UNIVERSITAT DE BARCELONA

New targets in tumor angiogenesis to block tumor re-growth and therapeutic resistance

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Facultat de Farmacia

Programa de Doctorat en Biomedicina

NEW TARGETS IN TUMOR ANGIOGENESIS TO BLOCK TUMOR RE-GROWTH AND THERAPEUTIC RESISTANCE.

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NEW TARGETS IN TUMOR ANGIOGENESIS TO BLOCK TUMOR RE-GROWTH AND THERAPEUTIC RESISTANCE.

Doctoral Thesis of Nicklas Bassani
University of Barcelona

This thesis has been done under the supervision of Dr. Oriol Casanovas Casanovas in the Laboratori de Recerca Translacional 1, part of the Institut Catala d'Oncologia (ICO) and of the Institut d'Investigació Biomèdica de Bellvitge.

Barcelona, October 2017

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Introducion.

1. Angiogenesis.

The formation of new blood vessels is a fundamental process that occurs during the embryonic and post-natal development, indispensable to allow immune surveillance by the hematopoietic cells, oxygen and nutrients supply or to dispose of waste. However, although beneficial for tissue growth and regeneration, vessels can also fuel inflammatory and malignant diseases, are exploited by tumor cells to promote cancer growth and metastasize and deviation from normal vessel growth contribute to numerous diseases. Indeed, insufficient vessel growth or maintenance can lead to stroke, myocardial infraction, ulcerative disorders and neurodegeneration, whereas abnormal vessel growth or remodeling fuel cancer, inflammatory disorders, pulmonary hypertension and blinding eye diseases (Folkman, 2007; Carmeliet, 2003).

Even if several modes of vessel formation have been identified, schematically it could be divided into three processes: vasculogenesis, angiogenesis and arteriogenesis (Fig. 1).

Vasculogenesis is the *in situ* differentiation of undifferentiated precursors, called angioblasts, into endothelial cells that assemble into a vascular labyrinth (Risau, 1997). It occurs in the yolk sac during embryo formation, where vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2) and basic fibroblast growth factor (bFGF) promote angioblast differentiation, whereas VEGFR1 suppresses their commitment (Ferrara, 1999).

Angiogenesis is defined as the sprouting of new capillaries form a pre-existing vessel resulting in a new capillary network and is one, but not the only, mechanisms of blood vessel formation in the adult. In healthy adults, endothelial cells are normally in long-life quiescent state, covered by pericytes which suppress their proliferation and protect against insults by releasing VEGF, angiopoietin-1 (ANG-1) and fibroblast growth factors (FGFs), and usually form a monolayer of phalanx cells interconnected by junctional molecules, such as vascular-endothelial (VE)-cadherin and claudins (Carmeliet and Jain, 2011).

Since effective oxygen diffusion is limited to short distances, endothelial cells are equipped with oxygen sensors, such as prolyl hydroxylase domain 2 (PHD2), and hypoxia-inducible factors (HIFs) which allow the vessels to re-adjust their shape and optimize the blood flow to increase perfusion and maintain or restore local oxygen and nutrient supply. In fact, when a quiescent vessel senses angiogenic signals, such as VEGF, FGFs or chemokine released by hypoxic or inflammatory cells, took place a multi-steps process characterized by vasodilation, endothelial permeability and differentiation ending in new branching and lumen formation. First, in response to angiopoietin-2 (ANG-2) pericytes detach from the vessel wall and liberate themselves from the basement membrane by matrix metalloproteinases (MMPs)-mediated proteolytic degradation. Consequently, endothelial cells lose their junction, the vessels dilate and, in response to VEGF, increase the cell layer permeability causing plasma proteins to extravasate and lay down a provisional extracellular matrix (ECM) scaffold (Carmeliet and Jain, 2011). In response to integrin signaling, endothelial cells migrate into this ECM surface which is remodeled into an angio-competent milieu by the action of proteases which liberate the angiogenic molecules stored inside. Next, a very important process occurs: to build a perfused tube and prevent endothelial cells to migrate in masse towards the angiogenic signal, one endothelial cell, known as tip cell, is selected in response to the NOTCH ligands DLL4 and JAGGED1, and neuropilins (NRPs), become equipped with filopodia to sense the environment and lead the vessel elongation (Fig. 2A). Consequently, the neighbors stalk cells stimulated by NOTCH, WNTs, placental growth factor (PlGF) and FGFs divide to elongate the stalk and establish the lumen (Fig. 2B). Finally, myeloid bridge cells aid fusion between vessels branch allowing the initiation of blood flow, and endothelial cells resume their quiescent phalanx state and, in response to platelet-derived growth factor B (PDGF-B), ANG-1, transforming growth factor-β (TGF-β) and ephrin-B2, become covered by pericytes (Fig. 2C). Moreover, the tissue inhibitor

of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) cause the deposition of a basement membrane, help to re-established junctions to ensure an optimal flow distribution (Carmeliet and Jain, 2011).

Arteriogenesis describes the growth of functional collateral arteries from pre-existing arterio-arteriolar anastomoses after stenosis or the occlusion of a major artery (Heil et al., 2006).

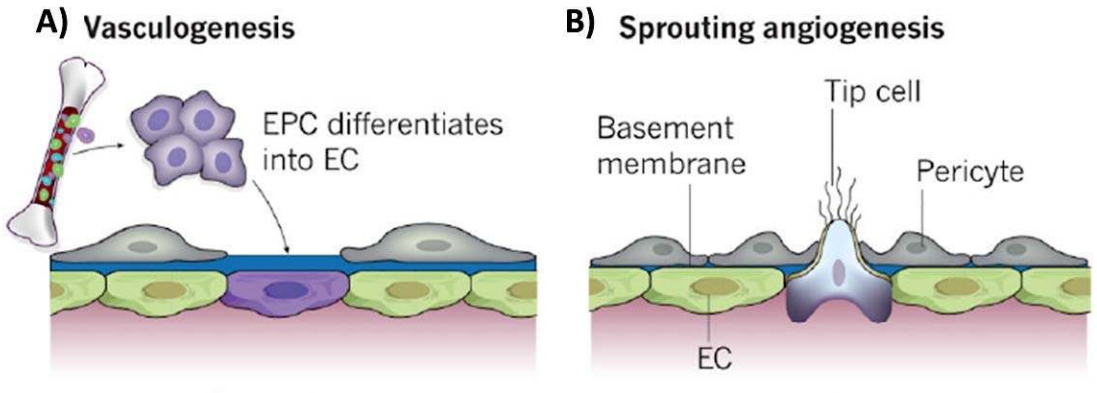

Fig. 1. **Methods of blood vessel formation.** Vessel formation can occur by the recruitment of bone-marrow-derived and/or vascular-wall-resident endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs) (A), or by sprouting angiogenesis (B). Adapted from Carmeliet and Jain, 2011.

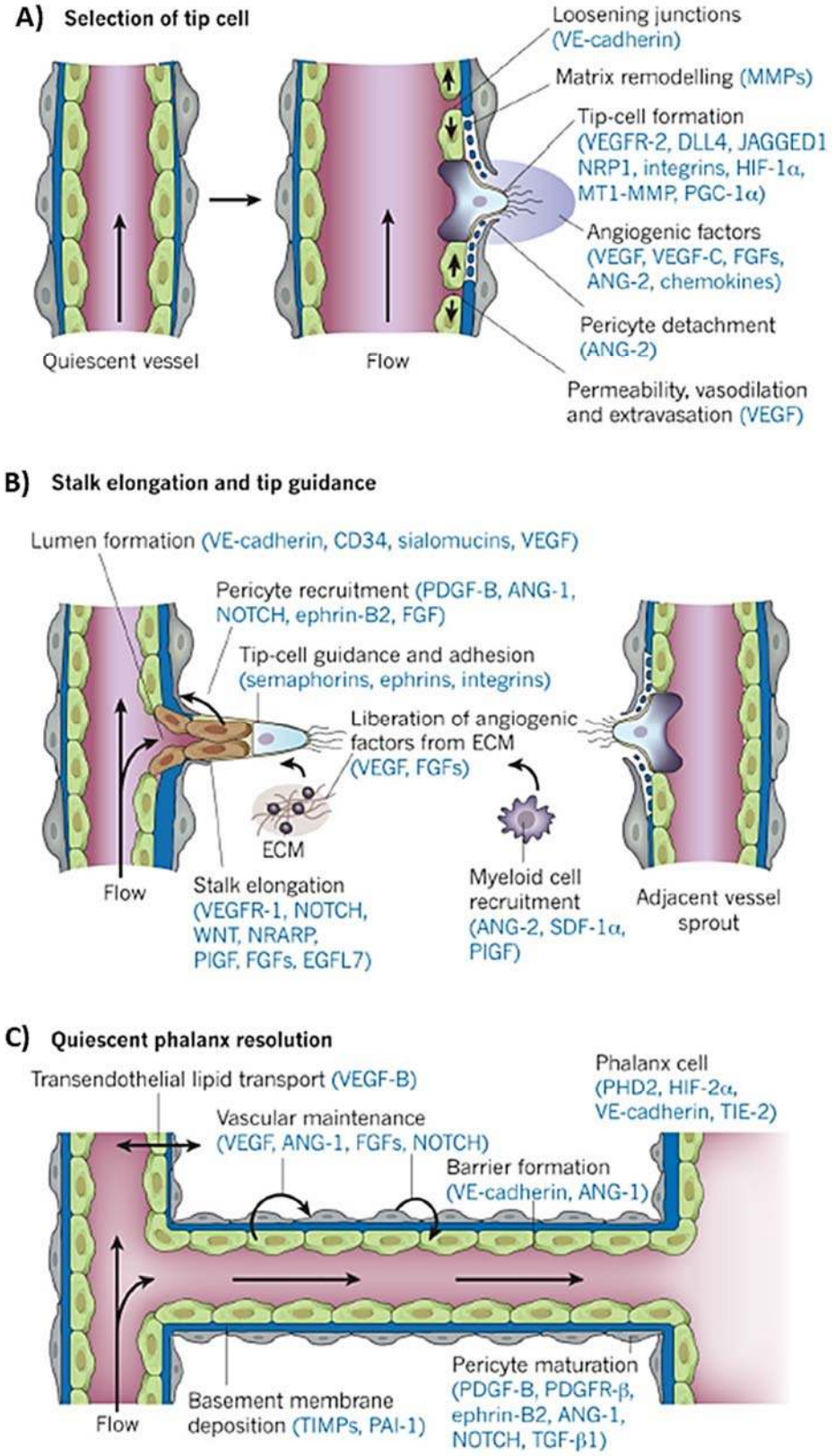

Fig. 2. Molecular basis of sprouting angiogenesis. Vessel branching consisting in the different consecutive steps of tip cells selection (A), stalk elongation and tip guidance (B) and quiescent phalanx resolution (C) are shown with the key molecular players involved in each phase. Adapted from Carmeliet and Jain 2011.

1.1. Angiogenesis in cancer.

The introduction of the concept of angiogenesis in tumors goes back to the pioneering work of Ide and colleagues, who in 1939 described for the first time that tumors implanted into the ears of rabbits were accompanied by the formation of new capillaries, but it was not until the seventies that the subject started to be systematically studied by Judah Folkman (Ide et al., 1939; Folkman, 1971). Like normal tissues, tumors growth depend on oxygen and nutrients as well as the ability to eliminate wastes and carbon dioxide, and since passive tissue diffusion is effective only for few millimeters, tumor-associated angiogenesis is indispensable to address these needs (Hanahan and Weinberg, 2011). As previously described, during embryogenesis the development of the vasculature involved the birth of new endothelial cells and their assembly into tubes (vasculogenesis) and the sprouting of new vessels from existing ones (angiogenesis), but following morphogenesis the normal vasculature becomes largely quiescent. Indeed, endothelial cells are among the longest-lived in the body, only 1 every 10,000 endothelial cells (0.01%) is in cell division cycle at any given time, and exclusively in response to an appropriate stimulus as part of physiological processes such as wound healing and female reproductive cycling, the quiescent vasculature can be transiently turn on to grow new capillaries (Engerman et al., 1967). In contrast, during tumor progression an “angiogenic switch” is almost constantly activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help to sustain the expanding neoplastic growth (Hanahan and Folkman, 1996). Moreover, blood and lymphatic vessels are used by tumor cells to circulate, spread and metastasize to different organs. It’s consequently not surprising that in the last decades the characterization of the molecular pathways underling tumor angiogenesis has been a central goal in oncology and different inducers and inhibitors have been identified. Emerging evidences indicate that angiogenesis is regulated by both activator and inhibitors, and the up-regulation of the activity

of angiogenic factors is itself not sufficient to turn on the quiescent vasculature, but only changes in the balance of positive and negative signals mediate the angiogenic switch. A net balance of inhibitors over activators would maintain the switch in the off position, whereas a shift to an excess of activating stimuli would turn on angiogenesis (Fig. 3) (Hanahan and Folkman, 1996). Classically, the well-known prototypes of inducers and inhibitors are respectively vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1).

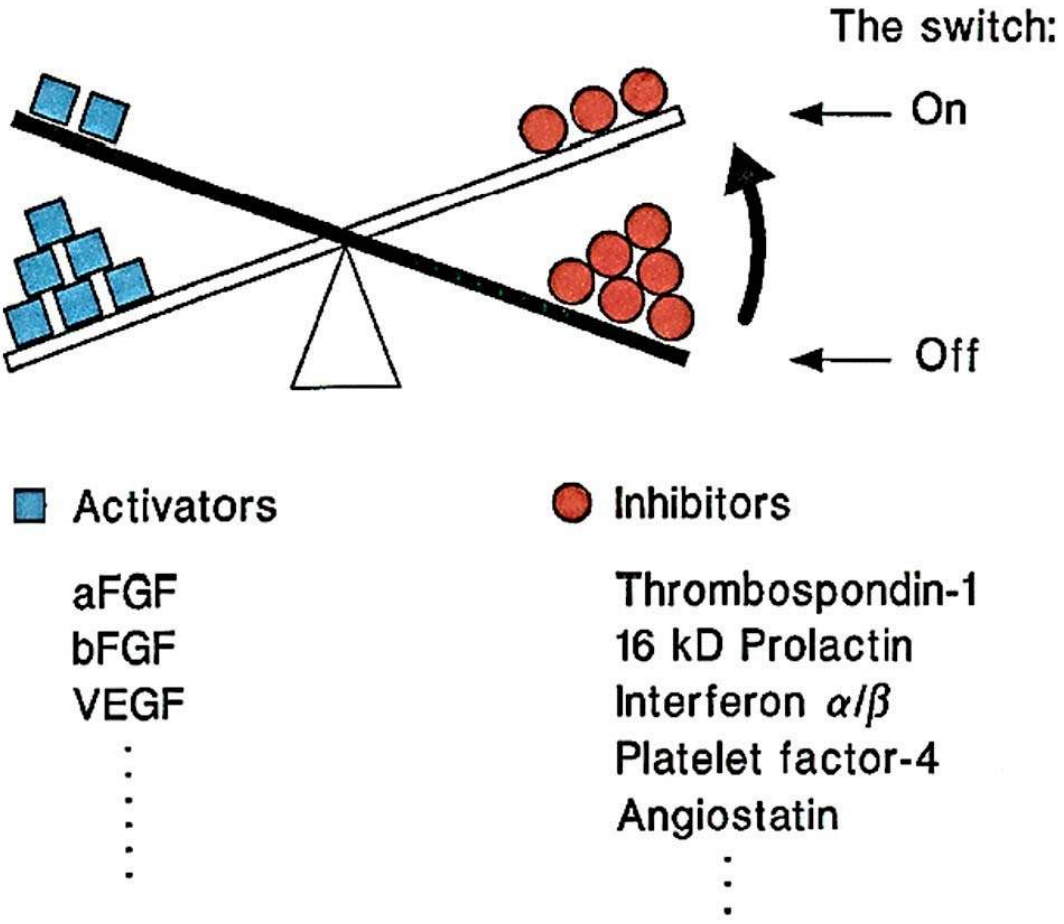

Fig. 3. The balance hypothesis for the angiogenic switch. The normally quiescent vasculature can be activated to sprout new capillaries (angiogenesis) only by chances in the balance of inducers over inhibitors of angiogenesis. Adapted from Hanahan and Folkman, 1996.

1.2. Activators of Angiogenesis.

More than a dozen different proteins have been identified as angiogenic activators, including VEGF, basic fibroblast growth factor (bFGF), transforming

growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, platelet-derived endothelial growth factor (PDGF), granulocyte colony-stimulating factor (G-CSF), placenta growth factor (PlGF), angiopoietins, interleukine-8 (IL-8), hepatocyte growth factor (HGF) and epidermal growth factor (EGF). Some of them will be described in more details in the following paragraphs.

1.2.1. The VEGF family.

VEGF-A, also known as vascular permeability factor (VPF), is the key member of a large family of polypeptides, including VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placenta growth factor (PlGF), all related to platelet-derived growth factor (PDGF) (Dvorak et al., 1995; Ferrara, 2009). Being a powerful angiogenic agent in normal and neoplastic tissues, VEGF expression can be upregulated both by hypoxia via hypoxia-inducible factor-1α (HIF-1α) or by oncogenes, and binding as functional homo- or heterodimer three different tyrosine kinase receptors (VEGFR-1-3) and one co-receptor (Neuropilin-1), it controls vascular processes (Ferrara, 2009). Specifically, VEGFR-1 binds VEGF-A, -B and PlGF, VEGFR-2 interacts mainly with VEGF-A and –E, while VEGFR-3 is exclusive for VEGF-C and –D. Neuropilin-1 (NRP1), originally described as a semaphorin receptor involved in neuronal guidance, acts as a co-receptor for VEGFR-2 and bind only certain VEGF-A splicing isoforms (Ferrara, 2009). The crucial role of VEGF/VEGF-A is underscored by profound vascular defects ending in embryonic lethality in mouse embryos lacking even a single *vegf* allele (haploinsufficiency) (Carmeliet et al., 1996; Ferrara, 2009). Indeed, VEGF-A acts as a potent mitogen, motility, and survival factor for ECs, and as chemoattractant for their progenitors (Ferrara, 2009). These powerful influences are regulated by alternative splicing of the VEGF-A transcript resulting in several protein isoforms, named according to the number of their constituent amino acids as VEGF121 (121 amino acids), VEGF145, VEGF165, VEGF189, VEGF206, and showing reduced heparin binding and association with both cellular membranes

and the ECM, and increased solubility, depending on their size (Ferrara, 2009). Thus, the shortest VEGF121 isoform is the highly soluble, while VEGF189 is mostly cell bound, and VEGF165 expressing intermediate proprieties is the one with the greatest angiogenic activity. Notably, the splicing process can also result in the expression of an alternative ligand endowed with antagonist anti-angiogenic activity, such as VEGF165b (Nowak et al., 2008.). The remaining VEGF family's members play minor roles in vascular process and germline deletions of the respective genes do not have haploinsufficient consequences. In fact, while VEGF-B is widely expresses in the heart and skeletal muscle providing additional survival protection to ECs, VEGF-C and –D expression appear to be restricted to the early development where binding VEGFR-3 control lymphangiogenesis (Bellomo et al., 2000; Baldwin et al., 1998).

1.2.2. PDGF Isoforms and Their Receptors.

The PDGF family include four different polypeptides (PDGF-A, -B, -C, and –D) inactive in their monomeric form, that become active and produce their biological effects building disulphide-linked dimers (Fredriksson et al., 2004; Chen et al., 2013). Till now, four homodimers (PDGF-AA, -BB, -CC and –DD) and one heterodimer (PDGF-AB), have been identified and described to promote angiogenesis by specific binding to the homodimeric and heterodimeric PDGF receptors -αα, -ββ and -αβ (Tallquist and Kazlauskas, 2004). Among them, homodimeric PDGFR-αα is activated by all PDGF ligands except PDGF-DD, PDGFR-αβ by all isoforms with the exception of PDGF-AA, while the activation of PDGFR-ββ occurs only by binding of PDGF-BB and PDGF-DD, exerting overlapping biological function (Cao, 2013). In fact, it is difficult to separate the signaling pathways because all activated receptors initiate a complex Ras/MAP-kinase signaling cascade which is responsible of the transcription of genes involved in angiogenesis, proliferation, invasion and metastasis (Cao, 2013).

1.2.3. Fibroblast Growth Factors.

Acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (bFGF, FGF-2) signaling network paly a ubiquitous role in normal cell growth, survival, differentiation and angiogenesis, but have also been implicated in tumor development. In fact, it has been shown that upon binding their cell surface tyrosine kinase receptors (FGFR), act synergistically with VEGF to amply tumor angiogenesis or promote autocrine and paracrine survival of malignant tumors, often resulting in chemotherapy resistance (Kork and Friesel, 2009; Lindner et al., 1990).

1.2.4. Transforming Growth Factor-β.

One of the most interesting paradoxes regarding tumor growth factors involves TGF-β. In fact, it has been shown that low concentration of tumor-secreted TGF-β triggers both VEGF and bFGF expression in stromal fibroblast and tumor cells stimulating angiogenesis. Conversely, when expressed at higher levels, TGF-β may act directly on endothelial cells inhibiting their proliferation and consequently angiogenesis (Borgi et al., 1994; Pertovaara et al., 1994). Moreover, to confirm how tightly tuned is angiogenesis, TGF-β was also found to stimulate the expression of thrombospondin-1 (Tsp-1) and, in turn, is activated by Tsp-1 (Penttinen et al., 1988). Intriguingly, TGF-β accomplished these diverse activities through the activation of different transcription factors: while the induction of Tsp-1 expression is achieved via the activation of Smad2, stimulation of VEGF is mediated by the activation of Smad3. Thus, depending on the contextual environment within the tumor-associated stroma, TGF-β can stimulate the expression of VEGF, Tsp-1 or both proteins (Nakagawa et al., 2004).

1.2.4. The Notch Pathway.

The Notch pathway plays a central role in cell proliferation, differentiation, development and homeostasis, and activating mutation or amplification that

contribute to tumor progression are commonly found in human cancer (Ranganathan et al., 2011). Consisting in a family of four related proteins (Notch 1 to 4), Notch receptors are single-pass transmembrane receptors that interact with 1 of 5 membrane-bound ligands: Jagged 1 and 2, and Delta-likes 1, 3 and 4. At the cell surface, Notch is presented as an extracellular and intracellular heterodimeric receptor held together by non-covalent bonds. Ligand binding to the extracellular peptide alters the receptor confirmation, leading to two proteolytic cleavages resulting in the release and translocation into the nucleus of the Notch Intracellular Domain (NICD) where directly or indirectly regulates a cohort of genes involved in epithelial to mesenchymal transition (EMT) and angiogenesis, like VEGF/VEGFR expression or EphrineB2 (Capaccione and Pine, 2013; Andersson and Lendahl, 2014).

1.2.5. Matrix Metalloproteinases.

The dissolution of the basement membrane and the consequent release of VEGF and other growth factors from their ECM stores, are indispensable processes during blood vessel formation and involve various classes of proteases. Moreover, local invasion across a degraded basement membrane and within the tissue microenvironment is a critical step in tumor progression and metastasis (Watnick, 2012). The main matrix metalloproteinases (MMPs) involved in tumor angiogenesis are MMP-1, -2, -9 and -14. Notably, in addition to cleaving matrix proteins, MMP-9 has also been shown to be cleaved and convert TGF-β in its active form promoting tumor growth in mammary tumor model (Yu and Stamenkovic, 2000).

1.3. Endogenous Inhibitors of Angiogenesis.

The endogenous inhibitors of angiogenesis are thought to maintain blood vessels in their quiescent state, or limit the magnitude of angiogenic responses both locally and systemically. Belonging to different classes of molecules, including ECM proteins (thrombospondin 1 and 2), their proteolytic fragments

(endostatin, tumstatin and arresten), fragments of enzymes (angiostatin and PEX domain), fragments of coagulation-related chemokines (such as platelet factor 4 (PF4)), hormones and cytokine (including prolactin 16-kDa fragment, interferons α, β and γ), these endogenous inhibitors orchestrate the formation of a barrier to counteract the effects of physiologic levels of proangiogenic growth factors, and might constitute a critical line of defense against the conversion of dormant neoplastic events into a malignant cancer (Folkman and Kalluri, 2003). Recently it has been suggested that genetic defects in the sequences of proteins or enzymes that generate angiogenic inhibitors could determine how fast a neoplastic event switch from a non-lethal lesion to a malignant angiogenic tumor, and the potential therapeutic application of these endogenous inhibitors started to be considered in the clinic (Ryan et al., 1999, Thomas et al., 2003; Xiao et al., 2002).

1.3.1. Thrombospondins.

Thrombospondin-1 (TSP-1) is a large multifunctional ECM glycoprotein regulating various biological events, including cell adhesion, proliferation and survival, or TGF-β activation, and was the first protein to be recognized as a naturally occurring inhibitor of angiogenesis (Good et al., 1990). In fact, it has been shown that overexpression of TSP-1 in mice suppresses wound healing and tumorigenesis, whereas the lack of functional TSP-1 results in increased vascularization. Moreover, expression of TSP-1 has been inversely correlates with malignant progression of breast and lung carcinomas and melanomas (Streit et al., 1999; Zebrenetzky et al., 1994). Functionally, TSP-1 is able to distinguish pathologic neovascularization from preexisting vasculature and promotes the expression of FasL expression on proliferating endothelial cells resulting in Fas/FasL mediated apoptosis (Volpert et al., 2002).

Thrombospondin-2 (TSP-2) also shows anti-angiogenic activity probably inhibiting VEGF-induced endothelial cell migration and tube formation. In fact,

extensive areas of necrosis, reduced vessels density and size were observed in TSP-2-expressing tumors, whereas tumor angiogenesis was significantly enhanced in TSP-2-deficient mice (Streit et al., 1999; Hawighorst et al., 2001).

1.3.2. Endostatin.

Endostatin corresponds to a 20-kDa fragment derived from the COOH-terminal NC1 domain of type XVIII collagen, and has been shown to efficiently block angiogenesis and suppress primary tumor growth and metastasis in experimental animal models (O'Reilly et al., 1997). In fact, interfering with FGF-2 signal transduction, causing G1 arrest of endothelial cells through inhibition of cyclin D1, blocking VEGF signaling via direct interaction with the VEGFR-2/Flk-1 receptor tyrosine kinase or inhibiting the activation of MMP-2 and -9, recombinant endostatin has been shown to inhibit tumor growth (Dixelius et al., 2002; Hanai et al., 2002; Kim et al., 2002).

1.3.3. Angiostatin.

Cleavage of plasminogen by proteases results in the formation of 38- to 45-kDa anti-angiogenic peptides, collectively called angiostatin (Cornelius et al., 1998). Several members of the human MMP family, including MMP-7, gelatinase A and B or MMP-12 have been reported to hydrolyze human plasminogen to generate angiostatin fragments, and several targets of angiostatin action have been proposed. For instance, angiostatin could directly bind the ATP synthase on the surface of endothelial cells, causing the drop of intracellular pH and leading to endothelial cell apoptosis. Moreover, it has been shown that angiostatin may interfere with the binding of plasmin to $\alpha_v\beta_3$ integrin blocking endothelial cell migration (Moser et al., 1999, Tarui et al., 2002).

1.3.4. Platelet Factor-4.

Released by platelet α-granules during platelet aggregation, PF-4 is a protein that has been shown to inhibit angiogenesis by associating directly with FGF-2, blocking its dimerization and binding to endothelial cells (Maione et al., 1990).

1.3.5. Interleukins and Interferons.

Interleukins are a family of leukocyte-derived proteins displaying broad-ranging effects on multiple physiologic events, including angiogenesis. In fact, while IL-8 tends to be mainly pro-angiogenic, IL-1, mainly secreted as IL-1β, has been shown to inhibit FGF-stimulated angiogenesis by an autocrine pathway (Cozzolino et al., 1990). Interferons are pleiotropic cytokines that binding cell surface receptors and initiating the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway regulate antiviral, antitumor, apoptotic and cellular immune responses. Among the different roles, it has been shown that low doses of IFN-α significantly reduces the secretion of IL-8 by tumor cells, and MMP-9 enzymatic activity and protein expression (Lingen et al., 1998; Ma et al., 2001). Moreover, IFN-α and -β treatment also inhibit angiogenesis directly down-regulating bFGF expression (Dinney et al., 2001).

Even if tumor angiogenesis is a well-established hallmark of cancer (Hanahan and Weinberg, 2011), it doesn't have the same impact in all tumor types and among them kidney cancer is surely one of the most vascularized.

2. Kidney Cancer.

Kidney cancer, usually named renal cell carcinoma (RCC), is the most lethal of all urologic conditions. Accounting for almost 2% of all cancers, approximately 295,000 new cases are diagnosed and 134,000 deaths are recorded annually worldwide. RCC incidence is commonly higher in developed countries, increases markedly with age, with men more affected than women (a 2:1 ratio of new

cases), and excess body weight, hypertension or cigarette smoking are considered the major risk factors for RCC (Hsieh et al., 2017).

Kidney cancer can occur in an inherited or in a sporadic no-inherited form and genetic and clinical studies during the past two decades have shown the clinico-pathologically heterogeneous nature of this disease. It comprises a number of different cancers that occur in this organ, each characterized by a unique histology, clinical course and alterations in different genes (Fig. 4; Tables 1,2) (Linehan et al., 2004). For these reasons big efforts were put into the characterization of the different subtypes, that lead the World Health Organizations (WHO) to classify the renal neoplasm into clear cell (ccRCCs) (accounting for almost 70% of all RCCs), papillary RCC (10 – 15%), chromophobe (4 – 6%), collecting duct carcinoma and medullary carcinoma (less than 1%) or unclassified categories (Table 1 and 2) (Prasad el al., 2006).

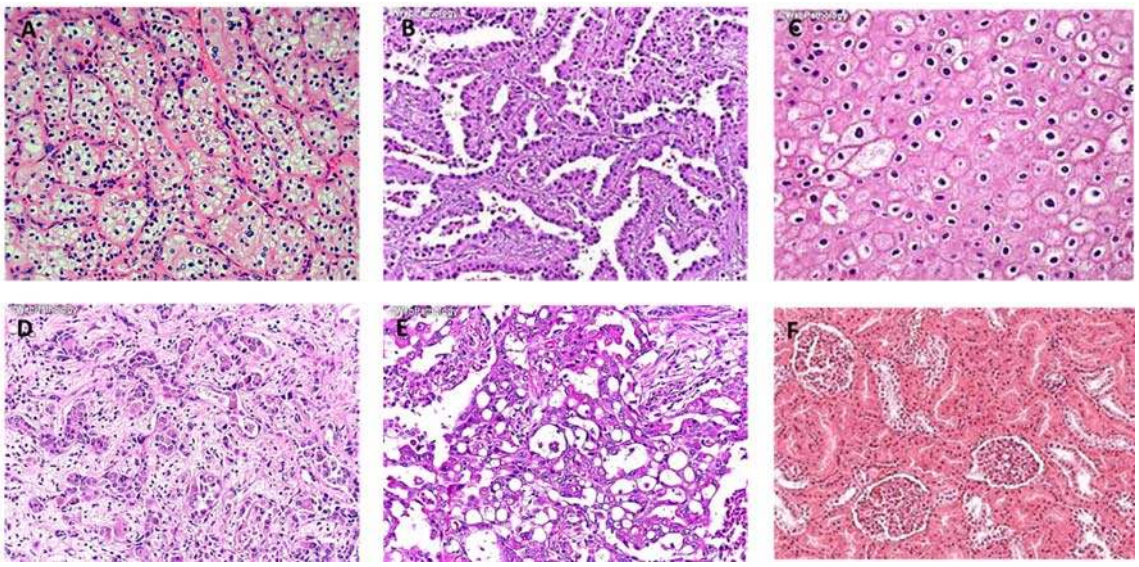

Fig. 4. Histological subtypes of renal cell carcinoma (A-E) and normal kidney (F). A) Clear cell RCC. B) Papillary RCC. C) Chromophobe RCC. D) Collecting duct carcinoma. E) Medullary carcinoma. F) Normal kidney tissue. Pictures adapted from pathologyoutlines.com.

