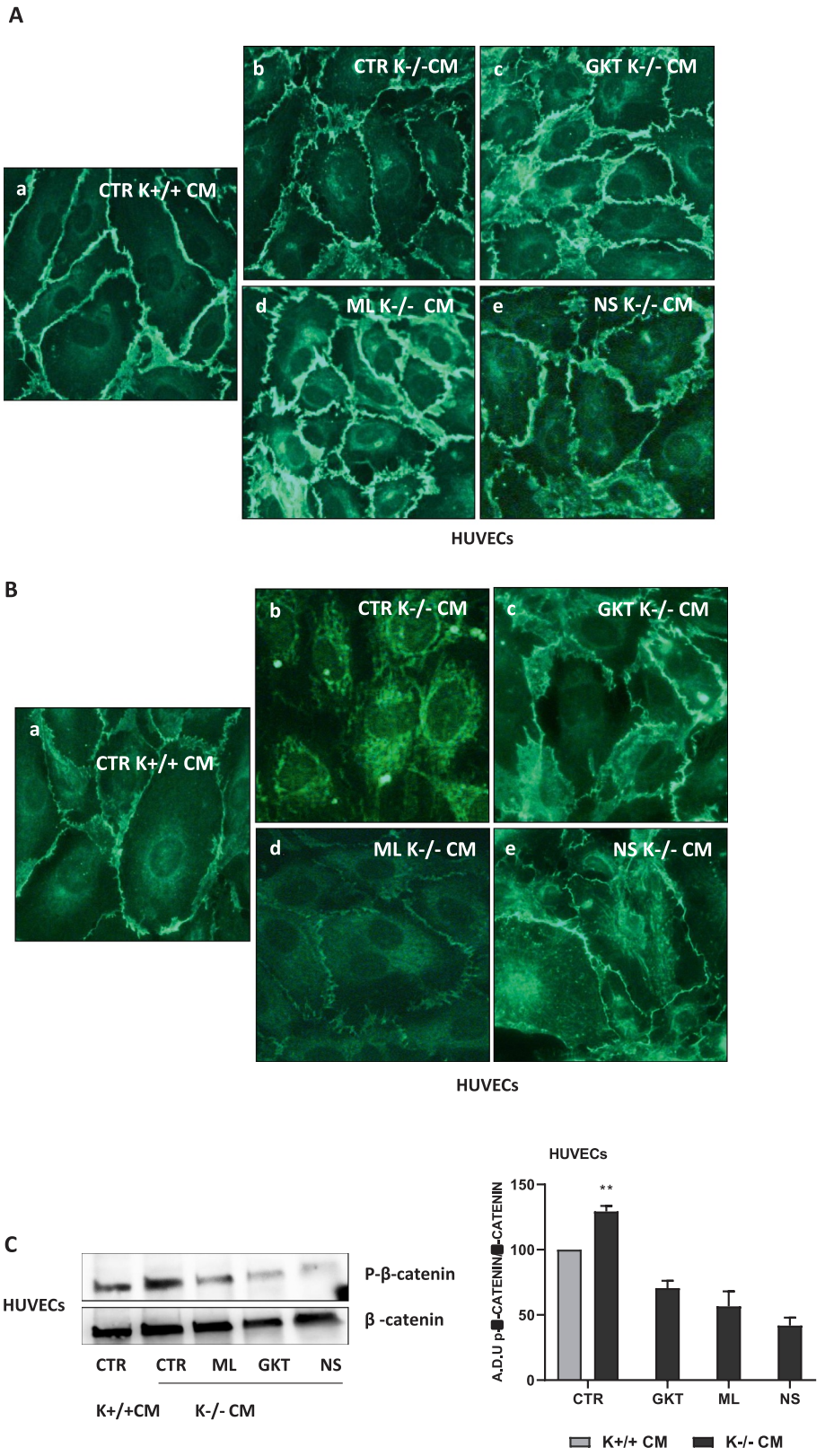


Fig. 6. NOX1 overexpression in stromal cells regulates VE-cadherin distribution and β-catenin activation in endothelial cells. (A) VE-cadherin and (B) β-catenin expression in HUVEC cells cultured with CM isolated from K+/+ or K−/− MEFs pre-treated or not with NOX1 or COX-2 inhibitor. HUVECs were grown for 24 h with (a) K+/+ CM; (b) K−/− CM; (c) GKT K−/− CM; (d) ML K−/− CM or (e) NS K−/− CM and then stained with (A) anti-VE-cadherin or (B) anti-β-catenin primary antibody. Images are representative of three different experiments. (C) Representative images and quantification of β-catenin phosphorylation in HUVEC cells. Protein extracts of HUVECs grown with K+/+ CM; K−/− CM; GKT K−/− CM; ML K−/− CM or NS K−/− CM were analyzed by western blot with anti-β-catenin and anti-P-β-catenin primary antibodies. The gel shown is representative of three independent experiments (A.D.U.: arbitrary densitometry units).

phenotype with increased proliferation, migration, ability to form pseudocapillaries, and activation of angiogenic pathways [3,36,37]. However, while it is established that KRIT1 loss-of-function predisposes to the development of CCM lesions by affecting endothelial cell-autonomous mechanisms, emerging evidence suggests that in the central nervous system (CNS) the endothelium is part of complex units, called neurovascular units (NVU), where it is in close contact with other cell types including pericytes, astrocytes and neurons. All components of this unit interact with each other by releasing signals that safeguard the integrity of the structure itself [49]. However, until now the effect of

the absence of CCM genes on NVU integrity and functionality is poorly investigated. By using neural specific conditional mouse mutants, Louvi et al. demonstrated that in the familial form of CCM, the CCM3 protein has both neural cell autonomous and nonautonomous functions. CCM3 neural deletion leads to increased proliferation, increased survival, and activation of astrocytes through cell autonomous mechanisms. In addition, loss of neural CCM3 results in the formation of multiple vascular lesions that closely resemble human cavernomas, probably through cell nonautonomous mechanisms, indicating that the primary defects in CCM disease need not be endothelial specific [13]. In addition, in recent papers, the authors hypothesized that CCM lesions develop as a consequence of clonal expansion of a single mutant cell, proposing a mechanism in which the first clonal expansion of somatically mutated endothelial cells is followed by the incorporation of normal non mutated endothelium that leads to the formation of the abnormal vessels [14,50], indicating a possible autocrine/paracrine mechanism of endothelial cell recruitment. In our model, conditioned media from KRIT1 null stromal cells induce endothelial cell proliferation, migration, MMP activation and loss of endothelial junctions, recapitulating endothelial cells behavior described in CCM lesions. These data indicate that the anomalous microenvironment derived from KRIT1 loss is able to induce a proangiogenic response in quiescent endothelial cells, and strongly suggest that for the formation of the abnormal vessels present in CCM lesions is sufficient a mutation in any of the cell components of NVU, which in turn may act on neighboring endothelial cells via a paracrine mechanism.

It is well known that the angiogenic process is tightly regulated by a balance between pro- and anti- angiogenic factors; this balance results altered in CCM vascular malformations as a consequence of a sort of threshold overcoming of environmental angiogenic signaling. For instance, alteration of VEGF, PDGF, TGF-β and NOTCH pro-angiogenic signaling pathways in CCM models has already been described [3], supporting the idea that KRIT1 loss of function could lead to a proangiogenic microenvironment. Here we showed that in addition to inducing overexpression of VEGF, stromal cells lacking KRIT1 (K−/−) are able to produce increased amounts of PGE2, a potent inflammatory and proangiogenic product of arachidonic acid pathway [32,48] that contributes to the proangiogenic threshold overcoming.

It is known that a failure in the ability of NVU cells to maintain the proper balance between ROS production and their neutralization causes the disruption of NVU itself, and that this event is associated with many CNS diseases. In this context, NADPH oxidases play a major role in the maintenance of the ROS levels, and NOX family over-activation/expression causes the disruption of vascular homeostasis, which can underlie the development of CNS diseases [51]. In a recent paper, we reported that NOX4 is upregulated in KRIT1 silenced endothelial cells and in KRIT1−/− mice, and controls vascular permeability [29]. However, while NOX4 have been described to play a central role in the control of ROS homeostasis in endothelial cells, very recent papers showed that NOX1 overexpression drives the angiogenic switch in in vitro and in vivo models of tumor [21–23], indicating that this isoform of NADPH oxidase may regulate the release of vasoactive and proangiogenic factors. Here, we demonstrated that K−/− MEFs exhibited higher levels of NOX1 and that COX-2/PGE2 and VEGF expression levels were reduced in K−/− cells treated with NOX1 inhibitors. Consistently, proliferation, migration, MMP2 activation and junction alteration of endothelial cells induced by CMs from K−/− MEFs were significantly affected when endothelial cells were cultured with CMs from K−/− MEFs treated with NOX1 and COX-2 selective inhibitors. These data indicate that oxidative stress consequent to KRIT1 loss mediated upregulation of NOX1 plays a key role in the modulation of microenvironment composition that leads to the acquisition of an angiogenic phenotype by endothelial cells.

It has been extensively reported that CCM onset and progression are linked to increased oxidative stress and inflammation subsequent to CCM genes loss [3]. Here we further outlined the central role of ROS increase in this disease by describing NOX1 as the major player of the angiogenic process induced by KRIT1 loss, and as the regulator of COX-2 expression and PGE2 production, suggesting a sort of hierarchy between oxidative stress and inflammation in CCM pathogenesis. In particular, as our data show that NOX1 inhibition is able to revert the COX-2 upregulation observed in K−/− cells, while COX-2 inhibition did not prevent NOX1 upregulation, but completely reverted the activation of endothelial cells, we suggest the prevalent role of NOX1/COX-2/PGE2 axis rather than NOX1/VEGF in the angiogenic process induced by stromal KRIT1 loss.

5. Conclusions

Our findings provide novel insights into CCM pathogenesis, and suggest novel promising therapeutic options for CCM prevention and treatment.

Currently, the only therapy available for CCM is surgical excision or radiological destruction of the lesions. While several compounds are being investigated in preclinical studies, only a few agents have reached clinical testing and to date CCM has no pharmacological options [52]. Several studies have been conducted to identify new CCM-related cellular mechanisms and some progresses to link basic and translational science have been made allowing to hypothesize new putative treatment targets, including angiogenesis. At the present, the potential efficacy of targeting angiogenesis in CCM is only supported by case reports where bevacizumab, the anti–VEGF A antibody, and propranolol, a β-adrenergic blocker and antiangiogenic agent used to treat hypertension and infantile hemangioma, are described to induce lesion regression or resolution [53,54]. Despite these encouraging observations, to our knowledge, the efficacy of antiangiogenic drugs in CCM has not been confirmed and further clinical studies are necessary.

The novel findings reported here show that KRIT1 loss-of-function in stromal cells affects the surrounding microenvironment through a NOX1-mediated induction and release of angiogenic factors that are able to promote paracrine proangiogenic responses in endothelial cells. These data strongly suggest a novel role for endothelial cell-nonautonomous effects of KRIT1 mutations in CCM pathogenesis, and point to NOX1 as a major regulator of these effects thus opening new perspectives for the identification of novel potential therapeutic targets for disease prevention and treatment.

Funding

This work was supported by the Telethon Foundation (grant GGP15219) to SFR and LT and MIUR (Progetto Dipartimento di Eccellenza 2018-2022) to LT and FF.

Acknowledgements

The authors are grateful to the Italian Research Network for Cerebral Cavernous Malformation (CCM Italia, http://www.ccmitalia.unito.it), and the Associazione Italiana Angiomi Cavernosi (AIAC, http://www.ccmitalia.unito.it/aiac) for fundamental collaboration and support.

References

[1] I.A. Awad, S.P. Polster, Cavernous angiomas: deconstructing a neurosurgical disease, J. Neurosurg. 131 (2019) 1–13, https://doi.org/10.3171/2019.3.JNS181724.
[2] A. Padarti, J. Zhang, Recent advances in cerebral cavernous malformation research, Vessel Plus. 2 (2018) 21, https://doi.org/10.20517/2574-1209.2018.34.
[3] S.F. Retta, A.J. Glading, Oxidative stress and inflammation in cerebral cavernous malformation disease pathogenesis: Two sides of the same coin, Int. J. Biochem. Cell Biol. (2016), https://doi.org/10.1016/j.biocel.2016.09.011.
[4] C. Antognelli, E. Trapani, S. Delle Monache, A. Perrelli, M. Daga, S. Pizzimenti, G. Barrera, P. Cassoni, A. Angelucci, L. Trabalzini, V.N. Talesa, L. Goitre, S.F. Retta, KRIT1 loss-of-function induces a chronic Nrf2-mediated adaptive homeostasis that sensitizes cells to oxidative stress: implication for cerebral cavernous malformation

disease, Free Radic. Biol. Med. (2018), https://doi.org/10.1016/j.freeradbiomed.2017.11.014.
[5] H. Choquet, L. Pawlikowska, M.T. Lawton, H. Kim, Genetics of cerebral cavernous malformations: current status and future prospects, J. Neurosurg. Sci. 59 (2015) 211–220 http://www.ncbi.nlm.nih.gov/pubmed/25900426 (accessed April 29, 2019).
[6] I.J.H.M. de Vos, M. Vreeburg, G.H. Koek, M.A.M. van Steensel, Review of familial cerebral cavernous malformations and report of seven additional families, Am. J. Med. Genet. A 173 (2017) 338–351, https://doi.org/10.1002/ajmg.a.38028.
[7] E. Trapani, S.F. Retta, Cerebral cavernous malformation (CCM) disease: from monogenic forms to genetic susceptibility factors, J. Neurosurg. Sci. 59 (2015) 201–209 http://www.ncbi.nlm.nih.gov/pubmed/25896717 (accessed April 29, 2019).
[8] D.A. McDonald, C. Shi, R. Shenkar, C.J. Gallione, A.L. Akers, S. Li, N. De Castro, M.J. Berg, D.L. Corcoran, I.A. Awad, D.A. Marchuk, Lesions from patients with sporadic cerebral cavernous malformations harbor somatic mutations in the CCM genes: evidence for a common biochemical pathway for CCM pathogenesis, Hum. Mol. Genet. 23 (2014) 4357–4370, https://doi.org/10.1093/hmg/ddu153.
[9] J. Gault, I.A. Awad, P. Recksiek, R. Shenkar, R. Breeze, M. Handler, B.K. Kleinschmidt-DeMasters, Cerebral cavernous malformations, Neurosurgery. 65 (2009) 138–145, https://doi.org/10.1227/01.NEU.0000348049.81121.C1.
[10] L. Huang, Y. Nakamura, E.H. Lo, K. Hayakawa, Astrocyte signaling in the neurovascular unit after central nervous system injury, Int. J. Mol. Sci. 20 (2019) 282, https://doi.org/10.3390/ijms20020282.
[11] C. Iadecola, The neurovascular unit coming of age: a journey through neurovascular coupling in health and disease, Neuron. 96 (2017) 17–42, https://doi.org/10.1016/j.neuron.2017.07.030.
[12] E.A. Winkler, A.Y. Lu, K.P. Raygor, J.R. Linzey, S. Jonzzon, B.V. Lien, W.C. Rutledge, A.A. Abla, Defective vascular signaling & prospective therapeutic targets in brain arteriovenous malformations, Neurochem. Int. 126 (2019) 126–138, https://doi.org/10.1016/j.neuint.2019.03.002.
[13] A. Louvi, L. Chen, A.M. Two, H. Zhang, W. Min, M. Günel, Loss of cerebral cavernous malformation 3 (Ccm3) in neuroglia leads to CCM and vascular pathology, Proc. Natl. Acad. Sci. 108 (2011) 3737–3742, https://doi.org/10.1073/pnas.1012617108.
[14] M. Malinverno, C. Maderna, A. Abu Taha, M. Corada, F. Orsenigo, M. Valentino, F. Pisati, C. Fusco, P. Graziano, M. Giannotta, Q.C. Yu, Y.A. Zeng, M.G. Lampugnani, P.U. Magnusson, E. Dejana, Endothelial cell clonal expansion in the development of cerebral cavernous malformations, Nat. Commun. 10 (2019) 2761, , https://doi.org/10.1038/s41467-019-10707-x.
[15] K.M. Draheim, O.S. Fisher, T.J. Boggon, D.A. Calderwood, Cerebral cavernous malformation proteins at a glance, J. Cell Sci. 127 (2014) 701–707, https://doi.org/10.1242/jcs.138388.
[16] L. Goitre, B. Pergolizzi, E. Ferro, L. Trabalzini, S.F. Retta, Molecular crosstalk between integrins and cadherins: do reactive oxygen species set the talk? J. Signal Transduct. 2012 (2012) 1–12, https://doi.org/10.1155/2012/807682.
[17] D.I. Brown, K.K. Griendling, Nox proteins in signal transduction, Free Radic. Biol. Med. 47 (2009) 1239–1253, https://doi.org/10.1016/j.freeradbiomed.2009.07.023.
[18] G. Teixeira, C. Szyndralewiez, S. Molango, S. Carnesecchi, F. Heitz, P. Wiesel, J.M. Wood, Therapeutic potential of NADPH oxidase 1/4 inhibitors, Br. J. Pharmacol. 174 (2017) 1647–1669, https://doi.org/10.1111/bph.13532.
[19] A. Konior, A. Schramm, M. Czesnikiewicz-Guzik, T.J. Guzik, NADPH oxidases in vascular pathology, Antioxid. Redox Signal. 20 (2013) 2794–2814, https://doi.org/10.1089/ars.2013.5607.
[20] M.W. Ma, J. Wang, Q. Zhang, R. Wang, K.M. Dhandapani, R.K. Vadlamudi, D.W. Brann, NADPH oxidase in brain injury and neurodegenerative disorders, Mol. Neurodegener. 12 (2017) 1–28, https://doi.org/10.1186/s13024-017-0150-7.
[21] A. Juhasz, S. Markel, S. Gaur, H. Liu, J. Lu, G. Jiang, X. Wu, S. Antony, Y. Wu, G. Melillo, J.L. Meitzler, D.C. Haines, D. Butcher, K. Roy, J.H. Doroshow, NADPH oxidase 1 supports proliferation of colon cancer cells by modulating reactive oxygen species-dependent signal transduction, J. Biol. Chem. 292 (2017) 7866–7887, https://doi.org/10.1074/jbc.M116.768283.
[22] E. Zulato, F. Ciccarese, G. Nardo, M. Pinazza, V. Agnusdei, M. Silic-Benussi, V. Ciminale, S. Indraccolo, Involvement of NADPH oxidase 1 in liver kinase B1-mediated effects on tumor angiogenesis and growth, Front. Oncol. 8 (2018), https://doi.org/10.3389/fonc.2018.00195.
[23] S. Liang, H.Y. Ma, Z. Zhong, D. Dhar, X. Liu, J. Xu, Y. Koyama, T. Nishio, D. Karin, G. Karin, R. Mccubbin, C. Zhang, R. Hu, G. Yang, L. Chen, S. Ganguly, T. Lan, M. Karin, T. Kisseleva, D.A. Brenner, NADPH oxidase 1 in liver macrophages promotes inflammation and tumor development in mice, Gastroenterology. 156 (2019) 1156–1172.e6, https://doi.org/10.1053/j.gastro.2018.11.019.
[24] L. Cianfruglia, A. Perrelli, C. Fornelli, A. Magini, S. Gorbi, A. Salzano, C. Antognelli, F. Retta, V. Benedetti, P. Cassoni, C. Emiliani, G. Principato, A. Scaloni, T. Armeni, S. Retta, KRIT1 loss-of-function associated with cerebral cavernous malformation disease leads to enhanced S-Glutathionylation of distinct structural and regulatory proteins, Antioxidants. 8 (2019) 27, https://doi.org/10.3390/antiox8010027.
[25] C. Antognelli, E. Trapani, S. Delle Monache, A. Perrelli, C. Fornelli, F. Retta, P. Cassoni, V.N. Talesa, S.F. Retta, Data in support of sustained upregulation of adaptive redox homeostasis mechanisms caused by KRIT1 loss-of-function, Data Br. 16 (2018) 929–938, https://doi.org/10.1016/j.dib.2017.12.026.
[26] L. Goitre, E. De Luca, S. Braggion, E. Trapani, M. Guglielmotto, F. Biasi, M. Forni, A. Moglia, L. Trabalzini, S.F. Retta, KRIT1 loss of function causes a ROS-dependent upregulation of c-Jun, Free Radic. Biol. Med. (2014), https://doi.org/10.1016/j.freeradbiomed.2013.11.020.
[27] E. Ferro, L. Goitre, S.F. Retta, L. Trabalzini, The interplay between ROS and Ras GTPases: physiological and pathological implications, J. Signal Transduct. 2012 (2011) 1–9, https://doi.org/10.1155/2012/365769.
[28] L. Goitre, F. Balzac, S. Degani, P. Degan, S. Marchi, P. Pinton, S.F. Retta, KRIT1 regulates the homeostasis of intracellular reactive oxygen species, PLoS ONE (2010), https://doi.org/10.1371/journal.pone.0011786.
[29] L. Goitre, P.V. Distefano, A. Moglia, N. Nobiletti, E. Baldini, L. Trabalzini, J. Keubel, E. Trapani, V.V. Shuvaev, V.R. Muzykantov, I.H. Sarelius, S.F. Retta, A.J. Glading, Up-regulation of NADPH oxidase-mediated redox signaling contributes to the loss of barrier function in KRIT1 deficient endothelium, Sci. Rep. (2017), https://doi.org/10.1038/s41598-017-08373-4.
[30] H. Choquet, E. Trapani, L. Goitre, L. Trabalzini, A. Akers, M. Fontanella, B.L. Hart, L.A. Morrison, L. Pawlikowska, H. Kim, S.F. Retta, Cytochrome P450 and matrix metalloproteinase genetic modifiers of disease severity in cerebral cavernous malformation type 1, Free Radic. Biol. Med. (2016), https://doi.org/10.1016/j.freeradbiomed.2016.01.008.
[31] F. Finetti, A. Basile, D. Capasso, S. Di Gaetano, R. Di Stasi, M. Pascale, C.M. Turco, M. Ziche, L. Morbidelli, L.D. D'Andrea, Functional and pharmacological characterization of a VEGF mimetic peptide on reparative angiogenesis, Biochem. Pharmacol. 84 (2012) 303–311, https://doi.org/10.1016/j.bcp.2012.04.011.
[32] F. Finetti, R. Solito, L. Morbidelli, A. Giachetti, M. Ziche, S. Donnini, Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1, J. Biol. Chem. 283 (2008), https://doi.org/10.1074/jbc.M703090200.
[33] T. Sacco, E. Boda, E. Hoxha, R. Pizzo, C. Cagnoli, A. Brusco, F. Tempia, Mouse brain expression patterns of Spg7, Afg3l1, and Afg3l2 transcripts, encoding for the mitochondrial m-AAA protease, BMC Neurosci. 11 (2010), https://doi.org/10.1186/1471-2202-11-55.
[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods. 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[35] F. Finetti, E. Terzuoli, S. Donnini, M. Uva, M. Ziche, L. Morbidelli, Monitoring endothelial and tissue responses to cobalt ferrite nanoparticles and hybrid hydrogels, PLoS ONE 11 (2016), https://doi.org/10.1371/journal.pone.0168727.
[36] J. Wüstehube, A. Bartol, S.S. Liebler, R. Brütsch, Y. Zhu, U. Felbor, U. Sure, H.G. Augustin, A. Fischer, Cerebral cavernous malformation protein CCM1 inhibits sprouting angiogenesis by activating DELTA-NOTCH signaling, Proc. Natl. Acad. Sci. 107 (2010) 12640–12645, https://doi.org/10.1073/pnas.1000132107.
[37] P.V. DiStefano, J.M. Kuebel, I.H. Sarelius, A.J. Glading, KRIT1 protein depletion modifies endothelial cell behavior via increased vascular endothelial growth factor (VEGF) signaling, J. Biol. Chem. 289 (2014) 33054–33065, https://doi.org/10.1074/jbc.M114.582304.
[38] E. De Luca, D. Pedone, M. Moglianetti, D. Pulcini, A. Perrelli, S.F. Retta, P.P. Pompa, Multifunctional platinum@BSA–rapamycin nanocarriers for the combinatorial therapy of cerebral cavernous malformation, ACS Omega. 3 (2018) 15389–15398, https://doi.org/10.1021/acsomega.8b01653.
[39] S. Marchi, M. Corricelli, E. Trapani, L. Bravi, A. Pittaro, S. Delle Monache, L. Ferroni, S. Patergnani, S. Missiroli, L. Goitre, L. Trabalzini, A. Rimessi, C. Giorgi, B. Zavan, P. Cassoni, E. Dejana, S.F. Retta, P. Pinton, Defective autophagy is a key feature of cerebral cavernous malformations, EMBO Mol. Med. 7 (2015) 1403–1417, https://doi.org/10.15252/emmm.201505316.
[40] P. Guazzi, L. Goitre, E. Ferro, V. Cutano, C. Martino, L. Trabalzini, S.F. Retta, Identification of the Kelch family protein Nd1-L as a novel molecular interactor of KRIT1, PLoS ONE 7 (2012) e44705, , https://doi.org/10.1371/journal.pone.0044705.
[41] V.W.M. Van Hinsbergh, P. Koolwijk, Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead, Cardiovasc. Res. 78 (2008) 203–212, https://doi.org/10.1093/cvr/cvm102.
[42] K. Schröder, N. Weissmann, R.P. Brandes, Organizers and activators: cytosolic Nox proteins impacting on vascular function, Free Radic. Biol. Med. 109 (2017) 22–32, https://doi.org/10.1016/j.freeradbiomed.2017.03.017.
[43] R.P. Brandes, N. Weissmann, K. Schröder, Nox family NADPH oxidases: molecular mechanisms of activation, Free Radic. Biol. Med. 76 (2014) 208–226, https://doi.org/10.1016/j.freeradbiomed.2014.07.046.
[44] D.I. Brown, K.K. Griendling, A. Juhasz, S. Markel, S. Gaur, H. Liu, J. Lu, G. Jiang, X. Wu, S. Antony, Y. Wu, G. Melillo, J.L. Meitzler, D.C. Haines, D. Butcher, K. Roy, J.H. Doroshow, A. Konior, A. Schramm, M. Czesnikiewicz-Guzik, T.J. Guzik, S. Liang, H.Y. Ma, Z. Zhong, D. Dhar, X. Liu, J. Xu, Y. Koyama, T. Nishio, D. Karin, G. Karin, R. Mccubbin, C. Zhang, R. Hu, G. Yang, L. Chen, S. Ganguly, T. Lan, M. Karin, T. Kisseleva, D.A. Brenner, M.W. Ma, J. Wang, Q. Zhang, R. Wang, K.M. Dhandapani, R.K. Vadlamudi, D.W. Brann, H. Yokota, A. Tsuzuki, Y. Shimada, A. Imai, D. Utsumi, T. Tsukahara, M. Matsumoto, K. Amagase, K. Iwata, A. Nakamura, C. Yabe-Nishimura, S. Kato, NADPH oxidase 1 supports proliferation of colon cancer cells by modulating reactive oxygen species-dependent signal transduction, Free Radic. Biol. Med. 12 (2017) 2794–2814, https://doi.org/10.1124/jpet.116.235580.
[45] D.I. Brown, K.K. Griendling, Regulation of signal transduction by reactive oxygen species in the cardiovascular system, Circ. Res. 116 (2015) 531–549, https://doi.org/10.1161/CIRCRESAHA.116.303584.
[46] M. Ushio-Fukai, VEGF signaling through NADPH oxidase-derived ROS, Antioxid. Redox Signal. 9 (2007) 731–739, https://doi.org/10.1089/ars.2007.1556.
[47] F. Corti, F. Finetti, M. Ziche, M. Simons, The syndecan-4/protein kinase Cα pathway mediates prostaglandin E2 – induced extracellular regulated kinase (ERK) activation in endothelial cells and angiogenesis in vivo, J. Biol. Chem. 288 (2013), https://doi.org/10.1074/jbc.M113.452383.
[48] F. Finetti, S. Donnini, A. Giachetti, L. Morbidelli, M. Ziche, Prostaglandin E_2 primes the angiogenic switch via a synergic interaction with the fibroblast growth factor-2 pathway, Circ. Res. 105 (2009) 657–666, https://doi.org/10.1161/CIRCRESAHA.

109.203760.
[49] S. Liebner, R.M. Dijkhuizen, Y. Reiss, K.H. Plate, D. Agalliu, G. Constantin, Functional morphology of the blood–brain barrier in health and disease, Acta Neuropathol. 135 (2018) 311–336, https://doi.org/10.1007/s00401-018-1815-1.
[50] M.R. Detter, D.A. Snellings, D.A. Marchuk, Cerebral cavernous malformations develop through clonal expansion of mutant endothelial cells, Circ. Res. 123 (2018) 1143–1151, https://doi.org/10.1161/CIRCRESAHA.118.313970.
[51] C. Carvalho, P.I. Moreira, Oxidative stress: a major player in cerebrovascular alterations associated to neurodegenerative events, Front. Physiol. 9 (2018) 1–14, https://doi.org/10.3389/fphys.2018.00806.
[52] M.O. Chohan, S. Marchiò, L.A. Morrison, R.L. Sidman, W.K. Cavenee, E. Dejana, H. Yonas, R. Pasqualini, W. Arap, Emerging pharmacologic targets in cerebral cavernous malformation and potential strategies to alter the natural history of a difficult disease: a review, JAMA Neurol. 76 (2019) 492–500, https://doi.org/10.1001/jamaneurol.2018.3634.
[53] D. Aguilera, T. Tomita, S. Goldman, J. Fangusaro, Incidental resolution of a radiation-induced cavernous hemangioma of the brain following the use of bevacizumab in a child with recurrent medulloblastoma, Pediatr. Neurosurg. 46 (2010) 303–307, https://doi.org/10.1159/000321801.
[54] J.M. Zabramski, M.Y.S. Kalani, A.S. Filippidis, R.F. Spetzler, Propranolol treatment of cavernous malformations with symptomatic hemorrhage, World Neurosurg. 88 (2016) 631–639, https://doi.org/10.1016/j.wneu.2015.11.003.

Archives of Biochemistry and Biophysics 691 (2020) 108483

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

KRIT1 as a possible new player in melanoma aggressiveness

Jasmine Ercoli[a], Federica Finetti[a], Brittany Woodby[b], Giuseppe Belmonte[c], Clelia Miracco[c], Giuseppe Valacchi[b,d,e,1,*], Lorenza Trabalzini[a,1,**]

[a] Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Italy
[b] Plants for Human Health Institute, NC Research Campus, NC State University, NC, USA
[c] Unit of Pathological Anatomy, Department of Medicine, Surgery, and Neurosciences, University of Siena, Siena, Italy
[d] Dept. of Biomedical and Specialist Surgical Sciences, University of Ferrara, Ferrara, Italy
[e] Department of Food and Nutrition, Kyung Hee University, Seoul, South Korea

ARTICLE INFO

Keywords:
KRIT1
Melanoma
Tumor suppressor
N-cadherin
Inflammation
β-catenin

ABSTRACT

Krev interaction trapped protein 1 (KRIT1) is a scaffold protein known to form functional complexes with distinct proteins, including Malcavernin, PDCD10, Rap1 and others. It appears involved in several cellular signaling pathways and exerts a protective role against inflammation and oxidative stress. KRIT1 has been studied as a regulator of endothelial cell functions and represents a determinant in the pathogenesis of Cerebral Cavernous Malformation (CCM), a cerebrovascular disease characterized by the formation of clusters of abnormally dilated and leaky blood capillaries, which predispose to seizures, neurological deficits and intracerebral hemorrhage. Although KRIT1 is ubiquitously expressed, few studies have described its involvement in pathologies other than CCM including cancer. Cutaneous melanoma represents the most fatal skin cancer due to its high metastatic propensity. Despite the numerous efforts made to define the signaling pathways activated during melanoma progression, the molecular mechanisms at the basis of melanoma growth, phenotype plasticity and resistance to therapies are still under investigation.

The hypothesis driving this work is that KRIT1 may control melanoma progression by acting as a tumor suppressor. Here, we show that KRIT1 is expressed in normal human melanocyte but is lacking in melanoma. In human cell model of melanoma KRIT1 silencing induced increased growth and invasion and a switch toward a malignant cell phenotype. These data candidate KRIT1 as a new possible player of a complex signalling network that regulates melanoma progression and offer a new possible target for melanoma therapies.

1. Introduction

KRIT1 (Krev interaction trapped protein-1) is an intracellular protein isolated by a two-hybrid screening through its interaction with the Ras-family GTPase Krev1/Rap1a [1]. KRIT1 lacks defined catalytic domains while contains a well characterized protein-protein interaction motifs and domains, including three NPXY/F motifs, three ankyrin repeats and a FERM domain. It has been extensively described that KRIT1 is able to form functional complexes with distinct proteins, comprising Malcavernin (CCM2), PDCD10 (CCM3), ICAP1, Rap1, Nd1-L and others [2–6].

KRIT1 is ubiquitously expressed during early embryogenesis, followed by a restricted expression which persists up to adult life. During this second phase, KRIT1 is mainly expressed in neuronal cells of central and peripheral nervous system and in various epithelia (epidermal, digestive, respiratory, uterine and urinary) both in mouse and human [7].

KRIT1 signalling pathway has been extensively studied since its mutation causes Cerebral Cavernous Malformations (CCMs), a rare vascular disease typically found in the central nervous system with a prevalence of 0.3%–0.5% of the population [8,9]. CCMs are characterized by thin walled vessels lacking of normal structure [10–12]. In the CCM disease, endothelial cells of brain vessels acquire characteristics comparable to tumors including increased growth and migration, reduced apoptosis, endothelial mesenchymal transition, loss of cell-cell junction, pro-inflammatory status, altered redox homeostasis and activation of growth factors receptor signalling.

The mechanisms by which the loss of KRIT1 leads to vascular malformations has not yet been fully clarified, although many

* Corresponding author. Plants for Human Health Institute, NC Research Campus, NC State University, NC, USA.
** Corresponding author. Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Italy.
E-mail addresses: gvalacc@ncsu.edu (G. Valacchi), lorenza.trabalzini@unisi.it (L. Trabalzini).
[1] These authors equally contributed to the work.

https://doi.org/10.1016/j.abb.2020.108483
Received 3 February 2020; Received in revised form 30 June 2020; Accepted 2 July 2020
Available online 28 July 2020
0003-9861/

Table 1
Main clinicopathological data of melanocytic lesions used in this study
A): BCN, benign common nevi; DN, dysplastic nevi
B): RGPM, radial growth phase melanomas; VGPM, vertical growth phase melanomas; MelMet, melanoma metastases; MM, malignant melanomas.

	BCN	DN
(n)	5	5
Male/female	3/2	5/0
Median age (range)	40 (29–55)	45(41–64)
Site (HN/T/L/F)	1/3/1/0	0/5/0/0
AML (BCN/DN/MM/No)	3/0/0/2	0/4/2/0*

	RGPM	VGPM	MEL MET
(n)	5	23	11
Male/female	2/3	10/13	3/8
Median age (range)	72 (44–87)	67 (33–86)	59 (25–86)
MM Site (HN/T/L)	2/2/1/0	7/7/9	
MelMet Site (S/LN/V)	–	–	4/4/3
MM type (ALM/LMM/NM/NeM/ SSM)	0/2/0/0/3	1/3/6//1/12	–
pT (is/1a/1b/2/3/4)	3/2/0/0/0/0	0/2/0/8/7/6	–
CSDS/non-CSDS	3/2	4/19	–

M(n)/wt(n)/nd(n):

BRAF	wt (1)/nd (4)	p.V600E3(14)/wt(3)	p.V600E(3)/p.V600K(1) p.G466(1)/wt(6)
NRAS	wt(1)/nd (4)	p.Q61R(3); p.Q61K(2)/wt (18)	p.Q61R(1)/wt (10)
C-KIT	wt(1)/nd (4)	p.L576P(1)/wt(22)	wt(11)
Progression (Smet/LNmet/NoP)	0/0/5	8/7/8	–
Status(A/D/U)	5/0/0	17/6/0	7/3/1

HN, head and neck; T, trunk; L, limbs; F, foot; AML, associated melanocytic lesions (BCN, benign common nevi, DN, dysplastic nevi. MM, malignant melanomas; No, none).
(n), number of cases.
* a patient with Dysplastic nevus syndrome had both DN and MM.
HN, head and neck; T, trunk; L, limbs.
S, skin; LN, lymph node; V, visceral.
ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; NeM, nevoid melanoma; SSM, superficial spreading melanoma.
pT according to AJCCC, 8th ed.
CSDs, chronically sun damaged skin; non-CSDs, not chronically sun damaged skin.
M, mutation; wt, wild type; nd, not done.
Smet, skin metastases; LNmet, lymph node metastases; NoP, not progressed; U, unknown.
A, alive; D, dead; U, unknown.

evidences support pleiotropic effects related to the eventual ability of KRIT1 to modulate several mechanisms involved in angiogenesis, vascular homeostasis, cell responses to oxidative stress and inflammation, including cytoskeleton dynamics [2,13–19]. KRIT1 is associated with microtubules, membranes, adherents junctions and cellular nucleus [20]. In particular, as Rap1 GTPase effector, KRIT1 interacts with cell junctions in a complex with VE-cadherin, p120-catenin and β-catenin [5]. Consequently, KRIT1 depletion could induce the loss of a stable cellular architecture and the activation of TGFβ/BMP signalling downstream of β-catenin [21].

Previous studies demonstrated that KRIT1 is an antiangiogenic protein able to keep the human endothelium quiescent and to inhibit proliferation, migration, lumen formation and sprouting angiogenesis [17,22–24]. In addition, KRIT1 negatively coordinates cell cycle by controlling FoxO-mediated downregulation of cyclin D1 and upregulation of p27Kip1 levels through the modulation of intracellular reactive oxygen species (ROS) levels [25].

Based on its ability to regulate several signaling pathways and its ubiquitous expression, KRIT1 is likely to be involved in others pathological conditions than CCM. To date, very few studies have demonstrated that KRIT1 is implicated in other pathologies as cardiovascular disease [26], diabetes [16], intestinal epithelial barrier dysregulation [14] and cancer [27,28]. In particular, the involvement of KRIT1 as tumor suppressor in cancer has been hypothesized since the oncogenic miR-21 expression anticorrelates with KRIT1 [27]. No other data are available in the literature on the contribution of KRIT1 in cancer progression.

Cutaneous malignant melanoma (CMM), which comprises 5% of all skin cancers, is the most aggressive and highly lethal form of skin cancer [29,30]. Several factors contribute to the formation of melanoma, including exposure to ultraviolet (UV) radiation and the malignant transformation of nevus, along with a variety of genetic factors [31]. Most forms of melanoma are sporadic, so the somatic mutations are acquired during person's lifetime and are present only in the melanocytes that give rise to the melanoma. In some cases this is the result of environmental insults followed by proto-oncogene activation coupled with suppression of tumor suppressor genes and defects in DNA repair mechanism [32]. The malignancy is more likely to spread in patients with deep primary tumors or regional lymph node metastases, which leads to a median survival of only 6–9 months [33]. Significant progress has been made in the identification of genetic markers and cellular pathways involved with the melanoma progression to identify novel therapeutic targets; nevertheless, additional key players underlying melanoma onset and progression need to be identified. Recently, mutations of genes involved in human melanoma have also been found in angiomas [34,35].

Taken together, all these elements gave rise to the idea that KRIT1 could be involved in the onset and progression of melanoma. To demonstrate that we investigated the role of KRIT1 in the growth and metastasis of melanoma by using KRIT1 knockdown melanoma cells

and specimens from patients. We hypothesized that KRIT1 may play an important role in the formation of melanoma metastasis and may control tumor progression by acting as a suppressor. This novel hypothesis opens the avenue to the study of a completely new role of KRIT1 in cancer development giving new perspectives for melanoma prevention and treatment.

2. Materials and methods

2.1. Cell culture

A375 melanoma cells (ATCC, Rockville, MD, USA) were cultured in DMEM (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Chicago, IL, USA), 100 U/ml penicillin/streptomycin (Gibco), and 4 mM L-glutamine (Corning, NY, USA) [36].

2.2. Human melanocytic samples analysis

Taking advantage of the rich collection of samples kept at the Section of Pathological Anatomy of the Azienda Ospedaliera Universitaria Senese (Siena, Italy), we choose 57 consecutive samples of melanocytic lesions with a 5-year minimum follow up (see Tab le1). They included 5 benign common nevi (BCN); 5 dysplastic nevi (DN); 5 radial growth phase melanomas (RGPMM), 23 vertical growth phase malignant melanomas (VGPMM) and 11 melanoma metastases (MelMet). As inclusion criteria, we chose frozen samples with mutations for BRAF, NRAS, and C-KIT. Control samples were represented by healthy skin from the lesion margins.

2.3. Immunohistochemistry

As previously described [37], tissue slides were deparaffinised in xylene and dehydrated in ethanol. Microwave pre-treatment in 10 mM citrate buffer (pH 6.0) was performed for 30 min. Non-specific binding was blocked for 20 min with 2.5% normal horse serum (NHS). The slides were incubated with primary antibodies targeting KRIT1 (1:100; ThermoFisher, Waltham, MA, USA) followed by chromogenic visualization using ImmPress-AP (Vector). In particular, sections were incubated for 15 min with NBT-BCIP added with Levamisole. After counterstaining with nuclear fast red (NFR), slides were washed thoroughly, dehydrated, cleared in xylene and mounted. Staining intensity was scored as negative (no staining) or positive (blue colour). Fluorescence quantification was calculated as ratio between the area of each cell and the fluorescence intensity.

A semiquantitative evaluation of the immunostaining was performed by using the software ImageJ (version 1.52v, https://imagej.nih.gov/ij/). KRIT1-immunostained cytoplasm of normal melanocytes served to choose the threshold. All tumor sections were examined.

2.4. siRNA transfection

A375 cells, were plated (5.0×10^5 cells/well) on 6 well multiplates and after adhesion transfected with 50 nM of control or KRIT1 siRNA (Qiagen, Hilden, Germania) using 4 μL of lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions and as previously described [38]. 6 h post-transfection, cells were washed with PBS, and media was changed. Cells were used for experiments 24 h post-transfection.

2.5. MTT assay

Cells were plated (2.0×10^4 cells/well) in 96 wells multiplates in DMEM 10% FBS. After 24 h, the medium was removed, and cells were incubated for 4 h with fresh medium in the presence of 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich,St. Louis, MO, USA). The MTT solution was removed and 100 μL of DMSO were added to each well to dissolve the blue formazan crystals. The absorbance of the formazan dye was measured at 570 nm with a microplate reader (Spectramax M5 multimode microplate reader, Molecolar Device, San Jose, CA, USA). Data were expressed as a percentage of the basal control [39].

2.6. BrdU assay

For 5-bromo-2-deoxyuridine (BrdU) experiments, A375 cells were seeded (5.0×10^3 cells/well) in a 96-well plate and incubated overnight. The next day, cells were transfected with 50 nM concentration of either control or KRIT1 siRNA using 0.3 μL of lipofectamine 3000 in 200 μL total volume of media, using the same method as previously mentioned (see siRNA transfection section for method). Six hours post-transfection, medium was changed. BrdU experiments were performed 24 h post-transfection, using a BrdU assay kit (Roche, catalog no. 11,647,229 001), and BrdU was added to the cells for 1 h (as suggested by the manufacturer for this cell line). After labeling, cell culture medium was removed. Next, the cells were fixed, and the DNA was denatured by adding FixDenat (from kit). Then, anti-BrdU-POD, an anti-BrdU antibody conjugated with peroxidase, was added to the samples. This antibody binds to newly, synthesized cellular DNA with BrdU incorporated. Next, the cells were washed with PBS, and the peroxidase substrate was added. Absorbance was measured at λ 370 nm (reference wavelength: 492 nm) [38].

2.7. Migration assay

A375 cells were seeded (1.0×10^6 cells/well) in 24-well multiplate in DMEM 10% FBS. After 12 h, cell monolayers were scored vertically down the center of each well with a sterile tip. Each well was washed with PBS to remove detached cells. Fresh medium (1% serum), with ARA C (Sigma–Aldrich) (2.5 mg/ml) to inhibit cell proliferation, was added. Images of the wound in each well were acquired at time 0 and after 6 and 12 h (magnification of 10×). Results were expressed as arbitrary units of wound and percentage of healing taking as reference the area at time 0 [39].

2.8. Invasion assay

Chemotaxis experiments were performed using *trans*-well system. The day before, the *trans*-well was coated with gelatin 0.25% (100 μL/well) and maintained overnight at 4 °C. A375 cells were plated (1.0×10^5 cell/well) in coated *trans*-well in DMEM without FBS, and 650 μL of DMEM 10% FBS were added in the lower chamber. The cells were incubated and after 12, 18 and 24 h were fixed in EtOH 70%, colored with Coomassie Blue solution (10% Acetic acid, 10% ethanol and 0,25% Blue Coomassie) and washed with DDI water.

The number of migrated cells present in five fields/well was counted at 20× magnification. Data were reported as percentage of migrated cells [41].

2.9. Protein extraction and Western blotting

Cells silenced with siKRIT1 and siCTRL were seeded at the density of 500,000 cells/well in 6 well multiplates in DMEM with 10% FBS. After 24 h, cells were lysated and briefly centrifuged at 15,000×*g* for 20 min at 4 °C.

Protein content was measured using a BCA protein assay kit (Thermo Scientific). For Western blotting analysis, aliquots of cell extract supernatants containing an equal amount of proteins (50 μg) were treated with Laemmli buffer, boiled for 10 min, resolved on 4–20% stain-free gel and then blotted onto a nitrocellulose membrane. Membranes were incubated with 1:1000 dilutions of anti-KRIT1 (Millipore, Burlington, MA, USA), anti-β actin (Sigma Aldrich), anti-β-catenin (Santa Cruz, Dallas, TX, USA), anti-vimentin (Santa Cruz), anti-

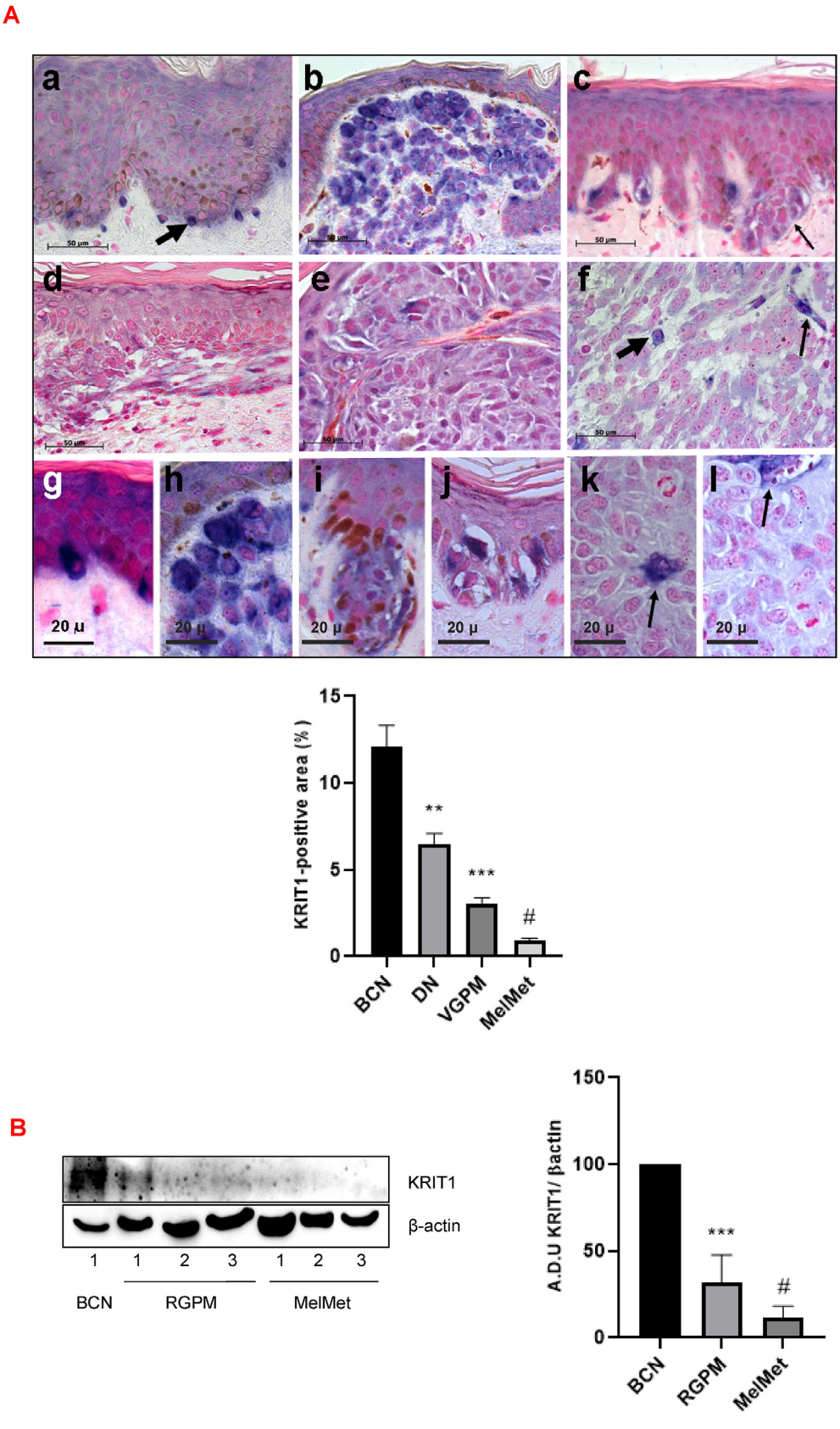


Fig. 1. KRIT1 expression in melanoma and healthy tissues. (A) Strongly immunopositive melanocytes (as the one indicated by the arrow) in normal skin (a), and in a benign common nevus, BCN (b). Marked decrease in immunopositivity in a group of melanocytes of a dysplastic nevus, DN (c, arrow), and in a radial growth phase melanoma, RGPM (d). Almost completely immunonegative melanocytes in a vertical growth phase melanoma, VGPM (e) and in a melanoma metastasis, MelMet (f; the thick arrow points on an isolated immunopositive melanocyte, and the thin arrow on immunopositive vessels). Details of KRIT1 immunohistochemistry: normal skin (g); benign common nevus (h); dysplastic nevus (i); early growth phase melanoma (j); vertical growth phase melanoma (k), and melanoma metastases (l). The arrows in "k" and "l" boxes indicate immunopositive vessels. (B) Western blot analysis of KRIT1 levels in patient samples. The histogram represents the KRIT1 relative expression, normalized with β-actin expression (A.D.U: arbitrary densitometry units) (**p < 0.01; ***p < 0.001; #p < 0.0001). The scale bar in figures (a–f) represents 50 µm; in figures (g–l) represents 20 µm.

N-cadherin (Bioss, Woburn, MA, USA), anti-COX-2 (Cell signaling, Danvers, MA, USA), anti-mPGES-1 (Santa Cruz) or anti-cyclin D1 (BioRad, Hercules, CA, USA) antibody. The membranes were then incubated with 1:10,000 dilutions of horseradish peroxidase-conjugated secondary antibody (BioRad) for 1 h at RT. Chemiluminescence was detected by ChemiDoc imager (BioRad), and quantification was performed using Image J [43].

2.10. MMP activation assay

5 × 10^4 cells/well (A375) were cultured in 96-well cell culture plates in 10% fetal bovine serum medium. After adhesion, cells were incubated with 50 μL of serum-free conditioned media. After 48 h, media were collected, clarified by centrifugation and assayed by zymography. Media were subjected to electrophoresis in 8% SDS-PAGE containing 1 mg/ml gelatin under non-denaturing conditions, by using Sample Buffer w/o β-ME and sample boiling. After electrophoresis, gel were washed with 2.5% Triton X-100 to remove SDS and incubated for 48 h at 37 °C in 50 mM Tris buffer containing 200 mM NaCl and 20 mM CaCl2, pH 7.4. Gels were stained with 0.05% Coomassie brilliant blue R-250 in 10% acetic acid and 10% ethanol and destained with 10% acetic acid and 10% ethanol. Bands of gelatinase activity appeared as transparent areas against a blue background. Gelatinase activity was then evaluated by quantitative densitometry [15,42].

2.11. Proliferation assay

A375 cells were seeded in 96-well microplates (2.0 × 10^4 cells/well) in DMEM 10% FBS and after 12 and 18 and hours they were fixed for 10 min with 70% EtOH and colored with Coomassie Blue solution (10% Acetic acid, 10% ethanol, and 0,25% Blue Coomassie).

The number of proliferated cells present in five fields/well was counted at 20× magnification. Data were reported as percentage of proliferating cells [40].

2.12. Immunofluorescence analysis

A375 cells (2.5 × 10^5 cells/well) were seeded on glass cover-slips. After 24 h cells were fixed with formalin for 10 min and permeabilized with PBS 0.25% Triton ×100 for 10 min, incubated with 1% BSA for 30 min and stained overnight at 4 °C with primary antibody for β-catenin, Vimentin and N-cadherin (Santa Cruz). Slips were washed three times with PBS and then incubated 1 h at room temperature with Alexa Fluor 568 or 488 secondary antibodies (ThermoFisher Scientific, Waltham, MA, USA). Nuclei were stained with 1 μg/ml DAPI (D1306 Invitrogen, USA) for 1 min after removal of secondary antibody. Microscopy imaging was performed on Olympus IX71/X51 (Olympus Life science) inverted microscope using a 60× objective [43].

The Corrected Total Cell Fluorescence (CTCF) was measured in at least 15 view fields at 600× magnification. The images were taken at the same exposition time and light intensity. Then, the data were evaluated using the ImageJ software measuring total cell area and a region next to selected cell as background. The following equation was used:

CTCF = integrated density − (area of selected cell × mean fluorescence of background readings).

To study β-catenin cellular localization, the intensity of nuclear and cytoplasmic fluorescence was measured in siCTRL and siKRIT1 cells and the following formula was applied: siCTRL([intensity in the cytoplasm]/[intensity in the nucleus]) versus siKRIT1([intensity in the cytoplasm]/[intensity in the nucleus]).

2.13. Statistical analysis

Data were generated from three independent experiments and expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test for unpaired data; $p < 0.05$ was considered statistically significant.

3. Results

3.1. KRIT1 expression in benign melanocytes and in melanoma

In the adult life the expression levels of KRIT1 are higher in endothelium and in neuronal cells of central and peripheral nervous system but it is known that KRIT1 is also present in endothelia [44] and in various epithelia such as epidermal, digestive, respiratory, uterine and urinary [7]. Although, several studies have been performed in order to define the physio-pathological role of KRIT1 in Cerebral Cavernous Malformation models, very few studies report the involvement of KRIT1 in other pathologies. To explore a possible role of KRIT1 in cancer, and in particular in melanoma progression, in this study we evaluated the expression levels of KRIT1 by immunohistochemical and Western blot analysis in human specimens (Fig. 1A). In all sections, the endothelium was intensely positive to KRIT1, and it represented our internal positive control. KRIT1 immunopositivity was also observed in the luminal side of the sweat glands epithelium. Normal melanocytes, as well as benign common nevi (BCN), were strongly decorated by KRIT1 (Fig. 1A, panel a). In dysplastic nevi (DN) and in radial growth phase melanomas (RGPM), single melanocytes and groups of negative melanocytes were observed, disorderly mixed with positive melanocytes. The quantification of immunohistochemical images shows a significant and progressive decrease of KRIT1 protein expression in DN, vertical growth phase melanomas (VGPM), and melanoma metastasis (MelMet), when compared to BCN, with the lowest values registered in MelMet. In addition, these data were confirmed by Western blot analysis of the corresponding frozen samples (Fig. 1B). These data indicated a possible regulatory role of KRIT1 in melanoma metastasis.

3.2. KRIT1 knockdown stimulates cellular proliferation in A375 cells

In order to investigate the role of KRIT1 in melanoma progression, we knocked down KRIT1 in A375 cells using siRNA transfection (siKRIT1 A375) (Fig. 2A). We observed that KRIT1 knockdown increased cellular viability measured by MTT assay **(**Fig. 2B**)** and, consistently, stimulated cellular proliferation (Fig. 2C and D). Furthermore, we observed increased in cyclin D1 and decrease in p27 levels in siKRIT1 A375 cells respect to siCTRL A375 cells (Fig. 2E).

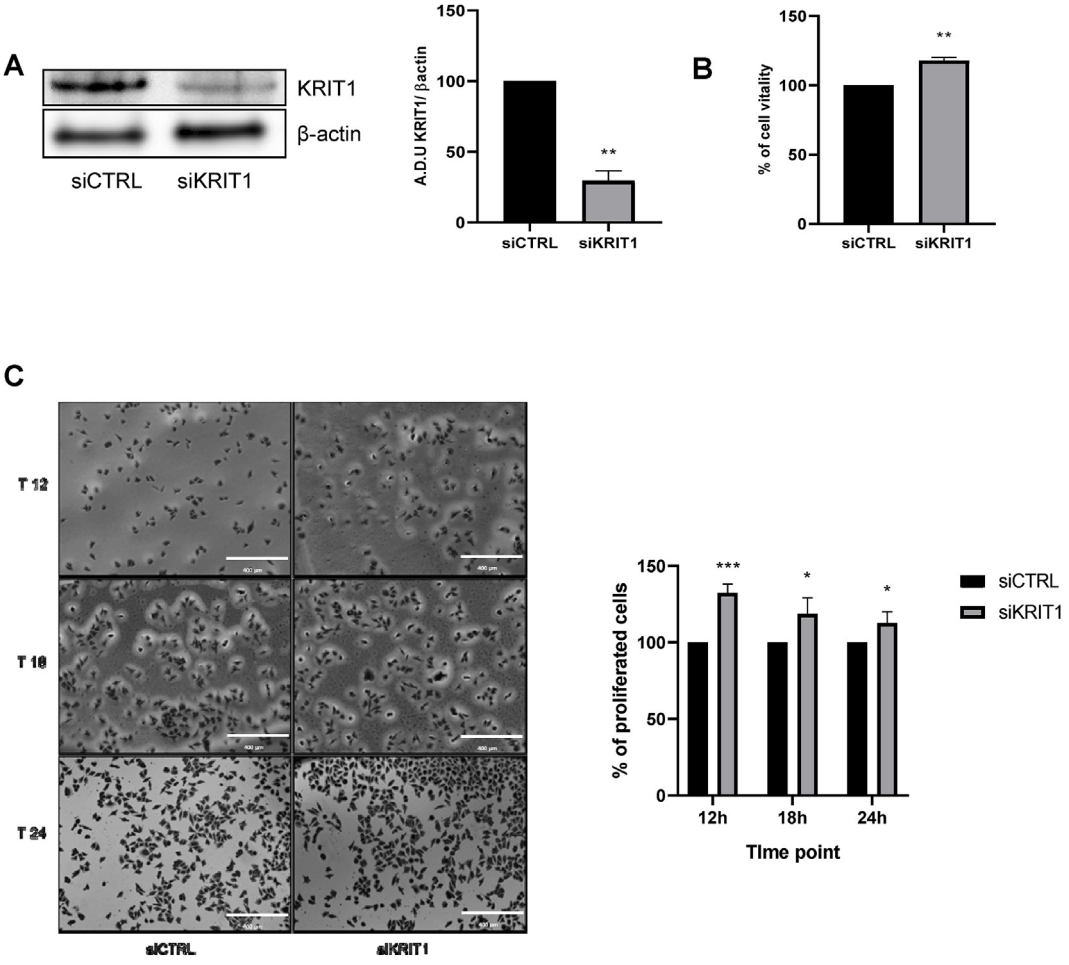
3.3. KRIT1 downregulation increases migration and invasion in A375 melanoma cells

In view of the observation reported for human melanoma samples, we evaluated whether loss of KRIT1 affected cell migration and invasion by using a wound closure *in vitro* scratch assay. As reported in Fig. 3A, the rate of wound closure in siKRIT1 A375 cells was faster than in siCTRL A375 cells. Additionally, we performed a migratory assay to assess whether knockdown of KRIT1 affected the ability of the cells to directionally move towards a selected chemoattractant. We observed that knockdown of KRIT1 promoted cell invasion (Fig. 3B**).** It is known that the invasion process is linked to activation of metalloproteinases (MMPs), key enzymes involved in extracellular matrix (ECM) degradation and in the regulation of cancer cells invasion into surrounding tissue/circulation [45]. In addition, the ability of KRIT1to affect MMP2 activity was also assessed and as shown in Fig. 3C, silencing KRIT1 increased MMP-2 activity in melanoma cells.

3.4. KRIT1 knockdown induces β-catenin nuclear translocation and the expression of metastatic markers of A375 melanoma cells

Since we observed increased migration and invasion in siKRIT1 cells, we hypothesized that knockdown of KRIT1 may affect also phenotype plasticity through regulation of the Wnt/β-catenin signalling pathway [46]. Perturbations in Wnt-β-catenin signalling and elevated β-catenin levels are positively correlated with melanoma aggressiveness and are associated with melanoma malignant phenotype [47]. In addition, it has been already demonstrated that knockdown of KRIT1 increases nuclear β-catenin localization and activation of β-catenin-dependent transcription [24].

As shown in Fig. 4A, siKRIT1 A375 cells showed higher levels of β-



(caption on next page)

Fig. 2. KRIT1 knockdown stimulates cellular proliferation in A375 cells (A). Western blot analysis of KRIT1 levels in siKRIT1 and siCTRL A375 cells. The histogram represents the relative expression, normalized with β-actin expression (A.D.U: arbitrary densitometry units) (**p < 0.01). (B) Cell viability of siKRIT1 and siCTRL A375 cells by MTT assays. These results are representative of three independent experiments (**p < 0.01). (C) Cellular proliferation in siKRIT1 and siCTRL A375 cells. Images are obtained at 20× magnification. Results are reported as percentage of proliferating cells above the control (*p < 0.05; ***p < 0.001). (D) BrdU incorporation assay was performed in A375 cells transfected with either control siRNA (siCtrl) or KRIT1-targeting siRNA (siKRIT1) 24 h post-transfection. Data shown are the averages of results from three independent experiments. *p < 0.05. (E) Western blot analysis of cyclin D1 and p27 in siKRIT1 and siCTRL A375 cells. The histogram represents the relative expression, normalized to β-actin expression. (A.D.U: arbitrary densitometry units) (*p < 0.05, **p < 0.01). The scale bar in figure above represents 400 μm.

catenin expression in respect to siCTRL cells. Moreover, immunofluorescence analysis of β-catenin localization indicated a shift of the protein from cytoplasm to nucleus (Fig. 4B).

As nuclear translocation of β-catenin leads to transcription of β-catenin genes [48], which are involved in EMT associated protein network and in melanoma plasticity, we measured the levels of EMT proteins as vimentin and N-cadherin in siKRIT1 and siCTRL A375 cells [49]. As shown in Fig. 5, silencing KRIT1 increased the levels of both mesenchymal markers, evaluated by both, Western blot (Fig. 5A) and immunofluorescence (Fig. 5B).

Recently it has been reported that melanoma progression and metastatization is linked to inflammatory pathways [50–53]. In addition, elevated expression of cyclooxigenase-2 (COX-2) plays an important role in tumorigenesis as mediating the progression and metastasis of many epithelial cancer and melanoma [54]. As reported in Fig. 6 siKRIT1 A375 expressed higher COX-2 levels than control cells (Fig. 6A). Moreover, we also measured the expression levels of microsomal Prostaglandin E synthase 1 (mPGES-1), the enzyme responsible

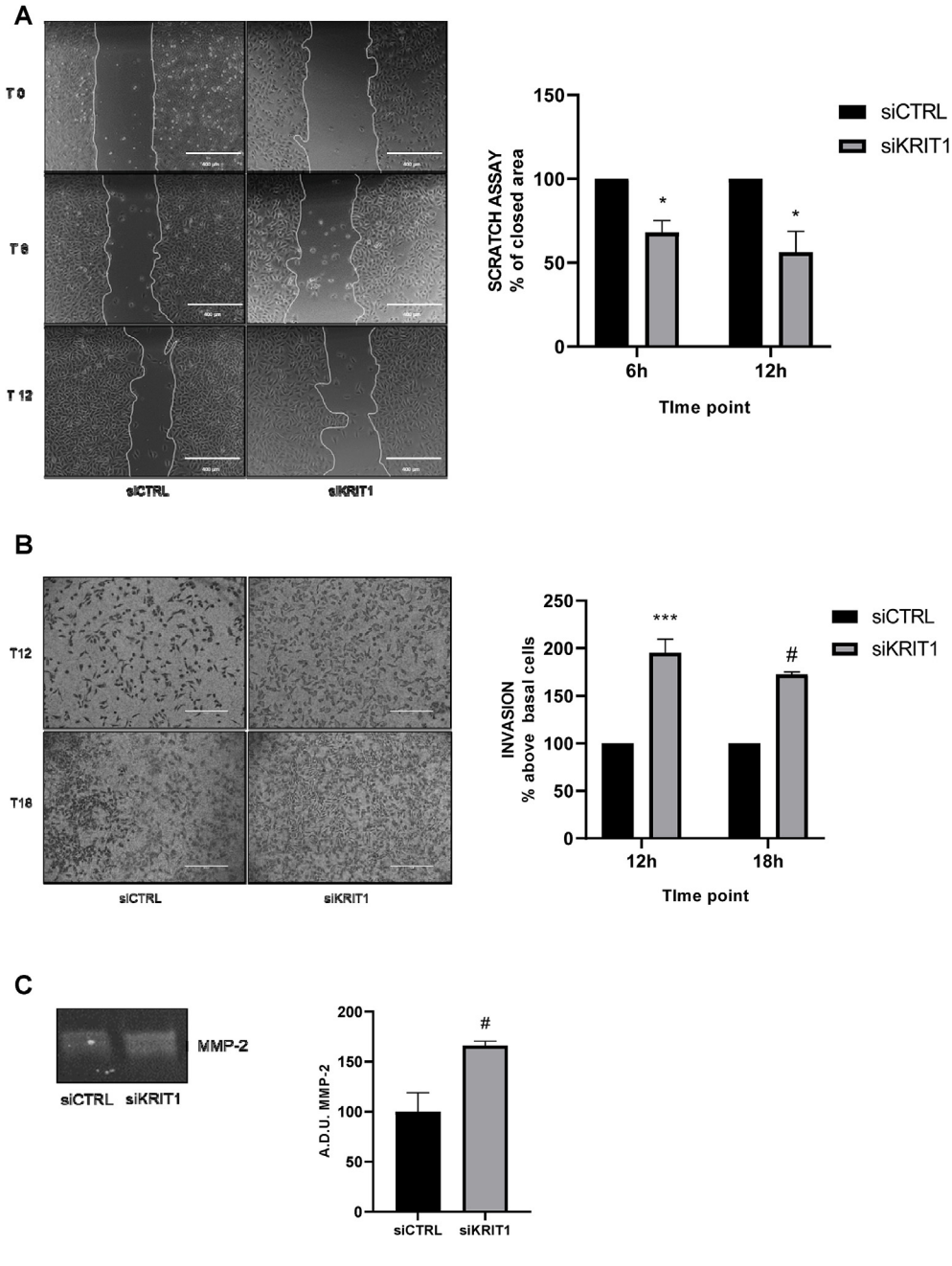


Fig. 3. KRIT1 downregulation increases migration and invasion in A375 melanoma cells. (A) Confluent monolayers of siKRIT1 and siCTRL A375 cells were scratched, and images were taken 6 and 12 h post-wounding at 20× magnification. Data are reported as percentage of wound closed area for well and are representative of two independent experiments (*p < 0.05). (B) Invasion assay. Image (20× magnification) of siKRIT1 and siCTRL A375 cells. Data are reported as percentage of migrated cells for well and are representative of three independent experiments (***p < 0.001; #p < 0.0001) (C) Enzymatic activity of MMP-2 in siKRIT1 and siCTRL A375 cells. Percentage of MMP-2 band was evaluated by quantitative densitometry and normalized to the number of cells/well (A.D.U: arbitrary densitometry units). Data are representative of three independent experiments (#p < 0.001). The scale bar in figures (A) and (B) represents 400 μm.

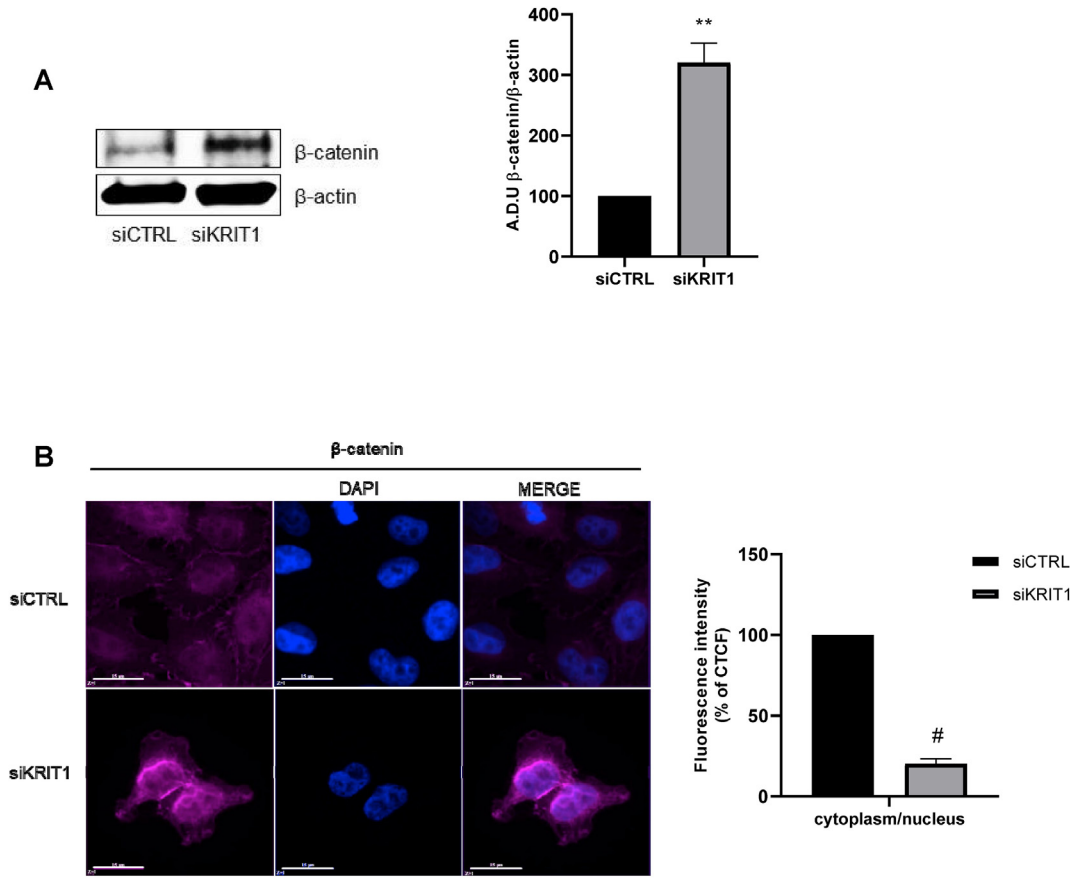


Fig. 4. KRIT1 silencing induces β-catenin overexpression and nuclear translocation of A375 melanoma cells. (A) Western blot analysis of β-actin levels in siKRIT1 and siCTRL A375 cells. Quantification of β-catenin levels normalized to β-actin (A.D.U: arbitrary densitometry units) (**$p < 0.01$). (B) Immunofluorescence images of β-catenin (40× magnification) in siKRIT1 and siCTRL A375 cells. Quantification of nuclear and cytoplasmatic β-catenin fluorescence intensity (CTCF: corrected total cell fluorescence) #$p < 0.001$). The scale bar in figures above represents 15 μm.

of prostaglandin E2 synthesis and downstream to COX-2, and we observed that loss of KRIT1 promoted mPGES-1 upregulation (Fig. 6B), indicating that KRIT1 is able to regulate the inflammatory status in melanoma cells possibly contributing to melanoma malignancy.