Histologic Subtype	Prevalence (%)	Putative Cell of Origin
Clear cell RCC	70	Epithelium of proximal convoluted tubule
Papillary RCC	10	Epithelium of proximal convoluted tubule
Chromophobe RCC	5	Cortical collecting duct, type B intercalated c cell
Collecting duct carcinoma	<1	Medullary collecting duct
Medullary carcinoma	<1	Medullary collecting duct
Hereditary cancer syndromes	5	…
Unclassified lesions	4	…

Table 1. 2004 WHO Histologic Classification of RCC.

Histologic Subtype	Cytogenetic Findings
Clear cell RCC	3p deletions, von Hippel-Lindau gene mutations
Papillary RCC	Trisomy of chromosomes 7 and 17, loss of Y chromosome
Hereditary papillary RCC syndrome	7q34 chromosome abnormality
Chromophobe RCC	Loss of multiple chromosomes: 1, 2, 6, 10, 13, 17, 21
Collecting duct carcinoma	Loss of multiple chromosomes: 1, 2, 14, 15 22; gain of chromosome 3
Medullary carcinoma	Extracellular matrix gene loss

Table 2. Cytogenetic Findings in Histologic Subtypes of RCC.

2.1.1. Clear Cell RCC.

Previously referred to as conventional RCC, clear cell RCC is the most common histologic subtype accounting for almost the 70% of all RCCs. It originates from the malignization of the epithelium of the proximal convoluted tubules: the part of the nephron that by exchanging hydrogen ions in the interstitium, for bicarbonate ions in the filtrate, regulates the pH of the filtrate (Polascik et al., 2002).

Extremely rich in intracytoplasmic glycogen and lipids that get dissolved during the histologic processing rendering this cells "clear", clear cell RCC appears heterogeneous at imaging due to the presence of hemorrhage, necrosis and cysts, and a profuse network of small, thin-walled blood vessels is a characteristic diagnostic feature (Fig. 5A,B) (Polascik et al., 2002).

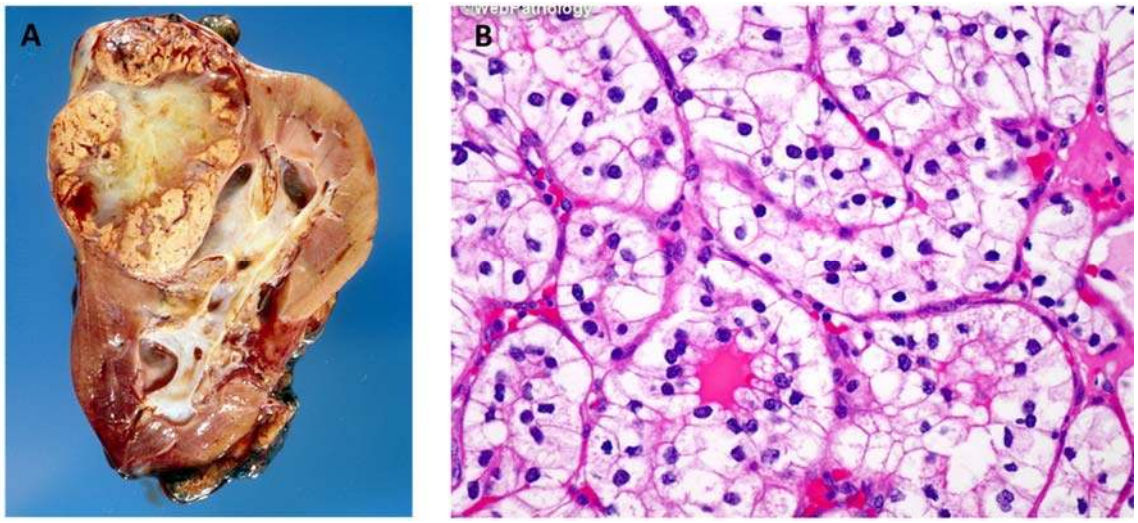

Fig. 5. Gross specimen (A) and photomicrograph of hematoxylin-eosin staining (B) of a clear cell RCC. A) Radical nephrectomy specimen showing a sharply demarcated tumor with golden-yellow color due to abundant lipid content in the tumor cells. The central portion of the tumor shows a scarring with necrosis and hyalinization. B) H&E staining showing clear cells border by a prominent vasculature. Pictures adapted from pathologyoutlines.com.

Clear cell RCC typically exhibits an expansile growth patter, with multicentricity or bilaterality rare and observed only in sporadic cases (less than 5%), and it has been ascribed to inactivating mutation in the von Hippel-Lindau (*VHL*) gene (Linehan et al., 2010). Located on the short arm of chromosome 3, germline

mutations of the *VHL* gene (intragenic, deletions or splicing defects) lead to von Hippel-Lindau syndrome: an inherited disorder characterized by the formation of tumors and fluid-filled sacs (cysts) in many different parts of the body that usually appear during the young adulthood. At a molecular level, patients with VHL disease have inherited a defective *VHL* allele from one of their parents and pathology develops when the remaining wild-type *VHL* allele is mutated, silenced or lost (Kaelin, 2017). People with von Hippel-Lindau syndrome are at an increased risk of developing up to 600 clear cell renal carcinomas per kidney and somatic biallelic inactivating mutations of *VHL* have been reported in up to 91% of patients with sporadic clear cell renal cell carcinoma (Gnarra et al., 1994; Walther et al., 2010). Moreover, mutations in the *VHL* gene are a unique feature of ccRCC and are not shared with any other kidney cancer (Linehan et al., 2004).

The *VHL* gene has the characteristic of a tumor suppressor gene encoding for a protein (pVHL) part of the complex responsible of the oxygen-dependent degradation of Hypoxia-inducible Factor 1α and 2α (HIF1α, -2α).

pVHL is a multifunctional protein that shuttles between the nucleus and cytoplasm and its best-documented function, and the one firmly linked with pathogenesis, relates to its ability to form an ubiquitin ligase complex with Elongin B, Elongin C, Cullin 2 (Cul2) and Ring Box 1 (RBX1) (Lee et al., 1999). pVHL contain two mutational hotspots, the alpha and the beta domain, serving as the substrate recognition unit: in fact, while the alpha domain recruits the Elongins, Cul2 and RBX1, the beta domain works as HIF1α and -2α binding domain (Kealin, 2002). HIF-1 is a heterodimeric transcription factor, composed of HIF-1α or -2α and the constitutively expressed HIF-1β subunit, that mediates response to and it's regulated by hypoxia (Semenza, 2013).

During normaxia, HIF prolyl hydroxylase (PHD) using oxygen, 2-oxoglutarate, ascorbate and Fe^{2+} as cofactors, hydroxylates HIF-α on two critical proline residue (Pro^{402} and Pro^{564}) facilitating the pVHL binding and the consequent HIF-α proteasomal degradation by the E3 ubiquitin ligase complex. Under low

oxygen conditions, prolyl hydroxylation is reduced, the pVHL complex does not bind leading to increased HIF-α stability, accumulation and consequent to dimerization with HIF-1β and association with co-activators results in a complex that is competent to bind the core DNA sequence 5'-RCGTG-3' for transcriptional activation (Fig. 6) (Semenza, 2008). Similarly, when *VHL* gene is mutated, as occur in clear cell carcinoma, pVHL can no longer bind HIF-α resulting in its over accumulation and increased transcription of downstream targets genes, currently stated in more than 800 (i.e. at least 1 out of every 30 human genes) (Linehan et al., 2010; Xia et al., 2009).

Although there is considerable overlap in the genes that are transcriptionally regulated by HIF1α and -2α, in vitro and in vivo studies have indicated that HIF2-α is the critical HIF for tumorigenesis in clear cell kidney cancer. In fact, while downregulation of HIF2-α using specific short hairpin RNA is sufficient to suppress tumor formation by pVHL-defective renal carcinoma cells, forced expression of a HIF2-α variant lacking both of its prolyl hydroxylation/pVHL-binding sites (thereby preventing VHL-mediated ubiquitination) leads to tumor xenograft formation even in VHL-competent cells (Kondo et al., 2003). Consequently, it is not surprising that HIF-1 activity is the earliest sign of pVHL loss of function detected in biopsies from patients with von Hippel-Lindau syndrome and considerable progress has been made in elucidating the molecular mechanism by which HIF-1 activity contributes to the pathogenesis of renal cancer (Mandriota et al., 2002).

Among the first changes that distinguish neoplastic from normal cells are an increased rate of cell proliferation and decreased rate of cell death, due to increased expression of secreted growth/survival factors. Often, the same cells express the cognate membrane receptors for these factors, resulting in autocrine signaling. In ccRCC HIF-1 accumulation determines the expression of transforming growth factor α (TGF-α), which, considering the fact that the epithelial cells target for transformation also express the epidermal growth

factor receptor (EGFR) as a result of pVHL loss of function, promotes dysregulated cell proliferation and survival (Ananth et al., 1999). Metabolic adaptation is another of the first aspects studied in cancer. In the 1920s Warburg noted that glycolysis, which normally increases under anaerobic conditions, was often enhanced in cancers in the presence of abundant oxygen, a phenomenon known as aerobic glycolysis or the "Warburg effect" (Warburg et al., 1927). This switch from oxidative phosphorylation to glycolysis with its concomitant accumulation of lactate by-products in the tumor microenvironment represents the best-known alteration of tumor cell metabolism, and it is thought that the resulting increased acidity in the microenvironment promotes tumor cell adaptation and may spur the evolution of the tumor niche (Gillies et al. 2008). In this facet, HIF-1 mediates the expression of genes encoding the glucose transporters *GLUT1* and *GLUT3* which convert extracellular to intracellular glucose, glycolytic enzymes (ALDOA, ENO1, GAPDH, HK1, HK2, PFKL, PGK1, PKM2) essentials to convert glucose to pyruvate and lactate dehydrogenase A (LDHA) resulting in an increase flux from glucose to lactate (Semenza, 2008). Moreover, HIF-1 by increasing the expression of pyruvate dehydrogenase kinase 1 (PDK1), responsible of the phosphorylation and inactivation of pyruvate dehydrogenase (PDH), thereby preventing the conversion pyruvate to acetyl coA for enter into the mitochondrial tricarboxyl acid (TCA) cycle, and by activating the expression of Bcl-2 nineteen-kilodalton interacting protein 3 (BNIP3) and its homolog BNIP3L, actively suppresses mitochondrial oxidative metabolism (Semenza et al., 2012).

In cancer cell hypoxia and of HIF-1 pathways are considered the main responsible of the activation of different pro-tumoral responses that include blood vessel growth, epithelial to mesenchymal transition, invasion and metastasis. In fact, HIF-1α and HIF-2α directly regulate the expression of multiple genes encoding pro-angionenic growth factor, like vascular endothelia growth factor (*VEGF*), stromal-derived factor 1(*SDF1*), placental growth factor

(*PlGF*), platelet-derived growth factor B (*PDGFB*) or angiopoietin (*ANGPT*) 1 and 2, all essential for endothelial cell survival, proliferation, migration and tube formation (Krock et al., 2011). Moreover, by activating the expression of genes encoding repressors, such as Inhibitor of differentiation 2 (*ID2*), Snail 1 and 2 (*SNAI1*, *2*), that block the expression of E-cadherin and other proteins that contribute to cytoskeleton rigidity and cell-cell adhesion, and by mediating the expression of genes that promote cytoskeleton flexibility, like vimentin (*VIM*), HIF-1 regulates the epithelial to mesenchymal transition (Semenza, 2012).

Finally, HIF-1 targets genes which encode proteases, like matrix metalloproteinase 2 and 9 (*MMP2, 9*) or lysyl oxidase (*LOX*), responsible of the degradation and remodeling of the extracellular matrix, or inducing the expression of motility and permeability factors, including Met proto-oncogene (*MET*) and autocrine motility factor (*AMF*), HIF-1 promotes intravasation of cancer cell into the blood stream and metastasis (Semenza et al., 2012).

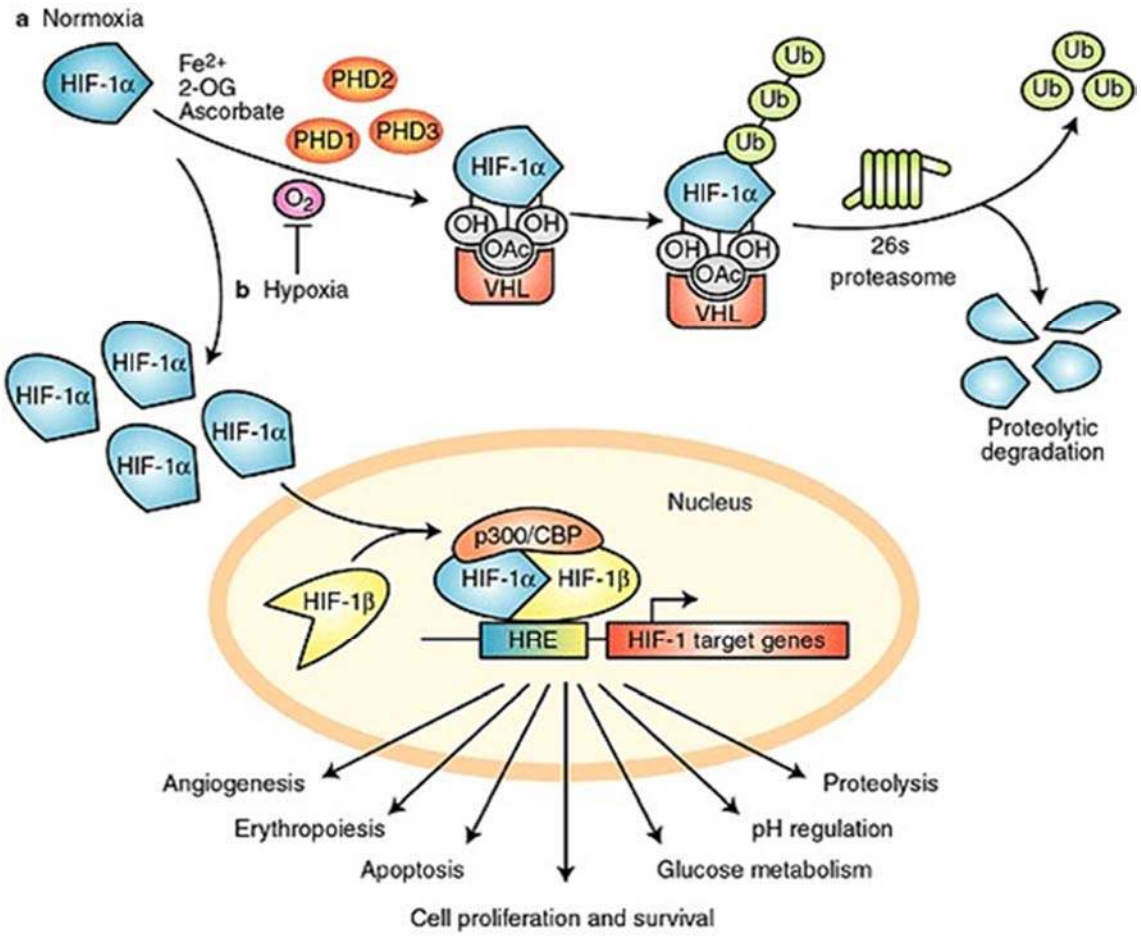

Fig. 6. HIF-1α regulation by proline hydroxylation. Schematic representation of HIF-1α pHVL-mediated degradation in normoxia (a), and its accumulation and transcriptional activation in hypoxia (b). Picture adapted from Carroll and Ashcroft, 2005.

2.1.2. Papillary RCC.

Papillary RCC is the second most common histologic subtype making up 10 – 15% of RCCs and is frequently associated with trisomies of chromosome 3 and 7, or the loss of Y chromosome (Prasad et al., 2006). As ccRCC, papillary RCC affects the epithelium of the proximal convoluted tubules and macroscopically often contains areas of hemorrhage, necrosis and cystic degeneration. Histologically it's characterized by a papillary growth pattern consisting in a fibrovascular core with stromal aggregates of foamy macrophages with cholesterol crystals that form the tumor papilla (Fig. 7). Two histomotphologic subtypes have been identified depending on whether tumors are characterized by a monolayer of small cells with scanty cytoplasm (type 1) (Fig. 7A), or

contains high-nuclear-grade cells with abundant eosinophilic cytoplasm (type 2) (Fig. 7B) (Prasad et al., 2006). Differently to ccRCC, papillary RCCs typically appear hypovascular and homogeneous on imaging studies, bilateral and multifocal tumors are more common than in other subtypes of kidney cancer and usually affects end-stage kidneys (Polascik et al., 2002).

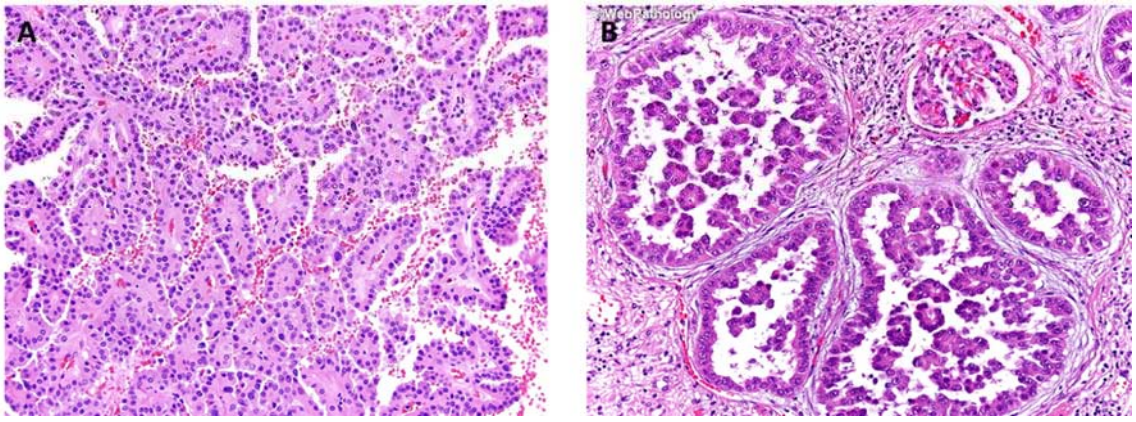

Fig. 7. Photomicrograph of papillary RCCs. A, B) H&E stainings of type 1 (A) and type 2 (B) papillary RCC. Pictures adapted from pathologyoutlines.com.

2.1.3. Chromophobe RCC.

Chromophobe RCC accounts for less than 5% of RCCs, it is caused by the loss of multiple chromosomes and postulated to differentiate toward type B intercalated cells of the cortical collecting duct (Thoenes et al., 1988). Chromophobe RCC shows a mean age of incidence in the 6th decade and equally affects men and women. Macroscopically it is well circumscribed and solid, and it is histopathologically characterized by large polygonal cells with prominent cell membranes, and contrastingly to ccRCC the tumor blood vessels are thick walled and eccentrically hyalinized (Fig. 8A) (Prasad et al., 2006).

2.1.4. Collecting Duct Carcinoma.

Collecting duct carcinoma of the kidney is a high aggressive subtype of RCC that accounts for less than 1% of all malignant renal neoplasm. It origins from the medullary collecting duct, typically appears as a gray-white infiltrative neoplasm with its epicenter in the pelvicaliceal system and it is histologically characterized by a tubule-papillary growth pattern, the presence of inflammatory or

desmoplastic stroma and mucin production (Fig. 8B) (Eble et al., 2004). Collecting duct carcinoma commonly shows an infiltrative growth pattern, tubular epithelial dysplasia in the adjacent renal parenchyma and it is considered a high aggressive neoplasm with one third of patients that already have metastatic dissemination at presentation, and less than one-third that survive more than 2 years after diagnosis (Eble et al., 2004).

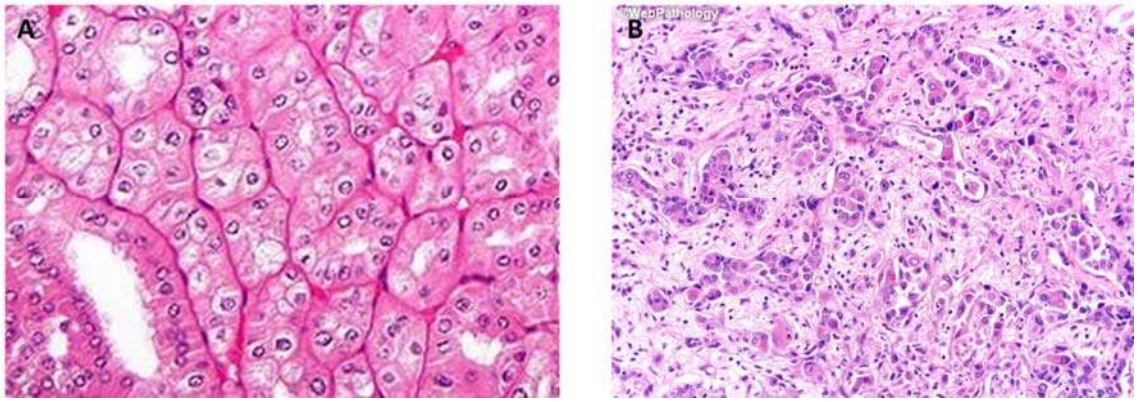

Fig. 8. Photomicrograph of chromophobe (A) and collecting duct (B) RCCs. A) H&E staining of a tissue section of chromophobe RCC showing characteristic large polygonal cells surrounded by thick walled blood vessels. B) H&E staining of a tissue section of collecting duct RCC histologically characterized by a papillary growth pattern. Pictures adapted from pathologyoutlines.com.

2.1.5. Renal Medullary Carcinoma.

Renal medullary carcinoma is an extremely rare malignant neoplasm occurring almost exclusively in patients with sickle cell trait, an inherited blood disorder in which a person has one abnormal allele of the hemoglobin beta gene (*HBB*) (Davis et al., 1995). Hypothesized to arise from the medullary collecting duct, renal medullary carcinoma is always found in young patients with a typical age range between 10 and 40 years and a male-female ration of 2:1. At histologic analysis, it shows sheets of poorly differentiated, mucin-producing eosinophilic cells associated with inflammatory, fibrous or edematous stroma and a reticular pattern of growth consisting of tumor cell aggregates forming spaces of different sizes (Fig.9) (Davis et al., 1995). Typically hypovascularized, renal medullary carcinoma manifests as an infiltrative neoplasm, in which

hemorrhage and necrosis contribute to tumor heterogeneity, and it is characterized by a really poor prognosis with a mean duration of survival of only 15 weeks due to early metastization in liver and lungs (Eble et al., 2004).

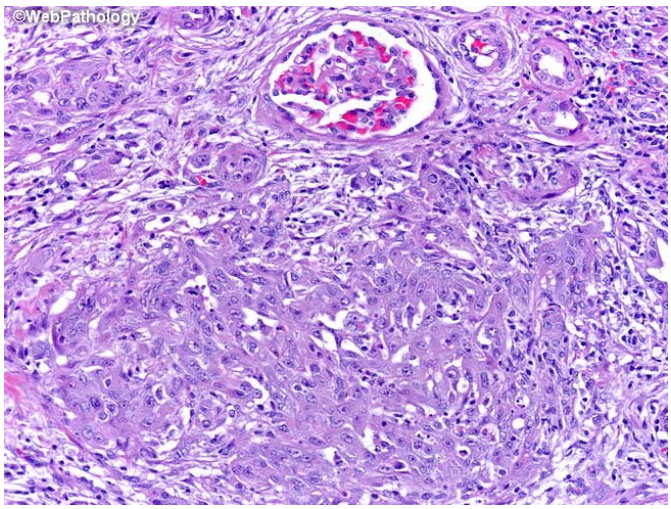

Fig. 9. Photomicrograph of renal medullary carcinoma. H&E staining of a tissue section of renal medullary carcinoma showing solid nests of tumor cells deeply infiltrating the renal parenchyma. Picture adapted from pathologyoutlines.com.

2.1.6. Hereditary RCC syndromes.

Hereditary RCC syndromes are characterized by early development of bilateral and multicentric renal neoplasm in both sexes with a family history of RCC. Specific syndromes are associate with development of a distinct histological subtype, the most studied example is clear cell RCC occurring in patients with von Hippel-Lindau syndrome (Prasad et al., 2006).

2.2. Diagnosis and stage.

Historically patients were diagnosed with RCC after presenting with flank pain, gross haematuria or a palpable abdominal mass, but nowadays non-invasive radiological techniques, such as magnetic resonance imaging (MRI) of abdominal computed tomography (CT), are commonly used to diagnose and stage renal cell carcinomas according to the TNM system. Describing the size and whether the primary tumor has grown into nearby areas (T), the extent of spread to regional lymph nodes (N) and if it has metastasized to other part of the body (M), this system stages RCC in 1 to 4 indicating increasing severity (Fig. 10) (Hsieh et al., 2017).

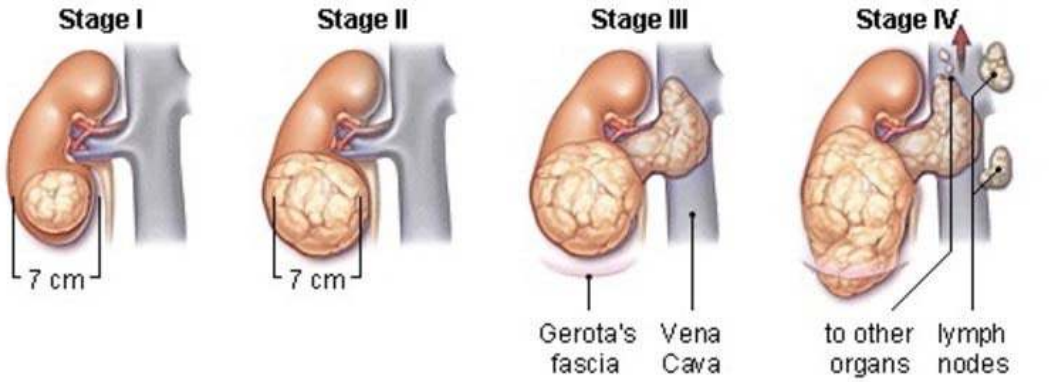

Fig. 10. Stages of kidney cancer. Stage I: tumor limited to the kidney and smaller than 7 cm. **Stage II**: tumor bigger than 7 cm but still limited to the kidney. **Stage III**: tumor extends into the major vain or adrenal glands within Gerota's fascia (the layer of connective tissue encapsulating the kidney and adrenal glands) or can involve regional lymph nodes. **Stage IV**: tumor invades beyond Gerota's fascia and has distant metastasis. Picture adapted from newhealthadvisor.com.

Nonetheless, pathologists pointed out that there are other factors than the extent of the cancer that should be considered for prognosis determination, and in 2012 the International Society of Urological Pathology (ISUP) introduced a novel grading system based upon the "Fuhrman-nuclear grade". This system categorizes renal cell carcinoma on a scale of 1 through 4 reflecting inverse prognosis and it's based on nuclear size, shape and pleomorphism of the neoplasm with hematoxylin and eosin staining. Grade 1 indicates tumors in which the nuclei are small (< 10 μm), round, basophilic, hyperchromatic with no visible nucleoli and little detail in chromatin. In grade 2 are gather tumors which nuclei are slightly larger (15 μm) with finely granular chromatin but small, inconspicuous nucleoli. If the nuclei of the tumor cells are larger than 20 μm, oval in shape with coarsely granular chromatin and the nucleoli are eosinophilic and clearly visible at 100X magnifications, RCC are group in grade 3. Finally, grade 4 reflected the presence of pleomorphic nuclei with open chromatin and the presence of multiple macronucleoli and tumor giant cells (Saramatunga et al., 2014).

2.3. Renal Cell Carcinoma main treatments.

Deciding which treatment undertakes and the length and frequency of clinical follow-up is largely guided by TNM stage and Fuhrman-nuclear grade. For patients with surgically resectable RCC, partial or radical nephrectomy is the standard care; meanwhile those with inoperable or metastatic RCC typically undergo systemic treatment with targeted agents and/or immune checkpoint inhibitors (Hsieh et al., 2017). Moreover, key prognostic factors, including hemoglobin, calcium and lactate dehydrogenase levels or neutrophil and platelets blood count, have been identified and adopted to guide and stratify patients with metastatic RCC for systemic treatment.

The goal of partial nephrectomy is to completely remove the primary tumor while preserving the largest possible amount of healthy renal parenchyma and it is indicated for patients with T1 tumor (according the TNM staging system) and a normal contralateral kidney, or for patients with von Hippel-Lindau syndrome. Otherwise, cases with multiple renal tumors or in which tumor extends into the vasculature (stages II and III) go for classical radical nephrectomy, consisting in the complete removal of the kidney, perirenal fat tissue, adrenal gland and regional lymph nodes (Hsieh et al., 2017).

Stage IV, relapse or recurrent diseases with predominantly clear cell histology usually undergo systemic therapy, and during the last decade the treatment of metastatic RCC was revolutionized with the advent of anti-angiogenic drugs and tyrosine-kinase inhibitors (TKI). In fact, before 2005 the only option available for patients with metastatic RCC was immunotherapy that by using cytokines such as interferon-α and high-dose interleukin 2 (IL-2) enhance the anti-tumor immune activity (Hsieh et al., 2017). However, both drugs typically benefit only a small subset of patients and are associated with substantial toxicity, particularly in the case of high-dose IL-2. Hence, given the highly vascular nature of RCCs it is not surprising that several of these new drugs adopted in the clinic are inhibitors of the VEGF signaling axis that, by hitting different parts of the

angiogenic pathway, allow the possibility of combinatory therapies or first and second-line strategies. Usually, sunitinib (Sutent; Pfizer Inc., New York, NY) or pazopanib (GlaxoSmithKline, Research Triangle Park, NC) both TKI of VEGF receptors 1, 2 and 3, PDGFR and c-kit; or the combination of bevacizumab (Avastin; Genetech, South San Francisco, Calif.), a monoclonal neutralizing antibody of circulating VEGF protein, with interferon-α are approved as first-line option; whereas the simultaneous inhibitor of VEGFRs and PDGFR-β axitinib (AG-0137336; Pfizer Inc.), and cabozantinib (Cometriq; Exelixis Inc.) which targets VEGFRs and the mesenchymal epithelial transition (MET) receptor, are approved as second-line settings (Rini, 2009). Finally, the mammalian target of rampamycin (mTOR) inhibitors everolimus (Afinitor; Novartis; Sw) and temsirolimus (Torisel; CCL-779; Wyeth; NJ) have been approved as single agents in second-line settings and as first-line in patients with a poor risk status (Hsieh et al., 2017). As a result, the survival of patients with advance RCC has significantly improved from a median overall survival (OS) of approximately 12 months in the cytokines era to a more than 26 months with first-line VEGF inhibitors (Escudier et al., 2007; Motzer et al., 2007).

However, none of these anti-angiogenic agents maintained durable tumor responses and most patients, despite an initial period of response, will ultimately experience disease progression due to the development of treatment resistance.

3. Anti-angiogenic Resistance.

It is well established that the angiogenic switch is a critical step in carcinogenesis, and because of the key role of the vascular endothelial growth factor (VEGF) in this process, anti-angiogenic research has been focused largely on this molecule. In fact for two main reasons VEGF provided an attractive target for therapeutic intervention: it serves as a point of integration of a variety of upstream and downstream signals, leading ultimately to endothelial cells

stimulation; and it promotes angiogenesis acting directly on genetically stable endothelial cells, rather than on genetically labile tumor cells (Kerber et al., 2002). Consequently, it has been proposed that drugs that directly inhibit the VEGF signaling may be less susceptible to selection of mutation that confers resistance, resulting in Food and Drug Administration (FDA) approval of different drugs like the first-generation anti-VEGF monoclonal antibody bevacizumab in 2004, or the second-generation TKI sunitinib in 2006. However, translating the preclinical successes into clinical practice, identifying the optimal clinical indication and maximizing the efficacy of the anti-angiogenic therapies has been more challenging than anticipated. For instance, bevacizumab improves clinical results mostly when combined with chemotherapy probably because normalizing the residual tumor vessels it optimizes drug delivery (Jain, 2005; Kerbel, 2006). In contrast, even if the TKI are capable of suppress tumor growth as monotherapies, likely because they inhibit multiple targets, the resulting clinical benefits variably depend on tumor type, stage and treatment history. Moreover, it has been reported that VEGFR inhibitors, mainly if combined with TKI, cause endothelial dysfunction and vessel pruning in healthy tissues that even if usually manageable and rarely outweighing the clinical benefits, may result in dose reduction, drug holidays or discontinuous therapy (Azad et al., 2008). Nonetheless, the greatest challenge today is that a substantial number of cancer patients are intrinsic refractory to anti-angiogenic drugs, such as pancreatic cancer patients, or even if initially responders, escape from anti-angiogenic therapy after experiencing clinical benefits in the range of month but without permanent cure (Kerbel, 2008; Kindler, 2007). Thus, despite transient disease stabilization or tumor regression reflected in a favorable progression free survival (PFS), the overall survival (OS) is often not prolonged, like reported in renal cancer patients treated with bevacizumab monotherapy; debacle that could be explain by the rapid regrowth of tumor vessels (Yang et al., 2003).