4. Discussion

In the present study we described for the first time evidences on the possible contribution of KRIT1 in melanoma progression. Even if most of the current literature links KRIT1 functions to endothelial cells and to cardiovascular development, KRIT1 is ubiquitously expressed. As previously reported, KRIT1 modulates endothelial cell functions by inducing proliferation, migration, angiogenesis and vascular permeability [22,55]. KRIT1 expression affects endothelial functions and its loss induces an endothelial phenotype highly invasive. It is well known that KRIT1 is an intracellular protein that interacts strongly and specifically with the tumor suppressor Rap1 [56], and it has been demonstrated that, in endothelial cells, KRIT1/Rap1 interaction regulates VE-cadherin/β-catenin association and KRIT1 loss increases the translocation of β-catenin to the nucleus [5,57]. In addition, Glading et al. showed that through this mechanism, KRIT1 mutations may lead to increased intestinal tumorigenesis [57]. Furthermore, Orso et al. have reported an anti-correlation between miR21 and KRIT1 that regulates epithelial tumor growth [27], supporting the idea that KRIT1 could play a role as tumor suppressor.

Cutaneous melanoma accounts for less than 5% of all skin cancer but it causes the majority of skin cancer deaths [58]. The main reason for the melanoma lethality is its high metastatic capacity even when the primary tumor is still significantly small in size. Intriguingly, we found that melanocytes constitutively express KRIT1, while in melanoma tissue, cancerous cells did not express KRIT1, or KRIT1 expression was limited to a focal immunopositivity. These findings support the idea that KRIT1 could be involved in melanoma progression and led us to explore the consequence of KRIT1 downregulation in A375 melanoma cell line. Our data indicate that KRIT1 deficiency increased cell survival, proliferation, migration and invasion, sustaining our original hypothesis.

Wnt/β-catenin signalling plays an important role in melanocyte biology, especially in the early stages of melanocyte transformation. β-catenin is an intracellular signal transducer of Wnt signalling and triggers transcription of genes involved in cell proliferation and invasion. Wnt/β-catenin signalling is frequently activated in melanoma and elevated β-catenin levels are positively correlated with melanoma aggressiveness. However, mechanisms that regulate β-catenin expression in melanoma are not fully understood. In our melanoma model, loss of KRIT1 induced increased β-catenin expression and its nuclear translocation, supporting the idea that KRIT1 could play a role as a tumor suppressor in melanoma. Additionally, since in epithelial cancers β-catenin controls the expression of proteins mainly involved in EMT, we explored the possibility that KRIT1 loss could induce the upregulation of mesenchymal markers that are associated to melanoma phenotype plasticity. Our data confirmed also this hypothesis being N-cadherin and vimentin upregulated after knocking down KRIT1.

Moreover, it is recognized that inflammatory process may drive melanoma cancer progression, and inhibition of COX-2 may inhibit melanoma metastasis [50,52,53]. In this work we showed that COX-2 and m-PGES1 are up-regulated after KRIT1 silencing in melanoma cells, supporting the idea that KRIT1 loss could increase melanoma

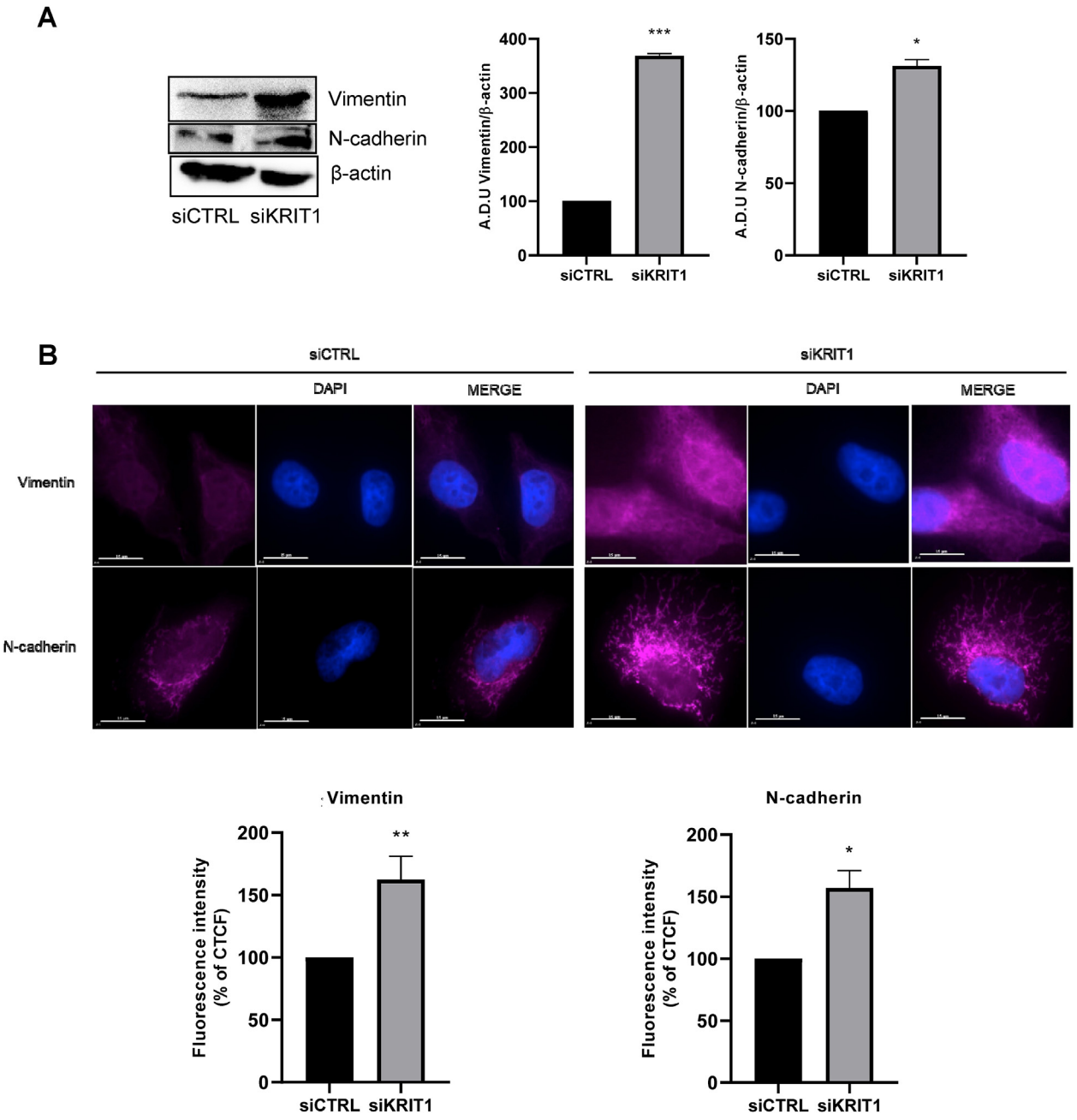


Fig. 5. KRIT1 silencing induces epithelial mesenchymal transition of A375 melanoma cells. (A) Western blot analysis of vimentin and N-cadherin expression in siKRIT1 and siCTRL A375 cells. Quantification of vimentin and N-cadherin levels normalized to β-actin (A.D.U:arbitrary densitometry units) (*p < 0.05; ***p < 0.001). (B) Immunofluorescence images of vimentin and N-cadherin (40× magnification) in siKRIT1 and siCTRL A375 cells. Quantification of N-cadherin and vimentin fluorescence intensity (CTCF: corrected total cell fluorescence) (*p < 0.05; **p < 0.01). The scale bar in figures above represents 15 μm.

aggressiveness also by modulating the inflammatory pathways.

5. Conclusions

In the present work, we have identified a novel pathway that could play a role in melanoma progression. Depletion of KRIT1 increased melanoma cells growth, migration and invasion, improved the β-catenin expression and its translocation into the nucleus, and induced the expression of markers of inflammation and melanoma plasticity.

Data deriving from the analysis of oncological databases, including canSAR Black (https://cansarblack.icr.ac.uk), COSMIC (https://cancer.sanger.ac.uk), and cBioPortal (https://www.cbioportal.org), showed an incidence of KRIT1 mutations in melanoma ranging between 3 and 12.5% versus a prevalence of 0% in benign melanocytic nevi, supporting our hypothesis that KRIT1 mutations play a role in melanoma development. Among the three mutated genes in the CCM disease, KRIT1 is by far the most involved in melanoma. Furthermore, data from the Cavernous Angioma patient registry (Angioma Alliance association, www.angioma.org) showed that the incidence of melanoma in CCM patients is about 1.2% versus an incidence of about 0.02% in general population (https://cancerstatisticscenter.cancer.org, https://seer.cancer.gov). Thus, even though specific information about the type of CCM gene (KRIT1/CCM1, CCM2 or CCM3) involved in CCM patients with melanoma is not available to date, the analysis of the different databases strongly suggest that patients bearing mutations in any of the three CCM genes are more likely to develop melanoma. The greater involvement of mutations of the KRIT1 gene in melanoma compared to the CCM2 and CCM3 genes, however, suggests that CCM patients

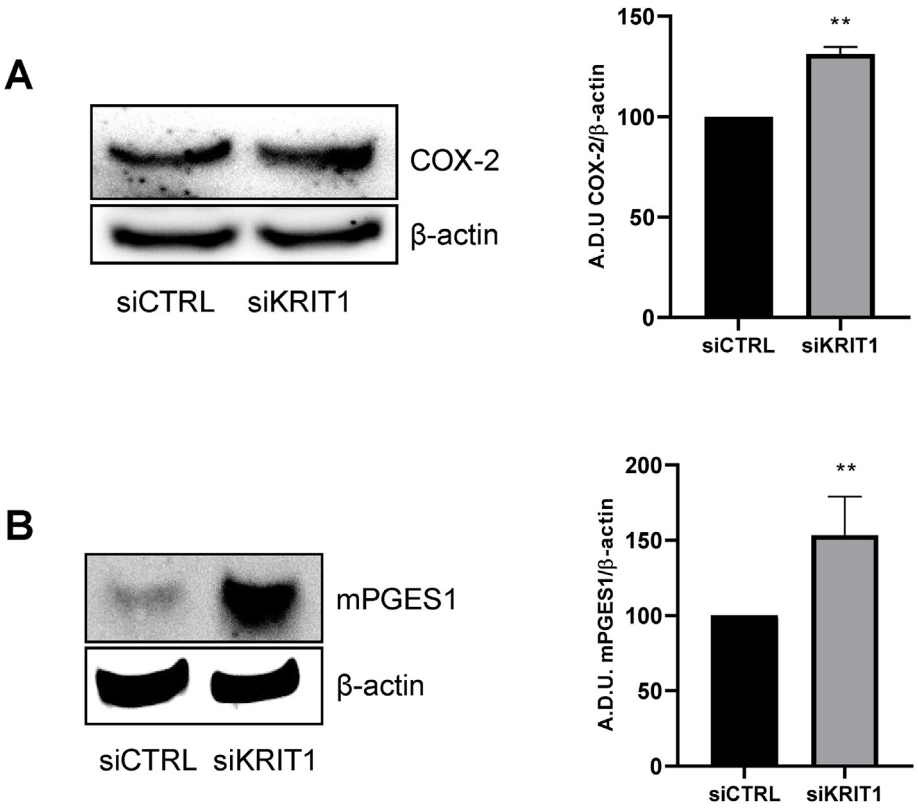


Fig. 6. KRIT1 silencing induces COX-2 and mPGES-1 expression. (A, B) Western blot analysis of COX-2 and, mPGES-1 expression in siKRIT1 and siCTRL A375 cells. Quantification of COX-2 and mPGES-1 levels normalized to β-actin (A.D.U: arbitrary densitometry units) (**p < 0.01).

bearing KRIT1 heterozygous mutations might be at higher risk of developing melanomas, possibly due to local effects in skin cells.

In conclusion, the data reported here strongly support the hypothesis that KRIT1 acts as a tumor suppressor in melanoma and its loss increases melanoma aggressiveness. Thank to this fundamental function, KRIT1 has the potentiality to be a future therapeutic target and diagnostic marker for melanoma.

Even though more in-depth epidemiological studies will be necessary to sustain and better elucidate these data, they will have important implications and relapses for both CCM and melanoma patients.

Funding

This work was supported by the Telethon Foundation (grant GGP15219) to LT, MIUR (Progetto Dipartimento di Eccellenza 2018–2022) to LT, FF and JE, and FAR'19 to GV.

CRediT authorship contribution statement

Jasmine Ercoli: Methodology, Writing - original draft. **Federica Finetti:** Conceptualization, Supervision, Writing - original draft. **Brittany Woodby:** Conceptualization, Supervision, Writing - original draft. **Giuseppe Belmonte:** Data curation, Investigation, Methodology. **Clelia Miracco:** Data curation, Investigation, Methodology. **Giuseppe Valacchi:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Lorenza Trabalzini:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Acknowledgments

The authors are grateful to Giulia Macrì and Paola Nezi for providing help in some experiments, and Angioma Alliance for allowing access to the Cavernous Angioma patient registry.

References

[1] I. Serebriiskii, J. Estojak, G. Sonoda, J.R. Testa, E.A. Golemis, Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22, Oncogene 15 (1997) 1043–1049, https://doi.org/10.1038/sj.onc.1201268.

[2] P. Guazzi, L. Goitre, E. Ferro, V. Cutano, C. Martino, L. Trabalzini, S.F. Retta, Identification of the kelch family protein Nd1-L as a novel molecular interactor of KRIT1, PloS One 7 (2012) e44705, , https://doi.org/10.1371/journal.pone.0044705.

[3] J.S. Zawistowski, I.G. Serebriiskii, M.F. Lee, E.A. Golemis, D.A. Marchuk, KRIT1 association with the integrin-binding protein ICAP-1: a new direction in the elucidation of cerebral cavernous malformations (CCM1) pathogenesis, Hum. Mol. Genet. 11 (2002) 389–396, https://doi.org/10.1093/hmg/11.4.389.

[4] J.S. Zawistowski, L. Stalheim, M.T. Uhlik, A.N. Abell, B.B. Ancrile, G.L. Johnson, D.A. Marchuk, CCM1 and CCM2 protein interactions in cell signaling: implications for cerebral cavernous malformations pathogenesis, Hum. Mol. Genet. 14 (2005) 2521–2531, https://doi.org/10.1093/hmg/ddi256.

[5] A. Glading, J. Han, R.A. Stockton, M.H. Ginsberg, KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell–cell junctions, J. Cell Biol. 179 (2007) 247–254, https://doi.org/10.1083/jcb.200705175.

[6] E. Faurobert, C. Albiges-Rizo, Recent insights into cerebral cavernous malformations: a complex jigsaw puzzle under construction, FEBS J. 277 (2010) 1084–1096, https://doi.org/10.1111/j.1742-4658.2009.07537.x.

[7] C. Denier, J.M. Gasc, F. Chapon, V. Domenga, C. Lescoat, A. Joutel, E. Tournier-Lasserve, Krit1/cerebral cavernous malformation 1 mRNA is preferentially expressed in neurons and epithelial cells in embryo and adult, Mech. Dev. (2002), https://doi.org/10.1016/S0925-4773(02)00209-5.

[8] T. Sahoo, E.W. Johnson, J.W. Thomas, P.M. Kuehl, T.L. Jones, C.G. Dokken, J.W. Touchman, C.J. Gallione, S.Q. Lee-Lin, B. Kosofsky, J.H. Kurth, D.N. Louis, G. Mettler, L. Morrison, A. Gil-Nagel, S.S. Rich, J.M. Zabramski, M.S. Boguski, E.D. Green, D.A. Marchuk, Mutations in the gene encoding KRIT1, a Krev-1/rap1a binding protein, cause cerebral cavernous malformations (CCM1), Hum. Mol. Genet. 8 (1999) 2325–2333, https://doi.org/10.1093/hmg/8.12.2325.

[9] S.L. Couteulx, H.H. Jung, P. Labauge, J. Houtteville, C. Lescoat, M. Cecillon, E. Marechal, A. Joutel, J. Bach, E. Tournier-lasserve, Cause hereditary cavernous angiomas, Nat. Genet. (1999), https://doi.org/10.1038/13815.

[10] S. Batra, D. Lin, P.F. Recinos, J. Zhang, D. Rigamonti, Cavernous malformations: natural history, diagnosis and treatment, Nat. Rev. Neurol. 5 (2009) 659–670, https://doi.org/10.1038/nrneurol.2009.177.

[11] J. Gault, H. Sarin, N.A. Awadallah, R. Shenkar, I.A. Awad, Pathobiology of human cerebrovascular malformations: basic mechanisms and clinical relevance, Neurosurgery 55 (2004) 1–16 discussion 16-7 http://www.ncbi.nlm.nih.gov/

pubmed/15214969 accessed September 3, 2019.
[12] D. Rigamonti, Cavernous Malformations of the Nervous System, (2011), https://doi.org/10.1017/CBO9781139003636.
[13] A. Padarti, J. Zhang, Recent advances in cerebral cavernous malformation research, Vessel Plus 2 (2018) 21, https://doi.org/10.20517/2574-1209.2018.34.
[14] Y. Wang, Y. Li, J. Zou, S.P. Polster, R. Lightle, T. Moore, M. Dimaano, T.C. He, C.R. Weber, I.A. Awad, L. Shen, The cerebral cavernous malformation disease causing gene KRIT1 participates in intestinal epithelial barrier maintenance and regulation, Faseb. J. (2019), https://doi.org/10.1096/fj.201800343R.
[15] F. Finetti, I. Schiavo, J. Ercoli, A. Zotta, E. Boda, S.F. Retta, L. Trabalzini, KRIT1 loss-mediated upregulation of NOX1 in stromal cells promotes paracrine pro-angiogenic responses, Cell, Signals 68 (2020) 109527, https://doi.org/10.1016/j.cellsig.2020.109527.
[16] C. Antognelli, E. Trapani, S. Delle Monache, A. Perrelli, M. Daga, S. Pizzimenti, G. Barrera, P. Cassoni, A. Angelucci, L. Trabalzini, V.N. Talesa, L. Goitre, S.F. Retta, KRIT1 loss-of-function induces a chronic Nrf2-mediated adaptive homeostasis that sensitizes cells to oxidative stress: implication for Cerebral Cavernous Malformation disease, Free Radic. Biol. Med. (2018), https://doi.org/10.1016/j.freeradbiomed.2017.11.014.
[17] L. Goitre, P.V. DiStefano, A. Moglia, N. Nobiletti, E. Baldini, L. Trabalzini, J. Keubel, E. Trapani, V.V. Shuvaev, V.R. Muzykantov, I.H. Sarelius, S.F. Retta, A.J. Glading, Up-regulation of NADPH oxidase-mediated redox signaling contributes to the loss of barrier function in KRIT1 deficient endothelium, Sci. Rep. 7 (2017) 8296, https://doi.org/10.1038/s41598-017-08373-4.
[18] L. Goitre, E. De Luca, S. Braggion, E. Trapani, M. Guglielmotto, F. Biasi, M. Forni, A. Moglia, L. Trabalzini, S.F. Retta, KRIT1 loss of function causes a ROS-dependent upregulation of c-Jun, Free Radic. Biol. Med. (2014), https://doi.org/10.1016/j.freeradbiomed.2013.11.020.
[19] A.J. Glading, F. Finetti, L. Trabalzini, Disease models in cerebral cavernous malformations, Drug Discov. Today Dis. Model. xxx (2019) 1–9, https://doi.org/10.1016/j.ddmod.2019.10.009.
[20] F. Francalanci, M. Avolio, E. De Luca, D. Longo, V. Menchise, P. Guazzi, F. Sgrò, M. Marino, L. Goitre, F. Balzac, L. Trabalzini, S.F. Retta, Structural and functional differences between KRIT1A and KRIT1B isoforms: a framework for understanding CCM pathogenesis, Exp. Cell Res. 315 (2009) 285–303, https://doi.org/10.1016/j.yexcr.2008.10.006.
[21] L. Maddaluno, N. Rudini, R. Cuttano, L. Bravi, C. Giampietro, M. Corada, L. Ferrarini, F. Orsenigo, E. Papa, G. Boulday, E. Tournier-Lasserve, F. Chapon, C. Richichi, S.F. Retta, M.G. Lampugnani, E. Dejana, EndMT contributes to the onset and progression of cerebral cavernous malformations, Nature 498 (2013) 492–496, https://doi.org/10.1038/nature12207.
[22] M.G. Lampugnani, F. Orsenigo, N. Rudini, L. Maddaluno, G. Boulday, F. Chapon, E. Dejana, CCM1 regulates vascular-lumen organization by inducing endothelial polarity, J. Cell Sci. (2010), https://doi.org/10.1242/jcs.059329.
[23] J. Wüstehube, A. Bartol, S.S. Liebler, R. Brütsch, Y. Zhu, U. Felbor, U. Sure, H.G. Augustin, A. Fischer, Cerebral cavernous malformation protein CCM1 inhibits sprouting angiogenesis by activating DELTA-NOTCH signaling, Proc. Natl. Acad. Sci. Unit. States Am. 107 (2010) 12640–12645, https://doi.org/10.1073/pnas.1000132107.
[24] P.V. DiStefano, J.M. Kuebel, I.H. Sarelius, A.J. Glading, KRIT1 protein depletion modifies endothelial cell behavior via increased vascular endothelial growth factor (VEGF) signaling, J. Biol. Chem. 289 (2014) 33054–33065, https://doi.org/10.1074/jbc.M114.582304.
[25] L. Goitre, F. Balzac, S. Degani, P. Degan, S. Marchi, P. Pinton, S.F. Retta, KRIT1 regulates the homeostasis of intracellular reactive oxygen species, PloS One (2010), https://doi.org/10.1371/journal.pone.0011786.
[26] F. Vieceli Dalla Sega, R. Mastrocola, G. Aquila, F. Fortini, C. Fornelli, A. Zotta, A.S. Cento, A. Perrelli, E. Boda, A. Pannuti, S. Marchi, P. Pinton, R. Ferrari, P. Rizzo, S.F. Retta, KRIT1 deficiency promotes aortic endothelial dysfunction, Int. J. Mol. Sci. 20 (2019), https://doi.org/10.3390/ijms20194930.
[27] F. Orso, F. Balzac, M. Marino, A. Lembo, S.F. Retta, D. Taverna, MiR-21 coordinates tumor growth and modulates KRIT1 levels, Biochem. Biophys. Res. Commun. 438 (2013) 90–96, https://doi.org/10.1016/j.bbrc.2013.07.031.
[28] J. Abou-Fadel, Y. Qu, E.M. Gonzalez, M. Smith, J. Zhang, Emerging roles of CCM genes during tumorigenesis with potential application as novel biomarkers across major types of cancers, Oncol. Rep. 43 (2020) 1945–1963, https://doi.org/10.3892/or.2020.7550.
[29] E. Erdei, S.M. Torres, A new understanding in the epidemiology of melanoma, Expert Rev. Anticancer Ther. (2010), https://doi.org/10.1586/era.10.170.
[30] Z. Kutlubay, B. Engin, S. Serdaroglu, Y. Tuzu, Current management of malignant melanoma: state of the art, Highlights Ski. Cancer (2013), https://doi.org/10.5772/55304.
[31] M. Rastrelli, S. Tropea, C. Rossi, M. Alaibac, Melanoma risk factors, Vivo, 2012.
[32] V.C. Gorantla, J.M. Kirkwood, State of melanoma. An historic overview of a field in transition, Hematol. Oncol. Clin. N. Am. (2014), https://doi.org/10.1016/j.hoc.2014.02.010.
[33] A. Sandru, S. Voinea, E. Panaitescu, A. Blidaru, Survival rates of patients with metastatic malignant melanoma, J. Med. Life. 7 (4) (2014) 572–576.
[34] N. Klebanov, W.M. Lin, M. Artomov, M. Shaughnessy, C.N. Njauw, R. Bloom, A.K. Eterovic, K. Chen, T.B. Kim, S.S. Tsao, H. Tsao, Use of targeted next-generation sequencing to identify activating hot spot mutations in cherry angiomas, JAMA Dermatology 155 (2019) 211–215, https://doi.org/10.1001/jamadermatol.2018.4231.
[35] A.J. Parish, V. Nguyen, A.M. Goodman, K. Murugesan, G.M. Frampton, R. Kurzrock, GNAS, GNAQ, and GNA11 alterations in patients with diverse cancers, Cancer 124 (2018) 4080–4089, https://doi.org/10.1002/cncr.31724.
[36] C. Cavicchio, M. Benedusi, E. Pambianchi, A. Pecorelli, F. Cervellati, V. Savelli, D. Calamandrei, E. Maellaro, G. Rispoli, E. Maioli, G. Valacchi, Potassium ascorbate with ribose: promising therapeutic approach for melanoma treatment, Oxid. Med. Cell. Longev. (2017), https://doi.org/10.1155/2017/4256519 2017.
[37] F. Ferrara, E. Pambianchi, A. Pecorelli, B. Woodby, N. Messano, J.P. Therrien, M.A. Lila, G. Valacchi, Redox regulation of cutaneous inflammasome by ozone exposure, Free Radic. Biol. Med. (2019), https://doi.org/10.1016/j.freeradbiomed.2019.11.031 0–1.
[38] X.M. Muresan, M.S. Narzt, B. Woodby, F. Ferrara, F. Gruber, G. Valacchi, Involvement of cutaneous SR-B1 in skin lipid homeostasis, Arch. Biochem. Biophys. (2019), https://doi.org/10.1016/j.abb.2019.03.005.
[39] A. Fallacara, S. Vertuani, G. Panozzo, A. Pecorelli, G. Valacchi, S. Manfredini, Novel artificial tears containing cross-linked hyaluronic acid: an in vitro re-epithelialization study, Molecules 22 (2017) 1–13, https://doi.org/10.3390/molecules22122104.
[40] F. Finetti, E. Terzuoli, E. Bocci, I. Coletta, L. Polenzani, G. Mangano, M.A. Alisi, N. Cazzolla, A. Giachetti, M. Ziche, S. Donnini, Pharmacological inhibition of microsomal prostaglandin E synthase-1 suppresses epidermal growth factor receptor-mediated tumor growth and angiogenesis, PloS One 7 (2012), https://doi.org/10.1371/journal.pone.0040576.
[41] I. Crivellari, C. Sticozzi, G. Belmonte, X.M. Muresan, F. Cervellati, A. Pecorelli, C. Cavicchio, E. Maioli, C.C. Zouboulis, M. Benedusi, C. Cervellati, G. Valacchi, SRB1 as a new redox target of cigarette smoke in human sebocytes, Free Radic. Biol. Med. 102 (2017) 47–56, https://doi.org/10.1016/j.freeradbiomed.2016.11.021.
[42] F. Finetti, R. Solito, L. Morbidelli, A. Giachetti, M. Ziche, S. Donnini, Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1, J. Biol. Chem. 283 (2008) 2139–2146, https://doi.org/10.1074/jbc.M703090200.
[43] G. Allavena, B. Del Bello, P. Tini, N. Volpi, G. Valacchi, C. Miracco, L. Pirtoli, E. Maellaro, Trehalose inhibits cell proliferation and amplifies long-term temozolomide- and radiation-induced cytotoxicity in melanoma cells: a role for autophagy and premature senescence, J. Cell. Physiol. 234 (2019) 11708–11721, https://doi.org/10.1002/jcp.27838.
[44] O. Guzeloglu-Kayisli, U.A. Kayisli, N.M. Amankulor, J.R. Voorhees, O. Gokce, M.L. DiLuna, M.S.H. Laurans, G. Luleci, M. Gunel, Krev1 interaction trapped-1/cerebral cavernous malformation-1 protein expression during early angiogenesis, J. Neurosurg. 100 (2004) 481–487, https://doi.org/10.3171/ped.2004.100.5.0481.
[45] E.S. Radisky, D.C. Radisky, Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer, J. Mammary Gland Biol. Neoplasia (2010), https://doi.org/10.1007/s10911-010-9177-x.
[46] T. Reya, H. Clevers, Wnt signalling in stem cells and cancer, Cellular Signal. 434 (2005) 843–850 2005.
[47] K. Prabhakar, C.I. Rodríguez, A.S. Jayanthy, D.M. Mikheil, A.I. Bhasker, R.J. Perera, V. Setaluri, Role of miR-214 in regulation of β-catenin and the malignant phenotype of melanoma, Mol. Carcinog. (2019), https://doi.org/10.1002/mc.23089.
[48] K.M. Cadigan, Wnt-β-catenin signaling, Curr. Biol. (2008), https://doi.org/10.1016/j.cub.2008.08.017.
[49] C.Y. Liu, H.H. Lin, M.J. Tang, Y.K. Wang, Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation, Oncotarget (2015), https://doi.org/10.18632/oncotarget.3862.
[50] K.M. Kim, A.R. Im, S.H. Kim, J.W. Hyun, S. Chae, Timosaponin AIII inhibits melanoma cell migration by suppressing COX-2 and in vivo tumor metastasis, Canc. Sci. 107 (2016) 181–188, https://doi.org/10.1111/cas.12852.
[51] A.-C. Goulet, J.G. Einsphar, D.S. Alberts, A. Beas, C. Burk, A. Bhattacharyya, J. Bangert, J.M. Harmon, H. Fujiwara, A. Koki, M.A. Nelson, Analysis of cyclooxygenase 2 (COX-2) expression during malignant melanoma progression., Canc. Biol. Ther. 2 (n.d.) 713–8. http://www.ncbi.nlm.nih.gov/pubmed/14688483 (accessed January 30, 2020).
[52] E. Panza, P. De Cicco, G. Ercolano, C. Armogida, G. Scognamiglio, A.M. Anniciello, G. Botti, G. Cirino, A. Ianaro, Differential expression of cyclooxygenase-2 in metastatic melanoma affects progression free survival, Oncotarget 7 (2016) 57077–57085, https://doi.org/10.18632/oncotarget.10976.
[53] P. Zhou, J. Qin, Y. Li, G. Li, Y. Wang, N. Zhang, P. Chen, C. Li, Combination therapy of PKCζ and COX-2 inhibitors synergistically suppress melanoma metastasis, J. Exp. Clin. Canc. Res. 36 (2017) 1–12, https://doi.org/10.1186/s13046-017-0585-2.
[54] N. Hashemi Goradel, M. Najafi, E. Salehi, B. Farhood, K. Mortezaee, Cyclooxygenase-2 in cancer: a review, J. Cell. Physiol. 234 (2019) 5683–5699, https://doi.org/10.1002/jcp.27411.
[55] S.F. Retta, A.J. Glading, Oxidative stress and inflammation in cerebral cavernous malformation disease pathogenesis: two sides of the same coin, Int. J. Biochem. Cell Biol. (2016), https://doi.org/10.1016/j.biocel.2016.09.011.
[56] X. Li, R. Zhang, K.M. Draheim, W. Liu, D.A. Calderwood, T.J. Boggon, Structural basis for small G protein effector interaction of ras-related protein 1 (Rap1) and adaptor protein krev interaction trapped 1 (KRIT1), J. Biol. Chem. 287 (2012) 22317–22327, https://doi.org/10.1074/jbc.M112.361295.
[57] A.J. Glading, M.H. Ginsberg, Rap1 and its effector KRIT1/CCM1 regulate -catenin signaling, Dis. Model. Mech. 3 (2010) 73–83, https://doi.org/10.1242/dmm.003293.
[58] M. Potrony, C. Badenas, P. Aguilera, J.A. Puig-Butille, C. Carrera, J. Malvehy, S. Puig, Update in genetic susceptibility in melanoma, Ann. Transl. Med. 3 (2015), https://doi.org/10.3978/j.issn.2305-5839.2015.08.11.

antioxidants

MDPI

Article

Phaseolus vulgaris L. var. Venanzio Grown in Tuscany: Chemical Composition and In Vitro Investigation of Potential Effects on Colorectal Cancer

Federica Finetti [1,*,†], Marco Biagi [2,†], Jasmine Ercoli [1], Giulia Macrì [1], Elisabetta Miraldi [2] and Lorenza Trabalzini [1,*]

[1] Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy; ercoli3@student.unisi.it (J.E.); giulia.macri@student.unisi.it (G.M.)

[2] Department of Physical Sciences, Earth and Environment, University of Siena, Via Laterina 8, 53100 Siena, Italy; marco.biagi@unisi.it (M.B.); elisabetta.miraldi@unisi.it (E.M.)

* Correspondence: finetti2@unisi.it (F.F.); lorenza.trabalzini@unisi.it (L.T.)

† These authors contributed equally to this work.

Received: 9 September 2020; Accepted: 24 November 2020; Published: 26 November 2020

Abstract: *Phaseolus vulgaris* L. (common bean) is a leguminous species that is an important dietary component due to its high content of proteins, unsaturated fatty acids, minerals, dietary fibers and vitamins. Due to the high content of polyphenols, several biological activities have been described for bean extracts, making it possible to include *P. vulgaris* among food with beneficial effects for human health. Moreover, more than 40,000 varieties of beans have been recognised with different nutraceutical properties, pointing out the importance of food biodiversity. In this work, we describe for the first time the chemical composition and biological activity of a newly recognized Italian variety of *P. vulgaris* grown in a restricted area of the Tuscany region and named "Fagiola di Venanzio". Fagiola di Venanzio water extract is rich in proteins, sugars and polyphenols and displays antioxidant, anti-inflammatory and antiproliferative activities in *in vitro* assays on colon cancer cellular models. Our data indicate that this variety of *P. vulgaris* appears to be a promising source of bioactive compounds and encourage more in-depth studies to better elucidate the implications of its consumption for public health.