So far, it has been challenging to match optimally the pharmacological profile with patients selection or to readjust anti-angiogenic therapy upon disease progression; mainly because we insufficiently understand the mechanisms underlying intrinsic and acquired resistance and for the lack of well-validated biomarkers to monitor efficacy, optimal dosing or predict toxicity and resistance to anti-angiogenics. However, an increasing list of emerging preclinical studies revealed that intrinsic refractoriness and evasive escape are driven by different mechanisms attributable not only to tumor cells but also to the host microenvironment, offering new therapeutic avenues (Loges et al., 2010).

3.1. Role of Malignant Cells in Anti-Angiogenic Escape.

Malignant tumor cells contribute to resistance against anti-angiogenic therapy via several mechanisms. For instance, human cancers express a myriad of angiogenic factors that can substitute for one another at consecutive steps of tumor progression or metastasis and are in turn changed again by the type and stage of treatment (Relf et al., 1997; Yoshiji et al., 1997). In fact, important biological processes require more than one mediator, and angiogenesis in no exception. Although there is no question about the key role of VEGF, there are multiple additional angiogenic factors that have distinct or compensatory function. Indeed, in preclinical and clinical studies the inhibition of VEGF activity leads to an increase in the plasma levels of a number of cytokines including VEGF itself, placental growth factor (PlGF) family member, angiopoietin-1 stroma-derived factor 1 (SDF-1) and others that, probably induced by tumor hypoxia or pre-existing as in the case of intrinsic resistant patients, are responsible of tumor revascularization and escape (Motzer et al., 2006; Willett et al., 2005).

Moreover, our and other laboratories have shown, using preclinical models, that VEGF target therapy could lead to a more aggressive, invasive tumor phenotype and, despite controlling primary tumor growth, increased the rate of

metastasis by different mechanisms (Paez-Ribes et al., 2009; Ebos et al., 2009). First, the low oxygen levels in tumors regularly approaches anoxic conditions representing a hostile microenvironment from which tumor cells try to escape. Besides hypoxia, VEGF inhibitors also induce the release of a "cytokine storm" by healthy tissues resulting in a systemic "pseudo-inflamed" state that may promote tumor cell extravasation, metastatic lodging and recruitment of angio-competent myeloid cells to primary tumors or pre-metastatic niches (Ebos et al., 2009; Gupta et al., 2006). Moreover, Ebos and colleagues have also shown that VEGF signaling blockade can also lead to an increased cytokine production in non-tumor bearing mice and furthermore that pretreatment of mice prior to tumor cell injection preconditioned the animals for metastasis formation (Ebos et al., 2009). Even if these experimental observations need to be further evaluated in the clinic, these findings call into question whether depriving tumor cells from vascular supply as maximally as possible is desirable or, instead, should be balanced to prevent tumor invasiveness and metastasis.

Nonetheless, although certain tumor cells try to escape from the hostile hypoxic environment, others become more hypoxia tolerant, like cancer stem cells (CSM) which contribute to metastasis recurrence homing into the hypoxic tumor regions where they can sustain self-renewal (Dayan et al., 2008; Keith et al., 2007).

3.2. Vascular Co-option, Vasculogenesis and Vascular Mimicry.

The hypothesis that solid tumors are dependent on sprouting angiogenesis, the formation of new branches, for outgrowth and metastasis has acquired a central position in cancer research and inspired scientists for several decades, but more recently it has become clear that tumors may use alternative ways to obtain blood supply. Indeed, while certain tumors by hijacking the existing vasculature allow tumor cells migration along the vessels (vascular co-option), others form new blood vessels by the postnatal differentiation of circulating

endothelial progenitor cells (EPCs) to (vasculogenesis) or by vascular mimicry, a mechanism by which highly aggressive tumor cells form vessel-like structure themselves in virtue of their high plasticity. Obviously, one can intuitively assume that a single anti-angiogenic agent may not be capable of neutralizing all these non-overlapping mechanisms of vessel growth (Longes et al., 2010; Leenders et al., 2002; Donnem et al., 2013).

Co-option of preexisting blood vessels has been well documented in human pancreatic carcinomas, non-small cell lung cancer (NSCLCs) or pulmonary metastasis, and suggested to be due to the interplay of angiopoietins and VEGF. During development while VEGF acting via VEGFR-2 promotes endothelial cell differentiation, proliferation and primitive vessel formation, angiopoietin-1 (Ang-1) activating the Tie2 receptor on endothelial cells coordinately remodels these primitive vessels, promoting maturation and interaction with the surrounding supporting cells leading to the formation of a stable vasculature. In adults, only at sites of vascular remodeling, the pattern changed and angiopoietin-2 (Ang-2) is primarily expressed to block the Tie2 receptor and antagonize the constitutively stabilizing action of Ang-1 and destabilize the blood vessels facilitating the angiogenic response (Holash et al., 1998). As can be imagine, in tumors Ang-2 and VEGF reprise to roles they play during vascular remodeling in normal tissue: cancer cell striking induce Ang-2 and VEGF expression coopting existing host vessels to form a well-vascularized tumor mass (Holash et al., 1998).

Vascularization of tumors via vasculogenesis occurs when circulating endothelial progenitor cells (EPCs) are recruited into the tumor mass, secrete a plethora of angiogenic cytokines and differentiate to endothelial cells into nascent vessels. Experimental and clinical studies indicate that circulating EPCs levels correlate with tumor size, and moreover, consistent with their physiologic role in tissue regeneration, EPCs are recruited when the tumor vasculature is destroyed

mediating resistance against anti-angiogenic therapies (Brunner et al., 2008; Shaked et al., 2008; Ding et al., 2008).

Finally, vascular mimicry (VM) refers to the plasticity of aggressive cancer cells to form *de novo* non-endothelial vascular channels that contained red blood cells within a tumor mass, and thereby contribute to the perfusion of rapidly growing tumors (Seftor et al., 2012). Intuitively, tumor cells capable of VM exhibit a remarkable degree of plasticity that confers the ability to trans-differentiate to ECs, and a molecular signature characterized by the up-regulated expression of genes associated with embryonic progenitors, endothelial cells, vessel formation, matrix remodeling and coagulation inhibitors. However, unlike normal embryonic progenitors, these tumor cells lack critical regulatory checkpoint, a characteristic that underlies their multipotent phenotype and contributes to unregulated growth and aggressive behavior (Seftor et al., 2012). Even if the molecular mechanism promoting VM is still unclear, recent studies in melanoma have highlighted that the hypoxic microenvironment, activating the Notch and Nodal signaling pathway, seems to contribute to the phenotype switch allowing cancer cells to form blood vessel (Delgado-Bellido et al., 2017).

3.3. Tumor Stroma in Anti-Angiogenic Resistance.

In addition to tumor cells intrinsic or acquired resistance, it is nowadays well assumed that also extrinsic mechanisms, mainly dwelling in the stroma, are involved in resistance to anti-angiogenic therapy. Composed by different cell types, including pericytes, smooth muscle cells, fibroblast and immune cells, tumor stroma is the target of several promising third-generation drug candidates with potential to induce less or overcome resistance.

Pericytes and smooth muscle cells promote maturation and vessels stabilization and can consequently influence the responsiveness of tumors to anti-angiogenic therapy. In fact, by releasing platelet-derived growth factor-B (PDGF-B)

endothelial cells recruit pericytes to render nascent vessels more stable, quiescent and mature and this may explain why immature "naked" growing blood vessels are more susceptible to VEGF-targeted agents, whereas mature pericyte-covered persist (Bergers et al., 2003). However, even if pre-clinical studies documented that a combination of VEGF and PDGF receptor (PDGFR) inhibitors is more effective in blocking tumor growth, loss of pericytes coverage can enhance vessel leakage and consequently decrease drug delivery or promote tumor cell intravasation and fuel metastasis (Gerhardt et al., 2008). Thus, the precise clinical consequences of pericyte-targeted therapy require further study.

Cancer-associated fibroblast (CAFs) are the major components of tumor stroma and are similar in morphology to myofibroblasts, which are large spindle-shaped cells that are activated and mediate inflammation responses during the wound healing process. However, while during wound healing, when the process is completed, activated fibroblast decrease; in cancer CAFs are perpetually activated and neither revert to a normal phenotype nor undergo apoptosis and elimination, creating a microenvironment that promotes tumor growth, angiogenesis and metastasis via secretion of various cytokines, chemokines and degradation of extracellular matrix (ECM) proteins (Shiga et al., 2015). Moreover, besides stimulating angiogenesis by producing angiogenic cytokines, CAFs also secrete SDF-1, which mobilizing and recruiting bone marrow derived EPCs in a VEGF-independent manner, promotes vasculogenesis (Orimo et al., 2005). Finally, CAFs of anti-angiogenic resistant tumors up-regulate PDGF-C increasing the perycites coverage and rescuing vascularization (Crowford et al., 2009).

Among the different myeloid cell types, the ones that express the surface markers Gr1 and CD11b ($Gr1^+CD11b^+$ cells) are thought to be the main regulators of tumor refractoriness to anti-VEGF treatment (Shojaei et al., 2007). In fact, tumor mass producing great amount of the granulocyte colony-

stimulating factor (G-CSG) induces mobilization and recruitment of these myeloid cells, which secreting the matrix metalloproteinase 9 (MMP-9), which has been associated with increased bioavailability of VEGF-A for its receptors, and suppressing the immune response of different subtypes of T cells, including CD4+ and CD8+ cells, confers resistance to anti-angiogenic treatments (Shojaei et al., 2007).

Finally, also tumor-associated macrophages (TAMs) contribute to tumor escape form anti-angiogenic therapies. Macrophages are an essential component of the innate immune system and play a pivotal role in inflammation and hose defense. Based on immunological responses, macrophages have been classified into “classical activated” M1 macrophages which mediate the host response to pathogens, or into “alternative activated” M2 macrophages, which stimulate angiogenesis, lymphoangiogenesis, invasion and immunosuppression (Martinez and Gordon, 2014). Tumor-associated macrophages belong predominantly to the M2 phenotype and are associated with tumor progression, metastasis and poor prognosis in cancer patients (Mantovani et al., 2008). In fact, it has been found that TAMs depletion or blockade of their recruitment using a monoclonal antibody against PlGF, enhance murine tumor responsiveness to different VEGF targeting agents. Because PlGF is upregulated upon anti-VEGF treatments, increased recruitment of pro-angiogenic and resistance-conferring TAMs by PlGF might be a mechanism used by tumor to compensate for the vessel pruning and contribute to resistance (Fisher et al., 2007).

Another subtype of myeloid cells that are scarcely present in healthy tissues but enrich in human solid tumors are the Tie2-expressing monocytes (TEMs). TEMs are recruited by angiopoietin-2 (Ang2) which is upregulated in hypoxic tumors in response to anti-angiogenic treatments, and are thought to stimulate angiogenesis mainly by releasing bFGF and downregulating the anti-angiogenic cytokine IL-12 (De Palma et al., 2005; Murdoch et al, 2007).

4. Thymidine Phosphorylase/Platelet-derived Endothelial Cell Growth Factor.

The history of thymidine phosphorylase (TP)/platelet-derived endothelial cell growth factor (PD-ECGF1) started in 1953, when a nucleoside metabolism enzyme was first characterized as involved in the nucleic acid homoeostasis, and responsible of the reversible conversion of thymidine (TdR) to thymine and 2-deoxy-α-D-ribose-1-phosphate (dRib-1-P) or the phosphorolysis of deoxyuridine to uracil and 2-deoxy-α-D-ribose-1-phosphate (Fig. 11) (Friedkin and Roberts, 1954). More than thirty years later a "novel" angiogenic factor was isolated from platelet lysate, named platelet-derived endothelial cell growth factor (PD-ECGF1) and described as a classical growth factor that binding a cognate cell-surface receptor would elicit a cellular response (Miyazono et al., 1987). However, in 1992 a protein sequence search revealed that PD-ECGF1 shared significant sequence identity with *E. coli* TP, concluding that TP and the angiogenic factor PD-ECGF1 are different names for the same molecule (Moghaddam et al., 1992; Usuki et al., 1992).

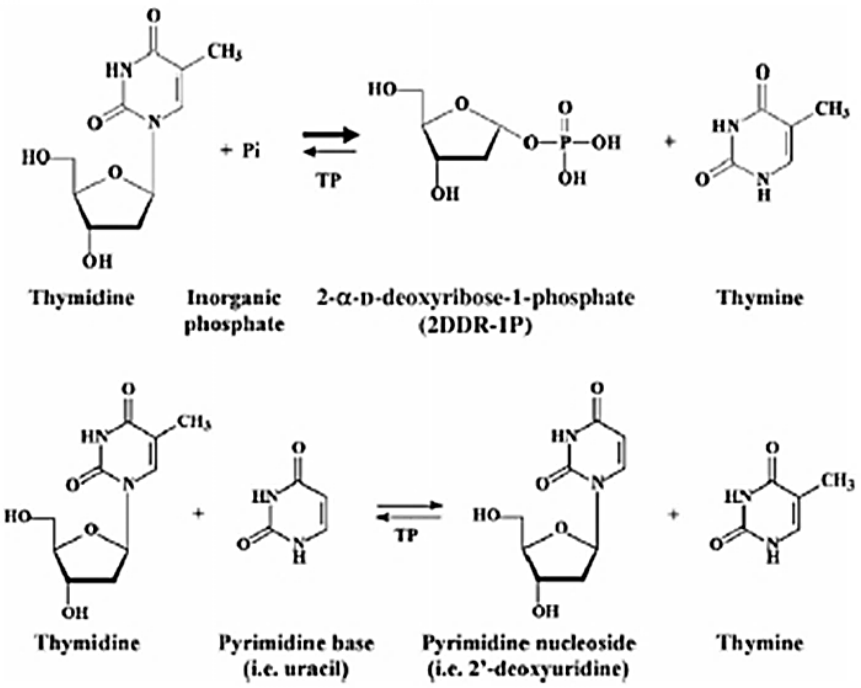

Fig. 11. Enzymatic reaction catalyzed by TP. TP catalysis the conversion of thymidine (TdR) to thymine and 2-deoxy-α-D-ribose-1-phosphate (dRib-1-P) or the phosphorolysis of deoxyuridine to uracil and 2-deoxy-α-D-ribose-1-phosphate. Figure adapted from Elamin et al., 2015.

TP/PD-ECGF1, that from now on I'll simply refer to as PD-ECGF1, is a homodimeric enzyme consisting of two identical subunits non-covalently associated with a dimeric molecular mass of 51 kDa (El Omari et al., 2006). Each subunit is composed of a small α-helical domain containing the thymidine binding site, and a large α/β domain where the phosphate binding site is located, and although the reaction is reversible, the main function of PD-ECGF1 is catabolic, ensuring a pyrimidine-nucleotide pool sufficiently large for efficient DNA repair and replication (El Omari et al., 2006). In healthy tissues, it is expressed in both nucleus, where it modulates the pool of pyrimidine nucleosides for DNA synthesis, and cytoplasm of macrophages, stromal cells, glial cells and some epithelia but most of its activity resides in the cytoplasm of platelets (Shaw et al., 1988). In fact, even if platelets lack nuclei, they possess functional mitochondria and remain capable of both anaerobic and aerobic

energy metabolism for which they utilize a variety of substrates many of which cytotoxic or genotoxic for other nucleated cells, like nucleic acid precursors. In particular, platelets actively scavenge adenine and adenosine which they convert to nucleosides and use in energy metabolism, and rapidly phosphorylase thymidine and liberate thymine into the extracellular medium, playing an important role in nucleic acid precursor metabolism (Shaw et al., 1988). Thymidine homeostasis is in fact of great importance and imbalances caused by elevated thymidine concentrations can alter cell growth and viability, as reported in patients affected by mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), in which mutations in the gene encoding PD-ECGF1 cause a complete loss of its catalytic activity, producing systemic accumulation of its substrates. This deoxyribonucleotide imbalance impairs mitochondrial DNA (mtDNA) replication and results in mtDNA depletion, multiple deletions and somatic point mutations resulting in mitochondrial dysfunction, progressive external ophthalmoplegia, gastrointestinal dysmotility, cachexia, peripheral neuropathy and diffuse leukoencephalopathy (Hirano et al., 2004).

4.1. The Role of PD-ECGF1 in Angiogenesis.

PD-ECGF1 enzymatic activity, as mutagenesis of the enzyme's active-site residues had established, is involved in physiological and pathological, including cancer, angiogenesis (Miyadera et al., 1995). PD-ECGF1 lacks a signal sequence necessary for cell secretion, and its angiogenic effects are mediated through the released of the cell-permeant 2-deoxy-D-ribose, which results from the desphosphorylation of 2-deoxy-D-ribose-1-phosphate, and could stimulate endothelial cell migration by several mechanisms (Hemalatha et al., 2009; Pula et al., 2009). Firstly, 2-deoxy-D-ribose has been shown to activate cell surface integrins $\alpha_5\beta_1$ and $\alpha_V\beta_1$ and to stimulate the phosphorylation of focal adhesion kinases (FAK) on tyrosine 397 (Tyr^{397}), leading to focal adhesion formation

(Hotchkiss et al., 2003). Focal adhesion kinases are a 125 kDa non-receptor protein-tyrosine kinases that are recruited by integrins to focal adhesion and activated by phosphorylation at potentially six tyrosines, of which phosphorylation on Tyr^{397} is consider to be the first to occur. In fact, its phosphorylation creates a binding site for the Src homology 2 domain allowing the recruitment of Src, that phosphorylating the other tyrosine residues on FAK lead to the recruitment of the cytoplasmic and cytoskeletal proteins tallin, paxillin, vinculin and actin, mediating cell attachment and migration (Hotchkiss et al., 2003). Secondly, 2-deoxy-D-ribose can regulate cell proliferation and angiogenesis activating the downstream kinase of the mTOR pathway p70/s6K, and providing an energy source for migrating endothelial cells could induce pseudopodia formation (Bjinsdorp et al., 2010; Brown and Bicknell, 1998). Finally, PD-ECGF1 can stimulate angiogenesis also acting indirectly by enhancing the release of angiogenic factors. In fact, as Bjinsdorp and collegues have shown, colon cancer cells with high PD-ECGF1 expression secreting the angiogenic factors IL-8, bFGF and TNF-α stimulate human umbilical endothelial cells (HUVECs) migration and invasion, but not proliferation (Bjinsdorp et al., 2011). Importantly, PD-ECGF1 did not increase VEGF secretion, and has no effect on invasiveness or migration capacity of cancer cells (Bjinsdorp et al., 2011).

4.2. PD-ECGF1 and Cancer.

More complex is the role of PD-ECGF1 in cancer. In fact, whilst its overexpression by several human cancers, including gastric, breast, colorectal, kidney, and cervical cancer, correlates with angiogenic and anti-apoptotic roles that aid cancer progression, PD-ECGF1 is also a key factor in determining the response to some chemotherapy agents (Elamin et al., 2015). Transcriptionally, several studies have shown that different factors, including inflammatory cytokines, cellular stresses like hypoxia and low pH, chemotherapy and

radiotherapy, lead to PD-ECGF1 overexpression. In fact, having a SP1 transcription binding site within its promoter region, the inflammatory cytokine TNF-α by activating SP1, which in turn binds to the promoter region, can induce PD-ECGF1 protein expression (Ikuta et al., 2012). Moreover, interferons α, β and γ activating the IFN-stimulated response element (ISRE) and the signal transduction and activator of transcription 1 (STAT1) can promote PD-ECGF1 expression, or by stabilizing its mRNA lead to a rise in its enzymatic activity (Bijnsdorp et al., 2008). Finally, also chemotherapy agents like docetaxel, paclitaxel, cyclophosphamide, and oxaliplatin can increase PD-ECGF1 levels possibly by inducing inflammatory cytokines like IFNs and TNFs (De Bruin et al., 2006). Importantly, the fact that PD-ECGF1 levels were reported to be elevated also non-malignant cells of the tumor microenvironment and chronic inflammatory conditions, like arthritis and psoriasis, suggest that raise in its expression is influenced by local inflammation and stress conditions rather than genetic changes (Fig. 12) (Ikuta et al., 2012).

Beyond its described angiogenic effects, PD-ECGF1 has been reported to inhibit hypoxia induced apoptosis, enhance the expression of genes involved in the epithelial to mesenchymal transition (EMT) and promote cancer cell evasion from immune cells (Elamin et al., 2015). One of the best documented mechanisms involved in PD-ECGF1 anti-apoptotic role regards the suppression of p38 MAPK phosphorylation and activation. In fact, depending on the stimulus, the p38 MAPK pathway plays a dual role mediating either cell proliferation and survival, or death through the phosphorylation of the pro-apoptotic protein BAX and subsequent release of mitochondrial cytochrome c and activation of caspase 3 and 9 (Trempolec et al., 2013). Indeed, while under hypoxic conditions human leukemia cell lines HL-60 and Jurkat cells are affected by chromatin condensation, p38 MAPK activation and BAX translocation from the cytosol to mitochondria resulting in cell death, 2-doexy-D-ribose was found to protect from hypoxia-induce morphological changes and to reduce the levels

of BAX attached to the mitochondria, suggesting that 2-deoxy-D-ribose inhibits hypoxia-induced apoptosis by suppressing p38 MAPK phosphorylation (Ikeda et al., 2006). Moreover, PD-ECGF1 has been reported to play a role in tumor invasiveness supporting the expression of the matrix metalloproteinases MMP-1, -7 and -9, probably via the activation of the PI3K-Akt pathway (Gotanda et al., 2006). In line with the previously described functions, its enzymatic inhibition or the counteraction of the stereoisomer 2-deoxy-L-ribose, decreases MMP-9 mRNA levels resulting in a reduce tumor cell invasiveness (Nakajima et al., 2006). Finally, PD-ECGF1 can indirectly suppress the innate immune response to tumor cells by increasing the secretion of IL-10, which modulating the expression of cytokines, soluble mediators and surface receptors by macrophages and dendritic cells can inhibit their anti-inflammatory functions (Fig. 12) (Bjinsdorp et al., 2008).

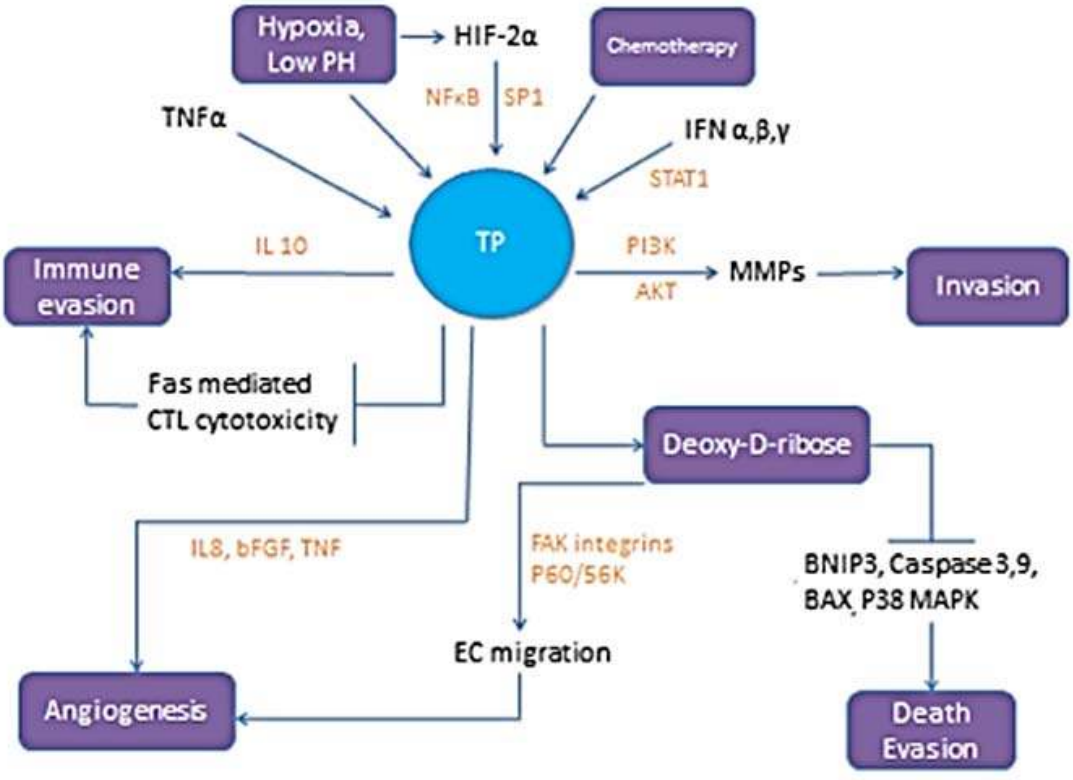

Fig. 12. PD-ECGF1 transcriptional regulation and biological effects. PD-ECGF1 is overexpressed in response to cellular stresses like hypoxia, low pH, inflammatory

cytokines and chemotherapy, and plays a role in angiogenesis and cancer progression. Figure adapted from Elamin et al., 2015.

Considering the emerging role in cancer progression and angiogenesis, it is not surprising that PD-ECGF1 is an attractive therapeutic target and several PD-ECGF1 targeting molecules have been synthetized, even if no specific inhibitor has been approved for clinical use at present. The most potent inhibitors to date are the pyrimidine analogue, 5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)uracil hydrochloride and the uracil derivate, 6-(2-aminoethyl)amino-5-chlorouracil (AEAC), which in preclinical studies have been shown to decrease tumors growth affecting the MVD and apoptotic index (Fukushima et al., 2000; Lu et al., 2006).

However, PD-ECGF1 also plays a central role in the activation of the antimetabolite drug 5-flouropyrimidine (5-FU), and PD-ECGF1 increased tumor levels opposed to normal tissues has been used as a strategy for selective toxicity and to minimize side effects (O'Neil, 2006). Widely used as the principal chemotherapy agents in colorectal, breast, head and neck carcinomas, 5-FU is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen, and its cytotoxicity is due to the inhibition of the enzyme thymidylate synthase (TS) required for DNA synthesis by its active metabolites fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) (Longley et al., 2014). In this pathway PD-ECGF1 is responsible of the conversion of 5-FU to fluorodeoxyuridine (FUDR), which can then be turn into the active metabolite FdUMP (Fig. 13) (Longley et al., 2014). Unfortunately, two main problems occurred: given the cytotoxic non-selectivity most of the patients suffered systemic toxicity, including neutropenia, stomatitis and diarrehea, and moreover, more than the 80% of administered drug is catabolized in the liver by dihydropyrimidine dehydrogenase (DPD) into dihydrofluorouracil (DHFU) and eliminated (Daisio and Harris, 1989). To improve tolerability and overcome this DPD-mediated liver

degradation, different prodrugs, like capecitabine, have been designed and are currently used in the clinic (Walko and Lindley, 2005). Capecitabine is an oral fluropyrimidine that is absorbed unchanged through the gastrointestinal wall, is first converted to 5'-deoxy-5-fluorouridine (5'DFUR) in the liver by the sequential action of carboxylesterase and cytidine deaminase, and is ultimately converted into 5-FU by PD-ECGF1 within the tumor tissue (Fig. 13) (Longley et al., 2014).

In conclusion, PD-ECGF1 could be seen as a double-edged sword that from one side is promoting tumor angiogenesis, invasion, evasion from the immune system and resistance to apoptosis, but from the other also plays a kay role in cancer treatment.

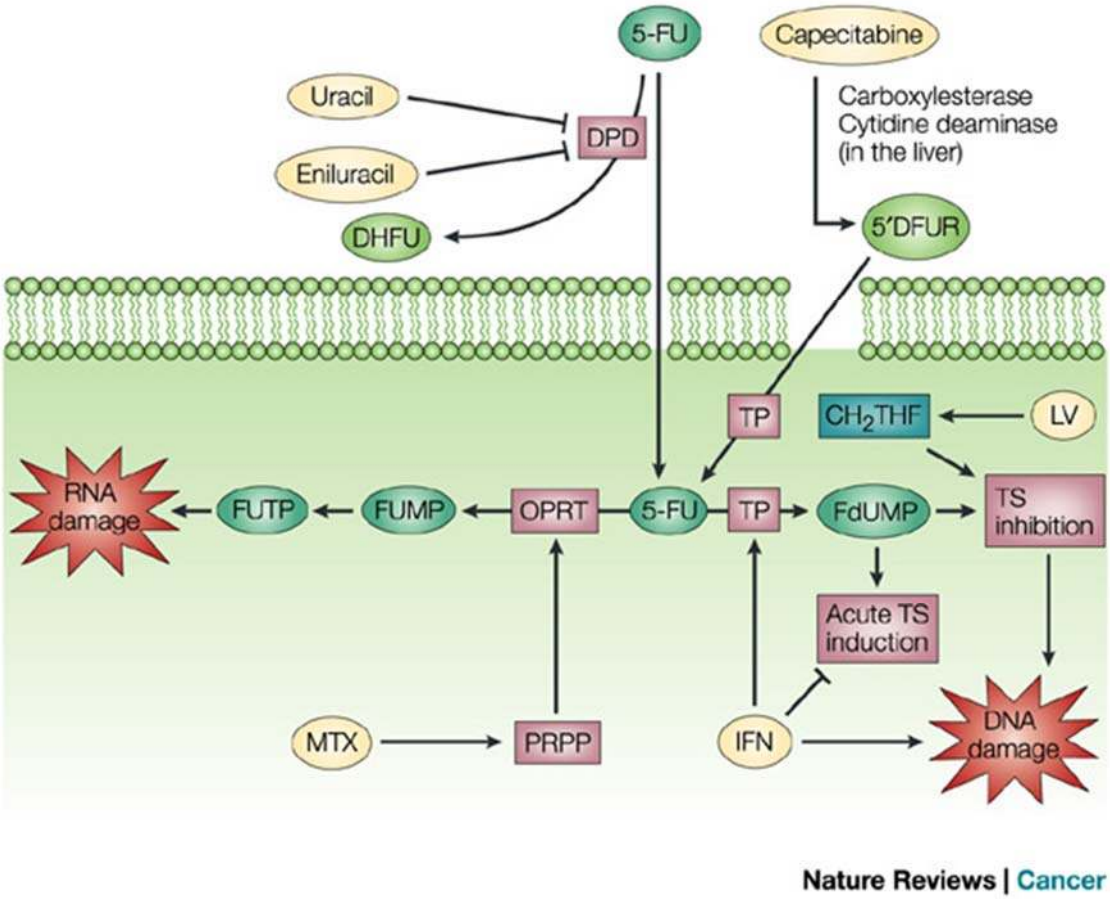

Fig. 13. 5-fluorouracil and capecitabine activity. Summary of 5-FU and capecitabine modulation and cytotoxic activity. Figure adapted from Longley et al., 2014.

Antecedents and Objectives.