Keywords: *Phaseulus vulgaris* L.; biodiversity; nutraceuticals; oxidative stress; inflammation; functional food; COX-2; colon cancer

1. Introduction

Biodiversity and dietary habits are crucially important to prevent the development of lifestyle-associated diseases. In the last few years, there has been an increased interest in the consumption of bioactive foods, capable of exerting biological effects at different cellular levels and endowed with beneficial properties for public health [1]. *Phaseolus vulgaris* L. (common bean) is the most important edible legume in the diet and gastronomy of many countries in the world. Numerous species of *Phaseolus* are cultivated and more than 12 million tons of dry beans are produced worldwide [2]. *P. vulgaris* exists in many variations regarding growth characteristics, maturation and adaptation, accounting for more than 40,000 varieties. Common beans play an important role in human nutrition as a fundamental source of plant proteins, unsaturated fatty acids, minerals, dietary fibers and vitamins [3,4]. Moreover, in the past years beneficial effects for human health have been described and have been associated with the high content of phenolic compounds. In the majority of characterized bean extracts, the phenolic content is mainly represented by phenolic acids, hydroxycinnamic acids, flavones, flavanols, flavanones, isoflavonoids, anthocyanins, chalcones and dihydrochalcones [4,5].

The consumption of beans have received increased attention due to their beneficial health effects in the prevention and control of numerous chronic and degenerative diseases, that are the main causes of mortality in the world. In particular, beans consumption has proven to be effective in reducing the risk of cardiovascular diseases (for the antioxidant, anti-inflammatory and hypolipidemic properties), obesity and diabetes (for the presence of α-amilase inhibitors and phytohemagglutinin and for the presence of starch) and cancer [2,4,6,7].

Several epidemiological studies suggest a link between a diet rich in beans and reduced risk of numerous types of cancer. Beans consumption for two or more times per week reduced the risk of colon cancer (up to 47%) [8] and prostate cancer (about 22%) [9]. These data were also confirmed in animal models in which a diet rich in beans reduced the incidence of tumors as colon cancer [3,10–16] and breast cancer [17,18] by interfering with multiple signaling pathways. Moreover, in vitro cellular models showed that bean extracts exert antiproliferative, anti-inflammatory, and pro-apoptotic activities in different types of cancer cells [3,13,14,19].

In this work, we studied for the first time an endangered Italian variety of *P. vulgaris*, grown in a restricted area of the municipality of Murlo (Siena, Tuscany) named "Fagiola di Venanzio" (FV) (Figure 1). FV has been cultivated since the mid-nineteenth century by the Burresi family in their farm located near Murlo and has been recognized as a specific variety in 2017 (N. VE_145 20-12-2017, Regione Toscana, Italy). FV beans are characterized by a white seed coat color, with a relatively small size, very flattened and elliptical-wide shape (Figure 1). Here, we first determined the chemical composition of FV extracts and then we studied antioxidant, anti-inflammatory and antiproliferative activity on colorectal cancer cellular models.

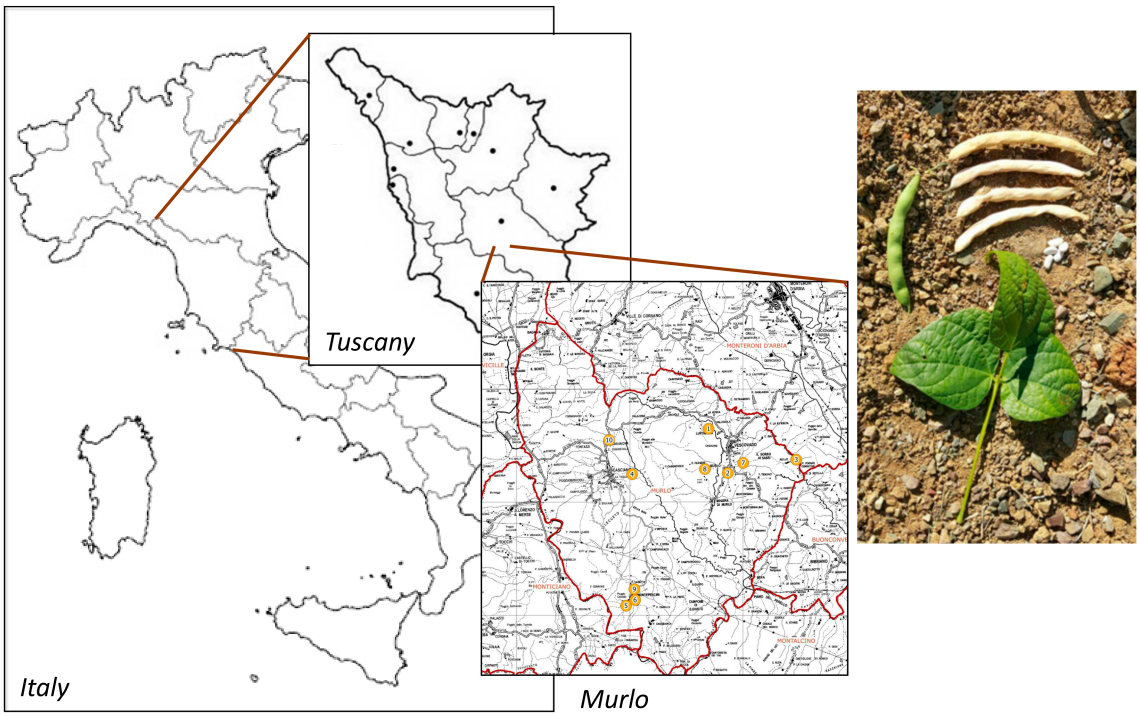


Figure 1. Production area of Fagiola di Venanzio (FV). Colored circles indicate the micro areas within Murlo municipality where FV is cultivated.

2. Material and Methods

2.1. Preparation of Extracts of FV

Phaseolus vulgaris L. var. "Fagiola di Venanzio" (FV) dried seeds (beans), were harvested in the municipality of Murlo (Siena, Tuscany, Italy, Latitude 43°10'16"32 N, longitude 11°23'32"28 E) and were identified by botanists in the Siena University Botanical Garden. Four samples of FV provided by different growers were used (Table 1).

Table 1. "Fagiola di Venanzio" samples

Grower	# Sample
Società Agricola Aiellino	1
Nicola Ulivieri	2
Burresi family	3
Azienda Agricola Podere Vignali	4

In order to preserve the whole phytocomplex of beans and to extract polyphenols, proteins and carbohydrates at the same time, the extractive procedure was accomplished by briefly soaking 10 g of manually grinded beans in water at 50 °C, discarding the liquid and then performing a maceration at 35 °C with 100 mL of distilled water for 48 h. The extract was adjusted to a 1:10 final drug:extract ratio.

2.2. Polyphenols Content

Total polyphenols content (TPP) of FV extracts was evaluated by Folin–Ciocalteau (FC) colorimetric assay, optimizing the procedure reported in Biagi et al., 2019 [20]. Briefly, 100 µL of extract was diluted to 3 mL with distilled water; 500 µL of 1:10 FC reactive in water (Sigma-Aldrich, Milan, Italy) were added and the mixture was gently shaken for 1 min. A quantity of 1000 µL of 30% *w/v* sodium carbonate water solution was added and, after incubation for 1 h in the dark at RT, absorbance of samples was read at 750 nm, using distilled water as blank. Gallic acid (Sigma-Aldrich) was used as reference standard. A calibration curve was created using gallic acid 5000 to 78 mg/L.

2.3. Soluble Carbohydrates Content

Total soluble carbohydrates of extract were quantified using the acid phenol assay described for the first time by Dubois et al., 1951 [21], with optimization of the method. A quantity of 100 µL of the supernatant was added to 190 µL of water and 100 µL of a 6% *w/v* phenol (Sigma-Aldrich) water solution. The solution was gently shaken for 30 s and 500 µL of concentrated sulfuric acid (Sigma-Aldrich) were added. The mixture was heated at 80 °C for 15 min and cooled to RT. Absorbance was read at 490 nm. D-glucose (Sigma-Aldrich) was used from 80 to 1.25 mg/L as reference standard. Total saccharide content was calculated, interpolating the data on the calibration curve of D-glucose.

2.4. Protein Content

Total proteins of extracts were determined spectrophotometrically using the BCA protein assay kit (Euroclone, Milan, Italy). Briefly, 2 µL of different dilutions of FV extract were added to 100 µL of bicinchoninic acid and, after incubation at 37 °C for 30 min, the absorbance was measured at 562 nm with a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Protein concentration was determined and reported with reference to standards of a bovine serum albumin (BSA).

2.5. HPLC-DAD Analysis on Main Polyphenolic Constituents

HPLC-DAD analysis was performed by using a Shimadzu Prominence LC 2030 3D instrument equipped with a Bondapak® C18 column, 10 μm, 125 Å, 3.9 mm × 300 mm column (Waters Corporation, Milford, MA, USA).

Water solutions containing 0.1 % (*v*/*v*) formic acid (A) and 0.1% (*v*/*v*) acetonitrile (B) were used as mobile phase. The following program was applied: B from 10% at 0 min to 35% at 20 min, then B 50% at 25 min; flux was set at 0.8 mL/min. Chromatograms were recorded at 254, 280, 330 and 350 nm. Analyses were performed using 10 μL of FV extract; gallic acid, chlorogenic acid, caffeic acid, catechin, genistein, daidzein, quercetin and kaempferol (Sigma-Aldrich) were used as external standards. Calibration curves were established using reference standards ranging from 0.008 mg/mL to 0.500 mg/mL. The correlation coefficient (R^2) of each curve was >0.99.

2.6. HPLC-DAD-DPPH (2,2-Diphenyl-1-picrylhydrazyl)

To evaluate the different role of FV polyphenols in exerting antiradical activity, the HPLC-DAD run described above was repeated after having incubated the FV extract with a 1×10^{-2} M DPPH (2,2-diphenyl-1-picrylhydrazyl) methanolic solution for 15 min. Each chromatogram peak area was compared before and after DPPH reaction. Ascorbic acid was used to validate the test.

2.7. Cell Culture

HCT116, colorectal carcinoma cells, (ATCC, Rockville, MD, USA) were cultured in DMEM (Euroclone) supplemented with 10% fetal bovine serum (FBS, Euroclone), 100 U/mL penicillin/streptomycin (Euroclone), and 4 mM L-glutamine (Euroclone).

HT29, colorectal adenocarcinoma cells, (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 (Euroclone) medium supplemented with 10% FBS with 100 U/mL penicillin/streptomycin. Both cell lines were grown at 37 °C and 5% CO_2.

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Lonza, Basel, Switzerland). All experiments were performed on low passage cell cultures. Cells were grown on gelatin-coated dishes in Endothelial Growth Medium (EGM-2) (EBM-2, FBS 10%, VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000) (Lonza) at 37 °C and 5% CO_2.

2.8. MTT Assay

Either 3.5×10^3 (HT29) or 2.5×10^3 (HCT116) cells/well were seeded in 96-multiwell plates in medium with 10% FBS and, after adherence, were maintained for 18 h in medium without phenol red (0.1% serum) with different concentrations of the FV extract (5, 10, 50 and 100 μM) and then exposed to IL1β (10 ng/mL) for 24 h.

After 24 h, the medium was removed, and cells were incubated for 4 h with fresh medium in the presence of 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich). The MTT solution was then removed and 100 μL of DMSO were added to each well to dissolve the blue formazan crystals. The absorbance of the formazan dye was measured at 570 nm with a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Data were expressed as a percentage of the basal control [22].

2.9. Western Blotting Analysis

HT29 or HUVEC (3.0×10^5 cells/well) were seeded in 6-well multiplates in medium added with 10% serum. After 24h cells were starved for 6 h in medium containing 0.1% serum, and then were treated with extracts for 18 h (10 and 50 μM) and then exposed to IL1β (10 ng/mL). After 24 h, extraction of total proteins was performed by lysing cells in precooled radioimmunoprecipitation assay (RIPA) lysis buffer.

Protein concentration of cell extracts was determined spectrophotometrically using the BCA protein assay kit (Euroclone). For western blotting analysis, aliquots of cell extract supernatants containing an equal amount of proteins (50 µg) were treated with Laemmli buffer, boiled for 10 min, resolved on 4–20% stain-free gel and then blotted onto a nitrocellulose membrane using Semidry Electro-blotter System (Galileo Bioscience, Cambridge, MA, USA). To determine glutathionylated proteins western blotting of total extract was performed under non reducing conditions. The blots were blocked with 5% defatted dry milk (Euroclone) in tris-buffered saline (TBS) containing 0.5% tween 20 for 1 h, at RT, and incubated overnight at 4 °C with appropriate dilutions of primary antibodies. Subsequently, membranes were incubated for 1h with horseradish peroxidase (HRP)-conjugated secondary antibodies. Proteins were then visualized by an enhanced chemiluminescence detection system (Euroclone). The primary antibodies used in the present study included anti-COX-2 (Cell Signaling Technology, Leiden, The Netherlands), anti-p-ERK1/2 (Cell Signaling Technology), p-NFkB (Cell signaling Technology), anti-GSH (Virogen, Watertown, MA, USA), and anti-GAPDH (EMD Millipore, Darmstadt, Germany). Affinity-purified HRP-conjugated secondary antibodies were from Sigma-Aldrich. Protein bands from western blots were quantified by densitometry using the ImageJ software, and their relative amounts were normalized to the levels of housekeeping proteins serving as internal loading controls [23].

2.10. ROS Measurement

ROS levels were evaluated as previously reported [24]. 3.5 or 2.5 × 10^3 cell/well (HT29, HCT116 or HUVEC) were seeded in 96-multiwell plates and, after adherence, were maintained for 18 h in medium without phenol red (0.1% serum) with different concentrations of the extract (10 and 50 µM) and then exposed to IL1β (10 ng/mL) for 24 h. $DCFH_2$-DA (2,-7-dichlorodihydrofluorescein diacetate) (Invitrogen, Milan, Italy) was added (10 µM, 1h) and intracellular levels of ROS were evaluated photometrically with a microplate reader (excitation/emission 495/527) (EnVision, PerkinElmer).

2.11. Statistical Analysis

Data were generated from three independent experiments and expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t test for unpaired data; differences in dataset with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Chemical Composition of FV Extracts

Chemical analyses of FV extracts obtained from four different samples provided by different growers (Table 1) were performed. To investigate the polyphenolic fraction of FV extracts and to identify the main chemical constituents, a high-performance liquid chromatography-diode array detection (HPLC-DAD) analysis was carried out. FV was found to be rich in polyphenols (Table 2), in accordance with other published papers on the species [4].

Table 2. Chemical composition of FV extracts

Components	Quantification (mg/g)			
	#1	#2	#3	#4
Total polyphenols	0.142 ± 0.018	0.123 ± 0.011	0.129 ± 0.016	0.120 ± 0.015
Total hydroxycinnamic derivatives	0.054 ± 0.004	0.051 ± 0.003	0.046 ± 0.005	0.052 ± 0.005
Isoflavones	<0.005	<0.005	<0.005	<0.005

HPLC-DAD analysis revealed that two main polyphenols subclasses could be identified in FV extracts, namely simple phenolic acids and hydroxycinnamic derivatives. As a water extraction was performed, the prevalence of hydrophilic compounds in the extract was expected and consistent with

a previous work on common beans endemic of Southern Italy [3]. Gallic acid (Figure 2 related to #1 FV extract, Retention Time, RT = 4.41 min) and chlorogenic acid (RT = 7.94 min.) resulted the main phenolic and hydroxycinnamic acid, respectively. The other main peaks before gallic acid (RT = 3.88–4.18 min) could be assigned to phenolic acids by monitoring UV spectra for their typical λmax at 270–280 nm. Other hydroxycinnamic derivatives, different from caffeic acid, were recognized by UV spectra (λmax at 270–280 nm and 320–330 nm) and linked with the peak at 6.47 min. Flavonoids were found only in small amounts. At RT = 11.33, 11.71 and 12.40 min, the zone of the chromatogram where isoflavones are recorded, two constituents with UV spectrum similar to genistein and daidzein (λmax at 250–255 nm), but with different RTs, were found. Other flavonoids referable to used standards and their derivatives were not detected as present in concentrations below the detection limits of the method. Table 3 shows the quantification of gallic and chlorogenic acid identified in FV extracts and total phenolic and hydroxycinnamic derivatives, expressed as gallic and chlorogenic acid, respectively.

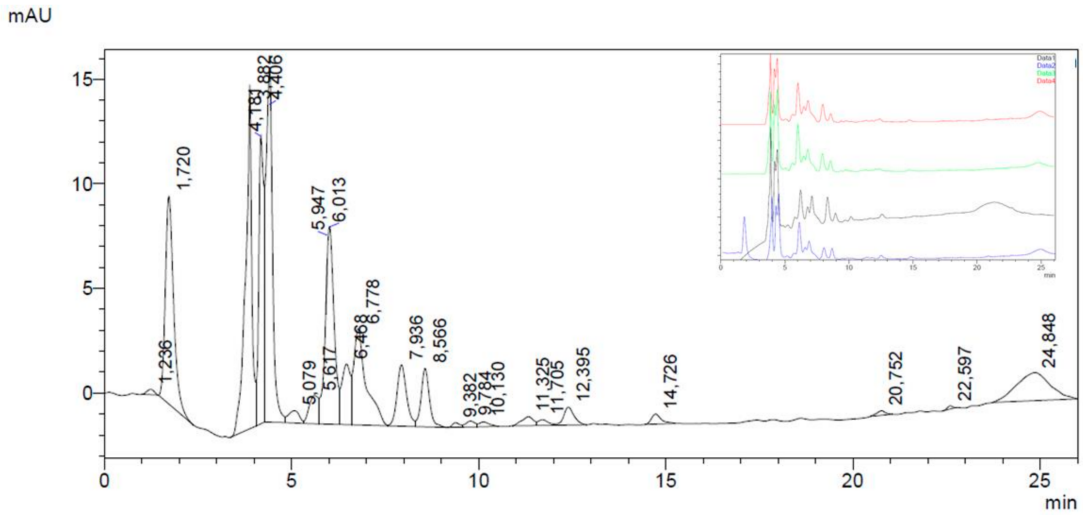


Figure 2. HPLC-DAD profile of polyphenols present in FV extract. Chromatogram of sample #1. In the top box, chromatograms of all samples are reported: blue = sample #1, grey = sample #2, green = sample #3 and red = sample #4.

Table 3. Chemical composition of #1 FV extract.

Composition	Quantification
Total polyphenols	0.131 ± 0.016 mg/g
Total hydroxycinnamic derivatives	0.046 ± 0.004 mg/g
Gallic acid	0.052 ± 0.005 mg/g
Chlorogenic acid	0.011 ± 0.002 mg/g
Isoflavones	<0.005 mg/g
Total soluble carbohydrates	10.032 ± 0.820 mg/g
Total proteins	15.190 ± 2.020 mg/g

Due to the minimum variance in polyphenols content and a strong similarity in chromatogram profiles of the four different samples (see Table 2 and Figure 2), only the extract obtained from sample #1, available in highest amount, was further analyzed and investigated in chemical and biological tests. Table 3 summarizes the chemical composition of the selected FV extract of sample #1. In accordance with known nutritional data and literature on *P. vulgaris* (nutritiondata.com), carbohydrates represent the main class of metabolites: the concentration of soluble carbohydrates in the FV extract was shown to be 10.032 mg/g. The concentration of total proteins was 15.190 mg/g.

3.2. DPPH Test and HPLC-DAD-DPPH

As previously reported [3], extracts of common beans obtained from different Italian varieties of *P. vulgaris* possess antioxidant and antiradicalic properties. According to that, the FV extract showed antiradicalic capacity, monitored by DPPH reduction. The comparison between the HPLC-DAD chromatograms obtained before and after adding DPPH to FV extract showed that differences in peak areas related to polyphenols occur (Figure 3a), thus demonstrating that some constituents of FV extract were able to react with DPPH and underwent oxidative degradation.

Chlorogenic acid was the most degraded molecule, more than 75.0% after DPPH reaction, meaning that this hydroxycinnamic derivative primarily contributed to the antiradicalic activity of FV extract. The other main hydroxycinnamic derivative displayed a minor degradation (−39.4%); among phenolic acids, only gallic acid seemed to participate in DPPH reaction and its recorded degradation was 37.6%. Other polyphenolic constituents of FV extract did not show a significant degradation after DPPH reaction.

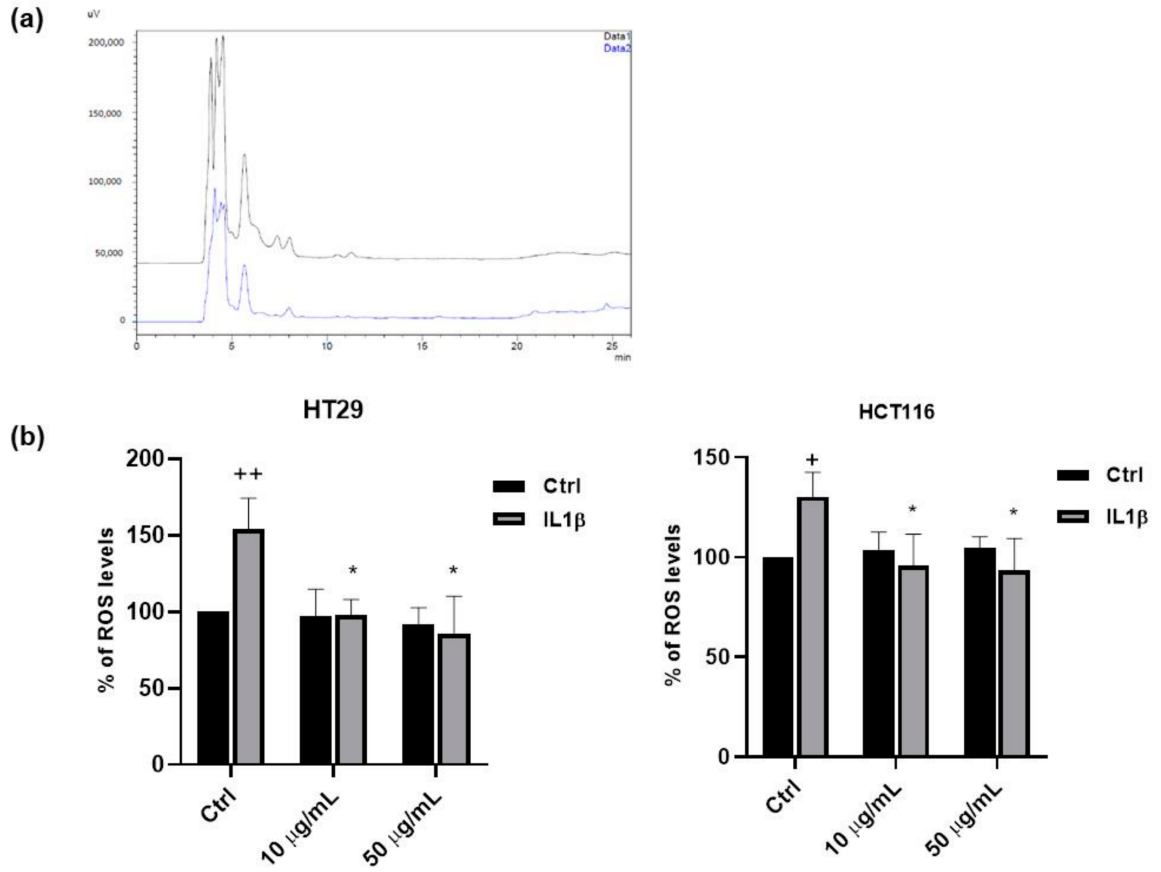


Figure 3. *Cont.*

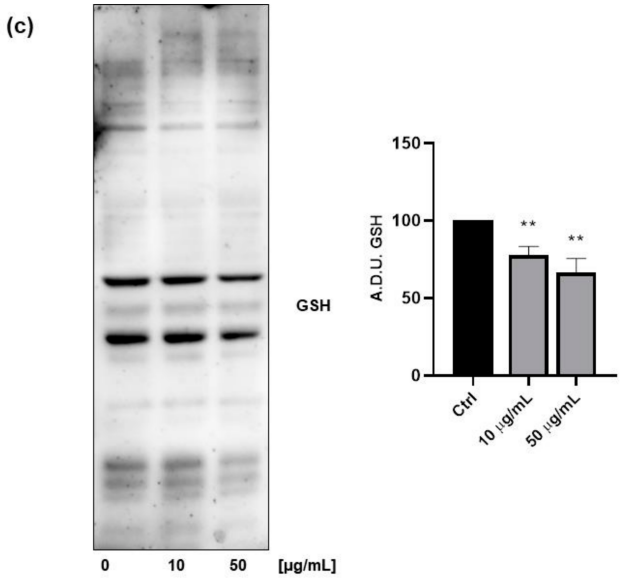


Figure 3. Antioxidant activity of FV extract. (**a**) HPLC-DAD-DPPH test. Chromatograms showed the differences in peak areas between the chromatograms obtained before (black) and after (blue) mixing DPPH and FV extract. (**b**) ROS measurement in HT29 and HCT116 cells after 18 h of exposure with the different concentrations of FV extract (10–50 µM) followed by a 24 h incubation with IL1β (10 ng/mL). Data are expressed as relative fluorescence units (++ $p < 0.01$ vs. Ctrl; + $p < 0.05$ *vs*. Ctrl; * $p < 0.05$ vs. IL1β). (**c**) Evaluation of the levels of glutathionylated proteins. HT29 cells were treated with different concentrations of FV extract (10–50 µM) for 36 h and then analyzed by western blot under non reducing conditions using an anti-GSH primary antibody. The gels are representative of three independent experiments (A.D.U.: arbitrary densitometry units). ** $p < 0.01$ vs. Ctrl.

3.3. Antioxidant Properties of FV Extract

To further analyse the antioxidant properties of FV extract, we performed in vitro experiments on different cellular models. Due to the high nutraceutical impact and to the findings that common bean consumption is associated with in vivo chemoprotective effects at the early stages of colon cancer [16] and pro-apoptotic and anti-proliferative activities in vitro [14], we selected two different cellular models of colorectal adenocarcinoma, HT29 and HCT116 cells. To mimic a pro-oxidant and pro-inflammatory milieu, we stimulated colon cancer cells with interleukin 1β (IL1β, 10 ng/mL, 48 h) in the presence and in the absence of different concentrations of FV extract (10, 50 µg/mL), and we measured ROS levels by means of $DCFH_2$-DA assay. As reported in Figure 3b, the ability of IL1β to promote ROS production was inhibited by the FV extract, at both concentrations and in both cell lines. It is well known that, in addition to triggering traditional post-translational protein modifications (including phosphorylation, acetylation, ubiquitination, etc.), ROS can directly modify cellular proteins, adding another layer of protein regulation to the proteome classified as oxidative post-translational modifications (OPTMs). In particular, ROS may cause various types of chemical modifications of proteins, including glutathionylation [25,26]. To explore the possibility that FV extract could affect these mechanisms, we treated colon cancer cells with the FV extract for 36 h and we measured the levels of glutathion–protein complexes by western blotting.

Figure 3c shows that the FV extract was able to significantly reduce the levels of total glutathionylated proteins, indicating the antioxidant activity of the FV extract.

3.4. Anti-Inflammatory Properties of FV Extract

To further explore the biological properties of the FV extract, we investigated its ability to reduce the inflammation related to cancer. It is well known that inflammation is a key component in colon cancer onset and progression and that the cyclooxygenase 2 (COX-2) pathways play a major role

in modulating cell growth, apoptosis and epithelial mesenchymal transition (EMT) [27–29]. Recent reports indicate that a direct interplay exists between inflammation and carcinogenesis. In fact, the risk of developing colon cancer is increased by chronic inflammatory diseases (such as inflammatory bowel disease), chronic infections or inflammations caused by environmental exposures. In addition, administration of COX inhibitors, such as aspirin and other non-steroidal, anti-inflammatory drugs (NSAIDs), is connected with a lower risk of developing colon cancer and its recurrence [30–32]

It has been demonstrated that IL1β, a pro-inflammatory cytokine, induces COX-2 expression in colorectal cells and that COX-2 drives colon cancer progression [33,34]. In this context, we evaluated the activity of FV extract on COX-2 expression induced by IL1β. We stimulated HT29 cells with IL1β (10 ng/mL, 48 h), in presence of 10 and 50 μg/mL of FV extract and we observed that at the higher concentration FV extract was able to strongly inhibit IL1β-induced COX-2 expression (Figure 4a). These data clearly indicate that FV extract could reduce the inflammation related to cancer.

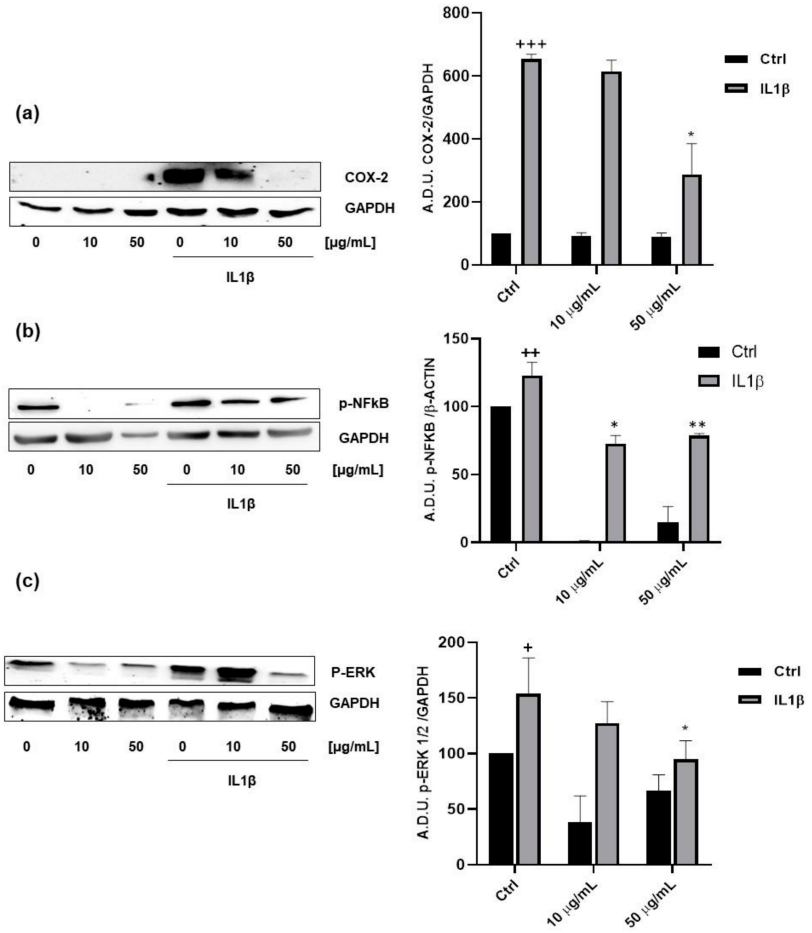


Figure 4. Anti-inflammatory activity of the FV extract. (**a**) Western blot analysis and quantification of COX-2 expression in HT29 cells after 18 h of exposure with different concentrations of FV extract (10–50 μM) followed by a 24 h incubation with IL 1β (10 ng/mL). +++ $p < 0.001$ vs. Ctrl, * $p < 0.05$ vs. IL1β. (**b**) Western blot analysis and quantification of NFκB phosphorylation in HT29 cells after exposure with FV extract (10–50 μM) followed by incubation with IL 1β (10 ng/mL). ++ $p < 0.01$ vs. Ctrl, ** $p < 0.01$ vs. IL1β, * $p < 0.05$ vs. IL1β (**c**) Western blot analysis and quantification of ERK 1/2 phosphorylation in HT29 cells. (A.D.U.: arbitrary densitometry units). + $p < 0.05$ vs. Ctrl, * $p < 0.05$ vs. IL1β. The gels showed in the figure are representative of four independent experiments.

It has been described that natural compounds, and in particular dietary polyphenols, exhibit a relevant anti-inflammatory activity linked to the inhibition of NFκB, MAPK and iNOS signalling [35]. In this light, we investigated the activity of the FV extract on NFkB and MAPK activation. By analysing

the phosphorylation levels of NFkB and ERK1/2, we showed that the FV extract was able to reduce both p-NFkB and p-ERK1/2 levels in IL1β-stimulated colon cancer cells (Figure 4b,c), indicating once again an anti-inflammatory activity of FV components.

In order to further investigate the activity of FV extract, we measured the ROS levels and COX-2 expression in non-cancer cells. To this aim we used Human Umbilical Vein Endothelial Cells (HUVEC) as a model in which it is well known the activity of IL1β in inducing ROS production and COX-2 expression [36]. As reported in Figure 5, while IL1β treatment induced both ROS production and COX-2 expression, FV extract was shown to be inactive (Figure 5a,b), indicating a potential selective role of FV extract on cancer cells.

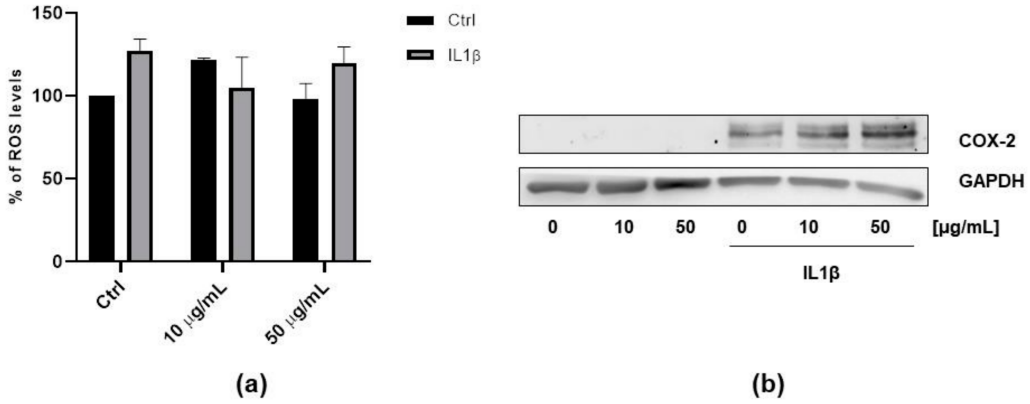


Figure 5. FV extract activity in endothelial cells. (**a**) ROS measurement in HUVEC cells after 18 h of exposure with the different concentrations of FV extract (10–50 μM) followed by a 24 h incubation with IL1β (10 ng/mL). Data are expressed as relative fluorescence units. (**b**) Western blot analysis of COX-2 expression in HUVEC cells after 18 h of exposure with different concentrations of FV extract (10–50 μM) followed by a 24 h incubation with IL 1β (10 ng/mL).

3.5. Anti-Proliferative Activity of FV Extract

In order to evaluate whether the FV extract could modulate tumor progression, we studied the proliferation of colon cancer cells by MTT test. HT29 and HCT116 were treated with 10 ng/mL IL1β for 48 h in the presence of increasing concentrations of the FV extract (from 5 to 100 μg/mL). According to previous studies conducted on white beans [3], the FV extract did not modify cancer cells' growth in basal conditions. However, when the cells were exposed to an inflammatory milieu (IL1β), FV extract was able to reduce cell growth in a concentration-dependent manner (Figure 6a,b). Taken together, these data strongly support a potential biological activity of the FV extract, especially in inflammatory conditions.