Before my enrollment in the lab, performing different studies on both cell derived (786O$^-$ and RCC4) and primary (Ren13 and Ren28) orthoxenograft models of ccRCC, the group described that anti-angiogenic drugs (DC101 and Bevacizumab) were able to control tumor growth only transitory and unfortunately, as described in the clinic, tumor adaptation and relapse frequently occurred. Moreover, performing a TaqMan assay on a subset of angiogenic and invasion genes, we observed how several pro-angiogenic molecules were upregulated during anti-angiogenic therapies and therefore possible mediators of the process of acquisition of resistance. Among the other genes, for its known endothelial-restricted pro-angiogenic function we focused our attention on Endothelial Cell Growth Factor 1 (ECGF1), also known as Platelet-derived Endothelial Cell Growth Factor (PD-ECGF1) or TYMP.
Starting from this solid background, in this project we postulated to pursue and achieved the following objectives:

1. **Functional validation *"in vivo"* of PD-ECGF1 as a new therapeutic target of resistance to anti-angiogenic drugs.** Using several independent lines of RCC tumorgraft models, we will evaluate the short- and long-term response to the antiangiogenic drug DC101, and consequently validate PD-ECGF1 as new possible therapeutic target.

2. **Functional validation *"in vitro"* of PD-ECGF1 as a candidate gene of tumor resistance to anti-angiogenic therapies.** We will validate in human ccRCC cell lines PD-ECGF1's role in tumor resistance to anti-angiogenics, and study the biological pathways in which it's involved.

3. **Application of the preclinical results obtained in the previous objectives in clinical samples of Renal Cell Carcinoma patients.** Validation, together with Dr Maria Ochoa de Olza from the Medical Oncology Department (ICO), of PD-ECGF1 as a preclinical molecular marker of resistance in

clinical samples of RCC patients gathered in two clinical studies that are currently on-going in the institute.

Experimental Procedures.

Cell Lines.

pVHL-deficient 786O cell line was kindly provided by B. Jimenez (Instituto de Investigaciones Biomédicas CSIC-UAM, Madrid, Spain). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 µg/mL streptomycin sulfate, and 2 mmol/L glutamine (all Gibco, Life technologies, California, USA).

SN12C cell line was established in the lab from a piece of tumor tissues and grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 µg/mL streptomycin sulfate, and 2 mmol/L glutamine (all Gibco, Life technologies, California, USA).

pTRIPZ-shECGF Lentiviral Production.

293FT cells were plate in 15cm cell culture dishes previously coated with poly-L-Lysine (Sigma), and transfected with 100 µg total DNA (50 µg shRNAs + 37,5 µg pPAX2 + 12,5 µg pMD2-G, which represent a 4:3:1 ratio) in linear polyethylenimine (PEI) (Sigma) at 1:4. Three different targeting shRNAs, named sh89, sh91 and sh93, and a non-targeting sequence (shNS) have been used. At day 4 the viral supernatant has been harvest, centrifuged at 2500 rpm for 10 minutes, filtered and conserved at 4 ºC.

786O$^-$ and SN12C Cell Transduction.

786O$^-$ and SN12C cells have been plated in RPMI 1640 (786O$^-$) or DMEM (SN12C) medium supplemented as described in point 4, with the addition of polybrene 800ug/mL and when the confluency was approximately of 70% transduced with viral supernatants. After 24 hours each plate was washed in PBS1X, medium was freshly replaced and puromycin 2ug/mL (Sigma) added to select the cells that have successfully transduced. Fresh puromycin-supplemented media was replaced every 3 or 4 days and shRNA expression induced by doxycycline dose at 5ug/mL.

786O$^-$ Cell Proliferation and Apoptosis Analysis.

To examine cell proliferation, 786O cells were seeded on glass coverslip at the density of 3 x 10^5 per wells, let recover for 6 to 8 hours, wash in PBS1X and culture in RPMI full medium (control condition) or in the presence of AEAC 100uM, 2-deoxy-D-ribose 50 µM, and the combination of the two. After 24h proliferating cells were labelled performing an Edu Assay (FlouProbes) according to manufacture instructions; while total cells marked with DAPI. Five photos for each field were taken at 20X magnification using a Nikon Eclipse 80i microscope and the % of proliferating cells quantified. To examine cell apoptosis, 786O cells were seeded in the same conditions described above but labelled using a TUNEL assay (R&D Systems).

786O$^-$ sh91 and shNS Cell Proliferation Analysis.

Differences in 786O$^-$ sh91 and shNS cells proliferation was determined applying the same procedure described for the parental cell line, with the difference that cells were treated with doxycycline dosed at 2 µg/mL for 24h instead of AEAC. Pictures and counting were performed in an identical way.

PD-ECGF1 protein levels quantification.

PD-ECGF1 protein levels were quantified using a dual set Elisa Assay (Sigma) and when indicated expressed as fold changed to untreated conditions (cell growth in DMEM complete media).

Hypoxia and Nutrient Deprivation.

SN12C sh91 cells were maintained in normoxia at 21% O_2, 5% CO_2, 37 ºC or in hypoxia at 3% O_2, 5% CO_2, 37 ºC in full reconstituted DMEM medium (100% nutrients) or a dilution of 25% DMEM not FBS-supplemented in PBS1X (25% nutrients, FBS free) for 24 hours. To verify the pathway specificity, in parallel shRNA expression was induced treating with doxycycline dosed at 5 µg/ml for 24h.

SN12C sh91 and shNS thymidine proliferation assay.

SN12C sh91 and shNS were seeded on glass coverslip at the density of 3 x 10^5 per wells, let recover for 6 to 8 hours, wash in PBS1X and culture in DMEM full media (control condition) or in the presence of doxycycline (2 µg/mL) to silence PD-ECGF1 expression. At day 2, cells were maintained in DMEM full media (control condition), growth under nutrient deprivation condition (25% nutrients, FBS free), starved of nutrients but supplemented of thymidine dose at 1mM (25% nutrients, FBS free + thym), and starved of nutrients, supplemented of thymidine and maintained under doxycycline pressure (25% nutrients, FBS free + thym + dox). At day 3, proliferating cells were labelled performing an Edu Assay (FlouProbes) according to manufacture instructions; while total cells marked with DAPI. Five photos for each field were taken at 20X magnification using a Nikon Eclipse 80i microscope and the % of proliferating cells quantified.

786O$^-$-Derived Orthoxenograft Model.

786O$^-$ human cancer cells were directly injected in the right kidney (original neoplastic organ) of male athymic nude mice (Harlan Laboratoris). Once injected, tumor cells were able to colonize the kidney and develop in approximately 20 days a palpable tumor mass then perpetuated into successive passages to obtain an experimental cohort. All animal experiments were developed according to our Institute's Animal Research Committee acceptance, and following Spanish laws and European directives on ethical usage of rodents for animal research (approval DARP-#4899). This method was used for parental, and 786O$^-$ sh91 and shNS as well.

786O$^-$ Tumor Bering Mice Treatments Schedule.

Once tumors started to be palpable (approximate volume of 1000 mm^3) mice were initially randomized in untreated controls (n = 8) or received the anti-angiogenic DC101 twice a week dosed at 1mg/kg/i.p. (n = 25). Tumor growth was followed by palpation twice a week, and the treatment was consider

effective until the primary tumors incremented by a 50% their initial volume, consequently approximately 1500mm^3 by palpation. In the moment of resistance DC101 treated animals were randomized in the following groups:

- DC101, scheduled as previously reported (n = 5);
- DC101 + AEAC, prepared and dosed at 50 mg/kg/day orally (n = 5);
- DC101 + AEAC + recombinant 2-deoxy-D-ribose, prepared and dosed at 100 mg/kg/day i.p. (n = 5);
- DC101 + recombinant 2-deoxy-D-ribose (n = 5);
- DC101 off, group in which the treatment was stopped (n= 5).

AEAC was purchased by Tractus Company Limited (London, England) while recombinant 2-deoxy-D-ribose from Sigma (D5899). Mice were scarified after 14 days of treatment, the tumor tissue weight and processed for histology or molecular analysis.

786O$^-$ sh91 Tumor Bering Mice Treatments Schedule.

When tumors started to be palpable (approximate volume of 1000mm^3) to verify PD-ECGF1 knock down a first experiment was performed. A group of four animals was randomized into untreated controls (n = 2) or a group that received doxycycline (n = 2) dosed at 2 mg/ml and administer in water + 3% of sucrose (the latter added to increase palatability). Animals were killed after 7 days of treatment and tumor tissue weight and processed for histology or molecular analysis.

To study the impact of PD-ECGF1 genetic silencing after anti-angiogenic resistance, mice received DC101 as first therapy (n=22), and at resistance were randomized into the groups:

- DC101, scheduled as previously reported (n = 6);
- DC101 + doxycycline (n = 8);
- DC101 + doxycycline + recombinant 2-deoxy-D-ribose (n = 8);

Animals were scarified after 14 days of treatment, the tumor tissue weight and processed for histology or molecular analysis.

786O$^-$ shNS Tumor Bering Mice Treatments Schedule.

When tumors started to be palpable (approximate volume of 1000mm^3) animals were randomized into untreated controls (n = 8) or a group that received doxycycline (n = 8) prepare and dosed as previously described. Tumor progression was followed by palpation twice a week and animals were killed after 14 days of treatment. Tumor tissue weight and processed for histology or molecular analysis.

Patient-Derived Orthoxenograft Model from RCC Human Biopsy.

A fresh surgical specimen of RCC (chronologically named Ren28) was obtained from the Bellvitge Hospital under local ethics committee's approved protocols (CEIC approvals ref. PR322/11). Surgical implantation was done orthotopically in the right kidney (original neoplastic organ) of male athymic nude mice (Harlan Laboratories) and then perpetuated into successive passages. All animal experiments were developed according to our Institute's Animal Research Committee acceptance, and following Spanish laws and European directives on ethical usage of rodents for animal research (approval DARP-#4899).

Ren28 Tumor Bering Mice Treatments Schedule

Experiments started, and first line of treatments scheduled as described for 786O$^-$ tumor bearing mice. At resistance DC101 treated animals were identically randomized with the addition of a group that received only AEAC, and a group treated with AEAC + recombinant 2-deoxy-D-ribose. Drugs were purchased, prepared and dosed as previously mentioned. Mice were killed after 37 days of treatment, the tumor tissue weight and processed for histology or molecular analysis.

Immunohistology and Quantifications

Tumor sections were either OCT-embedded and frozen or fixed in formalin buffer and embedded in paraffin. Primary antibodies used for specific tissue immunostaining were mouse anti-ECGF1 (Abcam, ab3151), rabbit anti-CD31 (Abcam, ab28364) and rabbit anti-Ki67 (SP6) (Thermo Fisher, RM9106 S-1). Apoptotic cells were stained performing a TUNEL assay (R&D Systems) in accordance to manufacture instruction. EnVision system of labeled polymer-HRP anti-mouse or –rabbit IgG (Dako-Cytomation) were used as a secondary antibody. DAPI (Vector Laboratories) was used for nuclear counterstaining, and finally tissue sections were mounted in DPX new (Merk). To quantify CD31, Ki67 and TUNNEL positivity four to six hotspot fields in viable tissue zones at 10X or 20X magnifications were capture for each tumor. Microvessel area (MVD) was obtained counting the number of CD31-positive vessels in each field, whereas the % of proliferating and apoptotic tumor cells were calculated as the ratio of Ki67 or TUNNEL positive tumor cells to the total number of tumor cells in each field. Stroma- or immuno-cells were not counted.

Western Blot Analyses.

Western blot analyses utilized monoclonal anti-vinculin (V9131, Sigma-Aldrich) and monoclonal anti-thymidine phosphorylase (P-GF.44C, ab3151, Abcam). Quantification was performed using Quantity One software (version 4.6.1; Bio-Rad).

Patient Series and Clinical Data.

The series of pre- and post-samples from patients with RCC was gather prospectively and retrospectively between 2007 and 2014 under clinical ethics committee approval (IDIBELL CEIC approval ref. PR322/11) and acceptance by the Spanish Government (AEMPS). A total of 10 clear-cell RCC pre- and post-treatment biopsies were collected and PD-ECGF1 expression detected by IHC. Moreover, a total of 12 plasma samples from patients affected by ccRCC was

selected and PD-ECGF1 expression detected using and Elisa kit (R&D). These patients were treated with antiangiogenic therapy (mostly bevacizumab and a few sunitinib at standard dose) and the response evaluated by RECIST guidelines (tumor progression PD, resistance; or tumor response SD, in efficacy) at the end of the antiangiogenic treatment.

Statistical Analysis.

Statistical comparison was done using Mann Statistical comparison was done using Mann-Whitney U test (2 Whitney U test (2-tailed) performed in tailed) performed in GraphPad GraphPad Prism (GraphPad Software, Inc. USA). Differences were considered statistically significant at $p<0.05$. Statistic coding: *p < 0.05, **, p< 0.01; ***, p< 0.001, ****, p< 0.0001.

Results.

Results.

1 – Anti-angiogenic response in 786O$^-$ Renal Cell Carcinoma (RCC) tumors.

According to what has been reported happen in the clinic we can easily describe two consecutive phases. Initially, animals respond to the therapy showing a significant reduction in tumor growth (Fig. 14, dotted blue vs black lines), but unfortunately these effects appear to be only temporary, and all treated tumors reached resistance (set as described in materials and methods and indicated by a red dotted vertical line in fig 1). From this point on, displacing or continuing DC101 treatment had a similar effect on tumor growth (Fig. 14, full black vs light red lines), meaning that DC101 is no more effective in confining tumor progression and confirming the quality of this animal model to study resistance to anti-angiogenics in RCC.

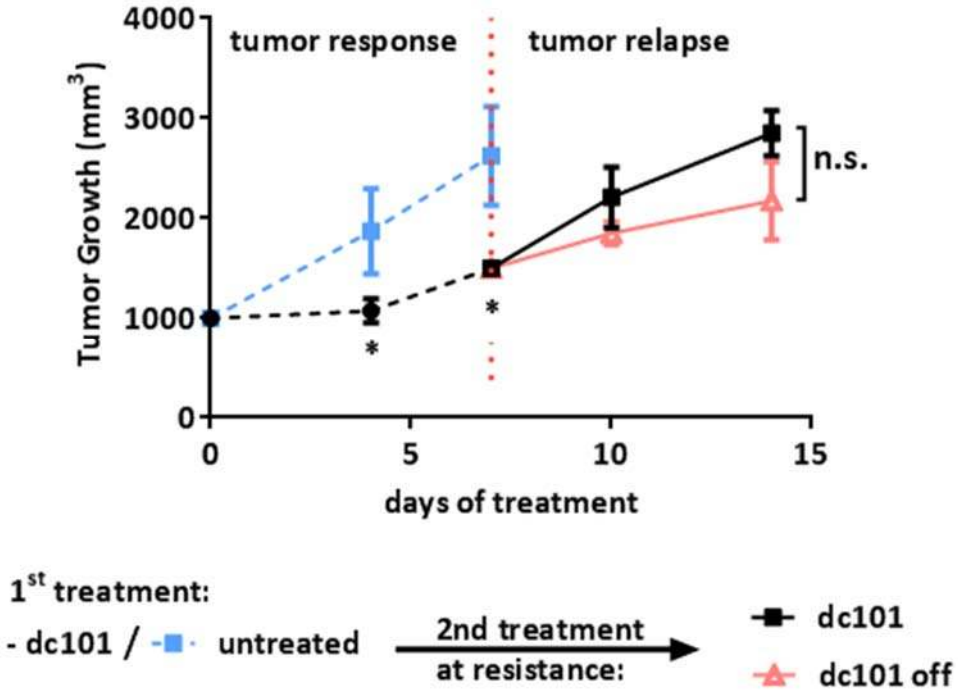

Fig. 14. 786O$^-$ orthoxenograft tumors developed resistance to DC101 treatment. Tumor progression of 786O$^-$ tumor bearing mice revealed two consecutive phases. After a transitory reduction of tumor growth, all treated animals reached resistance (indicated by the red vertical dotted line in the graph) and relapse. *: $p < 0.05$. Bars

show standard deviation. n = 8 for the control group; n = 20 for the DC101 group until resistance; n = 5 for the DC101 group after resistance and for the DC101 off group.

1.1 – Tumor resistance is vessel independent.

Laboratory and clinical research has associated resistance to anti-VEGF therapies to different mechanisms, including upregulation of VEGF, co-option of existing vessels that are less VEGF-dependent or the activation of alternative angiogenic pathways leading to vessels recruitment, tumor re-vascularization and malignization.

Consequently, to investigate the reasons for the described therapeutic failure, the microvessel density (MVD) was quantified. Surprisingly, even if DC101 doesn't affect tumor progression, the drug doesn't lose its ability to hit the vasculature, suggesting the idea that the described anti-angiogenic resistance is vessels independent (Fig. 15).

The partial recovery resulting when the treatment was stopped indicates that probably seven days of "off treatment" are not enough to observe a complete re-vascularization, or the consequence of the remaining therapeutic effects ahead of the complete drug clearance.

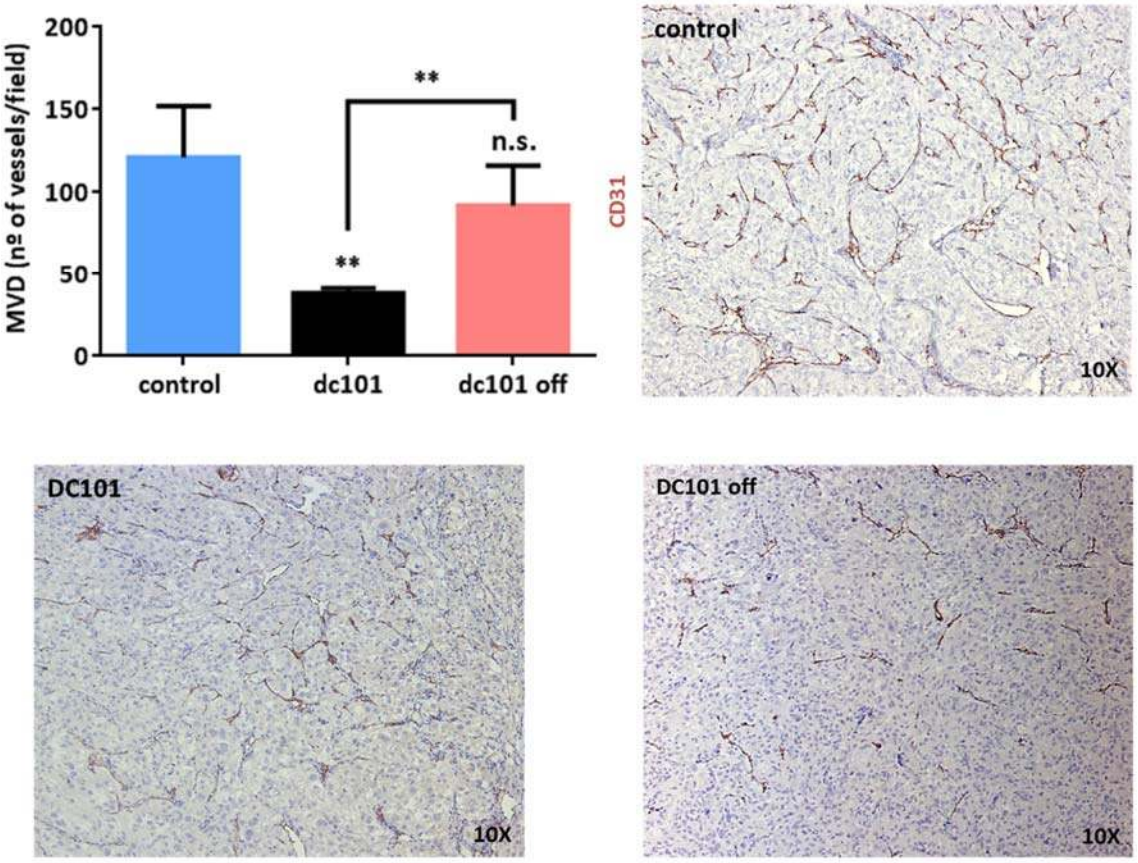

Fig. 15. DC101 treatment reduces MVD. In comparison with untreated control, DC101 treatment significantly decreases MVD, even if in the same animals tumor growth is no more affected. When the anti-angiogenic is displaced new-angiogenesis occurs. Pictures show representative images for each group. *: $p < 0.05$. **: $p < 0.005$. Bars show standard deviation. n = 8 for the control group; n = 5 for the DC101 group after resistance and for the DC101 off group.

1.2 – Genetic silencing of PD-ECGF1 expression.

To verify our preliminary data of PD-ECGF1 as a possible mediator of resistance to anti-angiogenics, we decided to adopt a genetic approach to specific knock down the gene expression in the moment of DC101-resistance. In fact, we didn't go for a constitutively knock out, to avoid the possibility that tumor cells would adapt and circumvent the anti-angiogenics through a different mechanism.

Consequently, three different silencing (sh89, 91 and 93) and a control non-silencing (shNS) clones have been used, and the efficacy of infection and PD-ECGF1 knock down verified *in vitro*. All three silencing clones worked similarly, reducing PD-ECGF1 protein expression in a time-dependent way upon

doxycycline activation (Fig. 16). However, we notice some diversity in cell growth with the clone 93 slower and sometimes different in shape under microscopy observation, hence opted for the clone 91 to embark further studies.

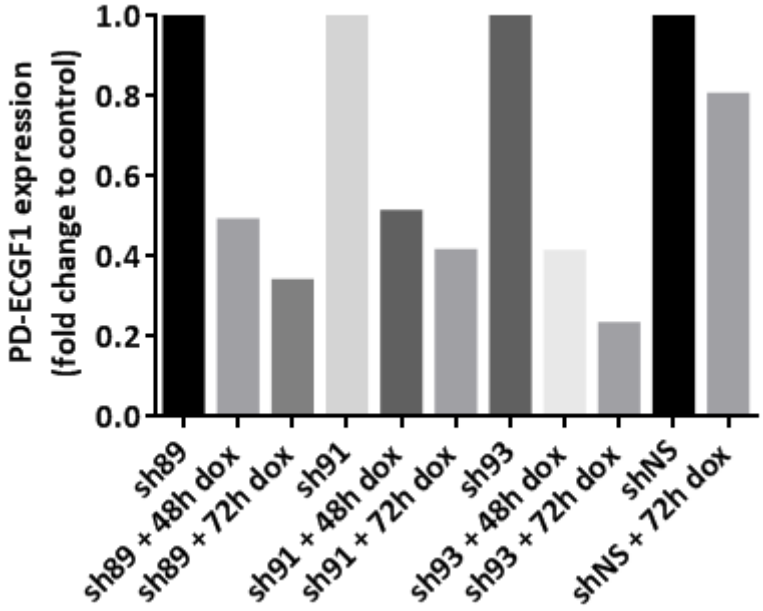

Fig. 16. *In vitro* PD-ECGF1 genetic knock. Quantification of PD-ECGF1 protein levels detected by Elisa Assay and express as fold change to untreated control. Upon doxycycline activation (2 µg/ml) the three clones similarly knock down PD-ECGF1 expression in 786O^{-} sh91 cells.

1.2.1 – In vivo tumorigenic capacity and silencing efficacy.

Before starting more complex and tedious investigations, the clone 91 capacity to generate solid tumors and the *in vivo* knock down efficacy were examined in a pivotal experiment in which a group of four animals were randomized into untreated controls (n = 2) and a group treated with doxycycline as described in the methodology (n = 2). Even if was not the aim of the experiment, doxycycline treatment resulted in a promising anti-tumor effect (Fig. 17A-C) due to a drop down of PD-ECGF1 protein levels of approximately 60% (Fig. 17D). Satisfied by the clone 91 capacity to generate RCCs tumors and to silence PD-ECGF1

expression if needed, we moved to study the impact of PD-ECGF1 genetic knock down in anti-angiogenic resistant tumors.

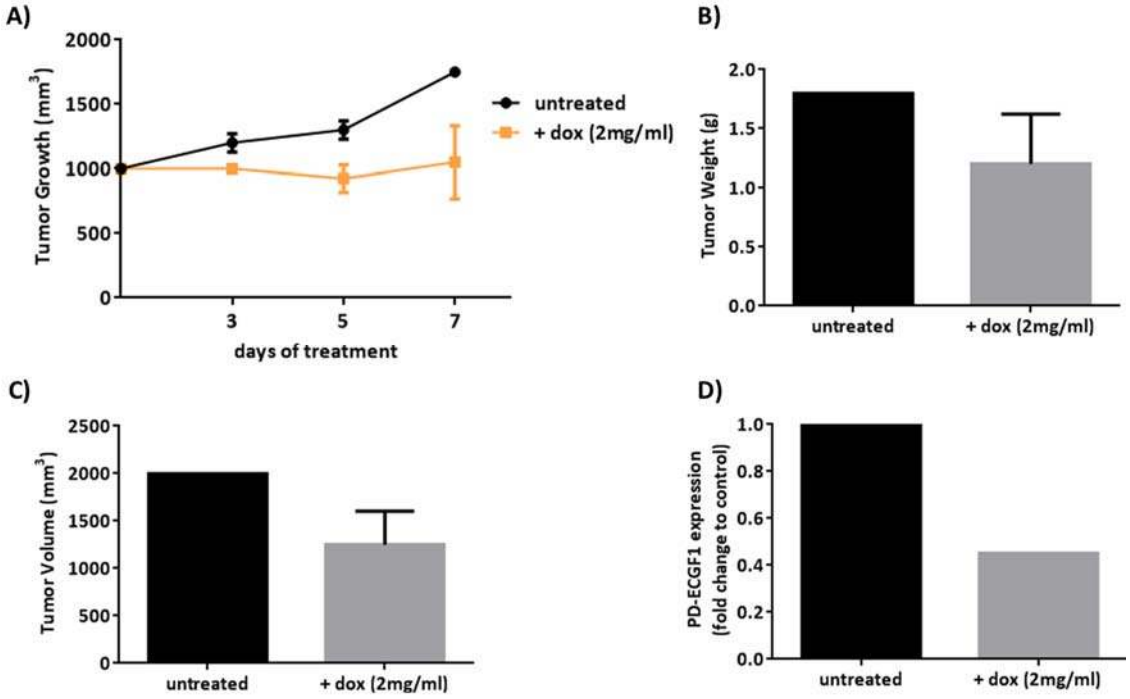

Fig. 17. Effects of *in vivo* PD-ECGF1 genetic knock down. **A**) Tumor progression by palpation of untreated (n = 2) and doxycycline treated (n = 2) 786O^{-} sh91 tumor bearing mice showed, as a result of PD-ECGF1 genetic silencing, a promising anti-tumor effect. **B** – **C**) Effect of PD-ECGF1 genetic silencing on tumor weight (**B**) and volume (**C**) at sacrifice. **D**) Quantification of PD-ECGF1 protein levels determined by Elisa Assay and express as fold change to untreated controls. Bars show standard deviation. n = 2 for both groups.

1.2.2 – PD-ECGF1 genetic knock down in combination with DC101 arrest tumor re-growth.

Still having DC101 anti-angiogenic effectiveness (Fig. 15), as second line of treatment after resistance, the genetic silencing of PD-ECGF1 was combined with DC101 treatment. PD-ECGF1 genetic knock down stopped tumor progression (Fig. 18A) significantly affecting tumor weight and volume measured at sacrifice (Fig. 18B, C). Consequently, knock down efficacy was evaluated, finding that, in comparison with DC101-treated animals, upon doxycycline activation PD-ECGF1 protein level was reduced by approximately an 80% (Fig. 19).

To dissect the molecular mechanism responsible of the described tumor arrest, MVD, tumor cell proliferation and apoptosis have been deeply analyzed.

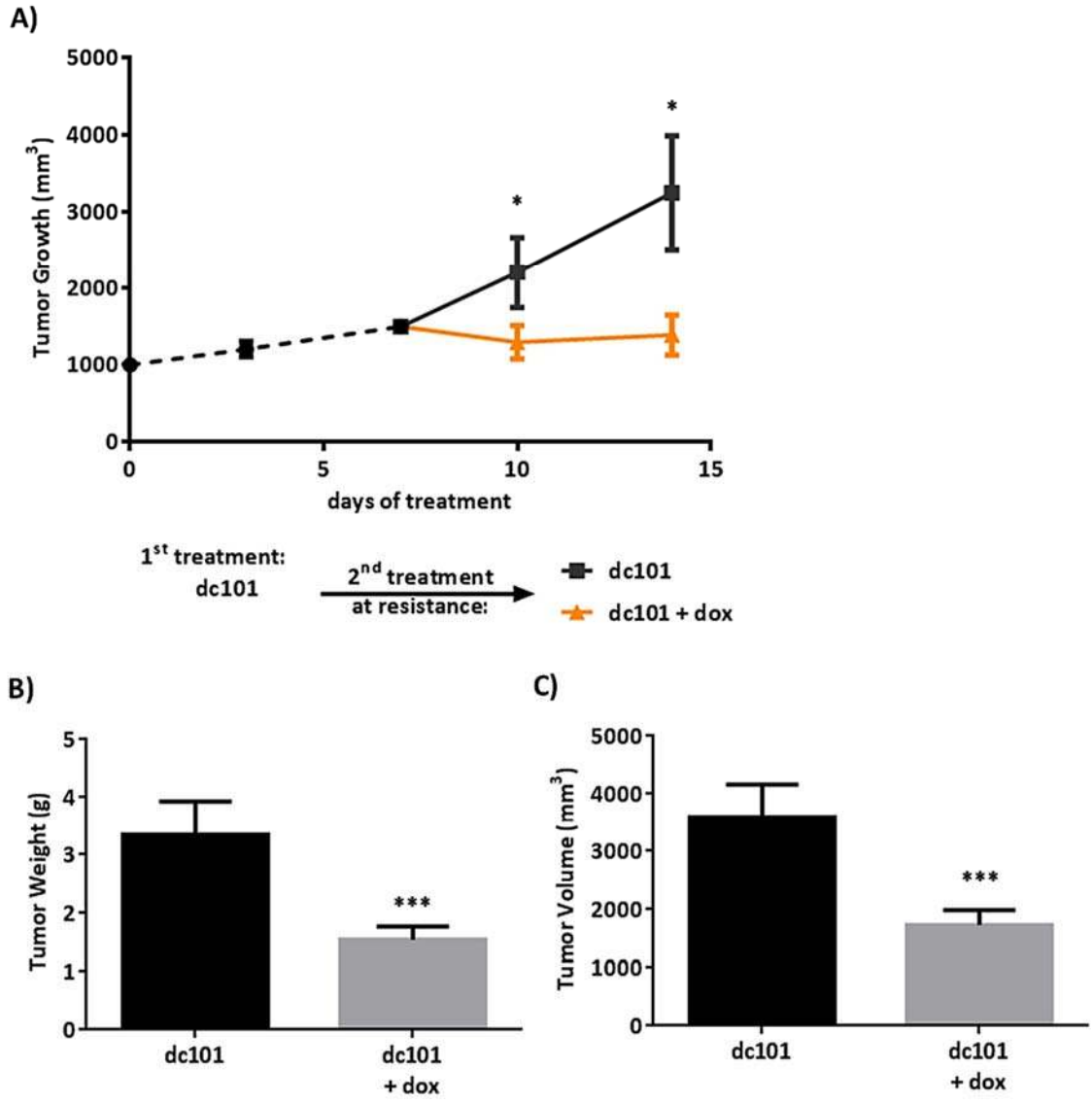

Fig. 18. PD-ECGF1 silencing arrests tumor re-growth. **A**) Tumor progression by palpation shows a statistically significant inhibition of tumor growth upon PD-ECGF1 silencing. **B**, **C**) Tumor weight and volume measured at sacrifice. Bars show standard deviation. * = < 0.05. n = 22 for the DC101 treated group until resistance; n = 6 for the DC101 group after resistance; n = 8 for the DC101 + dox group.

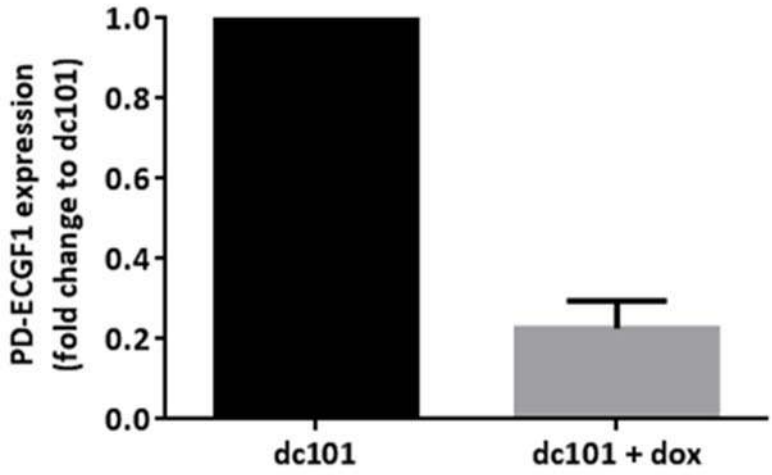

Fig. 19. PD-ECGF1 protein expression. Quantification of PD-ECGF1 protein levels in tumor tissue measured by Elisa Assay and expressed as fold change to DC101 treated animals. Bars show standard deviation. n = 2 for each group.

1.2.3 – PD-ECGF1 genetic knock down doesn't affect MVD.

The pro-tumoral role of PD-ECGF1 and its link with endothelial cells migration and angiogenesis has been well documented by different studies (Brown et al., 2000; Bijnsdrop et al., 2011; Elamin et al., 2015). Consequently, we wondered if PD-ECGF1 genetic silencing was blocking tumor re-growth acting on tumor vasculature. Surprisingly, we didn't notice any difference in terms of MVD between the two groups, suggesting that in this model PD-ECGF1 promotes tumor relapse not through the recruitment of new blood vessels (Fig. 20).

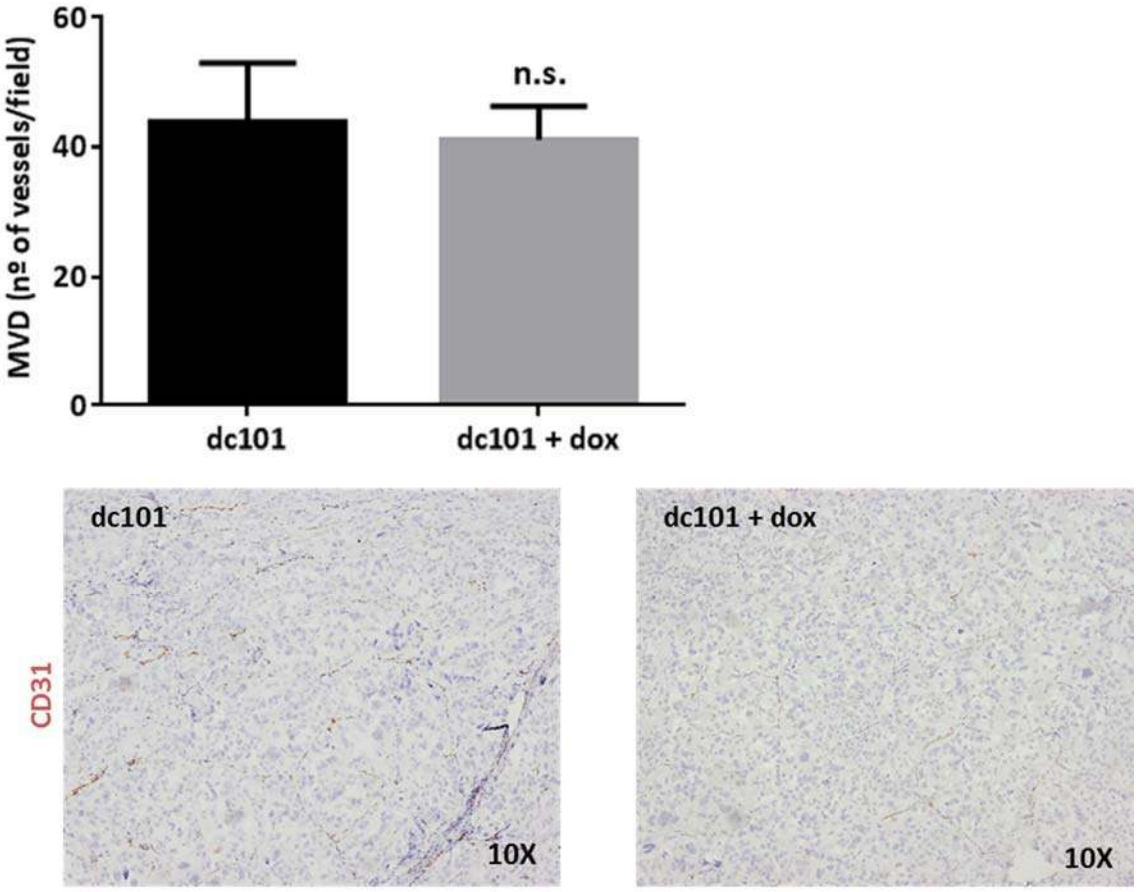

Fig. 20. PD-ECGF1 genetic silencing doesn't affect MVD. MVD quantification shows no differences between treatments. Pictures show representative images for each group. Bars show standard deviation. n = 6 for the DC101 group after resistance; n = 8 for the DC101 + dox group.

1.2.4 – PD-ECGF1 genetic knock down directly affects tumor cells proliferation and apoptosis.

Beyond its described angiogenic effects, PD-ECGF1 tumor cell expression has been associated to autocrine protection from hypoxia induced apoptosis (Ikeda et al., 2006). Consequently we wondered if the anti-tumoral effect upon PD-ECGF1 silencing could be tumor cell intrinsic. In comparison with DC101 resistant tumors, the genetic knock down of PD-ECGF1 significantly decreases tumor cells proliferation (Fig. 21A) and increases apoptosis (Fig. 21B), supporting the hypothesis that PD-ECGF1 in this model mainly acts intracellularly to promote tumor cell proliferation and protect from apoptosis.

Doxycycline has been reported to inhibit tumorigenesis acting on proliferation and migration of different cancer cell lines (Wu et al., 2014; Qin et al., 2015). To verify its toxicity we proceed to evaluate the response of 786O^{-} shNS tumor bearing mice to doxycycline.

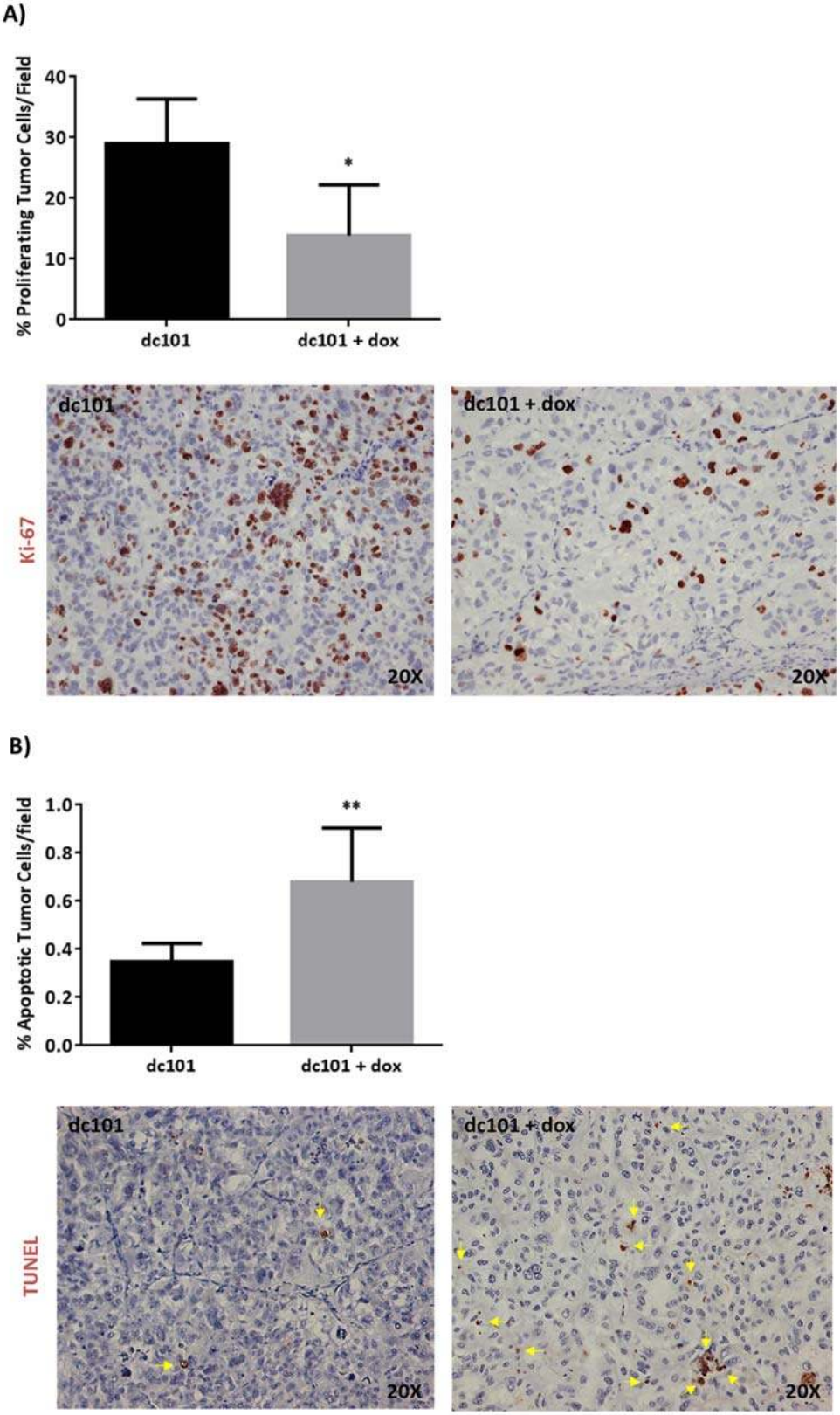

Fig. 21. PD-ECGF1 genetic knock down significantly affects tumor cell proliferation and apoptosis. A, B) Quantification and representative pictures of the percentage of proliferating (**A**) and apoptotic tumor cells (**B**). Yellow arrows mark the apoptotic

tumor cells counted. *: < 0.05. **: p < 0.005. ***: p < 0.0005. Bars show standard deviation. n= 8 for each group.

1.2.5 – Doxycycline doesn't affect tumor progression.

To evaluate possible toxicity and long term side effects a group of 786O$^-$ shNS tumor bearing mice (n = 16) was randomazed into untreated control (n = 8) or put on doxycycline regimen (n = 8), and tumor growth followed for 14 days. We didn't observe any sign of toxicity (i.e. cachexia, body weight lost) and, even if we noticed considerable variation, tumor progression and final tumor weight were not affected by the treatment (Fig. 22A, B). Consequntly, changes in the MVD, percentage of proliferating and apoptotic tumor cells were investegated without reporting any difference between the two groups (Fig. 22C-E). We can conclude that, at the dose used, doxycyline didn't affect tumor cells and the anti-tumoral effect seen in 786O$^-$ sh91 tumor bering mice was due only to PD-ECGF1 knock down (Fig. 18, 21).

PD-ECGF1 has been reported to play an important role in tumorigenesis thank to its catabolic activity (Elamin et al., 2015), and our findings demonstrate that its genetic silencing blocks tumor re-growth. Consequently, using the small molecule AEAC, we proceeded to investigate if the inhibition of its enzymatic activity would confirm these results and highlight the molecular mechanism responsible.

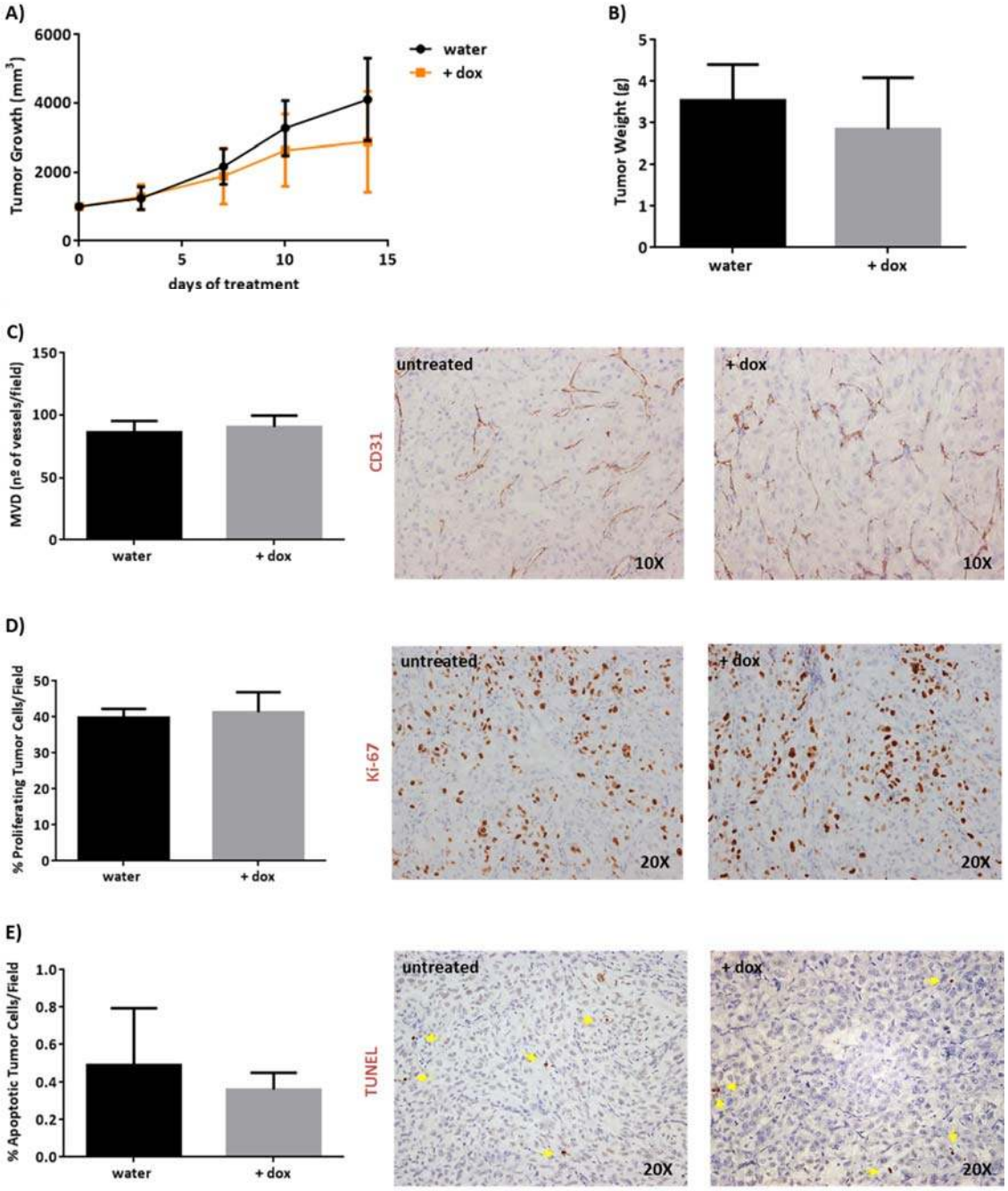

Fig. 22. Doxycycline isn't toxic for tumor cells and doesn't affect tumor progression. A, B) Tumor progression by palpation (**A**) and weight at sacrifice (**B**) showed a considerable variation but not statistically differences between the two groups. C, D, E) Quantifications and representative images of MVD (**C**), the percentage of proliferating (**D**) and apoptotic (**E**) tumor cells. In comparison with untreated tumors, doxycycline alone doesn't affect any of the parameters exanimated. Yellow arrows marks the apoptotic tumor cells counted. Bars show standard deviation. n = 8 for both groups.

1.3 – The enzymatic inhibition of PD-ECGF1 blocks tumor re-growth.

To verify the importance of PD-ECGF1's enzymatic activity in the failure of anti-angiogenic therapies, at resistance a group of animals received as second line the combination of DC101 and AEAC treatments. AEAC has been reported to inhibit the enzymatic activity of PD-ECGF1 and reduce MVD in several *in vivo* and *in vitro* studies (Lu et al., 2009; Kylie et al., 2003), and previous data from the lab confirmed, using a mouse retina model, a reduction in endothelial cell migration and low toxicity *in vitro*. In comparison with DC101-treated animals, as second line of treatment the combination of the enzymatic inhibition of PD-ECGF1 with the anti-angiogenic blocks tumor re-growth, resulting at end point in a significant reduction of tumor weight and volume (Fig. 23A-C).

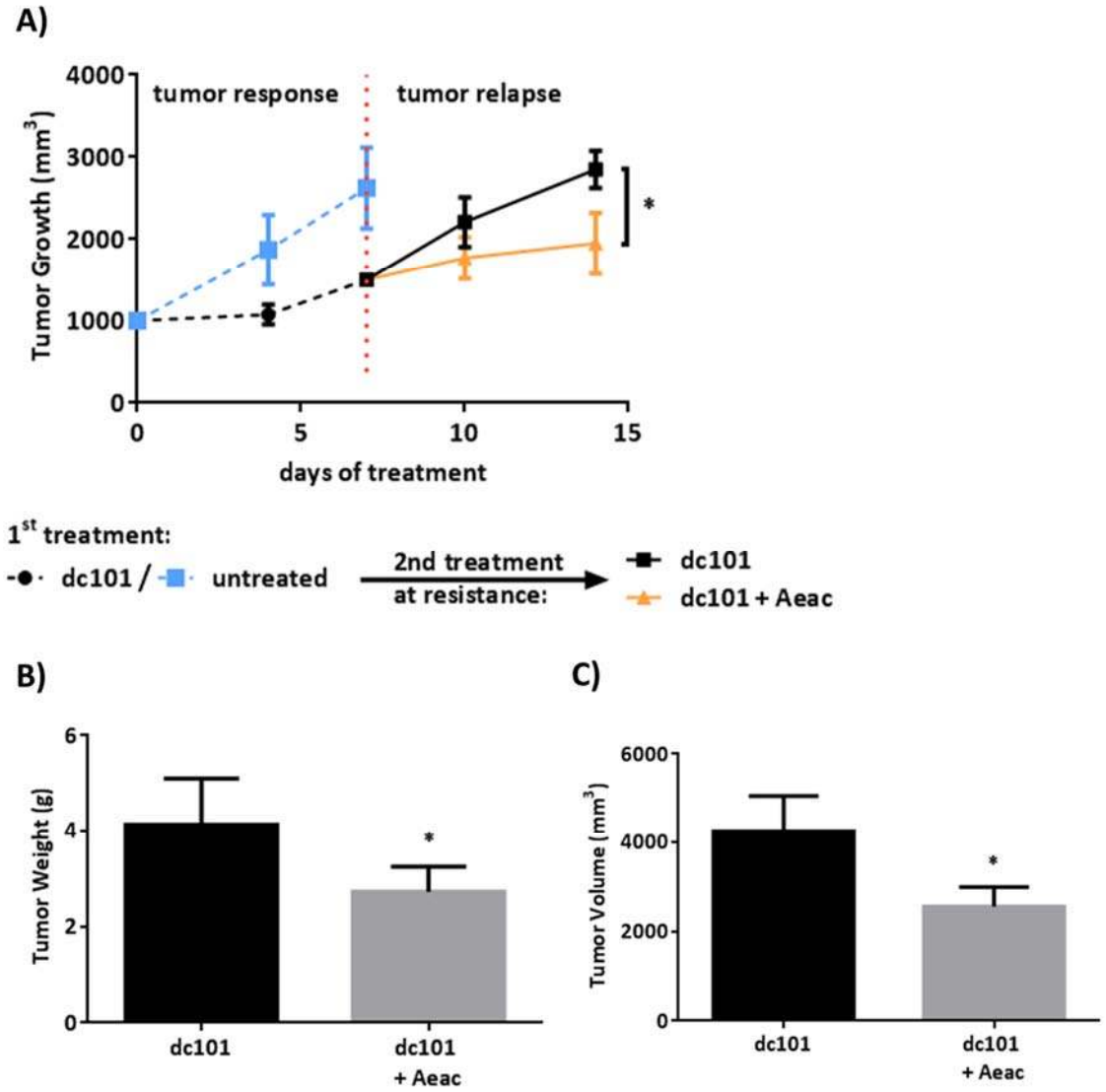

Fig. 23. PD-ECGF1 enzymatic inhibition blocks tumor re-growth. A) In comparison with DC101 treated mice, as second line of treatment DC101 plus AEAC results in a significant reduction of tumor growth (**A**), weight (**B**) and volume (**C**) measured at sacrifice. *: $p < 0.05$. Bars show standard deviation. n = 5 for each group.

1.3.1 – PD-ECGF1 enzymatic inhibition directly acts on tumor cells without affecting tumor vasculature.

Even if AEAC is one of the most potent competitive inhibitor of PD-ECGF1, its mechanism of action still needs clarifications and its anti-tumoral activity attributed only to a reduction of tumor vasculature without direct effects on tumor cells (Lu et al., 2009). Surprisingly, we didn't notice any difference in terms of MVD between the two groups (Fig. 24A), whilst the enzymatic inhibition of PD-ECGF1 significantly decreases tumor cells proliferation (Fig. 24B)

and increases apoptosis (Fig. 24C), suggesting that in this model PD-ECGF1 promotes tumor relapse acting intracellularly to promote tumor cell proliferation and protect from apoptosis.

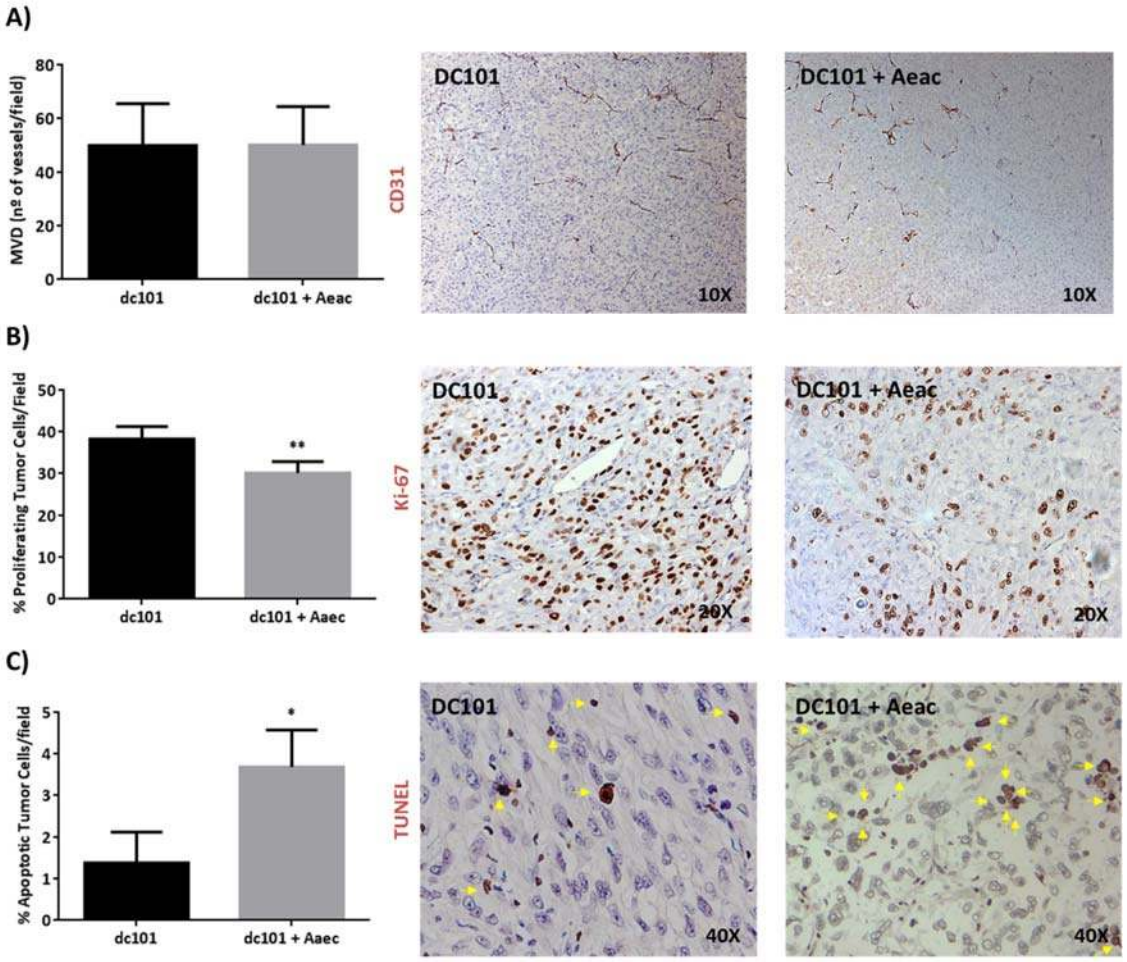

Fig. 24. PD-ECGF1 enzymatic inhibition affects tumor cell proliferation (A) and apoptosis (B), without reducing tumoral vasculature (C). A, B) In comparison with DC101 resistant tumors, the enzymatic inhibition of PD-ECGF1 significantly reduces the percentage of proliferating tumor cells and increases the percentage of apoptotic tumor cells. C) The enzymatic inhibition of PD-ECGF1 does not affect MVD. Pictures show representative images for each group in which the apoptotic tumor cells have been marked with yellow arrows. *: < 0.05. **: p < 0.005. Bars show standard deviation. n = 5 for each group.

1.3.2 – Tumor arrest is rescued by 2-doexy-D-ribose.

Different studies reported that PD-ECGF1 exerts its pro-tumoral and angiogenic effects by its enzymatic activity and final metabolite 2-deoxy-D-ribose (Ikeda et al., 2002; Seelier et al., 2004). Moreover, in pathology caused by PD-ECGF1 deficiencies like MNGIE, thymidine accumulation results toxic impairing mtDNA replication altering cell growth and viability (Hirano et al., 2004). Therefore, to discriminate whereas the anti-tumoral effects described upon PD-ECGF1 enzymatic inhibition were due to the lack of its final metabolites, or to nucleotide imbalance, at resistance mice were treated with 2-deoxy-D-ribose.

Strikingly we found that 2-deoxy-D-ribose rescued tumor growth, weight and volume as in DC101-resistant animals (Fig. 25A orange vs green lines; 18B). Importantly, if PD-ECGF1 was not inhibited, 2-deoxy-D-ribose didn't promote tumor growth (Fig. 25A light purple line) suggesting that even if the enzymatic activity of PD-ECGF1 is critical to bypass the anti-angiogenic therapy, the pathway is tightly regulated and tumor cells can't consume 2-deoxy-D-ribose boundless.

To investigate if 2-deoxy-D-ribose rescued tumor growth by acting directly on tumor cell proliferation and apoptosis or alternatively, given its pro-angiogenic nature, promoting re-vascularization, tumor sections were stained for CD31, Ki-67 and TUNEL. Consistently with the cell intrinsic mechanism postulated, 2-deoxy-D-ribose did not overrule DC101 anti-angiogenic effects (Fig. 26A) whereas it directly affects tumor cells, and by promoting proliferation and protecting cancer cells from apoptosis leads tumor relapse (Fig. 26B, C). Finally, no significant differences were seen when PD-ECGF1 was not inhibited confirming what has been already described regarding tumor growth.

Next, we decided to further confirm the biological effects of 2-deoxy-D-ribose in tumors lacking PD-ECGF1.

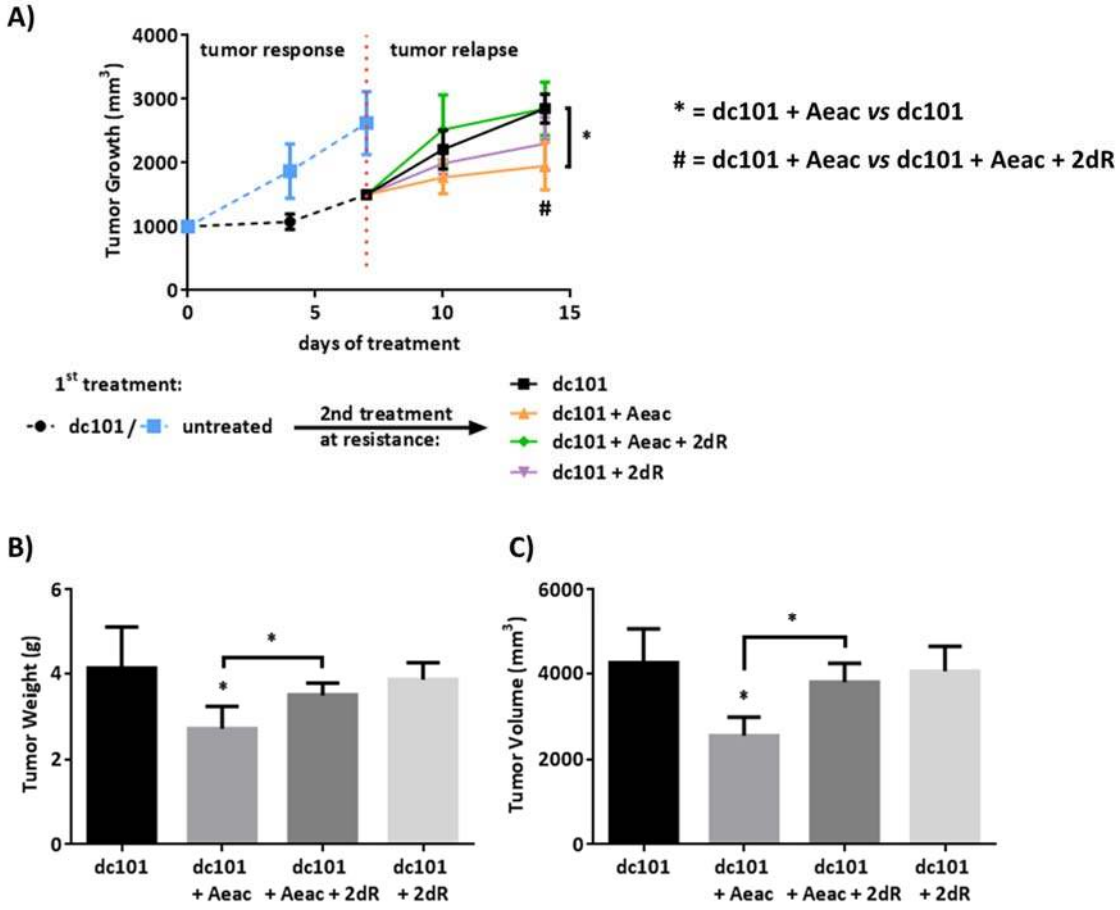

Fig. 25. 2-deoxy-D-ribose rescues tumor growth (A), weight (B) and volume (C). A) 2-deoxy-D-ribose counteracts the arrest of tumor growth resulted from AEAC inhibition (green vs orange lines). No pro-tumoral effects were noticed if PD-ECGF1 was not inhibited (light purple vs black lines). B, C) Effects of 2-deoxy-D-ribose on tumor weight and volume confirmed what described for tumor growth. *, #: $p < 0.05$. **: $p < 0.005$. Bars show standard deviation. n = 5 for each group.