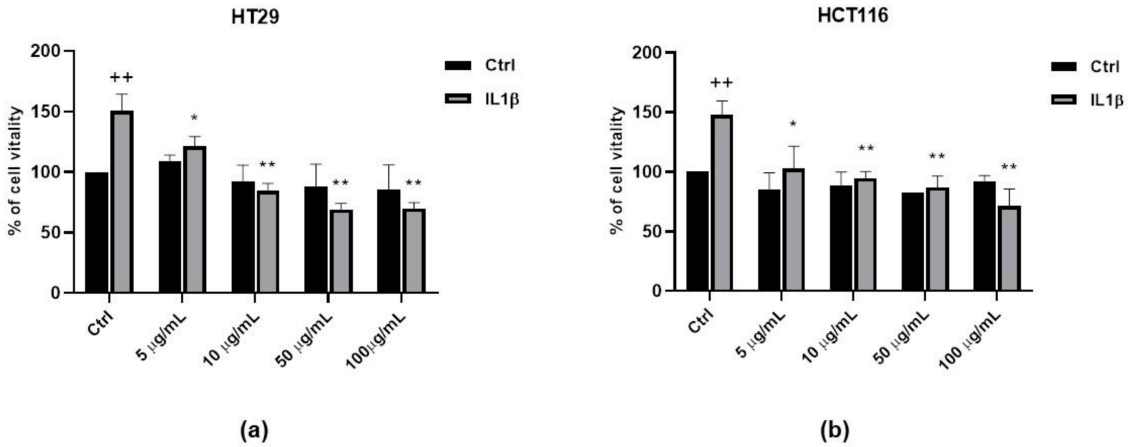


Figure 6. FV extract inhibits colon cancer cell growth. Cell proliferation induced by IL 1β (10 ng/mL) in the presence or absence of FV extract (5, 10, 50 and 100 μg/mL) was measured by MTT assay. HT29 (**a**) and HCT116 (**b**) cells were exposed to IL 1β for 48h. Data are reported as % of cell viability and are the means of 4 experiments run in triplicate. ++ $p < 0.01$ vs. Ctrl, * $p < 0.05$ and ** $p < 0.01$ vs. IL 1β.

4. Discussion

The identification of new species and varieties of vegetables is of fundamental importance to produce safe food and to obtain nutritional supplements and functional foods.

Food biodiversity has a high impact on public health and can offer more nutritious and healthier foods for rural and urban consumers, and provides opportunities to generate income and contribute to sustainable rural development. Therefore, there is an urgent need to develop and promote strategies for sustainable diets, mainstreaming biodiversity and nutrition as a common path, promoting nutrition-sensitive development and food-based approaches to solving health problems.

Phaseolus vulgaris is cultivated all over the world and is the most important edible legume for direct consumption. However, beans are more than a foodstuff since they are rich in many compounds with biological activity.

In the present work, we analysed for the first time the "Fagiola di Venanzio" (FV), a recently identified Italian variety of *P. vulgaris,* with the aim to characterize its chemical composition and potential biological activities. We initially analyzed four different samples of beans cultivated in four different areas within Murlo, a small municipality in the south of Tuscany. The areas of origin of the four samples are quite close to each other, but they differ in terms of soil composition and exposure. Nevertheless, the four samples did not demonstrate significant differences in chemical composition. We found that FV beans are rich in polyphenols including phenolic acids and hydroxycinnamic derivatives, as previously reported for other Italian varieties of *P. vulgaris.*

Since the beneficial effects of polyphenols on human health are expressed mainly through the reduction in oxidative stress, we evaluated the antioxidant activity of FV extract in in vitro assays and we reported that FV possesses antiradicalic activity *in vitro* and reduces ROS production promoted by interleukin 1β in two different models of colon cancer cells. In addition, the DPPH assay suggested that, in FV extract, chlorogenic acid primarily acts as a radicalic neutralizer. We also showed that the FV extract is able to reduce the expression of the inflammatory marker COX-2, the activation of NFkB and ERK1/2 MAPK and colon cancer cell growth promoted by IL1β, a well known pro-inflammatory cytokine. Interestingly, while FV extract did not inhibit cell growth in basal conditions, according to the results reported in the previous literature for white beans [3], we observed a strong reduction in proliferation induced by IL1β. It is well known that IL1β mimics inflammatory conditions that occur in intestinal tract and that may drive the development of such inflammatory chronic diseases as cancer, or such inflammatory bowel diseases (IBD) as ulcerative colitis and Crohn's disease. Our results, showing that FV extract is able to reverse the effects of IL1β on colon cancer cells, strongly suggest that FV may play an important role in preventing the alteration of molecular processes characterizing

the inflammatory microenvironment that leads to cancer and chronic diseases. In this scenario, these data suggest that bean consumption may be helpful in the prevention or treatment of inflammatory diseases of intestinal tract, and outline once again the importance of nutrition for human health.

5. Conclusions

In this work, we characterized the Fagiola di Venanzio, recently recognized and classified as a novel variety of *P. vulgaris* but never studied before, by determining its chemical composition and potential biological activity. This initial work on FV has been preparatory to a comparative work aimed at determining differences and similarities with other varieties of *P. vulgaris*, which is currently in progress. FV is cultivated in a very restricted area of a small municipality in the South of Tuscany, by a very restricted number of growers, and for this reason is in danger of extinction due to the spread of commercial varieties. As FV appears to be a promising source of bioactive compounds and rich in nutraceutical properties, more in-depth studies aimed to further elucidate its biological and nutraceutical potential will be fundamental to safeguard and promote this specific variety of *Phaseolus*, and to better understand the implications of its consumption for public health.

Author Contributions: Conceptualization, L.T., F.F., and M.B.; Methodology, J.E., and G.M.; Validation, E.M.; Formal Analysis, J.E. and M.B.; Investigation, J.E., G.M. and M.B.; Data Curation, J.E. and M.B.; Writing—Original Draft Preparation, F.F. and M.B.; Writing—Review and Editing, L.T. and E.M.; Supervision, L.T.; Project Administration, L.T.; Funding Acquisition, L.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by MIUR (Progetto Dipartimento di Eccellenza 2018-2022) to L.T., F.F., J.E. and G.M.

Acknowledgments: The authors are grateful to the Murlo Municipality and Associazione Culturale di Murlo for fundamental collaboration and support. We would like to thank Nicola Ulivieri, Società Agricola Aiellino, Burresi's family and Azienda Agricola Podere Vignali for providing beans of Fagiola di Venanzio.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Teodoro, A.J. Bioactive compounds of food: Their role in the prevention and treatment of diseases. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 3765986. [CrossRef] [PubMed]
2. Suárez-Martínez, S.E.; Ferriz-Martínez, R.A.; Campos-Vega, R.; Elton-Puente, J.E.; Carbot, K.d.; García-Gasca, T. Bean seeds: Leading nutraceutical source for human health. *CYTA J. Food* **2016**, *14*, 131–137. [CrossRef]
3. Ombra, M.N.; D'Acierno, A.; Nazzaro, F.; Riccardi, R.; Spigno, P.; Zaccardelli, M.; Pane, C.; Maione, M.; Fratianni, F. Phenolic Composition and Antioxidant and Antiproliferative Activities of the Extracts of Twelve Common Bean (*Phaseolus vulgaris* L.) Endemic Ecotypes of Southern Italy before and after Cooking. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 1398298. [CrossRef] [PubMed]
4. Ganesan, K.; Xu, B. Polyphenol-rich dry common beans (*Phaseolus vulgaris* L.) and their health benefits. *Int. J. Mol. Sci.* **2017**, *18*, 2331. [CrossRef]
5. Yang, Q.Q.; Gan, R.Y.; Ge, Y.Y.; Zhang, D.; Corke, H. Polyphenols in Common Beans (*Phaseolus vulgaris* L.): Chemistry, Analysis, and Factors Affecting Composition. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 1518–1539. [CrossRef]
6. Hayat, I.; Ahmad, A.; Masud, T.; Ahmed, A.; Bashir, S. Nutritional and Health Perspectives of Beans (*Phaseolus vulgaris* L.): An Overview. *Crit. Rev. Food Sci. Nutr.* **2014**, *54*, 580–592. [CrossRef]
7. Thompson, S.V.; Winham, D.M.; Hutchins, A.M. Bean and rice meals reduce postprandial glycemic response in adults with type 2 diabetes: A cross-over study. *Nutr. J.* **2012**, *11*, 1. [CrossRef]
8. Correa, P. Epidemiological Correlations Between Diet and Cancer Frequency. *Cancer Res.* **1981**, *41*, 3685–3689.
9. Kolonel, L.N.; Hankin, J.H.; Whittemore, A.S.; Wu, A.H.; Gallagher, R.P.; Wilkens, L.R.; John, E.M.; Howe, G.R.; Dreon, D.M.; West, D.W.; et al. Vegetables, fruits, legumes and prostate cancer: A multiethnic case-control study. *Cancer Epidemiol. Biomark. Prev.* **2000**, *9*, 795–804.

10. Hughes, J.S.; Ganthavorn, C.; Wilson-Sanders, S. Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *J. Nutr.* **1997**, *127*, 2328–2333. [CrossRef]
11. Hangen, L.; Bennink, M.R. Consumption of black beans and navy beans (Phaseolus vulgaris) reduced azoxymethane-induced colon cancer in rats. *Nutr. Cancer* **2002**, *44*, 60–65. [CrossRef] [PubMed]
12. Feregrino-Pérez, A.A.; Berumen, L.C.; García-Alcocer, G.; Guevara-Gonzalez, R.G.; Ramos-Gomez, M.; Reynoso-Camacho, R.; Acosta-Gallegos, J.A.; Loarca-Piña, G. Composition and chemopreventive effect of polysaccharides from common beans (*Phaseolus vulgaris* L.) on azoxymethane-induced colon cancer. *J. Agric. Food Chem.* **2008**, *56*, 8737–8744. [CrossRef] [PubMed]
13. Chan, Y.S.; Xia, L.; Ng, T.B. White kidney bean lectin exerts anti-proliferative and apoptotic effects on cancer cells. *Int. J. Biol. Macromol.* **2016**, *85*, 335–345. [CrossRef]
14. Moreno-Jiménez, M.R.; López-Barraza, R.; Cervantes-Cardoza, V.; Pérez-Ramírez, I.F.; Reyna-Rojas, J.A.; Gallegos-Infante, J.A.; Estrella, I.; Rojas-Contreras, J.A.; González-Laredo, R.F.; Rocha-Guzmán, N.E. Mechanisms associated to apoptosis of cancer cells by phenolic extracts from two canned common beans varieties (*Phaseolus vulgaris* L.). *J. Food Biochem.* **2019**, *43*, 1–10. [CrossRef] [PubMed]
15. Feregrino-Perez, A.A.N.; Piñol-Felis, C.; Gomez-Arbones, X.; Guevara-González, R.G.; Campos-Vega, R.; Acosta-Gallegos, J.; Loarca-Piña, G. A non-digestible fraction of the common bean (*Phaseolus vulgaris* L.) induces cell cycle arrest and apoptosis during early carcinogenesis. *Plant Foods Hum. Nutr.* **2014**, *69*, 248–254. [CrossRef]
16. Vergara-Castañeda, H.A.; Guevara-González, R.G.; Ramos-Gómez, M.; Reynoso-Camacho, R.; Guzmán-Maldonado, H.; Feregrino-Pérez, A.A.; Oomah, B.D.; Loarca-Piña, G. Non-digestible fraction of cooked bean (*Phaseolus vulgaris* L.) cultivar Bayo Madero suppresses colonic aberrant crypt foci in azoxymethane-induced rats. *Food Funct.* **2010**, *1*, 294–300. [CrossRef]
17. Mensack, M.M.; McGinley, J.N.; Thompson, H.J. Metabolomic analysis of the effects of edible dry beans (*Phaseolus vulgaris* L.) on tissue lipid metabolism and carcinogenesis in rats. *Br. J. Nutr.* **2012**, *108*, S155–S165. [CrossRef]
18. Thompson, M.D.; Mensack, M.M.; Jiang, W.; Zhu, Z.; Lewis, M.R.; McGinley, J.N.; Brick, M.A.; Thompson, H.J. Cell signaling pathways associated with a reduction in mammary cancer burden by dietary common bean (*Phaseolus vulgaris* L.). *Carcinogenesis* **2012**, *33*, 226–232. [CrossRef]
19. Campos-Vega, R.; García-Gasca, T.; Guevara-Gonzalez, R.; Ramos-Gomez, M.; Oomah, B.D.; Loarca-Piña, G. Human gut flora-fermented nondigestible fraction from cooked bean (*Phaseolus vulgaris* L.) modifies protein expression associated with apoptosis, cell cycle arrest, and proliferation in human adenocarcinoma colon cancer cells. *J. Agric. Food Chem.* **2012**, *60*, 12443–12450. [CrossRef]
20. Biagi, M.; Noto, D.; Corsini, M.; Baini, G.; Cerretani, D.; Cappellucci, G.; Moretti, E. Antioxidant Effect of the Castanea sativa Mill. Leaf Extract on Oxidative Stress Induced upon Human Spermatozoa. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 8926075. [CrossRef]
21. Dubois, M.; Gilles, K.; Hamilton, J.K.; Rebers, P.A.; Smith, F. A colorimetric method for the determination of sugars. *Nature* **1951**, *168*, 167. [CrossRef] [PubMed]
22. Finetti, F.; Terzuoli, E.; Donnini, S.; Uva, M.; Ziche, M.; Morbidelli, L. Monitoring endothelial and tissue responses to cobalt ferrite Nanoparticles and Hybrid Hydrogels. *PLoS ONE* **2016**, *11*, e0168727. [CrossRef] [PubMed]
23. Finetti, F.; Moglia, A.; Schiavo, I.; Donnini, S.; Berta, G.N.; di Scipio, F.; Perrelli, A.; Fornelli, C.; Trabalzini, L.; Retta, S.F. Yeast-derived recombinant avenanthramides inhibit proliferation, migration and epithelial mesenchymal transition of colon cancer cells. *Nutrients* **2018**, *10*, 1159. [CrossRef] [PubMed]
24. Finetti, F.; Schiavo, I.; Ercoli, J.; Zotta, A.; Boda, E.; Retta, S.F.; Trabalzini, L. KRIT1 loss-mediated upregulation of NOX1 in stromal cells promotes paracrine pro-angiogenic responses. *Cell. Signal.* **2020**, *68*, 109527. [CrossRef]
25. Dalle-Donne, I.; Rossi, R.; Colombo, G.; Giustarini, D.; Milzani, A. Protein S-glutathionylation: A regulatory device from bacteria to humans. *Trends Biochem. Sci.* **2009**, *34*, 85–96. [CrossRef]
26. Hsieh, H.J.; Liu, C.A.; Huang, B.; Tseng, A.H.; Wang, D.L. Shear-induced endothelial mechanotransduction: The interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. *J. Biomed. Sci.* **2014**, *21*, 3. [CrossRef]
27. Goradel, N.H.; Najafi, M.; Salehi, E.; Farhood, B.; Mortezaee, K. Cyclooxygenase-2 in cancer: A review. *J. Cell. Physiol.* **2019**, *234*, 5683–5699. [CrossRef]

28. Donnini, S.; Finetti, F.; Terzuoli, E.; Giachetti, A.; Ĩiguez, M.A.; Hanaka, H.; Fresno, M.; Rådmark, O.; Ziche, M. EGFR signaling upregulates expression of microsomal prostaglandin e synthase-1 in cancer cells leading to enhanced tumorigenicity. *Oncogene* **2012**, *31*, 3457–3466. [CrossRef]
29. Wang, Y.; Wang, W.; Sanidad, K.Z.; Shih, P.A.; Zhao, X.; Zhang, G. Eicosanoid signaling in carcinogenesis of colorectal cancer. *Cancer Metastasis Rev.* **2018**, *37*, 257–267. [CrossRef]
30. Piotrowski, I.; Kulcenty, K.; Suchorska, W. Interplay between inflammation and cancer. *Rep. Pract. Oncol. Radiother.* **2020**, *25*, 422–427. [CrossRef]
31. Nasef, N.A.; Mehta, S. Role of inflammation in pathophysiology of colonic disease: An update. *Int. J. Mol. Sci.* **2020**, *21*, 4748. [CrossRef]
32. Patrignani, P.; Patrono, C. Aspirin and Cancer. *J. Am. Coll. Cardiol.* **2016**, *68*, 967–976. [CrossRef] [PubMed]
33. Liu, W.; Reinmuth, N.; Stoeltzing, O.; Parikh, A.A.; Tellez, C.; Williams, S.; Jung, Y.D.; Fan, F.; Takeda, A.; Akagi, M.; et al. Cyclooxygenase-2 is up-regulated by interleukin-1β in human colorectal cancer cells via multiple signaling pathways. *Cancer Res.* **2003**, *63*, 3632–3636. [PubMed]
34. Gupta, R.A.; Dubois, R.N. Colorectal Cancer Prevention of Cyclooxygenase-2. *Nat. Rev. Cancer* **2001**, *1*, 11–21. [CrossRef]
35. Owczarek, K.; Lewandowska, U. The Impact of Dietary Polyphenols on COX-2 Expression in Colorectal Cancer. *Nutr. Cancer* **2017**, *69*, 1105–1118. [CrossRef]
36. Monti, M.; Terzuoli, E.; Ziche, M.; Morbidelli, L. H_2S dependent and independent anti-inflammatory activity of zofenoprilat in cells of the vascular wall. *Pharmacol. Res.* **2016**, *113*, 426–437. [CrossRef]

biology

MDPI

Review

Prostaglandin E2 and Cancer: Insight into Tumor Progression and Immunity

Federica Finetti [1,*,†], Cristina Travelli [2,†], Jasmine Ercoli [1], Giorgia Colombo [3], Erica Buoso [2] and Lorenza Trabalzini [1,*]

1 Department of Biotechnology, Chemistry and Pharmacy, University of Siena, 53100 Siena, Italy; ercoli3@student.unisi.it
2 Department of Pharmaceutical Sciences, University of Pavia, 27100 Pavia, Italy; cristina.travelli@unipv.it (C.T.); erica.buoso@unipv.it (E.B.)
3 Department of Pharmaceutical Sciences, University of Piemonte Orientale, 28100 Novara, Italy; giorgia.colombo@uniupo.it
* Correspondence: finetti2@unisi.it (F.F.); lorenza.trabalzini@unisi.it (L.T.)
† These authors contributed equally to the work.

Received: 30 October 2020; Accepted: 29 November 2020; Published: 1 December 2020

Simple Summary: Inflammation is assessed as a hallmark of cancer and it is now widely recognized that there exists a direct causal link between inflammation and tumors. Among the inflammatory mediators, prostaglandin E2 (PGE2), the major product of cyclooxygenases (COXs), plays a pivotal role in tumor progression. Numerous pieces of evidence suggest that drugs, such as aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit PGE2 production, may exert a protective effect against tumor initiation and may play a role during tumor progression. In fact, a number of studies suggest that PGE2 increases tumor growth and invasion, reduces apoptosis, increases metastasis and angiogenesis, and suppresses antitumor immunity. In this review, we describe the current knowledge on the pro-tumoral activity of PGE2 focusing on its role in cancer progression and in the regulation of the tumor microenvironment.

Abstract: The involvement of inflammation in cancer progression has been the subject of research for many years. Inflammatory milieu and immune response are associated with cancer progression and recurrence. In different types of tumors, growth and metastatic phenotype characterized by the epithelial mesenchymal transition (EMT) process, stemness, and angiogenesis, are increasingly associated with intrinsic or extrinsic inflammation. Among the inflammatory mediators, prostaglandin E2 (PGE2) supports epithelial tumor aggressiveness by several mechanisms, including growth promotion, escape from apoptosis, transactivation of tyrosine kinase growth factor receptors, and induction of angiogenesis. Moreover, PGE2 is an important player in the tumor microenvironment, where it suppresses antitumor immunity and regulates tumor immune evasion, leading to increased tumoral progression. In this review, we describe the current knowledge on the pro-tumoral activity of PGE2 focusing on its role in cancer progression and in the regulation of the tumor microenvironment.

Keywords: prostaglandin E2; tumor inflammation; angiogenesis; metastasis; EP receptor; tumor microenvironment; cancer-related inflammation; immunosuppression

1. Introduction

The involvement of inflammation in cancer progression was first described in 1863 by Rudolf Virchow. He observed that infiltrated immune cells reflect the place where cancer lesions appear in the

inflamed tissue and hypothesized that chronic inflammation is a condition that predisposes one to cancer development. Most recent observations revealed that there is a direct causal link between inflammation and cancer: it is estimated that primary infections (such as *Helicobacter pylori*, hepatitis B and C viruses) and inflammatory responses are linked to 7% to 30% of cancer deaths worldwide [1,2]. Consistently, epidemiological observation showed that daily aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) reduced deaths due to several common cancers [3–7], indicating that cyclooxygenase (COX) inhibition and the reduction of its main metabolic product, prostaglandin E2 (PGE2), may prevent solid-organ cancers.

2. Prostaglandin E2 Biosynthesis and Functions

Several biological activities have been attributed to PGE2 both in physiological and pathological conditions. In physiological processes, PGE2 regulates fever, kidney function, pain, mucosal integrity, blood vessel homeostasis, and inflammation. In pathological conditions, as in cancer, PGE2 is produced by cancerous stromal cells and enhances tumor cell proliferation and survival, promotes angiogenesis, and induces metastasis. During tumor progression, PGE2 exerts its activity through ligation with four E-type prostanoid (EP) receptors 1–4 (EP 1–4), by acting on releasing cells (autocrine mechanism) and neighboring cells (paracrine mechanism) [8].

PGE2 belongs to the prostanoid family of lipids, a subclass of eicosanoids produced by oxidation of 20-carbon essential fatty acids that are localized within cell membranes. Prostanoids are synthesized by sequential actions of different and highly specific enzymes. Their synthesis is initiated after the release of arachidonic acid (AA) from membrane lipids by phospholipases A2 (PLA2) family members.

Membrane-released AA is rapidly oxidized into the unstable metabolite, prostaglandin G2 (PGG2), which is subsequently reduced to PGH2. Both steps are sequentially catalyzed by the COX enzymes. COX-1 is constitutively expressed at basal levels in many cells, generating low levels of PGs that are cytoprotective and maintain body homeostasis. In contrast, COX-2 is normally absent in most cells and it is induced in response to a variety of stimuli including growth factors and cytokines [9–11]. Once synthesized, PGH2 is rapidly converted into PGE2 by three distinct terminal synthases (Figure 1).

These synthases include microsomal PGE synthase-1 (mPGES-1), mPGES-2, and cytosolic PGE synthase (cPGES) and are tightly regulated under various conditions. mPGES-1 is frequently induced concomitantly with COX-2 by several proinflammatory stimuli to generate a transient increase of PGE2 levels [12,13]. The levels of PGE2 can also be regulated by its metabolic turnover. The activation of two key catabolic enzymes, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and 15-ketoprostaglandin-13-reductase (13-PGR), can essentially eliminate the biological activity of PGE2 [14].

Following synthesis, the physiological activity of PGE2 is mediated by the activation of downstream signaling cascades via seven transmembrane G-protein coupled receptors (GPCRs), referred as the EP receptors. The EP receptor family consists of four isoforms (EP1-4) coupled to heterotrimeric G proteins containing stimulatory (GαS) or inhibitory (Gαi) subunits that can modulate the levels of Ca2+, cyclic AMP (cAMP), and inositol phosphate, thus, activating divergent downstream signaling pathways [15] (Figure 1). The interaction between PGE2 and EP receptors is dependent on cell and tissue type and location. On cancer cells, the expression and localization of EP receptors may be variable and may influence cell response to PGE2. Specific effects depending on the activation of the different EP receptor subtype have been described, including EP1-dependent tumor cell migration and invasion, EP2-induced angiogenesis and suppression of the anti-tumor immune response, and, finally, EP4-related tumor cell migration and metastasis. The role of the EP3 receptor has yet to be clarified [15].

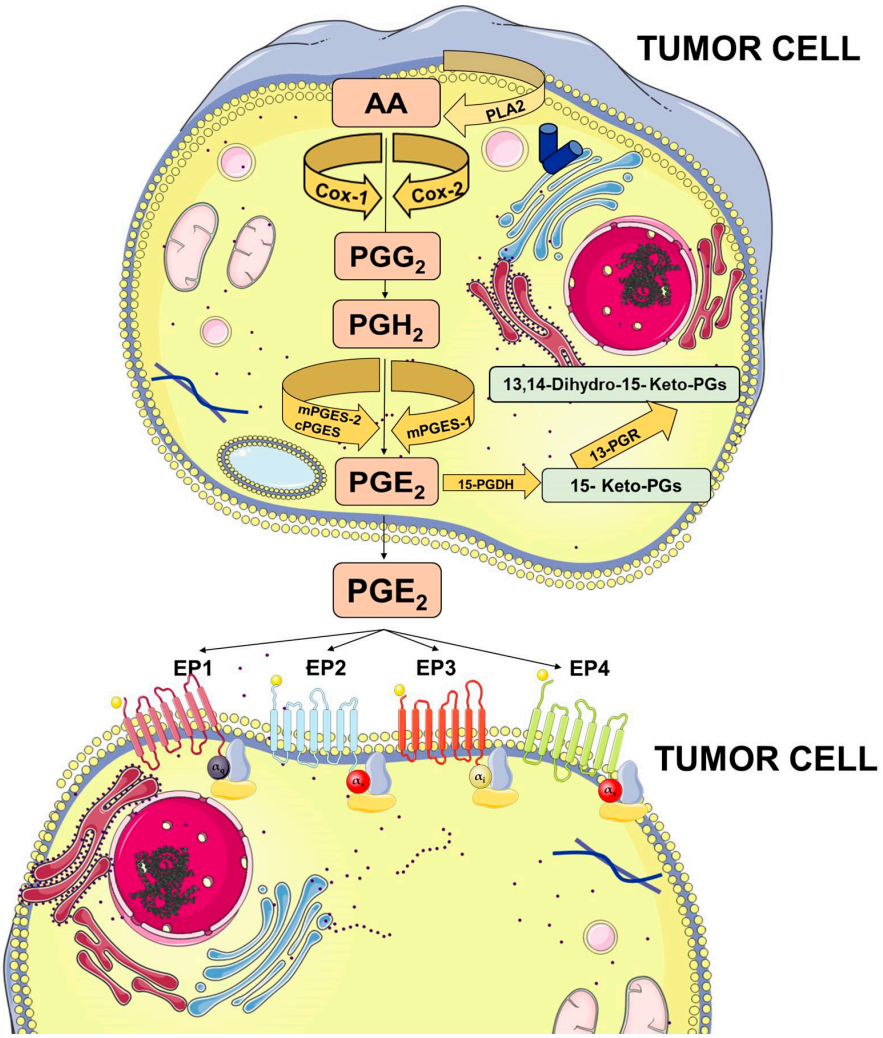


Figure 1. Prostaglandin E2 (PGE2) biosynthesis. Arachidonic acid (AA) is a polyunsaturated fatty acid that constitutes the phospholipid domain of most cell membranes and is released from the cellular membranes by cytoplasmic phospholipases A2 (PLA2). Free AA can be metabolized to PGE2 through the cyclooxygenase (COX) pathway. In this pathway, the key step is the enzymatic conversion of AA to the intermediate prostaglandin G2 (PGG2), which is then reduced to the intermediate PGH2 by the peroxidase activity of COX. PGH2 is sequentially metabolized to PGE2 by specific PGE synthases (cytosolic PGE synthase (cPGES), microsomal PGE synthase-1 (mPGES1), and mPGES2). PGE2 exerts its effects through ligation with four G-protein-coupled receptors (GPCRs), EP1–EP4. Each E-type prostanoid (EP) receptor couples to distinct signaling pathways. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

3. Prostaglandin E2 and Cancer

PGE2 is the most abundant prostaglandin that has been found in various human malignancies. Inhibition of its production by unselective COX inhibitors such as aspirin or other NSAIDs have been associated with a reduced risk of colon, breast, lung, prostate, and other solid cancers and their recurrence (see Table 1) [16–18]. Furthermore, it has been reported that COX-2 and/or mPGES-1 are constitutively expressed in several cancers, including non-small cell lung cancer [19,20], colorectal cancer [21,22], breast cancer [23,24], prostate cancer [25,26], melanoma [27], and hepatocellular carcinoma [28,29], suggesting that the COX-2/mPGES-1/PGE2 pathway is linked to the neoplastic progression. To outline the importance of this pathway, several efforts have been conducted to develop

selective COX-2 inhibitors lacking the side effects of unselective COX inhibitors and provided with specific activities [30]. Among these, celecoxib showed antitumoral activity (see Table 1), being able to reduce the risk of colon, breast, prostate, and lung cancer [31]. However, long-term consumption of COX-2 inhibitors presents important side effects [30] and their use as anticancer agents has to be better investigated.

Table 1. Studies related to the role of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) in cancer.

NSAID	Cancer	References
Aspirin	Colorectal cancer	[3,18,32–45]
	Breast cancer	[17,41,46–51]
	Head and neck squamous cell carcinoma	[52–60]
	Lung cancer	[3,37,38,43,61]
	Prostate cancer	[3,62]
	Ovarian cancer	[37,63–65]
	Gastric cancer	[3,37,66–70]
Coxib	Breast cancer	[31,71–76]
	Colon cancer	[31,76–79]
	Prostate cancer	[31,76]
	Lung cancer	[31,76,80–83]

Several animal models have been developed and used to assess the efficacy of COX inhibition in vivo by using low-dose aspirin or NSAIDs, and to elucidate the molecular mechanisms of PGE2-induced tumor progression.

A large number of studies have been conducted to reveal the role of PGE2 in colon cancer carcinogenesis and progression. For example, regression of small intestinal adenomas in ApcMin/+ mice induced by NSAIDs is blocked by PGE2 treatment [84]. Moreover, the adenoma-preventive activity of celecoxib is abrogated in 15-PGDH KO mice that possess increased endogenous PGE2 levels [85]. Genetic deletion of 15-PGDH increases endogenous PGE2 levels and promotes colon tumor growth also in ApcMin/+ and azoxymethane (AOM) mouse models [86]. In addition, intraperitoneal PGE2 treatment boosts the AOM-induced colon tumor incidence and multiplicity and significantly increases proliferative index and reduces apoptotic index [87]. Likewise, it has been demonstrated in three different mouse models of intestinal tumorigenesis that chronic low-dose aspirin prevents tumor formation and that the aspirin antitumor effect is most pronounced when treatment is started before tumor initiation [88].

To further outline the important role of PGE2 in colon carcinogenesis, different studies showed that mPGES-1 deletion suppresses the development of intestinal tumors in ApcMin/+ and AOM models [89,90]. Moreover, Sasaki et al. have reported that mPGES-1 deletion reduces AOM-induced colon polyp and aberrant crypt foci (ACF) formation [91]. Similar results were reported in animal models of breast cancer [92].

Additional information regarding the role of PGE2 signaling has been obtained from xenograft animal models, in which reduction of PGE2 production results in decreased tumor growth [15,26,93,94].

4. Molecular Mechanisms Linking PGE2 and Tumor Progression

Inflammation has been reported as one of the hallmarks of cancer, due to its capability to supply bioactive molecules that promote cancer proliferation, invasion, and metastasis; limit cell apoptosis; and induce the angiogenic process [95]. Molecular and cellular inflammatory pathways that sustain cancer progression have been identified and are reported as intrinsic and extrinsic

inflammatory pathways [96,97]. In the intrinsic pathway, genetic events that are able to induce neoplastic transformation promote the expression of inflammatory mediators that guide the construction of an inflammatory microenvironment and sustain tumor progression processes. Instead, the extrinsic pathway is driven by inflammatory leukocytes and soluble mediators that establish inflammatory conditions that increase cancer risk [96]. The upregulation of the COX-2/mPGES-1/PGE2 axis appears to be fundamental for both processes. In this review, we will provide a detailed overview of the principal molecular pathways activated by PGE2 (Figure 2).

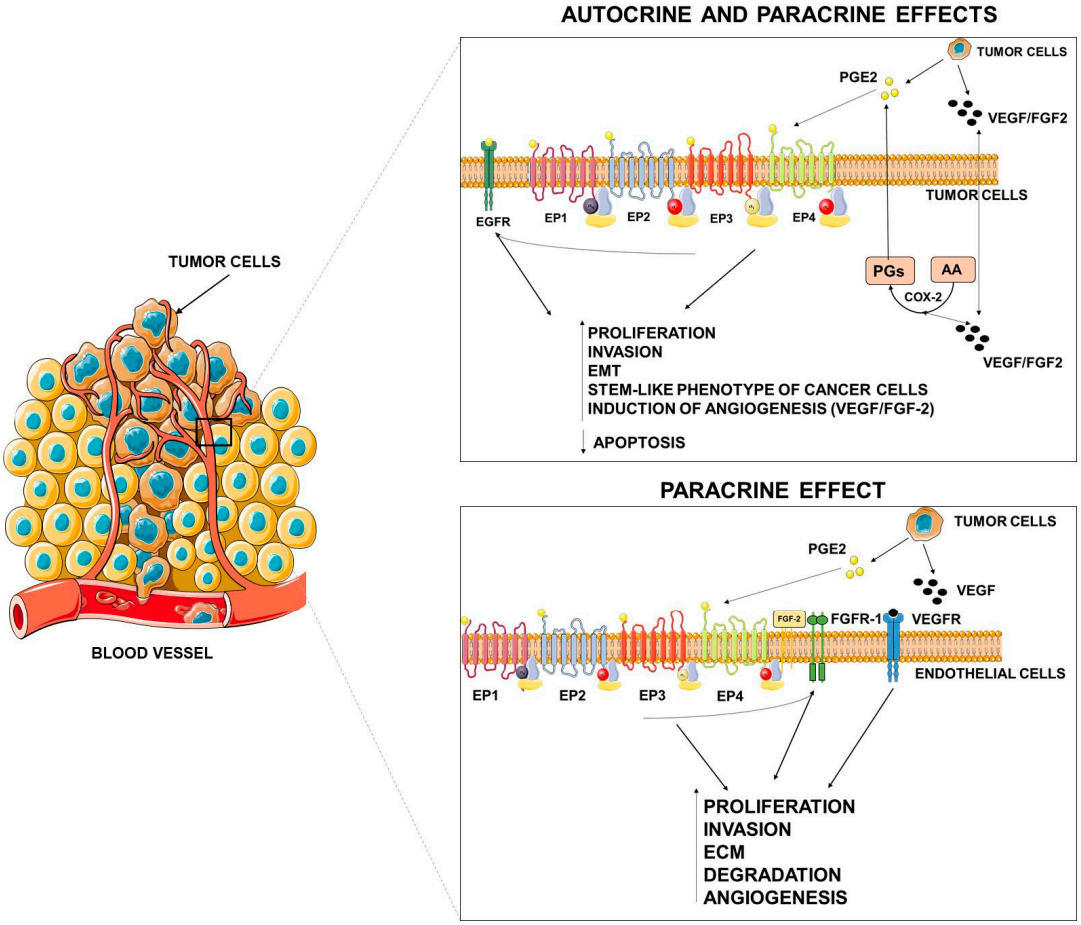


Figure 2. Autocrine and paracrine effects of PGE2. PGE2, released by tumor cells, may elicit autocrine and paracrine effects on tumor or stromal cells either by specific activation of its receptors or by tyrosine kinase (TK) receptor transactivation. VEGF = vascular endothelial growth factor, FGF = fibroblast growth factor, EMT = epithelial mesenchymal transition, ECM = extracellular matrix. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

As occurs in extrinsic inflammation, an increasingly large body of evidence indicates that PGE2 promotes tumor growth through autocrine and paracrine mechanisms by activating EP receptors present both in cancer cells and in stromal cells and by transactivating growth factor receptor tyrosine kinases (RTKs) frequently upregulated in cancer cells [15,98]. EP receptors activate a range of intracellular signaling pathways that mediate the effects of PGE2 on cell functions.

The EP1 receptor is coupled to the Gαq protein subunit that is linked to phosphoinositide-PLC activation. This signaling leads to an increase of intracellular Ca2+ and PKC activation that finally induce gene transcription through the activation of nuclear factor of activated T cells (NFAT), nuclear factor-kappaB (NFκB), and the MAPK pathways [99].

The involvement of the EP1 receptor in cancer has been documented by several reports showing that EP1 signaling inhibition obtained by selective antagonists or by KO mice reduce the number of azoxymethane-induced aberrant crypt foci formation [100–102]. Consistently, EP1 receptor antagonists inhibit polyp formation in APC KO mice [103], decrease the number of UVB-induced skin tumors in mice [104], and diminish the incidence of tongue cancer in rats [105].

Instead, both the EP2 and EP4 receptors are linked to Gαs proteins that activate adenylate cyclase and generate cAMP which in turn activates the protein kinase A (PKA) pathway [106]. EP2 and EP4 receptors also mediate glycogen synthase kinase 3β (GSK3β)-β catenin pathways. PGE2 is able to promote colon cancer cell growth through EP2 signaling that involves the activation of phosphoinositide 3-kinase (PI3K) and the protein kinase Akt and the inactivation and release of GSK3β from its complex with axin, thereby activating the β-catenin signaling pathway [107]. Moreover, in similar models of colon cancer cells, PGE2 induces CREB phosphorylation by the PKA pathway and regulates β-catenin and cyclin D1 cellular localization via EP2 and EP4 receptors [108]. In addition, Akt pathway activation promoted by EP4 receptor results in the activation of mTORC1 [109]. Several other studies have shown that EP2 and EP4 induce the activation of multiple signaling cascades that are associated with squamous cell carcinoma (SCC) [110,111]; human hepatocellular carcinoma (HCC) [112]; glioma [113]; prostate [114], bladder [115], endometrial [116], and breast cancer [117] cell growth.

By using EP2 receptor KO mice, it has been demonstrated that the EP2 receptor controls the progression of lung [118], skin [119,120], and breast [121,122] cancer. Moreover, genetic ablation of the EP2 or EP4 receptors also decrease both the size and number of intestinal polyps in APC mice [123,124]. Moreover, the inhibition of the EP4 receptor with either AH23848 or ONO-AE3-208 reduces metastasis in breast cancer models [125].

The human EP3 gene consists of ten exons and nine introns, encoding at least eight distinct EP3 splice variants [126]. This could partially explain the different effects of EP3 in different tumors. The EP3 receptor is able to couple with a number of G-protein subunits including Gi, Gs, and G13, thus, stimulating or inhibiting adenylyl cyclase (AC), as well as stimulating Ca2+ mobilization, possibly via PLC. The major EP3 splice variant is thought to be coupled to an inhibitory (Gi) protein. Therefore, the primary outcome of EP3 receptor signaling is inhibition of AC and activation of the Ras/Raf and MAPK signaling pathway [106,127,128]. EP3 has been reported to mediate the carcinogenesis in numerous tumors with conflicting effects [15].

In addition to the canonical activation of EP receptors, PGE2 has been shown to promote cancer progression through the interaction with oncogenic signals, including epidermal growth factor (EGF) and its receptor (EGFR) [26,110,129–132]. In particular, PGE2 and EGF/EGFR may cooperate to promote growth, invasion, epithelial mesenchymal transition (EMT), and a stem-like phenotype of cancer cells [110,130,131,133]. It was also demonstrated that EGF induces the upregulation of mPGES-1 expression and PGE2 production, and that mPGES-1 inhibition significantly reduces the EGF-mediated tumorigenicity [26,93,134], indicating a cooperative loop between the two signaling pathways.

PGE2 and Angiogenesis

Numerous in vitro and in vivo studies have indicated that the COX-2/mPGES-1/PGE2 pathway plays a pivotal role in promoting the angiogenic switch in cancer (Figure 2) [11]. COX-2/mPGES-1 overexpression in tumor cells has been reported to promote the production of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2) [11].

In cancer cells, PGE2 stimulates VEGF expression through several mechanisms including the activation of the hypoxia inducible factor-1α (HIF-1α) [135,136] or the cAMP signaling pathway [137]. Similarly, mPGES-1/PGE2 drive the angiogenic phenotype of cancer cells through the Dicer downregulation, and the subsequent PGE2-mediated downregulation of miR-15a and miR-186 that appear to be specifically related to VEGF production [138]. PGE2 can also induce VEGF secretion through the transactivation of EGFR mediated by EP2 and EP4 receptors [139–141]. Similarly, PGE2 can

activate EP2-mediated FGF2 expression in endometrial adenocarcinoma cells through the activation of PKA, Src, EGFR, and ERK1/2 signaling [142].

Intriguingly, VEGF and FGF2 induce COX-2 expression and PGE2 production in endothelial cells. These data suggest that the effects of PGE2 on regulation of VEGF and FGF2 are probably amplified through a positive feedback loop [143,144].

In animal models, the EP2 receptor that controls the number and size of intestinal polyps in Apc (Delta 716) mice increases cellular cAMP and stimulates the expression of COX-2 and VEGF in the polyp stroma, demonstrating that PGE2 induction of VEGF is important for tumor growth in vivo [123]. Similar data have been reported also for breast cancer [122].

In addition to direct effects elicited by cancer-produced PGE2 on cancer and endothelial cells (autocrine/paracrine actions), several observations reported that PGE2 produced by stromal cells and present in the microenvironment may affect tumor niche to promote tumor progression. PGE2 signaling appears as a node of chronic inflammation which shapes the tumor microenvironment [145,146].

For example, PGE2 signaling in stromal cells also contributes to angiogenesis. A host PGE2–EP2 signal is required for tumor angiogenesis by enhancing endothelial cell motility and vascular hyperpermeability, as demonstrated in a mouse model of breast cancer in which the deletion of the EP2 receptor impairs tumor angiogenesis [147]. Similarly, the host PGE2–EP3 signal is a prerequisite for tumor-stromal angiogenesis that was markedly suppressed in mice EP3-/- and was linked to a reduced expression of VEGF [148].

PGE2 can also directly act on endothelial cells to promote a pro-angiogenic phenotype through the activation of fibroblast growth factor receptor-1 (FGFR1) signaling [149]. Indeed, it was reported that PGE2 synergizes with fibroblast growth factor 2 (FGF2) and induces the endothelial autocrine/paracrine FGF2/FGFR1 signaling through upregulation of FGF2 expression and its mobilization from the extracellular matrix [150]. In this context, PGE2 acts as a primer of the angiogenic switch by promoting the activation of the FGF2/FGFR1 system by multiple mechanisms [149,150].

Given the pivotal role played by the microenvironment in tumor metastasis, some observations are available on the possible molecular processes by which the primary tumor controls the pre-metastatic niche in the secondary site prior to the formation of metastasis. Liu et al. have determined that primary tumor-derived VEGF tumors can alter the lung microenvironment through PGE2 production in a model of breast cancer, leading circulating tumor cells to localize preferentially in these regions [151]. Similarly, PGE2 appears to regulate tumor metastasis in non-small-cell lung cancer (NSCLC) [152], colorectal cancer [153,154], breast cancer [155], and hypopharyngeal squamous cell carcinoma [156].

5. PGE2 and Its Roles in the Regulation of the Tumor Microenvironment

It is now well accepted that a typical hallmark of tumors is an important immunosuppressive niche composed of suppressive immune cells which play a major role in the regulation of tumor progression, supporting cancer stemness and helping the tumor in the metastatic process. In the last decade, therapeutic strategies targeting these cellular populations have been developed and found to be beneficial in clinical practice (e.g., anti-PD1 and anti-CTLA4 therapies) [157]. The immune subsets that orchestrate tumor immunosuppression include myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), dendritic cells (DCs), natural killer (NK) T cells, and regulatory T-cells (Tregs). Herein, as depicted in Figure 3, the major roles of PGE2 in the fine regulation of these cells are summarized, with the aim of immunoregulation.

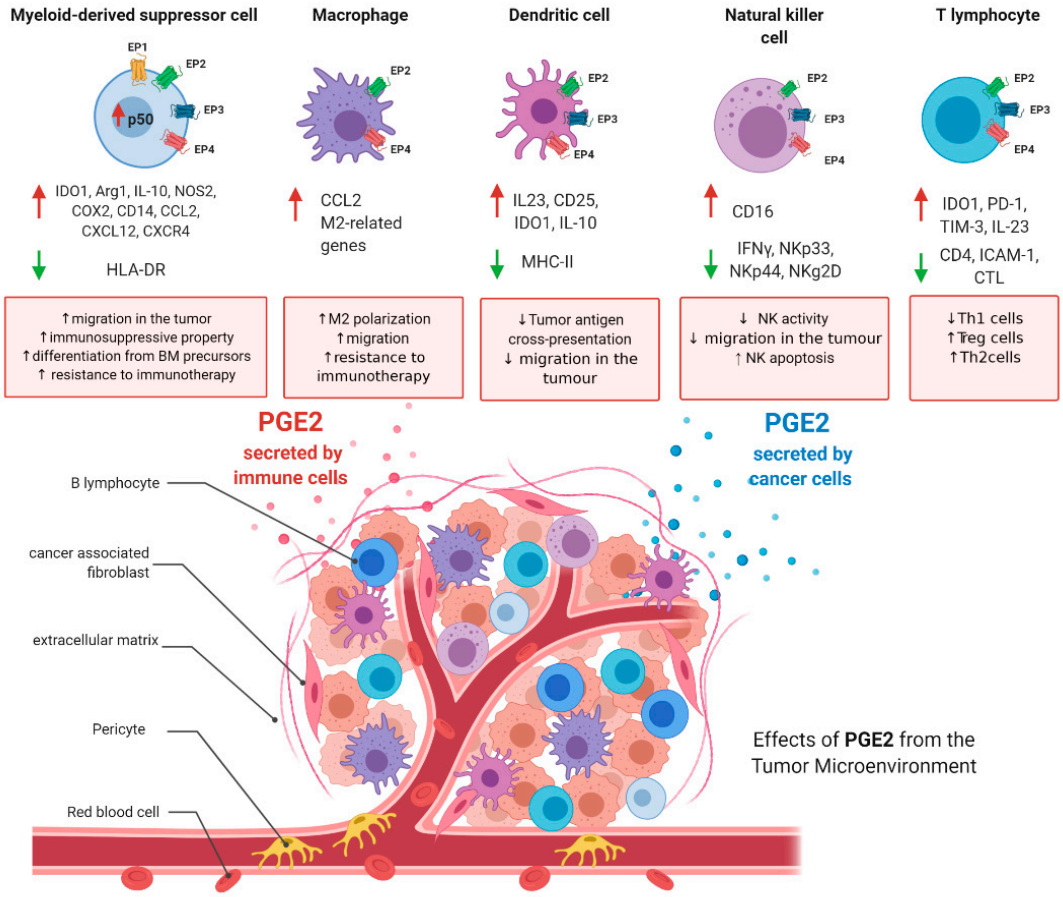


Figure 3. PGE2 roles in immunosuppressive tumoral niche. PGE2 levels are increased in the tumoral microenvironment. PGE2 is secreted by cancer cells and immune cells. In the figure are depicted the main functions of PGE2 in myeloid-derived suppressor cells, tumor-associated macrophages, dendritic cells, natural killer (NK) cells, and T-cells. Figure has been generated using BioRender.

5.1. PGE2 and Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells, enriched in cancers, with a crucial role in the maintenance of an immunosuppressive microenvironment [158]. Physiologically, in the bone marrow (BM), the hematopoietic stem cells (HSCs) give rise to immature myeloid cells (IMCs) which differentiate into mature myeloid cells. In the context of cancer, the tumor microenvironment releases mediators/cytokines that determine the development of IMCs to MDSCs. Furthermore, cancer cells secrete different types of chemokines that signal MDSC migration to tumors. MDSCs are schematically subdivided into monocytic-like cells (M-MDSCs) and granulocytic-like cells (PMN-MDSCs). These two subsets of MDSCs share the same ability to suppress adaptive immunity via different mechanisms of action. In brief, MDSCs suppress anti-tumor immunity through multiple mechanisms, including (i) the release of factors able to stimulate Treg activation and differentiation; (ii) the blockade of migration of naïve-T cells to lymphoid organs and the formation of effector T cells; (iii) production of high levels of reactive oxygen species (ROS) and nitric oxide (NO), up-regulating arginase 1 (ARG-1), and inducible nitric oxide synthase/ nucleotide-binding oligomerization domain-containing protein 2 (iNOS/NOD2). Of note, the induction of MDSCs' development can be triggered by several factors including interleukin-1β (IL-1β), interleukin 10 (IL-10), macrophage-colony stimulating factor (M-CSF), interleukin-6 (IL-6), metabolic enzymes (e.g., NAMPT, indoleamine 2,3-dioxygenase (IDO1)), toll like receptor (TLR)-ligands, or VEGF [158–160]. Importantly, PGE2 has emerged as an important player in MDSCs' activation and migration.

5.1.1. PGE2 and MDSCs Differentiation

The primary role of PGE2 in MDSCs was first characterized by Sinha and collaborators demonstrating how PGE2 controls MDSC differentiation in a preclinical model of mammary carcinoma. They highlighted that PGE2 induces a 3-fold increase in levels of suppressive $Gr1^{+}CD11b^{+}$ cells in vitro, therefore, prompting the idea that PGE2 is involved in the differentiation of MDSCs from bone marrow progenitors. Of note, by analyzing spleens of 4T1 tumor-bearing mice, they found that MDSCs express all the four receptors for PGE2 (i.e., EP1, EP2, EP3, EP4) [161]. Importantly, using butaprost (EP2 agonist), AH6809 (EP1 and EP2 antagonist), and AH23848 (EP4 antagonist), they clearly demonstrated that PGE2 mediates MDSC differentiation through either the EP1, EP2, and/or EP4 receptors. Using EP2 KO mice they also proved that the ablation of EP2 receptor retards mammary carcinoma growth by reducing MDSC levels, indicating that PGE2 mediates MDSC accumulation also in vivo [161]. Importantly, the treatment with the COX-2 inhibitors (SC58236 and SC58236) delays primary carcinoma tumor burden with an important reduction of MDSCs levels.

In accordance with these findings, it is known that tumor exosomes are taken up by bone marrow myeloid cells and contribute to the development of MDSCs [162]. Furthermore, Xiang et al. demonstrated that the promotion of tumor growth by MDSCs is dependent on tumoral exosomes which are enriched of PGE2. Of note, antibodies against exosomal PGE2 block the activity of these exosomes on MDSCs' induction and attenuate tumor growth in vivo [163]. In agreement with this study, the direct role of PGE2 in the activation of monocyte differentiation has been proven since the stimulation of monocytes with PGE2 induces several immunosuppressive factors leading to MDSC phenotype (e.g., IDO1, Arg1, IL-10, NOS2). Moreover, PGE2 per se induces COX-2 leading to autocrine production of endogenous PGE2 in MDSCs [164]. Obermayer and collaborators discovered a positive feedback loop between PGE2 and COX-2 in the differentiation of monocytes, redirecting to functional differentiation of DCs toward monocytic MDSCs, a mechanism undertaken by cancer cells to locally produce suppressive MDSCs. Alone, the administration in vitro of PGE2 redirects the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 to the differentiation of dendritic cells toward MDSCs [164,165]. The ability of EP2 and EP4 agonists, but not of EP3/1 agonists, to reproduce PGE2-induced effects demonstrates the key role of EP2 and EP4 in mediating the MDSCs. Of note, Rodriguez-Ubreva et al. studied the comparison between MDSC and DC DNA methylomes and revealed extensive demethylation with specific gains of DNA methylation. In particular, they found that PGE2 leads to highly specific and DNA methyltransferase 3 alpha (DNMT3A)-dependent hypermethylation and downregulation of a subset of myeloid-associated genes. Although the mechanisms by which MDSCs suppress T-cells have been extensively studied, less is known about MDSCs' regulation of NK-cell activity [166]. Direct involvement of PGE2 during the initiation and maintenance of NK-cell suppression by MDSCs has been proven by Mao et al. They demonstrated that PGE2 increases the immature status of monocytes. Indeed, PGE2 increases the expression of CD14, while the expression of HLA-DR is reduced [167]. They found that the co-culture of MDSCs isolated from melanoma patients with NK cells induces interferon (IFN)γ release and IL2-activated NK cells. The stimulation with PGE2 suppresses NK-cell responses, TGFβ production, and the activation of the EP2 or EP4 receptors [167].

5.1.2. PGE2 and MDSCs Recruitment

Several reports have suggested a key role of PGE2 in the recruitment of MDSCs in the tumor site. It is well known that the CXCR4-CXCL12 axis is the key pathway involved in the recruitment of MDSCs into the tumor. Obermajer et al. have observed a correlation between the expression of COX-2, CXCL12 concentrations, and the production of PGE2 in ovarian cancer. Importantly, the secretion of CXCL12 in ovarian cancer ascites was hindered by COX-2 inhibitors [164]. They also found that PGE2 induces both CXCL12 production in the ovarian cancer environment and CXCR4 expression in MDSC precursors, therefore, promoting the attraction and retention of MDSCs in the tumor microenvironment [164]. Similarly, in mouse models of glioma, it has been demonstrated

that treatment with COX-2 inhibitors reduces the PGE2 production which results in a decrease of MDSC-attracting chemokine CCL2 in the tumor. These data suggest that COX-2 blockade hampers the development of MDSCs and their accumulation in the tumor with a CCL2-dependent mechanism [168]. The role of COX-2 and PGE2 in the recruitment of MDSCs has been validated also in lung cancer. The authors demonstrated that cytotoxic T lymphocytes (CTLs) induce tumor cells to secrete PGE2 as a mediator of MDSCs' recruitment into the tumor. Indeed, both the COX-2 inhibitor and the PGE2 neutralizing antibody (2B5) reduce the number of MDSCs recruited by tumor cells [169]. All these data suggest that the chemoattraction of MDSCs by tumor cells is mediated first by the up-regulation of COX-2 and, secondly, by the PGE2 synthesis.

5.1.3. PGE2 and MDSCs Activation

Within the tumors, MDSCs are activated and are able to suppress T-cell proliferation, resulting in the impairment of the anti-tumor immunity. Activated MDSCs increase the expression of iNOS/NOD2, arginase, and IDO1 leading to a significant increase of ROS and NO levels and finally to a decrease of T-cells' proliferation [170].

Ochoa et al. have described for the first time that PGE2 secreted into the tumor microenvironment is a signal to increase arginase expression in MDSCs, resulting in the block of T-cells' proliferation [171]. In addition, the Kiessling group found that the axis COX-2/PGE2 is at the basis of the mechanism by which melanoma cells arrest monocytes in an immature stage, similar to the stage of tumor MDSCs, associated with the ability to impair T-cell functions. Indeed, they found that treatment of melanoma-patient-derived monocytes with PGE2 leads to the loss of T-cell proliferation and IFNγ production. Of note, the suppression is mediated by the direct activation of monocytes despite T cells, because PGE2 per se does not affect T-cell proliferation in this cellular model [172].

Recently, Porta et al. clearly demonstrated that tumor-derived PGE2 leads to the nuclear accumulation of p50-NF-κB in M-MDSCs, resulting in a reduction of tumor necrosis factor (TNF)α expression and diverting their response to IFNγ. In agreement, the treatment with butaprost (PGE2 receptor antagonist) is able to reprogram M-MDSCs towards a "NOS2^{low}/TNFα^{high} phenotype", thus, restoring the antitumor activity of IFNγ [160]. An important role of MDSCs in the tumor is also to control cancer stem-like cells. It has been demonstrated both in vitro and in vivo that MDSCs induced by tumor-derived G-CSF enhance the stemness of cervical cancer cells by producing PGE2. Moreover, PGE2 produced by MDSC increases tumor PD-L1 expression in ovarian cancer cells, in an mTOR-dependent mechanism. Accordingly, the treatment with celecoxib inhibits the induction of cancer stem-like cells and enhances the efficacy of common chemotherapy (e.g. cisplatin). Importantly, the authors have translated these pieces of evidence for humans and they found a positive correlation between MDSCs, PGE2, and CSCs in clinical samples [173,174].

5.1.4. PGE2, MDSCs, and Resistance to Immunotherapy

Although immunotherapies have been found effective in several types of cancers (e.g., anti–PD-L1 and anti-PD-1 therapies), a portion of patients still fail to respond to therapy, and there is an urgent need to discover the mechanism at the basis of this resistance. In this setting, Prima et al. found that co-culture of BM cells with bladder tumor cells promotes strong expression of PD-L1 in M-MDSCs [161]. Tumor-infiltrating PD-L1^{+} cells isolated from tumor-bearing mice express high levels of PGE2 synthase 1 (mPGES1) and COX-2. Interestingly, the treatment with mPGES1/COX-2 inhibitors reduces the PD-L1 expression in MDSCs, suggesting that reprogramming PGE2 metabolism in a tumor microenvironment provides an opportunity to reduce immune suppression and may increase the efficacy of immunotherapy. Moreover, Hou et al. demonstrated that tumoral PGE2 is a key mediator of immunotherapy resistance (including virotherapy). Indeed, they have highlighted a decrease of Treg, but not of the number of MDSCs, after viral therapy, in different tumor models (e.g., 4T1, MC38), suggesting that MDSCs can block the immunotherapeutic activity of these vectors. The authors found a correlation between elevated levels of PGE2 and the suppressive profiles of tumors as well as with

high levels of PMN-MDSCs, and using viral vectors engineered to target PGE2, they were able to alter the cancer immune status [175].

5.2. PGE2 and Tumor-Associated Macrophages

Macrophages display marked plasticity since they can polarize to M1- (pro-inflammatory) and M2-like (anti-inflammatory) phenotypes by various stimulations (e.g., IFNγ, LPS, IL-4) in inflamed tissues or in cancers. In a tumoral setting, it is well accepted that macrophages acquire the M2-like phenotype and become the so-called tumor-associated macrophages (TAMs). They could promote migration, metastatization, and angiogenesis and suppress regulatory immune circuits.

5.2.1. Role of PGE2 in Controlling Macrophage Polarization

The key role of PGE2 in the regulation of macrophage polarization has been extensively described. Indeed, it has been demonstrated that human peripheral blood mononuclear cells (PBMCs) cultured in the presence of GM-CSF and IL-4 normally differentiate into DCs, while the stimulation with PGE2 suppresses the formation of DCs and shifts the differentiation into the M2-like macrophages. The mechanism at the basis of this switch in the differentiation process seems to be dependent on the activation of EP4, since the treatment with E7046 (EP4 antagonist) is able to revert the differentiation to M2-like macrophages [176–179]. These data prove that PGE2 promotes M2 polarization, therefore, contributing to the enhancement of the anti-tumor immunity. In accordance, macrophages usually express EP2 and EP4, but not EP1 or EP3. The treatment with EP4 antagonist (E7046) of ApcMin/+ mice determines the change of TAM phenotype from M2 to M1 polarization, suggesting that EP4 is essential for PGE2-dependent M2 polarization [177]. Similarly, it has been proved in a glioblastoma model that PGE2, secreted by glioblastoma cancer stem cells, is able to convert macrophages into M2-TAMs [176]. Finally, as already reported in the MDSC section, the activation of COX-2/mPGES1/PGE2 pathways promotes PD-L1 expression by TAMs [180].

5.2.2. Role of PGE2 in Controlling Macrophage Migration

PGE2 controls also macrophage migration into the tumor. Indeed, it has been demonstrated that PGE2 leads to CCL2 up-regulation, a key chemokine involved in macrophage recruitment in the tumor. Moreover, in a mouse model of gastric cancer, the treatment with EP4 antagonist reduces the recruitment of M2-like macrophages and consequently the tumoral growth [179].

5.3. PGE2 and Dendritic Cells

Dendritic cells (DCs) are able to exert different activities including being sentinels of the immune system, checking continuously the immune niche. DCs are not effector cells against pathogens, but they control adaptive immunity, presenting the foreign antigens [181]. All DCs originate from a macrophage/dendritic cell progenitor (MDP) present in the bone marrow which further differentiates itself into the monocyte/macrophage lineage or to the common dendritic cell progenitor (CDP). CDP in the bone marrow differentiates in turn to both plasmacytoid DC (pDC) and pre-DC progenitors. pDCs go to the bloodstream as mature functional cells. Therefore, pre-DCs migrate through the vascular system to their final location in the tissues or lymph nodes, where they differentiate into conventional DCs (cDCs) [182,183]. Apart from antigen presentation, DCs deliver co-stimulatory signals and produce cytokines which are necessary for instructing appropriate effector or regulatory T-cell responses. Antigen-presenting cells, as dendritic cells, are able to guide immune response to tumor antigens. Circulating DC levels and activity are reduced in cancer patients as compared with healthy subjects, correlating with the severity of disease.

5.3.1. PGE2 and DC Differentiation

Notably, a key role of PGE in controlling DC differentiation has been highlighted by different authors. Indeed, in tumor models of colon cancers, PGE2 promotes tumor growth by suppressing DC differentiation from bone marrow progenitors [79]. Furthermore, PGE2 inhibits the antigen presentation ability of BM-derived DCs by reduction of MHC II expression and upregulation of IL-10 through EP2 and EP4 [79]. PGE2 has also been shown to switch the function of DCs from induction of immunity to T-cell tolerance via upregulation of CD25 and indoleamine 2,3-dioxygenase (IDO1). Furthermore, PGE2 unbalances the IL-12/IL-23 axis using EP2 and EP4 receptors in favor of IL-23 which brings an increase of the number of Th17 cells in vitro [79].

Importantly, Ogawa et al. have reported that COX-2-derived PGE2 is essential for the formation of a premetastatic niche and lymph node metastasis (LNM). Using a murine model of Lewis lung carcinoma (LLC), they have shown COX-2 overexpression in cDCs. The administration of a COX-2 inhibitor, plus a stromal cell-derived factor 1 (SDF-1) antagonist and a CXCR4 neutralizing antibody, determine the reduction of LNM. Moreover, LNM is reduced in KO mice for EP3, suggesting that the effect of PGE2 on DCs could be mediated by the activation of EP3, despite other receptors. Indeed, compared with WT $CD11c^+$ DCs, injection of EP3-deficient $CD11c^+$ DCs dramatically reduces accumulation of SDF-1^+CD11c^+ DCs in regional lymph nodes (LNs) and lymph nodes metastasis (LNM) in LLC-injected mice, showing a COX-2/EP3-dependent signaling [184].

5.3.2. PGE2 and DC Migration

Migration and storing of cDCs to lymphoid organs are essential for T-cell-induced response against tumors. However, tumor niche might let some tumor cells escape from the immune response by reducing DC migration. A key role of PGE2 in this setting has been proven. The medium of murine prostate cancer cells inhibits migration of BM-DCs and splenic cDCs through the activation of CC chemokine receptor-7 (CCR7) ligand CCL19 in vitro, and migration to draining lymph nodes in vivo [185]. However, the treatment with PGE2 rescues this impairment of DC migration with upregulation of CCR7 and inhibition of LXRα. Moreover, in prostate-cancer-bearing mice, PGE2 treatment inhibits tumor growth and induces more tumor-infiltrating T cells and CD11c dendritic cells in tumor niche [185].

5.4. PGE2 and Natural Killer Cells

Natural killer (NK) cells are innate immune lymphocytes, with a role in the regulation of innate and adaptive immune response which primarily function to lyse tumor cells. NK cells targeting tumor-mediated mechanisms include granule exocytosis, death receptor-mediated killing, and interferon (IFN)-γ release [186]. Activation receptors are NK group 2, member D (NKG2D) and natural cytotoxicity receptors NKp44, NKp46, and NKp30. NK cells produce perforin and granzyme B to penetrate into target cells and bring them to death. Activated NK cells also secrete IFN-γ to stimulate other immune cell types and activate an immune response [186]. Various types of tumor cells express ligands that are recognized by NK cells and stimulate their cytotoxic activity. NKG2D binds major histocompatibility complex class I polypeptide-related sequence A/B expressed on the surface of cancer cells, proliferating cell nuclear antigen binds to NKp44, while B7-H6 molecule is recognized by NKp30. Usually, ligands are more expressed in tumor cells than in normal cells [187].

PGE2 and NK Activity

The general idea is that NK cells could reject human tumors, influencing clinical outcome. In 1992, Fulton et al. have firstly evaluated that PGE2 receptor, without distinguishing the isoform, is overexpressed in metastatic murine mammary tumor, and PGE2 itself is able to induce NK activity inhibition [188]. Of note, NK cells express EP2, EP3, and EP4, but not EP1 [189]. Several manuscripts, and several years later, it has been highlighted the efficacy of NK to target cancer cells, whose activity is punctually suppressed by PGE2 production, usually secreted by tumoral cells. Suppressed NK cell

activity has been found in human colorectal cancer and it is an important prognostic factor for the development of metastases [79]. Similarly, tumor-infiltrating NK cell levels are associated with an improved rate of survival in gastric cancer, negatively correlating with COX-2 levels, and enhancing lung metastases in rats [79]. Moreover, modulation of EP4 receptor signaling mediates the effects of PGE2 on the promotion of breast cancer metastasis and suppression of NK cell function in a murine model of metastatic breast cancer, suggesting that EP4 may be crucial for the activation of NK by PGE2. In this case, PGE2 suppresses NK cell function via multiple mechanisms: (i) downregulating NK receptors via a cAMP/PKA pathway, (ii) inhibiting production of IFN-γ by NK cells and IL-12–induced or IL-18–induced IFN-γ expression in NK cells via EP2 receptor, and (iii) inhibiting NK cell proliferation and inducing apoptosis [79]. PGE2 inhibits the killing of target cells by NK cells activated through NCR, CD16, or NKG2D. Moreover, the percentage of $CD107a^+$ NK cells is significantly inhibited by increasing doses of PGE2 in a dose-dependent manner [189]. Both melanoma and hepatocellular carcinoma cells are able to inhibit the expression of NK receptors that trigger their immune function, including NKp30, NKp44, and NKG2D, with the impairment of NK-cell-mediated cytolytic activity against melanoma cells, through IDO1 and PGE2 expression [190,191]. Moreover, Park et al. have also highlighted that thyroid cancer cells suppress the cytolytic activity of NK cells through PGE2 secretion, downregulating NKp44 and NKp30 receptors. They have described that PGE2 and COX-2 are over-expressed in anaplastic thyroid cancer cells [192] and that also cancer-associated fibroblasts (CAFs) are responsible for PGE2 production [193]. Several NK functions, such as lysis, migration, and cytokine production, are compromised in tumor-bearing (injected with 66.1 cells) mice, producing PGE2. Indeed, PGE2 interferes with the potential of NK cells to migrate, exerting cytotoxic activity, and secreting IFNγ, with a mechanism dependent on the activation of EP2 and EP4 receptors. Importantly, NK cells are susceptible to inhibition after the treatment with EP4 and EP2 agonists compared to NK cells from healthy mice. Holt et al. have reported that an EP4 antagonist (frondoside A) inhibits breast tumor metastasis in an NK-dependent manner and protects IFNγ production by NK cells from PGE2 mediated suppression [194,195]. Similarly, the EP4 antagonist AH23848 reduces the ability of tumor cells to colonize the lungs or to spontaneously metastasize from the mammary gland. Of note, metastasis inhibition is lost in mice lacking either functional NK cells or interferon-γ [196]. Lastly, unique molecule EP4 antagonist (RQ-15986) is able to reduce tumoral mass in a syngeneic murine model of metastatic breast cancer. NK-cell functions are markedly depressed in mice bearing murine mammary tumor 66.1 or 410.4 cells due to the actions of PGE2 on NK cell EP4 receptors [197]. Taken together, all these reports clearly demonstrate that the activation of the EP4 receptor is essential for the PGE2 activation of NK cells.

Another emerging role of NKs is that they are able to crosstalk with both T cells and dendritic cells. The cytokine- and chemokine-producing capacity, T-cell polarization, migration, and stimulatory functions of DCs are finely regulated by activated NK cells. NK functions require close interactions with activated DCs. Cell-membrane-associated molecules and soluble mediators, including cytokines and prostaglandins, contribute to the bidirectional crosstalk between DCs and NK cells, usually inhibited by PGE2 [198].

5.5. PGE2 and T-Cells

HSCs differentiate into multipotent progenitors (MPPs) which could be differentiated in both myeloid and lymphoid cells. MPPs differentiate to a common lymphoid progenitor (CLP) which converts exclusively into T, B, or NK cells. CLPs migrate to the thymus, where they implant themselves. First, cells that implant in the thymus are the double-negative ones, as they express neither the CD4 nor the CD8 co-receptor, while double positive CD4/CD8 migrate into the cortex of the thymus and present self-antigens. If they interact with MHC-I or MHC-II, they survive; if they do not, they will be discarded. If the double-positive cells interact with MHC class II, molecules will differentiate in $CD4^+$ cells—$CD8^+$ cells if they interact with MHC class I [199]. Differentiated T cells have an important role in adaptive immunity and are subdivided into (i) $CD8^+$ T cells that have cytotoxic activity, able to

kill infected or tumor cells; (ii) $CD4^+$ T cells, or "helper cells", that release cytokines and activate indirectly regulatory B cells; and (iii) regulatory T cells (Treg) usually activated by tumor cells to prevent their killing.

Several studies have underlined the effect of PGE2 on T cells. For example, it has been reported that PGE2 is able to inhibit the proliferation of T cells in a dose-dependent manner in vitro. A comparison between IFN-γ and IL-4 production showed that PGE2 increases the relative ratio of IL-4 to IFN-γ in $CD4^+$ T cell culture and regulates $CD4^+$ T cells toward Th2 development. This mechanism seems to be primarily dependent on IDO-overexpression, determining Tregs formation and development [200]. In support of these findings, it has been reported that murine renal carcinoma (Renca) cells over-secrete PGE2 and consequently inhibit antitumor cytotoxic T lymphocyte (CTL) responses in vivo by preventing the IFNγ-dependent upregulation of ICAM-1 responsible for the initial priming of naïve $CD8^+$ T cells. In addition, exogenous IFNγ abolishes PGE2-mediated suppression on naïve CD8 T-cell priming, and overexpression of ICAM-1 by tumor cells re-establishes IFNγ production [201]. PGE2 immunosuppression may be an indirect consequence determined by COX-2 overexpression, as in melanoma. In a number of tumor cell lines, constitutive IDO1 expression depends on COX-2 and PGE2 which upon autocrine signaling through the EP receptor activates IDO1 via the PKC and PI3K pathways. Moreover, most of these tumors have been associated with PI3K or MAPK mutations which may support constitutive IDO1 expression, and usually lack T-cell infiltration and fail to immunotherapy [202].