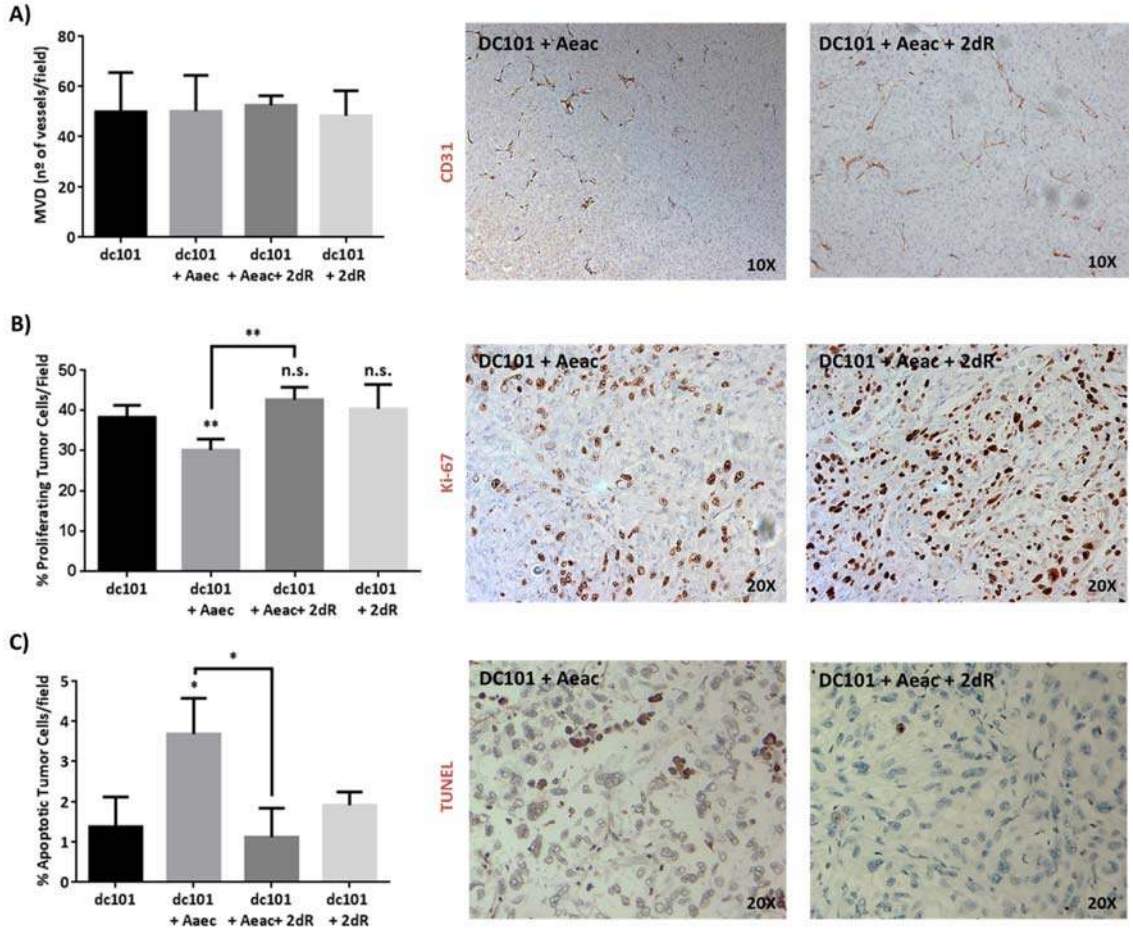

Fig. 26. Effects of 2-deoxy-D-ribose on MVD (A), tumor cell proliferation (B) and apoptosis (C). A) MVD quantification shows no differences between treatments. B) 2-deoxy-D-ribose significantly rescued the percentage of proliferating tumor cells when PD-ECGF1 is inhibited. No statistically difference was notice in comparison with the DC101 group. C) 2-deoxy-D-ribose protected cancer cells from apoptosis. Pictures show representative images for each group. *: < 0.05. **: p < 0.005. Bars show standard deviation. n = 5 for each group.

1.4. – 2-deoxy-D-ribose counteracts the loss of PD-ECGF1 promoting tumor cell proliferation and protecting from apoptosis.

Upon PD-ECGF1 genetic silencing, 2-deoxy-D-ribose promotes tumor progression (Fig. 27A orange vs green lines) even if we observed not a full reversion but an intermediate phenotype, confirmed by tumor weight (Fig. 27B) and volume (Fig. 27C) measured at sacrifice. Effects on tumor cell proliferation and apoptosis were analyzed, validating the role of 2-deoxy-D-ribose in supporting proliferation (Fig. 28A) and protecting from apoptosis (Fig. 28B).

Using genetic and pharmacological approaches, we have dissect a now role of PD-ECGF1 as a possible mediator of resistance to anti-angiogenics in ccRCC. However, the animals models used in the study derived from orthotopic inoculation of immortalized human cancer cell lines, a limitation that needs to be consider. Consequently, to verify these findings in a more clinical relevant animal model, we decided to study the anti-angiogenic response in patient-derived Ren28 primary tumor bearing mice.

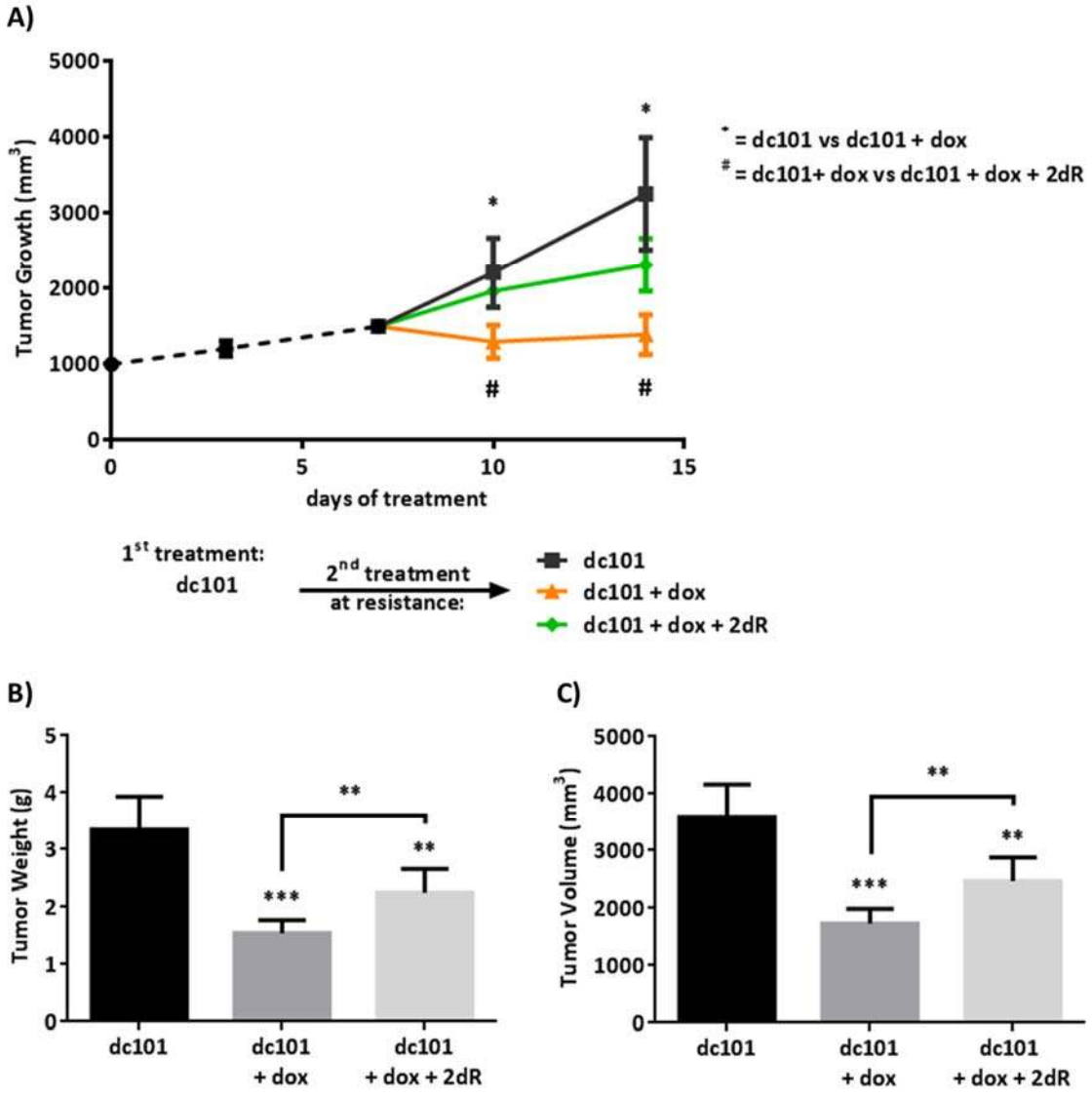

Fig. 27. 2-deoxy-D-ribose promotes tumor resistance in 786O- sh91 tumors. A) In comparison with doxycycline treated animals, daily injections of 2-deoxy-D-ribose promote tumor growth. B, C) Tumor weight and volume at sacrifice. *, #: < 0.05. **: p < 0.005. ***: p < 0.0005. Bars show standard deviation. n = 22 for the DC101 treated

group until resistance; n = 6 for the DC101 group after resistance; n = 8 for the DC101 + dox, and DC101 + dox + 2dR groups.

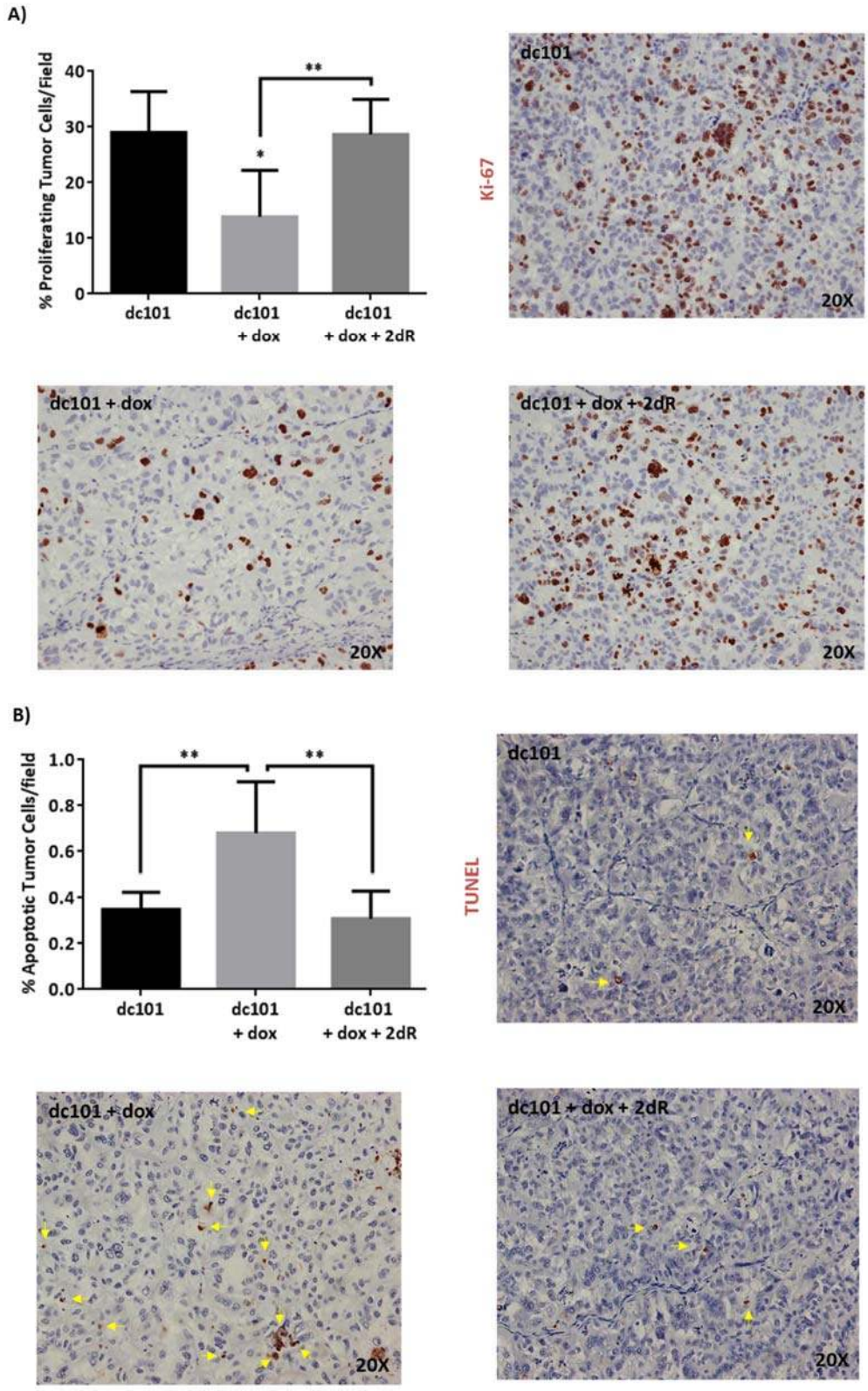

Fig. 28. Effects of 2-deoxy-D-ribose on tumor cell proliferation (A) and apoptosis (B) in 786O^{-} sh91 tumor bearing mice. A, B) 2-deoxy-D-ribose counteracts the loss of PD-

ECGF1 supporting proliferation (A) and protecting from apoptosis (B) tumor cells of 786O- sh91 tumor bearing mice. *: < 0.05. **: p < 0.005. ***: p < 0.0005. Bars show standard deviation. n = 6 for the DC101 group after resistance; n = 8 for the DC101 + dox, and DC101 + dox + 2dR groups.

2 – Ren28 primary tumors respond transitory to anti-angiogenics.

Ren28 is an orthoxenograft tumor model derived from a human biopsy originally chirurgical implanted into the right kidney of an athymic nude mouse, and then successfully perpetuated into successive passages.

Previously to my arrival in the lab, to certify that the model was able to truthfully reproduce the human ancestor and do not lose its intrinsic characteristics, hematoxylin and eosin (H&E) analysis were performed. Histologically, derived tumors resemble the original biopsy maintaining the specifics features upon all the passages (Fig. 29). Moreover, starting from the first passage the human stroma is replaced by the murine counterpart, allowing the use of DC101 as anti-angiogenic therapy.

As described in material and methods section, anti-angiogenic response was evaluated. In comparison with cell derived tumors, Ren28 tumors grow slower and the treatment seems more effective in time, however the group confirmed the existence of an early phase in which tumor growth is stabilized by the anti-angiogenic, unfortunately followed by resistance and evasion to the treatment (Fig. 30A, black *vs* red lines). As previously observed, when tumor started to relapse (Fig 30A, black dotted vertical line) continuing or interrupting the treatment has the same effect on tumor growth, meaning that some adaptation has occurred and the tumor mass is no longer sensible to the treatment (Fig. 30A red *vs* orange lines). Moreover, even if unable to reduce tumor burden, DC101 maintains anti-angiogenics effects on endothelial cells, validating the hypothesis that resistance is vessel independent (Fig. 30B).

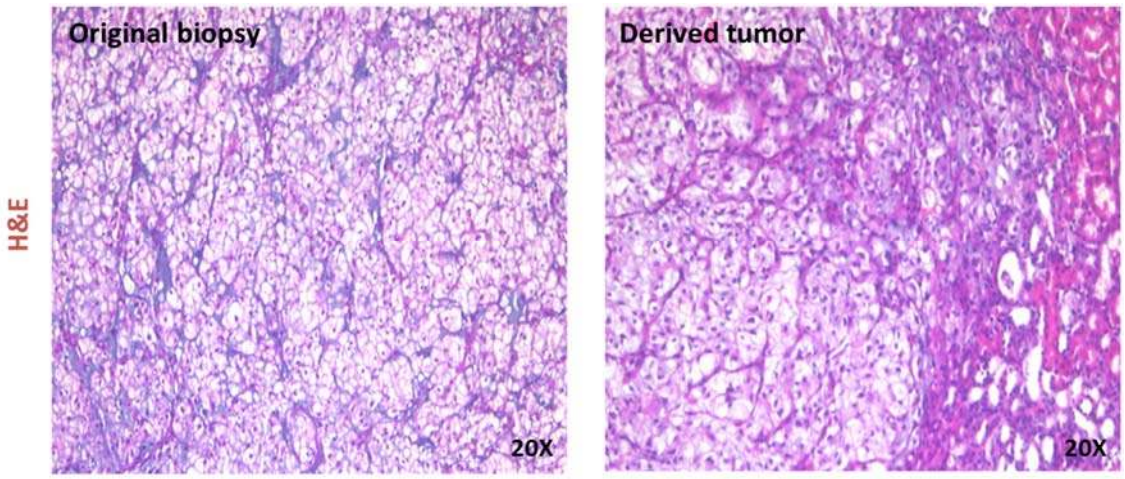

Fig. 29. Hematoxylin and eosin (H&E) staining of the original biopsy and the derived orthoxenograft tumor. Representative images of FFPE tumor sections stained for H&E.

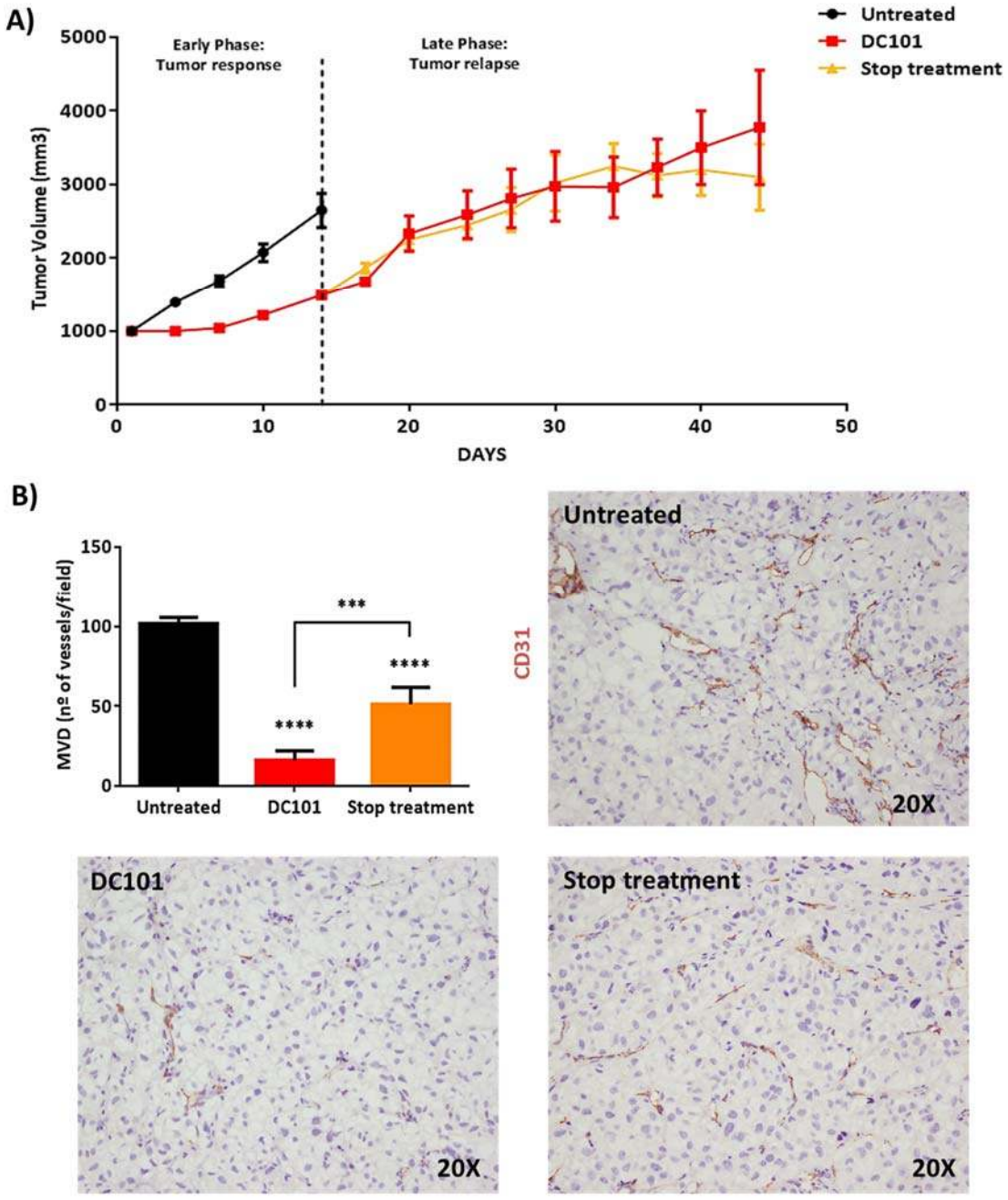

Fig. 30. Ren28 tumor bearing mice develop a vessel independent resistance to DC101. A) Tumor progression by palpation of Ren28 tumor bearing mice. B) MVD quantification shows that DC101 treatment (red bar) significantly decreases MVD, even if in the same animals tumor growth is not affected. Displace the treatment (orange bar) results in a partial revascularization. Pictures show representative images for each group. ***: $p < 0.0005$. ****: $p < 0.00005$. Bars show standard deviation. n = 8 for the control group; n = 20 for the DC101 group until resistance; after resistance n = 7 for the two groups. The experiment was performed by Jimenez-Valerio G.

2.1 – PD-ECGF1 drives anti-angiogenic resistance.

To confirm PD-ECGF1 as a putative target of resistance, its tumor tissue expression and protein levels have been detected by IHC and WB analysis, respectively. As reported for 786O$^-$ tumors, DC101-resistant tumors show, in comparison with untreated controls, a significant up-regulation of PD-ECGF1 tissue and protein expression (Fig. 31 A-C). Consequently, to study its role in the process of acquisition of resistance we adopted the same strategy successful in 786O$^-$ tumor bearing mice using the small molecule AEAC.

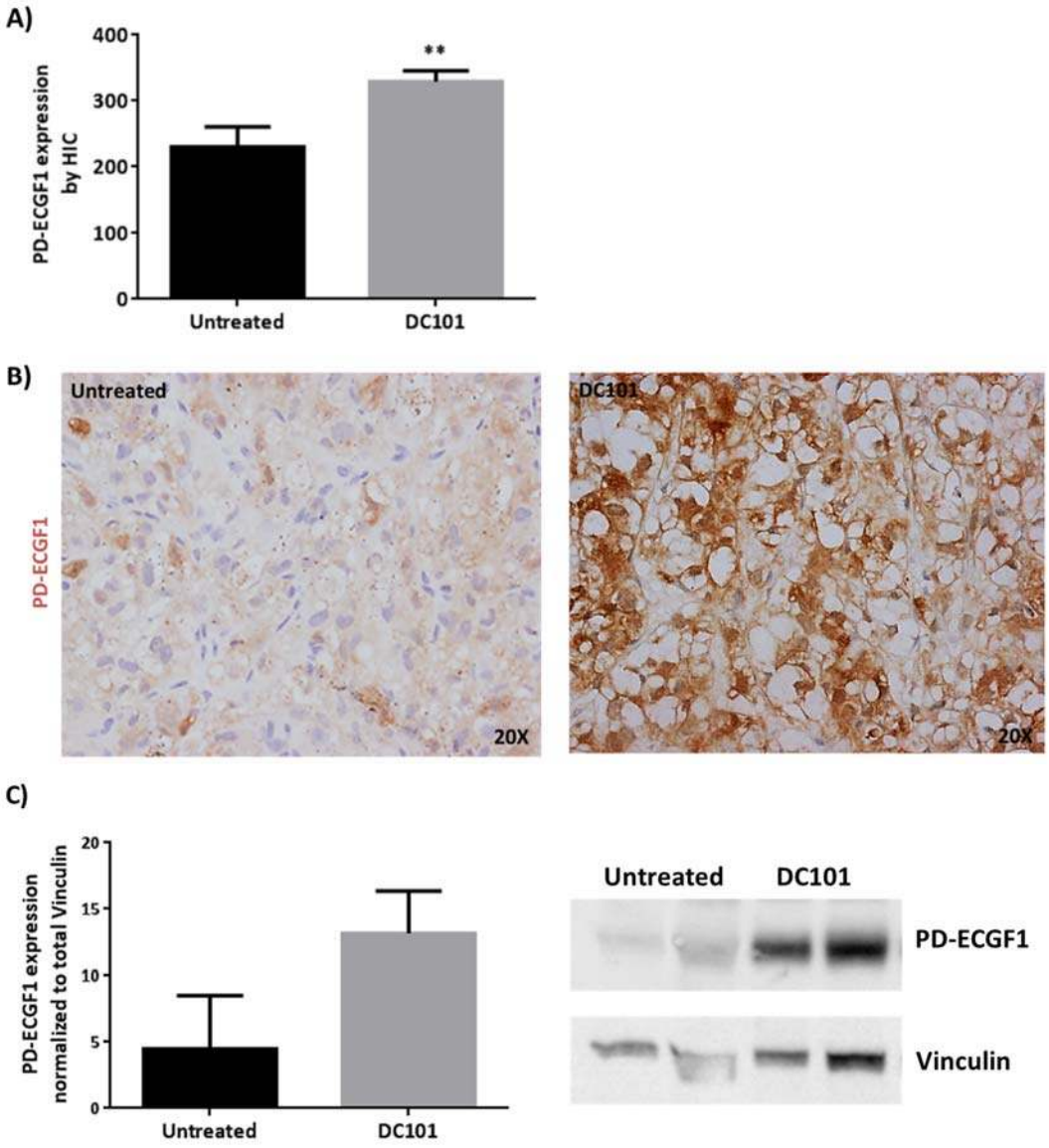

Fig. 31. PD-ECGF1 is significantly over-expressed in DC101-resistant tumors. A) Quantification of PD-ECGF1 tumor tissue expression detected by IHC. B)

Representative images for each group. C) Quantification of PD-ECGF1 protein levels detected by western blot analysis confirms the PD-ECGF1 over-expression noticed by IHC. **: $p < 0.005$. Bars show standard deviation. n = 5 for each group in the IHC detection. n = 2 for each group in the BW analysis. IHC analysis and quantification was performed by Jimenez-Valerio G.

2.2 – PD-ECGF1 enzymatic inhibition in combination with DC101 overcomes resistance.

The enzymatic inhibition of PD-ECGF1 overcomes anti-angiogenic resistance (Fig. 32A, orange vs black lines), emphasizing the effects already described for 786O$^-$ tumors. In fact, tumor progression is stabilized for more than 20 days and is reflected in a final tumor weight (Fig. 32C) and volume (Fig. 32B) that rarely exceed the initial volume at resistance (tumor volume by palpation = 1500mm^3) when the second line of treatment was applied. As stated in 786O$^-$ tumors, 2-deoxy-D-ribose rescued tumor growth (Fig. 32A, green line), weight (Fig. 32C) and volume (Fig. 32B) confirming AEAC specificity and suggesting that PD-ECGF1 exerts pro-tumoral effects by its enzymatic activity.

To confirm that PD-ECGF sustain tumor progression by an autocrine mechanism, changes in tumor cell proliferation and in the number of apoptotic cells were examined. In comparison with untreated controls and DC101 treated animals, the percentage of proliferating (Fig. 33A) and apoptotic tumor cells (Fig. 33B) were dramatically affected by PD-ECGF1 enzymatic inhibition and rescued by 2-deoxy-D-ribose. We also noticed an increased in the number of apoptotic tumor cells in DC101 treated animals (Fig. 33B), likely the consequence of the vascular trimming and the resulting lack of nutrients and oxygen. To understand if the anti-angiogenic pressure was indispensable to achieve these results, and if 2-deoxy-D-ribose had pro-tumoral effects independently of PD-ECGF1 inhibition, at resistance mice were further randomized into AEAC, AEAC plus 2-deoxy-D-ribose and DC101 plus 2-deoxy-D-ribose groups.

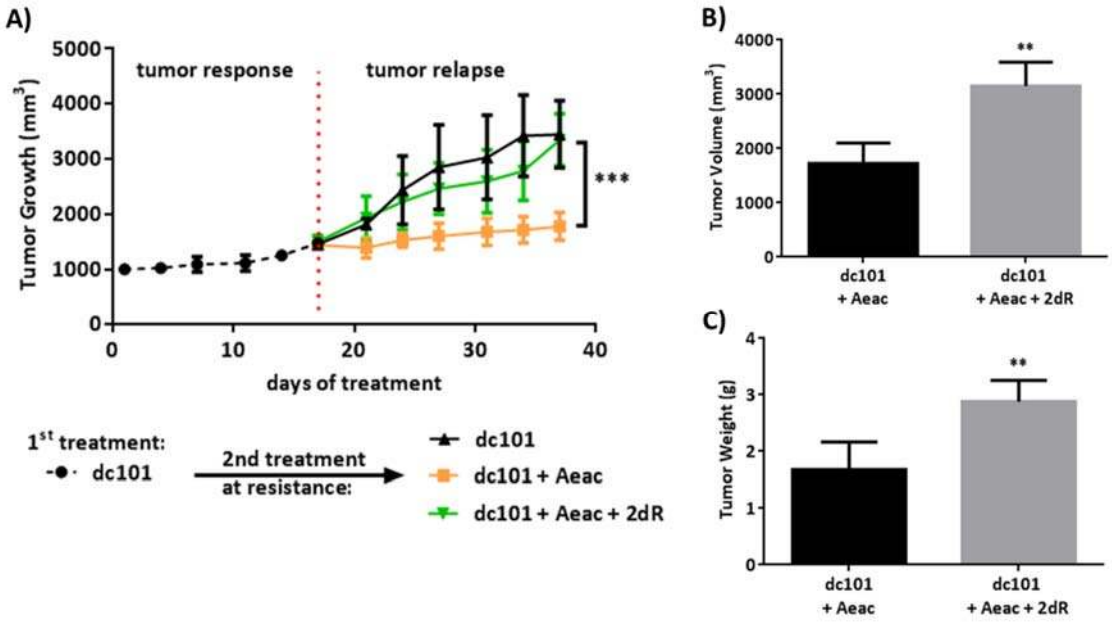

Fig. 32. PD-ECGF1 enzymatic activity promotes Ren-28 resistant tumor progression. A) Tumor progression by palpation shows that the enzymatic inhibition of PD-ECGF1 (orange line) significantly reduces tumor growth overcoming anti-angiogenic resistance. 2-deoxy-D-ribose fully reverts this phenotype (green line). B, C) Tumor volume and weight are completely rescue by 2-deoxy-D-ribose. **: $p < 0.005$. ***: $p < 0.0005$. Bars show standard deviation. n = 33 for the DC101 group until resistance; n = 6 for the DC101 group after resistance; n = 8 for the DC101 + AEAC treated group; n = 6 for the DC101 + AEAC + 2dR treated group.

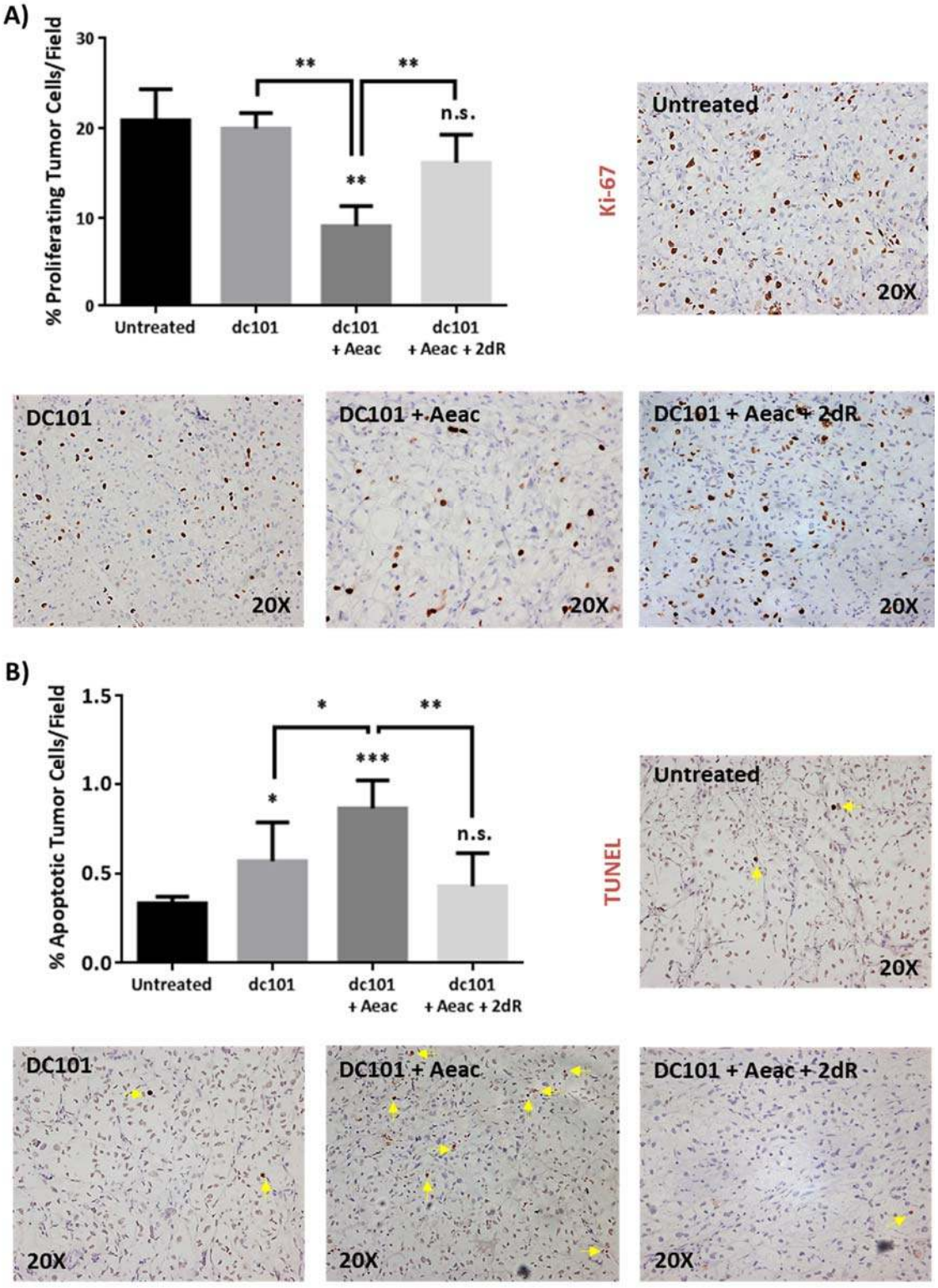

Fig. 33. PD-ECGF1 enzymatic inhibition affects tumor cell proliferation (A) and apoptosis (B). A) Quantification and representative images of the percentage of proliferating tumor cells. B) Quantification and representative images of the percentage of apoptotic tumor cells. Yellow arrows marked apoptotic tumor cells. *: < 0.05. **: $p < 0.005$. ***: $p < 0.0005$. Bars show standard deviation. n = 5 for each group.