$CD4^+CD25^+$ T reg cells play an important role in the maintenance of immunologic self-tolerance in non-small cell lung cancer. $CD4^+CD25^+$ T reg cell activities increase in lung cancer and appear to play an important role in suppressing antitumor immune responses. COX-2/PGE2 signaling induces expression of Foxp3 and increases Treg properties. PGE2-mediated induction of Treg cell Foxp3 gene expression is significantly down-expressed in the absence of the EP4 receptor and totally eliminated in the absence of the EP2 receptor. In vivo, COX-2 inhibition reduces Treg cell frequency and activity, attenuates Foxp3 expression in tumor-infiltrating lymphocytes (TILs), and decreases tumor weight. Adoptive Treg cells transfer or administration of PGE2 to mice treated with COX-2 inhibitor are able to overturn these effects [203]. Another important piece of evidence is reported by Kim et al. who have emphasized GM-CSF potential in cancer vaccines through IL9-producing Th (Th9) cells. GM-CSF improves Th9 cell differentiation by regulating the COX-2–PGE2 pathway and it is able to inhibit the differentiation of induced regulatory T cells in vitro and in vivo. GM-CSF-activated monocyte-derived dendritic cells convert tumor-specific naïve Th cells into Th9 cells and delay tumor growth by inducing antitumor CTLs in an IL9-dependent manner [204].

Moreover, fibroblastic reticular cells (FRCs) in the T-cell zone of lymph nodes are essential for T-cell survival, mobility, and tolerance. FRCs are able to limit T-cell activation, secreting PGE2 due to COX-2 overexpression in immune cells [205]. Another important role of PGE2, mainly related to immunotherapy resistance, is its ability to promote the expression of PD-1 and TIM-3 in T-cells increasing the interaction with PD-L1 and PD-L2, highly expressed on CAFs. Blocking the activity of PGE2 partially restores the proliferative capacity of both $CD4^+$ and $CD8^+$ T-cells [206].

All these reports demonstrate an important function of PGE2 in controlling the balance between Th1, Th2, and Treg, with a key role in the induction of Treg and Th2 phenotype resulting in a promotion of immunosuppressive niche and tumor growth.

6. Conclusions

The importance of inflammation in driving the predisposition to cancer has been widely documented. Starting from this observation, in recent years the scientific community has begun to repurpose anti-inflammatory agents not only for the prevention but also as a therapeutic option in cancer. Among the inflammatory mediators, PGE2 is known to play a pivotal role in regulating the inflammatory milieu that drives cancer onset and progression. Several studies have demonstrated that PGE2 is able to activate growth factor signaling; promote cancer cell growth and resistance to apoptosis

and metastasis; and to modulate immune response. Extensive clinical and epidemiological studies support the idea that PGE2 level reduction could be useful to prevent tumor initiation, to reprogram anti-tumor immunity, to inhibit tumor growth and metastasis, and, finally, to increase the efficacy of current pharmacological and immunological therapies.

To date, the anti-tumoral potential of aspirin and coxibs has been well studied in preclinical and clinical settings. Notably, several clinical trials aimed to study the efficacy of coxibs alone or in combination are under investigation. For example, the clinical efficacy of celecoxib alone is under phase I and II clinical trials for treating patients with stage I, stage II, or stage IIIA non-small cell lung cancer and advanced carcinoma of the cervix (NCT00030407 and NCT00023660 clinicaltrials.gov). Further, the combination of celecoxib with irinotecan, cisplatin, and radiation therapy is under investigation in esophageal cancer (NCT00023660 clinicaltrials.gov).

Of note, the current therapies targeting PGE2 using NSAIDs or COX-2 inhibitors have sometimes failed due to the global prostanoid suppression which in turn results in severe side effects [207]. It is, therefore, more plausible and clinically relevant to act not on PGE2 biosynthesis, but mainly on the antagonism of EP receptors. For this reason, numerous small-molecule ligands targeting EP receptors have been developed and are under investigation both as conventional anticancer agents and as immunomodulating drugs (e.g., ONO-8711 for EP1; PF-04418948 for EP2; ONO-AE3-240 for EP3; AH23848b for EP4). For example, the antagonist of EP4, RQ-00000007, is under clinical evaluation in combination with gemcitabine for prostate cancer, non-small cell lung cancer, and breast cancer (NCT02538432 www.clinicaltrials.gov). The TPST-1495 dual EP2 and EP4 antagonist is in phase 1a/1b as single treatment or in association with pembrolizumab in solid tumors (bladder cancer, triple negative breast cancer, gastric cancer) (NCT04344795 clinicaltrials.gov).

This review has highlighted the pleiotropic role of PGE2 in controlling tumor cells but also the tumor microenvironment (TME), suggesting that targeting PGE2 could be a good strategy to both act on cancer cells but also on immune cells. Accordingly, the putative immunomodulatory effect of blocking the PGE2 pathway is under investigation. For example, a phase I clinical study has already been started with E7046, an EP4 inhibitor, with a potent immunosuppressive effect on myeloid cells in the TME (NCT02540291: https://clinicaltrials.gov/ct2/show/study/NCT02540291).

In conclusion, PGE2 represents an old target with pleiotropic functions in TME with a new potential clinical impact. The usage of NSAIDS and COX-2-inhibitors as anti-cancer agents may be bypassed by selective EP antagonists which may overcome severe side effects and, therefore, increase the real potential of targeting the PGE2 pathway as a novel therapeutic approach in the clinical setting.

Author Contributions: Conceptualization, F.F., C.T., and L.T.; writing—original draft preparation, F.F., C.T., J.E., G.C., and E.B.; writing—review and editing, F.F., C.T., and L.T.; supervision, L.T. All authors have read and agreed to the published version of the manuscript.

Funding: MIUR (Progetto Dipartimento di Eccellenza 2018-2022) to L.T., F.F., and J.E., Fondi FRG 2019 (Università degli Studi di Pavia) to C.T.; Fondazione Umberto Veronesi to C.T.

Acknowledgments: The authors thank Servier Medical Art (SMART) for the preparation of Figures 1 and 2, and BioRender for the preparation of Figure 3.

Conflicts of Interest: The authors declare no conflict of interest

References

1. De Martel, C.; Ferlay, J.; Franceschi, S.; Vignat, J.; Bray, F.; Forman, D.; Plummer, M. Global burden of cancers attributable to infections in 2008: A review and synthetic analysis. *Lancet Oncol.* **2012**, *13*, 607–615. [CrossRef]
2. Schottenfeld, D.; Beebe-Dimmer, J. The cancer burden attributable to biologic agents. *Ann. Epidemiol.* **2015**, *25*, 183–187. [CrossRef] [PubMed]
3. Rothwell, P.M.; Fowkes, F.G.R.; Belch, J.F.; Ogawa, H.; Warlow, C.P.; Meade, T.W. Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials. *Lancet* **2011**, *377*, 31–41. [CrossRef]

4. Cho, M.H.; Yoo, T.G.; Jeong, S.-M.; Shin, D.W. Association of aspirin, metformin, and statin use with gastric cancer incidence and mortality: A nationwide cohort study. *Cancer Prev. Res.* **2020**. [CrossRef]
5. Lin, Y.S.; Yeh, C.C.; Huang, S.F.; Chou, Y.S.; Kuo, L.T.; Sung, F.C.; Muo, C.H.; Su, C.T.; Su, F.H. Aspirin associated with risk reduction of secondary primary cancer for patients with head and neck cancer: A population-based analysis. *PLoS ONE* **2018**, *13*, e0199014. [CrossRef]
6. Ma, Y.; Brusselaers, N. Maintenance use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) and prostate cancer risk. *Prostate Cancer Prostatic Dis.* **2018**, *21*, 147–152. [CrossRef]
7. Veettil, S.K.; Lim, K.G.; Ching, S.M.; Saokaew, S.; Phisalprapa, P.; Chaiyakunapruk, N. Effects of aspirin and non-aspirin nonsteroidal anti-inflammatory drugs on the incidence of recurrent colorectal adenomas: A systematic review with meta-analysis and trial sequential analysis of randomized clinical trials. *BMC Cancer* **2017**, *17*, 763. [CrossRef]
8. Donnini, S.; Finetti, F.; Terzuoli, E.; Bazzani, L.; Ziche, M. Targeting PGE2 signaling in tumor progression and angiogenesis. *For. Immunopathol. Dis. Therap.* **2014**. [CrossRef]
9. Dubois, R.N.; Abramson, S.B.; Crofford, L.; Gupta, R.A.; Simon, L.S.; van de Putte, L.B.A.; Lipsky, P.E. Cyclooxygenase in Biology and Disease. *FASEB J.* **1998**, *12*, 1063–1073. [CrossRef]
10. Simmons, D.L.; Botting, R.M.; Hla, T. Cyclooxygenase Isozymes: The Biology of Prostaglandin Synthesis and Inhibition. *Pharmacol. Rev.* **2004**, *56*, 387–437. [CrossRef]
11. Wang, D.; Dubois, R.N. Eicosanoids and cancer. *Nat. Rev. Cancer* **2010**, *10*, 181–193. [CrossRef] [PubMed]
12. Murakami, M.; Nakatani, Y.; Tanioka, T.; Kudo, I.; Synthase, P.E. Molecular Biology of the Arachidonate Cascade. *Prostaglandins Other Lipid Mediat.* **2002**, *68–69*, 383–399. [CrossRef]
13. Samuelsson, B.; Morgenstern, R.; Jakobsson, J. Membrane Prostaglandin E Synthase-1: A Novel Therapeutic Target. *Pharmacol. Rev.* **2007**, *59*, 207–224. [CrossRef] [PubMed]
14. Tai, H.H. Prostaglandin catabolic enzymes as tumor suppressors. *Cancer Metastasis Rev.* **2011**, *30*, 409–417. [CrossRef] [PubMed]
15. O'Callaghan, G.; Houston, A. Prostaglandin E2 and the EP receptors in malignancy: Possible therapeutic targets? *Br. J. Pharmacol.* **2015**, *172*, 5239–5250. [CrossRef] [PubMed]
16. Cai, Y.; Yousef, A.; Grandis, J.R.; Johnson, D.E. NSAID therapy for PIK3CA-Altered colorectal, breast, and head and neck cancer. *Adv. Biol. Regul.* **2020**, *75*, 100653. [CrossRef]
17. Harris, R.E.; Beebe-Donk, J.; Doss, H.; Doss, D.B. Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: A critical review of non-selective COX-2 blockade (review). *Oncol. Rep.* **2005**, *13*, 559–583. [CrossRef]
18. Drew, D.A.; Schuck, M.M.; Magicheva-Gupta, M.V.; Stewart, K.O.; Gilpin, K.K.; Miller, P.; Parziale, M.P.; Pond, E.N.; Takacsi-Nagy, O.; Zerjav, D.C.; et al. Effect of Low-dose and Standard-dose Aspirin on PGE 2 Biosynthesis Among Individuals with Colorectal Adenomas: A Randomized Clinical Trial. *Cancer Prev. Res.* **2020**. [CrossRef]
19. Fang, H.Y.; Lin, T.S.; Lin, J.P.; Wu, Y.C.; Chow, K.C.; Wang, L.S. Cyclooxygenase-2 in human non-small cell lung cancer. *Eur. J. Surg. Oncol.* **2003**, *29*, 171–177. [CrossRef]
20. Yoshimatsu, K.; Subbaramaiah, K.; Dannenberg, A.J.; DeLellis, R.A.; Golijanin, D.; Paty, P.B.; Soslow, R.A.; Jakobsson, P.J. Inducible microsomal prostaglandin E synthase is overexpressed in colorectal adenomas and cancer. *Clin. Cancer Res.* **2001**, *7*, 3971–3976.
21. Sano, H.; Kawahito, Y.; Wilder, R.L.; Hashiramoto, A.; Mukai, S.; Asai, K.; Kimura, S.; Kato, H.; Kondo, M.; Hla, T. Expression of Cyclooxygenase-1and -2 in Human Colorectal Cancer1. *Cancer Res.* **1995**, *55*, 3785–3790. [PubMed]
22. Roelofs, H.M.J.; Morsche, R.H.M.t.; van Heumen, B.W.H.; Nagengast, F.M.; Peters, W.H.M. Over-expression of COX-2 mRNA in colorectal cancer. *BMC Gastroenterol.* **2014**, *14*, 3–8. [CrossRef] [PubMed]
23. Half, E.; Tang, X.M.; Gwyn, K.; Sahin, A.; Wathen, K.; Sinicrope, F.A. Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. *Cancer Res.* **2002**, *62*, 1676–1681. [PubMed]
24. Mehrotra, S.; Morimiya, A.; Agarwal, B.; Konger, R.; Badve, S. Microsomal prostaglandin E2 synthase-1 in breast cancer: A potential target for therapy. *J. Pathol.* **2006**, *208*, 356–363. [CrossRef]
25. Khor, L.Y.; Bae, K.; Pollack, A.; Hammond, M.E.H.; Grignon, D.J.; Venkatesan, V.M.; Rosenthal, S.A.; Ritter, M.A.; Sandler, H.M.; Hanks, G.E.; et al. COX-2 expression predicts prostate-cancer outcome: Analysis of data from the RTOG 92-02 trial. *Lancet Oncol.* **2007**, *8*, 912–920. [CrossRef]

26. Finetti, F.; Terzuoli, E.; Giachetti, A.; Santi, R.; Villari, D.; Hanaka, H.; Radmark, O.; Ziche, M.; Donnini, S. MPGES-1 in prostate cancer controls stemness and amplifies epidermal growth factor receptor-driven oncogenicity. *Endocr. Relat. Cancer* **2015**, *22*. [CrossRef]
27. Panza, E.; de Cicco, P.; Ercolano, G.; Armogida, C.; Scognamiglio, G.; Anniciello, A.M.; Botti, G.; Cirino, G.; Ianaro, A. Differential expression of cyclooxygenase-2 in metastatic melanoma affects progression free survival. *Oncotarget* **2016**, *7*, 57077–57085. [CrossRef]
28. Koga, H.; Sakisaka, S.; Ohishi, M.; Kawaguchi, T.; Taniguchi, E.; Sasatomi, K.; Harada, M.; Kusaba, T.; Tanaka, M.; Kimura, R.; et al. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: Relevance to tumor dedifferentiation. *Hepatology* **1999**, *29*, 688–696. [CrossRef]
29. Zang, S.; Ni, M.; Lian, Y.; Zhang, Y.; Liu, J.; Huang, A. Expression of microsomal prostaglandin E2 synthase-1 and its role in human hepatocellular carcinoma. *Hum. Pathol.* **2013**, *44*, 1681–1687. [CrossRef]
30. Zarghi, A.; Arfaei, S. Selective COX-2 Inhibitors: A Review of Their Structure-Activity Relationships. *Iran. J. Pharm. Res.* **2011**, *10*, 655–683.
31. Harris, R. Beebe, Alshafie, Reduction in cancer risk by selective and nonselective cyclooxygenase-2 (COX-2) inhibitors. *J. Exp. Pharmacol.* **2012**, *91*. [CrossRef]
32. Liao, X.; Lochhead, P.; Nishihara, R.; Morikawa, T.; Kuchiba, A.; Yamauchi, M.; Imamura, Y.; Qian, Z.R.; Baba, Y.; Shima, K.; et al. Aspirin Use, Tumor PIK3CA Mutation, and Colorectal-Cancer Survival. *N. Engl. J. Med.* **2012**, *367*, 1596–1606. [CrossRef] [PubMed]
33. Paleari, L.; Puntoni, M.; Clavarezza, M.; DeCensi, M.; Cuzick, J.; DeCensi, A. PIK3CA Mutation, Aspirin Use after Diagnosis and Survival of Colorectal Cancer. A Systematic Review and Meta-analysis of Epidemiological Studies. *Clin. Oncol.* **2016**, *28*, 317–326. [CrossRef] [PubMed]
34. Patrignani, P.; Patrono, C. Aspirin and Cancer. *J. Am. Coll. Cardiol.* **2016**, *68*, 967–976. [CrossRef] [PubMed]
35. Patrono, C.; Rocca, B. Aspirin: Promise and resistance in the new millennium. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, 25–32. [CrossRef]
36. Soriano, L.C.; Soriano-Gabarró, M.; Rodríguez, L.A.G. Trends in the contemporary incidence of colorectal cancer and patient characteristics in the United Kingdom: A population-based cohort study using The Health Improvement Network. *BMC Cancer* **2018**, *18*, 402. [CrossRef]
37. Thun, M.J.; Jacobs, E.J.; Patrono, C. The role of aspirin in cancer prevention. *Nat. Rev. Clin. Oncol.* **2012**, *9*, 259–267. [CrossRef]
38. Algra, A.M.; Rothwell, P.M. Effects of regular aspirin on long-term cancer incidence and metastasis: A systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol.* **2012**, *13*, 518–527. [CrossRef]
39. Andreotti, F.; de Caterina, R.; Crea, F. Aspirin and the prevention of a common disease: Colorectal cancer. *Int. J. Cardiol.* **2017**, *248*, 394–395. [CrossRef]
40. Cole, B.F.; Logan, R.F.; Halabi, S.; Benamouzig, R.; Sandler, R.S.; Grainge, M.J.; Chaussade, S.; Baron, J.A. Aspirin for the chemoprevention of colorectal adenomas: Meta-analysis of the randomized trials. *J. Natl. Cancer Inst.* **2009**, *101*, 256–266. [CrossRef]
41. Elwood, P.C.; Morgan, G.; Pickering, J.E.; Galante, J.; Weightman, A.L.; Morris, D.; Kelson, M.; Dolwani, S. Aspirin in the treatment of cancer: Reductions in metastatic spread and in mortality: A systematic review and meta-analyses of published studies. *PLoS ONE* **2016**, *11*, e0152402. [CrossRef] [PubMed]
42. Giovannucci, E. Aspirin Reduces the Risk of Colorectal Cancer in Women. *Nurse Pract.* **1995**, *20*, 90. [CrossRef]
43. Jonsson, F.; Yin, L.; Lundholm, C.; Smedby, K.E.; Czene, K.; Pawitan, Y. Low-dose aspirin use and cancer characteristics: A population-based cohort study. *Br. J. Cancer* **2013**, *109*, 1921–1925. [CrossRef] [PubMed]
44. Joharatnam-Hogan, N.; Cafferty, F.; Hubner, R.; Swinson, D.; Sothi, S.; Gupta, K.; Falk, S.; Patel, K.; Warner, N.; Kunene, V.; et al. Aspirin as an adjuvant treatment for cancer: Feasibility results from the Add-Aspirin randomised trial. *Lancet Gastroenterol. Hepatol.* **2019**, *4*, 854–862. [CrossRef]
45. Kune, G.A.; Kune, S.; Watson, L.F. Colorectal cancer risk, chronic illnesses, operations and medications: Case-control results from the Melbourne colorectal cancer study. *Int. J. Epidemiol.* **2007**, *36*, 951–957. [CrossRef] [PubMed]
46. Chen, W.Y.; Holmes, M.D. Role of Aspirin in Breast Cancer Survival. *Curr. Oncol. Rep.* **2017**, *19*. [CrossRef]
47. Fraser, D.M.; Sullivan, F.M.; Thompson, A.M.; McCowan, C. Aspirin use and survival after the diagnosis of breast cancer: A population-based cohort study. *Br. J. Cancer* **2014**, *111*, 623–627. [CrossRef]

48. Holmes, M.D.; Olsson, H.; Pawitan, Y.; Holm, J.; Lundholm, C.; Andersson, T.M.L.; Adami, H.O.; Askling, J.; Smedby, K.E. Aspirin intake and breast cancer survival-a nation-wide study using prospectively recorded data in Sweden. *BMC Cancer* **2014**, *14*, 391. [CrossRef]
49. Huang, X.Z.; Gao, P.; Sun, J.X.; Song, Y.X.; Tsai, C.C.; Liu, J.; Chen, X.W.; Chen, P.; Xu, H.M.; Wang, Z.N. Aspirin and nonsteroidal anti-inflammatory drugs after but not before diagnosis are associated with improved breast cancer survival: A meta-analysis. *Cancer Causes Control.* **2015**, *26*, 589–600. [CrossRef]
50. Zhong, S.; Zhang, X.; Chen, L.; Ma, T.; Tang, J.; Zhao, J. Association between aspirin use and mortality in breast cancer patients: A meta-analysis of observational studies. *Breast Cancer Res. Treat.* **2015**, *150*, 199–207. [CrossRef]
51. Zhao, Y.S.; Zhu, S.; Li, X.W.; Wang, F.; Hu, F.L.; Li, D.D.; Zhang, W.C.; Li, X. Association between NSAIDs use and breast cancer risk: A systematic review and meta-analysis. *Breast Cancer Res. Treat.* **2009**, *117*, 141–150. [CrossRef] [PubMed]
52. Ahmadi, N.; Goldman, R.; Seillier-Moiseiwitsch, F.; Noone, A.-M.; Kosti, O.; Davidson, B.J. Decreased Risk of Squamous Cell Carcinoma of the Head and Neck in Users of Nonsteroidal Anti-Inflammatory Drugs. *Int. J. Otolaryngol.* **2010**, *2010*, 424161. [CrossRef] [PubMed]
53. Bosetti, C.; Talamini, R.; Franceschi, S.; Negri, E.; Garavello, W.; la Vecchia, C. Aspirin use and cancers of the upper aerodigestive tract. *Br. J. Cancer* **2003**, *88*, 672–674. [CrossRef] [PubMed]
54. Hedberg, M.L.; Peyser, N.D.; Bauman, J.E.; Gooding, W.E.; Li, H.; Bhola, N.E.; Zhu, T.R.; Zeng, Y.; Brand, T.M.; Kim, M.O.; et al. Use of nonsteroidal anti-inflammatory drugs predicts improved patient survival for PIK3CA-altered head and neck cancer. *J. Exp. Med.* **2019**, *216*, 419–427. [CrossRef] [PubMed]
55. Lumley, C.J.; Kaffenberger, T.M.; Desale, S.; Tefera, E.; Han, C.J.; Rafei, H.; Maxwell, J.H. Post-diagnosis aspirin use and survival in veterans with head and neck cancer. *Head Neck.* **2019**, *41*, 1220–1226. [CrossRef]
56. Macfarlane, T.V.; Macfarlane, G.J.; Thakker, N.S.; Benhamou, S.; Bouchardy, C.; Ahrens, W.; Pohlabeln, H.; Lagiou, P.; Lagiou, A.; Castellsague, X.; et al. Role of medical history and medication use in the aetiology of upper aerodigestive tract cancers in Europe: The ARCAGE study. *Ann. Oncol.* **2012**, *23*, 1053–1060. [CrossRef]
57. Macfarlane, T.V.; Lefevre, K.; Watson, M.C. Aspirin and non-steroidal anti-inflammatory drug use and the risk of upper aerodigestive tract cancer. *Br. J. Cancer* **2014**, *111*, 1852–1859. [CrossRef]
58. Macfarlane, T.V.; Murchie, P.; Watson, M.C. Aspirin and other non-steroidal anti-inflammatory drug prescriptions and survival after the diagnosis of head and neck and oesophageal cancer. *Cancer Epidemiol.* **2015**, *39*, 1015–1022. [CrossRef]
59. Panje, W.R. Regression of Head and Neck Carcinoma With a Prostaglandin-Synthesis Inhibitor. *Arch. Otolaryngol.* **1981**, *107*, 658–663. [CrossRef]
60. Wilson, J.C.; Anderson, L.A.; Murray, L.J.; Hughes, C.M. Non-steroidal anti-inflammatory drug and aspirin use and the risk of head and neck cancer: A systematic review. *Cancer Causes Control.* **2011**, *22*, 803–810. [CrossRef]
61. Hochmuth, F.; Jochem, M.; Schlattmann, P. Meta-analysis of aspirin use and risk of lung cancer shows notable results. *Eur. J. Cancer Prev.* **2016**, *25*, 259–268. [CrossRef] [PubMed]
62. Shebl, F.M.; Sakoda, L.C.; Black, A.; Koshiol, J.; Andriole, G.L.; Grubb, R.; Church, T.R.; Chia, D.; Zhou, C.; Chu, L.W.; et al. Aspirin but not ibuprofen use is associated with reduced risk of prostate cancer: A PLCO Study. *Br. J. Cancer* **2012**, *107*, 207–214. [CrossRef]
63. Cuzick, J.; Otto, F.; Baron, J.A.; Brown, P.H.; Burn, J.; Greenwald, P.; Jankowski, J.; la Vecchia, C.; Meyskens, F.; Senn, H.J.; et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: An international consensus statement. *Lancet Oncol.* **2009**, *10*, 501–507. [CrossRef]
64. Bosetti, C.; Gallus, S.; la Vecchia, C. Aspirin and cancer risk: An updated quantitative review to 2005. *Cancer Causes Control.* **2006**, *17*, 871–888. [CrossRef] [PubMed]
65. Prizment, A.E.; Folsom, A.R.; Anderson, K.E. Nonsteroidal anti-inflammatory drugs and risk for ovarian and endometrial cancers in the Iowa women's health study, Cancer Epidemiol. *Biomarkers Prev.* **2010**, *19*, 435–442. [CrossRef]
66. Kim, M.H.; Chang, J.; Kim, W.J.; Banerjee, S.; Park, S.M. Cumulative Dose Threshold for the Chemopreventive Effect of Aspirin Against Gastric Cancer. *Am. J. Gastroenterol.* **2018**, *113*, 845–854. [CrossRef]
67. Niikura, R.; Hirata, Y.; Hayakawa, Y.; Kawahara, T.; Yamada, A.; Koike, K. Effect of aspirin use on gastric cancer incidence and survival: A systematic review and meta-analysis. *JGH Open* **2020**, *4*, 117–125. [CrossRef]

68. Venerito, M.; Vasapolli, R.; Rokkas, T.; Malfertheiner, P. Gastric cancer: Epidemiology, prevention, and therapy. *Helicobacter* **2018**, *23*, 2–7. [CrossRef]
69. Yang, P.; Zhou, Y.; Chen, B.; Wan, H.W.; Jia, G.Q.; Bai, H.L.; Wu, X.T. Aspirin use and the risk of gastric cancer: A meta-analysis. *Dig. Dis. Sci.* **2010**, *55*, 1533–1539. [CrossRef]
70. Wang, X.; Luo, Y.; Chen, T.; Zhang, K. Low-dose aspirin use and cancer-specific mortality: A meta-analysis of cohort studies. *J. Public Health (Bangkok)* **2019**, fdz114. [CrossRef]
71. Falandry, C.; Debled, M.; Bachelot, T.; Delozier, T.; Crétin, J.; Romestaing, P.; Mille, D.; You, B.; Mauriac, L.; Pujade-Lauraine, E.; et al. Celecoxib and exemestane versus placebo and exemestane in postmenopausal metastatic breast cancer patients: A double-blind phase III GINECO study. *Breast Cancer Res. Treat.* **2009**, *116*, 501–508. [CrossRef] [PubMed]
72. Li, J.; Hao, Q.; Cao, W.; Vadgama, J.V.; Wu, Y. Celecoxib in breast cancer prevention and therapy. *Cancer Manag. Res.* **2018**, *10*, 4653–4667. [CrossRef] [PubMed]
73. Chow, L.W.C.; Yip, A.Y.S.; Loo, W.T.Y.; Lam, C.K.; Toi, M. Celecoxib anti-aromatase neoadjuvant (CAAN) trial for locally advanced breast cancer. *J. Steroid Biochem. Mol. Biol.* **2008**, *111*, 13–17. [CrossRef] [PubMed]
74. Martin, L.A.; Davies, G.L.S.; Weigel, M.T.; Betambeau, N.; Hills, M.J.; Salter, J.; Walsh, G.; A'Hern, R.; Dowsett, M. Pre-surgical study of the biological effects of the selective cyclo-oxygenase-2 inhibitor celecoxib in patients with primary breast cancer. *Breast Cancer Res. Treat.* **2010**, *123*, 829–836. [CrossRef]
75. Arun, B.; Goss, P. The role of COX-2 inhibition in breast cancer treatment and prevention. *Semin. Oncol.* **2004**, *31*, 22–29. [CrossRef]
76. Tołoczko-Iwaniuk, N.; Dziemiańczyk-Pakieła, D.; Nowaszewska, B.K.; Celińska-Janowicz, K.; Miltyk, W. Celecoxib in Cancer Therapy and Prevention—Review. *Curr. Drug Targets* **2018**. [CrossRef]
77. Arber, N.; Eagle, C.J.; Spicak, J.; Rácz, I.; Dite, P.; Hajer, J.; Zavoral, M.; Lechuga, M.J.; Gerletti, P.; Tang, J.; et al. Celecoxib for the Prevention of Colorectal Adenomatous Polyps. *N. Engl. J. Med.* **2006**, *355*, 885–895. [CrossRef]
78. Baron, J.A.; Sandler, R.S.; Bresalier, R.S.; Quan, H.; Riddell, R.; Lanas, A.; Bolognese, J.A.; Oxenius, B.; Horgan, K.; Loftus, S.; et al. A Randomized Trial of Rofecoxib for the Chemoprevention of Colorectal Adenomas. *Gastroenterology* **2006**, *131*, 1674–1682. [CrossRef]
79. Wang, D.; Dubois, R.N. Role of prostanoids in gastrointestinal cancer. *J. Clin. Investig.* **2018**, *128*, 2732–2742. [CrossRef]
80. Liu, R.; Xu, K.P.; Tan, G.S. Cyclooxygenase-2 inhibitors in lung cancer treatment: Bench to bed. *Eur. J. Pharmacol.* **2015**, *769*, 127–133. [CrossRef]
81. Mao, J.T.; Roth, M.D.; Fishbein, M.C.; Aberle, D.R.; Zhang, Z.F.; Rao, J.Y.; Tashkin, D.P.; Goodglick, L.; Holmes, E.C.; Cameron, R.B.; et al. Lung cancer chemoprevention with celecoxib in former smokers. *Cancer Prev. Res.* **2011**, *4*, 984–993. [CrossRef] [PubMed]
82. Csiki, I.; Morrow, J.D.; Sandler, A.; Shyr, Y.; Oates, J.; Williams, M.K.; Dang, T.; Carbone, D.P.; Johnson, D.H. Targeting cyclooxygenase-2 in recurrent non-small cell lung cancer: A phase II trial of celecoxib and docetaxel. *Clin. Cancer Res.* **2005**, *11*, 6634–6640. [CrossRef] [PubMed]
83. EKim, S.; Hong, W.K.; Lee, J.J.; Mao, L.; Morice, R.C.; Liu, D.D.; Jimenez, C.A.; Eapen, G.A.; Lotan, R.; Tang, X.; et al. Biological activity of celecoxib in the bronchial epithelium of current and former smokers. *Cancer Prev. Res.* **2010**, *3*, 148–159. [CrossRef]
84. Hansen-Petrik, M.B.; McEntee, M.F.; Jull, B.; Shi, H.; Zemel, M.B.; Whelan, J. Prostaglandin E2 protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apcmin/+ mice. *Cancer Res.* **2002**, *62*, 403–408. [PubMed]
85. Yan, M.; Myung, S.J.; Fink, S.P.; Lawrence, E.; Lutterbaugh, J.; Yang, P.; Zhou, X.; Liu, D.; Rerko, R.M.; Willis, J.; et al. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to celecoxib chemoprevention of colon tumors. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9409–9413. [CrossRef] [PubMed]
86. Myung, S.J.; Rerko, R.M.; Yan, M.; Platzer, P.; Guda, K.; Dotson, A.; Lawrence, E.; Dannenberg, A.J.; Lovgren, A.K.; Luo, G.; et al. 15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12098–12102. [CrossRef]
87. Kawamori, T.; Uchiya, N.; Sugimura, T.; Wakabayashi, K. Enhancement of colon carcinogenesis by prostaglandin E2 administration. *Carcinogenesis* **2003**, *24*, 985–990. [CrossRef]

88. Rohwer, N.; Kühl, A.A.; Schebb, N.H.; Zopf, D.; Mcdonald, F.M.; Weylandt, K.-H. Effects of chronic low-dose aspirin treatment on tumor prevention in three mouse models of intestinal tumorigenesis. *Cancer Med.* **2020**. [CrossRef]
89. Nakanishi, M.; Montrose, D.C.; Clark, P.; Nambiar, P.R.; Belinsky, G.S.; Claffey, K.P.; Xu, D.; Rosenberg, D.W. Genetic deletion of mPGES-1 suppresses intestinal tumorigenesis. *Cancer Res.* **2008**, *68*, 3251–3259. [CrossRef]
90. Nakanishi, M.; Menoret, A.; Tanaka, T.; Miyamoto, S.; Montrose, D.C.; Vella, A.T.; Rosenberg, D.W. Selective PGE 2 suppression inhibits colon carcinogenesis and modifies local mucosal immunity. *Cancer Prev. Res.* **2011**, *4*, 1198–1208. [CrossRef]
91. Sasaki, Y.; Nakatani, Y.; Hara, S. Role of microsomal prostaglandin E synthase-1 (mPGES-1)-derived prostaglandin E2 in colon carcinogenesis, Prostaglandins Other Lipid Mediat. *Prostaglandins Other Lipid Mediat.* **2015**, *121*, 42–45. [CrossRef] [PubMed]
92. Howe, L.R.; Subbaramaiah, K.; Kent, C.V.; Zhou, X.K.; Chang, S.H.; Hla, T.; Jakobsson, P.J.; Hudis, C.A.; Dannenberg, A.J. Genetic deletion of microsomal prostaglandin e synthase-1 suppresses mouse mammary tumor growth and angiogenesis. *Prostaglandins Other Lipid Mediat.* **2013**, *106*, 99–105. [CrossRef] [PubMed]
93. Finetti, F.; Terzuoli, E.; Bocci, E.; Coletta, I.; Polenzani, L.; Mangano, G.; Alisi, M.A.; Cazzolla, N.; Giachetti, A.; Ziche, M.; et al. Pharmacological inhibition of microsomal prostaglandin E synthase-1 suppresses epidermal growth factor receptor-mediated tumor growth and angiogenesis. *PLoS ONE* **2012**, *7*, e40576. [CrossRef] [PubMed]
94. Bergqvist, F.; Morgenstern, R.; Jakobsson, P.J. A review on mPGES-1 inhibitors: From preclinical studies to clinical applications. *Prostaglandins Other Lipid Mediat.* **2020**, *147*, 106383. [CrossRef] [PubMed]
95. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef] [PubMed]
96. Del Prete Annalisa, M.A. Allavena Paola, Santoro Giuseppe, Fumarulo Ruggiero, Corsi Massimi liano M, Infl ammation Mo le cu lar pat hways in can ce r-re la ted in fl am ma tion. *Biochem. Medica* **2011**, *21*, 264–275.
97. Rajendran, P.; Chen, Y.F.; Chen, Y.F.; Chung, L.C.; Tamilselvi, S.; Shen, C.Y.; Day, C.H.; Chen, R.J.; Viswanadha, V.P.; Kuo, W.W.; et al. The multifaceted link between inflammation and human diseases. *J. Cell Physiol.* **2018**, *233*, 6458–6471. [CrossRef]
98. Wang, D.; Dubois, R.N. Prostaglandins and cancer. *Gut* **2006**, *55*, 115–122. [CrossRef]
99. Sugimoto, Y.; Narumiya, S. Prostaglandin E receptors. *J. Biol. Chem.* **2007**, *282*, 11613–11617. [CrossRef]
100. Watanabe, K.; Kawamori, T.; Nakatsugi, S.; Ohta, T.; Ohuchida, S.; Yamamoto, H.; Maruyama, T.; Kondo, K.; Ushikubi, F.; Narumiya, S.; et al. Role of the prostaglandin E receptor subtype EP1 in colon carcinogenesis. *Cancer Res.* **1999**, *59*, 5093–5096.
101. Watanabe, K.; Kawamori, T.; Nakatsugi, S.; Ohta, T.; Ohuchida, S.; Yamamoto, H.; Maruyama, T.; Kondo, K.; Narumiya, S.; Sugimura, T.; et al. Inhibitory effect of a prostaglandin E receptor subtype EP1 selective antagonist, ONO-8713, on development of azoxymethane-induced aberrant crypt foci in mice. *Cancer Lett.* **2000**, *156*, 57–61. [CrossRef]
102. Kawamori, T.; Kitamura, T.; Watanabe, K.; Uchiya, N.; Maruyama, T.; Narumiya, S.; Sugimura, T.; Wakabayashi, K. Prostaglandin E receptor subtype EP1 deficiency inhibits colon cancer development. *Carcinogenesis* **2005**, *26*, 353–357. [CrossRef] [PubMed]
103. Kitamura, T.; Itoh, M.; Noda, T.; Tani, K.; Kobayashi, M.; Maruyama, T.; Kobayashi, K.; Ohuchida, S.; Sugimura, T.; Wakabayashi, K. Combined effects of prostaglandin E receptor subtype EP1 and subtype EP4 antagonists on intestinal tumorigenesis in adenomatous polyposis coli gene knockout mice. *Cancer Sci.* **2003**, *94*, 618–621. [CrossRef] [PubMed]
104. Tober, K.L.; Wilgus, T.A.; Kusewitt, D.F.; Thomas-Ahner, J.M.; Maruyama, T.; Oberyszyn, T.M. Importance of the EP1 receptor in cutaneous UVB-induced inflammation and tumor development. *J. Investig. Dermatol.* **2006**, *126*, 205–211. [CrossRef] [PubMed]
105. Makita, H.; Mutoh, M.; Maruyama, T.; Yonemoto, K.; Kobayashi, A.; Fujitsuka, H.; Toida, M.; Shibata, T.; Miyamoto, S.; Yasui, Y.; et al. A prostaglandin E2 receptor subtype EP1-selective antagonist, ONO-8711, suppresses 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Carcinogenesis* **2007**, *28*, 677–684. [CrossRef] [PubMed]
106. Narumiya, S.; Sugimoto, Y.; Ushikubi, F. Prostanoid receptors: Structures, properties, and functions. *Physiol. Rev.* **1999**, *79*, 1193–1226. [CrossRef]