2.3 – PD-ECGF1 enzymatic Inhibition *per se* fails to produce enduring effects.

As the graph of tumor progression shows (Fig 26A) and previously reported by the group, *per se* the enzymatic inhibition of PD-ECGF1 (red line) failed to produce enduring effects. In fact, even if in the first weeks after resistance mice treated only with AEAC (Fig. 34A, red line), and the ones that received the combination DC101 plus AEAC (Fig. 34A, orange line) seemed to behaved similarly, in the last part of the experiment AEAC single treatment was no more sufficient to block tumor re-growth. This misstep was reflected in a rise in final tumor weight (Fig. 34B) and volume (Fig 34C), confirming the importance of a simultaneous blockage of the VEGF and PD-ECGF1 pathways as second line therapy to overcome tumor re-growth. Surprisingly, AEAC's effects on tumor cell proliferation and apoptosis are not influenced by the absence of DC101 and are rescued by 2-deoxy-D-ribose (Fig. 35A-B), reinforcing the importance of PD-ECGF1 as a tumor progressing factor. Consequently to explain the differences in tumor growth, we did a step backward and analyzed tumor vasculature. Even if AEAC has been reported to have anti-tumoral effect decreasing MVD (Lu et al., 2009; Kylie et al., 2003); we observed a partial, but statistically significant, re-vascularization in mice that after resistance did not receive DC101, that is probably promoting tumor re-growth (Fig. 36).

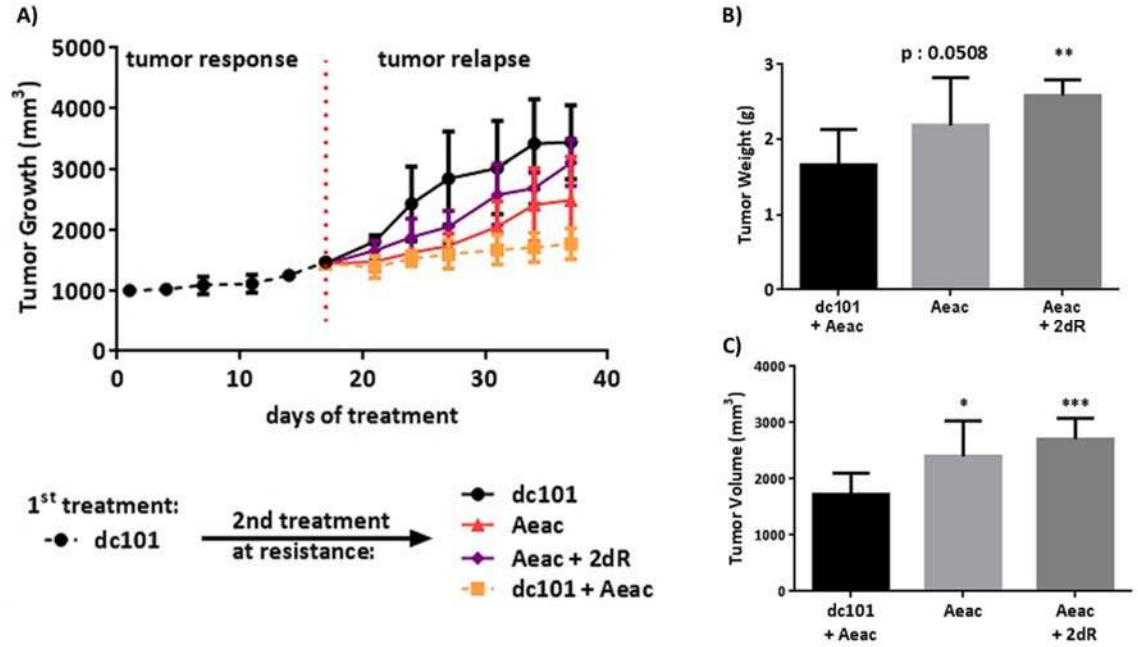

Fig. 34. AEAC as a single treatment post-resistance fails to block tumor re-growth. A) The enzymatic inhibition of PD-ECGF1 as a single treatment post-resistance (red line) stabilized tumor progression only temporarily, ending in tumor re-growth. However this transitory effect was not noticed when 2-deoxy-D-ribose was delivered, confirming AEAC specificity and the role of PD-ECGF1 as a tumor progressing factor. B, C) End point tumor weight (B) and volume (C) reflected the differences noticed in terms of tumor progression. *: < 0.05. **: p < 0.005. ***: p < 0.0005. Bars show standard deviation. n = 33 for the DC101 group until resistance; n = 6 for the DC101 group after resistance; n = 8 for the DC101 + AEAC treated group; n = 5 for the AEAC and AEAC + 2dR treated groups.

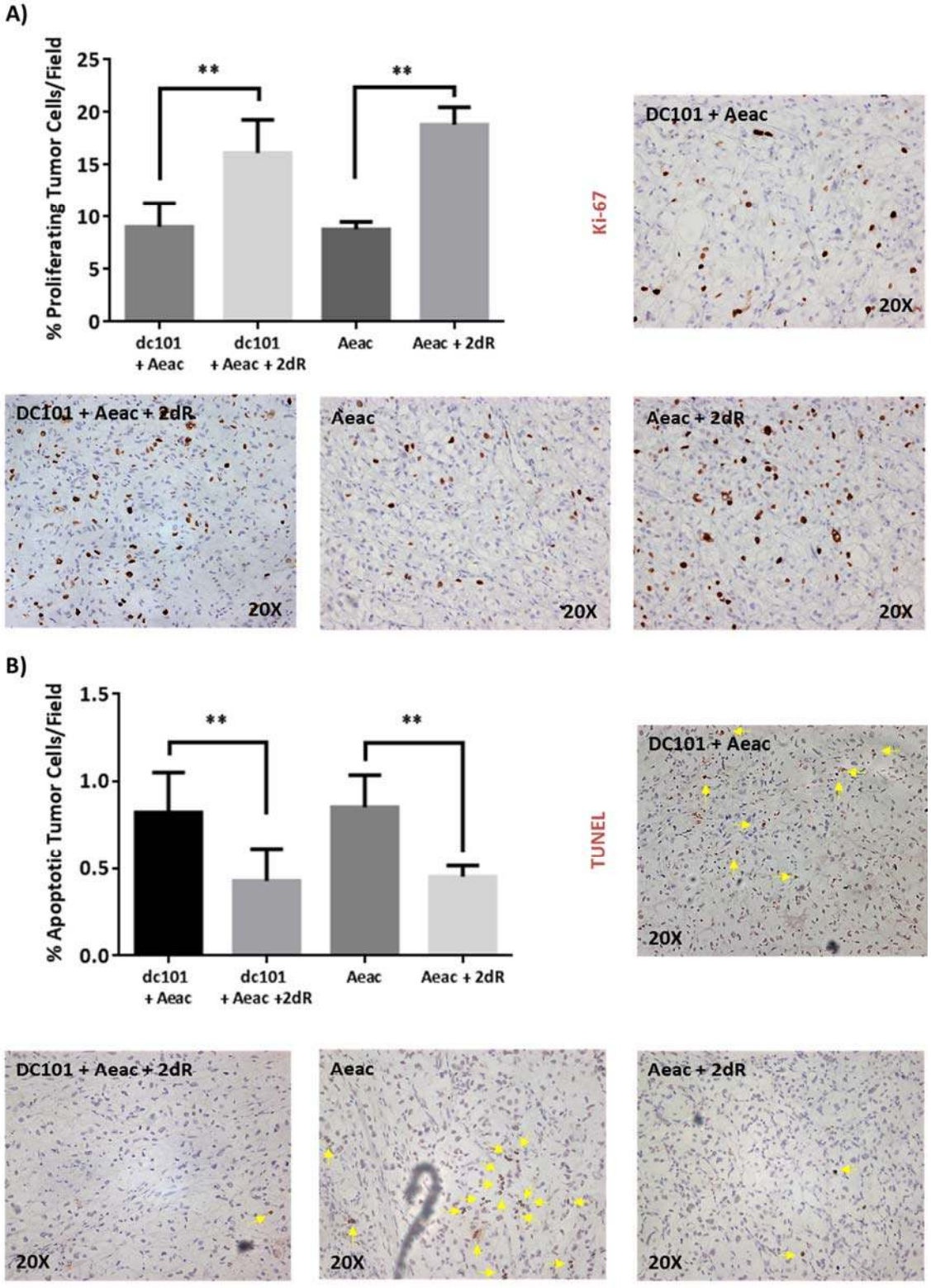

Fig. 35. AEAC as a single treatment affects tumor cell proliferation (A) and apoptosis (B). A, B) Quantification and representative images of the percentage of proliferating and apoptotic tumor cells. AEAC's effect on tumor cell proliferation and apoptosis were not influenced by the absence of DC101 and are rescued by 2-deoxy-D-ribose. **: $p < 0.005$. Bars show standard deviation. n = 6 for the DC101 group after resistance; n

= 8 for the DC101 + AEAC treated group; n = 5 for the AEAC and AEAC + 2dR treated groups.

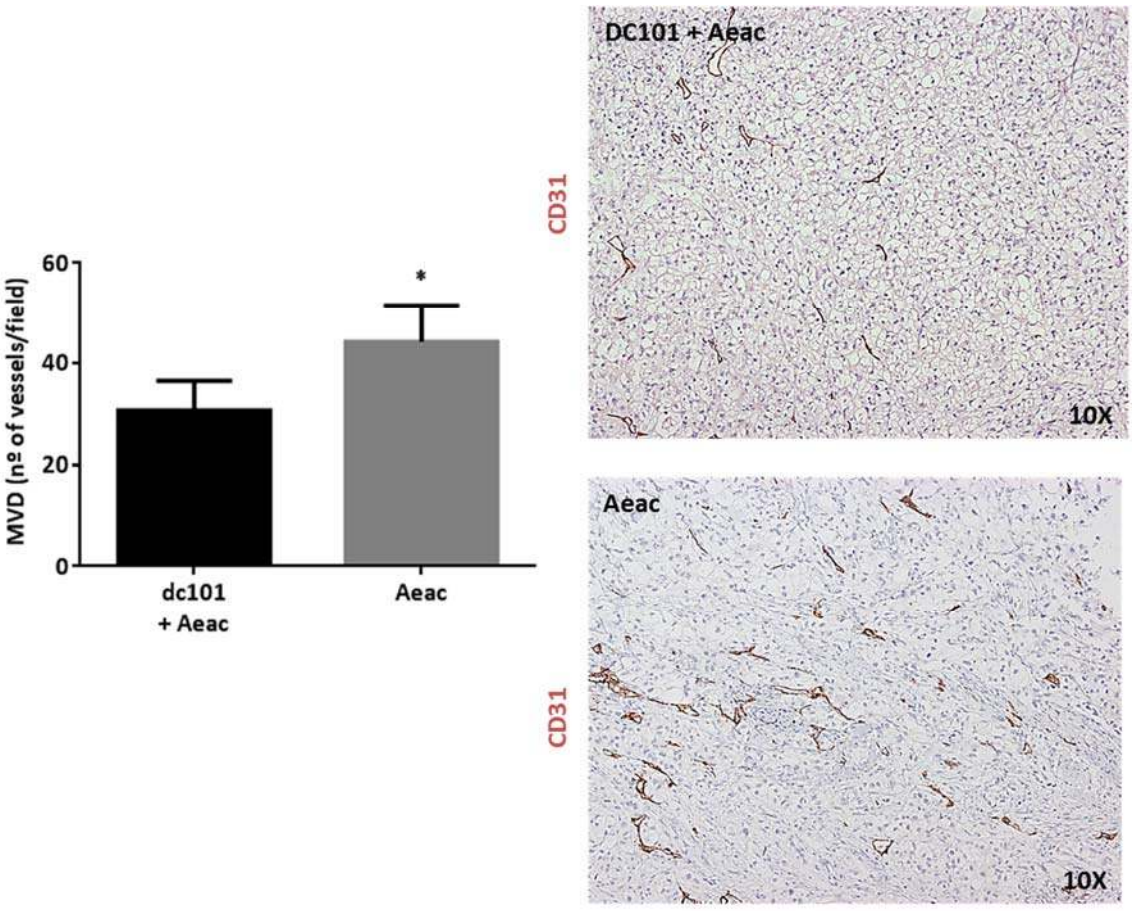

Fig. 36. AEAC single treatment effect on tumor vasculature. MVD quantification shows that in comparison with DC101 + AEAC treated animals, in tumors treated only with AEAC vessels partially re-colonized the tumors. Pictures show representative images for each group. *: $p < 0.05$. Bars show standard deviation. n = 8 for the DC101 + AEAC treated group; n = 5 for the AEAC group.

2.4 – 2-deoxy-D-ribose doesn't promote tumor re-growth.

In terms of tumor growth, animals that received as second line of therapy after resistance DC101 in combination with 2-deoxy-D-ribose did not show differences in comparison with the ones only treated with the anti-angiogenic (Fig. 37), suggesting that probably when PD-ECGF1 is upregulated and fully working, tumor cells are already exponentially growing and can't be forced forward.

To summarize this section of the results, using two mouse orthoxenograft models of RCCs we have described a new possible mechanism of resistance to anti-angiogenics, in which tumor cell after a period of adaptation characterized by drug effectiveness, escape the treatment up-regulating PD-ECGF1 that by its enzymatic activity promote cell growth and survival in an autocrine way. Even if our results seemed to exclude tumor stroma from this process, to assure AEAC tumor cells specificity, that no other components of the tumor microenvironment were involved, and to further understand the molecular mechanisms responsible of tumor cell adaptation, we decided to verify *in vitro* our findings on 786O^{-} and Ren28 primary tumor cells.

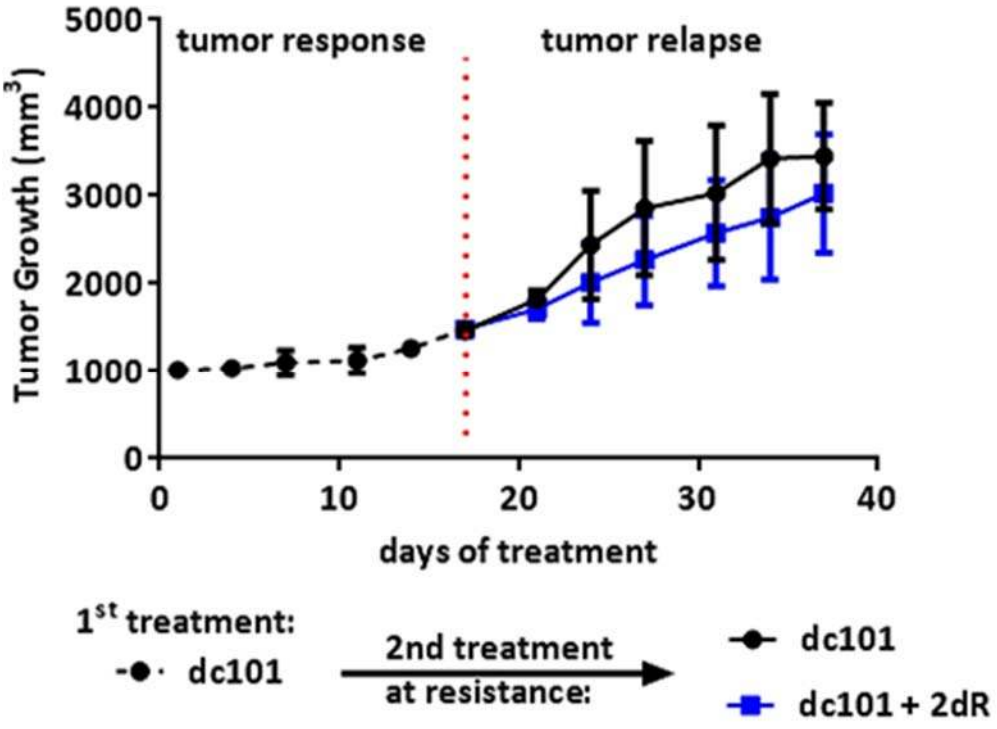

Fig. 37. Effects of 2-deoxy-D-ribose on tumor growth. Tumor progression by palpation shows that in comparison with DC101 treated animals, DC101 + 2-deoxy-D-ribose do not produce any significant effect on tumor growth. No further analysis has been done. Bars show standard deviation. n = 6 for the DC101 group after resistance; n = 4 for the DC101 + 2dR group.

3 – PD-ECGF1 promotes in vitro tumor cells growth.

To confirm the hypothesis that PD-ECGF1 supports tumor cell growth possibly playing a metabolic role, we silence its expression and investigate differences in cell proliferation. In comparison with cells growing in full media where the shRNA it's not induced, PD-ECGF1's knock down resulted in a significant inhibition of 786O$^-$ cell proliferation (Fig. 38A). 2-deoxy-D-ribose is only able to partially rescue this effect, suggesting that probably other intermediate or final products of PD-ECGF1 pathway are important to preserve cell growth at its normal kinetic (Fig. 38A). To evaluate the possibility of doxycycline *in vitro* toxicity the same experiment was repeated using 786O$^-$ shNS cells. We took notice of non-statistically significant decrease (Fig. 38B), but overall concluded that the anti-proliferative effects were PD-ECGF1 specific.

Previous data from the group stated AEAC ability to reduce vascular plexus formation in mouse retinal model and *in vitro* HUVEC migration, but not direct effects on cancer cells have been described (Lu et al., 2006). However our *in vivo* findings suggest that renal cancer cells might behave differently, and to verify our hypothesis we proceed to evaluate *in vitro* AEAC effects on 786O$^-$ cells.

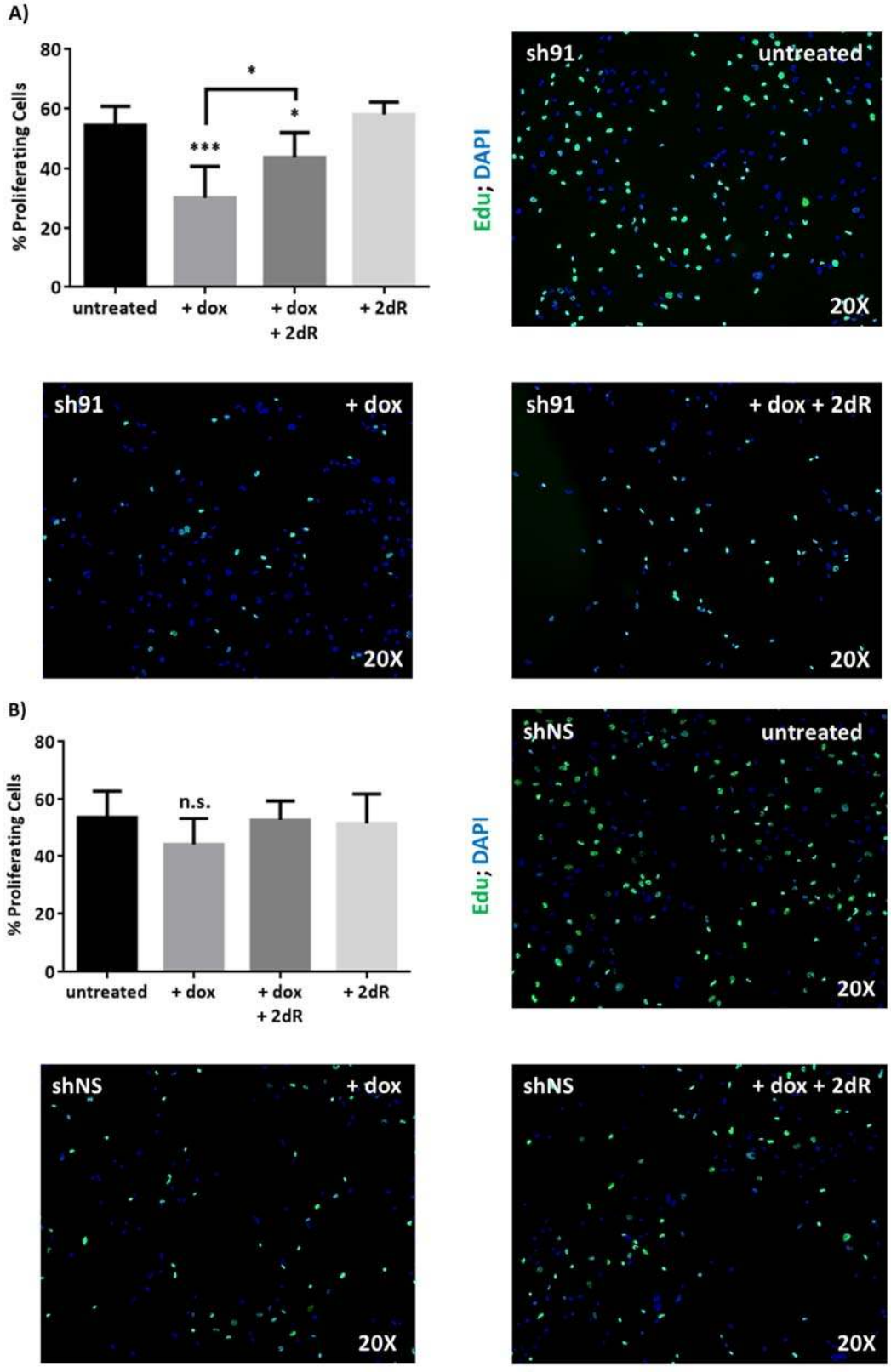

Fig. 38. PD-ECGF1 genetic knock down affect tumor cell proliferation. A, B) Quantification and representative images of the percentage proliferating 786O$^-$ sh91 (A) and shNS (B) cells after 24h of induction with doxycycline (2 µg/mL), 2-deoxy-D-ribose (50uM), the combination of the two or growing in complete RPMI medium. Bars

show standard deviation. *: < 0.05. ***: < 0.0005. Graphs are the pull of two independent experiments.

3.1 - AEAC affects *in vitro* tumor cell proliferation promoting apoptosis.

786O$^-$ cells were treated *in vitro* with AEAC and/or 2-deoxy-D-ribose and effects on tumor cell proliferation and apoptosis quantified. As seen in full tumors, the enzymatic inhibition of PD-ECGF1 significantly affected 786O$^-$ cells proliferation (Fig. 39A) and apoptosis (Fig. 39B) confirming the role played by PD-ECGF1 in tumor cell proliferation and survival and that AEAC's effects are tumor cells intrinsic. Moreover, 2-deoxy-D-ribose successfully rescued both phenotypes strengthening the importance of PD-ECGF1 enzymatic activity.

Different factors have been reported to be possible inducers of PD-ECGF1, like interferon-γ in macrophages (Goto et al., 2001), tumor necrosis factor in colon cancer (Zhu et al., 2002), or interleukin-1 alpha in colorectal carcinoma (Takebayashi et al., 1995), but unfortunately none of them worked in our setting. Consequently we decided to mimic the stresses generated by an anti-angiogenic treatment from a different prospective.

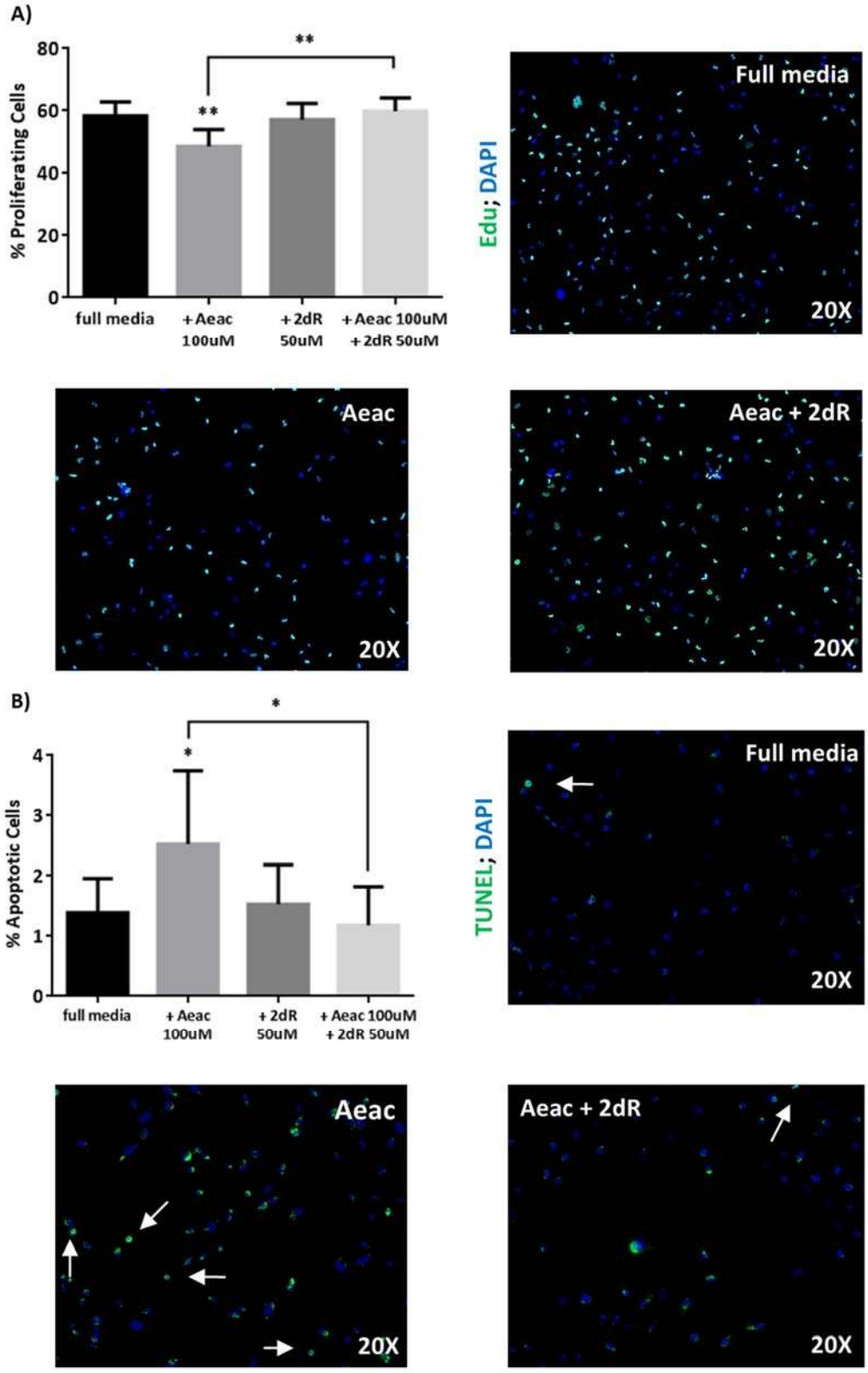

Fig. 39. AEAC affects directly 786O$^-$ cell proliferation and apoptosis. A, B) Quantification and representative images of the percentage of proliferating (**A**) and apoptotic (**B**) 786O$^-$ cells after 24h of treatment with AEAC, 2-deoxy-D-ribose, the combination of the two or growing in complete RPMI medium. White arrows mark the

apoptotic 786O$^-$ cells counted. Bars show standard deviation. *: < 0.05. Graphs are the pull of three independent experiments.

3.2 – Lack of nutrients drives PD-ECGF1 upregulation.

We have demonstrated that DC101 anti-angiogenic treatment efficiently decreases the number of tumoral vessels essential for the delivery of nutrients and oxygen required for tumor cells proliferation, but paradoxically it does not prevent tumor progression. Consequently, we hypothesized that tumoral cells sense this "hostile environment" and, after a period of adaptation, respond up-regulating ECGF1 renewing their exponential growth. Unfortunately, anti-angiogenics doesn't target directly tumor cells, and therefore we fought to mimic the anti-angiogenic effects starving of nutrients, or growing in hypoxia, SN12C tumor cells transfected with the shRNA expression vector 91 (as described in materials and methods section). The decision to use this different cell line was based on the consideration that among the different human kidney cancer cell lines available in the lab (786O$^-$, RCC4 and SN12C), SN12C cells expressed the highest levels of PD-ECGF1, and consequently we presumed might be more susceptible to changes in its expression. In comparison with cell growing in complete full media, tumor cells sensed the lack of nutrients and responded up-regulating PD-ECGF1 protein levels by approximately 2 fold, while hypoxia seemed to play a minor role in this pathway (Fig. 40). Moreover, upon doxycycline activation PD-ECGF1 protein levels regressed to normal demonstrating the pathway specificity.

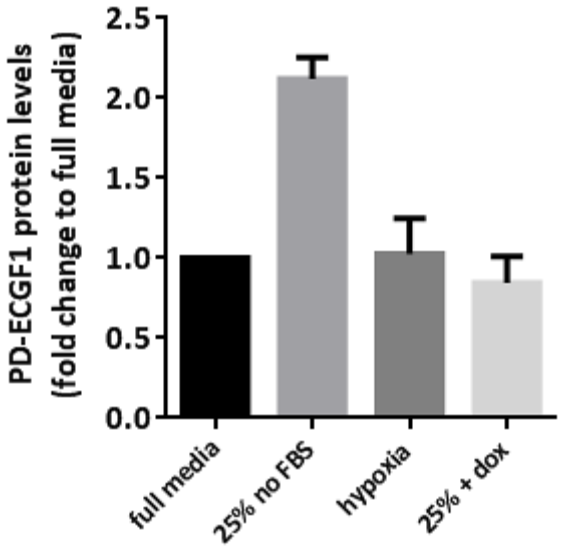

Fig. 40. SN12C sh91 tumor cells responds to nutrients deprivation up-regulating PD-ECGF1. Quantification of PD-ECGF1 protein levels measured by Elisa Assay and expressed as fold change to cell growth in complete DMEM medium condition. Bars show standard deviation. *: < 0.05. Graph is the pull of two independent experiments.

3.3 – Thymidine is recycled by PD-ECGF1 to promote cell proliferation under conditions of nutrient deprivation.

Next, using SN12C cells transfected with the shRNA expression vectors 91 and non-silencing (NS) (Fig. 42), we examined if, under conditions of nutrient deprivation, thymidine could be metabolized by PD-ECGF1 and contribute to cell proliferation. Under low nutrients condition both cell types show a dramatically decrease in the number of proliferating cells, rescued by thymidine (Fig. 41A, B). PD-ECGF1 genetic silencing suppressed thymidine effect in SN12C sh91 tumor cells (Fig. 41A), whereas doxycycline treatment did not produce any effects on SN12C shNS tumor cells (Fig. 41B), supporting the hypothesis that under condition of nutrient deprivation PD-ECGF1 recycling thymidine provides growth advantages. Moreover, 2-deoxy-D-ribose overstepped PD-ECGF1 silencing partially supporting cell growth (Fig. 41A, B) suggesting that other metabolites of this pathway are implicated, or that in this extreme condition the dose used was not adequate.