107. Castellone, M.D.; Teramoto, H.; Williams, B.O.; Druey, K.M.; Gutkind, J.S. Medicine: Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-β-catenin signaling axis. *Science* **2005**, *310*, 1504–1510. [CrossRef]
108. Kisslov, L.; Hadad, N.; Rosengraten, M.; Levy, R. HT-29 human colon cancer cell proliferation is regulated by cytosolic phospholipase A2α dependent PGE2 via both PKA and PKB pathways. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2012**, *1821*, 1224–1234. [CrossRef]
109. Dufour, M.; Faes, S.; Dormond-Meuwly, A.; Demartines, N.; Dormond, O. PGE2-induced colon cancer growth is mediated by mTORC1. *Biochem. Biophys. Res. Commun.* **2014**, *451*, 587–591. [CrossRef]
110. Donnini, S.; Finetti, F.; Solito, R.; Terzuoli, E.; Sacchetti, A.; Morbidelli, L.; Patrignani, P.; Ziche, M. EP2 prostanoid receptor promotes squamous cell carcinoma growth through epidermal growth factor receptor transactivation and iNOS and ERK1/2 pathways. *FASEB J.* **2007**, *21*. [CrossRef]
111. Cui, F.B.; Huang, D.F.; Zhang, F.L.; Gao, E.Y.; Zhang, Y.; Cao, Y.M.; Ding, S.; Wang, Y.; Cao, Q.S.; Cao, X.M. Investigation on the regulatory effect of PGE2 on ESCC cells through the trans-activation of EGFR by EP2 and the relevant mechanism. *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21*, 5668–5676. [CrossRef] [PubMed]
112. Zang, S.; Ma, X.; Wu, Y.; Liu, W.; Cheng, H.; Li, J.; Liu, J.; Huang, A. PGE2 synthesis and signaling in malignant transformation and progression of human hepatocellular carcinoma. *Hum. Pathol.* **2017**, *63*, 120–127. [CrossRef] [PubMed]
113. Qiu, J.; Li, Q.; Bell, K.A.; Yao, X.; Du, Y.; Zhang, E.; Yu, J.J.; Yu, Y.; Shi, Z.; Jiang, J. Small-molecule inhibition of prostaglandin E receptor 2 impairs cyclooxygenase-associated malignant glioma growth. *Br. J. Pharmacol.* **2019**, *176*, 1680–1699. [CrossRef] [PubMed]
114. Xu, S.; Zhou, W.; Ge, J.; Zhang, Z. Prostaglandin E2 receptor EP4 is involved in the cell growth and invasion of Prostate cancer via the cAMP-PKA/PI3K-Akt signaling pathway. *Mol. Med. Rep.* **2018**, *17*, 4702–4712. [CrossRef] [PubMed]
115. Kashiwagi, E.; Inoue, S.; Mizushima, T.; Chen, J.; Ide, H.; Kawahara, T.; Reis, L.O.; Baras, A.S.; Netto, G.J.; Miyamoto, H. Prostaglandin receptors induce urothelial tumourigenesis as well as bladder cancer progression and cisplatin resistance presumably via modulating PTEN expression. *Br. J. Cancer* **2018**, *118*, 213–223. [CrossRef]
116. Ke, J.; Yang, Y.; Che, Q.; Jiang, F.; Wang, H.; Chen, Z.; Zhu, M.; Tong, H.; Zhang, H.; Yan, X.; et al. Prostaglandin E2 (PGE2) promotes proliferation and invasion by enhancing SUMO-1 activity via EP4 receptor in endometrial cancer. *Tumor Biol.* **2016**, *37*, 12203–12211. [CrossRef]
117. Fredika, M.C. M Robertson 1, Ann-Marie Simeone, Abhijit Mazumdar, Ashish H Shah, John S McMurray, Sukhen Ghosh, Molecular and pharmacological blockade of the EP4 receptor selectively inhibits both proliferation and invasion of human inflammatory breast cancer cells-PubMed. *J. Exp. Ther. Oncol.* **2008**, *7*, 299–312.
118. Keith, R.L.; Geraci, M.W.; Nana-Sinkam, S.P.; Breyer, R.M.; Hudish, T.M.; Meyer, A.M.; Malkinson, A.M.; Dwyer-Nield, L.D. Prostaglandin E2 receptor subtype 2 (EP2) null mice are protected against murine lung tumorigenesis. *Anticancer Res.* **2006**, *26*, 2857–2861.
119. Sung, Y.M.; He, G.; Fischer, S.M. Lack of expression of the EP2 but not EP3 receptor for prostaglandin E2 results in suppression of skin tumor development. *Cancer Res.* **2005**, *65*, 9304–9311. [CrossRef]
120. Sung, Y.M.; He, G.; Hwang, D.H.; Fischer, S.M. Overexpression of the prostaglandin E2 receptor EP2 results in enhanced skin tumor development. *Oncogene* **2006**, *25*, 5507–5516. [CrossRef]
121. Chang, S.H.; Ai, Y.; Breyer, R.M.; Lane, T.F.; Hla, T. The prostaglandin e 2 receptor EP 2 is required for cyclooxygenase 2-mediated mammary hyperplasia. *Cancer Res.* **2005**, *65*, 4496–4499. [CrossRef] [PubMed]
122. Chang, S.H.; Liu, C.H.; Wu, M.T.; Hla, T. Regulation of vascular endothelial cell growth factor expression in mouse mammary tumor cells by the EP2 subtype of the prostaglandin E2 receptor. *Prostaglandins Other Lipid Mediat.* **2005**, *76*, 48–58. [CrossRef] [PubMed]
123. Sonoshita, M.; Takaku, K.; Sasaki, N.; Sugimoto, Y.; Ushikubi, F.; Narumiya, S.; Oshima, M.; Taketo, M.M. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in ApcΔ716 knockout mice. *Nat. Med.* **2001**, *7*, 1048–1051. [CrossRef] [PubMed]
124. Mutoh, M.; Watanabe, K.; Kitamura, T.; Shoji, Y.; Takahashi, M.; Kawamori, T.; Sugimura, T.; Wakabayashi, K.; Tani, K.; Kobayashi, M.; et al. Involvement of prostaglandin E receptor subtype EP4 in colon carcinogenesis. *Cancer Res.* **2002**, *62*, 28–32. [PubMed]

125. Ma, X.; Kundu, N.; Rifat, S.; Walser, T.; Fulton, A.M. Prostaglandin E receptor EP4 antagonism inhibits breast cancer metastasis. *Cancer Res.* **2006**, *66*, 2923–2927. [CrossRef] [PubMed]
126. Kotani, M.; Tanaka, I.; Ogawa, Y.; Usui, T.; Tamura, N.; Mori, K.; Narumiya, S.; Yoshimi, T.; Nakao, K. Structural organization of the human prostaglandin EP3 receptor subtype gene (PTGER3). *Genomics* **1997**, *40*, 425–434. [CrossRef]
127. Orie, N.N.; Clapp, L.H. Role of prostanoid IP and EP receptors in mediating vasorelaxant responses to PGI2 analogues in rat tail artery: Evidence for Gi/o modulation via EP3 receptors. *Eur. J. Pharmacol.* **2011**, *654*, 258–265. [CrossRef]
128. Ma, J.; Chen, M.; Xia, S.K.; Shu, W.; Guo, Y.; Wang, Y.H.; Xu, Y.; Bai, X.M.; Zhang, L.; Zhang, H.; et al. Prostaglandin E2 promotes liver cancer cell growth by the upregulation of FUSE-binding protein 1 expression. *Int. J. Oncol.* **2013**, *42*, 1093–1104. [CrossRef]
129. Bazzani, L.; Donnini, S.; Finetti, F.; Christofori, G.; Ziche, M. PGE2/EP3/SRC signaling induces EGFR nuclear translocation and growth through EGFR ligands release in lung adenocarcinoma cells. *Oncotarget* **2017**, *8*. [CrossRef]
130. Pai, R.; Soreghan, B.; Szabo, I.L.; Pavelka, M.; Baatar, D.; Tarnawski, A.S. Prostaglandin E2, transactivates EGF receptor: A novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat. Med.* **2002**, *8*, 289–293. [CrossRef]
131. Buchanan, F.G.; Wang, D.; Bargiacchi, F.; DuBois, R.N. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J. Biol. Chem.* **2003**, *278*, 35451–35457. [CrossRef] [PubMed]
132. Zhang, M.; Zhang, H.; Cheng, S.; Zhang, D.; Xu, Y.; Bai, X.; Xia, S.; Zhang, L.; Ma, J.; Du, M.; et al. Prostaglandin E2 accelerates invasion by upregulating Snail in hepatocellular carcinoma cells. *Tumor Biol.* **2014**, *35*, 7135–7145. [CrossRef] [PubMed]
133. Terzuoli, E.; Finetti, F.; Costanza, F.; Giachetti, A.; Ziche, M.; Donnini, S. Linking of mPGES-1 and iNOS activates stem-like phenotype in EGFR-driven epithelial tumor cells. *Nitric Oxide Biol. Chem.* **2017**, *66*. [CrossRef] [PubMed]
134. Donnini, S.; Finetti, F.; Terzuoli, E.; Giachetti, A.; Ĩiguez, M.A.; Hanaka, H.; Fresno, M.; Rådmark, O.; Ziche, M. EGFR signaling upregulates expression of microsomal prostaglandin e synthase-1 in cancer cells leading to enhanced tumorigenicity. *Oncogene* **2012**, *31*. [CrossRef]
135. Terzuoli, E.; Donnini, S.; Giachetti, A.; Iñiguez, M.A.; Fresno, M.; Melillo, G.; Ziche, M. Inhibition of hypoxia inducible factor-1α by dihydroxyphenylethanol, a product from olive oil, blocks microsomal prostaglandin-E synthase-1/vascular endothelial growth factor expression and reduces tumor angiogenesis. *Clin. Cancer Res.* **2010**, *16*, 4207–4216. [CrossRef]
136. Fukuda, R.; Kelly, B.; Semenza, G.L. Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. *Cancer Res.* **2003**, *63*, 2330–2334.
137. Wang, X.; Klein, R.D. Prostaglandin E2 induces vascular endothelial growth factor secretion in prostate cancer cells through EP2 receptor-mediated cAMP pathway. *Mol. Carcinog.* **2007**, *46*, 912–923. [CrossRef]
138. Terzuoli, E.; Donnini, S.; Finetti, F.; Nesi, G.; Villari, D.; Hanaka, H.; Radmark, O.; Giachetti, A.; Ziche, M. Linking microsomal prostaglandin E Synthase-1/PGE-2 pathway with miR-15a and -186 expression: Novel mechanism of VEGF modulation in prostate cancer. *Oncotarget* **2016**, *7*. [CrossRef]
139. Spinella, F.; Rosanò, L.; di Castro, V.; Natali, P.G.; Bagnato, A. Endothelin-1-induced prostaglandin E3-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. *J. Biol. Chem.* **2004**, *279*, 46700–46705. [CrossRef]
140. Jain, S.; Chakraborty, G.; Raja, R.; Kale, S.; Kundu, G.C. Prostaglandin E2 regulates tumor angiogenesis in prostate cancer. *Cancer Res.* **2008**, *68*, 7750–7759. [CrossRef]
141. Madrigal-Martínez, A.; Fernández-Martínez, A.B.; Cazaña, F.J.L. Intracrine prostaglandin E2 pro-tumoral actions in prostate epithelial cells originate from non-canonical pathways. *J. Cell. Physiol.* **2018**, *233*, 3590–3602. [CrossRef] [PubMed]
142. Battersby, S.; Sales, K.J.; Williams, A.R.; Anderson, R.A.; Gardner, S.; Jabbour, H.N. Seminal plasma and prostaglandin E2 up-regulate fibroblast growth factor 2 expression in endometrial adenocarcinoma cells via E-series prostanoid-2 receptor-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase pathway. *Hum. Reprod.* **2007**, *22*, 36–44. [CrossRef] [PubMed]

143. Salcedo, R.; Zhang, X.; Young, H.A.; Michael, N.; Wasserman, K.; Ma, W.H.; Martins-Green, M.; Murphy, W.J.; Oppenheim, J.J. Angiogenic effects of prostaglandin E2 are mediated by up-regulation of CXCR4 on human microvascular endothelial cells. *Blood* **2003**, *102*, 1966–1977. [CrossRef] [PubMed]
144. Inada, M.; Takita, M.; Yokoyama, S.; Watanabe, K.; Tominari, T.; Matsumoto, C.; Hirata, M.; Maru, Y.; Maruyama, T.; Sugimoto, Y.; et al. Direct melanoma cell contact induces stromal cell autocrine prostaglandin E2-EP4 receptor signaling that drives tumor growth, angiogenesis, and metastasis. *J. Biol. Chem.* **2015**, *290*, 29781–29793. [CrossRef]
145. Karpisheh, V.; Nikkhoo, A.; Hojjat-Farsangi, M.; Namdar, A.; Azizi, G.; Ghalamfarsa, G.; Sabz, G.; Yousefi, M.; Yousefi, B.; Jadidi-Niaragh, F. Prostaglandin E2 as a potent therapeutic target for treatment of colon cancer. *Prostaglandins Other Lipid Mediat.* **2019**, *144*. [CrossRef]
146. Aoki, T.; Narumiya, S. Prostaglandin E2-EP2 signaling as a node of chronic inflammation in the colon tumor microenvironment. *Inflamm. Regen.* **2017**, *37*. [CrossRef]
147. Kamiyama, M.; Pozzi, A.; Yang, L.; DeBusk, L.M.; Breyer, R.M.; Lin, P.C. EP2, a receptor for PGE2, regulates tumor angiogenesis through direct effects on endothelial cell motility and survival. *Oncogene* **2006**, *25*, 7019–7028. [CrossRef]
148. Amano, H.; Hayashi, I.; Endo, H.; Kitasato, H.; Yamashina, S.; Maruyama, T.; Kobayashi, M.; Satoh, K.; Narita, M.; Sugimoto, Y.; et al. Host prostaglandin E2-EP3 signaling regulates tumor-associated angiogenesis and tumor growth. *J. Exp. Med.* **2003**, *197*, 221–232. [CrossRef]
149. Finetti, F.; Solito, R.; Morbidelli, L.; Giachetti, A.; Ziche, M.; Donnini, S. Prostaglandin E_2 Regulates Angiogenesis via Activation of Fibroblast Growth Factor Receptor-1. *J. Biol. Chem.* **2008**, *283*, 2139–2146. [CrossRef]
150. Finetti, F.; Donnini, S.; Giachetti, A.; Morbidelli, L.; Ziche, M. Prostaglandin E2 primes the angiogenic switch via a synergic interaction with the fibroblast growth factor-2 pathway. *Circ. Res.* **2009**, *105*. [CrossRef]
151. Liu, S.; Jiang, M.; Zhao, Q.; Li, S.; Peng, Y.; Zhang, P.; Han, M. Vascular endothelial growth factor plays a critical role in the formation of the pre-metastatic niche via prostaglandin E2. *Oncol. Rep.* **2014**, *32*, 2477–2484. [CrossRef] [PubMed]
152. Wang, T.; Jing, B.; Sun, B.; Liao, Y.; Song, H.; Xu, D.; Guo, W.; Li, K.; Hu, M.; Liu, S.; et al. Stabilization of PTGES by deubiquitinase USP9X promotes metastatic features of lung cancer via PGE2 signaling. *Am. J. Cancer Res.* **2019**, *9*, 1145–1160. [PubMed]
153. Wang, D.; Fu, L.; Sun, H.; Guo, L.; Dubois, R.N. Prostaglandin E2 Promotes Colorectal Cancer Stem Cell Expansion and Metastasis in Mice. *Gastroenterology* **2015**, *149*, 1884–1895.e4. [CrossRef] [PubMed]
154. Cen, B.; Lang, J.D.; Du, Y.; Wei, J.; Xiong, Y.; Bradley, N.; Wang, D.; DuBois, R.N. Prostaglandin E2 Induces miR675-5p to Promote Colorectal Tumor Metastasis via Modulation of p53 Expression. *Gastroenterology* **2020**, *158*, 971–984.e10. [CrossRef]
155. Li, S.; Xu, X.; Jiang, M.; Bi, Y.; Xu, J.; Han, M. Lipopolysaccharide induces inflammation and facilitates lung metastasis in a breast cancer model via the prostaglandin E2-EP2 pathway. *Mol. Med. Rep.* **2015**, *11*, 4454–4462. [CrossRef]
156. Watanabe, Y.; Imanishi, Y.; Ozawa, H.; Sakamoto, K.; Fujii, R.; Shigetomi, S.; Habu, N.; Otsuka, K.; Sato, Y.; Sekimizu, M.; et al. Selective EP2 and Cox-2 inhibition suppresses cell migration by reversing epithelial-to-mesenchymal transition and Cox-2 overexpression and E-cadherin downregulation are implicated in neck metastasis of hypopharyngeal cancer. *Am. J. Transl. Res.* **2020**, *12*, 1096–1113.
157. Topalian, S.L.; Taube, J.M.; Anders, R.A.; Pardoll, D.M. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nat. Rev. Cancer* **2016**. [CrossRef]
158. Condamine, T.; Gabrilovich, D.I. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends Immunol.* **2011**, *32*, 19–25. [CrossRef]
159. Travelli, C.; Consonni, F.M.; Sangaletti, S.; Storto, M.; Morlacchi, S.; Grolla, A.A.; Galli, U.; Tron, G.C.; Portararo, P.; Rimassa, L.; et al. Nicotinamide phosphoribosyltransferase acts as a metabolic gate for mobilization of myeloid-derived suppressor cells. *Cancer Res.* **2019**, *79*, 1938–1951. [CrossRef]
160. Porta, C.; Consonni, F.M.; Morlacchi, S.; Sangaletti, S.; Bleve, A.; Totaro, M.G.; Larghi, P.; Rimoldi, M.; Tripodo, C.; Strauss, L.; et al. Tumor-Derived Prostaglandin E2 Promotes p50 NF-κB-Dependent Differentiation of Monocytic MDSCs. *Cancer Res.* **2020**, *80*, 2874–2888. [CrossRef]
161. Sinha, P.; Clements, V.K.; Fulton, A.M.; Ostrand-Rosenberg, S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res.* **2007**, *67*, 4507–4513. [CrossRef] [PubMed]

162. Yu, S.; Liu, C.; Su, K.; Wang, J.; Liu, Y.; Zhang, L.; Li, C.; Cong, Y.; Kimberly, R.; Grizzle, W.E.; et al. Tumor Exosomes Inhibit Differentiation of Bone Marrow Dendritic Cells. *J. Immunol.* **2007**. [CrossRef] [PubMed]
163. Xiang, X.; Poliakov, A.; Liu, C.; Liu, Y.; Deng, Z.B.; Wang, J.; Cheng, Z.; Shah, S.V.; Wang, G.J.; Zhang, L.; et al. Induction of myeloid-derived suppressor cells by tumor exosomes. *Int. J. Cancer* **2009**, *124*, 2621–2633. [CrossRef] [PubMed]
164. Obermajer, N.; Muthuswamy, R.; Lesnock, J.; Edwards, R.P.; Kalinski, P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood* **2011**, *118*, 5498–5505. [CrossRef] [PubMed]
165. Obermajer, N.; Kalinski, P. Generation of myeloid-derived suppressor cells using prostaglandin E2. *Transplant. Res.* **2012**, *1*, 2–7. [CrossRef]
166. Rodríguez-Ubreva, J.; Català-Moll, F.; Obermajer, N.; Álvarez-Errico, D.; Ramirez, R.N.; Company, C.; Vento-Tormo, R.; Moreno-Bueno, G.; Edwards, R.P.; Mortazavi, A.; et al. Prostaglandin E2 Leads to the Acquisition of DNMT3A-Dependent Tolerogenic Functions in Human Myeloid-Derived Suppressor Cells. *Cell Rep.* **2017**, *21*, 154–167. [CrossRef]
167. Mao, Y.; Sarhan, D.; Steven, A.; Seliger, B.; Kiessling, R.; Lundqvist, A. Inhibition of tumor-derived prostaglandin-E2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity. *Clin. Cancer Res.* **2014**, *20*, 4096–4106. [CrossRef]
168. Fujita, M.; Kohanbash, G.; Fellows-Mayle, W.; Hamilton, R.L.; Komohara, Y.; Decker, S.A.; Ohlfest, J.R.; Okada, H. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res.* **2011**, *71*, 2664–2674. [CrossRef]
169. Yang, F.; Wei, Y.; Cai, Z.; Yu, L.; Jiang, L.; Zhang, C.; Yan, H.; Wang, Q.; Cao, X.; Liang, T.; et al. Activated cytotoxic lymphocytes promote tumor progression by increasing the ability of 3LL tumor cells to mediate MDSC chemoattraction via Fas signaling. *Cell. Mol. Immunol.* **2015**, *12*, 66–76. [CrossRef]
170. Gabrilovich, D.I. Myeloid-derived suppressor cells. *Cancer Immunol. Res.* **2017**. [CrossRef]
171. Ochoa, A.C.; Zea, A.H.; Hernandez, C.; Rodriguez, P.C. Arginase, Prostaglandins, and Myeloid-Derived Suppressor Cells in Renal Cell Carcinoma. *Clin. Cancer Res.* **2007**. [CrossRef] [PubMed]
172. Mao, Y.; Poschke, I.; Wennerberg, E.; de Coaña, Y.P.; Brage, S.E.; Schultz, I.; Hansson, J.; Masucci, G.; Lundqvist, A.; Kiessling, R. Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. *Cancer Res.* **2013**, *73*, 3877–3887. [CrossRef] [PubMed]
173. Kuroda, H.; Mabuchi, S.; Yokoi, E.; Komura, N.; Kozasa, K.; Matsumoto, Y.; Kawano, M.; Takahashi, R.; Sasano, T.; Shimura, K.; et al. Prostaglandin E2 produced by myeloid-derived suppressive cells induces cancer stem cells in uterine cervical cancer. *Oncotarget* **2018**, *9*, 36317–36330. [CrossRef] [PubMed]
174. Komura, N.; Mabuchi, S.; Shimura, K.; Yokoi, E.; Kozasa, K.; Kuroda, H.; Takahashi, R.; Sasano, T.; Kawano, M.; Matsumoto, Y.; et al. The role of myeloid-derived suppressor cells in increasing cancer stem-like cells and promoting PD-L1 expression in epithelial ovarian cancer. *Cancer Immunol. Immunother.* **2020**. [CrossRef]
175. Hou, W.; Sampath, P.; Rojas, J.J.; Thorne, S.H. Oncolytic Virus-Mediated Targeting of PGE2 in the Tumor Alters the Immune Status and Sensitizes Established and Resistant Tumors to Immunotherapy. *Cancer Cell.* **2016**. [CrossRef]
176. Yin, J.; Kim, S.S.; Choi, E.; Oh, Y.T.; Lin, W.; Kim, T.H.; Sa, J.K.; Hong, J.H.; Park, S.H.; Kwon, H.J.; et al. ARS2/MAGL signaling in glioblastoma stem cells promotes self-renewal and M2-like polarization of tumor-associated macrophages. *Nat. Commun.* **2020**, *11*. [CrossRef]
177. Chang, J.; Vacher, J.; Yao, B.; Fan, X.; Zhang, B.; Harris, R.C.; Zhang, M.Z. Prostaglandin E receptor 4 (EP4) promotes colonic tumorigenesis. *Oncotarget* **2015**, *6*, 33500–33511. [CrossRef]
178. Heusinkveld, M.; van Steenwijk, P.J.d.; Goedemans, R.; Ramwadhdoebe, T.H.; Gorter, A.; Welters, M.J.P.; van Hall, T.; van der Burg, S.H. M2 Macrophages Induced by Prostaglandin E 2 and IL-6 from Cervical Carcinoma Are Switched to Activated M1 Macrophages by CD4 + Th1 Cells. *J. Immunol.* **2011**, *187*, 1157–1165. [CrossRef]
179. Oshima, H.; Hioki, K.; Popivanova, B.K.; Oguma, K.; van Rooijen, N.; Ishikawa, T.; Oshima, M. Prostaglandin E2 2 signaling and bacterial infection recruit tumor-promoting macrophages to mouse gastric tumors. *Gastroenterology* **2011**, *140*, 596–607.e7. [CrossRef]

180. Prima, V.; Kaliberova, L.N.; Kaliberov, S.; Curiel, D.T.; Kusmartsev, S. COX2/mPGES1/PGE2 pathway regulates PD-L1 expression in tumor-associated macrophages and myeloid-derived suppressor cells. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 1117–1122. [CrossRef]
181. Huang, L.; Nakamura, Y.; Lo, E.H.; Hayakawa, K. Astrocyte Signaling in the Neurovascular Unit After Central Nervous System Injury. *Int. J. Mol. Sci.* **2019**, *20*, 282. [CrossRef] [PubMed]
182. Waisman, A. Dendritic cells as gatekeepers of tolerance. *Semin. Immunopathol. Vol.* **2017**, *39*, 153–163. [CrossRef] [PubMed]
183. Böttcher, J.P.; Bonavita, E.; Chakravarty, P.; Blees, H.; Cabeza-Cabrerizo, M.; Sammicheli, S.; Rogers, N.C.; Sahai, E.; Zelenay, S.; e Sousa, C.R. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell* **2018**, *172*, 1022–1037.e14. [CrossRef] [PubMed]
184. Ogawa, F.; Amano, H.; Eshima, K.; Ito, Y.; Matsui, Y.; Hosono, K.; Kitasato, H.; Iyoda, A.; Iwabuchi, K.; Kumagai, Y.; et al. Prostanoid induces premetastatic niche in regional lymph nodes. *J. Clin. Investig.* **2014**, *124*, 4882–4894. [CrossRef] [PubMed]
185. Youlin, K.; Weiyang, H.; Simin, L.; Xin, G. Prostaglandin E 2 Inhibits Prostate Cancer Progression by Countervailing Tumor Microenvironment-Induced Impairment of Dendritic Cell Migration through LXR α/CCR7 Pathway. *J. Immunol. Res.* **2018**, *2018*, 5808962. [CrossRef] [PubMed]
186. Caligiuri, M.A. Human natural killer cells. *Blood* **2008**, *112*, 461–469. [CrossRef]
187. Fruci, D.; Monaco, E.L.; Cifaldi, L.; Locatelli, F.; Tremante, E.; Benevolo, M.; Giacomini, P. T and NK cells: Two sides of tumor immunoevasion. *J. Transl. Med.* **2013**, *11*, 30. [CrossRef]
188. Fulton, A.M.; Chong, Y.C. Prostaglandin E2 receptor activity and susceptibility to natural killer cells. *J. Leukoc. Biol.* **1992**, *51*, 176–180. [CrossRef]
189. Martinet, L.; Jean, C.; Dietrich, G.; Fournié, J.J.; Poupot, R. PGE2 inhibits natural killer and $\gamma\delta$ T cell cytotoxicity triggered by NKR and TCR through a cAMP-mediated PKA type I-dependent signaling. *Biochem. Pharmacol.* **2010**, *80*, 838–845. [CrossRef]
190. Pietra, G.; Manzini, C.; Rivara, S.; Vitale, M.; Cantoni, C.; Petretto, A.; Balsamo, M.; Conte, R.; Benelli, R.; Minghelli, S.; et al. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res.* **2012**, *72*, 1407–1415. [CrossRef]
191. Asghar, K.; Farooq, A.; Zulfiqar, B.; Rashid, M.U. Indoleamine 2,3-dioxygenase: As a potential prognostic marker and immunotherapeutic target for hepatocellular carcinoma. *World J. Gastroenterol.* **2017**, *23*, 2286–2293. [CrossRef] [PubMed]
192. Park, A.; Lee, Y.; Kim, M.S.; Kang, Y.J.; Park, Y.J.; Jung, H.; Kim, T.D.; Lee, H.G.; Choi, I.; Yoon, S.R. Prostaglandin E2 secreted by thyroid cancer cells contributes to immune escape through the suppression of natural killer (NK) cell cytotoxicity and NK cell differentiation. *Front. Immunol.* **2018**, *9*, 1859. [CrossRef] [PubMed]
193. Li, T.; Yi, S.; Liu, W.; Jia, C.; Wang, G.; Hua, X.; Tai, Y.; Zhang, Q.; Chen, G. Colorectal carcinoma-derived fibroblasts modulate natural killer cell phenotype and antitumor cytotoxicity. *Med. Oncol.* **2013**, *30*. [CrossRef]
194. Holt, D.M.; Ma, X.; Kundu, N.; Collin, P.D.; Fulton, A.M. Modulation of Host Natural Killer Cell Functions in Breast Cancer via Prostaglandin E2 Receptors EP2 and EP4. *J. Immunother.* **2012**, *35*, 179–188. [CrossRef]
195. Holt, D.; Ma, X.; Kundu, N.; Fulton, A. Prostaglandin E2 (PGE2) suppresses natural killer cell function primarily through the PGE2 receptor EP4. *Cancer Immunol. Immunother.* **2011**, *60*, 1577–1586. [CrossRef]
196. Kundu, N.; Ma, X.; Holt, D.; Goloubeva, O.; Ostrand-Rosenberg, S.; Fulton, A.M. Antagonism of the prostaglandin E receptor EP4 inhibits metastasis and enhances NK function. *Breast Cancer Res. Treat.* **2009**, *117*, 235–242. [CrossRef]
197. Ma, X.; Holt, D.; Kundu, N.; Reader, J.; Goloubeva, O.; Take, Y.; Fulton, A.M. A prostaglandin E (PGE) receptor EP4 antagonist protects natural killer cells from PGE2-mediated immunosuppression and inhibits breast cancer metastasis. *Oncoimmunology* **2013**, *2*, e22647. [CrossRef]
198. Harizi, H. Reciprocal crosstalk between dendritic cells and natural killer cells under the effects of PGE2 in immunity and immunopathology. *Cell. Mol. Immunol.* **2013**, *10*, 213–221. [CrossRef]
199. Kondo, M. One Niche to Rule Both Maintenance and Loss of Stemness in HSCs. *Immunity* **2016**, *45*, 1177–1179. [CrossRef]

200. Shimabukuro-Vornhagen, A.; Liebig, T.M.; Koslowsky, T.; Theurich, S.; von Bergwelt-Baildon, M.S. The ratio between dendritic cells and T cells determines whether prostaglandin E2 has a stimulatory or inhibitory effect. *Cell. Immunol.* **2013**, *281*, 62–67. [CrossRef]
201. Basingab, F.S.; Ahmadi, M.; Morgan, D.J. IFNg-Dependent Interactions between ICAM-1 and LFA-1 counteract prostaglandin E2-mediated inhibition of antitumor CTL responses. *Cancer Immunol. Res.* **2016**, *4*, 400–411. [CrossRef] [PubMed]
202. Hennequart, M.; Pilotte, L.; Cane, S.; Hoffmann, D.; Stroobant, V.; de Plaen, E.; van den Eynde, B.J. Constitutive IDO1 expression in human tumors is driven by cyclooxygenase-2 and mediates intrinsic immune resistance. *Cancer Immunol. Res.* **2017**, *5*, 695–709. [CrossRef] [PubMed]
203. Sharma, S.; Yang, S.C.; Zhu, L.; Reckamp, K.; Gardner, B.; Baratelli, F.; Huang, M.; Batra, R.K.; Dubinett, S.M. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+CD25+ T regulatory cell activities in lung cancer. *Cancer Res.* **2005**, *65*, 5211–5220. [CrossRef] [PubMed]
204. Kim, I.K.; Koh, C.H.; Jeon, I.; Shin, K.S.; Kang, T.S.; Bae, E.A.; Seo, H.; Ko, H.J.; Kim, B.S.; Chung, Y.; et al. GM-CSF promotes antitumor immunity by inducing Th9 cell responses. *Cancer Immunol. Res.* **2019**, *7*, 498–509. [CrossRef]
205. Yu, M.; Guo, G.; Zhang, X.; Li, L.; Yang, W.; Bollag, R.; Cui, Y. Fibroblastic reticular cells of the lymphoid tissues modulate T cell activation threshold during homeostasis via hyperactive cyclooxygenase-2/prostaglandin E2 axis. *Sci. Rep.* **2017**, *7*, 3350. [CrossRef] [PubMed]
206. Gorchs, L.; Moro, C.F.; Bankhead, P.; Kern, K.P.; Sadeak, I.; Meng, Q.; Rangelova, E.; Kaipe, H. Human pancreatic carcinoma-associated fibroblasts promote expression of co-inhibitory markers on CD4+ and CD8+ T-cells. *Front. Immunol.* **2019**, *10*. [CrossRef]
207. Mizuno, R.; Kawada, K.; Sakai, Y. Prostaglandin E2/EP signaling in the tumor microenvironment of colorectal cancer. *Int. J. Mol. Sci.* **2019**, *20*, 6254. [CrossRef]