Finally, in collaboration with Dr Maria Ochoa de Olza from the Medical Oncology Department (ICO), we validate our preclinical results in clinical samples of ccRCC samples.

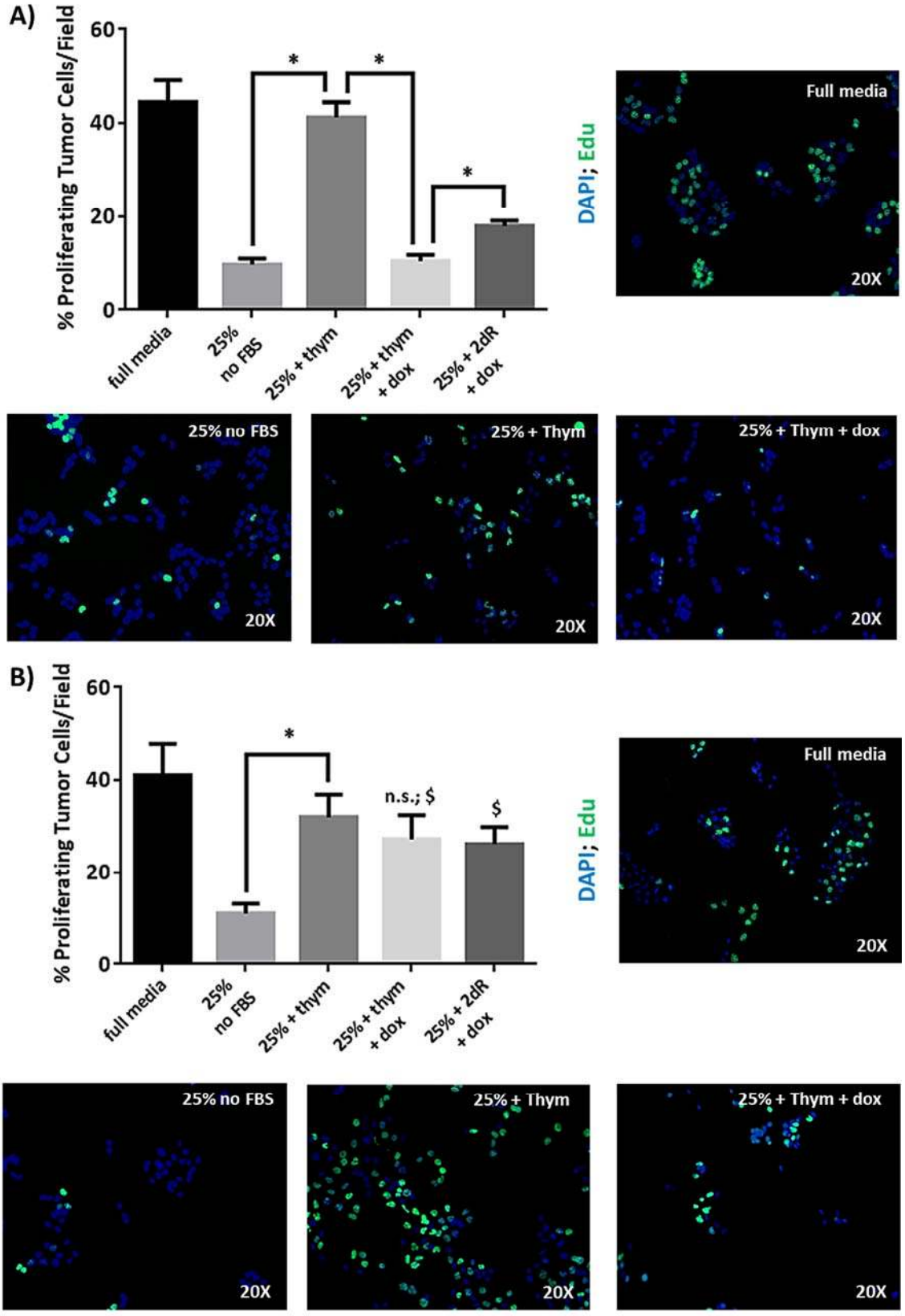

Fig. 41. PD-ECGF1 and thymidine effects on SN12C tumor cell proliferation under conditions of nutrient deprivation. A, B) Quantification and representative images of the percentage of proliferating SN12C sh91 (**A**) and SN12C shNS cells (**B**) growth for

24h in complete full DMEM media, under conditions of nutrients deprivation, nutrient deprivation and thymidine (1mM), nutrient deprivation, thymidine (1mM) and doxycycline (2 µg/mL), or nutrient deprivation, 2-deoxy-D-ribose (50µM) and doxycycline (2 µg/mL) The percentage of proliferating tumor cells/filed have been counted. Bars show standard deviation. *: < 0.05. $^{\$}$: < 0.05 vs nutrients deprivation condition. Graphs are the pull of two independent experiments.

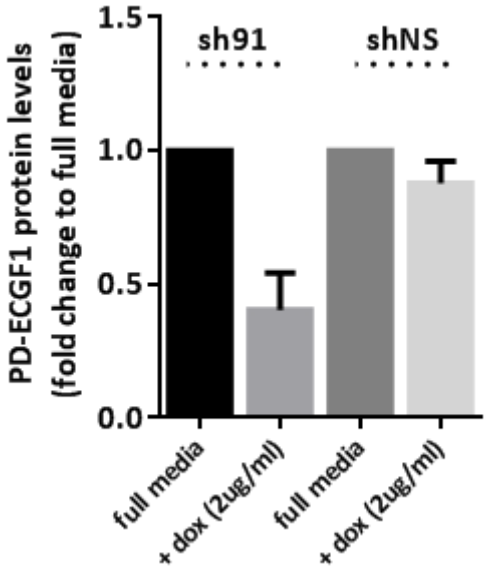

Fig. 42. PD-ECGF1 genetic silencing in SN12C sh91 and shNS tumor cells. Quantification of PD-ECGF1 protein levels detected by Elisa Assay and express as fold change to untreated control. Upon doxycycline activation (2 µg/ml), SN12C sh91 tumor cells show a 50% knock down down PD-ECGF1 expression, whereas resulted not affected in SN12C shNS line.

4 – Clinical relevance of PD-ECGF1 in RCC patients.

Before my enrollment in the lab, PD-ECGF1 tissue expression was evaluated in pre- and post-treatment (sunitinib) tumor biopsies of a group of 10 patients from the Bellvitge Hospital. We observed a general increase (Fig. 43A), even if considering that the patients underwent different treatment schedules these results are not conclusive. Consequently, the fold change in PD-ECGF1 expression was individually calculated, and patients grouped as responders or resistant according to their clinical outcome. Overall, resistant patients show a tendency to up-regulate PD-ECGF1 (Fig. 43C).

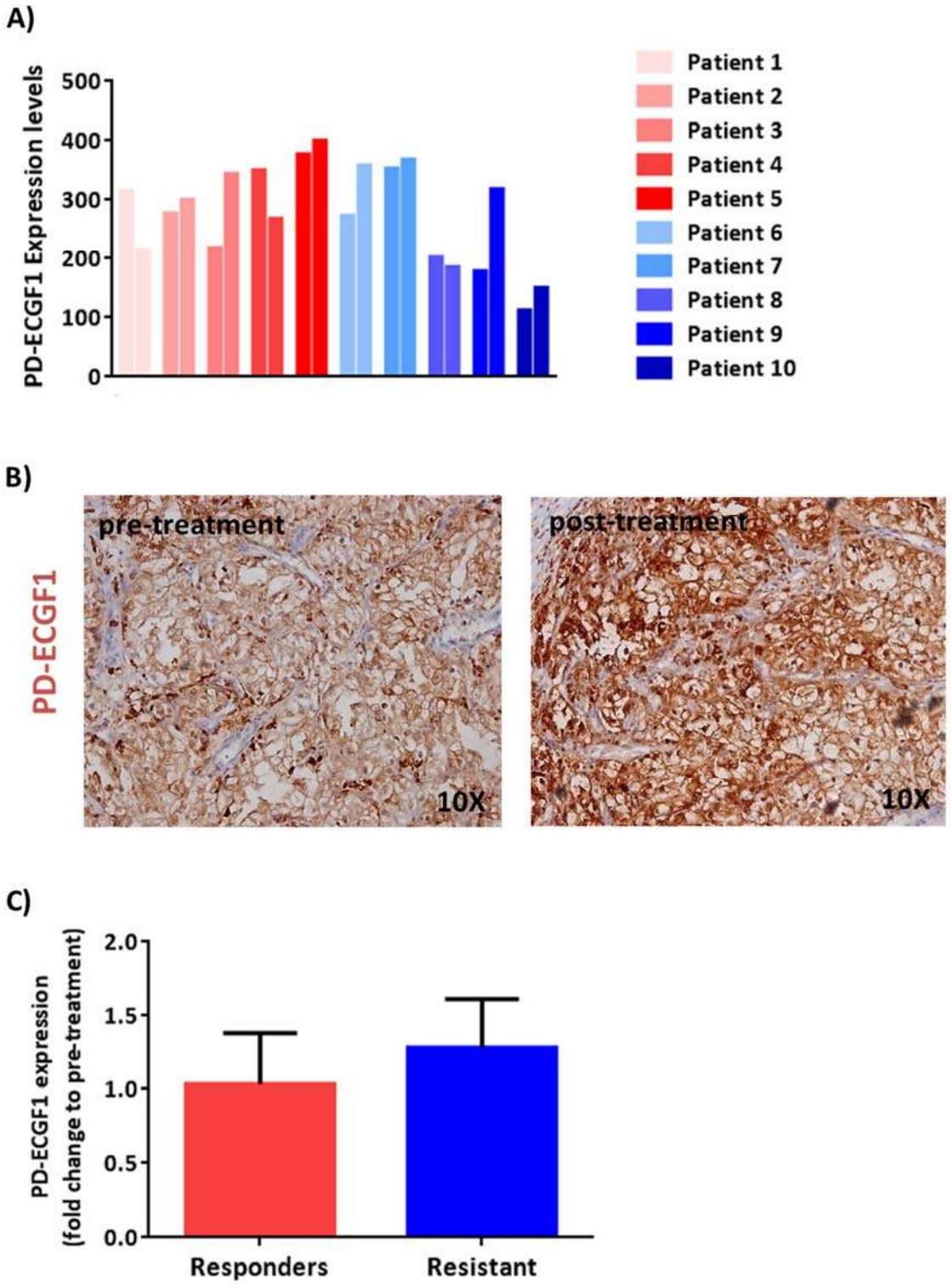

Fig. 43. PD-ECGF1 expression in pre- and post-treatment tumor biopsies. A) PD-ECGF1 tissue expression in pre- and post-treatment tumor biopsies of a group of 10 ccRCC patients. B) Representative images for each group. C) Fold change in PD-ECGF1 expression calculated for each patient and grouped according to the clinical outcome. Analysis performed by Jimenez-Valerio G.

4.1. – PD-ECGF1 expression in RCC patients correlates with worse PFS.

PD-ECGF1 plasma levels have been analyzed in a cohort of RCC patients from the Bellvitge Hospital and in healthy donors. PD-ECGF1 was not detected in any of the healthy donors (Fig. 44A), meanwhile 10 out of 12 RCC patients (83,33 %) showed detectable and heterogeneous levels (Fig. 44A and B). Consequently, patients were ranked as low and high expressing (PD-ECGF1 plasma level respectively lower or higher than the average of the entire data set) and the percentage progression-free survival calculated. PD-ECGF1 expression correlates with a worse progression-free survival (PFS) (Fig.44C).

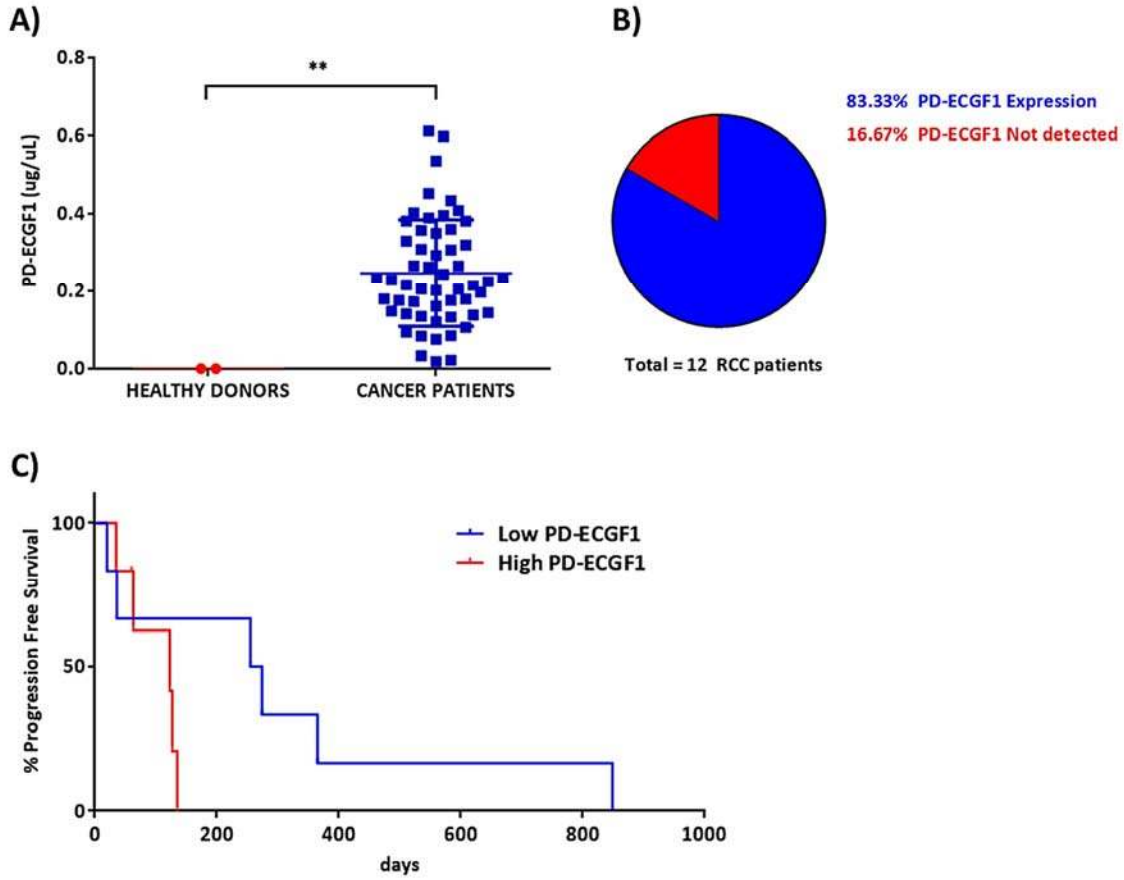

Fig. 44. PD-ECGF1 expression in plasma of RCC patients. A, B) PD-ECGF1 levels were analyzed in plasma from RCC patients or healthy donors using an Elisa kit (R&D). None of the healthy donor showed detectable levels of PD-ECGF1, meanwhile 10 out of 12 RCC patients showed heterogeneous levels. C) Percentage of progression-free survival of RCC patients indicates that PD-ECGF1 expression correlates with a worse prognosis. **: $p < 0.005$.

Discussion.

Discussion.

Cancer cells can be easily defined as cells that lose their identity, gain progenitor characteristics and divide relentlessly forming a solid mass, called tumor. In this process, the vascular supply of nutrients and oxygen, as well as wastes' elimination, are indispensable to grow a large mass, and since passive tissue diffusion is effective only for few millimeters, tumors require the recruitment of new blood vessels. In fact, in the absence of a blood supply, a tumor can grow a mass of about 10^6 cells, roughly a sphere of 2 mm in diameter. At this point, division of cells on the outside of the tumor mass is balanced, due to inadequate supply of nutrients, by death of those in the center and such tumors cause few problems. Unfortunately, the majority of cancers early overstep this barrier inducing the formation of new blood vessels that invade and nourish the tumors. Moreover, angiogenesis is induced surprisingly early during the development of cancer, and, as histological analysis have elucidated, it is not important just for rapidly growing macroscopic tumors but also contributes to the microscopic premalignant phase of neoplastic progression, further cementing its status as in in integral hallmark of cancer (Hanahan and Weinberg, 2011). Probably being the only characteristic shared by all human tumors, and considering the genetic stability of endothelial cells, targeting tumor angiogenesis seemed to be a uniquely attractive target to oncologists, and extensive basic and clinical research had led to the FDA approval of different drugs, like the first-generation anti-VEGF monoclonal antibody bevacizumab in 2004 or the second-generation TKI sunitinib in 2006, for the treatment of solid tumors. However, translating the preclinical successes into clinical practice, identifying the optimal clinical indication and maximizing the efficacy of the anti-angiogenic therapies has been more challenging than anticipated. In fact, despite transient disease stabilization reflected in a favorable progression free survival (PFS) of patients treated with anti-angiogenics in comparison with placebo or cytotoxic drugs, the overall survival (OS) is often not prolonged, and nonetheless a considering number of patients developed acquired resistance in

the range of months without a permanent cure (Yang et al., 2003; Kerbel, 2008; Loges et al., 2010).

Overall, the ability of the available anti-angiogenic drugs to target the angiogenic pathway is not under question, but further elucidations to understand how solid tumors tackle these treatments determining a therapeutic failure could be decisive for the fate of many cancer patients. Consequently, in this project we decided to evaluate the anti-angiogenic response in animal orthoxenograft models of ccRCC, for which inhibitors of the angiogenic axis are currently the first therapeutic option.

Anti-angiogenic response in RCC tumor-bearing mice.

Using two different orthoxenograft animal models of renal cell carcinoma (RCC), we have demonstrated that DC101, a monoclonal antibody directed against mouse VEGFR-2, has a limited therapeutic window of efficacy in which, in comparison with untreated animals, tumor growth is stabilized (Fig. 14 and 30A). Unfortunately, we didn't notice any shrinkage, and in all treated animals tumors inexorably progressed provoking the failure of the treatment, confirming what has been reported in different clinical studies in which benefits were only of few months (Yang et al., 2003). However, using the same antibody other groups have in the past reported complete inhibition of tumor growth and metastasis (Prewett et al., 1999; Izumi et al., 2003) even though the drug was administered in the first days of tumor development, before the angiogenic switch, in a prospective improbable clinical situation.

We determined that in both animal models, once resistance had occurred, continuing or interrupting the treatment have the same effect on tumor growth (Fig. 14 and 30A,) meaning that tumor cells during the period of drug susceptibility have taken a breath from uncontrolled growth, evolved and adopted some permanent strategy to tackle the drug. Nevertheless, histological analysis surprisingly reveled that DC101's anti-angiogenic effects, even if not

sufficient to control tumor progression, were not lessen in resistant tumors suggesting that in both models resistance was vascular independent (Fig. 15 and 30B). Besides, these data confirm the insult-dependent response of tumor cells which do not lose time trying to alter the target availability, decrease the drug activation or increase its degradation like in chemotherapy (Lugmani, 2005), but opt to bypass the need of a vascular supply suggesting that non-canonical mechanisms of resistance needs further considerations. In fact, even if resistance to anti-angiogenics has been extensively studied and a variety of possible mechanisms, including tumor cells release of alternative angiogenic factors, vascular co-option, induction of vasculogenesis and vascular mimicry, proposed (Motzer et al., 2006; Longes et al., 2010; Holash et al., 1998; Seftor et al., 2012), all these mechanisms explain relapse to anti-angiogenic treatments pointing to the same final biological effect: vessels recruitment and tumor re-vascularization. Only recently a new mechanism of adaptive resistance in the context of continuing suppression of tumor neo-vascularization, involving a functional compartmentalization of energy metabolism has been described (Jimenez-Valerio et al., 2016; Allan et al., 2016; Pisarsky et al., 2016). Confirmed in pancreatic, renal and mammary cancer these studies propose metabolic symbiosis as a new mechanism of resistance, in which hypoxic and peri-necrotic tumor cells proliferate activating anaerobic glycolysis and exporting the accumulated lactate, that is coordinated imported and metabolized aerobically by perivascular and better oxygenated tumor cells to obtain energy (Sonveaux et al., 2008).

Our observations of a vascular independent resistance to anti-angiogenics, the identification of *PD-ECGF1* highly enrich gene expression in resistant versus responsive tumors, and the consideration that the majority of the oncogene associated with the development of kidney cancer are involved in single cell's ability to respond to nutrient deprivation by metabolic alterations (Linehan and Ricketts, 2013), suggested that tumor might circumvent the treatment by novel

possible unconventional mechanisms, and identified PD-ECGF1 as a new possible target.

This enzyme, classically known as involved in the nucleic acid homeostasis, has received increasing attention during the last years for its controversial role in cancer (Friedkin and Roberts, 1954; Elamin et al., 2015). In fact, usually found overexpressed in tumor tissues, whilst in some cancers is involved in angiogenesis, evasion from apoptosis resulting in poor prognosis and tumor progression, its enzymatic activity it's also been used for the design of different chemotherapeutic agents (Bijnsdorp et al., 2008; Elamin et al., 2015; Longley et al., 2014).

The role of PD-ECGF1 in tumor resistance.

Using two different approaches, we demonstrated that, in both cell derived and primary orthoxenograft animal models of RCC, PD-ECGF1 by its enzymatic activity is responsible of the acquisition of resistance to anti-angiogenics. In fact, the first insights of resistant-tumor growth stabilization resulting from the genetic silencing of PD-ECGF1 protein expression (Fig. 18), where further confirmed by its enzymatic inhibition using the small molecule AEAC (Fig. 23), suggesting that its pro-tumoral actions relay on its catabolic activity.

AEAC specificity (Klein et al., 2001) and anti-tumoral activity, alone or in combination with VEGF-traps, has been well documented (Lu et al., 2006), but always associated with a direct effect on endothelial cells resulting in increased vascular trimming when combine with antiangiogenics. Here, we demonstrated that PD-ECGF1 inhibition did not increase the anti-vascular effect of DC101 (Fig. 24A), but dramatically affected tumor cell proliferation and apoptosis (Fig. 24B and 24C), validating the idea that PD-ECGF1 works autocrinally by its enzymatic activity, and not as a classical ligand-like growth factor, as could be expected by the fact that it lacks a signal sequence required for cell secretion (Elamin et al., 2015). Considering the numerous proprieties ascribed to its final metabolite 2-

deoxy-D-ribose (Ikeda et al., 2006; Bjinsdorp et al., 2008), we evaluated its role, finding that 2-deoxy-D-ribose counteracted the effect of AEAC on 786O$^-$ and Ren28 tumor growth, rescuing tumor cell proliferation and apoptosis without promoting re-vascularization (Fig. 25, 26, 32, 33). Overall these findings, confirmed also in an *in vitro* setting (Fig. 38 and 39), demonstrate that in this model of anti-angiogenic resistance tumor stroma's role is marginal, and that PD-ECGF1 acts mainly intracellularly supporting tumor cell proliferation and protecting from apoptosis by its enzymatic activity and final metabolite 2-deoxy-D-ribose. Moreover, when 2-deoxy-D-ribose was administer in the absence of any enzymatic inhibition, it didn't promote tumor growth (Fig. 37) indicating that if cancer cells have plenty of substrate that can flow into the PD-ECGF1 pathway and be recycle for energy production do not require external sources. Nevertheless, PD-ECGF1 showed different grades of tissue expression, and the possibility tumor cell secretion of accumulated 2-deoxy-D-ribose to nourish other tumor areas can not be rule out.

Moreover, the fact that AEAC when administer alone failed to produce enduring effect as a result of a partial tumor re-vascularization (Fig.34, 35 and 36), suggests that vessel recruitment, if possible, remain the principal mechanism of resistance, confirming the importance of a combinatory treatment.

However, the fact that recombinant 2-deoxy-D-ribose had reduced rescuing effects favors different considerations. First, we can reasonably postulate that AEAC inhibitory potency is ways less efficient that PD-ECGF1 genetic silencing, and consequently determine milder effects that are easier relief by recombinant 2-deoxy-D-ribose. Moreover, these results suggest that also other products of the PD-ECGF1 pathway are required to promote optimal tumor growth, and consider 2-deoxy-D-ribose the only player is probably simplistic. Finally, the intra-tumoral accumulation of thymidine that could not be recycled and possibly causes adverse effects, as reported in MNGIE (Hirano et al., 2004), it's worth to be mentioned.

To verify these findings in vitro, where VEGFR-blockers are harmless on cancer cells, we decided to mimic the final effects of anti-angiogenic treatments, describing that under nutrient deprivation conditions SN12C tumor cells significantly up-regulate PD-ECGF1 expression (Fig. 40), and metabolizing thymidine acquired considerable growth advantages (Fig. 41). Even if the thymidine catabolic pathway in mammalian cells remains unclarified, in certain prokaryotes thymidine-derived 2-deoxy-D-ribose-1-phosphate is converted into 2-deoxy-D-ribose-5-phosphate by phosphopentose mutase (PPM), which is later converted into glyceraldehyde 3-phosphate (GAP) an intermediate of both the glycolytic and pentose phosphate pathways (Hoffee, 1968). Here we hypothesized that under condition of nutrient deprivation resulting from anti-angiogenic treatment, the activation of thymidine catabolism by PD-ECGF1 overexpressing cells could supply the carbon source for those pathways. This unexpected pro-tumoral function of PD-ECGF1 as a recycling metabolic enzyme agrees with the finding that elevated thymidine concentrations within the tumor microenvironment are the result of necrotic cell DNA hydrolysis (Brown et al., 2000), a common characteristic of anti-angiogenic treated tumors, and with the recently publish work of Tabata and colleagues in which demonstrated how human epidermoid carcinoma KB cells, under serum free conditions, metabolized thymidine by PD-ECGF1 enzymatic activity as a carbon source for glycolysis (Tabata et al., 2017).

Finally, the analysis of tissue and plasma samples of ccRCC patients from the Bellvitge Hospital confirm in a clinical set that PD-ECGF1 is exclusively found in cancer patients' plasma, where if highly expressed correlates with poor prognosis, and a tendency to be up-regulated upon anti-angiogenic treatment (Fig. 43, 44). So far, the lack of well-established biomarkers for patient's selection or to monitor disease progression and resistance has made difficult for oncologists to supervise anti-angiogenics efficacy, usually assessed by CT scans after several cycles of drug. Even if more comprehensive studies are surely

mandatory, our results suggest that PD-ECGF1 could be a useful biomarker for patients and treatment selection, and a possible second-line target to avoid treatment resistance.

Working model and future outlook.

Overall, we hypothesized the following model of tumor adaptation via PD-ECGF1 up-regulation. Anti VEGF/R therapies generates a hostile environment characterized by vessels, and consequently nutrients shortage inadequate for exponential growth typical of malignant cells (Fig. 45). Unprepared for this unexpected turnover, cancer cells hit the wall undergoing death by necrosis that results in the control growth characteristic of the initial tumor response phase. Unfortunately a second phase rapidly appears in which some perinecrotic tumor cells respond to the lack of nutrients up-regulating PD-ECGF1 expression, and "cannibalizing" thymidine coming from necrotic cell DNA hydrolysis supply the need of carbon sources to ensure proliferation resulting in tumor rebound and therapeutic failure.

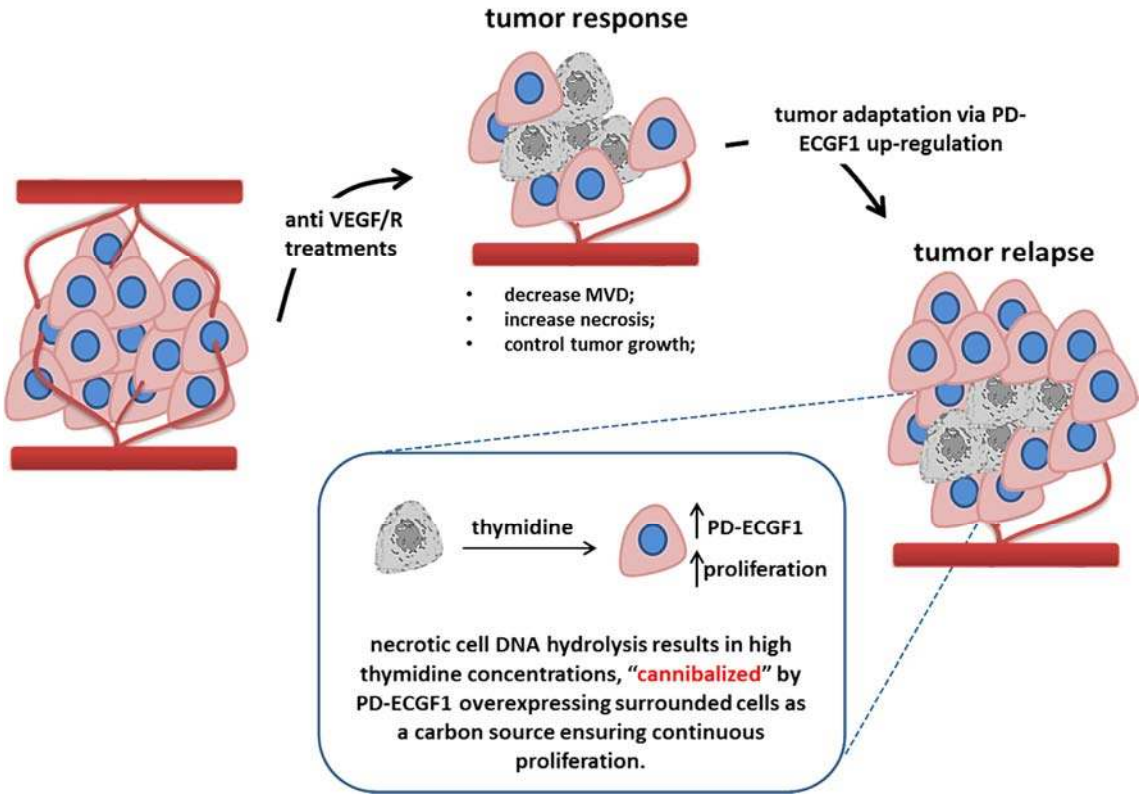

Fig. 45. Hypothesized model of tumor adaptation via PD-ECGF1 up-regulation.

But, why thymidine? And can other nucleotides follow the same route? Our data and bibliographic researches only allow speculations. In MNGIE the lack of PD-ECGF1 results in systemic accumulation of deoxyribonucleosides thymidine (dThd) and deoxyuridine (dUrd) (Yadak et al., 2017), that can both be converted in 2-deoxy-D-ribose-1-phosphate and theoretically enter into the glycolytic and pentose phosphate pathways. However, uridine plasma and tissue concentration are tightly regulated by uridine phosphorylase (UPase) activity, responsible of its reversible phosphorylation essential for RNA synthesis (Pizzorno et al., 2002). Even if we haven't direct evidences of UPase tumor up-regulation, its gene expression is known to be strictly controlled at the promoter level by oncogenes and the activity usually elevated in various cancer tissues (Yan et al., 2006), suggesting that this second nucleotide might not be available for PD-ECGF1 degradation and undergo a different faith.

These and other questions are surely challenging and open the possibility of further and deeper investigations.

Conclusions.

- Using two different orthoxenograft animal models of ccRCC we have demonstrated that anti-angiogenics have a short therapeutic window, followed by drug resistance and tumor relapse;

- In our study resistance shows to be vessel independent and the result of PD-ECGF1 enzymatic activity;

- Using two different approaches we have demonstrated that targeting the PD-ECGF1 and VEGF axis can be a useful strategy to block tumor re-growth;

- Mechanistic studies demonstrate that, under condition of nutrient deprivation, kidney cancer cells up-regulating PD-ECGF1 expression and metabolizing thymidine acquired considerable growth advantages, suggesting a novel metabolic escape from anti-angiogenics.

- The analysis of clinical samples from the Bellvitge Hospital supports our pre-clinical data and suggests that PD-ECGF1 might be a good therapeutic predictor factor.

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